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Protective effect of CV247 against cisplatin nephrotoxicity in rats

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Conflict of Interest: None

Abbreviations

Crea	serum creatinine
BUN	blood urea nitrogen
COX-2	prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2)
C	control
CV	CV247
CDDP	cis-diammine-dichloroplatinum; trade name: Cisplatin
ICP-OES	inductively coupled plasma optical emission spectrometry

Abstract

CV247, an aqueous mixture of copper and manganese gluconates, vitamin C and sodium salicylate, increased the anti-tumour effects of cisplatin *in vitro*. We hypothesized that the antioxidant and cyclooxygenase (COX-2) inhibitory components of CV247 can protect the kidneys from cisplatin nephrotoxicity in rats.

Cisplatin (6.5 mg/kg, ip.) slightly elevated serum creatinine (Crea) and blood urea nitrogen (BUN) 12 days after treatment. Kidney histology demonstrated extensive tubular epithelial damage, and COX-2 immunoreactivity increased 14 days after treatment. Cisplatin increased renal platinum (Pt) but decreased iron (Fe), copper (Cu), manganese (Mn), molybdenum (Mo) and zinc (Zn) concentrations, and increased plasma Fe and Cu concentrations. Cisplatin elevated plasma free radical concentration. Treatment with CV247 alone for 14 days (twice 3 ml/kg/day p.o.) did not influence these parameters.

Chronic CV247 administration after cisplatin reduced renal histological damage and almost significantly decreased COX-2 immunoreactivity, while failed to improve Crea and BUN. Blood free radical concentration was decreased, i.e. CV247 improved redox homeostasis. CV247 restored plasma Fe and renal Fe, Mo and Zn, while decreased renal Pt and elevated Cu and Mn concentrations.

Besides the known synergistic anti-tumour effects with cisplatin, CV247 partially protected the kidneys from cisplatin nephrotoxicity probably through its antioxidant effect.

Keywords: antioxidant, cisplatin, COX-2, manganese, nephrotoxicity, salicylate

Introduction

Cisplatin is a highly effective chemotherapeutic agent used for the treatment of various malignancies.[1-5] High-dose cisplatin-based combination chemotherapy regimens are used as first-line treatment of small-cell and non-small cell lung cancers.[6-8] However, the use of cisplatin is limited by serious side effects. Despite the use of different hydration protocols allowing dose escalation to therapeutic levels, nephrotoxicity is the main dose-limiting side effect of cisplatin.[10-14] About 20% of acute renal failure cases was due to cisplatin among hospitalised patients. High-dose cisplatin-induced nephrotoxicity (>25% decrease in eGFR) was diagnosed in 29 % of patients, following a single dose of cisplatin, and temporary elevation of serum creatinine concentration above the upper normal limit was observed in 41% of 400 cisplatin-treated patients with different solid tumours.[17] Comorbidities in lung cancer patients greatly increased the incidence of cisplatin-induced nephrotoxicity from 7.5% without co-morbidities to 20.9% with concurrent hypertension with or without ischemic heart disease, and to 30.8% with diabetes mellitus and ischaemic heart disease[18]. Due to the superior efficacy of cisplatin against a variety of human carcinomas, intensive efforts have been undertaken to weaken the side effects especially the nephrotoxic effect of cisplatin.

During treatment, cisplatin accumulates in the kidney and thus, by far the highest cisplatin concentration is measured in the kidney after treatment.[19] Copper transporter 1 (Ctr1) and the organic cation transporter 2 (OCT2) are critically involved in cisplatin uptake into renal tubular epithelial cells consequently determining nephrotoxicity[20-23]. Oxidative stress, induction of an inflammatory response and direct DNA damage are also implicated in the mechanisms of cisplatin-induced nephrotoxicity. The importance of oxidative stress has been highlighted by numerous studies demonstrating, that antioxidant agents ameliorate cisplatin nephrotoxicity in experimental animals.[24-26] The organic thiophosphate amifostine is an FDA approved protective agent against cisplatin nephrotoxicity[27] although its efficacy could not be

unequivocally demonstrated in animal experiments. Alkaline phosphatase converts amifostine to a free thiol compound, which is citoprotective by binding free radicals.[30] Acute or chronic treatment with vitamin C produced encouraging results in rat experiments.[31-34] Co-administration of Vitamin E and selenium were also beneficial[35]. Acetylsalicylic acid and sodium salicylate were also protective.[36-38]

CV247 is composed of manganese and copper gluconates, sodium salicylate and ascorbic acid (exact composition is given in the methods section), which are known to have antioxidant (ascorbic acid, manganese and copper), and TNFα inhibitor (sodium salicylate) effects. CV247 was shown to decrease the viability of six cancer cell lines in culture and to augment the cytotoxic effect of cisplatin against human breast and especially colon carcinoma.. As some constituents of CV247 protected against kidney injury as shown above, it may also alleviate the cisplatin-induced nephrotoxicity by synergistically acting at several target sites. Therefore, the aim of this study was to investigate the net effects of CV247 on renal function, antioxidant status and kidney histology after cisplatin treatment.

Materials and methods

Animals

The study was conducted on 40 male, 8-week old Wistar rats weighing 175-190 g. The animals were randomly divided into 4 groups (n=10/group). They were kept individually under standard conventional conditions according to European Council Directive 123. The study conformed to the Declaration of Helsinki guidelines and was approved by the local Animal Ethic Committee.

Test materials

Cisplatin (10 mg in 20 ml) was obtained from TEVA, Israel. The composition of CV247 (Pharmaserve Ltd, Manchester UK) was the following: 40 mg ascorbic acid, 2 mg manganese gluconate (USP), 2 mg copper gluconate, 35 mg sodium salicylate per millilitre solution (www.ivymedical.com). Methyl cellulose mucilage (Dow Chemicals) was prepared in distilled water (1 %).

Protocol of the study

The control group (C) received 1% methyl cellulose at 10 ml/kg body weight, p.o. by gastric gavage twice daily for 14 days. Another group of rats received CV247 at 3 ml/kg body weight, p.o. twice daily for 14 days (CV). The dose given was 2x120 mg/kg/day vitamin C, 2x105 mg/kg/day sodium salicylate, 2x6 mg/kg/day copper gluconate and 2x6 mg/kg/day manganese gluconate. Two groups were intraperitoneally injected with a single dose of cisplatin (CDDP) at 6.5 mg/kg body weight.[41] CDDP was suspended in 10 ml/kg 1% methyl cellulose. One of the groups injected with CDDP was subsequently treated with vehicle (C) or CV at 3 ml/kg body weight, p.o. twice daily for 14 days (CDDP+CV). All rats were weighed and food and water consumptions were also measured daily. On day 12 1.5 ml blood samples were taken from all rats by retro orbital puncture under isoflurane anaesthesia after a 20-hour food deprivation. The blood was anticoagulated with citrate and centrifuged twice at 2500

rpm for 10 min at +4 °C to obtain plasma. Plasma creatinine (Crea) and blood urea nitrogen (BUN) were determined from the plasma by colorimetric tests using commercially available kits. Rats were terminally anaesthetised with pentobarbitone on day 14. Blood was collected by aortic puncture and the kidneys were removed and weighed.

Induced chemiluminescence in the plasma

A small volume of plasma samples (50-100 µl) were assayed with a H_2O_2/·OH–microperoxidase-luminol system for 30 sec as described previously.[42] Chemiluminescence was detected in a Berthold Lumat 9501 luminometer (Berthold GmbH, Germany).

Metal contents in the plasma and kidney

After digestion of the samples in nitric acid (5 ml 65%) and hydrogen peroxide (2 ml 30%), an inductively coupled plasma optical emission spectrometric (ICP-OES) method was used for measuring metal content[43] in a Spectro Genesis ICP equipment (Kleve, Germany). For the standardization of equipment and measurements of elements Spectro multielement and Spectrum 3D standards were used. A computer guided TraceLab 50 type polarographic-voltammetric analyser was used for the voltammetric determination of selenium at -550 mV.[44]

Histology and immunohistochemistry

The kidneys were fixed in 8% buffered formalin (pH 7.4) and paraffin sections were prepared and stained with haematoxylin–eosin. Renal histological changes were blindly evaluated using a 5-grade severity scale (0 = no change; 1 = minimal changes; 2 = mild changes; 3 = moderate changes; 4 = severe changes). The cyclooxygenase-2 (COX-2) immunohistochemistry was done using a mouse monoclonal COX-2 primary antibody (Novocastra, UK) at 1:100 dilution. The secondary antibody was a peroxidase-conjugated mouse/rabbit polymer (Dako Real™ Envision™ /HRP, Rabbit/Mouse). Diaminobenzidine was used for visualisation.

Statistical analysis

Means±SD are given throughout. The statistical comparisons were performed by two-way repeated measures ANOVA with Bonferroni post hoc test or Mann-Whitney U test using GraphPad Prism 5 for Windows, or by two-way ANOVA using the SPSS 17 for Windows, when appropriate. The level of significance was set at $p<0.05$.

Results

Bodyweight

Body weight of rats steadily increased in group C from 171±8 g to 234±15 g over the 14 days of the study with a drop on day 11 after the overnight food deprivation (Fig. 1). In comparison to the baseline value, CDDP caused a 5.5 % peak body weight loss ($p<0.001$) from 167±6 g to 158±9 g on day 3 after treatment. Thereafter, body weight gain returned to a rate similar to that seen in group C. CV treatment did not influence body weight in comparison to groups C and CDDP, respectively. Consequently, on the last day of the study, body weight of rats treated with CDDP and CDDP+CV was 12-15 % lower ($p<0.001$) than body weight of rats in groups C and CV.

Food and water consumptions

CV consistently increased water consumption in comparison to that in group C (**Fig. 1**), which was statistically significant on days 8, 9 and 11 ($p<0.05$, all). Cisplatin caused a short, non-significant decrease in water consumption on day 2 after its administration. Thereafter, from day 4 rats in the CDDP and CDDP+CV groups drank significantly more water than rats in group C. Co-administration of CV to cisplatin did not alter water consumption in comparison to the group treated with cisplatin only. Neither CDDP nor CV alone or in combination altered food consumption (data not shown).

Clinical chemistry

Crea and BUN values were within physiological limits in groups C and CV (Crea: 17.0-22.5 μmol/l; BUN: 6.63- 10.48 mmol/l). CDDP increased both Crea and BUN concentrations at day 12 after its administration, while CV did not alter these effects of CDDP on renal function (**Fig. 2**).

Plasma reactive oxidant levels increased 14 days after CDDP administration, as measured by chemiluminescence. CV did not alter chemiluminescence in comparison to group C while CV attenuated the CDDP-induced elevation in plasma reactive oxidant levels (**Fig. 3**).

Metal concentrations in the plasma and kidneys

Kidney Cu, Fe, Mn, Mo and Zn concentrations were lower in the kidney 14 days after treatment with CDDP, while Co and Se concentrations did not change ($p<0.05$). Treatment with CV increased Mo concentrations in the kidney while it did not change other element concentrations (**Table 1**). Co-administration of CV with CDDP restored renal Fe and Zn concentrations to control levels, and also increased renal Cu and Mn concentrations significantly, although Cu and Mn remained below the control levels. The effect of CDDP on renal Mo concentration was restored by CV. Surprisingly, kidney Pt concentration was 3 μg/g in the group treated with CDDP. CV strikingly reduced kidney Pt concentration by 30 % ($p<0.05$).

Plasma concentrations of Pt and Se were undetectable in untreated control animals. CDDP increased plasma Co and Fe concentrations (**Table 2**). Coadministration of CV with CDDP restored plasma Fe concentrations while CV did not alter the effect of CDDP on plasma Co concentrations.

Kidney histology and immunohistochemistry

No histological changes were seen in the kidneys in groups C and CV. Varying degrees of pathological changes were found in the kidneys of CDDP and CDDP+CV groups. Renal tubular epithelial cell atrophy presented as cystic dilatations of the tubular lumina, in which accumulation of desquamated tubular epithelial cells were present as hialynacous material. Many tubular epithelial cells appeared apoptotic or necrotic. In addition, in the damaged tubular epithelium atypical, regenerating cells were visible. Tubulointerstitial inflammation presented as lymphocytic and macrophage infiltration in the interstitial space, accompanied

by interstitial fibrosis appearing as multiple focal presence of fibroblasts (**Fig. 4**). Blind assessment demonstrated a reduction in the mean score severity of histological kidney injury from 3.67±0.50 in group CDDP to 2.67±0.71 in group CDDP+CV ($p<0.01$). Immunohistochemistry revealed a moderate degree of focal COX-2 activity in the cytoplasm of the tubular epithelium, in the interstitial space and in the walls of major blood vessels (score, control: 1.20±0.42 and CV: 1.0±0.0). Blind assessment of COX-2 activity revealed that COX-2 immunoreactivity markedly increased in the groups treated with CDDP and CDDP+CV. Treatment with CV did not alter COX-2 immunoreactivity in comparison to that in group C but it almost significantly (3.00±0.71 vs. 2.44±0.53, $p=0.097$, Mann Whitney test) decreased the changes caused by CDDP (**Fig. 5**).

Discussion

In the present study we demonstrated, that CV247, a potent enhancer of the anti-neoplastic effects of cisplatin, effectively protected the kidney from cisplatin toxicity as demonstrated by renal histology and restoration of redox and trace metal homestasis. However, slight renal retention of Crea and BUN 14 days after CDDP injection was not prevented by CV247.

In group CDDP, histological damage was clearly present at day 14 after cisplatin injection, and immunohistochemistry revealed marked Cox-2 activation in the kidney, as well as an increase in plasma reactive oxidant levels, detected by chemiluminescence. Cisplatin decreased copper, manganese and zinc concentrations in the kidney, which minerals are essential cofactors of several enzymes. Chronic treatment of rats with CV247 offered protection against cisplatin-induced nephrotoxicity demonstrated by attenuation of histological injury, restored plasma reactive oxidant levels and kidney copper content, and improved kidney manganese, selenium and zinc contents at 14 days after cisplatin administration. However, slight plasma creatinine and BUN retention still present 14 days after CDDP injection and renal inflammation, as revealed by cox-2 immunohistochemistry, were not influenced by CV247.

It has been reported that CDDP accumulated in the kidney[45] and renal Pt content decreased very slowly after cisplatin administration.[46] It was an important observation in our study that chronic treatment with CV247 significantly decreased kidney platinum content by day 14 in comparison to the cisplatin alone group. Since administration of CV247 did not precede that of cisplatin, it can be excluded that CV247 interfered with renal cisplatin uptake. Therefore it seems likely that long-term administration of CV247 accelerated elimination of Pt from the kidney. This observation suggests that kidneys of CV247-treated rats recovered faster from the cisplatin-induced nephrotoxicity.

Most importantly, blind assessment of tissue pathology demonstrated that CV247 reduced the severity of renal histological injury. This observation seems to be in harmony with attenuation or full reversal of the cisplatin-induced decreases in trace mineral content of the kidney. These changes are compatible with the assumption that concentration of all those enzymes increased in the kidney, which use these minerals as cofactors for achieving their full activity. All these changes seem to suggest that manganese and copper constituents of CV247 contributed to improve the biochemical machinery of the kidney. Although, only manganese and copper were supplemented, it is well-known that trace mineral metabolism is subject to mutual synergisms and antagonisms.[47] Therefore, administration of one or few trace minerals may also consequently alter the concentrations of other minerals in the kidney.

A component of CV247, sodium salicylate inhibits the activity of cyclooxygenase (COX-1 and COX-2) isoenzymes[48]. COX-2 inhibition is anti-inflammatory and can be a renoprotective strategy as a highly selective COX-2 inhibitor (SC-58236) reduced urinary excretions of TGF-β, TNF-α, albumin, PGE2, 6-ketoPGF1α and TxB2 in streptozotocin-diabetic rats.[49] Our immunohistochemistry findings demonstrated that the cisplatin-induced elevation in COX-2 expression was almost significantly decreased by chronic treatment with CV247. In addition COX-2 enzyme activity may have been reduced by CV247 as sodium salicylate inhibits COX-2.[48]

The protective effect of sodium salicylate could be attributed to suppression of TNFα production as well.[37] Treatment with acetyl salicylic acid or sodium salicylate for 4-5 days decreased plasma BUN and creatinine at a daily dose similar to that given by us[38], restored the renal concentration of superoxide dismutase, and decreased oxidative stress as shown by renal malondialdehyde concentration in cisplatin nephrotoxicity.[36] Such effects of sodium salicylate may help explain why CV247 accelerated histological recovery of the kidney from cisplatin nephrotoxicity in our study.

Even a 10 min pretreatment with various doses of vitamin C restored cisplatin-induced increases in plasma creatinine, dose-dependently increased renal glutathione concentration and attenuated renal lipid peroxidation as assessed by malondialdehyde levels at one week after cisplatin injection.[34] One hour pretreatment with a medium dose of vitamin C (100 mg/kg/day) restored the changes in urinary 8-hydroxy-2'-deoxyguanosine caused by cisplatin suggesting that vitamin C prevented oxidative DNA damage.[33] A single high vitamin C dose given six hours prior to cisplatin also prevented the decreases in the renal cortical brush border membrane enzyme (alkaline phosphatase, leucine aminopeptidase, gamma-glutamyl transferase and maltase) activities and transport of inorganic phosphate 4 days after cisplatin administration.[32] However, the dose of 250 mg/kg was most effective in attenuating cisplatin-induced increases in plasma creatinine and BUN concentrations but lower and especially higher vitamin C doses were less effective.[32] In a light and electron microscopic study chronic daily treatment with vitamin C at low dose (8 mg/kg) decreased renal histological injury caused by three repeated cisplatin administrations at 21 day intervals.[31] These observations were similar to those seen in our study. Collectively we can conclude that pretreatment with vitamin C at medium-high doses of 100-250 mg/kg can attenuate cisplatin-induced nephrotoxicity.

Cisplatin elevated iron concentration in the plasma which may induce free radical reactions.[50] Treatment with CV247 restored the cisplatin-induced increase in plasma iron concentration. Otherwise plasma metal element content hardly differed from the control in the groups treated either with CV247 or cisplatin+CV247. The restoration of plasma iron concentration in the group treated with cisplatin+CV247 was very favourable and may explain the significant decrease of chemiluminescence intensity in the plasma.

There is always some concern whether the efficacy of a drug is impaired or not when its side effects are aimed to be reduced by administration of an adjuvant. CV247 has been

shown to have a cytotoxic effect at the G2 phase in 4 malignant human cells lines (breast, prostate, colon and lung), and CV247 produced a synergistic effect with cisplatin in 3 breast and 3 colon carcinomas by increasing the cytotoxic efficacy of cisplatin up to four-fold in cell cultures. These observations suggest that besides offering some protection from nephrotoxicity, CV247 may allow reducing the dose of cisplatin without altering its efficacy.

Cisplatin also blocks the normal accumulation of Cu and Zn in the kidney.[51] CV treatment was able to inhibit the depletion of Cu and Mn, but not of Zn.

In conclusion, the current study demonstrated that chronic CV247 administration after treatment with cisplatin offered some protection against nephrotoxicity at two weeks in rats. Since previous studies have shown that CV247 had direct toxic effects on malignant cells and also synergically increased the anticancer effect of cisplatin in cancer cell lines CV247 may enable dose reduction of cisplatin in cancer patients. A lower cisplatin dose and a partial protection against nephrotoxicity may considerably reduce the side effect of cisplatin in patients treated with CV247. However, further pharmacological studies are needed to demonstrate that CV247 also increases the anticancer effect of cisplatin in whole animal cancer models.

Acknowledgement

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Figure legends

Figure 1.

The effects of acute cisplatin and chronic CV247 administration on the body weight and daily water consumption in rats (n=10/group). Left panel: body weight. The asterisk (*) shows that from day 3 body weight was significantly decreased in the groups treated with cisplatin and cisplatin + CV247 in comparison to the groups treated with vehicle and CV247. Right panel: water consumption. The asterisk (*) shows that from day 5 water consumption was significantly increased in the groups treated with cisplatin and cisplatin + CV247 in comparison to the group treated with vehicle. The open circles show that on days 8, 9 and 11 water consumption was significantly increased in the group treated with CV247 in comparison to the group treated with vehicle. The statistical analysis was performed by two-way repeated measures ANOVA followed by Bonferroni post hoc test.

Figure 2.

The effects of acute cisplatin and chronic CV247 administration on plasma creatinine and blood urea nitrogen concentration on day 12 in rats (n=10/group). CDDP increased both plasma creatinine and blood urea nitrogen (BUN) concentrations on day 12 of the experiment. CV247 did not alter plasma creatinine and BUN concentrations compared to those of the groups treated either with vehicle or with CDDP. Group effects of CDDP and CV247 and their interaction were obtained from two-way ANOVA.

Figure 3.

The effects of acute cisplatin and chronic CV247 administration on the free radical- and reactive oxygen species (ROS)-scavenging ability of the serum of rats measured by

chemiluminescence (RLU%) on day 14 after cisplatin administration. Cisplatin increased while CV247 decreased plasma chemiluminescence. Group effects of CDDP and CV247 and their interaction were obtained from two-way ANOVA.

Figure 4.

The effects of acute cisplatin and chronic CV247 administration on kidney histology (haematoxylin–eosin staining). The structure of the kidney was normal in the C (A) and CV groups (C). Severe degree of tubulointerstitial abnormality was present in rats treated with CDDP (B), and similar but significantly less severe (CDDP: 3.67±0.50 vs. CDDP+CV: 2.67±0.71; $p<0.01$) alterations were detected in the group treated with CDDP+CV (D). See higher magnification of sections from the group CDDP (E) and CDDP+CV (F). Blinded scores of histological abnormalities were statistically compared by two-way ANOVA.

Figure 5.

The effects of acute cisplatin and chronic CV247 administration on COX-2 immunohistochemistry in the renal cortex. Mild activity in the interstitium and tubular epithelium was present in rats treated with vehicle (A) and CV (C). CDDP increased COX-2 activity in the damaged areas of the kidney (B), which effect was almost significantly decreased (3.00±0.71 vs. 2.44±0.53, $p=0.097$, Mann Whitney test) by CV (D). See higher magnification of sections from the group CDDP (E) and CDDP+CV (F). Blinded scores of COX-2 immunoreactivity were statistically compared by two-way ANOVA.

Figure 1

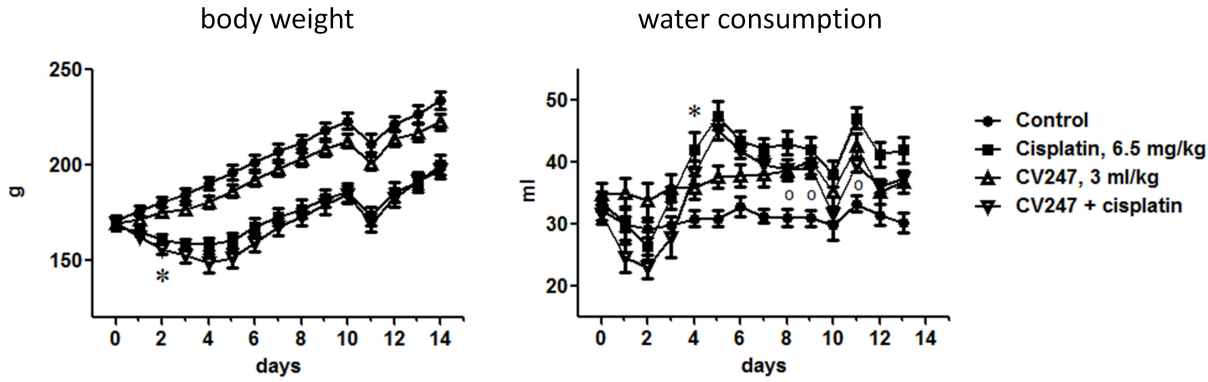
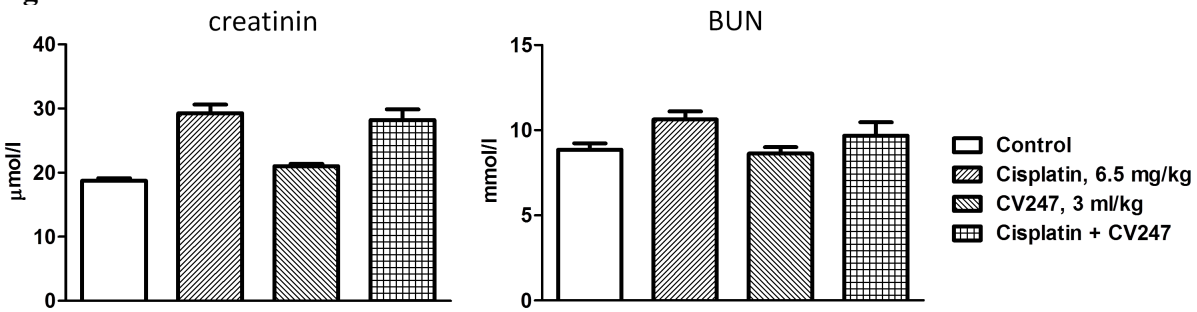

body weight
water consumption
g
ml
days
days
Control
Cisplatin, 6.5 mg/kg
CV247, 3 ml/kg
CV247 + cisplatin

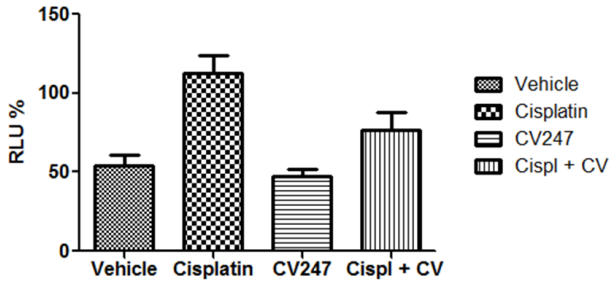
Figure 2



	p<
cisplatin	0,001
CV247	0,597
interaction	0,140

	p<
cisplatin	0,012
CV247	0,280
interaction	0,501

Figure 3



	p<
cisplatin	0,001
CV247	0,024
interaction	0,122

Figure 4

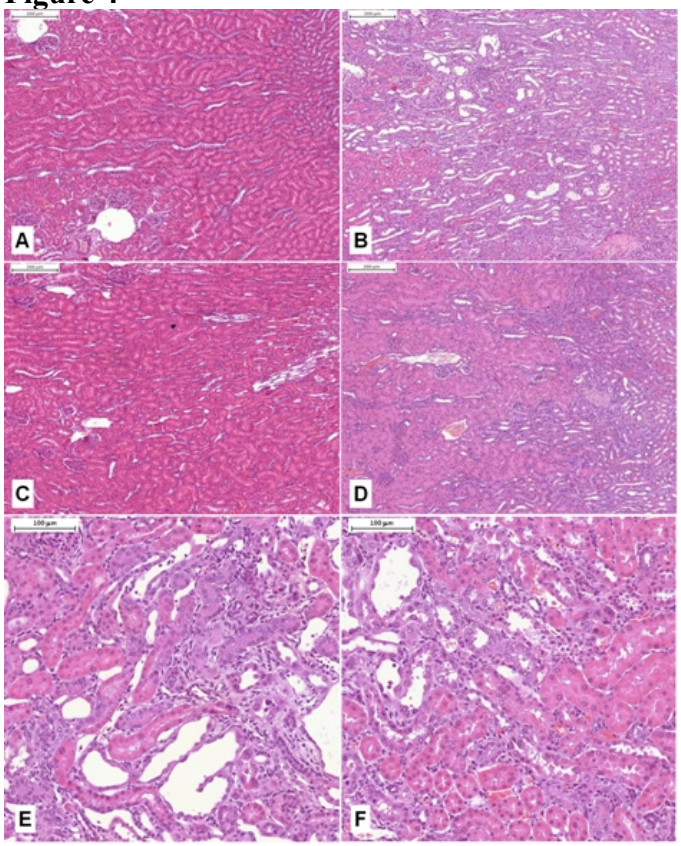


Figure 5

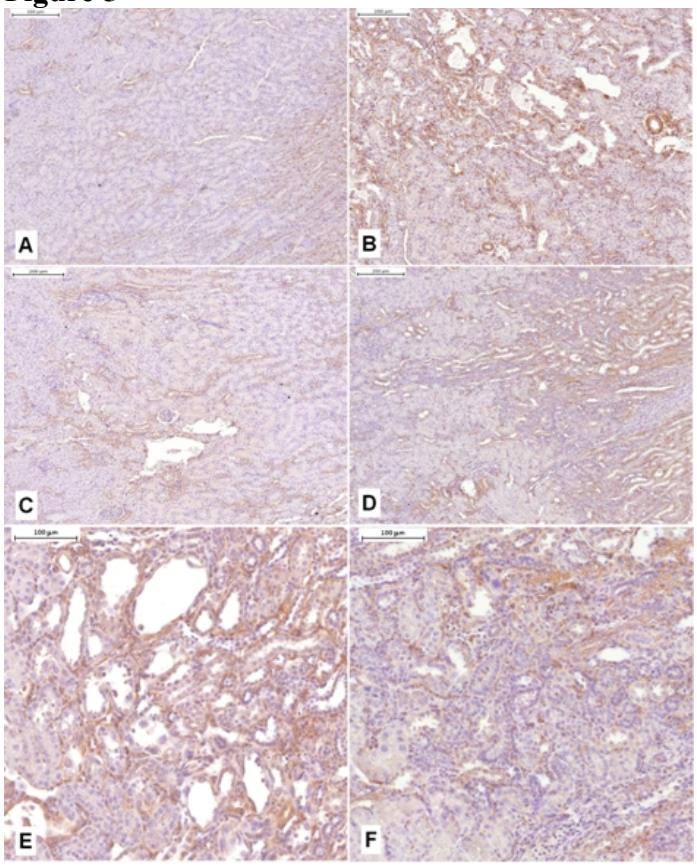


Table 1. Element concentrations of rat kidneys (µg/g) measured by ICP-OES

	Element concentration				**ANOVA**		
	C	**CDDP**	**CV**	**CDDP+CV**	**CDDP**	**CV**	**CDDP*CV**
	µg/g	**µg/g**	**µg/g**	**µg/g**	**p<**	**p<**	**p<**
Cobalt (Co)	0.25±0.03	0.21±0.05	0.23±0.03	0.21±0.05	0.053	0.384	0.382
Copper (Cu)	5.09±0.50	2.85±0.61	5.07±0.80	3.86±0.54	**0.001**	**0.024**	**0.016**
Iron (Fe)	42.5±4.1	33.4±3.4	38.2±2.8	38.7±4.4	**0.001**	0.743	**0.001**
Manganese (Mn)	0.81±0.12	0.51±0.10	0.79±0.08	0.60±0.07	**0.001**	**0.01**	**0.032**
Molybdenum (Mo)	0.23±0.02	0.19±0.03	0.26±0.02	0.23±0.04	**0.001**	**0.001**	0.662
Platinum (Pt)	BLQ	3.05±0.58	BLQ	2.07±0.25	-	**0.001**	-
Selenium (Se)	0.19±0.16	0.13±0.06	0.21±0.09	0.29±0.15	0.895	0.202	0.331
Zinc (Zn)	16.1±1.38	13.2±1.51	15.1±0.82	15.0±0.70	**0.001**	0.340	**0.001**

BLQ: below limit of quantitation. Statistical analysis was performed

Table 2. Element content in rat plasma (μg/g) measured by ICP-OES

	Element concentration				ANOVA		
	C	**CDDP**	**CV**	**CDDP+CV**	**CDDP**	**CV**	**CDDP*CV**
	μg/g	**μg/g**	**μg/g**	**μg/g**	**p<**	**p<**	**p<**
Copper	0.824±0.101	0.957±0.116	0.820±0.069	0.961±0.201	**0.002**	0.993	0.916
Iron	3.56±0.97	5.89±1.87	3.81±1.63	3.46±0.68	**0.046**	**0.029**	**0.008**
Manganese	0.026±0.012	0.041±0.025	0.024±0.012	0.029±0.016	0.082	0.208	0.378
Molybdenum	0.056±0.020	0.067±0.019	0.041±0.022	0.045±0.024	0.263	0.017	0.644
Zinc	1.37±0.27	1.48±0.18	1.33±0.12	1.27±0.17	0.738	0.076	0.233

Plasma cobalt and selenium concentrations were below limit of quantitation in most cases.

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