Protective Effects of N-Benzoyl Amino Acids on Cisplatin Nephrotoxicity in Rats

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The protective effects of N-benzoyl amino acids (NAAs) and piperacillin (PIP), anionic transport inhibitors, against the nephrotoxicity of cisplatin were examined in rats. Male Wistar rats were injected i.p. with 6 mg/kg of cisplatin combined with i.p. NAAs or PIP. Rats were sacrificed on day 5 after cisplatin injection to weight the kidney and liver, and to determine blood urea nitrogen (BUN) and serum creatinine (serum Cr) levels. Treatments with NAAs were an effective means of protection against cisplatin-induced nephrotoxicity. The combination of cisplatin with NAAs containing a short and straight chain significantly suppressed ($p < 0.05$) the changes in body, kidney and liver weights, BUN and serum Cr. Furthermore, betamipron (BP) at a 2000 mg/kg dose showed no apparent effect on the body, kidney and liver weights, BUN and serum Cr levels in rats. The combination of cisplatin with PIP caused a loss in body weight. The protective effects of PIP against cisplatin toxicity are inferior to those of BP when compared at 250 mg/kg doses.

Key words cisplatin; betamipron; N-benzoyl-$\beta$-alanine; N-benzoyl amino acid; nephrotoxicity

cis-Diaminedichlorodiammineplatinum(II) (cisplatin), is one of the most effective antineoplastic agents, with a wide spectrum of therapeutic activity against certain human neoplasms such as tumors of the testis, ovaries, bladder, head and neck. However, the clinical use of cisplatin is limited by its dose-dependent adverse effects such as severe nephrotoxicity, gastrointestinal toxicity, bone marrow toxicity, neurotoxicity and ototoxicity. With the use of animal models, many attempts have been made to prevent cisplatin nephrotoxicity using candidate protective agents. Most studies on the reduction in toxic effects by these antidotes have often been accompanied by a reduction in cisplatin’s antitumor activity, so that an improvement in therapeutic index is not achieved. In a previous paper, we demonstrated that N-benzoyl-$\beta$-alanine (betamipron, BP), one of a series of N-benzoyl amino acids (NAAs), reduced the renal toxicity of cisplatin, and histological analysis of the kidneys confirmed the protective effects of BP.

The damage observed in the kidney is localized to the proximal tubule cells. Nagunuma et al. reported on the nephroprotective effects of BP, an anionic transport inhibitor and its mechanism, in view of the renal transport of panipenem, a new carbapenem antibiotic, in rabbits. A concomitant i.v. dose of BP dose-dependently decreased the degree of renal tubular necrosis caused by the antibiotic administration. The decrement of nephrotoxicity correlated well with the reduction in the renal cortical accumulation of panipenem. Both BP and probenecid, organic anion transport inhibitors, practically prevented the $[1^{14}]$ panipenem uptake in an isolated renal tubule. These experiments indicate that BP inhibits the active transport of an organic anion in the same way as probenecid. BP has been used clinically to protect against panipenem-induced nephrotoxicity, as an injectable drug panipenem/BP, in Japan. Moreover, Hirouchi et al. have shown that NAAs remarkably suppress the histopathological damage in the kidney induced by cephaloridine. NAAs generally show low toxicity in laboratory animals [e.g., $LD_{50}$ of BP was more than 3000 mg/kg, i.v. in rats]. Therefore, we investigated the correlation of the side chain structures of NAAs with their protective effects. The structures of all tested NAAs are shown in Table 1. In addition, since piperacillin (PIP), an anionic transport inhibitor, has been reported to be protective against cisplatin-induced nephrotoxicity, we compared the protective effects of BP and PIP against the nephrotoxicity of cisplatin in rats.

MATERIALS AND METHODS

Animals Male Wistar rats (195—407 g) were obtained from Kyudo Co. (Tosu, Japan) and acclimatized for at least one week before the experiments. The animals were maintained on a 12 h light/dark cycle and the temperature of the animal care facilities was kept 23—26°C. Food and water were taken ad libitum.

Drugs and Chemicals Cisplatin was kindly supplied by Nippon Kayaku Co. (Tokyo, Japan). N-Benzoylglycine (hippuric acid, HA), N-benzoyl-$\beta$-alanine (BP), N-benzoyl-5-aminovaleric acid (BAV), N-benzoyl-6-amino-hexanoic acid (BAH), N-benzoyl-$\delta$-lalanine (BA), $\alpha\delta$-dibenzoyl-$\delta$-l-lysine (DBL), $\alpha\delta$-benzoyl-$\delta$-l-methionine (BM) and $\alpha\delta$-aminobenzoylglycine (p-aminohippuric acid, PAH) were purchased from Tokyo Kasei Ind. Co. (Tokyo, Japan). N-Benzoyl-$\delta$-l-valine (BV) and N-benzoyl-$\delta$-l-phenylalanine (BPA) were kindly supplied by Sanky Co. (Tokyo, Japan). PIP (sodium salt) was purchased from Toyama Chemical Co. (Tokyo, Japan). Cisplatin and other chemicals 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, then sterilized by filtration through a 0.22-μm filter unit (Nippon Millipore Co., Tokyo, Japan) within 3 h of injection.

Experimental Protocol The animals were randomly divided into 16 groups of three to six rats each, housed individually in cages, and injected i.p. with the following

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combinations of drugs: normal saline (6 ml/kg body weight); cisplatin soln. (6 ml/kg, 6 mg/kg body weight); NAAAs (10 ml/kg, 250 mg/kg body weight) 1 h after cisplatin soln. (6 ml/kg, 6 mg/kg body weight); BP (10 ml/kg, 250 mg/kg and 2000 mg/kg body weight) and PIP (10 ml/kg, 250 mg/kg and 1000 mg/kg body weight) just before cisplatin soln. (6 ml/kg, 6 mg/kg body weight). Since treatment with cisplatin followed 1 h later with BP (or PIP) most effectively suppressed changes in the body weight, blood urea nitrogen (BUN) and serum creatinine (serum Cr) levels among the various administration schedules of BP, as reported in a previous paper, the administration time of NAAAs was fixed at 1 h after cisplatin treatment. PIP was administered just before cisplatin treatment. Rats were sacrificed on day 5 after cisplatin injection to weigh the kidney and liver, and to determine BUN and serum Cr levels.

Analytical Procedures Renal toxicity was evaluated by BUN and serum Cr. Blood was obtained by heart puncture. 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 Inds., Osaka, Japan). Serum samples were stored at -20°C.

Statistical Procedures The standard error of mean (S.E.M.) was computed for each group. Analysis of data was carried out by a one-way ANOVA. When the F ratio in the ANOVA test was significant, Tukey’s multiple range test was employed. In these tests, differences among groups for which p was less than 0.05 were considered significant.

RESULTS

Effect of NAAAs and PIP Treatments on Body Weight The changes in body weight for each treatment are shown in Table 2. The values of body weight gain indicate the percent of body weight changed from day -2 to day 5. Animals treated with saline gained 12.0% compared with their weights prior to treatment. The animals treated with cisplatin lost 10.8%, with a difference (p < 0.05) in the changes from the saline group. There was a body weight gain following the administration of NAAAs containing a straight chain after cisplatin dose. In contrast, body weight was lost following the administration of NAAAs containing a branched group at the C2 position, except for BPA. Therefore, the most remarkable gain in body weight following the administration of 10 NAA groups, was observed in animals treated with cisplatin followed 1 h later with HA, and this differed (p < 0.05) from the animals that received cisplatin alone. However, the body weight changes in animals treated with cisplatin followed...
1 h later with BM and DBL, were different (p < 0.05) from the animals that received saline injection alone.

Animals treated with cisplatin followed 1 h later with 250 mg/kg BP dose, 250 mg/kg or 2000 mg/kg BP doses alone, gained. There were no significant differences in the changes among the three groups, but these groups differed (p < 0.05) from those in the animals that received cisplatin alone. Body weight was lost following the administration of 250 mg/kg or 1000 mg/kg PIP doses just after cisplatin, with a difference (p < 0.05) in the change from those in the animals that received saline injection.

Effect of NAAs and PIP Treatments on Kidney and Liver Weights The ratios of kidney weight to body weight for each treatment group are shown in Fig. 1(a). The ratio for the animals treated with saline was 0.688%, whereas the ratio for the animals treated with cisplatin was 1.08% with a difference (p < 0.05) between the two groups. The ratios of kidney weight to body weight after the administration of NAAs containing the straight chain were smaller (p < 0.05) than that with cisplatin alone. In contrast, the ratio after the administration of DBL was greater (p < 0.05) that than that with saline. The greatest suppression of kidney weight among the 10 NAA groups was observed in animals treated with cisplatin followed 1 h later with BPA, differing (p < 0.05) from the animals that received cisplatin alone.

There were no significant differences in the ratios of kidney weight to body weight among the three groups treated with cisplatin followed 1 h later with 250 mg/kg BP dose, 250 mg/kg or 2000 mg/kg BP doses alone, and these groups differed (p < 0.05) from those in the animals that received cisplatin alone. The ratio of kidney weight to body weight after the administration of cisplatin just before 250 mg/kg or 1000 mg/kg PIP doses was different (p < 0.05) from those in animals which received only cisplatin or saline injection, respectively.

The ratios of liver weight to body weight for each group are shown in Fig. 1(b). The ratio for the control animals injected with saline was 4.09%, whereas the ratio for the animals treated with cisplatin was 3.26%, without a significant difference between the two groups. The ratios of liver weight to body weight after the administration of NAAs containing the straight chain following cisplatin treatment tended to be larger than cisplatin alone. The greatest ratio of liver weight change among the 10 NAA groups was observed in animals treated with cisplatin followed 1 h later with HA, differing (p < 0.05) from the animals that received cisplatin alone.

There were no significant differences in the ratios of liver weight to body weight among the three groups treated with cisplatin followed 1 h later with 250 mg/kg BP dose, 250 mg/kg or 2000 mg/kg BP doses alone, and these groups differed (p < 0.05) from those in the animals that received cisplatin alone. The ratio of liver weight to body weight after the administration of cisplatin just before 250 mg/kg PIP dose was different (p < 0.05) from those in the animals that received cisplatin combined with BP. Furthermore, the ratio of liver weight to body weight after the administration of cisplatin just before the 1000 mg/kg PIP dose was different (p < 0.05) from that in the animals which received cisplatin combined with BP and saline injection.

Effect of NAAs and PIP Treatments on BUN and Serum Cr Levels The BUN levels for each group are shown in Fig. 2(a). The mean BUN value for the control animals injected with saline was 24.2 mg/dl, whereas that of the animals which received cisplatin alone was 114 mg/dl. This dramatic elevation in the BUN level 5d after the cisplatin treatment differed (p < 0.05) from the control group and agreed with previous studies by other laboratories. The BUN values after the administration of NAAs showed the same pattern as the changes in body weight (Table 2); the shorter the straight chain length of NAAs, the lower the BUN level, approaching the value after the injection of saline alone. The BUN levels in animals treated with cisplatin followed 1 h later with HA, BP, BAV, BAH and BPA differed (p < 0.05) from those in the animals that received cisplatin alone. The most remarkable decrease in BUN level among the groups was observed in the animals treated with cisplatin followed 1 h later with BPA.

The BUN values after the administration of BP alone
and cisplatin combined with PIP showed the same pattern of change in body weight. There were no significant differences in the BUN values among the three groups treated with cisplatin followed 1 h later with 250 mg/kg BP dose, 250 mg/kg or 2000 mg/kg BP doses alone, and these groups differed (p < 0.05) from those in the animals that received cisplatin alone. The mean BUN values after the administration of cisplatin just before 250 mg/kg or 1000 mg/kg PIP doses were different (p < 0.05) from those in the animals which received cisplatin alone. In addition, the mean BUN value following treatment with cisplatin combined with a 250 mg/kg PIP dose differed (p < 0.05) from the saline injection and from cisplatin combined with BP.

The serum Cr levels for each group are shown in Fig. 2(b). The serum Cr values showed a pattern of change that was different from the data obtained from the body, kidney and liver weights, and BUN values. The mean serum Cr value for the control animals treated with saline was 0.928 mg/dl, whereas that for the animals treated with cisplatin was 4.91 mg/dl with a difference (p < 0.05) between the two groups. In contrast, the serum Cr values after the administration of all tested NAAs following cisplatin treatment were smaller (p < 0.05) than those in the animals treated with cisplatin alone.

The serum Cr values after the administration of BP alone and cisplatin combined with BP or PIP indicate that serum Cr values after all the tested combinations were smaller (p < 0.05) than those in the animals treated with cisplatin. There were no significant differences in serum Cr among the three groups treated with cisplatin followed 1 h later with 250 mg/kg BP dose, 250 mg/kg or 2000 mg/kg BP doses alone. However, the protective effect of PIP at 250 mg/dl was inferior to that of PIP at 1000 mg/kg.

**DISCUSSION**

Many organic cations are transported actively in the renal proximal tubule via a specific saturable process.14-16 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.14,15,17 The organic cation transport system across the brush border membrane appears to be driven by a proton exchange mechanism.16 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.18-21 Hayashi et al.11 indicated the protective effect of piperacillin against the nephrotoxicity of cisplatin. In similar findings, it has already been reported, with probenecid, that an organic anion transport inhibitor prevents cephalosporin- and cisplatin-induced nephrotoxicity.22-25 In a previous paper,25 we demonstrated that BP, an organic anion transport inhibitor, reduced the renal toxicity of cisplatin. In this study, we investigated the correlation of the side chain structures of NAAs with their protective effects and the protective effects of BP and PIP against the nephrotoxicity of cisplatin in rats.

Five days after treatment with 6 mg/kg cisplatin in rats, changes in body weight following the administration of NAAs containing a straight chain after the treatment with cisplatin were positive. In contrast, following the administration of NAAs with a branched group at the C2 position of the NAAs, except for BPA, body weight was lost. In humans, treatment with cisplatin induces a severe loss of appetite as well as nausea and vomiting, which are all well-known adverse effects.26,27 Since cisplatin causes an appetite suppression which limits food intake in animals, treatment with cisplatin induces a loss in body weight. Though many attempts have been made to prevent cisplatin toxicity with protective agents such as fosfomycin28 and acivicin,29 they did not prevent changes in body weight. However, NAAs containing a short and straight chain eliminated the weight loss following treatment with cisplatin. The mechanism of weight loss is considered to be different from the mechanism of nephrotoxicity, therefore, the increase in body weight following the administration of NAAs containing the straight chain after the treatment with cisplatin may be attributable to an action other than organic anion
transport inhibition. These data suggest that NAAs containing a short and straight chain may alleviate the adverse side effects of appetite loss.

The ratio of kidney weight to body weight, as affected by treatment with cisplatin, increased ($p < 0.05$) compared to the treatment with saline. It has been reported that size of the kidney increased after cisplatin injection and decreased with recovery. The ratios of kidney weight to body weight following the administration of NAAs containing a straight chain, after treatment with cisplatin, were decreased ($p < 0.05$). This suppression of the increase in kidney weight indicated that the administration of NAAs containing a straight chain, and BPA, protected against the renal toxicity of cisplatin.

Hepatopathy, one of the cisplatin-induced adverse effects, has not been well understood. There have been reports on cisplatin hepatopathy, but clinically useful modalities are very few. In those studies, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were used as indicators for hepatopathic toxicity. We measured the ratio of liver weight to body weight after treatment with cisplatin in this study. Treatment with cisplatin induced a decrease ($p < 0.05$) in the ratio of liver weight to body weight compared with that treated with saline. The ratios of liver weight to body weight, following the administration of NAAs containing a straight chain after the treatment with cisplatin, tended to increase. The greatest suppression of the liver weight decrease among NAAs examined was observed in animals treated with cisplatin followed 1 h later with HA, differing ($p < 0.05$) from those animals that received cisplatin alone. These data suggest that HA may protect against the hepatopathy induced by cisplatin.

The protective effects of BP against cisplatin nephrotoxicity were observed in both serum markers. Five days after treatment with $6$ mg/kg cisplatin to rats, their BUN levels were elevated 4.7-fold compared with those of the control. The shorter the straight chain length of NAAs administered after the treatment with cisplatin, the lower the BUN level, approaching the value after the injection of saline alone. The BUN levels in animals treated with cisplatin followed 1 h later with HA, BP, BAV and BAH differed ($p < 0.05$) from those in the animals that received cisplatin alone. The BUN level, as well as the body, kidney and liver weights, revealed that treatment with cisplatin followed 1 h later with NAAs containing a short and straight chain and no branched group at the C2 position of NAAs, except for BPA containing an aromatic side chain, were effective against cisplatin toxicity.

The serum Cr values showed a pattern of change that is different from the data obtained from the body, kidney and liver weights, and BUN values. Serum Cr levels were also elevated 5.3-fold after cisplatin injection. Combinations of all the tested NAAs with the administration of cisplatin resulted in lowering the serum Cr levels. The serum Cr levels obtained from these treatments were not correlated with the body, kidney and liver weights, and BUN values, but all tested NAAs were effective against cisplatin toxicity.

Further, BP at a $2000$ mg/kg dose had no apparent effect on the body, kidney and liver weights, BUN and serum Cr levels in rats (Table 3, Figs. 3 and 4). Since PIP, an anionic transport inhibitor, has been reported to be protective against cisplatin-induced nephrotoxicity, we compared the protective effects of BP and PIP against the nephrotoxicity of cisplatin in rats. For changes in body weight, the administration of cisplatin combined with PIP caused a loss in body weight in the same pattern as the cisplatin alone. The ratios of kidney, and liver weights to body weight after the administration of PIP were different ($p < 0.05$) from those in the animals which received cisplatin alone or saline injection, and saline or cisplatin combined with BP, respectively. The BUN level after the administration of cisplatin combined with PIP was different ($p < 0.05$) from that with cisplatin alone. However, the mean BUN value, when cisplatin was combined with a $250$ mg/kg PIP dose, differed ($p < 0.05$) from those of the saline injection and cisplatin combined with BP. Hayashi et al. indicated that the administration of PIP at a $250$ mg/kg dose had no apparent effect on the nephrotoxicological parameters (e.g. BUN and serum Cr values) in rats. That is, the protective effect of PIP was inferior to that of BP at $250$ mg/kg doses. The serum Cr values after the administration of cisplatin combined with PIP were smaller ($p < 0.05$) than those treated with cisplatin alone. The serum Cr levels obtained from these treatments did not correlate with the body, kidney and liver weights, and BUN values. In conclusion, the co-administration of BP with cisplatin was more suppressive than cisplatin with PIP at $250$ mg/kg doses.

We showed that NAAs inhibit the nephrotoxicity of cisplatin in rats. However, these data imply that the protective effects of NAAs against cisplatin-induced damage to the proximal tubule cells may be due to the blockade of the active transport by organic anion transport inhibitors; the duration of plasma cisplatin concentration and an increase in action other than in kidney tissue may also be causes. Naganuma et al. reported on the tissue distribution of panipenem in rabbits after single i.v. administrations of panipenem alone or with concomitant BP. A decrease in kidney panipenem concentration was observed in animals treated with concomitant BP, with a significantly different concentration ($p < 0.05$) from the animals that received panipenem alone; however, there was no significant difference in plasma panipenem concentration between the two groups. Similarly, both BP and probenecid practically prevented panipenem uptake in an isolated renal tubule. Furthermore, Takahagi et al. showed that there was no significant difference in plasma panipenem concentration between panipenem alone and with concomitant BP in dogs.

Pharmacokinetic and pharmacodynamic investigations will be needed to clarify the mechanism. Future studies should reveal in more detail the mechanism of the preventive effects of NAAs against cisplatin-induced nephrotoxicity, and also explain how NAAs inhibit the uptake and accumulation of cisplatin in renal tubules.

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