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LABORATORY STUDY

Betamipron Reduces Cisplatin Nephrotoxicity in Rodents Without Modifying Its Antileukemic Activity in Mice

Jin Tokunaga, MS, Mie Kobayashi, BS,
Chizuko Nakamura, BS, Akira Kitagawa, MS,
Kazuhiko Arimori, PhD,
and Masahiro Nakano, PhD

Department of Pharmaceutical Services
Kumamoto University Hospital
Kumamoto, Japan

ABSTRACT

Protective effects of betamipron (BP, N-benzoyl-β-alanine), one of a series of N-acyl amino acids, on cisplatin-induced nephrotoxicity were examined. Since the damage observed in the kidney is localized to the proximal tubule cells, we investigated the influence of BP on urinary enzymes and excreta. Male Wistar rats and ddY mice were injected i.p. with 6 mg/kg and 16 mg/kg, respectively, of cisplatin combined with an i.p. 250 mg/kg BP dose. The toxicity of cisplatin as indicated by body weight gain, blood urea nitrogen, and serum creatinine levels was significantly ($p < 0.05$) suppressed by administration of BP after cisplatin treatment. The increase in urinary N-acetyl-β-D-glucosaminidase activity, increase and subsequent decrease in γ-glutamyl transferase activities, and increase in β_2-microglobulin level observed after treatment with cisplatin were suppressed by administration of BP after cisplatin

Address correspondence to: Dr. Masahiro Nakano, Department of Pharmaceutical Services, Kumamoto University Hospital, 1-1-1 Honjo, Kumamoto 860, Japan. Fax: +81-96-373-5906; E-mail: nakano@kaiju.medic.kumamoto-u.ac.jp

treatment. The combination of cisplatin and BP had no apparent effect on the efficacy of cisplatin against P388 leukemic cells in mice.

Key Words: Antileukemic activity; Betamipron; *N*-Benzoyl-β-alanine; Cisplatin; Nephrotoxicity.

INTRODUCTION

The clinical usefulness of *cis*-diamminedichloroplatinum(II) (cisplatin) in cancer treatment is limited by the occurrence of various adverse effects including gastrointestinal toxicity, bone marrow toxicity, neurotoxicity, and ototoxicity as well as a cumulative and dose-dependent nephrotoxicity (1–3). It is also generally accepted that high doses of cisplatin are more effective than low doses against various neoplasms, particularly tumors of the testis, ovaries, bladder, head, and neck (4,5). Thus, to try to control adverse effects induced by cisplatin, particularly the dose-limiting renal toxic effect, many different compounds have been tested. Unfortunately, in most studies the reduction in toxic effects by these antidotes has often been accompanied by a reduction in antitumor activity (6,7) so that an improvement in therapeutic index is not achieved.

The intrinsic function of the kidney as an excretory organ results in its inevitable exposure to high concentrations of endogenous and exogenous compounds that might induce nephrotoxicity due to direct action on the renal cells. A high intracellular concentration of cisplatin has been considered to be closely related to its nephrotoxicity, and therefore other compounds that have strong inhibiting activities against tubular transport of cisplatin have been investigated in a recent paper (8). It has already been reported on probenecid that an organic anion transport inhibitor prevents cephalosporin- and cisplatin-induced nephrotoxicity (9–12). However, since probenecid causes gastrointestinal reactions, headache, and hypersensitivity reactions, clinically useful modalities are limited, excluding the treatment of gout. In similar findings, betamipron (BP, *N*-benzoyl-β-alanine), one of a series of *N*-acyl amino acids, decreased dose dependently the degree of renal toxicity caused by carbapenem and cephalosporin antibiotics by the same mechanisms that inhibited active transport of organic anion (13,14). BP should be suitable for clinical use because of its generally low toxicity in the animals [e.g., LD_{50} was more than 3000 mg/kg i.v. in rats (15,16)]. BP has been used clinically to protect against panipenem-induced nephrotoxicity as in an injectable formulation of panipenem in Japan.

In a previous paper (17) we demonstrated that BP reduced the renal toxicity of cisplatin, and histological analysis of the kidneys confirmed the protective effect using blood urea nitrogen (BUN) and serum creatinine (serum Cr) levels as the indicators of nephrotoxicity. The damage observed in the kidney is localized to the proximal tubule cells (18,19). To test the protective effect of BP on the proximal tubule cells, we investigated the influence on urinary enzymes and excreta in the normal rats, using urinary *N*-acetyl-β-D-glucosaminidase (NAG) and γ-glutamyl transferase (γ-GT) levels as the indicators of damage to the proximal tubule cells; and BUN, serum Cr, urinary creatinine (urine Cr), and β_2-microglobulin (β_2-M) levels as the indicators of damage to the glomerulus. In addition, the effect of BP on antitumor activity of cisplatin in mice bearing P388 leukemic cells was examined.

MATERIALS AND METHODS

Drugs

Cisplatin was kindly supplied by Nippon Kayaku Co. (Tokyo, Japan). BP was purchased from Tokyo Kasei Ind. Co. (Tokyo, Japan). Cisplatin and BP were dissolved in isotonic saline (0.9% NaCl; Terumo Co., Tokyo, Japan) at 1 mg/mL and alkaline solution (pH 9) at 25 mg/mL, respectively (13). Alkaline soln. at pH 9 was prepared by addition of 1 N HCl soln. to 1 N NaOH soln. They were sterilized by filtration through a 0.22-μm filter unit (Nippon Millipore Co., Tokyo, Japan) within 3 h of injection.

Toxicity Studies in Rodents

Male Wistar rats (235–365 g) and ddY mice (25–32 g) were obtained from Kyudo, Co. (Tosu, Japan), and acclimatized for at least 1 week before the experiments. The animals were maintained on a 12-h light/dark cycle and the temperature range of the animal care facilities was 23°–26°C. Food and water were taken ad libitum.

The rats were randomly divided into 3 groups of 6 rats each and housed individually in metabolic cages, and injected i.p. with the following combinations of drugs: normal saline (6 mL/kg body weight); cisplatin soln. (6 mL/kg, 6 mg/kg body weight); BP (10 mL/kg, 250 mg/kg body weight) 1 h after cisplatin soln. (6 mL/kg, 6 mg/kg body weight). As reported in a previous paper (17), the treatment with cisplatin followed 1 h later with BP gave the most effective suppression in the changes of body weight, BUN, and serum Cr levels among various administration schedules of BP; therefore, the administration time was fixed at 1 h after cisplatin treatment. A blood sample (0.5 mL) was taken from the tail vein on day 5, and a urine sample was collected in a metabolic cage for 24 h following the various treatments. Seven days later, the animals were weighed and sacrificed. The renal toxicity was evaluated by NAG and γ-GT activities as the indicators of damage to the proximal tubule cells; and BUN, serum Cr, urine Cr, and β_2-M levels as the indicators of damage to the glomerulus. Water consumption, volume and pH of urine, NAG, γ-GT, β_2-M, and urine Cr were measured on days 0, 1, 3, and 7. NAG, γ-GT, and β_2-M levels were indicated in the following way: NAG (U/day)/urine Cr (mg/day) = NAG (U/mg Cr); γ-GT (IU/day)/urine Cr (mg/day) = γ-GT (IU/mg Cr); and β_2-M (ng/day)/urine Cr (mg/day) = β_2-M (ng/mg Cr), respectively. BUN and serum Cr levels were measured on day 5. Because Ward et al. (20) indicated that BUN level reached its peak on day 5 after cisplatin administration, measurement of BUN levels was made on day 5.

The mice were randomly divided into 3 groups of 3 mice each and housed in cages, and injected i.p. with the following combinations of drugs: alkaline soln. (10 mL/kg body weight) 1 h after normal saline (16 mL/kg body weight); alkaline soln. (10 mL/kg body weight) 1 h after cisplatin soln. (16 mL/kg, 16 mg/kg body weight); BP (10 mL/kg, 250 mg/kg body weight) 1 h after cisplatin soln. (16 mL/kg, 16 mg/kg body weight). Five days later, a blood sample (0.5 mL) was obtained by heart puncture and the animals were weighed and sacrificed. The renal toxicity was evaluated by BUN levels.

Urinary enzyme activities and excreta levels were measured colorimetrically using commercially available kits: Creatinine-test Wako (Wako Pure Chemical Ind., Osaka, Japan), Meiassay NAG (Sanko Pure Chemical Co., Tokyo, Japan), γ-GTP C-test Wako (Wako Pure Chemical Ind.), and Imzyme β_2-M (Fujirebio Co., Tokyo, Japan). BUN and

serum Cr levels were measured in serum colorimetrically using commercially available kits: Urea-N-Blood-test Wako and Creatinine-test Wako (Wako Pure Chemical Ind.). Urinary samples were filtered through a paper disk (Whatman Int. Co., Maidstone, England) and adjusted to pH 7 with 1 N NaOH soln. Samples were stored at −20°C except for urine for γ-GT measurement (4°C).

Effects of BP and Cisplatin Treatments on Survival Time in Leukemic Mice

C57BL/6 × DBA/2 hybrid male mice (BDF1), aged 5 weeks and weighing 21–24.5 g (Charles River Laboratories, Yokohama, Japan) were used and acclimatized for at least 1 week before the experiments. The mice were maintained as described above. Twenty-four mice were inoculated i.p. on day 0 with 10^6 P388 leukemic cells. The following day (day 1), the mice were randomly allocated to 4 groups of 6 rodents each in cages, and injected i.p. with the following combinations of drugs: alkaline soln. (10 mL/kg body weight) 1 h after normal saline (8 mL/kg body weight); BP (10 mL/kg, 250 mg/kg body weight) 1 h after normal saline (8 mL/kg body weight); alkaline soln. (10 mL/kg body weight) 1 h after cisplatin soln. (8 mL/kg, 8 mg/kg body weight); BP (10 mL/kg, 250 mg/kg body weight) 1 h after cisplatin soln. (8 mL/kg, 8 mg/kg body weight). In this experiment, dose of cisplatin was reduced to one half of that used in the toxicity study in mice. Because administration of cisplatin at a 16 mg/kg dose showed no significant differences in survival time between saline with alkaline soln. and cisplatin with alkaline soln. groups (data are not shown), we considered that the dose of cisplatin at a 16 mg/kg level was too large in the experiment for the antileukemic activity. Mortality was monitored daily for 40 days. The in vivo activity of each treatment against P388 leukemic cells was assessed by determining median survival time (MST). The results were expressed as a percentage of increase in life span (ILS).

Statistical Procedures

The standard error of mean (SEM) was computed for each group. Significant differences between groups were detected by applying the Tukey's multiple range test. Survival data were analyzed statistically by the Wilcoxon rank sum test of Breslow and used for testing survival differences between groups, as allowed by procedure of the SAS statistical software.

RESULTS

Protective Effects of BP Against the Toxicity of Cisplatin in Rats

The protective effects of BP against the toxicity of cisplatin, based on indicators such as body weight gain, BUN, and serum Cr levels in rats, are shown in Table 1. The values of body weight gain indicate percent of body weight changed from day −2 to day 5. The body weight gain for the animals treated with saline differed significantly ($p < 0.05$) from that of animals received only cisplatin. However, for administration of cisplatin plus BP, the loss of body weight with cisplatin treatment was not observed and significantly different ($p < 0.05$) from cisplatin alone.

The mean BUN and serum Cr values for the control animals injected with saline differed significantly ($p < 0.05$) from that received cisplatin group and agreed with previous studies

Table 1

Protective Effect of BP After Treatment with Cisplatin on Body Weight Gain, BUN, and Serum Cr Levels in Rats on Day 5

Treatment	No. of Animals	Body Weight Gain (%)[b]	BUN (mg/dL)	Serum Cr (mg/dL)
Saline[a]	6	12.0 ± 0.75	24.2 ± 1.05	0.93 ± 0.20
Cisplatin[a]	6	−10.8 ± 1.66[c]	114 ± 4.12[c]	4.91 ± 0.64[c]
Cisplatin + BP	6	9.37 ± 3.82[d]	32.4 ± 9.92[d]	0.88 ± 0.29[d]

Note. The values are mean ± SEM.

[a]Previous data (17).

[b]From day −2 to day 5.

[c,d]The mean for the group differs significantly ($p < 0.05$) from saline and cisplatin, respectively.

by other laboratories (21,22). The BUN and serum Cr levels in the animals treated with cisplatin plus BP differed significantly ($p < 0.05$) from the animals that received only cisplatin.

Figure 1 shows the water consumption, and volume and pH of urine after administration of cisplatin plus BP in rats. Water consumption of animals treated with cisplatin gradually decreased and reached a minimum on day 3, with a significant difference ($p < 0.05$) from the saline group, and it subsequently increased on day 7 [Fig. 1(a)]. But the loss of water consumption on day 3 with cisplatin treatment was not observed after administration of cisplatin plus BP. Urinary volume in the animals treated with cisplatin increased on day 7, but the increase was not significant compared to the saline group [Fig. 1(b)]. Increase in urinary volume on day 7 with cisplatin treatment was not observed after administration of cisplatin plus BP. Urinary pH of animals treated with cisplatin rapidly decreased on day 1 with a significant difference ($p < 0.05$) from the saline group, but it subsequently increased toward day 7 [Fig. 1(c)]. The decrease in urinary pH on day 1 after cisplatin treatment, however, was significantly ($p < 0.05$) suppressed by administration of cisplatin plus BP.

The protective effects of BP on the toxicity of cisplatin in rats as indicated by urine Cr, NAG, γ-GT, and β_2-M levels after administration of cisplatin plus BP in rats are shown in Figure 2. Urine Cr level treated with cisplatin decreased and reached a minimum on day 3, with no significant difference from the saline group, but it subsequently recovered on day 7 [Fig. 2(a)]. In contrast, the decrease in urine Cr level on day 3 for cisplatin treatment was not observed after administration of cisplatin plus BP, with a significant difference ($p < 0.05$) between the two groups.

NAG activity observed after treatment with cisplatin gradually increased and reached a maximum on day 3, with a significant difference ($p < 0.05$) from the saline group, but it subsequently decreased toward day 7, with a still significant difference ($p < 0.05$) from the saline group [Fig. 2(b)]. This increase in NAG is in agreement with previous studies by other investigators (23). In contrast, the increase in NAG activities on days 3 and 7 for cisplatin treatment was not observed after administration of cisplatin plus BP, with a significant difference ($p < 0.05$) from cisplatin alone.

γ-GT activity observed after treatment with cisplatin gradually increased and reached a maximum on day 3, with a significant difference ($p < 0.05$) from the saline group, and it

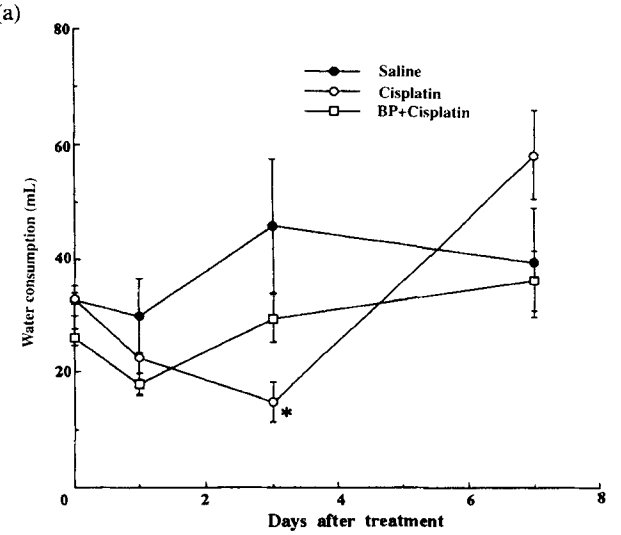


Figure 1. Water consumption (a), urinary volume (b), and urinary pH (c) in rats after cisplatin treatment followed 1 h later with BP. The values are mean ± SEM (bars) for 6 animals. *, §: the mean for the group differs significantly ($p < 0.05$) from saline, cisplatin, respectively.

subsequently decreased toward day 7, with a significant difference ($p < 0.05$) from the saline group [Fig. 2(c)]. This decrease may be attributed to depletion of γ-GT by destruction of the proximal tubule cells with cisplatin treatment and agreed with previous studies by other laboratories (24). In contrast, the increase and subsequent large decrease in γ-GT activities on days 3 and 7 for cisplatin treatment were not observed after administration of cisplatin plus BP, with a significant difference ($p < 0.05$) from the cisplatin group on day 7.

β_2-M level observed after treatment with cisplatin rapidly increased on day 1, but it subsequently decreased on days 3 and 7 [Fig. 2(d)]. This increase is in agreement with previous studies by other investigators (23). However, β_2-M levels on days 1, 3, and 7 after cisplatin treatment were not significantly different from the data obtained from the saline injection due to large standard deviation in values in animals treated with cisplatin. In contrast, the increase in β_2-M levels on days 1, 3, and 7 for cisplatin treatment was not observed after administration of cisplatin plus BP.

Protective Effects of BP Against the Toxicity of Cisplatin in Mice

The protective effects of BP against cisplatin toxicity, as indicated by body weight gain and BUN level in mice, are shown in Table 2. The values of body weight gain indicate percentage of body weight changed from day 0 to day 5. The value of body weight gain for the animals treated with saline plus alkaline soln. differed significantly ($p < 0.05$) from that of animals receiving cisplatin plus alkaline soln. In contrast, for administration of cisplatin plus BP, loss of body weight with cisplatin treatment was significantly ($p < 0.05$) less than cisplatin plus alkaline soln.

The mean BUN value for the control animals injected with saline plus alkaline soln. differed significantly ($p < 0.05$) from that for the cisplatin plus alkaline soln. group. The

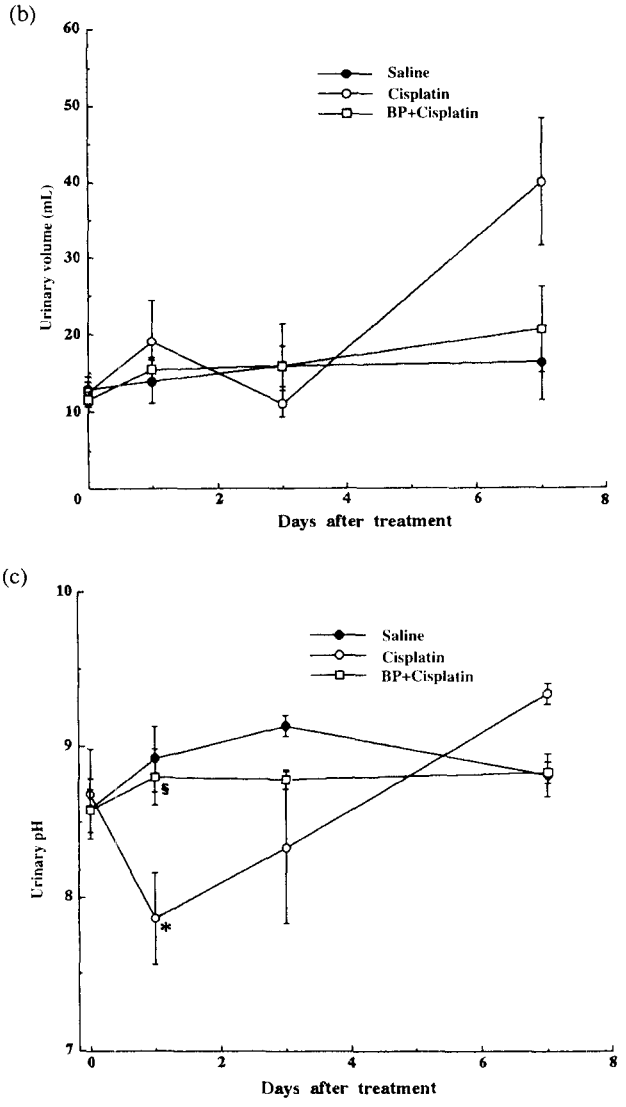
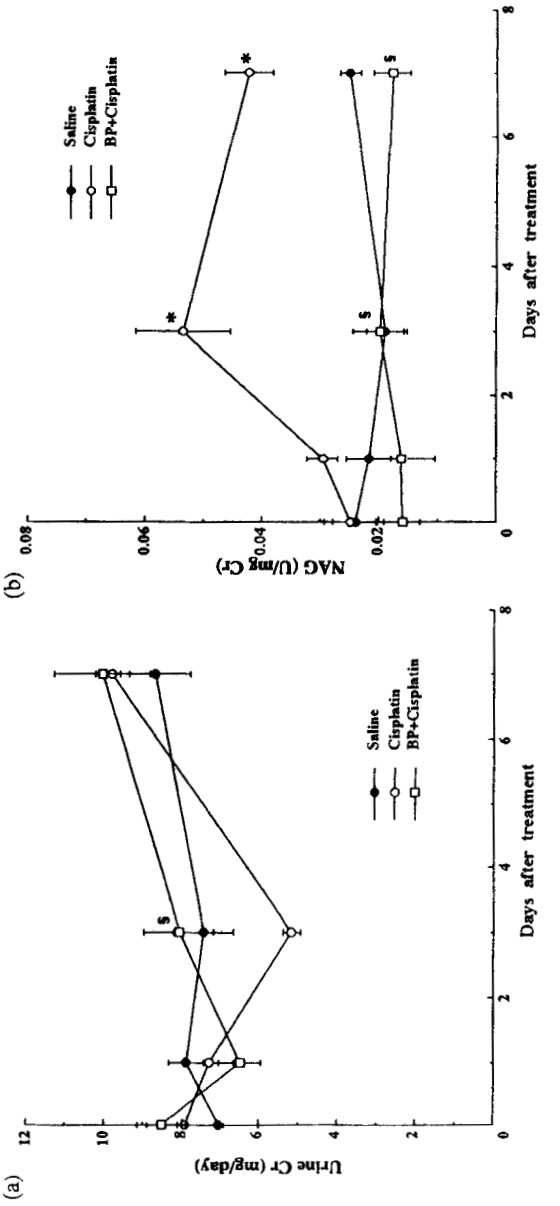


Figure 1. (Continued)

BUN level in the animals treated with cisplatin plus BP differed significantly ($p < 0.05$) from the animals that received cisplatin plus alkaline soln.

Effects of BP and Cisplatin Treatments on Survival Time in Leukemic Mice

The protective effects of BP after cisplatin treatment on survival time (MST) in leukemic mice are shown in Figure 3 and Table 3. Survival data in Table 3 were obtained from Figure 3. When alkaline soln. or BP was administered 1 h after saline treatment, there was no significant difference in MST between the two groups. When alkaline soln. or BP was


(a)
Urine Cr (mg/day)
12
10
8
6
4
2
0
Saline
Cisplatin
BP+Cisplatin
$
0
2
4
6
8
Days after treatment
(b)
NAG (U/mg Cr)
0.08
0.06
0.04
0.02
0
Saline
Cisplatin
BP+Cisplatin
*
$
0
2
4
6
8
Days after treatment

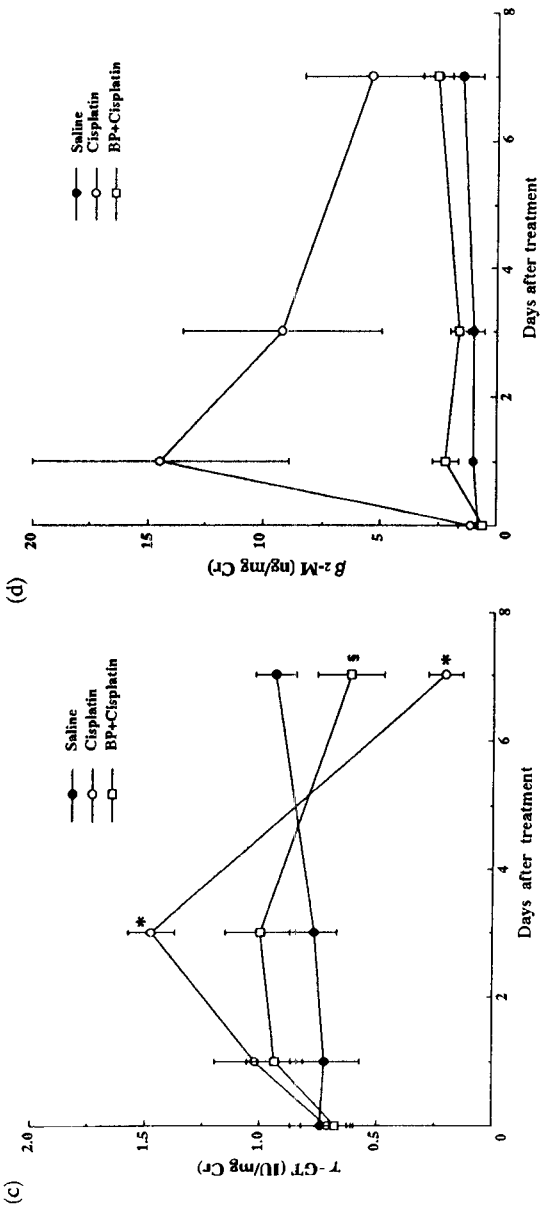


Figure 2. Urine Cr (a), NAG (b), γ-GT (c), and β_2-M (d) in rats after cisplatin treatment followed 1 h later with BP. The values are mean ± SEM (bars) for 6 animals. *, §: the mean for the group differs significantly ($p < 0.05$) from saline, cisplatin, respectively.

Table 2

Protective Effect of BP After Treatment with Cisplatin on Body Weight Gain and BUN Level in Mice on Day 5

Treatment	No. of Animals	Body Weight Gain (%)[a]	BUN (mg/dL)
Saline + alkaline soln.	3	2.56 ± 2.34	23.6 ± 2.01
Cisplatin + alkaline soln.	3	−36.6 ± 2.14[b]	46.8 ± 4.17[b]
Cisplatin + BP	3	−8.24 ± 9.85[c]	25.8 ± 1.33[c]

Note. The values are mean ± SEM.
[a]From day 0 to day 5.
[b,c]The mean for the group differs significantly ($p < 0.05$) from saline + alkaline soln. group and cisplatin + alkaline soln. group, respectively.

administered 1 h after cisplatin treatment, there was no significant difference in MST between the two groups. In addition, both groups differed significantly ($p < 0.05$) from control animals (saline plus alkaline soln. and saline plus BP). On the other hand, increase in life span (ILS) in the animals treated with cisplatin plus alkaline soln. or BP was greater than that in the animals treated with saline plus alkaline soln. or BP. Thus, the combination of cisplatin and BP had no apparent effect on the efficacy of cisplatin against P388 leukemic cells in mice.

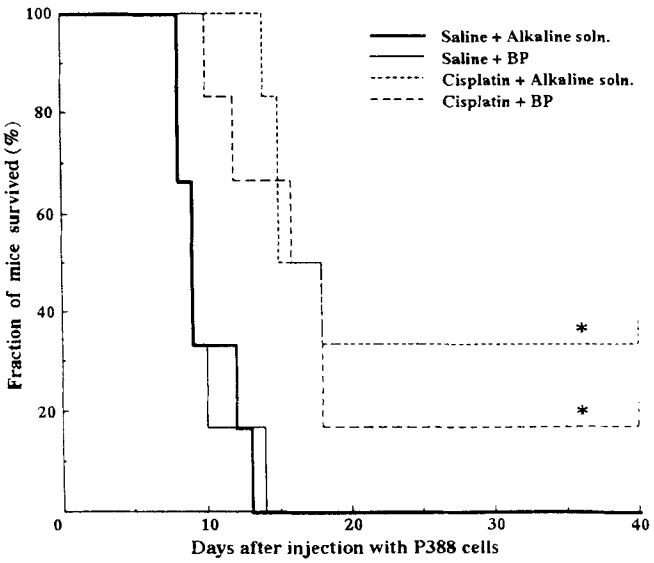


Figure 3. Effect of cisplatin and BP treatment on survival time in mice bearing P388 leukemic cells. The values are mean ± SEM (bars) for 6 animals. *: the mean for the group differs significantly ($p < 0.05$) from saline plus alkaline soln. or saline plus BP groups.

Table 3

Protective Effect of BP After Treatment with Cisplatin on Survival Time in Mice Bearing P388 Leukemic Cells

Treatment (Day 1)	No. of Animals	Cisplatin (mg/kg)	MST (Days)	ILS[a] (%)
Saline + alkaline soln.	6		9.83 ± 0.87	0
Saline + BP	6		9.67 ± 0.92	−1.63
Cisplatin + alkaline soln.	6	8	16.3 ± 0.80[c,d]	65.8
Cisplatin + BP	6	8	15.3 ± 1.46[b,d]	55.6

Note. The values are mean ± SEM.

[a]Calculated as the $[T - C)/C] \times 100$, where T and C are median survival time in treated animals and controls, respectively.

[b]Significantly different from saline + alkaline soln. group; $p < 0.05$.

[c]Significantly different from saline + alkaline soln. group; $p < 0.01$.

[d]Significantly different from saline + BP group; $p < 0.01$.

DISCUSSION

Many organic cations are transported actively in the renal proximal tubule via a specific saturable process (25–27). Distinct driving forces and mechanisms have been identified for the organic cation transport system in the brush-border and basolateral membrane of the proximal tubule (25,26,28). The organic cation transport system across the brush-border membrane appears to be driven by a proton exchange mechanism (29). It is generally assumed that the organic cation transporter is specific to organic cations and is not affected by organic anions. However, recently, some investigators have suggested the possible existence of interactions between organic anions and organic cations in excretion processes at the renal proximal tubule in several animals (30–32). Hayashi et al. (33) reported on the inhibitory effect of anionic piperacillin on the transport of gentamicin, one of the basic aminoglycoside antibiotics, in proximal tubular cells; coadministration of piperacillin resulted in reduced nephrotoxicity of gentamicin. Moreover, Hayashi et al. (8) indicated the protective effect of piperacillin against the nephrotoxicity of cisplatin. Although the protective effect of piperacillin was considered to be due to piperacillin itself (8,33), the mechanism of reduced nephrotoxicity due to piperacillin remains to be clarified. In addition, we demonstrated that BP, an organic anion transport inhibitor, reduced the renal toxicity of cisplatin (Tables 1 and 2). It is therefore necessary to establish firmly the mechanism and potential role of these interactions.

Umeki et al. (23) reported that fosfomycin reduces cisplatin-induced proximal tubular damage. They found that fosfomycin suppresses elevation in the NAG activity induced by cisplatin, possibly by enhancing the stability of the lysosomal membrane of the proximal tubular cells. Although the mechanisms of renal damage by cisplatin are unknown, histological findings and biochemical data indicate increased secretion into the urine of NAG and γ-GT, which exist maximally in the lysosomal membrane and brush-border membrane of the proximal tubular cells (34–36), and thus cisplatin may mainly act on the proximal tubular cells (37) and cisplatin nephrotoxicity may be correlated with the urinary activities

of NAG and γ-GT. The present report is designed to demonstrate that supplemental BP treatment prevents an increase in urinary NAG and γ-GT in the rats treated with cisplatin, suggesting that BP reduces cisplatin-induced renal proximal tubular damage [Figs. 2(b) and 2(c)]. In addition, we indicated that histological analysis of the kidneys confirmed the protective effect of BP as observed by BUN and serum Cr measurements (17). Since the decrement of nephrotoxicity is correlated with the inhibitory activity of the renal cortical accumulation of cisplatin, the increase in NAG activities after cisplatin treatment may be suppressed indirectly by BP after cisplatin treatment.

Several methods have been employed in an attempt to reduce the dose-limiting adverse effect, nephrotoxicity, of cisplatin (1–3). However, it is still difficult to obtain a sufficient protective effect against cisplatin-induced toxicity by these methods; bone marrow toxicity, nausea, or vomiting are still seen regularly (38,39). More importantly, these methods can reduce the antitumor activity of cisplatin as well as its toxicity (6,7). However, when alkaline soln. or BP was administered 1 h after cisplatin treatment, MST was not significantly different (Table 3). In addition, both groups differed significantly ($p < 0.05$) from control animals. Thus, the combination of cisplatin and BP had no apparent effect on the efficacy of cisplatin against P388 leukemic cells in mice.

In this study with animal models, we have confirmed that BP has a significant protective effect on cisplatin-induced nephrotoxicity without altering the antileukemic activity of cisplatin. Future studies should reveal a pharmacokinetic and pharmacodynamic detailed mechanism of the preventive effect of BP against cisplatin-induced nephrotoxicity and explain how BP inhibits the uptake and the accumulation of cisplatin in renal tubules. Also, the potential role of BP as a protective agent against the nephrotoxicity of cisplatin requires clinical confirmation.

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