Protecting Cisplatin-Induced Nephrotoxicity with Cimetidine Does Not Affect Antitumor Activity

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The present study examined the influence of cimetidine on the nephrotoxicity and antitumor effects of cisplatin *in vitro* and *in vivo*. When the serum concentration of cimetidine was maintained over 20 μg/ml for 4 h by bolus and continuous intravenous infusion, cimetidine prevented nephrotoxicity of cisplatin without influencing antitumor activity. Cimetidine and the antioxidant *N*-acetylcysteine (NAC) significantly inhibited the *in vitro* growth inhibition of cisplatin in cells originating from the kidney, but not in SOSN2 osteosarcoma cells. Cimetidine (1 mM) also did not influence platinum concentration in the cells, regardless of whether the organic cation transporter 2 (OCT2) was expressed. Cisplatin did induce reactive oxygen species (ROS) in the KN41 kidney cell line and cimetidine and NAC significantly reduced ROS production. However, cisplatin did not produce ROS in osteosarcoma cells. From these results, cimetidine clearly inhibits nephrotoxicity induced by cisplatin without any influence on the antitumor activity of cisplatin on osteosarcoma *in vitro* and *in vivo*.

Key words cisplatin; cimetidine; nephrotoxicity; inhibition; reactive oxygen species

Cisplatin is a potent antitumor drug used in many kinds of malignant tumors. In our hospital, cisplatin is an effective treatment for bone soft sarcomas in combination with high-dose caffeine.[1)] Cisplatin binds DNA, causing apoptosis and necrosis in growing tumor cells. However, cisplatin dosage is limited by its nephrotoxicity, and overcoming this limitation is an important issue in cisplatin cancer chemotherapy. Cisplatin has been reported to selectively damage proximal tubule cells through interaction with an organic cation transporter 2 (OCT2),[2,3)] and cisplatin-induced nephrotoxicity is induced by reactive oxygen species (ROS).[4—6)]

Cimetidine, a histamine H_2 antagonist, has been tested in a limited number of patients treated with cisplatin and shown to exert a beneficial effect on renal hemodynamics and to prevent a decline in renal function.[7)] However, the mechanism of cimetidine's action in the prevention of cisplatin-induced nephrotoxicity is not completely clear. One proposed possibility is competitive inhibition of cisplatin transport into OCT2-overexpressing kidney cells.[2,3)] Another is the blockade of iron release from cytochrome P450 and inhibition of ROS generation.[8)] To date, few studies have examined the *in vivo* effects of cimetidine on cisplatin-induced nephrotoxicity. This study confirmed the effect of cimetidine on cisplatin-induced nephrotoxicity and examined the antitumor effects of cisplatin in *in vivo* experiments.

MATERIALS AND METHODS

Materials *cis*-Diammineplatinum II dichloride (cisplatin) and *N*-acetylcystein (NAC) were purchased from Sigma (St. Louis, MO, U.S.A). Cimetidine (Tagamet®) was obtained from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). A Cell Counting Kit-8 was acquired from Dojindo Lab., Co. (Kumamoto, Japan) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Wako Pure Chemical Co. (Osaka, Japan). Oligonucleotide primers were custom-synthesized by Amersham Pharmacia Biotech (U.K.). Other chemicals were of reagent grade.

Animal Experiments All animal procedures were in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi campus of Kanazawa University. Seven-week-old male Fischer rats and male Wistar rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). The rats were divided into four groups (3 to 5 rats in a group): saline-injected control group (saline), cimetidine-alone group intravenously (i.v.) injected cimetidine *via* a jugular vein by bolus injection (19.3 mg/kg) and continuous infusion using a microsyringe pump (IC3100, ICIS, Osaka, Japan) at 27.1 mg/kg/h, according to the protocol as shown in Fig. 1 under light ether anesthesia (cimetidine alone), cisplatin-alone group intraperitoneally (i.p.) injected 1.75 mg/kg of cisplatin for tumor-bearing rats or 7 mg/kg of cisplatin for rats without tumor (cisplatin alone) and combined treatment group injected cisplatin just before cimetidine injection (cisplatin+cimetidine). The doses of cisplatin for tumor-bearing animals and

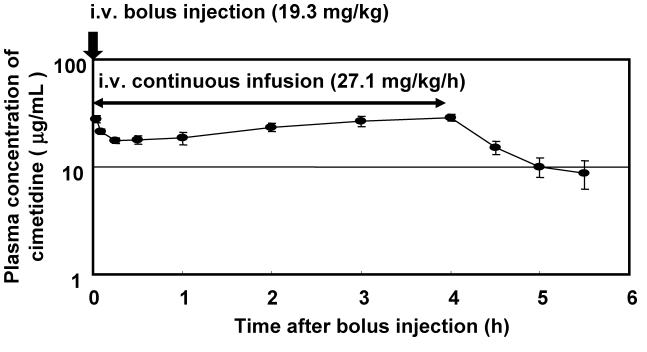

Fig. 1. Cimetidine Injection Protocol and Plasma Concentration

Cimetidine was injected i.v. into rats at a dose of 19.3 mg/kg and then continuously infused at 27.1 mg/kg/h. Plasma concentration of the drug was measured at the indicated times. Data are the means±S.E.

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unbearing animals were determined as the doses to show moderate antitumor effect against SOSN2 osteosarcoma and marked nephrotoxicity, based on the results of the preliminary experiments. The animals were housed in a climate- and light-controlled environment with free access to water and food.

Male Fischer rats were inoculated with a SOSN2 tumor block (about 10 mm^3) subcutaneously on the back. When tumor size reached about 300 mm^3, drug treatments (cisplatin; 1.75 mg/kg, i.p., cimetidine according to the protocol indicated in Fig. 1) were performed. The tumor size was measured every day for 16 d, as below.

$$\text{tumor size } (mm^3) = 1/2 \times \text{major axis} \times (\text{short diameter})^2$$

Male Wistar rats without tumors were treated with cisplatin (7 mg/kg) and cimetidine (according to the protocol indicated in Fig. 1), and blood samples (400 μl) were collected from the tail vein under light anesthesia every day; the rats were killed by cervical dislocation under deep ether anesthesia on day 5. Urine was collected for 24 h on the last day of the experiment. Creatinine and blood urine nitrogen (BUN) were measured in our laboratory using commercial kits (Wako), and other biochemical analyses were done by SRL Inc. (Tokyo, Japan).

Measurement of Cimetidine Serum Concentration One hundred microliters of the serum sample, 25 μl of the internal standard ranitidine (100 μg/ml), and 100 μl NaOH (5 N) were mixed and cimetidine was extracted with 3 ml methylene chloride, dissolved with 100 μl of the mobile phase (5% acetonitrile/0.002 M triethylamine and 0.025% acetic acid), and measured using high-performance liquid chromatography at 228 nm.

Cell Culture and Proliferation Test A human embryonic kidney (HEK293) cell line, human kidney cancer (KN41) cell line, and Madin–Darby canine kidney (MDCK) cell line, human osteosarcoma cell line (OST), and rat osteosarcoma (SOSN2) cell line were kindly provided by the Kanazawa University Cancer Institute. Cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 600 mg/ml kanamycin sulfate (Meiji Seika Co., Tokyo, Japan) in an atmosphere of 5% CO_2 at 37 °C.

Cells (2.5—5$\times 10^3$ cells/100 μl/well) were seeded onto 96-well plates, and after 24 h, treated with varying concentrations of cisplatin in the presence or absence of cimetidine (0.5, 1 mM) or NAC (3 mM) for 48 h. After treatment, cell viability was measured using the Cell Counting Kit-8, based on the reduction activity of mitochondria dehydrogenases, according to the manufacturer's instructions.

Cellular Uptake of Cisplatin For the measurement of cisplatin uptake, confluent growing cells in 100 mm culture dishes were incubated with medium containing cisplatin (500 μM) with or without cimetidine (1 mM) for 2 h. After treatment, the cells were rapidly washed twice with phosphate-buffered saline, then solubilized by 0.5 N NaOH, and the protein content of the cell was determined with a Dc Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Cell fluid solubilized with NaOH was diluted with deionized Milli-Q water (Millipore, Billerica, MA, U.S.A.) five times. Elemental platinum concentrations were measured by flame atomic absorption spectrometry on a model AA-6800 Atomic Absorption Spectrometer (Shimadzu Corporation, Kyoto, Japan). The absorbance of atomized platinum was measured at 14 mA and a wavelength of 265.9 nm with a 0.5-nm slit width. Integrated absorbance with a read time of 5 s was recorded. The standard curves were linear over a range of 0.5 to 50 μg/ml. All measurements were performed in triplicate. The cellular platinum levels were expressed as μg platinum per mg protein.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis Total RNA was isolated from cells with Isogen (Nippon Gene Co., Ltd., Toyama, Japan). cDNA was prepared by reverse transcription of each RNA sample at 37 °C for 2 h, and amplified with a Peltier Thermal Cycler PTC-200 (Bio-Rad Laboratories). The following primers were used:

OCT2[9]

Sense 5′-CAGGGACTGGTCAGCAAAGCAGGCTGGT-T-3′

Antisense 5′-GGCCATGGTGCCCATTCAACCCAAGC-3′

β-Actin[10]

Sense 5′-TTCTACAATGAGCTGCGTGTGGC-3′

Antisense 5′-CTCGTAGCTCTTCTCCAGGGAGGA-3′

The amplified products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide.

Measurement of Reactive Oxygen Species (ROS) ROS production in cells was assessed using the fluorescent probe H_2DCFDA. Cells (1×10^4/100 μl/well) were incubated for 24 h, washed, and incubated with cisplatin (100 μM or 300 μM) in the presence or absence of cimetidine (1 mM) or NAC (3 mM) and H_2DCFDA (10 μM) for 1 h. Intracellular ROS were detected at an excitation wavelength of 485 nm and emission wavelength of 530 nm using a fluorescence plate reader.

Statistical Analysis Statistical analysis were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, U.S.A.). For multiple comparisons, two-way ANOVA (analysis of variance) with Bonferroni's *post hoc* test was performed first. And if an interaction effect between two elements (the treatment, type of cell line and biometrics) was observed, one-way ANOVA with Dunnett's or Tukey's *post hoc* tests were performed for each cell lines or biometrics. Student's *t*-test was performed for comparison between two independent groups. The criterion of significance was taken to be $p<0.05$.

RESULTS

***In Vivo* Experiments** In our previous experiment, serum concentrations of cisplatin after i.p. injection (3.5 mg/kg) into rats resulted in a 24 h half-life, but cisplatin accumulated in the kidney for 48 h.[11] Due to this rapid clearance documented in rats,[12] high serum concentrations of cimetidine after cisplatin injection were maintained by an i.v. bolus injection and continuous infusion for 4 h, as shown in Fig. 1. The serum concentration of cimetidine was maintained over 20 μg/ml for 4 h using this injection protocol.

We initially examined whether this cimetidine dosage influences the antitumor effect of cisplatin. Figure 2 shows the changes in tumor mass after treatment with cisplatin

(1.75 mg/kg, i.p.) and cimetidine in SOSN2-bearing rats. Cisplatin significantly inhibited tumor growth, and at 16 d after treatment, the tumor mass of the cisplatin-alone group was one-third that of the control group. Cimetidine had little influence on tumor growth in the control group or in combination with cisplatin.

Next, a nephrotoxic dose of cisplatin (7 mg/kg, i.p.) was administered to non-tumor rats, and the effect of cimetidine on the kidney function was examined. After administering cisplatin, kidney weight was significantly elevated, and this increase tended to be restrained by the addition of cimetidine, which when administered alone did not affect kidney weight (Table 1). As shown in Figs. 3 and 4, the serum creatinine and BUN levels significantly increased after the third day of cisplatin injection, and cimetidine coadministration significantly inhibited the increase of these biomarkers. As a result, rats fell into serious renal damage 5 d after treatment with cisplatin, and the combined treatment with cimetidine clearly reduced the damage (Table 2). In histopathology, many necrotic and apoptotic changes were appeared in the epithelium cell of the renal tubule in the cisplatin treated group, but the degree and range of the injury was apparently decreased by the combination of cimetidine.

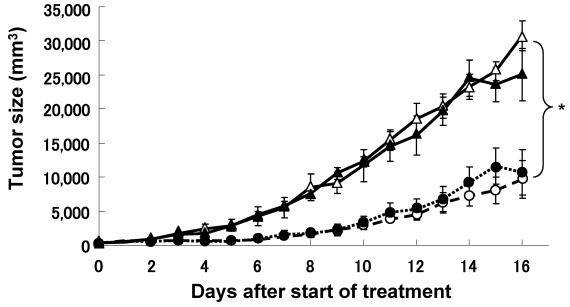

Fig. 2. Influence of Cimetidine on the Antitumor Effect of Cisplatin in Rats Bearing SOSN2 Osteosarcomas

Cisplatin (1.75 mg/kg) was injected i.p., and cimetidine immediately administered i.v. to rats, according to the protocol indicated in Fig. 1. Tumor volume was measured every 24 h for up to 16 d. △, Untreated control (n=5); ▲, cimetidine alone (n=3); ○, cisplatin alone (n=5); ●, cisplatin+cimetidine (n=4). Each data point represents the mean±S.E. $*p<0.05$; repeated-measures two-way ANOVA with Bonferroni's *post hoc* test (untreated control *vs.* cisplatin alone and *vs.* cisplatin+cimetidine).

Table 1. Body, Liver, and Kidney Weight at 5 d after Start of Treatment

Treatment	Body weight (g)	Liver weight (g) (% of body weight)	Kidney weight (g) (% of body weight)
Saline	252±32	10.2±1.0 (4.05±0.41) *	0.83±0.09 (0.33±0.04) *
Cisplatin alone	206±14	8.03±0.81 (3.90±0.35)	1.06±0.05 (0.51±0.03) *
Cimetidine alone	244±37	10.0±1.2 (4.10±0.42)	0.78±0.10 (0.32±0.01) *
Cisplatin+cimetidine	210±15	8.53±0.97 (4.06±0.16)	0.98±0.04 (0.46±0.03)

Each value represents the mean±S.D. (n=3—4). $*p<0.05$; One-way ANOVA with Tukey's *post hoc* test.

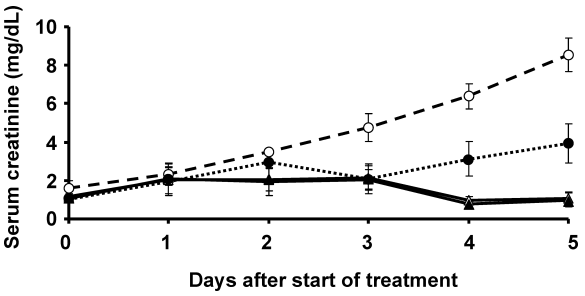

Fig. 3. Serum Creatinine Concentrations in Non-tumor-bearing Rats

The treatment protocol was the same as in Fig. 2 except for the cisplatin dose (7 mg/kg). △, Untreated control; ▲, cimetidine alone; ○, cisplatin alone; ●, cisplatin+cimetidine. Each data point represents the mean±S.E. (n=3—4).

***In Vitro* Experiments** Cimetidine significantly reversed the growth inhibitory effect of cisplatin on cells originating from the kidney (KN41, HEK293, MDCK) in a concentration-dependent manner (Table 3). The combination effect of cimetidine on the KN41 cell line was the most prominent among these kidney cell lines. The antioxidant NAC also inhibited cisplatin cytotoxicity in KN41 cells and the effect was greater than that of cimetidine. However, neither agent was able to inhibit cisplatin cytotoxicity in SOSN2 osteosarcoma cells (Fig. 5). Some investigators have suggested that cimetidine competitively inhibits cisplatin transport *via*

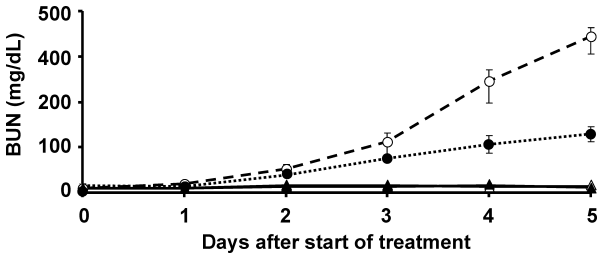

Fig. 4. Changes of BUN Concentration after the Start of Treatment in Non-tumor-bearing Rats

The treatment protocol was the same as in Fig. 2 except for the cisplatin dose (7 mg/kg). △, Untreated control; ▲, cimetidine alone; ○, cisplatin alone; ●, cisplatin+cimetidine. Each data point represents the mean±S.E. (n=3—4).

Table 2. Laboratory Data at 5 d after Start of Treatment

Treatment	AST (IU/ml)	ALT (IU/ml)	BUN (mg/dl)	CLcr (ml/h)
Saline	81.3±5.7	40.8±6.4	13.7±0.9 *	29.6±11.6 *
Cisplatin alone	101±21	48.1±9.2	443±38 * *	0.93±0.76 *
Cimetidine alone	86.1±5.8	42.8±7.3	11.3±1.5 * *	33.3±11.5 *
Cisplatin+cimetidine	88.5±7.6	44.2±7.3	129±29	12.5±3.4

Each value represents the mean±S.D. (n=3—4). $*p<0.05$; One-way ANOVA with Tukey's *post hoc* test.

Table 3. Effect of Cimetidine on Cell Growth Inhibition of Cisplatin in KN41, HEK293, MDCK, and SOSN2 Cells

Treatment	IC_{50} (μM)			
	KN41	HEK293	MDCK	SOSN2
Cisplatin alone (control)	11.8±3.1	2.42±0.65	16.1±5.6	20.3±6.8
Cisplatin+0.5 mM cimetidine	17.5±5.8	3.99±0.94*	28.0±8.1	—
Cisplatin+1 mM cimetidine	35.9±3.5*	5.16±0.53*	35.9±9.6*	25.5±7.2

Each value represents the mean±S.D. (n=3—4). $*p<0.05$; One-way ANOVA with Dunnett's *post hoc* test.

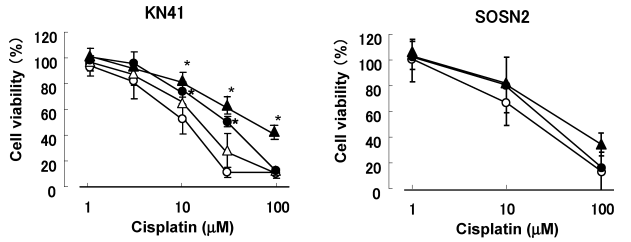

Fig. 5. Effect of Cimetidine on the Inhibition of Cell Growth by Cisplatin in KN41 Human Kidney Cancer Cells and SOSN2 Rat Osteosarcoma Cells

Cells were treated with varying concentrations of cisplatin in the presence or absence of cimetidine for 48 h. ○, Cisplatin alone (control); △, combined with 0.5 mM cimetidine; ●, 1 mM cimetidine or ▲, 3 mM NAC. Each data point represents the mean±S.E. $*p<0.05$; One-way ANOVA with Dunnett's *post hoc* test.

Table 4. Cellular Platinum Concentration after Treatment with Cisplatin and Cimetidine for 2 h in KN41, MDCK, and SOSN2 Cells

Treatment	KN41	MDCK (μg/mg protein)	SOSN2
Cisplatin (500 μM) alone	0.843±0.096	0.655±0.018	0.784±0.174
Cisplatin (500 μM)+1 mM cimetidine	0.731±0.059	0.753±0.021	0.781±0.040

Each value represents the mean±S.D. ($n=4$). No significance was observed by either two-way ANOVA or Bonferroni's test at $p<0.05$.

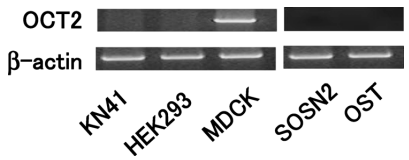

Fig. 6. mRNA Expression of OCT2 in KN41, HEK293, MDCK, SOSN2, and OST Cells

The size of the RT-PCR products is 599 bp (hOCT2) and 711 bp (cOCT2).

OCT2 into kidney cells.[2,3] To test this postulate, the effect of cimetidine on the intracellular platinum concentration was examined. Cellular platinum levels were not changed by 1 mM cimetidine (Table 4), even in MDCK cells that express OCT2 mRNA (Fig. 6). OCT2 mRNA expression was not detected in other kidney cells or osteosarcoma cells. Furthermore, mRNA expression of other transporters for cisplatin, such as the multidrug and toxic compound extrusion (MATE) family,[13] was not detected in any of the cells used in this study (data not shown).

We also examined ROS production by cisplatin and the influence of cimetidine in KN41 kidney cancer cells and SOSN2 osteosarcoma cells. As shown in Fig. 7, cisplatin induced ROS in a concentration-dependent manner in KN41 cells, and an antioxidant NAC significantly decreased ROS production. Cimetidine also inhibited the ROS production by cisplatin. In contrast, ROS were not produced by cisplatin in osteosarcoma cells and effects of cimetidine and NAC were also not observed.

DISCUSSION

This study clearly indicates that a high dose and continuous infusion of cimetidine reduces nephrotoxicity without having any influence on the antitumor activity of cisplatin *in vitro* and *in vivo*. Rats developed serious renal damage at 3 or

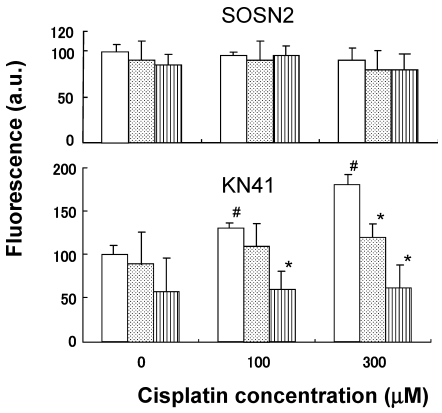

Fig. 7. ROS Production by Cisplatin and the Effects of Cimetidine and NAC *in Vitro*

Cells were treated with the indicated concentrations of cisplatin in the presence or absence of 1 mM cimetidine or 3 mM NAC together with H_2DCFDA for 1 h, and then fluorescence was measured. □, Cisplatin alone; ▨, combined with 1 mM cimetidine; ▥, 3 mM NAC. Each column represents the mean±S.E. ($n=3$—4). $\#p<0.05$; One-way ANOVA with Dunnett's *post hoc* test (*vs.* untreated control). $*p<0.05$; One-way ANOVA with Dunnett's *post hoc* test (*vs.* cisplatin alone).

4 d after treatment with cisplatin, and when the serum concentration of cimetidine was maintained over 20 μg/ml for 4 h, renal function clearly recovered. Moreover, cimetidine inhibited cisplatin cytotoxicity in cell lines originating from the kidney at 0.5 to 1 mM. The concentration of cimetidine in the kidney has been reported to reach 20- to 40-fold of the serum concentration 3 min after intravenous injection into rats.[14] Thus, in this study, the possibility exists that the concentration of cimetidine in the kidney reached the 1 mM concentration used *in vitro*.

Renal tubule cells express substantial OCT2 protein in the membrane and this transporter is believed to actively uptake cisplatin.[15] Cimetidine has been reported to competitively inhibit cisplatin transport through OCT2 and affects cell toxicity.[2,3] However, in this study, cimetidine inhibited cisplatin cytotoxicity in kidney cells but not in osteosarcoma cells (Table 3, Figs. 5, 6). Cimetidine also did not affect cellular platinum concentration in all cell lines, even in MDCK cells expressing OCT2 mRNA (Table 4). The established cell lines used in this study did not express the transporter or if the mRNA were expressed, the function of the transporter may have been lost. Therefore, determining whether the *in vitro* reduction of cisplatin-induced cytotoxicity by cimetidine is due to actions on the OCT2 transporter is difficult. However, we believe it likely that the *in vivo* nephrotoxicity of cisplatin and the inhibition by cimetidine might be provided through the OCT2 transporter since renal tubule cells in the body intrinsically express OCT2.

The antitumor activity of cisplatin is due to DNA alkylation, but undesirable side effects of cisplatin such as nephrotoxicity and hepatotoxicity at high doses have been reported to be induced by oxidative stress.[4—6,16] In this study, we showed that when cisplatin is administered at rates i.p. of 7 mg/kg, kidney function clearly decreases within days without liver damage, and nephrotoxicity is significantly inhibited by a combined treatment with cimetidine (Figs. 3, 4). Baliga *et al.* reported that renal damage was clearly observed 3 d after i.p. injection of cisplatin (10 mg/kg), but was significantly reduced by combination with a P450 inhibitor, piper-

onyl butoxide, accompanied with a decrease in P450 content in the kidney.[8] In that paper, they also reported that cisplatin increased catalytic iron, and cimetidine suppressed iron release and cytotoxicity in pig kidney LLC-PK1 cells. The iron ion released by cisplatin through P450 is thought to act on a peroxide and promote the Fenton reaction to produce ROS. Cimetidine may reduce cytotoxicity by binding to the heme protein in P450 and inhibiting enzyme activity and iron release. In the present study, intracellular ROS also increased after treatment with cisplatin in a concentration-dependent manner in KN41 kidney cancer cells, and combined treatment with cimetidine significantly decreased ROS production, as well as a control antioxidant agent NAC. However, these phenomena were not observed in SOSN2 osteosarcoma cells (Figs. 7, 8). Among cytochrome P450 (CYP) species, CYP2E1, which is poorly coupled with NADPH-cytochrome P450 reductase, recently became the most promising source of ROS in cisplatin-induced tissue injury.[17,18] Whether osteosarcoma cells express CYP2E1 is not known, although kidney cells are well-known to constantly express the CYP species. Taken together with results from the present study, cisplatin cytotoxicity *in vitro* may be caused by the actions of ROS induced by CYP2E1. Inhibition of cisplatin cytotoxicity by cimetidine may therefore stabilize CYP species and restrain iron release. A consideration of other mechanisms must also be considered to completely elucidate the inhibition of cisplatin-induced ROS by cimetidine.

In conclusion, cimetidine clearly inhibits nephrotoxicity and kidney cell damage induced by cisplatin without any influence on the antitumor activity of cisplatin *in vitro* and *in vivo*. Although the *in vitro* actions of cimetidine were primarily due to the inhibition of ROS production, the *in vivo* protective effect of cimetidine on the nephrotoxicity of cisplatin seems to be due to both inhibitory actions on ROS production and OCT2 intrinsically expressed in the kidney. Consequently, cimetidine may be useful to avoid nephrotoxicity in intensive chemotherapy containing cisplatin. However, because cisplatin is a potent kidney poison, there is not so large estrangement between the dose used for rats in this study and the clinical dose, but because the serum concentration of cimetidine in this study was dozens of times of the clinical effective concentration, for practical use we will need agents showing more high affinity to OCT2 and/or P450.

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