Improvement of Cisplatin Toxicity and Lethality by Juzen-taiho-to in Mice

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The effects of oral treatment with 1-, 5-, 10- and 20-fold the usual daily dose of Juzen-taiho-to on the nephrotoxicity, immunosuppression, hepatic toxicity and gastrointestinal toxicity caused by i.p. administration of 3.0 mg/kg cisplatin (CDDP) 9 times (on days 3, 4, 5, 6, 7, 8, 10, 11, and 12) were examined in ddY mice inoculated with sarcoma 180 (S-180) cells on day 1. The increase in blood urea nitrogen, serum creatinine, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase and relative stomach weight and decrease in white blood cell count, platelet count, relative spleen and thymus weight, food intake and body weight caused by CDDP were inhibited to nearly the control levels without reducing the antitumor activity of CDDP against S-180 by the oral treatment with either 10-fold (1.7 g/kg) or 20-fold (3.4 g/kg) the usual daily dose of Juzen-taiho-to 12 times (on days 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, and 15). All the mice receiving 4.5, 6.0, 7.5, 9.0, and 12.0 mg/kg CDDP died by day 12, while treatment with 3.4 g/kg Juzen-taiho-to efficiently prolonged the survival time.

These findings indicate that Juzen-taiho-to may provide protection against most clinical toxicity caused by CDDP, and Juzen-taiho-to may allow us to administer a much higher dose of CDDP in clinical therapy.

Keywords Juzen-taiho-to; cisplatin; nephrotoxicity; bone marrow toxicity; antitumor activity

cis-Diamminedichloroplatinum (II) (cisplatin, CDDP) is one of the most effective cancer chemotherapeutic agents, widely used against several types of tumors.^{1,2)} However, the clinical use of CDDP is limited by its severe side effects such as nephrotoxicity.^{3–5)} We demonstrated previously that the Kampo formulation Shi-Quan-Da-Bu-Tang (Juzen-taiho-to) prevented both the nephrotoxicity and bone marrow toxicity without reducing the antitumor effect of CDDP in mice when administered perorally (*p.o.*) 30 min before CDDP at a dose of only 10-fold the usual daily dose (1728 mg/kg).⁶⁾

In this experiment, we examined the dose-response effect of Juzen-taiho-to on CDDP-induced toxicity. Moreover, we examined whether Juzen-taiho-to could decrease or prevent the lethal toxicity of CDDP, when a high-dose of CDDP is administered.

MATERIALS AND METHODS

Animals Five-week-old, male, ddY mice (average weight, 25 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and kept in rooms with controlled temperature ($23 \pm 0.5^\circ\text{C}$), humidity ($50 \pm 5\%$), and 12-h light/12-h dark cycles. They were fed commercial mouse chow (MF: Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*, and were used after one week of acclimation (average weight, 30 g).

Preparation of CDDP Solution CDDP was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The toxicity of CDDP depends on its aquation reactions. Greene *et al.*⁷⁾ found that the aquation reactions reached an equilibrium 2 d after starting at room temperature, and the equilibrium in 0.9% saline was maintained for a long time. Therefore, the solution of CDDP was prepared more than one week before injection in sterile 0.9% saline at concentrations of 0.5 and 1.0 mg/ml: the solution of 1.0 mg/ml CDDP was used to examine the effects of Juzen-taiho-to on the lethal toxicity of 4.5–12.0 mg/kg CDDP.

Preparation of Juzen-taiho-to The ingredients of the formulation of Juzen-taiho-to were purchased from Yamamoto Yakuhin Kogyo Co., Ltd. (Tokyo). A normal daily dose⁸⁾ of a water extract of Juzen-taiho-to was prepared as follows: Angelicae Radix (3 g), Hoelen (3 g), Glycyrrhizae Radix (2 g), Ginseng Radix (3 g), Astragali Radix (3 g), Cinnamomi Cortex (3 g), Atractylodis Rhizoma (3 g), Paeoniae Radix (3 g), Cnidii Rhizoma (3 g), and Rehmanniae Radix (3 g) were blended, and then extracted with boiling water for 60 min. After cooling, the extract was filtered, and then lyophilized. The yield of the water extract was 8.5 g (29.3%). The lyophilized material was dissolved in water immediately before use.

Treatment of Animals Animal treatment using this method was a modification of the method reported previously.⁶⁾ Briefly, the animals in the test groups, each comprised of 10 mice, were inoculated with sarcoma 180 (S-180) cells (10^6 /mouse) subcutaneously in the left thigh on day 1. CDDP (3.0, 4.5, 6.0, 7.5, 9.0, or 12.0 mg/kg) was given i.p. to the mice on days 3, 4, 5, 6, 7, 8, 10, 11, and 12. Juzen-taiho-to was given orally to the mice on days 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, and 15. The mice in the control group were treated with water (*p.o.*) and 0.9% saline (i.p.). On day 17, the mice were anesthetized with ether, and blood was collected from the inferior vena cava using a heparinized syringe, and the number of red blood cells (RBC), white blood cells (WBC) and platelets (PLT) was immediately counted. After centrifugation of the remaining blood, the serum was analyzed for blood urea nitrogen (BUN), serum creatinine, serum glutamic-oxaloacetic transaminase (sGOT) and serum glutamic-pyruvic transaminase (sGPT). The liver, kidneys, spleen, thymus, stomach (organ and contents) and tumor were resected and weighed. The 24-h urine volume and 24-h intake of diet were measured on day 14. The inhibitory rate of the antitumor activity was calculated by the following formula: inhibitory rate (%) = $(1 - B/A) \times 100$, where *A* is the mean tumor weight of the non-treated group and *B* is that of the CDDP alone group or the Juzen-

taiho-to plus CDDP group.

Measurement of Functions RBC, WBC and PLT counts were made on a Celltac 4150 (Nihon Koden, Ltd., Tokyo). BUN, serum creatinine, sGOT and sGPT were measured on a COBAS FARA (Baxter, Ltd., Tokyo) spectrometrically using assay kits for urea nitrogen-HR II, creatinine-HA test Wako, GOT-FA test Wako and GPT-FA test Wako (Wako Pure Chemical Industries, Ltd., Tokyo), respectively.

Statistics The Student's *t* test was used to evaluate the significance of difference between the experimental groups.

RESULTS

Effects of Juzen-taiho-to against Toxicity Induced by 3.0 mg/kg CDDP 1) Nephrotoxicity Figure 1 shows the effects of Juzen-taiho-to on CDDP-induced nephrotoxicity. BUN and serum creatinine were increased significantly, to about 3- and 2-fold of the control levels, respectively, and urinary volume was decreased significantly to about 42% of the control level, by i.p. treatment

Fig. 1. Effect of Juzen-taiho-to on CDDP-Induced Nephrotoxicity

Juzen-taiho-to (0.17–3.4 g/kg/d $\times 12$) was administered *p.o.* to mice *s.c.* inoculated with S-180 cells 30 min before CDDP *i.p.* injection (3.0 mg/kg/d $\times 9$) once a day. BUN and serum creatinine levels and relative kidney weight were determined on day 17, and the 24 h-urinary volume was measured on day 14. Each value is the mean $\pm$ S.E. ($n = 10$). Significant difference from the CDDP(-) groups, *: $p < 0.05$ and ***: $p < 0.001$. Significant difference from the CDDP alone group, #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.001$.

with 3.0 mg/kg CDDP alone for 9 times. However, no significant change in relative kidney weight was observed compared to the control value: BUN, serum creatinine, urinary volume and relative kidney weight of mice treated with CDDP alone were 95.9 ± 14.1 , 0.79 ± 0.25 mg/dl, 4.0 ± 0.8 ml/100 g, and $1.73 \pm 0.12 (\times 10^{-2})$, respectively, and those of mice treated with neither CDDP nor Juzen-taiho-to were 29.0 ± 1.1 , 0.46 ± 0.05 mg/dl, 7.1 ± 0.9 ml/100 g, and $1.62 \pm 0.12 (\times 10^{-2})$, respectively. Treatment with more than 0.85 g/kg (5-fold the usual daily dose) Juzen-taiho-to significantly inhibited the increase of BUN and serum creatinine values and the decrease of urinary volume. Moreover, co-treatment with more than 1.7 g/kg Juzen-taiho-to recovered all the nephrotoxicity induced by CDDP to near the Juzen-taiho-to alone group. Juzen-taiho-to alone did not exhibit any significant changes in these parameters at any doses tested.

2) Bone Marrow Toxicity Figure 2 shows the effect of Juzen-taiho-to on CDDP-induced bone marrow toxicity. WBC count, PLT count, relative spleen and thymus

Fig. 2. Effect of Juzen-taiho-to on CDDP-Induced Bone Marrow Toxicity

All parameters were determined on day 17. Each result is the mean $\pm$ S.E. ($n = 10$). Significant difference from the CDDP(-) groups, **: $p < 0.01$ and ***: $p < 0.001$. Significant difference from the CDDP alone group, #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.001$.

weights decreased significantly to about 29%, 27%, 60% and 24% of the control level, respectively: RBC, WBC and PLT counts and the relative spleen and thymus weight of mice treated with CDDP alone were 9.7 ± 0.4 ($\times 10^6/\text{mm}^3$), 1.8 ± 0.3 ($\times 10^3/\text{mm}^3$), 2.1 ± 0.3 ($\times 10^5/\text{mm}^3$), 2.11 ± 0.19 ($\times 10^{-3}$) and 0.33 ± 0.09 ($\times 10^{-3}$), respectively, and those of mice treated with neither CDDP nor Juzen-taiho-to were 9.8 ± 0.4 ($\times 10^6/\text{mm}^3$), 6.2 ± 0.6 ($\times 10^3/\text{mm}^3$), 7.5 ± 1.0 ($\times 10^5/\text{mm}^3$), 3.49 ± 0.31 ($\times 10^{-3}$) and 1.35 ± 0.16 ($\times 10^{-3}$), respectively. The decreases in WBC, PLT, and relative thymus weight were significantly prevented in comparison with the CDDP alone group by treatment with more than 0.85 g/kg Juzen-taiho-to. The decrease in relative spleen weight was also inhibited significantly using more than 10-fold the usual daily dose (1.7 g/kg). Moreover, co-treatment with 1.7 g/kg Juzen-taiho-to did not exhibit any significant difference from the Juzen-taiho-to alone group {CDDP(-)}. Juzen-taiho-to alone did not significantly change those parameters at any doses tested.

3) Hepatic Toxicity Figure 3 shows the effect of Juzen-taiho-to on hepatic toxicity induced by 3.0 mg/kg CDDP 9 times. Administration of CDDP alone increased sGOT and sGPT values to about 3- and 5-fold of the control values, although no significant change in relative liver weight was observed: sGOT, sGPT and the relative liver weight of mice treated with CDDP alone were 61.2 ± 10.5 , 50.2 ± 6.9 U/l and 4.80 ± 0.80 ($\times 10^{-2}$), respectively, and those of mice treated with neither CDDP nor Juzen-taiho-to were 18.8 ± 1.4 , 9.5 ± 1.4 U/l and

Fig. 3. Effect of Juzen-taiho-to on CDDP-Induced Hepatic Toxicity

All parameters were determined on day 17. Significant difference from the CDDP(-) groups, *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$. Significant difference from the CDDP alone group, #: $p < 0.05$ and ##: $p < 0.01$.

5.50 ± 0.20 ($\times 10^{-2}$), respectively. The co-treatment with more than 1.7 g/kg Juzen-taiho-to kept those parameters to nearly the level in the Juzen-taiho-to alone group.

4) Other Toxicity Administration of CDDP alone decreased body weight and food intake to 65% and 31% of the control values, respectively, and significantly increased the relative stomach weight to about 4.5-fold of the control value: The body weight, food intake and relative stomach weight of mice treated with CDDP alone were 22.8 ± 0.8 , 1.1 ± 0.5 g, and 9.0 ± 1.0 ($\times 10^{-2}$), respectively, and those of mice treated neither CDDP nor Juzen-taiho-to were 35.0 ± 0.6 , 3.6 ± 0.9 g and 2.0 ± 0.2 ($\times 10^{-2}$), respectively. Both the increase and the decrease induced by CDDP were inhibited significantly by treatment with more than 0.85 g/kg Juzen-taiho-to. Co-treatment with 3.4 g/kg Juzen-taiho-to recovered these parameters to nearly the level in the Juzen-taiho-to alone group (Fig. 4).

5) Antitumor Activity Treatment with CDDP alone dose-dependently inhibited the growth of S-180 cells (ED_{50} : 2.30 mg/kg), and the inhibitory rate reached nearly the highest level (80.9%) at a dose of 3.0 mg/kg (Fig. 5). Table I summarizes the antitumor activity of CDDP and/or Juzen-taiho-to against S-180. Combined treatment with Juzen-taiho-to did not exert any significant effect on the antitumor effect of CDDP at any doses tested. Juzen-taiho-to alone also had no obvious antitumor effect on S-180 at any doses tested.

Fig. 4. Effect of Juzen-taiho-to on CDDP-Induced Body Weight Loss, Food Intake Loss and Relative Stomach Weight Increase

Body weight and relative stomach weight were determined on day 17, and 24-h food intake was measured on day 14. Each value is the mean $\pm$ S.E. ($n=10$). Significant difference from the CDDP(-) groups, *: $p < 0.05$ and ***: $p < 0.001$. Significant difference from the CDDP alone group, #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.001$.

Effect of Juzen-taiho-to on Lethal Toxicity of 3.0—12.0 mg/kg CDDP Figure 6 shows the effects of Juzen-taiho-to on the lethal toxicity induced by 3.0, 4.5, 6.0, 7.5, 9.0, and 12.0 mg/kg CDDP. During the first 17 d, all the

Fig. 5. Dose-Response Curve of Antitumor Effect of CDDP against S-180

S-180 cells (10^6 /mouse) were inoculated s.c. on day 1 and antitumor effect was determined on day 17. Each value is the mean ($n=10$). Tumor weight in the non-treated control and 3.0 mg/kg CDDP-treated group were 1.15 ± 0.17 and 0.25 ± 0.05 g, respectively. There are significant differences in tumor weight between the non-treated control and groups treated with more than 2.5 mg/kg CDDP.

mice receiving 3.0 mg/kg CDDP were alive, but about 40% of them died after day 18. Meanwhile, CDDP alone at a dose of 4.5, 6.0, 7.5, 9.0, and 12.0 mg/kg was very toxic and all the mice receiving these doses of CDDP died within 15, 12, 10, 7, and 5 d after the initial injection of CDDP, respectively, without any visual increase in the volume of S-180 cells inoculated in the left thigh. Treatment with 3.4 g/kg Juzen-taiho-to completely depressed the lethal toxicity of 3.0 and 4.5 mg/kg CDDP and efficiently prolonged the survival time of the S-180-bearing mice receiving 6.0, 7.5, 9.0, and 12.0 mg/kg CDDP. Treatment with 1.7 g/kg Juzen-taiho-to also depressed the lethal toxicity of 3.0—12.0 mg/kg CDDP, but the effect was weaker than that of 3.4 g/kg of Juzen-taiho-to (data not shown). Animals treated with 3.4 g/kg Juzen-taiho-to and 4.5 mg/kg CDDP exhibited no significant change in any parameters tested compared with the Juzen-taiho-to-only-treated group on day 17 (data not shown).

DISCUSSION

Several methods have been employed in an attempt to

TABLE I. Effect of Juzen-taiho-to on Antitumor Activity of CDDP against S-180

Dose of Juzen-taiho-to (g/kg, <i>p.o.</i>)	Juzen-taiho-to alone		Juzen-taiho-to + CDDP	
	Tumor wt. (g)	Antitumor activity (%)	Tumor wt. (g)	Antitumor activity (%)
0	1.123 ± 0.152	—	$0.215 \pm 0.056^{***}$	80.9
0.17	1.159 ± 0.095	-3.2	$0.223 \pm 0.042^{***}$	80.1
0.85	1.119 ± 0.126	0.4	$0.226 \pm 0.065^{***}$	79.9
1.7	1.109 ± 0.108	1.2	$0.219 \pm 0.092^{***}$	80.5
3.4	1.114 ± 0.125	0.8	$0.223 \pm 0.108^{***}$	80.1

S-180 cells (10^6 /mouse) were inoculated s.c. on day 1 and antitumor activity was determined on day 17. Juzen-taiho-to (0.17—3.4 g/kg/d $\times$ 12) was administered *p.o.* 30 min before 3.0 mg/kg CDDP i.p. injection 9 times. All values are mean $\pm$ S.E. ($n=10$). Significant difference from the non-treated control group, *** : $p < 0.001$. Significant difference from the only Juzen-taiho-to treated groups, *** : $p < 0.001$. There is no significant difference between the CDDP-treated only group and the Juzen-taiho-to and CDDP-treated groups.

Fig. 6. Effect of Juzen-taiho-to on Lethal Toxicity of CDDP

Juzen-taiho-to (3.4 g/kg/d) was administered *p.o.* to mice s.c. inoculated with S-180 cells (10^6 /mouse) 30 min before CDDP (3.0—12.0 mg/kg/d) i.p. injection once a day during the experimental period. All mice which received CDDP alone at doses of 4.5, 6.0, 7.5, 9.0 and 12.0 died within 15, 12, 10, 7 and 5 d after the initial CDDP injection, respectively. All mice that received Juzen-taiho-to and 3.0 and 4.5 mg/kg/d CDDP were alive 25 d after the initial CDDP injection. -----, CDDP alone; —, CDDP plus Juzen-taiho-to.

reduce the dose-limiting side effect, nephrotoxicity, of CDDP.⁹⁻¹⁵⁾ One of these methods, clinically, is the use of either hydration or diuresis by mannitol or furosemide (Lasix®).^{9,10)} However, it is still difficult to obtain a sufficient protective effect against CDDP induced toxicity by this method; bone marrow toxicity, nausea or vomiting are still seen regularly.^{16,17)} More importantly, this method reduces the antitumor effect of CDDP as well as its toxicity.⁶⁾ In the present study, the oral administration of more than 0.85 g/kg/d Kampo formulation Juzen-taiho-to, which has been used for patients with anorexia, anemia and/or fatigue,⁷⁾ and is known to have a function as a biological response modifier,¹⁸⁻²⁰⁾ resulted in significant protection against the nephrotoxicity, bone marrow toxicity, hepatic toxicity and gastrointestinal toxicity induced by multiple treatments using CDDP (3.0 mg/kg/d, 9 times, i.p.).

The multiple treatment with CDDP dose-dependently inhibited the growth of S-180 cells (ED₅₀: 2.30 mg/kg), and the inhibitory rate reached nearly the highest level at a dose of 3.0 mg/kg (Fig. 5). Moreover, the LD₅₀ by day 25 in multiple treatment with CDDP was about 3.0 mg/kg/d (Fig. 6), while the LD₅₀ of CDDP in a single treatment was 17.5 mg/kg. Therefore, we examined the effect of Juzen-taiho-to on CDDP-induced toxicity at a dose of 3.0 mg/kg/d. The nephrotoxicity induced by 3.0 mg/kg CDDP in this mouse model is in basic agreement with several prior studies utilizing CDDP in animal models,²¹⁻²³⁾ indicating that this nephrotoxicity is serious.

Stomach weight indicates the peculiar effect of CDDP on pyloric stasis, causing gastric distension.²⁴⁾ This is an immediate toxicity and may be linked to the nausea and vomiting that is rapidly induced by CDDP.^{25,26)} Stomach weight (organ and contents weight) increased significantly to 4.5-fold of the control value by the treatment with 3.0 mg/kg CDDP alone. Co-administration of more than 0.85 g/kg Juzen-taiho-to with CDDP significantly prevented gastric distension. This finding suggests that Juzen-taiho-to may prevent not only gastrointestinal toxicity but also vomiting caused by CDDP.

We examined whether Juzen-taiho-to could prevent the lethal toxicity induced by a high-dose of CDDP (4.5–12.0 mg/kg). Treatment with 3.4 g/kg Juzen-taiho-to completely depressed the lethal toxicity of 4.5 mg/kg CDDP, and efficiently prolonged the survival time of the S-180-bearing mice receiving 6.0 to 12.0 mg/kg CDDP (Fig. 6). Twenty-fold the usual daily dose of Juzen-taiho-to (3.4 g/kg) seems to be higher than that of other agents with a reducing effect on CDDP-induced toxicity,^{9,10)} but we consider these levels to be clinically applicable, because the CDDP-induced toxicity was very serious in our model.²¹⁻²³⁾ These findings therefore indicate that Juzen-taiho-to may allow us to administer a much higher dose of CDDP in clinical therapy.

The antitumor activity of CDDP may be explained by DNA cross-linking,²⁷⁻³¹⁾ interactions with cell surface nucleic acids,³²⁾ and/or interactions with the plasma membrane.^{33,34)} Also, CDDP inhibits amino acids, such as methionine uptake into tumor cells.^{33,34)} However, the molecular mechanism(s) of the nephrotoxicity induced by CDDP is still unknown. CDDP nephrotoxicity has been

shown to be reduced by the administration of free radical scavengers such as sodium selenite,³⁵⁾ superoxide dismutase,³⁶⁾ *O*-(β -hydroxyethyl)rutoside,³⁷⁾ and α -tocopherol.³⁸⁾ We considered at first that Juzen-taiho-to inhibits the toxicity induced by CDDP due partly to its anti-oxidative effect, because Kampo formulations do possess anti-oxidative effects.^{39,40)} However, recently Vermeulen *et al.*⁴¹⁾ examined whether CDDP can cause lipid peroxidation *in vitro*, either directly or indirectly, by reducing the microsomal or cytosolic defense mechanisms against lipid peroxidation, and demonstrated that CDDP is not able to induce lipid peroxidation *in vitro* in a variety of rat kidney test systems, either directly or indirectly, by damaging kidney microsomal or cytosolic glutathione-dependent protection mechanisms against lipid peroxidation. They concluded that the protective effect of anti-oxidants such as sodium selenite,³⁵⁾ superoxide dismutase,³⁶⁾ *O*-(β -hydroxyethyl)rutoside,³⁷⁾ and α -tocopherol,³⁸⁾ against CDDP-induced nephrotoxicity in rodents is not due to their anti-oxidative properties. In addition, we demonstrated in our previous report⁶⁾ that a diuretic, furosemide, reduced the antitumor effect of CDDP as well as its toxicity in this model, and we concluded that the protective effect of Juzen-taiho-to against CDDP-induced toxicity may not be due to its diuretic property. Therefore, the mechanism(s) of the protective effect of Juzen-taiho-to is still unknown, but we presume that multiple mechanisms are involved in this effect of Juzen-taiho-to, because Juzen-taiho-to possesses many pharmacological activities.¹⁸⁻²⁰⁾ Further studies to elucidate the multiple mechanisms involved in the protection of CDDP-induced toxicity by Juzen-taiho-to are being conducted in our laboratory.

Acknowledgment We thank Ms. Yoshie Miki and Mr. Yoshitomo Suhara for their excellent technical assistance.

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