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Inhibition of cisplatin-induced nephrotoxicity in rats by buthionine sulfoximine, a glutathione synthesis inhibitor

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Summary. DL-Buthionine-(*S,R*)-sulfoximine (BSO), a glutathione-depleting agent, was found to diminish the nephrotoxic effect of cisplatin (*cis*-diamminedichloroplatinum). Pretreatment of rats with BSO (4 mmol/kg s.c.) 2 h prior to cisplatin, either as a single dose of 5 mg/kg or at a daily dose of 2.5 mg/kg for 3 consecutive days, resulted in diminished elevations of plasma BUN concentration and decreased cisplatin-induced inhibition of renal γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase activity measured 6 days following treatment. Administration of BSO prior to cisplatin at 7.5 mg/kg did not significantly alter the effect of cisplatin on either BUN concentration or enzyme activity. The influence of BSO pretreatment on the antitumor activity of cisplatin was studied using implantation of a murine bladder cancer (MBT-2) in C3H mice. Pretreatment of mice with BSO (5 mmol/kg) did not influence cisplatin antitumor efficacy.

Introduction

Glutathione (GSH), a major nonprotein cellular thiol, is a substrate in a number of important reactions including cellular protection against both endogenous and exogenous toxic compounds [14]. Recently, reduction of GSH levels in tumor cells has been explored as a means of enhancing the cytotoxic effects of chemotherapeutic agents. Treatment with buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, has been reported to enhance the in vitro antitumor efficacy of a number of drugs including cisplatin [7], melphalan [7], and estramustine [22]. In vivo sensitization of tumors by thiol-depleting agents is accompanied by a decline in the GSH content of normal tissues as well [23]. Enhanced host toxicity may occur when systemic GSH depletion is combined with drugs such as doxorubicin [3] or acetominophen [16], since the adverse side effects of these drugs are caused in part by reducing GSH content in the target organs.

The effects of decreasing cellular thiols in vivo on either tumor sensitivity to cisplatin or its associated nephrotoxicity have not been extensively studied. Cisplatin nephrotoxicity does not appear to be mediated by a consumption of renal GSH [9, 10, 13]. However, coincidental depletion of GSH may still augment cisplatin renal toxicity if the thiol is required to play a significant role in renal cellular defenses.

Having noted that a variety of exogenous sulfur compounds such as sodium thiosulfate [17] can protect the kidney against cisplatin, Litterst et al. have recently examined the potential influence of GSH and other endogenous thiols on cisplatin toxicity [11]. They reported that stimulation of renal metallothionein levels by $CdCl_2$ was associated with improved survival after cisplatin treatment, but not with significant protection of renal function as measured by BUN. However, significant increases in both mortality and elevation of BUN were observed in animals in which cisplatin treatment followed depletion of renal GSH by diethylmaleate (DEM).

Our studies were designed to investigate whether glutathione depletion following BSO administration may alter cisplatin nephrotoxicity or the antitumor effect of cisplatin. BSO is a transient specific inhibitor of γ-glutamyl cysteine synthetase (GGS) [19], the rate-limiting enzyme of GSH synthesis in the γ-glutamyl cycle. The activities of GGS as well as γ-glutamyl transpeptidase (GGT) are also inhibited following cisplatin [13], and it was of interest to determine whether this effect would be influenced by BSO treatment. Compared with DEM, BSO has fewer toxic side effects [2, 4, 18, 20] and is therefore a more acceptable agent for in vivo studies or for potential clinical use.

Materials and methods

Animals. Male Sprague-Dawley rats 220–260 g, and C3H/He female mice all weighing 18–20 g were obtained from Charles River Laboratory, Pittsburg, Pa. They were housed in the University vivarium and provided with standard rodent chow and water ad libitum.

Drugs and chemicals. All drugs and chemicals were obtained through Sigma Chemical Company, St. Louis, Mo. Cisplatin was dissolved in 0.9% NaCl at 0.5 mg/ml. DL-Buthionine-(*S,R*)-sulfoximine (BSO) was dissolved in acidified 0.9% NaCl and subsequently readjusted to pH 7.0 with NaOH to obtain a 400-m*M* solution.

Experimental protocol. Rats were treated s.c. with saline or BSO at 4 mmol/kg 2 h prior to the cisplatin injection. Cisplatin was administered i.p. (or i.v.) at 5 or 7.5 mg/kg once, or i.p. at 2.5 mg/kg per day for 3 consecutive days. Blood for BUN was obtained from animals on day 3 and/

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or day 6. The activities of renal GGS and GGT were determined on day 6. Reported inhibition of these enzymes was not noted until 3 days following cisplatin treatment and became more prominent after 7 days [13]. A subset of rats was sacrificed 2 h following BSO or saline administration and renal GSH content was measured.

Assay procedures. For enzymatic assays, the kidneys were homogenized in a buffer consisting of KH_2PO_4 (20 m*M*), Na_2 EDTA (0.10 m*M*) and KCl (135 m*M*), pH 7.4. The tissue homogenate was used for determination of GGT. The cytosolic fraction for determination of GGS activity was prepared by differential centrifugation as follows: the homogenate was centrifuged at 10000 *g* for 20 min and the resulting supernatant centrifuged at 150000*g* for 1 h. Renal tissue for determination of GSH was homogenized in an ice-cold extraction mixture containing 10% trichloroacetic acid/1 m*M* Na_2 EDTA/0.01 *N* HCl (1/1/1, by volume). Protein was removed by centrifugation at 5000 *g* for 10 min.

GSH was measured fluorometrically based on the method of Cohn and Lyle [1]. Following homogenization of the tissue as described above, a 50-µl aliquot containing sample supernatant was added to a 1.0-ml mixture of 0.1 *M* Na HPO4 and 100 ul *o*-phthalaldehyde in methanol (1 mg/ml). GSH concentration was measured using exitation and emission wavelengths of 328 and 430 nm, respectively. Authentic GSH was used as the standard.

GGS activity was measured by the method of Sekura and Meister [19]. The reaction mixture (1.0 ml) contained: NaATP (5 m*M*), $MgCl_2$ (20 m*M*), L-glutamate (10 m*M*), L-α-aminobutyrate (10 m*M*), EDTA (1 m*M*), bovine serum albumin (0.10 mg), Tris-HCl buffer (10 m*M*, pH 8.2). The reaction was initiated by adding the enzyme preparation. The mixture was incubated for 30 min at 37° C. The reaction was terminated by the addition of 1 ml 10% trichloroacetic acid. Thereafter, the sample was centrifuged at 5000 *g* for 10 min to remove protein. Pi liberated was measured in the supernatant fraction by the method of Taussky and Shorr [21].

GGT activity was measured by the method of Meister [15]. The sample mixture contained the enzyme source (20–50 µg protein), glycylglycine (20 m*M*), and Tris-HCl buffer (0.05 *M*, pH 8.0). The reaction was started by the addition of L-γ-glutamyl-*p*-nitroanilide (1 m*M*), and the rate of formation of *p*-nitroanilide was measured at 410 nm.

Protein concentrations were measured by the method of Lowry et al. [12]. BUN levels were measured colorimetrically following liberation of ammonia by urease using a diagnostic kit from Sigma Chemical Co.

In vivo chemotherapy. The tumor cells utilized were an FANTF-induced undiffereniated transitional cell carcinoma (MBT-2) [8]. The influence of BSO treatment on the antitumor effect of cisplatin was assessed following implantation of MBT-2 tumor cells i.d. or beneath the renal capsule. For i.d. tumors, 5×10^5 viable MBT-2 cells were implanted into the superficial skin of each flank. The renal subcapsular implantation was accomplished by making a 1-cm vertical incision on the lt flank and exposing the peritoneum. Then 2×10^5 viable tumor cells in 50 µl were injected subcapsularly with a 27-G needle through the intact peritoneum. Following tumor inoculation, mice were randomized into treatment groups consisting of five to eight mice. Cisplatin (2.5–6 mg/kg) or 0.9% NaCl was given i.p. three times at 4-day intervals beginning on day 3. BSO (5 mmol/kg) or 0.9% NaCl was given s.c. 2 or 4–6 h prior to cisplatin administration. Intradermal tumor weight (TW) in milligrams was estimated by the following formula: $TW = 0.5 \times W^2 \times L$, where W is width and L is perpendicular length of tumor nodules in millimeters. The weights of renal subcapsular MBT-2 tumors were determined on day 13.

Statistical analysis. Results are reported as means ± SD. Data were compared with a Student's *t*-test, and a probability value of less than 0.05 was used to denote significance.

Table 1. Effect of BSO treatment on cisplatin-induced nephrotoxicity and body weight loss

Treatment (mg/kg)	BUN (mg/dl)		Body weight change (% of weight on day 0)
	Day 3	Day 6	Day 6
Control	22± 5	19± 3	+10
BSO	–	16± 1	+11
BSO 3X	–	18± 2	+ 5
DDP (5)	51±10 (i.v.)*[a]	56± 20*[a]	− 5*[a]
BSO + DDP (5)	26± 5 (i.v.)*[a, b]	25± 4*[a, b]	0*[a]
DDP (2.5 × 3)	34± 4*[a]	73± 28*[a]	−17*[a]
BSO + DDP (2.5 × 3)	27± 3*[a, b]	42± 17*[a, b]	−15*[a]
DDP (7.5)	60±10*[a]	216± 82*[a]	−22*[a]
BSO + DDP (7.5)	75±28*[a]	146±100*[a]	−18*[a]

Data were obtained from 5 separate experiments: the treatments with cisplatin (DDP) alone and BSO + DDP at the identical dose carried out simultaneously. Each treatment group consisted of 3–5 rats. BUN values and body weight change in controls are presented as the mean (±SD) of 12 animals from 3 separate experiments. BSO at 4 mmol/kg (or 0.9% NaCl) was given s.c. 2 h prior to DDP; DDP was given i.p. (or i.v. in one experiment) at 5 or 7.5 mg/kg once, or i.p. at 2.5 mg/kg daily for 3 consecutive days. Plasma BUN and body weight were determined 3 and/or 6 days following initial treatment

*$P < 0.05$; [a] control vs treatment; [b] DDP vs DDP plus BSO

Table 2. Effect of BSO and cisplatin on activities of renal γ-glutamylcysteine synthetase and γ-glutamyltranspeptidase

Treatment (mg/kg)	γ-Glutamylcysteine synthetase (% of control activity)	γ-Glutamyltranspeptidase (% of control activity)
BSO	90 ± 11	104 ± 2
DDP (5)	43 ± 14*[a]	52 ± 7*[a]
BSO + DDP (5)	96 ± 19*[b]	93 ± 7*[b]
DDP (2.5 × 3)	44 ± 6*[a]	61 ± 11*[a]
BSO + DDP (2.5 × 3)	70 ± 12*[a, b]	93 ± 8*[b]
DDP (7.5)	30 ± 2*[a]	60 ± 2*[a]
BSO + DDP (7.5)	25 ± 10*[a]	51 ± 19*[a]

The animals treated with cisplatin (DDP) i.p. and/or BSO as described in the legend to Table 1 were used. The kidneys were removed on day 6 and used for the estimation of enzyme activities. Assay procedures are described in detail in the Materials and Methods section. Control values for γ-glutamylcysteine synthetase were 57 ± 7 nmol Pi/min per mg protein and for γ-glutamyltranspeptidase were 1.15 ± 0.18 mmol product formed/min per mg protein. The values are means ± SD of 3–5 determinations; 1 rat was used for each determination

*$P < 0.05$; [a] treatment vs control values; [b] DDP vs DDP + BSO

Results

Renal GSH concentrations 2 h after BSO treatment were decreased to 47% of control values (316 ± 22 vs 678 ± 48 μg/g wet tissue weight, $n = 4$ in each group).

Table 1 shows the effect of BSO treatment on cisplatin-induced nephrotoxicity and changes in body weight. BUN levels of rats treated with BSO and cisplatin at either 5 mg/kg or 2.5 mg/kg × 3 were significantly lower than in animals receiving the identical dose of cisplatin and saline. Renal function in animals treated with BSO prior to cisplatin at 5 mg/kg remained nearly normal, whereas such pretreatment did not protect the animals against a higher dose of cisplatin (7.5 mg/kg). Animals lost body weight following cisplatin injection in a dose-dependent manner which was not significantly influenced by BSO administration.

Renal GGS and GGT activities following treatment are shown in Table 2. In rats treated with a lower dose of cisplatin (5 or 2.5 mg/kg × 3), the activity of GGS was inhibited by 56%–57% and that of GGT by 39%–48% of control values. BSO pretreatment resulted in restoration of GGS activity to 70%–96% of control values and nearly complete preservation of GGT activity. BSO pretreatment did not significantly alter the effect of cisplatin at 7.5 mg/kg on the activities of either GGS or GGT.

Figure 1 and Table 3 show the influence of BSO on the antitumor efficacy of cisplatin in i.d. and renal subcapsular MBT-2 tumors in mice. BSO treatment did not inhibit or enhance the effect of cisplatin against MBT-2 tumors.

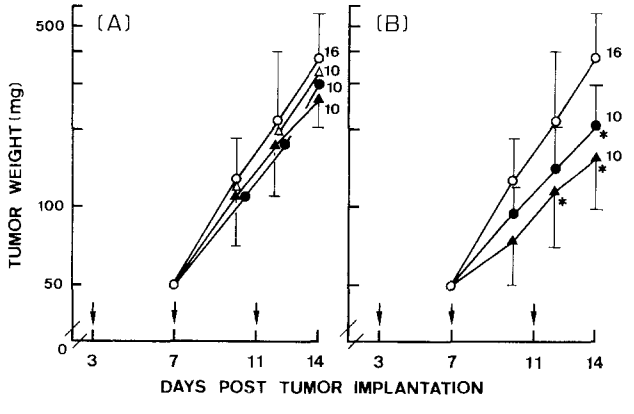

Fig. 1 A, B. Effect of BSO on antitumor effect of cisplatin. Mice were treated with saline (○), BSO alone (△), cisplatin alone (●), or both in combination (▲). Cisplatin was given i.p. at 2.5 **(A)** or 5.0 mg/kg **(B)** on days 3, 7, and 11. BSO was given s.c. at 5 mmol/kg 4–6 h prior to cisplatin. *, $p < 0.05$; cisplatin vs control, cisplatin + BSO vs control. Numbers on curves represent number of tumors in each group

Discussion

In this study, BSO was utilized to evaluate the effects of GSH depletion at the time of cisplatin administration. The activities of GGS and GGT were measured as a parameter of cisplatin toxicity on cellular function. Administration of BSO, a transient inhibitor of GGS, protected against the expected subsequent decline in activity of both GGS and GGT following cisplatin (Table 2). More importantly, global renal function as measured by BUN was also preserved (Table 1). These beneficial effects were not noted following cisplatin at a dose of 7.5 mg/kg as a single injection.

These studies contrast with a study by Litterst et al. [11], who reported increased cisplatin nephrotoxicity when DEM was used to reduce GSH levels. They suggested that depletion of renal GSH concentrations following DEM treatment compromised cellular defenses which resulted in enhanced cisplatin toxicity. Our data demonstrate that BSO treatment in rats significantly decreased the toxic ef-

Table 3. Effect of BSO and cisplatin on renal subcapsular MBT-2 tumors in mice

Treatment	n	Tumor weight (mg)
Control	5	579 ± 269
DDP (6 mg/kg)	5	317 ± 124
BSO + DDP (6 mg/kg)	5	219 ± 39*

Following renal subcapsular implantation of MBT-2 tumor cells mice were treated with saline, cisplatin or cisplatin and BSO. BSO (5 mmol/kg) was given s.c. 2 h before cisplatin injections i.p. on days 3, 7 and 11. Tumor weights were determined at sacrifice on day 13

*$P < 0.05$ (vs control value)

fects of cisplatin (Table 1, Table 2), even though renal GSH content was substantially suppressed at the time of cisplatin administration. This suggests that renal GSH concentration may not be a critical factor in the expression of cisplatin nephrotoxicity.

The apparent discrepancy between the data of Litterst's group [11] and our own findings regarding the effect of GSH depletion on cisplatin nephrotoxicity may reflect the chemical nature and/or action of the GSH-depleting agent used. DEM and BSO differ in the manner by which cellular GSH is depleted. In contrast to BSO, DEM rapidly stimulates GSH consumption and may also decrease stores of other intracellular thiols [5]. DEM is known to cause other systemic effects including suppression of protein synthesis and is considered more toxic than BSO [2, 4, 20]. Studies with rabbit proximal tubules in vitro have also shown BSO to be nontoxic and perhaps beneficial to cell function, while DEM was noted to have detrimental effects at higher concentrations [18].

The mechanism by which BSO treatment reduces cisplatin nephrotoxicity is unknown. Our experiments with tumored mice show prior treatment with BSO did not alter the chemotherapeutic effect of cisplatin; this suggests that BSO did not inactivate cisplatin in vivo. Maines speculated that rapid turnover of GSH by the γ-glutamyl cycle may contribute to the renal toxicity of cisplatin [13]. It is possible, then, that BSO may alter GSH metabolism in such a way as to inhibit cisplatin uptake into the kidney or formation of nephrotoxic metabolites [13]. Alternatively, BSO may protect cells by a mechanism distinct from its ability to deplete GSH, as was suggested by the studies with rabbit tubular cells [18]. It is apparent that the influence of GSH on cisplatin nephrotoxicity remains in question and further studies will be required to elucidate the protective action of BSO.

Our studies with tumored mice were conducted to determine not only whether BSO might suppress cisplatin cytotoxicity, but also whether such treatment might increase the therapeutic effect. The latter possibility was raised by the reports of enhanced chemosensitivity following depletion of cellular GSH in tumor cells [7, 22, 23]. In our experiments, GSH content in i.d. MBT-2 tumors was significantly reduced (50%–60% of control values) 4–8 h after BSO administration but not after 2 h (unpublished data). Cisplatin was therefore administered 4–6 h following BSO in an attempt to detect any enhancement of antitumor efficacy (Fig. 1). The results indicate that GSH depletion in MBT-2 tumor cells at the time of cisplatin administration does not increase detectable chemosensitivity.

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