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Enhancement of Antitumor Activity of Cisplatin on Human Gastric Cancer Cells *in vitro* and *in vivo* by Buthionine Sulfoximine

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An attempt was made to evaluate the enhancement of the antitumor activity of cisplatin (DDP) by buthionine sulfoximine (BSO) *in vitro* and *in vivo*. In the *in vitro* study, pre-treatment with BSO (5, 10 and 25 m*M*) increased the antitumor activity of DDP against the gastric cancer cell lines MKN-28 and MKN-45, whereas BSO alone exhibited only slight antitumor activity (inhibition rate, 20–30%). In the *in vivo* study, the antitumor effects of DDP against human gastric cancer xenografts St-15 and SC-1-NU in BALB/c nu/nu mice were enhanced pretreatment with BSO, which was administered intraperitoneally at a dose of 500 mg/kg according to a schedule of qd × 3. BSO alone showed no antitumor effects against these tumors in nude mice. The side effects (assessed in terms of death rate and body weight loss) associated with the maximum tolerated dose of DDP (9 mg/kg) were not increased by BSO pretreatment. As BSO increased the antitumor activity of DDP without a corresponding increment of its toxicity, BSO appears to be a promising agent for further study.

Key words: Buthionine sulfoximine — Cisplatin — Human gastric cancer

Buthionine sulfoximine (BSO) has been reported to reduce the intracellular level of glutathione (GSH),[1] which is associated with the detoxificaion of various xenobiotic compounds and the mechanism of resistance of malignant tumor cells to some antitumor agents.[2–5] Recently, BSO was demonstrated to be a novel anti-cancer agent, which enhances the antitumor activity of cisplatin (DDP) and cytostatic agents on cancer cells.[4, 5] In this study, we evaluated the antitumor effects of BSO *in vitro* and *in vivo*, in particular its enhancement of the antitumor activity of DDP.

MATERIALS AND METHODS

Drugs DDP was purchased from Bristol-Myers Squibb K.K., Tokyo, BSO, nicotinamide adenine dinucleotide phosphate (NADPH) and GSH reductase were purchased from Sigma Chemical Inc. (St. Louis, MO) and perchloric acid ($HClO_4$) and potassium carbonate (K_2CO_3) were purchased from Wako Pure Chemical Industries, Ltd., Osaka.

Cell lines MKN-28 and MKN-45, a well differentiated and a poorly differentiated gastric adenocarcinoma cell line, respectively, which were established at Niigata University,[6] were purchased from Immunological Biological Laboratory, Gumma; KATO III, a gastric cancer cell line, was supplied by Dr. M. Sekiguchi, Institute of Medical Science, University of Tokyo, Tokyo.

MTT assay The viability and survival of cancer cells *in vitro* were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay developed by Mosmann, with some modifications described elsewhere.[7] Cancer cells (10,000/100 μl/well) were plated into 96-well multiplates (MS-8096F Sumitomo Bakelite Co., Ltd., Tokyo) suspended in control culture medium, which consisted of Eagle's minimal essential medium, 10 n*M* insulin and 10% w/v fetal calf serum. Two hundred microliters of medium containing the required concentrations of drugs or control vehicles was incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. A mixture of MTT (4 mg/ml) and sodium succinate (0.1 mol/liter) in phosphate-buffered saline (PBS) was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA) and 10 μl solution/well was added, followed by incubation for a further 4 h at 37°C. Then 150 μl/well dimethyl sulfoxide was added to dissolve the formazan salt, and the plates were shaken for a few minutes. The absorbance of each resulting fluid was read on a model EAR easy reader (SLT-Labinstruments, Austria) at 540–630 nm.[8, 9] Survival rate was calculated as (absorbance of treated group − absorbance of blanks)/(absorbance of control − absorbance of blanks) × 100 as a percentage. The cells in control medium without a mixture of MTT and sodium succinate were measured as blanks. In order to assess the antitumor activity of BSO alone, tumor cells were incubated for 24 h in control medium as described above, followed by incubation with BSO (1, 5, 10 and 20 m*M*) for 12, 36 and 60 h at 37°C. In order to assess the effects of DDP and

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BSO in combination, 10,000 tumor cells were pre-incubated in control medium for 24 h, as described above, incubated with BSO (5, 10 and 25 m*M*) for 24 h, washed twice with PBS and incubated with DDP (0.1, 0.3, 1.0 and 3.0 μg/ml for MKN-28 and 0.01, 0.1, 0.3 and 1 μg/ml for MKN-45) for 48 h. In order to determine whether the order of drug treatment affected the anti-tumor activity of combination therapy against MKN-28 and MKN-45, 3 different BSO and DDP treatment regimens were tested using 5 m*M* BSO and 1, 3, 10 and 30 μg/ml DDP. In group (a), MKN-28 and MKN-45 tumor cells were incubated with BSO for 24 h, washed twice with PBS and then incubated with DDP for 24 h followed by incubation in control medium for 48 h. The tumor cells of group (b) were incubated wth DDP for 24 h, then with BSO for 24 h and finally in control medium for 24 h. In the simultaneous treatment group (c), the tumor cells were incubated with both BSO and DDP for 24 h followed by incubation in control medium for 48 h. All of the groups were incubated for 48 h after the removal of DDP.

Human tumor xenografts Two human gastric cancer xenografts, St-15 and SC-1-NU, were used for the *in vivo* experiments. The St-15 tumor strain was established at the Pathology Division, National Cancer Center, and SC-1-NU was supplied by Dr. M. Yamauchi, the Second Department of Surgery, Nagoya University. Histologically, St-15 and SC-1-NU are mucinous and poorly differentiated adenocarcinomas, respectively. These strains were maintained at our institute by serial transfer in the subcutaneous tissue of BALB/cA nude mice.

Mice Male nude mice with a BALB/cA genetic background were purchased from CLEA Japan, Inc., Tokyo, and those aged 6–8 weeks and weighing 20–22 g were used.

Tumor inoculation, measurement of tumors and drug activity evaluation One tissue fragment (approximately 3×3×3 mm) was inoculated into the subcutaneous tissue of the backs of nude mice under ether anesthesia using a trocar needle. The tumors were measured (length and width) with a sliding caliper three times weekly by the same observer. According to the method of Geran *et al.*,[10] the tumor weight was calculated from the linear measurements using the formula: tumor weight (mg) = length (mm) × (width in mm)^2/2. Drug treatment was initiated when the tumor weight reached 100–300 mg. On days 1, 2 and 3, BSO (500 mg/kg) dissolved in 0.2 ml of sterile water was administered intraperitoneally (i.p.), and DDP (6 or 9 mg/kg i.p.) on day 2. Mice given DDP or BSO treatment alone, or the control mice, were given 0.2 ml of saline instead of each drug according to the same schedule as that for the drug treatment. At the end of the experiment, all the mice were killed and the tumors and spleens were excised and weighed. The effects of the agents on the tumor, spleen and body weights were evaluated as the ratio (%) of the weight in the treated mice to that in the control mice (T/C ratio).[11] The effects of BSO and DDP combination therapy were evaluated by comparing the T/C ratio for the group treated with both BSO and DDP with the value (T/C ratio of BSO alone) × (T/C ratio of DDP alone). The combined therapy was considered to act synergistically if the T/C of BSO and DDP was lower than the value of (T/C of BSO alone) × (T/C of DDP alone). The results were expressed as means ± SD, and the significance of differences between them was analyzed by using Student's *t* test for unpaired samples. Differences at $P<0.05$ were considered to be significant.

Cellular GSH assay Five million cells of MKN-45 were suspended in a culture bottle with complete culture medium for 24 h before examination in the *in vitro* study. In the treated group, the cells were incubated with BSO at a concentration of 25 m*M* for 24 or 48 h. After trypsinization and counting of the cells, the cells of the control group and the treated group were homogenized in 2 ml of 0.6 *N* perchloric acid ($HClO_4$) as samples. In the *in vivo* study, 12 h after receiving the last dose of BSO (500 mg/kg i.p. on days 1, 2 and 3), nude mice bearing SC-1-NU were killed and the tumors were removed and weighed. Each tumor was homogenized in 0.6 *N* $HClO_4$.

The supernatant of the samples was neutralized by adding a 1/10 volume of 3 *M* potassium carbonate (K_2CO_3) on ice, followed by 10-fold dilution with 0.1 *M* phosphate beffer (pH 7.0) containing 5 m*M* EDTA. The total GSH, which comprised GSH and oxidized GSH, was measured as described by Tietze,[12] using the DTNB-GSH disulfide reductase recycling assay. The changes in the absorbance values (measured at 412 nm) during the reaction, which is based on the concurrent color change of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) induced by the cyclic reduction of GSH by GSH reductase and NADPH, were monitored for 10 min. The total GSH was determined by interpolation of a standard curve, which was constructed by using various known concentrations of GSH.

RESULTS

Antitumor activity *in vitro* The antitumor activities of BSO alone against MKN-28, MKN-45 and KATO III are shown in Fig. 1. Although BSO (1, 5, 10 and 20 m*M*) inhibited all these strains in a concentration-dependent manner, the minimum percent survival was more than 70% after incubation for 12, 36 and 60 h, and no marked time-dependence of inhibitory effect was observed (Fig. 1). The antitumor activity of DDP, i.e., the decrease of survival rate, against the gastric cancer lines MKN-28 and MKN-45 was greatly enhanced at all tested concen-

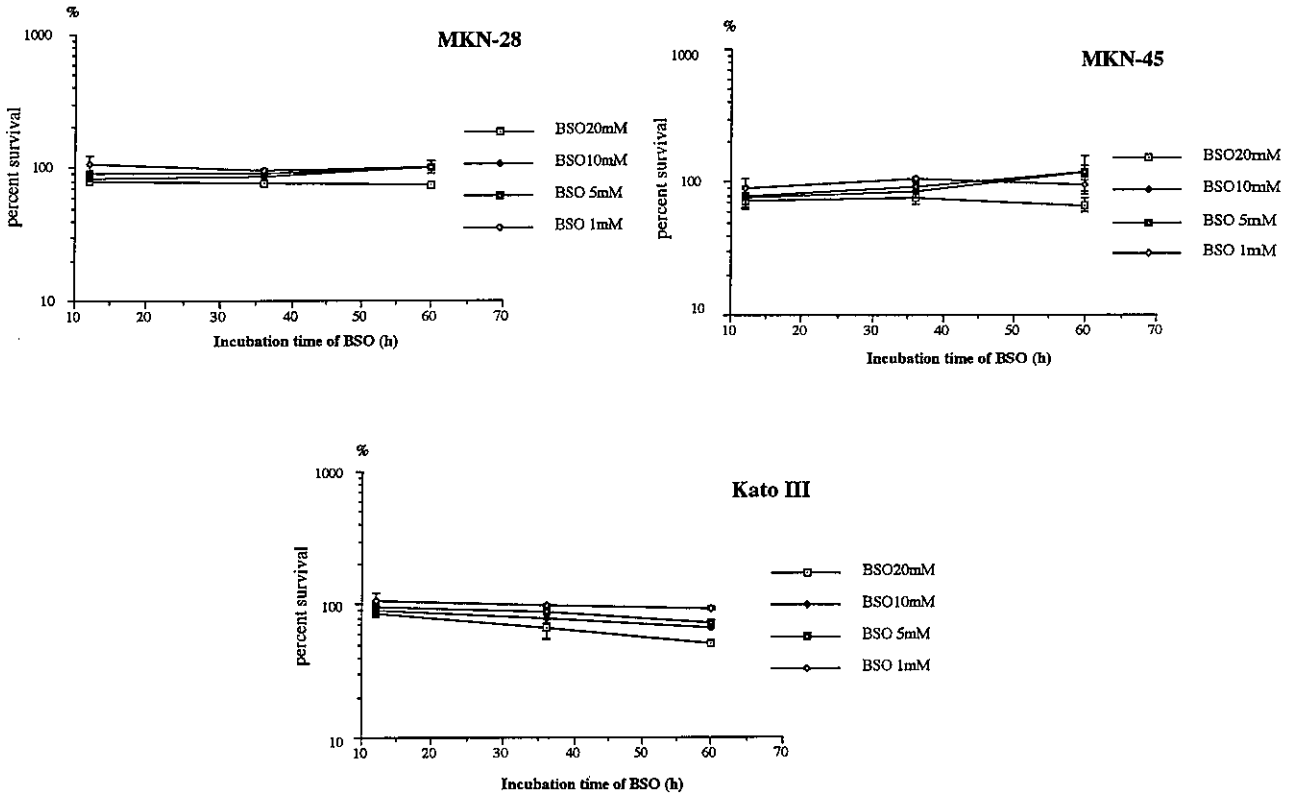

Fig. 1. *In vitro* antitumor activity of buthionine sulfoximine alone. After 24-h incubation of 10,000 tumor cells in the control medium, BSO was added for 12, 36 and 60 h at concentrations of 1, 5, 10, and 20 m*M*. The activity of BSO against these cell lines was limited, and no marked time dependency was observed in the antitumor activity of BSO alone.

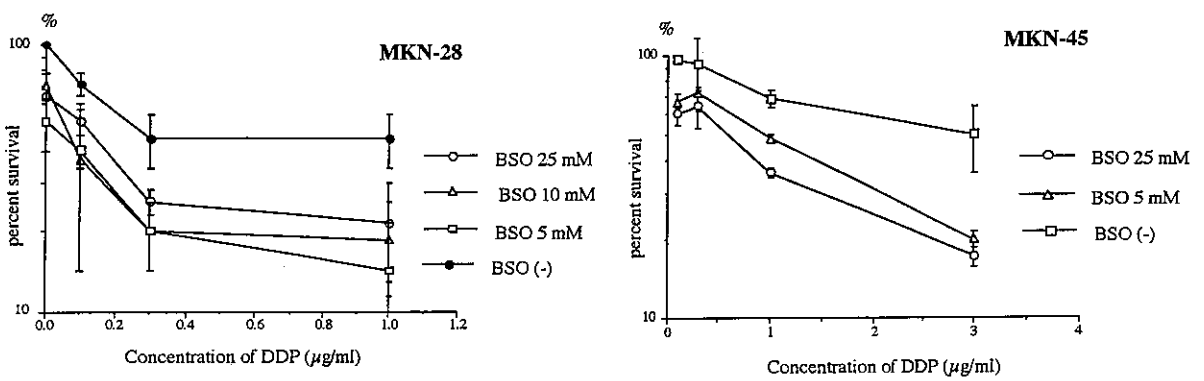

Fig. 2. Enhancement of cisplatin antitumor activity against gastric cancer cell lines *in vitro* by pretreatment with BSO. After 24-h preincubation of 10,000 tumor cells in the control medium, they were incubated with BSO for 24 h followed by 48-h incubation with DDP. All concentrations of BSO significantly enhanced the inhibition rate of DDP ($P<0.05$).

trations of the drug by pretreatment with BSO ($P<0.05$ for all concentrations of BSO) (Fig. 2). The concentration of BSO did not appear to correlate with the enhanced antitumor effect of DDP it induced. The antitumor activities for each of the three schedules against MKN-28 and MKN-45 are shown in Fig. 3. The sequence of BSO followed by DDP showed the highest antitumor activity among these treatment regimens

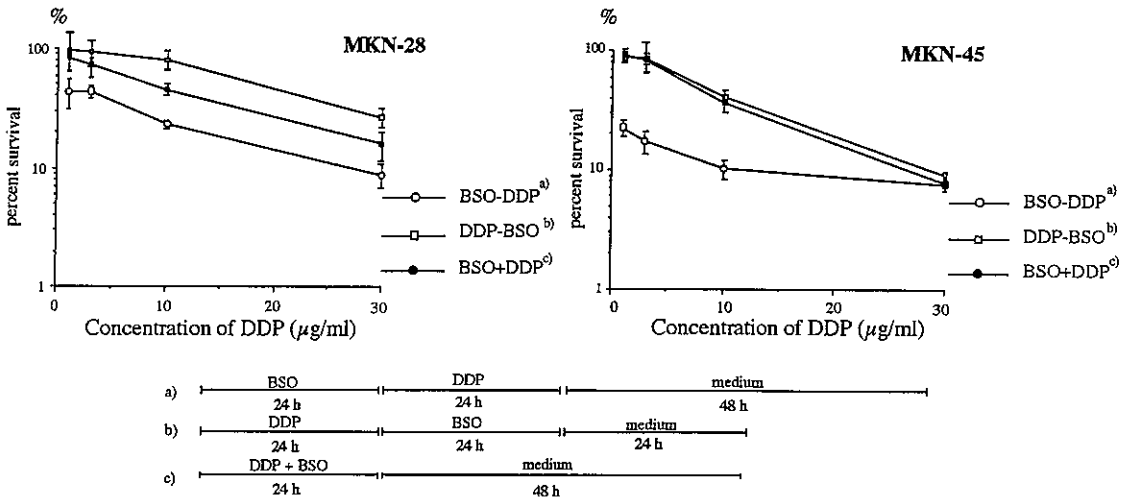

Fig. 3. Schedule-dependent combination antitumor activity of BSO and DDP. All tumor cells were preincubated in control medium for 24 h, followed by treatments according to the protocol shown in the figure. In group (a), the cells were incubated with BSO for 24 h, washed twice, incubated with DDP for 24 h, then washed again and incubated in control medium for 48 h (○). In group (b), the cells were incubated with DDP for 24 h, washed twice, and incubated with BSO followed by incubation in the control medium for 24 h (□). The cells in group (c) were incubated with both DDP and BSO simultaneously for 24 h (●). The concentration of BSO was 5 m*M* and the concentration of DDP was 1, 3, 10 or 30 μg/ml. Group (a) showed the highest antitumor activity among the three groups at every concentration of DDP (significant at $P<0.01$).

Table I. Enhancement of Antitumor Activity of Cisplatin by Pretreatment with BSO against Human Gastric Cancer Xenografts, St-15 and SC-1-NU

Tumor	Group	No. of mice	T/C (%)[a]	Tumor wt. (mg)	{T/C (%)}[b]	Body wt. loss[h]	T/C (spleen wt.)[i]
St-15	Control	5		810.9±595.9			
	BSO alone[c]	5	86.59	1118.3±1292.0	(121.77)	4.5	106.1
	DDP 6 mg/kg[d]	5	22.80	194.8±107.1*	(23.84)	11.1	99.5
	BSO+DDP[e]	5	11.26	47.8±24.7**	(6.42)	11.1	106.8
SC-1-NU	Control	4		5306.2±2630			
	BSO alone	4	101.21	5884.6±1855.5	(110.90)	4.5	106.1
	DDP 9 mg/kg[f]	4	56.32	4136.2±1453.4	(77.95)	6.0	85.3
	BSO+DDP[g]	4	31.50	1410.9±664.4**	(26.59)	17.0	54.0

a) The lowest T/C ratio during the experiment.
b) Actual tumor weight (T/C ratio) at the end of the experiment.
c) BSO at a dose of 500 mg/kg was administered intraperitoneally on days 1, 2 and 3.
d) DDP at a dose of 6 mg/kg was administered intraperitoneally on day 2.
e) *c*)+*d*).
f) DDP at a dose of 9 mg/kg was administered intraperitoneally on day 2.
g) *c*)+*f*).
h) The maximum body weight loss with respect to initial body weight (%) during experiment.
i) T/C ratio of spleen weight at the end of the experiment.
* Statistically significant difference from control at $P<0.05$. ** Statistically significant difference from control at $P<0.01$.
No mice died during the experiment.
The underline indicates synergism of the combination therapy of DDP and BSO. St-15: T/C of BSO+DDP (11.26%) < (T/C of BSO) × (T/C of DDP)(20.20%). SC-1-NU: T/C of BSO+DDP (31.50%) < (T/C of BSO) × (T/C of DDP)(57.00%).

against both cell lines for all the concentrations of DDP tested, with statistically significant differences at $P<0.01$, suggesting that BSO modulates antitumor activity of DDP against these cell lines.

Antitumor activity *in vivo* The antitumor activities of DDP and/or BSO against St-15 and SC-1-NU *in vivo* are shown in Table I. The lowest T/C ratios of BSO alone, DDP alone and DDP with BSO against St-15 were

Table II. Depletion of Total Intracellular Glutathione by BSO in MKN-45 *in vitro*

Incubation time (h)[a]	Total GSH (ng/10^7 cells)[b]
0	0.456 ± 0.073[c]
24	0.079 ± 0.021 *
48	undetectable

a) The cells were incubated control medium containing BSO at a concentration of 25 m*M* for 24 and 48 h.
b) Total intracellular GSH per 10^7 cells was assessed by DTNB-GSH reductase reaction after trypsinization of the cells.
c) Each value was obtained in triplicate, and the result is shown as mean±standard deviation.
* Statistically significant difference ($P<0.05$).

86.59%, 22.80% and 11.26%, respectively, and those against SC-1-NU were 101.21%, 56.32% and 31.56%. Although the antitumor activity of BSO alone was negligible, the antitumor effect of DDP was increased significantly by combined treatment with BSO, showing a synergistic effect against both xenografts.

***In vivo* toxicity** No death and no body weight loss in excess of 20% were encountered during these experiments when the mice were treated with 9 or 6 mg/kg DDP, which are equivalent to the maximum tolerated dose (MTD) and two-thirds of the MTD, respectively, and with BSO (500 mg/kg) in a schedule of qd×3 (Table I). We determined the spleen weights on completion of the experimens to assess the myelotoxicity of DDP and/or BSO. Cisplatin (6 mg/kg) with BSO showed no significant myelotoxic side effects (spleen weight T/C ratio of 106.8%), although DDP (9 mg/kg) with BSO suppressed spleen weight significantly (T/C ratio, 54.0%, Table I).

BSO-induced depletion of tumor tissue GSH The depletion of total intracellular GSH by BSO *in vitro* is shown in Table II, revealing 17.3% depletion of intracellular GSH by incubation with 25 m*M* BSO for 24 h, with a significant difference between the control group and the treated group. In the *in vivo* study, the mean and SD of GSH concentrations in SC-1-NU in the control and BSO-treated groups were 0.0437 ± 0.002 and 0.0011 ± 0.0004 mg/g wet tumor tissue, respectively, the difference between the two groups being significant ($P<0.01$). Therefore, we concluded that BSO treatment resulted in GSH depletion *in vitro* and *in vivo*.

DISCUSSION

BSO, an agent that depletes cellular GSH,[1,2] has been used in many studies on the roles of GSH, which detoxifies xenobiotic compounds, such as lead. Recently, it has been reported that the effects of antitumor drug, including methotrexate, cyclophosphamide and doxorubicin, are enhanced when these drugs are given in combination with BSO.[13-16] With regard to the combination of BSO with DDP, Meister[3] reported many and varied cellular functions of GSH and various toxicities arising from GSH depletion by BSO, and Ozols *et al.* observed an increase in the antitumor activity of DDP in the presence of BSO *in vitro*.[5] Furthermore, enhancement of the antitumor activity of DDP against *in vivo* cell lines by BSO was reported by Arrick and Nathan.[4] As the chemical structure of BSO resembles that of glutamate, BSO may antagonize γ-glutamylcysteine synthetase, an enzyme which regulates GSH synthesis, thereby resulting in intracellular GSH depletion. This intracellular GSH depletion is believed to be one of the mechanisms by which BSO increases the antitumor activity of anticancer drugs. Reducing the detoxifying action of GSH would be expected to increase the sensitivity of tumor cells to antitumor drugs. However, Kang and Enger have suggested that the magnitude of BSO-induced intracellular GSH depletion does not correlate with that of BSO-enhanced antitumor activity.[17] These discrepancies might be due to different mechanisms of sensitivity and resistance to DDP in the various cell lines used. Therefore the mechanism whereby BSO increases the antitumor activity of these agents is still controversial.

As the results of a recent study suggested that the drug resistance of malignant cells is attributable to high intracellular GSH concentrations, BSO may be useful for increasing the sensitivity of tumor cells that have acquired resistance to antitumor agents.[5] However GSH can account for only part of the resistance of tumor cells, since Richon *et al.* have reported that the accumulation of DDP in tumor cells and the processing after the platination of DNA represent the dominant mechanism of DDP resistance in the L1210 cell line, while the decrease of GSH by BSO does not sensitize DDP-resistant L1210,[18] and a decreased influx of DDP has also been observed in DDP-resistant L1210 cells by Hromas *et al.*[19]

In this study, we confirmed that the antitumor activity of DDP was enhanced by BSO *in vitro* and *in vivo* with little concomitant increase in toxicity, except for the myelotoxicity observed at the MTD of DDP plus BSO. We have also demonstrated that BSO can reduce tumor intracellular GSH levels, suggesting that the BSO-enhanced antitumor activity of DDP is associated with the depletion of intracellular GSH induced by BSO. If such glutathione depletion occurs in normal cells, BSO may also enhance the cytotoxicity of antitumor drugs on normal cells as well as on tumor cells. This has been reported to occur in DDP-induced renal failure, MMC-induced pancytopenia and cardiotoxicity caused by

doxorubicin.[20-24] The slight increment of toxicity with BSO observed in this study compared with the magnitude of its enhancement of DDP cytotoxicity may be attributable to differences of GSH concentrations and metabolism in normal and tumor cells.

The present study focused on the increase in antitumor activity of DDP induced by BSO against human gastric cancer xenografts, including its toxic effect on tumor-bearing nude mice. DDP is one of the key drugs used clinically against gastric cancers, and many attempts have been made to increase its antitumor activity and reduce its nephrotoxicity and emetic effects. The combination of DDP and BSO would be useful for treatment of gastric cancer, producing higher antitumor activity without a significant increment of toxicity. Thus, the clinical use of BSO may prove advantageous for combination chemotherapy with the conventional antitumor agents available at present, although careful observation of BSO-enhanced side effects may be necessary.

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