A novel free radical scavenger, Edarabone, protects against cisplatin-induced acute renal damage \textit{in vitro} and \textit{in vivo}

MINORU SATOH, NAOKI KASHIHARA, SOHACHI FUJIMOTO, HIDEYUKI HORIKE, TAKEHIKO TOKURA, TAMEHACHI NAMIKOSHI, TAMAKI SASAKI, and HIROFUMI MAKINO

Division of Nephrology, Department of Internal Medicine, Kawasaki Medical School, Kurashiki (M.S., N.K., S.F., H.H., T.T., T.N., T.S.), and Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine and Dentistry, Okayama (M.S., H.M.), Japan
Running title page

a) Running title: Radical scavenger in cisplatin nephropathy.

b) Corresponding author: Minoru Satoh, M.D., Ph.D.,
Division of Nephrology, Department of Internal Medicine,
Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan
Phone +81 (86) 464-1039
FAX +81 (86) 464-1039
E-mail: satoh-minoru@mx1.tiki.ne.jp

c) The number of Text page 20
   Table 3
   Figure 7
   References 40
The number of words in the Abstract 239 words
   Introduction 386 words
   Discussion 1455 words

d) A list of nonstandard abbreviations used in the paper
   AP, apurinic/apyrimidinic; BUN, blood urea nitrogen; Cer, creatinine clearance; COX, cytochrome-c oxidase; DCF, dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; EGTA, Ethyleneglycoltetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; GSH,
glutathione peroxidase; HEPES, N-2-hydroxyethylpiperazine-N’-2-ehanesulfonic acid; HNE, 4-hydroxy-2-nonenal; ICAM-1, intercellular adhesion molecule-1; i-NOS, inducible nitric oxide synthase; JC-1, 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolyl-carbocyanine iodide; ΔΨₘ, mitochondrial membrane potential; NAG, N-acetyl-ß-d-glucosaminidase; NF-κB, nuclear factor-kappa B; 8-OHdG, 8-hydroxy-deoxyguanosine; PBS, phosphate-buffered saline; PTC, proximal tubular cell; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; WST-1, tetrazolium salt.

e) A recommended section: Gastrointestinal, Hepatic, Pulmonary, & Renal
ABSTRACT

Accumulating evidence suggests that enhanced peroxidative damage caused by reactive oxygen species (ROS) may contribute to the pathogenesis of cisplatin-induced acute renal failure. However, little is known about the involvement of oxygen radicals in cisplatin nephropathy. Here we investigated the effects of a novel free radical scavenger, 3-methyl-1-phenyl-pyrazolin-5-one (MCI-186; edarabone), on murine proximal tubular cell (PTC) damage induced by exposure to cisplatin in vitro, and on renal function in an in vivo model of cisplatin-induced acute renal failure.

Edarabone inhibited cisplatin (40 µM, 24 h)-induced cytotoxicity in a concentration-dependent manner (10⁻⁵ to 10⁻³ M). Edarabone also attenuated cisplatin-induced mitochondrial transmembrane potential loss and ROS production of PTCs. In the in vivo study, male Wistar rats were co-treated with cisplatin (5 mg/kg, i.p.) and edarabone (1 or 5 mg/kg, i.v.). Effects of edarabone on the kidney were examined 5 days after treatment. Cisplatin resulted in renal dysfunction, renal tubular damage, mitochondrial damage (assayed by histochemical staining for respiratory chain complex IV), renal protein oxidation (examined by Western blot analysis using a specific antibody for carbonyl group-containing proteins) and tubular apoptosis (determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining). The above changes were attenuated by edarabone treatment. Thus, edarabone exhibited cytoprotective effects in PTCs and renoprotective effects against cisplatin. Our findings suggest that ROS, in particular hydroxyl radicals, are involved in cisplatin nephropathy and that edarabone may be potentially useful in protecting the kidneys and prevention of acute renal failure.
Cisplatin is a simple platinum complex, comprising a central platinum atom, a chlorine atom and ammonia molecule in the cis position, and is effective in the treatment of a wide variety of neoplastic diseases (Lebwohl and Canetta, 1998). While effective, cisplatin is associated with many adverse drug reactions, such as renal damage, gastrointestinal dysfunction, auditory toxicity and peripheral nerve toxicity (Cooley et al., 1994). Nephrotoxicity in particular is a major complication and a dose-limiting factor for cisplatin therapy (Safirstein et al., 1986). Administration of cisplatin is frequently associated with renal insufficiency and tubular dysfunction. The possible involvement of peroxidative damage caused by a reactive oxygen species (ROS) has been suggested in the pathogenesis of cisplatin-induced renal failure (Matsushima et al., 1998). In particular, the hydroxyl radical is highly reactive among oxygen radicals. Once excessive hydroxyl radicals are released, lipid peroxidation, which causes changes in the fluidity and permeability of membranes, is induced (Schmidley, 1990). Several antioxidants and oxygen radical scavengers have been reported to be effective in protection against these injuries (Rao M et al., 1999; Davis et al., 2001; Hara et al., 2001). However, the roles of hydroxyl radicals and other free radical species in cisplatin nephrotoxicity have not been fully elucidated.

The novel free radical scavenger, 3-methyl-1-phenyl-pyrazolin-5-one (MCI-186; edarabone), has been shown to trap both hydroxyl radicals and prevent iron-induced peroxidative injuries (Murota et al., 1990). It has also been shown to have protective effects against cerebral and myocardial ischemia in rats. Pharmacological
studies have suggested that the anti-cerebral ischemic action of edarabone is related to its antioxidant action (Watanabe et al., 1994a; Wu et al., 2000). Oxidative stress is also involved in the pathogenesis of glomerular and tubular injuries in various acute renal diseases (Baud and Ardaillou, 1986). Nevertheless, the usefulness of this compound in acute renal disease has not been investigated.

The present study was designed to examine whether edarabone would prevent cisplatin-induced cytotoxicity in cultured renal epithelial cells. Furthermore, we investigated whether edarabone provided protection against cisplatin-induced deterioration of renal function and structural injury in vivo. Our results indicate that edarabone may be of value in preventing cisplatin-induced cytotoxicity and decline of renal function. The beneficial effect of edarabone relates to reducing the mitochondrial damage, ROS generation and lipid, protein and nucleic acid oxidation, ultimately resulting in preservation of tubular cell structure.
Materials and Methods

In vitro studies

Cell culture

Murine renal proximal tubular epithelial mProx cells (PTCs; kindly provided by Dr. T. Sugaya, Discovery Research Laboratory, Tanabe Seiyaku Co., Osaka, Japan) were used. These PTCs were microdissected out from C57BL/6J adult mouse kidney and immortalized and transfected with the SV40 large T antigen gene. These cells were stained by cytokeratin, but not by α smooth muscle actin and showed albumin reabsorption (Takaya et al., 2003). Thus, this cell line expressed proximal tubular phenotype. PTCs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 4 mM glutamine, 5% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C under 95% room air - 5% CO₂ gas mixture on a 1% gelatin-coated dish. Cultured cells from the 4th to 30th passages were used for the experiments.

Cell viability assays

Cell viability was examined by trypan blue exclusion for living cells and the tetrazolium salt (WST-1) degradation assay for mitochondrial viability. For trypan blue exclusion, cells were seeded in 12-well plates followed by exposure to media containing phosphate-buffered saline (PBS) or 20 µM cisplatin (Sigma-Aldrich Japan Co., Tokyo, Japan) for 24 h with or without edarabone (a gift from Mitsubishi Pharma Corp., Osaka, Japan). Non-adherent cells were removed and adherent cells were
harvested by trypsin-EDTA digestion and stained with 0.4% trypan blue for 5 min at 37°C. The number of cells excluding trypan blue was counted in a hemocytometer and expressed as a percent of viable cells as compared to vehicle-treated cells. To assess mitochondrial viability, cells were plated in 96-well plates and incubated with 20 µM cisplatin for 24 h. WST-1 measurement was performed according to the manufacturer’s protocol (Cell Proliferation Assay System, TaKaRa Biomedicals, Tokyo, Japan).

**Measurement of mitochondrial membrane potential (ΔΨ<sub>m</sub>) and hydrogen peroxide production**

To assess changes in mitochondrial membrane potential (ΔΨ<sub>m</sub>), we used the potentiometric, fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Molecular Probes Inc., Eugene, OR) (Di et al., 1995). We determined changes in intracellular ROS levels by measuring the oxidative conversion of cell-permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes Inc.) to fluorescent dichlorofluorescein (DCF), by confocal laser scanning microscopy or fluorescence-activated cell sorting (FACS) (Mancini et al., 1998). Cells were seeded in 6-well plates followed by exposure to media containing PBS or 20 µM cisplatin for 6 h with or without edarabine. Cells were then incubated with 5 µg/ml JC-1 or 20 µM DCFH-DA in medium for 15 min at 37°C in the dark. Both floating and adherent cells were collected and pelleted by centrifugation at 500 × g for 5 min. In each sample, a minimum of 10,000 cells was subjected to FACS using a FACS Calibur (Nippon...
Becton Dickinson Co., Tokyo, Japan) and CellQuest software (Nippon Becton Dickinson). Intracellular distribution of the dye was assessed by confocal laser-scanning microscopy TCS-NT (Leica-microsystems Co., Tokyo, Japan).

**In vivo studies**

*Animal experiments*

Male Wistar rats (300 ± 30 g body weight; Charles River Japan, Inc. Kanagawa, Japan) were maintained on standard chow and tap water *ad libitum*. The experimental protocol was approved in advance by the Ethics Review Committee for Animal Experimentation of the Kawasaki Medical School, Kurashiki, Japan. Rats were assigned to four groups as follows: group 1, saline-treated controls (n=7); group 2, cisplatin-treated rats (n=7); group 3, rats treated with cisplatin plus edarabone at a dose of 1 mg/kg (n=7); and group 4, rats treated with cisplatin plus edarabone at a dose of 5 mg/kg (n=7). Under light ether anesthesia, animals were injected intraperitoneally with 1.0 ml of saline or 5 mg/kg of cisplatin (Matsushima et al, 1998; Ramesh and Reeves, 2002). At the same time, 0.1 ml of saline, 1 or 5 mg/kg of edarabone was administered by a single intravenous injection (Kawai et al, 1997). Animals from each group were placed into metabolic cages for 24 h urine collection for measurement of N-acetyl-ß-d-glucosaminidase (NAG) excretion and creatinine clearance (Ccr) at 4 days after injection of cisplatin or saline. At 5 days animals were humanely killed using pentobarbital anesthesia. Blood samples were obtained for measurement of blood urea nitrogen (BUN) and creatinine, and the kidneys were
dissected out. Surgically-removed kidney specimens were immediately frozen in liquid nitrogen and unfixed cryostat sections (5-µm thick) were prepared for cytochrome-c oxidase staining. Another kidney cross-section was collected in 4% neutral buffered paraformaldehyde for histopathological evaluation. The remaining portions of the kidney were frozen in liquid nitrogen and stored at -80°C for DNA and protein isolation.

**Histopathology**

The tissues collected in paraformaldehyde were processed, embedded in paraffin, sectioned (4-µm thick), and stained with hematoxylin and eosin. A pathologist performed a semiquantitative analysis of the kidney sections in a blinded fashion. Changes observed were limited to the tubules, especially the proximal straight S3 portion, the main site of cisplatin toxicity, such as cell swelling, vacuolization, necrosis, and desquamation. Tubular lesions were graded as follows: 0 = no damage; 1+ = lesion areas < 50%; 2+ = lesion areas > 50% with or without focal involvement of the S3 segment in the medullary rays; 3+ = lesion areas 100% with diffuse involvement of the medullary rays. The mean score for each group was calculated.

**Cytochrome-c oxidase staining**

To confirm mitochondrial activity *in vivo*, cytochrome-c oxidase (COX) staining methods (Seligman et al., 1968) were used. The percentages of COX-positive areas, with the exception of the glomerulus, were measured by using an image
analyzer (MCID image analyzer, Fuji Film, Tokyo, Japan). Ten consecutive fields from the renal cortex of each rat were randomly selected and evaluated at a magnification of $\times 100$.

**Western blot analysis**

Kidney samples were homogenized in lysis buffer containing 0.25 M sucrose, 50 mM DTT, 3 mM HEPES (pH 7.9), 500 µM EGTA, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.8 µM aprotinin, 21 µM leupeptin, 36 µM bestatin, 15 µM pepstatin A, 14 µM (4-guanidino)butane and 1% Triton X-100. After centrifugation ($8000 \times g$, 10 min, 4°C), the supernatants were used for Western blotting. Protein concentrations were determined using a protein concentration assay kit (QuantiPro BCA assay kit, Sigma). Western blot analysis for oxidative protein was performed with the Oncor Oxyblot kit according to the manufacturer’s protocol (Oncor, Gaithersburg, MD) as described previously (Keller et al., 1993). For the 4-hydroxy-2-nonenal (HNE) immunoblot, proteins (10 µg) were separated in a 12.5% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. The membranes were incubated for 1 h with anti-HNE monoclonal antibody (Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan, 1.0 µg/ml) followed by incubation with peroxidase-conjugated goat anti-mouse IgG antibody (0.1 µg/ml) for 1 h. Bands were visualized using the ECL-plus Western blotting detection system (Amersham Pharmacia Biotech in Japan, Tokyo, Japan).
Assessment of DNA oxidative injury

The amount of 8-hydroxy-deoxyguanosine (8-OHdG) in DNA was measured using a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check, Japan Institute for Control of Aging) (Toyokuni et al., 1997). Genomic DNA from the kidneys was extracted using DNAzol Reagent (Life Technologies Oriental Co., Tokyo, Japan). After hydrolyzing with DNA nuclease and alkaline phosphatase, 10 µg DNA were used for ELISA.

The number of apurinic/apyrimidinic (AP) sites in DNA was detected using a DNA damage quantification kit (Kumamoto Immunochemical Laboratory Co., Kumamoto, Japan). Purified DNA (1 µg) was incubated with N’-aminooxymethyl-carbonyl-hydrazino-d-biotin, which binds specifically to AP sites, for 1 h at 37°C. After fixing in 96-well plates overnight at room temperature, the plates were incubated with peroxidase-conjugated streptavidin for 1 h at 37°C. After color development with substrate solution, absorbances were measured at 660 nm.

Assessment of apoptosis in vivo

Apoptotic nuclei were detected with the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method, using an apoptosis detection kit (In situ apoptosis detection kit, TaKaRa Biomedicals) according to the protocol specified by the manufacturer. The analysis was performed in a blinded fashion. Random areas were viewed at a magnification of × 100 and scored for the number of apoptotic nuclei present in cortical renal tubules. The mean number of stained cells in at least 20 random fields was expressed as the number of TUNEL-positive cells.
Statistical analysis

All data are presented as mean ± SEM. The Student’s $t$ test was used for comparisons between two groups. Statistics were performed using StatView on a Macintosh computer. Significance was defined as $P < 0.05$. 
Results

In vitro studies

Cell viability

To test whether edarabone protected against cisplatin-induced cell death, cell viability was examined by trypan blue exclusion. The number of living PTCs decreased by treatment with 20 µM cisplatin for 24 h. Edarabone treatment significantly attenuated cisplatin-induced cytotoxicity in a dose-dependent manner. Treatment with $10^{-4}$M edarabone increased cell viability by 45.0% (cisplatin: 38.6 ± 4.3%; cisplatin + $10^{-4}$M edarabone: 83.6 ± 5.2%; $P < 0.01; n = 6$) (Fig. 1A).

Mitochondrial viability was determined by degradation of tetrazolium salt with mitochondria dehydrogenation enzyme. The results also revealed that cisplatin significantly decreased mitochondria dehydrogenation enzyme activity. Incubation with various concentrations of edarabone resulted in a concentration-dependent increase in survival rate (Fig. 1B). Treatment with $10^{-4}$M edarabone significantly decreased mitochondrial dysfunction induced by cisplatin (cisplatin: 55.1 ± 9.1%; cisplatin + $10^{-4}$M edarabone: 84.9 ± 12.6%; $P < 0.01; n = 6$).

Mitochondrial membrane potential ($\Delta \Psi_m$) change and reactive oxygen species production

The $\Delta \Psi_m$ is an important indicator of mitochondrial function and dysfunction. To assess mitochondrial damage induced by cisplatin, we monitored changes in $\Delta \Psi_m$ using the fluorescent dye JC-1. FACS analysis showed that high $\Delta \Psi_m$ was maintained
in control cells (68.9%) (Fig. 2). After cisplatin treatment, the rate of high \( \Delta \Psi_m \) was decreased (5.4%) and the rate of low \( \Delta \Psi_m \) was increased (46.5%). By co-incubation with edarabone, the \( \Delta \Psi_m \) was maintained at high levels (47.1%) and the rate of low \( \Delta \Psi_m \) was not increased (1.3%).

To determine intracellular ROS generation induced by cisplatin and the oxygen radical elimination ability of edarabone, cisplatin-treated PTCs with or without edarabone were incubated with DCFH-DA, and oxidation of intracellular DCF was measured by confocal laser-scanning microscopy and FACS. No ROS production was evident in the control cells (Fig. 3), whereas enhanced ROS production was observed in cisplatin-treated cells. Edarabone treatment reduced ROS production in cisplatin-treated PTCs. FACS analysis showed that cisplatin caused a significant increase in ROS generation (1.4% versus 74.5%). Pre-incubation of PTCs with \( 10^{-4} \)M edarabone significantly reduced ROS levels (22.3%).

**In vivo studies**

**Morphological evaluation**

Compared with controls at day 5, renal tissue sections from the cisplatin group exhibited increased evidence of acute structural damage, characterized by tubular necrosis, degeneration, casts, and red blood cell extravasation (Fig. 4). These changes usually involved the entire S3 segments in the outer stripe of the medulla zone. Semi-quantitative assessment of the histological lesions revealed a significantly higher score in cisplatin-treated rats versus controls at day 5 (Table 1, 2.7 \pm 0.2 versus 0.1 \pm 0.1, P
Edarabone treatment (5 mg/kg) significantly lowered the semi-quantitative score compared to cisplatin treatment only (cisplatin + 5 mg/kg edarabone: 1.7 ± 0.3, P < 0.05 versus cisplatin group).

Assessment of renal function

Injection of cisplatin induced a significant increase in plasma BUN levels at day 5 (Table 2). Administration of edarabone significantly prevented the increase in BUN in cisplatin-treated animals. Creatinine levels also improved following edarabone treatment. Five days after cisplatin administration, a marked and significant decrease in Ccr was observed in the cisplatin treatment group versus controls (0.05 ± 0.01 ml/min/100g BW versus 0.93 ± 0.05 ml/min/100 g BW, P < 0.05). As shown in Table 2, Ccr was significantly higher in the edarabone-treated group compared to the cisplatin-treated group. Urinary NAG excretion, a marker of tubular damage, also increased in the cisplatin group, but was significantly decreased by edarabone administration at a dose of 5 mg/kg (1.01 ± 0.15 IU/day versus 0.48 ± 0.07 IU/day, P < 0.05).

Assessment of renal mitochondrial function

To investigate the involvement of mitochondrial function in the pathogenesis of cisplatin-induced renal dysfunction, we analyzed COX activity by histochemical COX staining (Fig. 5A). Histochemical staining for COX yielded an intense, fine granular pattern in the tubular cells in controls. Proximal tubular COX reactivity was markedly decreased in the cisplatin-treated compared with the control group. However, the
edarabone-treated group exhibited equivalent COX reactivity to controls. The COX-positive area was significantly increased by edarabone treatment (Fig. 5B; cisplatin, 44.7%; cisplatin + edarabone 5 mg/kg, 79.6%).

**Analysis of oxidatively modified proteins, lipids and nucleic acids**

To characterize the effects of edarabone on the level of protein oxidation in the cytosolic and mitochondrial fractions of the kidney, we measured levels of carbonyl groups in specific proteins by Western blot analysis. As shown in Figure 6A, more carbonyl groups were present in cytosolic protein from the cisplatin-treated rats. Edarabone (5 mg/kg) reduced the protein oxidation by cisplatin in cytosol extracts. We also examined renal HNE content as an index of lipid peroxidation by Western blotting. The HNE content was increased by cisplatin treatment (Fig. 6B). The increase was completely prevented by treatment with 5 mg/kg of edarabone. DNA damage caused by cisplatin-induced ROS was examined by investigating the 8-OHdG and AP sites in DNA. Cisplatin treatment resulted in a significant increase in 8-OHdG and AP sites compared to controls (Table 3). The increases in 8-OHdG and AP sites were reduced by edarabone treatment in a dose-dependent manner.

**Quantification of apoptosis in vivo**

The degree of apoptosis in the kidney was assessed using the TUNEL assay at day 5 after administration of cisplatin. As shown in Figure 7A, significantly increased numbers of apoptotic nuclei were seen in the cisplatin group, but there were few apoptotic nuclei in the edarabone-treated group. Figure 7B shows the results of
quantification of apoptotic nuclei after 5 days. The cisplatin-treated and cisplatin +
edarabone-treated groups showed a 10.0-fold and 2.5-fold increase in apoptotic nuclei,
respectively, over the saline-treated group.
Discussion

Peroxidative damage caused by reactive oxygen species (ROS) has been implicated in the pathogenesis of cisplatin-induced renal failure. ROS induce modification of disulfide bonds of protein, oxidation of phospholipids, and DNA injury in cells such that ROS are considered cytotoxic. ROS contain a superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), singlet oxygen (O$_2^*$) or hydroxyl radical (·OH). Superoxide anion is the most common oxygen radical and is a molecular species generated in large amounts in the human body. Nevertheless, its reactivity is very low such that it is not involved in modification of protein or lipid. In contrast, the hydroxyl radical is one of the most reactive and aggressive chemical species. Once excessive hydroxyl radicals are released, lipid peroxidation, which causes changes in the fluidity and permeability of membranes, is induced. No enzymes exist to eliminate the hydroxyl radical from the cell. Edarabone, a free radical scavenger that traps hydroxyl radicals, was developed for the potential treatment of cardiovascular disease, cerebrovascular ischemia and cerebral edema. This compound has been registered in Japan for treatment of acute brain infarction. In the present study, we demonstrated that edarabone treatment protected kidneys against cisplatin-induced nephrotoxicity, both in vitro and in vivo. The present study suggested that enhanced peroxidative damage caused by ROS, especially the hydroxyl radical, might contribute to the pathogenesis of cisplatin-induced acute renal failure.

Mitochondrial dysfunction is a central component of cisplatin nephrotoxicity to proximal tubules in vivo (Gordon and Gattone, 1986; Davis et al., 2001) and in vitro
Renal PTCs, which accumulate significantly greater amounts of cisplatin in vivo and in vitro than other nephron segments (Goldstein and Mayor, 1983), undertake substance transport by consumption of large amounts of ATP. PTCs are therefore sensitive to ATP production disturbances. Almost all ATP is produced in mitochondria. Mitochondrial DNA does not bind histone protein, and, thus, it is readily susceptible to oxidative stress. Accumulation of oxidative damage in mitochondrial DNA may affect mitochondrial respiratory function and cause cellular dysfunction. In this study, we observed that mitochondria dehydrogenation enzyme activity was deteriorated by cisplatin in PTCs, and that mitochondrial complex IV activity (determined by COX staining) was also reduced. In vitro assays have revealed the generation of superoxide anion and hydroxyl radical during interaction of cisplatin with DNA (Masuda et al., 1994). We found that treatment with edarabone, which has hydroxyl radical but not superoxide anion scavenging activity, significantly ameliorated the mitochondrial dysfunction induced by cisplatin both in vitro and in vivo. These data indicate that hydroxyl radicals are important in the mechanism that underlies cisplatin-induced renal injury.

Mitochondria normally generate small quantities of superoxide by continuously converting 1 to 2% of consumed oxygen. Therefore, they are an important source of ROS. Mitochondria are generally protected from oxidative damage in several ways, including the activities of Mn-containing superoxide dismutase (SOD) and Se-containing glutathione peroxidase (GSH). However, expression of the SOD and GSH genes is downregulated by cisplatin (Rao et al., 1999; Huang et al., 2001; Saad et al., 2001), so that more ROS are generated. Furthermore, cisplatin-induced mitochondrial
injuries, especially inhibition of the electron transfer system, produce large amounts of ROS (Kruidering et al., 1997). Namely, once mitochondria are impaired by cisplatin-induced ROS, more ROS are generated, increasing the mitochondrial and cell damage. We demonstrated that cisplatin-induced ROS generation in vitro by measuring the oxidative conversion of DCFH-DA to DCF, and found that edarabone could reduce cisplatin-induced oxidation of intracellular DCF. The ROS detected by this system was superoxide anion and hydrogen. The hydroxyl radical does not react with DCFH-DA. Edarabone, which does not react with superoxide but scavenges hydroxyl radicals, may therefore prevent ROS generation by protection of mitochondria from hydroxyl radicals induced injury caused by cisplatin.

Although cells have developed various antioxidant defenses to protect against ROS, free radicals that escape such defenses non-specifically attack and modify subcellular components, including proteins and free amino acids, lipids and lipoproteins, nucleic acids and connective tissue macromolecules. These species may have an impact on cell activities such as membrane function, metabolism, and gene expression, which results in further mitochondrial and cell dysfunction. The oxidative proteins that have carbonyl groups at lysine, arginine, proline or threonine residues can modulate biochemical characteristics of proteins, such as enzymatic activity, DNA binding activities of transcription factors and the susceptibility to proteolytic degradation. We confirmed that many more carbonyl groups, which are formed in the amino side chains of protein by metal catalyzed oxidation and are standard markers of protein oxidation (Stadtman, 1992), were present in cisplatin-treated rats compared with control rats. The radical scavenger edarabone could block cisplatin-induced
protein oxidation. This indicates that edarabone might protect against cisplatin-induced functional disorders of renal metabolism.

It has been suggested that cisplatin-induced cytotoxicity may be due to peroxidation of cell membranes (Montine and Borch, 1988). The main content of cell membrane is lipid, and thus lipid peroxidation in renal tissue might cause nephrotoxicity. Several investigators have shown that cisplatin nephrotoxicity is associated with lipid peroxidation in renal cortical slices, and that antioxidants reverse cisplatin-induced lipid peroxidation (Rao et al., 1999; Saad et al., 2001). The present study also demonstrated that cisplatin causes lipid peroxidation. Edarabone was reported to inhibit iron-dependent lipid peroxidation and to depress the lipoxygenase pathway (Abe K et al., 1988; Watanabe et al., 1994a; Watanabe and Egawa, 1994b). Our data also indicate that edarabone is active in inhibiting lipid peroxidation, suggesting that edarabone might protect against cisplatin-induced renal functional disorders of membrane function.

Despite its toxic effects, cisplatin can induce DNA damage through the generation of active oxygen species, such as the superoxide anion and hydroxyl radicals (Crul et al., 1997). Hydroxyl radicals have very strong oxidative power and, therefore, can injure almost all bases and induce breakdown. It is known that 8-OHdG and AP site increase as markers of oxidative DNA injury (Lu et al., 2001). In this study, we analyzed 8-OHdG and AP sites in extracted DNA from renal cortical slices. Edarabone administration resulted in a suppression of 8-OHdG and AP sites increase. We infer from this that oxidative damage to DNA was diminished by edarabone.
treatment in cisplatin nephropathy, indicating that edarabone might protect against
 cisplatin-induced disordered gene expression in the kidneys.

Recent studies have shown that cisplatin causes apoptotic cell death in renal
tubule cells (Lieberthal et al., 1996; Lau, 1999). It has also been reported that
activation of mitochondrial pathways were important in apoptosis induced by cisplatin
(Davis et al., 2001; Park et al., 2002). We confirmed mitochondrial permeability
transition by cisplatin exposure \textit{in vitro}. This led to release of cytochrome c, activation
of caspase 9, and entry into the execution phase of apoptosis (Park et al., 2002).
Edarabone inhibited the cisplatin-induced decrease of $\Delta \Psi_m$. It should be noted that
mitochondrial dysfunction could induce necrotic cell death. Histological analysis of
apoptotic and necrotic cell death in prevention of cisplatin-induced renal dysfunction
by edarabone is currently under investigation in our laboratory.

Although edarabone did reduce the renal damage as evidenced on
histopathology, significant damage is still present. Another pathway, which is not
affected by edarabone, is thought to cause the cisplatin-induced nephrotoxicity. The
free radical scavenger, edarabone, can trap $\cdot$OH only, so that another ROS such as $O_2^-$
or $H_2O_2$ may cause renal damage. Recent findings suggest that $O_2^-$ can modulate
nuclear factor-\kappaB (NF-\kappaB) activation (Schreck et al., 1991). NF-\kappaB is a transcription
factor implicated in the inducible regulation of a wide range of genes involved in
inflammatory and immune responses (Baeuerle and Henkel, 1994; Barnes and Karin,
1997). Transcription of the intercellular adhesion molecule-1 (ICAM-1), inducible
nitric oxide synthase (i-NOS) and tumor necrosis factor-\alpha (TNF-\alpha) genes is regulated
at the NF-\kappaB site, and these genes have been shown to be upregulated and to
participate in renal injury (Deng et al., 2001; Ramesh and Reeves, 2002). So there is a possibility that these factors play a significant role in cisplatin-induced renal damage. Another reason that edarabone did not provide complete protection may be its short half-life. Edarabone has a half-life of about 6 to 8 hrs. Because of this short half-life, the cisplatin-induced nephropathy was not completely blocked by a single intravenous injection of edarabone.

In conclusion, we have demonstrated in the present study that edarabone attenuated cisplatin-induced PTC damage \textit{in vitro} and acute renal failure \textit{in vivo}. In addition to its excellent antioxidant effects, the promising features of edarabone are that it is lipophilic, readily accessible to tissue, and thus an effective tissue level can be maintained with a single intravenous bolus injection. Our results also suggest that ROS induced by mitochondrial injuries contribute to renal damage after cisplatin treatment. ROS are involved in many other acute and chronic renal injuries, suggesting that edarabone treatment might be beneficial in various renal diseases.
References


Footnotes

Reprint requests to:

Minoru Satoh, M.D., Ph.D.,
Division of Nephrology, Department of Internal Medicine,
Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan
Table 1. Semi-quantitative evaluation of tubular histological lesions.

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>0</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>Average Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (7)</td>
<td></td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>Cisplatin (7)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>2.7±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cisplatin + Ed (1)</td>
<td>(7)</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2.3±0.3&lt;sup&gt;a, b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cisplatin + Ed (5)</td>
<td>(7)</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1.7±0.3&lt;sup&gt;a, b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The grading system used for classifying tubular lesions is described in Material and Methods. Data are mean ± SEM. n, number of rats; Ed (1), edarabone 1 mg/kg; Ed (5), edarabone 5 mg/kg. Histopathological classification is described in Materials and Methods. <sup>a</sup>, P < 0.05 vs. Control group. <sup>b</sup>, P < 0.05 vs. Cisplatin group.
Table 2. Effects of edarabone on cisplatin-induced nephrotoxicity in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>BUN (mg/dl)</th>
<th>Cre (mg/dl)</th>
<th>Ccr (ml/min/100g BW)</th>
<th>Urinary NAG excretion (U/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.6±1.9</td>
<td>0.26±0.01</td>
<td>0.93±0.05</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>166.1±27.7</td>
<td>2.93±0.61</td>
<td>0.05±0.0</td>
<td>1.01±0.15</td>
</tr>
<tr>
<td>Cisplatin + Ed (1)</td>
<td>53.0±8.3</td>
<td>1.23±0.19</td>
<td>0.16±0.02</td>
<td>0.69±0.12</td>
</tr>
<tr>
<td>Cisplatin + Ed (5)</td>
<td>36.0±5.5</td>
<td>0.81±0.12</td>
<td>0.30±0.03</td>
<td>0.48±0.07</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Ed (1), edarabone 1 mg/kg; Ed (5), edarabone 5 mg/kg. a, P < 0.05 vs. Control group. b, P < 0.05 vs. Cisplatin group.
Table 3. Effects of edarabone on cisplatin-induced nucleic acid damage.

<table>
<thead>
<tr>
<th>Group</th>
<th>8-OHdG (ng/µg DNA)</th>
<th>AP site (/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.51 ± 0.6</td>
<td>10.2 ± 0.7</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>5.08 ± 5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.0 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cisplatin + Ed (1)</td>
<td>2.33 ± 4.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>16.2 ± 0.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cisplatin + Ed (5)</td>
<td>0.78 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.0 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Ed (1), edarabone 1 mg/kg; Ed (5), edarabone 5 mg/kg. <sup>a</sup>, P < 0.05 vs. Control group. <sup>b</sup>, P < 0.05 vs. Cisplatin group.
Figure Legends

**Fig. 1.** Cell viability assay. (A); Cell survival rate determined from trypan blue staining after 20 µM cisplatin treatment for 24 h with or without edarabone (10<sup>-5</sup> to 10<sup>-3</sup> M). *, P < 0.01 versus cisplatin (+) + edarabone (-). (B); Mitochondrial dehydrogenation enzyme activity determined from WST-1 assay after 20 µM cisplatin treatment for 24 h with or without edarabone (10<sup>-5</sup> to 10<sup>-3</sup> M). The trypan blue exclusion and WST-1 assays were both performed in triplicate and repeated at least twice. Data are expressed as mean percentages ± SEM, relative to non-cisplatin-treated cells. *, P < 0.01 versus cisplatin (+) + edarabone (-).

**Fig. 2.** Mitochondrial membrane potential (∆Ψ<sub>m</sub>) change. PTCs were incubated with 20 µM cisplatin with or without 10<sup>-4</sup> M edarabone for 6 h. The compound 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazo-carbocyanine iodide (JC-1) was used as a probe for ∆Ψ<sub>m</sub>. Flow cytometry analysis of the cells 6 h after exposure to 20 µM cisplatin with or without 10<sup>-4</sup> M edarabone were shown. The abscissa shows 530-nm emission values that reflect low ∆Ψ<sub>m</sub> cells. The ordinate shows 590-nm emission values that reflect high ∆Ψ<sub>m</sub> cells. Each experiment was performed in triplicate and repeated at least twice. Each panel represents the results from a total 10,000 input cells.

**Fig. 3.** Reactive oxygen species (ROS) production in vitro. PTCs were incubated with 20 µM cisplatin with or without 10<sup>-4</sup> M edarabone for 6 h. The compound 2′,7′-
dichlorofluorescin diacetate (DCFH-DA) was used as a probe for intracellular peroxide formation. ROS in the cells induce oxidation of DCFH, yielding the fluorescent product dichlorofluorescein (DCF). Changes in DCF fluorescence in cells were shown. The cells were analyzed by flow cytometry. The abscissa shows 530-nm emission values that reflect ROS-generating cells. The ordinate shows the number of cells. Each experiment was performed in triplicate and repeated at least twice.

**Fig. 4.** Light microscopy of kidneys stained with hematoxylin and eosin at day 5 after cisplatin (5 mg/kg) administration. Extensive tubular damage and intratubular cast formation in the outer stripe of the outer medulla was observed in the cisplatin group. In contrast, tubular damage was reduced by treatment with 1 or 5 mg/kg edarabone. Magnification, × 40.

**Fig. 5.** Histochemistry of the cytochrome-c oxidase (COX) respiratory complex. (A); COX staining in the kidneys of rats treated with saline, cisplatin (5 mg/kg) or cisplatin + edarabone (5 mg/kg) at day 5. A marked decrease in tubular reactivity of COX was observed in the cisplatin group, whereas COX levels were comparable in the edarabone and control groups. Magnification: × 100. (B); Percentage of the COX-positive area measured by image analyzer. Values are means ± SEM. *, P < 0.05 versus cisplatin-treated group. Cont, control group; Cis, cisplatin group; Cis+Ed (1), cisplatin + 1 mg/kg edarabone group; Cis+Ed (5), cisplatin + 5 mg/kg edarabone group.
**Fig. 6.** Western blot analysis. Oxidatively modified proteins containing carbonyl groups (A) or HNE (B) in renal protein. The extracts (10 µg) from each group were analyzed by Western blot using a polyclonal antibody developed against carbonyl groups or a monoclonal antibody against HNE. MW, molecular weight marker; Cont, control group; Cis, cisplatin group; Cis+Ed (1), cisplatin + 1 mg/kg edarabone group; Cis+Ed (5), cisplatin + 5 mg/kg edarabone group.

**Fig. 7.** Immunostaining for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL). (A); Apoptotic nuclei in the kidneys of rats treated with saline, cisplatin (5 mg/kg) or cisplatin + edarabone (5 mg/kg) at day 5. A significant number of TUNEL-positive cells was observed in the cisplatin group. In contrast, few TUNEL-positive cells were evident in the cisplatin + edarabone group. Magnification: × 100. (B); Quantitation of TUNEL-positive nuclei. Treatment with 5 mg/kg edarabone significantly reduced the number of TUNEL-positive nuclei. Values represent means ± SEM of apoptotic nuclei observed per field at a magnification of × 100 in kidneys prepared from each animal. *, P < 0.05 versus cisplatin group. Cont, control group; Cis, cisplatin group; Cis+Ed (1), cisplatin + 1 mg/kg edarabone group; Cis+Ed (5), cisplatin + 5 mg/kg edarabone group.
Figure 1
Figure 2

Control  Cisplatin  Cisplatin + 10^{-4} M Edarabone

68.9 % 2.6 %
28.4 % 0.1 %

5.4 % 11.1 %
37.0 % 46.5 %

47.1 % 13.2 %
38.4 % 1.3 %
Figure 4

Control

Cisplatin

Cisplatin + Edarabone 1mg/kg

Cisplatin + Edarabone 5mg/kg
Figure 5

A

Control  Cisplatin  Cisplatin + Edarabone 5mg/kg

B

Graph showing COX positive area (% of total) for different treatments:
- Control
- Cisplatin
- Cisplatin + Edarabone 1mg/kg
- Cisplatin + Edarabone 5mg/kg

* indicates a significant difference compared to the control group.
Figure 6

A

B

MW  Cont  Cis  Cis + Cis + Ed(1) Ed(5)

(kDa)

97.4
68.0
43.0
29.0
21.0
Figure 7

A

B