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ORIGINAL ARTICLE

Comparative nephrotoxicity of cisplatin and new octahedral Pt(IV) complexes

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Abstract
Purpose Previously, we have reported that the newly synthesized octahedral Pt(IV) compound, *trans*,*cis*-Pt(acetato)$_2$Cl$_2$(1,4-butanediamine), K101 and *trans*, *cis*-Pt(trifluoroacetato)$_2$Cl$_2$(1,4-butanediamine), K102 showed potent antitumor activities in vitro and in vivo. In order to compare the nephrotoxicity of the newly synthesized Pt(IV) complexes, K102 and K102 with cisplatin, we performed various tests.
Materials and methods We performed a single dose acute toxicity test for LD_{50} values determination, biochemical assays in blood serum, acid phosphatase enzyme histochemistry and transmission electron microscopic studies in renal proximal tubular cells in mice in vivo. The route of drugs administration is intraperitoneal injection.
Results In biochemical assays, the serum levels of BUN were significantly elevated at 6 h ($p < 0.001$), 1 day ($p < 0.05$) and 3 days ($p < 0.001$) after injection in cisplatin treated mice (6 mg/kg, single dose, i.p.). On the other hand, the serum levels of BUN were slightly elevated at 6 h ($p < 0.01$) only in K101 treated mice (8.2 mg/kg, single dose, i.p.), and were significantly raised at 6 h, 1 and 3 days ($p < 0.05$) after injection in K102 treated mice (6.2 mg/kg, single dose, i.p.). The higher serum BUN level in K102 treated mice is considered that K102 possesses more lipophilic fluoro group than acetyl group in K101. The values of creatinine and uric acid were similar in all groups. The ultrastructural morphological changes of K101- or K102-administrated mice were less remarkable than cisplatin-administrated mice. In acid phosphatase enzyme histochemistry, cisplatin treatment induced relevant changes in the distribution pattern of enzyme activity compared with K101 or K102 treatment at 7 days after injection.
Conclusions In conclusion, these results show that K101 is less nephrotoxic than cisplatin and a promising new platinum complex.

Keywords Cisplatin · Octahedral Pt(IV) complex · K101 · K102 · Nephrotoxicity · Ultrastructure · Acid phophatase activity

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Introduction

Cisplatin, *cis*-diamminedichloroplatinum(II) (Fig. 1) [1] is one of the most potent antitumor agents [2–4]. However, its spectrum of antitumor activity is narrow and its use in the clinical fields has been limited by its undesirable side effects, including nephrotoxicity, ototoxicity, neurotoxicity, nausea, vomiting and myelosuppression [5, 6]. The main dose-limiting side-effect is the impairment of kidney function [7–9]. Nephrotoxicity has been reported in more than 30% of patients after therapeutic dosage of cisplatin [7]. Although the mechanism underlying cisplatin-induced nephrotoxicity is still not clear,

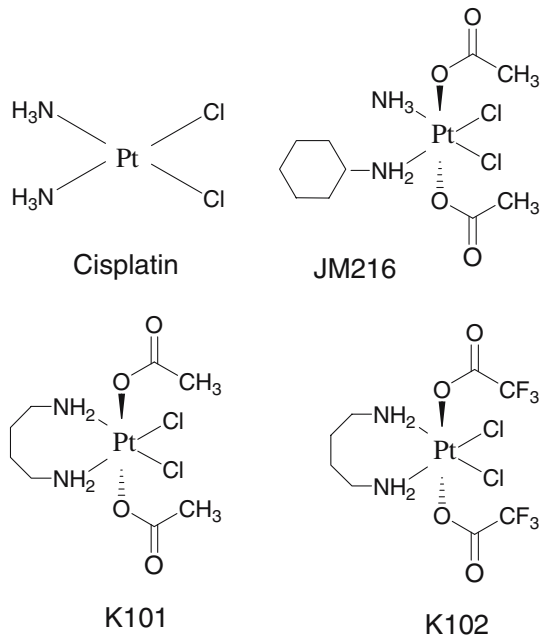

Fig. 1 Structures of cisplatin and Pt(IV) complexes as anticancer agents

experimental studies have confirmed via both light and electron microscopy, that cisplatin-induced cell injury and necrosis in the rat kidney are predominantly localized in the S3 segment of proximal tubules in the corticomedullary region, with or without accompanying distal tubule changes [10, 11]. Cisplatin nephrotoxic effect is probably related to its preferential uptake by proximal tubular cells [12, 13]. Efforts have been made to obtain analogs of cisplatin with reduced dose-limiting nephrotoxicity and the same or greater antitumor potency. Significantly, the prototype Pt(IV) complex, satraplatin (JM216) is mainly investigated in case of prostate cancer, and entered phase III clinical trials in 2001 as an orally active anticancer drug [14]. In lung cancer patients, oral JM216 showed no significant acute impairment of renal function [15]. The other cisplatin analog Pt(II) complex, carboplatin and nedaplatin showed reduced nephrotoxicity compared with cisplatin [16]. In our previous study, we reported that the series of (1,4-butanediamine) Pt(IV) complexes with axial acetato, trifluoroacetato ligands have the potent anticancer activities in vitro and in vivo [17]. In addition, K101 [*trans*,*cis*-Pt(IV)(acetato)$_2$Cl$_2$(1,4-butanediamine)] showed potent antitumor activity and induced apoptosis via ERK1/2 activation and p53 pathway [18]. In this paper, we aim to analyze the nephrotoxic effects of new octahedral Pt(IV) complexes, K101 and K102, compared with cisplatin by the methods of blood chemistry examination, morphological studies such as transmission electron microscopy and acid phosphatase enzyme histochemistry in ICR mice in vivo.

Materials and methods

Drugs

New octahedral Pt(IV) complexes, K101 [*trans*,*cis*-Pt(acetato)$_2$Cl$_2$(1,4-butanediamine)] and K102 [*trans*, *cis*-Pt(triflouroacetato)$_2$Cl$_2$(1,4-butanediamine)] were synthesized at STC life science center, Seoul, Korea [17]. Cisplatin was purchased from Sigma Chemical Co. (Louis, Missouri, USA) K101, K102 and cisplatin were dissolved in DMSO adding PBS immediately before use, then sterilized through 0.22 μm disc filter.

Animals

ICR strain male and female mice (about 25 g of weight, 6 weeks old) were purchased from Daehan Biolink Co. Ltd. (Chungbuk, Korea). The mice were housed in SPF (specific pathogen free) facility in College of Medicine, Hanyang University, and acclimated for a week. The animals were kept under standardized conditions (environmentally controlled room on a 12 h light/dark cycle) and had free access to food (purchased from Samyang Co. Ltd., Korea) and tab water up to the day of experiments.

In order to analyze the nephrotoxic effects, ICR male mice (7 weeks old) were used in this study. The mice were divided into control and experimental groups, and six mice were given to each group. The control mice were administrated only PBS by intraperitoneal injection (i.p.) and the experimental mice were administrated the half dose of LD_{50} (the half of lethal dose) of cisplatin, K101 and K102, respectively. The test for determination of LD_{50} values was performed according to 14-day toxicity protocol in National Toxicology Program (NTP).

Blood chemistry examination

Blood samples were obtained from the heart puncture using syringes. By 6 h, 1, 3 and 7 days after drugs treatment, the separated serum samples by centrifuging the blood from mice at 4,500 rpm for 15 min were kept at -20 °C. The serum levels of BUN (blood urea nitrogen), creatinine and uric acid were measured by autochemistry analyzer (Olympus Reply, Japan). The data represent the means ± SE.

Transmission electron microscopy

After the blood sampling for the chemistry study, the animals were sacrificed and the kidneys were removed promptly. In order to observe the ultrastructural changes of proximal renal tubule cells, parts of

renal cortex was finely cut in the size of 1 mm^3, and were fixed in cold fixative of 2% glutaraldehyde–2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After overnight fixation at 4 °C, the specimens were postfixed in 1% OsO_4, and dehydrated in graded alcohol. Decreasing concentrations of propylene oxide and increasing EmBed-812 (Electron Microscopy Services, Fort Washington, PA, USA) were employed. After pure fresh resin embedding and polymerization, 1 μm thick sections were initially cut and stained with methylene blue. Once the part of the renal cortex was confirmed in the same section, ultrathin sections, 60–80 nm thick, were made, stained with uranyl acetate and lead citrate, and observed with transmission electron microscope (TEM) at 80 kV using Hitachi H-7600S transmission electron microscope (Japan).

Acid phosphatase enzyme histochemistry

To perform acid phosphatase enzyme histochemistry, we modified Gomori's method [19]. The removed kidneys were fixed in 10% formalin for 24 h at 4 °C. Tissue sections, 14 μm in thickness, were cut on a cryostat at -20 °C cabinet (Rechurt-Jun, 2500, Germany). The sections were picked up onto clean poly-L-lysine coated glass slides and were stored in the cryostat cabinet until usage. The sections were incubated in the substrate medium (6 mL of 2% sodium-6-glycerol phosphate, 10 mL of 0.1 M acetate buffer pH 4.7, 4 mL of 5% lead citrate and 8 mL of DW) in order to demonstrate acid phosphatase activities. Control sections were incubated in substrate-free media. Acid phosphatase activity was observed under the light microscopy (400×). Enzyme histochemical staining intensities were represented the following arbitrary units such as '+++' (strong reaction), '++' (moderate reaction), and '+' (weak reaction).

Results

LD_{50} determination of drugs

The LD_{50} values of cisplatin, K101 and K102 were 12, 16.3, and 12.4 mg/kg, respectively (Table 1). The values were obtained using method of Probit analysis in StatsDirect statistical software (ver.2.5.5) and the data showed proportional response with 95% confidence intervals.

Serum levels of BUN, creatinine and uric acid

The serum levels of BUN were shown in Table 2. The results were represented as mean ± SE of six mice.

Table 1 LD_{50} values of cisplatin and new Pt(IV) complexes in ICR mice

Dose (mg/kg)	Survival mice number ($n = 10$)		
	Cisplatin	K101	K102
5	10	10	10
10	7	9	7
15	3	7	4
20	0	2	0
25	0	1	0
LD_{50} (mg/kg)	12	16.3	12.4

Cisplatin, K101 and K102 were administered by single intraperitoneal (i.p.) application as PBS to ICR mice. Each groups were comprised of ten mice ($n = 10$, five mice of male and five mice of female per group). The test for determination of LD_{50} values was performed in according to 14-day toxicity protocol in National Toxicology Program (NTP). During 14 days was observed for this test. LD_{50} values were calculated using method of Probit analysis in StatsDirect statistical software (ver.2.5.5) and the data showed proportional response with 95% confidence intervals

Among the K101-treated groups, serum BUN levels were slightly increased in only 6 h group ($p < 0.01$). On the other hand, the cisplatin-treated mice showed significantly increased serum BUN levels in 6 h ($p < 0.001$), 1 day ($p < 0.05$) and 3 days ($p < 0.001$) groups. The K102-treated mice also showed significantly raised levels in 6 h, 1 and 3 days ($p < 0.05$) groups. The levels of serum creatinine and uric acid were not changed in all experimental groups.

Ultrastructural changes by transmission electron microscopy

Epithelium of proximal renal tubule in control group

Proximal renal tubular epithelial cells were connected to neighboring cells by desmosome. Round nucleus was located in center of the cell. Numerous microvilli projected toward lumen and many endocytic vesicles and vacuoles in various sizes were located just beneath the microvilli. In the cytoplasm, mitochondria associated with rough endoplasmic reticulum, lysosomes and Golgi complex were found. At the bottom of the cytoplasm, well-developed basal folding disposed in parallel with mitochondria were observed.

Epithelium of proximal renal tubule at 6 h after treatment

In K101 treatment group, the general morphology was similar with the control group except big-sized vacuoles. In K102 treatment group, many lysosomes were found in apical cytoplasm and parallely

Table 2 Serum levels of BUN in ICR mice after a single i.p. of cisplatin or octahedral Pt(IV) complexes

Drugs	Dose (mg/kg)	6 h	1 day	3 day	7 day
Control	–	19.70 ± 1.27	22.13 ± 1.62	23.77 ± 1.37	18.85 ± 1.00
Cisplatin	6.0	31.13 ± 1.50***	31.83 ± 2.24**	33.05 ± 1.34***	19.65 ± 1.14
K101	8.2	28.06 ± 1.78*	23.87 ± 1.17	22.72 ± 1.84	22.38 ± 1.52
K102	6.2	30.24 ± 1.75**	31.73 ± 1.64**	31.93 ± 1.92**	20.32 ± 1.75

The mice were divided into control and experimental groups, and six mice were given to each group. Control mice were injected only PBS and test group mice were injected cisplatin or Pt(IV) complexes single intraperitoneal administration. Dose of drugs was designed as the half of LD_{50} value. The statistical data were obtained from one-way ANOVA. All data are represented as mean ± SE. Significant difference from corresponding control groups. * $p < 0.01$, ** $p < 0.05$, *** $p < 0.001$

arranged mitochondria were deteriorated in basal cytoplasm. Cisplatin induced cellular damage which is characterized by dark electron density, dilatation of perinuclear cistern, closely packed mitochondria and dilatation of cisternae of RER. In addition, numerous lysosomes and vacuolization in epithelium were observed.

Epithelium of proximal renal tubule at 1 day after treatment

In K101 treatment group, vacuolization and many secondary lysosomes were found in apical cytoplasm (Fig. 2, upper left). In K102 treatment group, vacuolization, alteration of several lysosomes as well as deterioration of basal foldings in parallel with mitochondria were observed (Fig. 2, upper center). In cisplatin treatment group, damaged cell (DC) was found in right side. It showed dark electron density, lysosomes and closely packed mitochondria. The other cell also showed vacuolization, many secondary lysosomes and deterioration basal mitochondrial arrangement (Fig. 2, upper right).

Epithelium of proximal renal tubule at 3 days after treatment

In K101 treatment group, big sized vacuoles and many secondary lysosomes were seen in the apical cytoplasm. The other cytoplasmic organelles look like the normal appearance. In K102 treatment group, alterations of the lysosomes and vacuolization were found. Some mitochondria showed morphological changes, that is, horse-shoe shaped, and swollen cristae. Cisplatin induced cell damage which showed many vacuoles and lysosomes as well as dilatation of perinuclear cistern and closely packed mitochondria. The other cells also possessed many vacuoles, primary and secondary lysosomes. In the basal cytoplasm, basal foldings and its associated mitochondria arrangement were disrupted.

Epithelium of proximal renal tubule at 7 days after treatment

In K101 treatment group, the proximal tubular cells appeared nearly normal morphology (Fig. 2, lower left). In K102 treatment group, vacuolization and numerous primary and secondary lysosomes were still found in the apical cytoplasm. Some portion of the basal cytoplasm, deterioration of arrangement of mitochondria and basal foldings were observed (arrow) (Fig. 2, lower center). In cisplatin group, damaged cell (DC) was still remained in the proximal tubule (Fig. 2, lower right).

Acid phosphatase enzyme histochemistry

The results are summarized in Table 3. In the control mice, strong positive reaction was shown in the proximal renal tubule cells. Cisplatin, K101 or K102 treatment induced relevant changes in the distribution pattern of acid phosphatase activity. At 1 day after treatment, K101 and K102 treatment group showed strong positive reaction (Fig. 3a and b), and cisplatin treatment group revealed weak positive activity (Fig. 3c). At 3 days after treatment, K101 and K102 were shown moderate to strong positive reaction, but weak to moderate reaction was revealed on cisplatin treatment group. At 7 days after treatment, K101 and K102 treatment groups revealed strong positive activity (Fig. 3d and e), on the other hand, cisplatin treatment group showed weak positive reaction (Fig. 3f).

Discussion

Nephrotoxicity represents one of the major set-backs in the use of the antineoplastic drug cisplatin. In order to reduce nephrotoxicity and increase antitumor activity of cisplatin, amounts of platinum analogs were synthesized and studied their toxicity. Among the analogs, Pt(IV) complex, satraplatin (JM216) showed potent

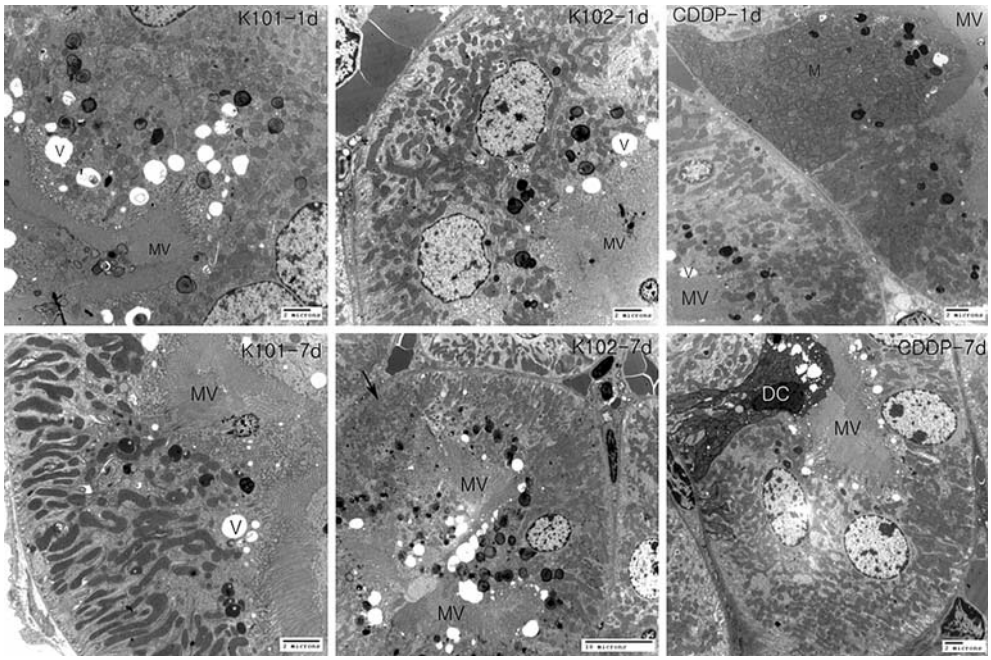

Fig. 2 Electron micrographs of proximal renal tubule segments from drugs injected groups after 1 day (*upper row*) and 7 days (*lower row*) treatment. Note vacuolization, numerous primary and secondary lysosomes, and cell debris in the lumen in K101 and K102 treatment after 1 day groups (*upper left* and *center*). Damaged cell is also observed in cisplatin treatment after 1 day group (*upper right*). After 7 days, K101 group appears almost normal (*lower left*). K102 group shows vacuolization, numerous primary and secondary lysosomes, cell debris in the lumen (*lower center*). In cisplatin group, apical portion of damaged cell (*DC*) is protruded into the lumen, and some cell shows deterioration of basal foldings associated with mitochondria (*lower right*)

antitumor activity and low nephrotoxicity in clinical trials [14, 15]. In previous papers, we have reported that new octahedral structure Pt(IV) complexes with axial ligands manifestate the potent anticancer activities [17]. And in the further study, we demonstrated that K101 induced apoptosis via ERK1/2 activation and p53 pathway in human colorectal cancer cell line [18]. In this paper, we aimed to analyze the functional and structural alterations in proximal renal tubular segments.

The blood levels of BUN, creatinine and uric acid are frequently monitored, but these parameters are not early indicators of nephrotoxicity [20]. Elevations of these parameters after cisplatin administration were confirmed in previous studies [21, 22]. In this study, the serum levels of BUN were significantly elevated in cisplatin- and K102-treated mice. On the other hand, K101-treated mice were not significantly changed. According to the results of blood chemistry examination in this study, we confirmed that K101 showed less nephrotoxicity than cisplatin.

Cisplatin primarily targets the proximal tubules at the renal cortex and corticomedullary region. Uehara et al. [23] or Ward and Fauvie [24] and Ward et al. [25] reported that cisplatin treated rats showed acute tubular necrosis in the proximal tubular regions at the

Table 3 Acid phosphatase enzyme histochemistry in control and drug-treated mice

Drugs	Dose (mg/kg)	1 day	3 days	7 days
Control	–	+++	+++	+++
Cisplatin	6.0	+	+/++	+
K101	8.2	+++	++/+++	+++
K102	6.2	+++	++/+++	+++

Control mice were injected only PBS and test group mice were injected cisplatin, K101 or K102 single intraperitoneal administration. Dose of drugs was designed as the half of LD_{50} value. The acid phosphatase histochemical intensities presented the following signs: +++ strong reaction; ++ moderate reaction; + weak reaction

cortex. According to transmission electron microscopic findings, more or less ultrastructural changes in the epithelium of proximal renal tubules were observed in all drugs treated ICR mice. However, damaged evidences began to appear in cisplatin treatment group as early as 6 h. In addition, damaged cells were found in all cisplatin treated groups from 6 h to 7 days after treatment. They are characterized by dark electron density, closely packed mitochondria, dilatation of perinuclear cistern and cisternae of RER, and numerous primary and secondary lysosomes. In K101 or K102 adminis-

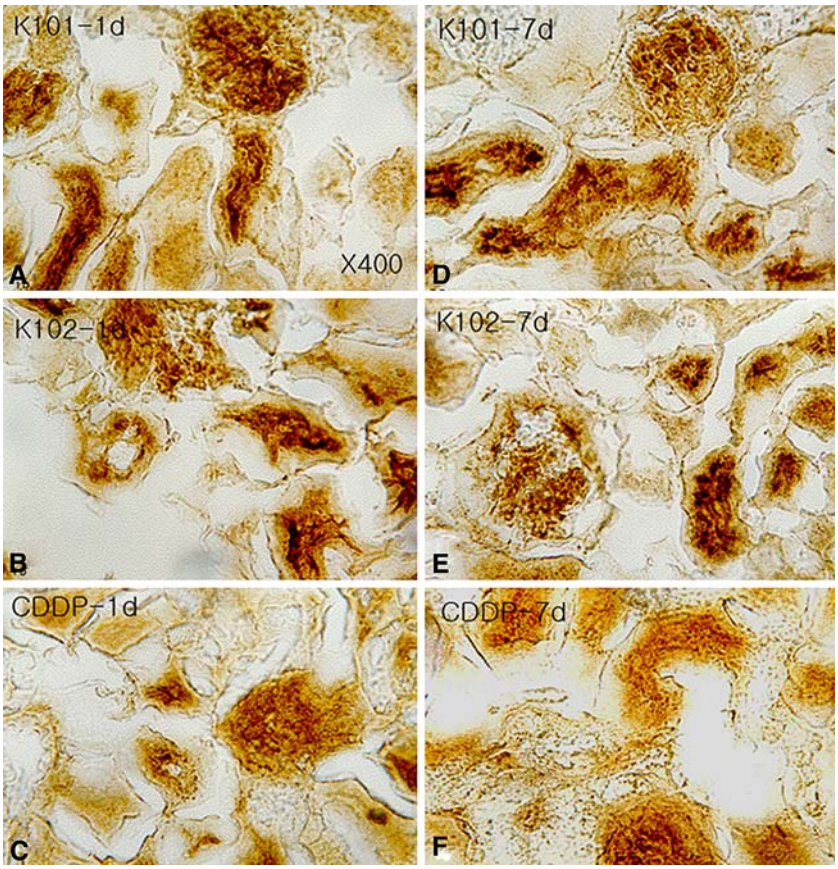

Fig. 3 Acid phosphatase activity of K101, K102 and cisplatin injected groups after 1 day (*left column*) and 7 days (*right column*) treatment. Note the reduction of positive reaction in cisplatin group (*left low*, **c**). Restoration of positive activity is observed in K101 (*right up*, **d**) and K102 (*right center*, **e**) groups and weak positive activity in cisplatin group (*right low*, **f**). Gomori's method, ×400

tered mice, the ultrastructural changes of the proximal tubular cells were less remarked than those of cisplatin treated mice. They also showed vacuolization, increase of lysosomes, alteration of various lysosomes, and deterioration of mitochondrial arrangement in parallel with the basal foldings. However, the most of organelles were recovered at 7 days after injection of K101 or K102, and even cisplatin except the existence of damaged cell.

The histochemical analysis of renal tissue at day 5 after treatment followed the indications reported in different papers, and demonstrated that, at toxic dose, cisplatin induces its maximal nephrotoxic activity between days 4 and 7 after treatment, as evidenced in mice and rats by histochemical and chemico-biological analyses [26–28]. Our study presents evidence regarding acid phosphatase activity in drugs treated samples. We observed in the proximal renal tubule cells that strong positive reaction were shown in non-drug treated mice samples. The drugs treatment induced relevant changes acid phosphatase activities. Especially, at 7 days after cisplatin treatment mice, weak reaction in acid phosphatase enzyme histochemical staining was observed. However, octahedral Pt(IV) complexes, K101 or K102 were showed moderate or strong reaction in proximal renal tubules.

In conclusion, the present study indicates that K101 is a promising new platinum analog with reduced nephrotoxicity and increased activity in vivo and in vitro.

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