Combining cisplatin with cationized catalase decreases nephrotoxicity while improving antitumor activity

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Cisplatin is frequently used to treat solid tumors; however, nephrotoxicity due to its reactive oxygen species-mediated effect limits its use. We tested the ability of cationized catalase, a catalase derivative, to inhibit nephrotoxicity in cisplatin-treated mice. Immunohistochemical analysis showed that the catalase derivative concentrated in the kidney more efficiently than native catalase. Repeated intravenous doses of cationized catalase significantly decreased cisplatin-induced changes in serum creatinine, blood urea nitrogen, nitrite/nitrate levels, lactic dehydrogenase activity, and renal total glutathione and malondialdehyde contents. In addition, cationized catalase effectively blunted cisplatin-induced proximal tubule necrosis but had no significant effect on the cisplatin-induced inhibition of subcutaneous tumor growth. Repeated doses of catalase, especially cationized catalase, significantly increased the survival of cisplatin-treated tumor-bearing mice preventing cisplatin-induced acute death. Our studies suggest that catalase and its derivatives inhibit cisplatin-induced nephrotoxicity, thus improving the efficiency of cisplatin to treat solid tumors.

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Cisplatin (*cis*-diammine-dichloroplatinum II) is one of the most frequently used anticancer agents for the treatment of solid tumors, including ovarian, testicular, bladder, head and neck, osteogenic, and uterine cervix carcinomas. Unfortunately, chemotherapy using high-dose cisplatin is often accompanied by serious side effects that affect the peripheral neurons and cochlea of the kidney, and these side effects sometimes lead to termination of the treatment or a reduction in the dose.[1,2] Although the mechanism underlying cisplatin-induced nephrotoxicity is not yet fully understood, reactive oxygen species (ROS) have been widely implicated in the toxicity.[3–5] Therefore, various antioxidant enzymes and antioxidants, including superoxide dismutase, catalase, glutathione peroxidase, glutathione (GSH), selenium, flavonoids, and diethyldithiocarbamates, have been investigated as compounds able to protect against cisplatin-induced nephrotoxicity.[6–8]

Although various ROS could be involved in the ROS-mediated, cisplatin-induced nephrotoxicity, hydrogen peroxide is considered to be an effective target molecule to inhibit this toxicity. This is because (i) superoxide anion, which is initially produced, is spontaneously or enzymatically (by superoxide dismutase) converted to hydrogen peroxide and (ii) hydrogen peroxide is stable and has a long half-life. If this is the case, catalase, an enzyme that degrades hydrogen peroxide into oxygen and water, would be a good candidate for the inhibition of cisplatin-induced nephrotoxicity. However, catalase (from bovine liver) is not significantly distributed to the kidney after intravenous injection[9,10] and this severely limits its therapeutic efficacy against such toxicity.

A series of catalase derivatives with different physicochemical properties have been developed in our laboratory, each of which showed unique tissue distribution characteristics.[10] Our previous studies using these catalase derivatives[10–21] clearly indicate that catalase can be highly effective in inhibiting various ROS-mediated injuries once delivered to the region of interest. Therefore, a catalase derivative targeting the kidney would be useful for inhibiting cisplatin-induced nephrotoxicity.

Cationic macromolecules have the ability to interact with the kidneys after entering the blood circulation, as clearly demonstrated with charged dextrans.[22] We have recently synthesized a cationized derivative of bovine liver catalase, that is, ethylenediamine (ED)-conjugated catalase (ED-catalase), and showed that it can protect hepatocytes from carbon tetrachloride-induced acute liver failure in mice.[14] Although ED-catalase does not accumulate to a high degree in the kidneys because of its extensive uptake by the liver, an increase in the amount of catalase delivered to the kidney may be sufficient to inhibit cisplatin-induced nephrotoxicity.

In this study, we first demonstrated that ED-catalase is more efficiently delivered to the kidneys than catalase. Then, the effects of ED-catalase on cisplatin-induced nephrotoxicity were examined in mice by evaluating blood urea nitrogen (BUN), serum creatinine, nitrite/nitrate levels, lactate dehydrogenase (LDH) activity, and the total GSH content and malondialdehyde (MDA) levels in the kidney. Its effects on the antitumor activity of cisplatin were also investigated in tumor-bearing mice.

RESULTS

Renal accumulation of catalase and ED-catalase in mice

Figure 1 shows the immunohistochemical staining of catalase derivatives in mouse kidneys. Compared with the kidney section from an untreated mouse (Figure 1a), one from a mouse receiving an intravenous injection of catalase at a dose of 1 mg kg^{-1} (40 000 U kg^{-1}) showed faint catalase staining in the proximal tubule regions of the kidney (Figure 1c). To confirm the renal distribution of catalase, a very high dose of 100 mg kg^{-1} (4 000 000 U kg^{-1}) was used, which gave detectable signals in the proximal tubules (Figure 1b). On the other hand, the injection of ED-catalase at the low dose of 1 mg kg^{-1} (18 000 U kg^{-1}, 450 U mouse^{-1}) resulted in an intense staining in the same regions (Figure 1d), suggesting that ED-catalase is more efficiently delivered to the kidney than unmodified catalase. The total renal catalase activity was hardly changed by administration of catalase derivatives (data not shown), because of a very high endogenous catalase activity in mouse kidneys ($14\,200 \pm 2200$ U g^{-1} kidney, 5230 ± 840 U kidney^{-1}).

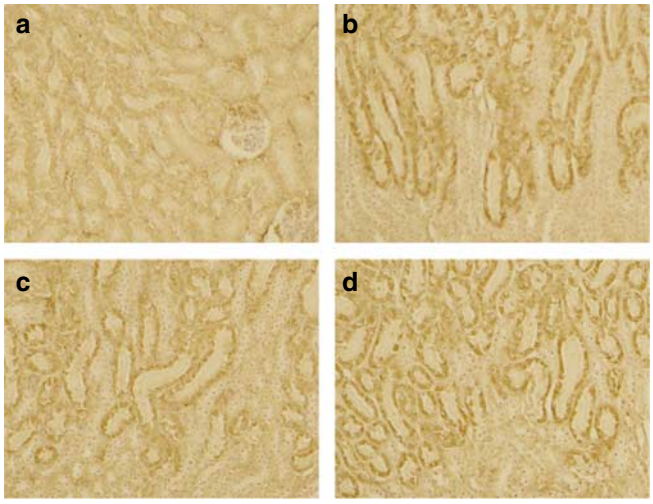

Figure 1 | Immunohistochemical staining of catalase and ED-catalase in mouse kidneys (× 100 original magnification). Catalase or ED-conjugated catalase was injected into the tail vein of mice at a dose of 1 mg kg^{-1}. At 30 min after injection, the kidneys were excised and paraffin-embedded sections were immunostained for bovine catalase using biotin-conjugated anti-bovine catalase antibody, followed by incubation with peroxidase-conjugated antibody. (**a**) Untreated, (**b**) high-dose catalase (100 mg kg^{-1}), (**c**) catalase, and (**d**) ED-catalase.

Single-dose cisplatin-induced nephrotoxicity and its inhibition by catalase

An intraperitoneal (i.p.) injection of cisplatin at a dose of 20 mg kg^{-1} induced serious injuries in mice, and all mice had died by 5 days after the injection. Cisplatin also caused significant weight loss in mice (Figure 2a). In addition, the BUN and serum creatinine levels were significantly increased by the injection of cisplatin 2 days after injection (Figure 2b and c). Administration of catalase (5000−50 000 U kg^{-1} shot^{-1}, seven injections) significantly reduced the cisplatin-induced changes in body weight (Figure 2d) and the BUN and serum creatinine levels (Figure 2e and f) in a dose-dependent manner.

Effect of ED-catalase on single-dose cisplatin-induced nephrotoxicity

Figure 3 shows the effects of catalase and ED-catalase on the nephrotoxicity induced by a single dose (20 mg kg^{-1}, i.p.) of cisplatin. Both catalase and ED-catalase (10 000 U kg^{-1} shot^{-1}, seven injections) significantly reduced the increase in BUN (Figure 3a), serum creatinine (Figure 3b), nitrite/nitrate (Figure 3c) levels, and LDH activity (Figure 3d). The reduction in the renal total GSH content was inhibited only by ED-catalase ($P<0.05$; Figure 3e). The increase in the renal MDA level was also significantly ($P<0.01$) inhibited only by ED-catalase (Figure 3f). For all parameters measured, ED-catalase produced greater changes than catalase, and the differences were significant for BUN ($P<0.01$) and renal MDA levels ($P<0.05$). These results indicate that ED-catalase is more effective than catalase in protecting against high-dose cisplatin-induced nephrotoxicity, and that the co-administration of ED-catalase allows an increase in the cisplatin dose for anticancer therapy. The administration of cisplatin (20 mg kg^{-1}, i.p.) to mice resulted in severe necrosis in the proximal tubules, with extensive epithelial vacuolization, swelling, and tubular dilatation (Figure 3h), which was markedly inhibited in catalase- or ED-catalase-treated mice (Figure 3i and j). The glomeruli appeared normal in all the groups studied. The graded histological changes are summarized in Table 1. Compared with the saline-treated mice, the catalase- and ED-catalase-treated groups exhibited much less significant changes. The platinum content in the kidneys of the saline-treated mice was 1.01 ± 0.13 μg kidney^{-1}, which was not significantly ($P=0.1$) different from that of the ED-catalase-treated mice (0.77 ± 0.18 μg kidney^{-1}).

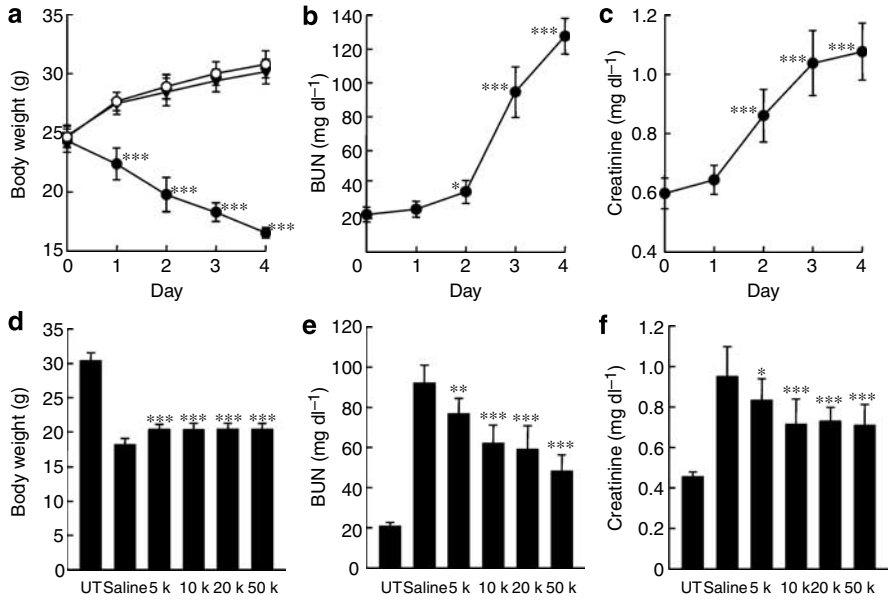

Figure 2 | Effect of catalase on single-dose cisplatin-induced nephrotoxicity. (**a**, **d**) Body weight, (**b**, **e**) BUN, and (**c**, **f**) serum creatinine levels in mice. (**a**–**c**) Mice received an i.p. injection of saline (vehicle) or cisplatin (20 mg kg^{-1}). ○, naïve mice; ▼, saline-injected; ●, cisplatin-injected. Results are expressed as the mean ± s.d. of five mice. *$P<0.05$, ***$P<0.001$; statistically significant difference compared with the saline-treated group. (**d**–**f**) Effect of catalase on cisplatin-induced changes 72 h after i.p. injection of cisplatin (20 mg kg^{-1}). Catalase (5000–50 000 U kg^{-1} shot^{-1}) was injected into the tail vein of mice twice a day, beginning 12 h before the cisplatin injection and continued for seven doses at 12-h intervals. Mice were euthanized 72 h after the cisplatin injection. UT, untreated; 5–50k, catalase-treated at a dose of 5000–50 000 U kg^{-1} shot^{-1}. Results are expressed as the mean ± s.d. of five mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$; statistically significant difference compared with the saline-treated group.

Effect of catalase derivatives on nephrotoxicity induced by repeated doses of cisplatin

Although ED-catalase was highly effective in inhibiting cisplatin-induced nephrotoxicity in mice treated with the high dose of cisplatin, all mice were dead in less than 5 days irrespective of the treatment. Then, a more moderate regimen for cisplatin administration was used for the following experiments. Repeated administration of low-dose cisplatin (5 mg kg^{-1}, three times) also resulted in significant changes in the parameters investigated (Figure 4). Again, catalase and ED-catalase (10 000 U kg^{-1} shot^{-1}, five injections) significantly attenuated the increase in BUN (Figure 4a) and LDH activity (Figure 4d). Furthermore, ED-catalase was also effective in inhibiting the elevation in the renal MDA level (Figure 4f). The platinum contents in the kidneys of the saline- and the ED-catalase-treated mice were 0.86 ± 0.24 and 1.19 ± 0.17 μg kidney^{-1}, respectively, and there was no significant difference between them ($P = 0.07$).

Effect of catalase derivatives on the antitumor activity of cisplatin in tumor-bearing mice

Figure 5a shows the number of B16-BL6/Luc cells in the subcutaneous tumor tissue 21 days after inoculation. Repeated doses of low-dose cisplatin (5 mg kg^{-1}, three times; the saline-treated group) were effective in significantly ($P<0.001$) reducing the number of tumor cells to less than 10% of those in the untreated group. Co-administration of catalase or ED-catalase (10 000 U kg^{-1} shot^{-1}, five injections) with cisplatin had little effect on the antitumor activity of cisplatin. The volume of subcutaneous S180 tumor tissues was also greatly inhibited by cisplatin (Figure 5b), and no significant differences were observed between the saline-treated group and the catalase- or ED-catalase-treated groups. These results indicate that, although the treatment with catalase or ED-catalase is effective in reducing cisplatin-induced nephrotoxicity, neither of them interferes with the antitumor activity of cisplatin.

Effect of catalase derivatives on the survival of tumor-bearing mice

Untreated B16-BL6-bearing mice started to die on day 37 after inoculation of tumor cells, and all had died by day 50 (Figure 5c). As reported previously, the repeated doses of cisplatin (5 mg kg^{-1}, three times) resulted in the acute death of mice, probably due to acute renal failure; 7 out of 12 B16-BL6-bearing mice, treated with saline, were dead by day 37. However, some of the animals survived longer than the untreated mice, and there was no significant difference in the survival between the untreated and the cisplatin-injected, saline-treated groups. The administration of catalase or ED-catalase (10 000 U kg^{-1} shot^{-1}, five injections) reduced the number of mice suffering from cisplatin-induced acute death, and the survival rates of the cisplatin/catalase- and cisplatin/ED-catalase-treated mice were significantly ($P<0.001$) longer than those of cisplatin/saline-treated mice. Furthermore, mice receiving ED-catalase lived significantly ($P<0.01$)

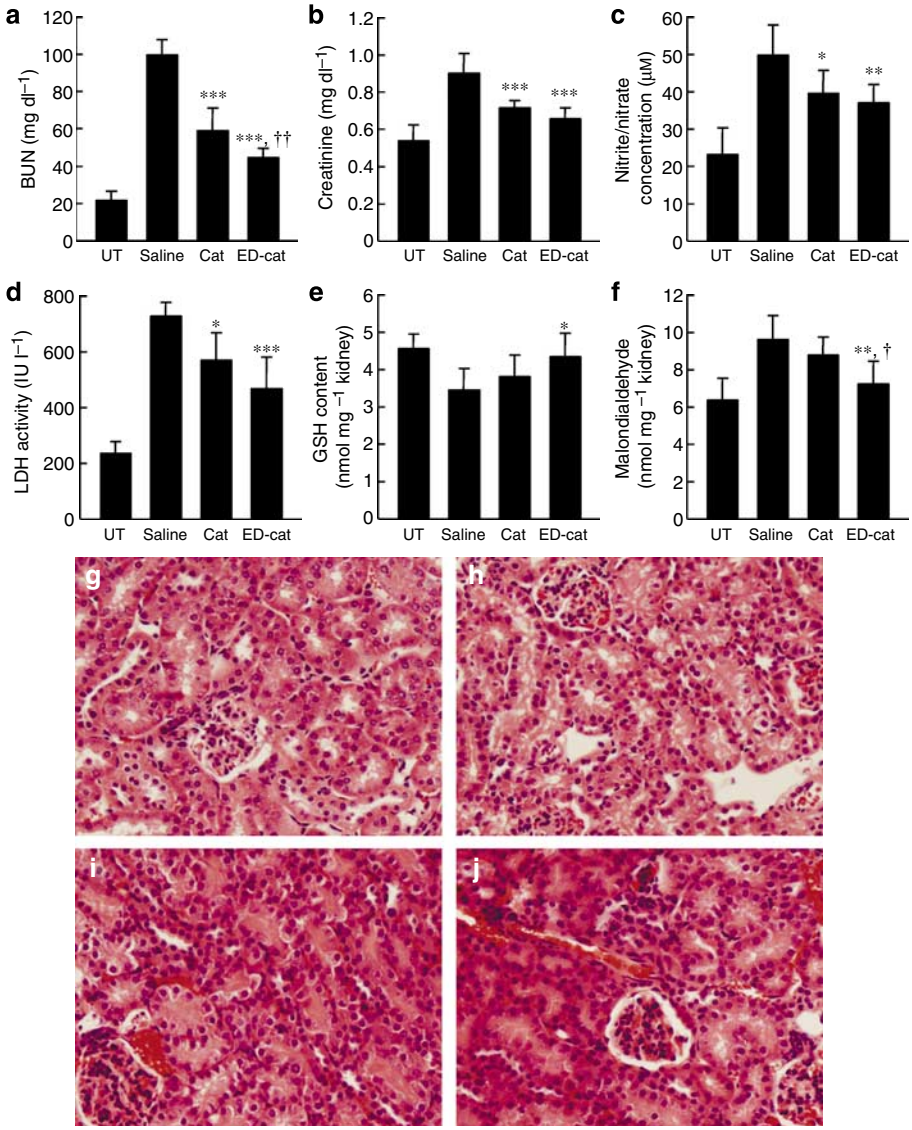

Figure 3 | Effect of catalase and ED-catalase on single-dose cisplatin-induced nephrotoxicity. Effect of catalase and ED-catalase on cisplatin-induced changes in (**a**) BUN, (**b**) serum creatinine, (**c**) nitrite/nitrate levels and (**d**) LDH activity, (**e**) renal total GSH content and (**f**) MDA level, and (**g–j**) hematoxylin and eosin staining of kidney sections (× 100 original magnification) 72 h after i.p. injection of cisplatin (20 mg kg^{-1}). Catalase or ED-conjugated catalase (10 000 U kg^{-1} $shot^{-1}$) was injected into the tail vein of mice twice a day, beginning 12 h before the cisplatin injection and continued for seven doses at 12-h intervals. Mice were euthanized 72 h after the cisplatin injection. UT, untreated; Cat, catalase; ED-cat, ED-catalase. Results are expressed as the mean ± s.d. of 4–6 mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; statistically significant difference compared with the saline-treated group. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$; statistically significant difference compared with the catalase-treated group. (**g–j**) At 72 h after i.p. injection of cisplatin at a dose of 20 mg kg^{-1}, kidneys were removed and 4-μm sections were prepared and stained with hematoxylin and eosin. (**g**) Naïve mice; (**h–j**) for mice receiving cisplatin, (**h**) saline, (**i**) catalase or (**j**) ED-catalase was injected into the tail vein twice a day, beginning 12 h before the cisplatin injection and continued for seven doses at 12-h intervals, at a dose of 10 000 U kg^{-1}.

longer than those given catalase, suggesting that ED-catalase is more effective than catalase in increasing the lifespan of B16-BL6-bearing, cisplatin-treated mice. Figure 5e shows the survival of mice that were alive at 37 days after cisplatin treatment: 5, 8, and 9 out of 12 mice for the saline-, catalase-, and ED-catalase-treated groups, respectively. As clearly demonstrated, ED-catalase significantly ($P<0.001$) increased the survival of these mice, suggesting that ED-catalase has some antitumor activity, in addition to its activity in preventing cisplatin-induced acute death. Figure 5d and f shows the survival of S180-bearing mice; similar but slightly less significant effects of catalase and ED-catalase were observed.

DISCUSSION

The mechanism of cisplatin-induced subcellular and molecular toxicity is not fully understood, but it has been shown that cisplatin is capable of binding to several cellular components, including membrane phospholipids, thiols, cytoskeletal microfilaments, proteins, RNA, and DNA.[23]

In addition, ROS have been implicated as important mediators of the acute renal failure induced by cisplatin and other toxic agents, such as gentamicin and cyclosporine A.[24] This study clearly demonstrated that the cisplatin-induced changes in the serum and renal parameters can be inhibited by catalase in a dose-dependent manner (Figure 2). These results suggest that hydrogen peroxide plays a major role in these toxic effects, which has also been demonstrated in previous *in vitro* studies.[25,26]

In general, high enzymatic activities of catalase and glutathione peroxidase resulting in degradation of hydrogen peroxide can be detected in various tissues and cells, especially in erythrocytes, liver, and kidneys. In this study, the renal catalase activity was estimated to be 5230 ± 840 U kidney^{-1}, which was much greater than the dose of catalase of 450 U mouse^{-1} (18 000 U kg^{-1}) or less. In spite of high endogenous activities of catalase and other antioxidant enzymes, antioxidant enzymes administered exogenously are needed to prevent the toxic effects induced by 100% oxygen[9] or by administration of hydrogen peroxide.[20] We have reported that targeted delivery of catalase to the liver is effective in inhibiting hepatic ischemia/reperfusion injuries,[10–13] carbon tetrachloride-induced acute liver failure,[14] and hepatic metastasis of tumor cells.[15–17] In separate investigations, polyethyleneglycol-conjugated catalase was highly effective in inhibiting pulmonary metastasis and peritoneal dissemination of tumor cells.[18–21] As the molecular weight of bovine liver catalase (220 000) is much greater than the threshold of glomerular filtration of the kidneys ($\sim$40 000), catalase derivatives may distribute to the kidneys, especially to the proximal tubules, from the capillary side of the epithelial cells in the tubules. This is similar to the situation of a cationized derivative of superoxide dismutase; using isolated perfused rat kidneys, we clearly demonstrated that cationized superoxide dismutase is taken up by the organ from the capillary side following an electrostatic interaction.[27,28] Renal accumulation of ED-catalase was clearly demonstrated by immunohistochemistry, but the total renal catalase activity was not significantly changed by the administration of ED-catalase because of the high endogenous catalase activity. However, a significant reduction in the cisplatin-induced increase in the renal MDA level, an indicator of oxidative stress, by ED-catalase strongly

Table 1 | Semiquantitative analysis of individual histological grades of kidney damage in mice.

Group	Total number analyzed	Histological grade				
		0	I	II	III	IV
Untreated	4	3	1			
Saline, cisplatin	5				2	3
Catalase, cisplatin	5			4	1	
ED-catalase, cisplatin	5			5		

ED, ethylenediamine.
Mice received intraperitoneal injections of 20 mg kg^{-1} cisplatin. Saline, catalase or ED-conjugated catalase (10 000 U kg^{-1} shot^{-1}) was administered into the tail vein of mice twice a day, beginning 12 h before the cisplatin injection and this was continued for seven doses at 12-h intervals. Animals were euthanized 72 h after the cisplatin injection.

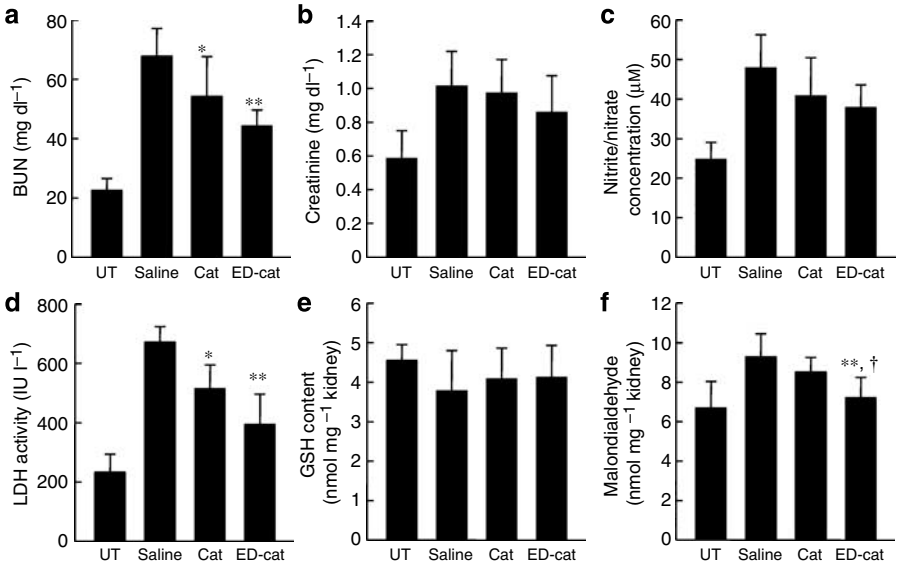

Figure 4 | Effect of catalase and ED-catalase on nephrotoxicity induced by repeated doses of cisplatin. Effect of catalase and ED-catalase on changes produced by repeated doses of cisplatin involving (**a**) BUN, (**b**) serum creatinine, (**c**) nitrite/nitrate levels and (**d**) LDH activity, and (**e**) the renal total GSH content and (**f**) MDA level 96 h after the first i.p. injection of cisplatin. Cisplatin was injected at a dose of 5 mg kg^{-1} into the peritoneal cavity on days 0, 1, and 2 at 24-h intervals. Catalase or ED-conjugated catalase (10 000 U kg^{-1} shot^{-1}) was injected into the tail vein of mice at 12 h before the first injection of cisplatin. The second injection was given to mice immediately after the first injection of cisplatin, and three additional doses were administered at 24-h intervals. Mice were euthanized 96 h after the first cisplatin injection. UT, untreated; Cat, catalase; ED-cat, ED-catalase. Results are expressed as the mean $\pm$ s.d. of 5–8 mice. *$P<0.05$, **$P<0.01$; statistically significant difference compared with the saline-treated group. $^{\dagger}P<0.05$; statistically significant difference compared with the catalase-treated group.

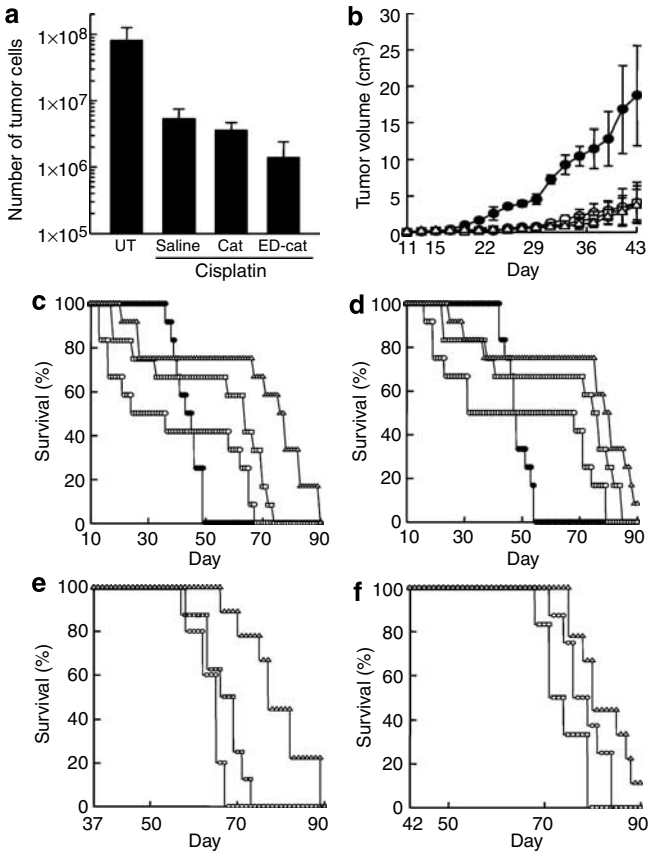

Figure 5 | Effect of catalase and ED-catalase on the antitumor activity of cisplatin in tumor-bearing mice. (**a**) B16-BL6/Luc (5×10^5) or (**b**) S180 (2.5×10^5) was inoculated subcutaneously into mice, and at 7 days after inoculation mice received three injections of 5 mg cisplatin per kg into the peritoneal cavity at 24-h intervals. Catalase or ED-conjugated catalase (10 000 U kg^{-1} shot^{-1}) was injected into the tail vein of mice at 12 h before the first injection of cisplatin. The second shot was administered to mice immediately after the first injection of cisplatin, and three additional doses were administered at 24-h intervals. (**a**) On day 21, B16-BL6/Luc-bearing mice were euthanized and the luciferase activity of the tumor tissues was measured. UT, untreated; Cat, catalase; ED-cat, ED-catalase. Results are expressed as the mean ± s.d. of five mice. (**b**) The dimensions of the subcutaneous S180 tumor tissues were measured every other day and the tumor volume was estimated. (●), untreated; (○), cisplatin-injected, saline-treated; (□) cisplatin-injected, catalase-treated; (△) cisplatin-injected, ED-catalase-treated. Results are expressed as the mean ± s.d. of 12 mice. (**c**, **d**) Survival of tumor-bearing mice. Groups of 12 B16-BL6 (**c**)- and S180 (**d**)-bearing mice were treated as described above. Differences in the survival of mice were significantly different between (**c**) saline and catalase ($P < 0.05$), saline and ED-catalase ($P < 0.001$), and catalase and ED-catalase ($P < 0.01$) and (**d**) saline and ED-catalase ($P < 0.01$). In both cases, no significant differences were obtained between the untreated and the saline-treated groups ($P = 0.48$ and 0.17, respectively). (**e**, **f**) Survival of tumor-bearing mice that escaped cisplatin-induced acute death. The survivors for the first 37 (**e**) and 42 days (**f**) in the experiments shown in (**c** and **d**) are plotted.

suggests that an increased catalase activity results in the inhibition of cisplatin-induced nephrotoxicity by ED-catalase (Figure 3f). These results clearly indicate that the distribution or location of catalase, not the total catalase activity, is important for inhibiting cisplatin-induced nephrotoxicity.

Unmodified catalase showed a marginal, but dose-dependent effect in inhibiting the cisplatin-induced changes (Figure 2), although an increase in the dose over 10 000 U kg^{-1} produced hardly any increase in the inhibitory effects. Hematoxylin and eosin staining of the kidney sections clearly showed cisplatin-induced necrosis in the proximal tubules, extensive epithelial vacuolization, swelling, and tubular dilatation (Figure 3g–j). As ED-catalase has the ability to distribute to those regions in the kidneys more efficiently than catalase, as shown in Figure 1, it would be more effective than catalase in inhibiting the cisplatin-induced nephrotoxicity; this was most clearly demonstrated by the reduced numbers of mice dying acutely (Figure 5c and d).

A very important observation is that catalase derivatives do not interfere with the antitumor activity of cisplatin. Furthermore, combined administration of catalase derivatives and cisplatin increased the survival of tumor-bearing mice (Figure 5c and d). Although the antitumor activity of cisplatin depends on its direct interaction with DNA, primarily by nucleotide excision repair,[29] it also damages the genetic material via generated ROS.[3,4] Solid tumor tissues are generally under hypoxic conditions, and tumor cells contain a greatly reduced number of mitochondria and they rely mostly on glycolysis for energy.[30] These conditions will lead to a reduced production of ROS within tumor tissues, so the cisplatin-induced oxidative damage would affect tumor tissues to a lesser degree than normal tissues, such as kidneys.

In addition to its cytotoxicity due to its oxidative activity, hydrogen peroxide acts as a second messenger that influences the expression of a number of genes and signal transduction pathways.[31] In fact, sublethal concentrations of hydrogen peroxide are reported to stimulate cell proliferation.[32,33] The administration of ED-catalase to cisplatin-treated B16-BL6-bearing mice tended to reduce the number of tumor cells determined at day 21 (Figure 5a), although the difference compared with saline-treated mice was not statistically significant: $5.3 \pm 2.1 \times 10^6$ cells and $1.4 \pm 1.0 \times 10^6$ cells for the saline- and the ED-catalase-treated mice, respectively. These results suggest that the proliferation of B16-BL6 cells in subcutaneous tumor tissues is inhibited to some extent by ED-catalase, which is in good agreement with previous reports demonstrating that cell proliferation is inhibited by co-expression of catalase.[34] We have recently reported that metastatic tumor growth aggravated by tumor removal is significantly inhibited by polyethyleneglycol-conjugated catalase.[20] Furthermore, in a series of studies, we have shown that the administration of catalase derivatives is highly effective in inhibiting experimental tumor metastasis to the liver,[15] lung,[18–20] and peritoneal organs.[21] Various processes, including the very early steps of tumor metastasis, such as adhesion of tumor cells to the endothelium, were inhibited by catalase derivatives.[19] We have recently reported that i.p. ED-catalase is effective in inhibiting experimental peritoneal dissemination of murine carcinoma cells in mice.[35,36] These

pieces of evidence suggest that not only the proliferation of tumor cells but also their metastasis is inhibited by catalase derivatives.

The survival data on mice that escaped cisplatin-induced acute death showed that ED-catalase can increase the survival of B16-BL6- or S180-bearing mice, with a greater effect in B16-BL6-bearing animals. The difference in the metastatic ability of these two cell lines (B16-BL6 is a highly metastatic clone) would explain the differences in the survival rates of the tumor-bearing mice treated with ED-catalase. Further studies are needed to confirm how catalase derivatives affect the proliferative and metastatic nature of tumor cells.

In conclusion, this study has demonstrated that cationization of catalase is an efficient method for targeting the antioxidant enzyme to renal tubule cells, thereby protecting against the renal dysfunction induced by cisplatin. The cationized catalase, ED-catalase, does not interfere with the antitumor activity of cisplatin, but significantly prevents drug-induced acute death, resulting in an increased survival of tumor-bearing mice treated with cisplatin.

MATERIALS AND METHODS

Chemicals

Cisplatin was obtained from Nippon Kayaku Co. (Tokyo, Japan). Bovine liver catalase (EC 1.11.1.6) and 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride were purchased from Sigma Chemical (St Louis, MO, USA). ED was obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). Dulbecco's modified Eagle's minimum essential medium and Hank's buffered salt solution were obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum was obtained from Biowhittaker (Walkersville, MD, USA). All other chemicals were of analytical grade.

Animals

Male ddY mice (4- to 5-week-old) and male C57BL/6 mice (6-week-old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. The protocols for animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University.

Synthesis and characterization of ED-catalase

The coupling of ED to catalase was performed as described previously.[14] The number of increased free amino groups of ED-catalase was 13.6, and its remaining enzymatic activity was about 44%. This reduction in enzymatic activity would be mainly due to changes in the structure of catalase by the coupling of ED.[14]

Immunohistochemical staining of catalase derivatives in kidney sections

Kidneys were collected from mice 30 min after intravenous injection of catalase or ED-catalase at a dose of 1 mg kg^{-1}. Then, the kidneys were fixed using an acetone–methylbenzoate–xylene method, and paraffin-embedded sections were prepared. Sections were also made by collecting kidneys after injection of catalase at a high dose of 100 mg kg^{-1}, and these sections were used as a positive control for the immunohistochemical staining of bovine liver catalase. The sections were immunostained for bovine catalase using a 1:500 dilution of antibovine catalase rabbit antibody (Rockland, Gilbertsville, PA, USA), followed by incubation with peroxidase-labeled secondary antibody (Histofine Simplestain Max PO; Nichirei, Tokyo, Japan). The sections were then incubated with 3,3′-diaminobenzidine tetrahydrochloride substrate solution to allow development of a brown color.

Measurement of renal catalase activity

Kidneys were collected from mice 30 min after intravenous injection of catalase or ED-catalase at a dose of 1 mg kg^{-1}. Then, the kidneys were homogenized with a lysis buffer (0.05% Triton X-100, 2 mM ethylenediaminetetraacetic acid, 0.1 M Tris (pH 7.8)). Catalase activity in the supernatant of tissue homogenates was measured using an Amplex® Red Catalase Assay Kit (Molecular Probes Inc., Eugene, OR, USA).

Nephrotoxicity of single-dose cisplatin and its inhibition by catalase derivatives

Groups of 4–6 mice received i.p. injections of 20 mg kg^{-1} cisplatin. Catalase (5000–50 000 U kg^{-1} shot^{-1}) or ED-catalase (10 000 U kg^{-1} shot^{-1}) was injected into the tail vein of mice twice a day, beginning 12 h before the cisplatin injection and this was continued for seven doses at 12-h intervals. The dosing schedule of catalase derivatives was determined based on previous reports in which attempts were made to inhibit cisplatin-induced nephrotoxicity.[37,38] The saline-treated (control) group received an intravenous injection of an isotonic saline solution. Animals were euthanized 3 days (72 h) after the cisplatin injection.

Nephrotoxicity of repeated doses of cisplatin and its inhibition by catalase derivatives

Groups of 5–8 mice received three injections of 5 mg cisplatin per kg into the peritoneal cavity on days 0, 1, and 2 at 24-h intervals. Catalase or ED-catalase (10 000 U kg^{-1}) was injected into the tail vein of mice 12 h before the cisplatin injection. Then, the second injection was given immediately after the first injection of cisplatin, and three additional doses were administered at 24-h intervals. Again, isotonic saline solution was given intravenously to the saline-treated (control) group instead of catalase derivatives. Animals were euthanized 4 days (96 h) after the first cisplatin injection.

Biochemical parameters of nephropathy

At indicated periods after treatments, mice were euthanized, blood was collected from the vena cava, and serum was separated by centrifugation for 20 min at 3000 *g*, 4°C. Serum LDH activity and the nitrite/nitrate level were determined using the Lactate Dehydrogenase C-II Test Wako (Wako Pure Chemical Co.) and the NO_2/NO_3 Assay Kit-C II (Dojindo Molecular Technologies Inc., Kumamoto, Japan), respectively. The kidneys were isolated, washed with saline, and weighed. Part of the kidney samples was homogenized in 5% sulfosalicylic acid and the total GSH content measured[39] using an assay kit (Total Glutathione Quantification Kit, Dojindo Molecular Technologies Inc.). The remaining part of the kidney was used to measure the MDA level by the method of Wasowicz *et al.*[40] based on the reaction of MDA with thiobarbituric acid at 95–100°C. The fluorescence intensity of MDA in the upper *n*-butanol phase was measured in a fluorescence spectrophotometer (Wallac 1420 ARVO$_{MX}$ Multilabel Counter, Perkin-Elmer, Finland) with excitation at 530 nm and emission at 560 nm. Arbitrary values

obtained were compared with a series of standard solutions of 1,1,3,3-tetramethoxypropane. Renal damage was assessed by monitoring serum levels of BUN and creatinine. The BUN and creatinine levels were measured by the diacetylmonoxime method[41] and Jaffe's method,[42] respectively, using assay kits purchased from Wako Pure Chemical Co. (Urea NB Test Wako and Creatinine Test Wako, respectively).

Histological evaluation of nephrotoxicity

At 72 h after injection of cisplatin at a dose of 20 mg kg^{-1}, kidneys were isolated from mice, fixed in 4% paraformaldehyde, dehydrated, and embedded in 70% ethanol. Then, 3 μm tissue sections were prepared, stained with hematoxylin and eosin, and examined for tubular necrosis and dilatation. The slides were coded, and a semiquantitative analysis of the sections was performed without knowledge of the treatment protocol, as described previously.[38] The changes observed were limited to the tubulointerstitial areas and graded as follows: 0, normal; I, areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving $<25\%$ of the cortical tubules; II, similar changes involving $>25\%$ but $<50\%$ of the cortical tubules; III, similar changes involving $>50\%$ but $<75\%$ of the cortical tubules; IV, similar changes involving $>75\%$ of the cortical tubules.

Measurement of platinum contents in kidneys

Groups of 3–4 mice were treated as described above with cisplatin (a single injection of 20 mg kg^{-1} or three injections of 5 mg kg^{-1}) and ED-catalase (seven doses of 10 000 U kg^{-1} or five doses of 10 000 U kg^{-1}, respectively). At 24 h after the last injection of ED-catalase, mice were euthanized and kidneys were harvested and homogenized in distilled water. Then, the tissue homogenates were centrifuged at 250 *g* for 10 min and the supernatant was collected for the measurement of platinum concentration by atomic absorption.

Tumor cells

Murine melanoma B16-BL6[43] and B16-BL6/Luc[19] cells were grown in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 0.15% sodium bicarbonate, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, at 37°C under an atmosphere of 5% CO_2 in air. Then, 5×10^5 cells were suspended in Hank's buffered salt solution and inoculated subcutaneously into mice. Separately, murine sarcoma 180 (S180) was maintained in ddY mice by weekly i.p. transfer of 2.5×10^5 cells. S180 cells (1×10^6) were inoculated subcutaneously to obtain tumor-bearing mice.

Effects of catalase derivatives on the antitumor activity of cisplatin

At 7 days after tumor inoculation, groups of 12 mice began to receive three injections of 5 mg cisplatin per kg into the peritoneal cavity at 24-h intervals. Catalase or ED-catalase (10 000 U kg^{-1}) was injected according to the same protocol as used for the repeated doses of cisplatin as described above. The dimensions of the subcutaneous S180 tumor tissues were measured with a caliper every other day, and the tumor volume was estimated from the following equation: volume $= 1/2LW^2$, where L is the long diameter and W is the short diameter of the tissue. Then, survival of S-180- or B16-BL6-bearing mice was evaluated. In different sets of mice, B16-BL6/Luc-bearing mice ($n = 5$ animals per group) were euthanized on day 21, and the luciferase activity of tumor tissues was measured using a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany) as reported previously.[19]

Statistical analysis

Differences were statistically evaluated by one-way analysis of variance followed by the Fisher PLSD multiple comparison text, and Kaplan–Meier analysis with a log-rank test to determine survival. The level of statistical significance was set at $P < 0.05$.

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