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Acute Systemic Toxicity of Combined *cis*-Diamminedichloroplatinum and Hyperthermia in the Rat

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Abstract—*To investigate the previously observed increased morbidity and mortality of combined* cis-*diamminedichloroplatinum* (cis-*DDP*) *and hyperthermia, BD IX rats were given* 4 mg/kg cis-*DDP i.p., waterbath hind leg heating* (44° C, 60 min) *with resultant whole body hyperthermia, or combined treatment with or without systemic cooling. Cardiac blood and histopathologic sections of kidney, small intestine and liver were examined in rats sacrificed* 2, 3 *and* 5 *days after and femur bone marrow* 5 *days after treatment. In a separate experiment, the effect of systemic hyperthermia on renal function was tested.*

The most significant finding was a marked increase in cis-*DDP induced renal damage by systemic hyperthermia, expressed as elevated creatinine levels and quantitatively enhanced proximal tubular necrosis. As both systemic hyperthemia and* cis-*DDP can result in primarily altered renal haemodynamics, it is postulated that relative tubular epithelial hypoxia and increased tubular exposure time to* cis-*DDP due to reduced tubular filtrate flow rate are likely mechanisms for the increased toxicity.*

INTRODUCTION

To obtain a therapeutic advantage when combining cytotoxic drugs with hyperthermia, the normal tissue toxicity has to be less increased than the effect on tumour. In previous experiments [1] we demonstrated an increased effect on the transplantable BT_4A tumour when *cis*-diamminedichloroplatinum (*cis*-DDP) i.p. was combined with waterbath hyperthermia. However, increased weight loss and a rise in toxic deaths to more than 50% of BD IX rats given combined modality treatment, compared to none in hyperthermia alone or drug alone groups, was also found. There are ongoing and planned clinical trials of combined *cis*-DDP and regional or whole body hyperthermia and disclosing the nature of the accentuated toxicity could be important for the clinical protocols. The present investigation was therefore initiated to examine the acute systemic toxicity of *cis*-DDP and hyperthermia in the BD IX rat. We postulated an accentuation of *cis*-DDP's dose limiting effects on rats during normothermia: renal damage, intestinal and myelotoxicity. As liver tissue is hyperthermia sensitive [2] and also has high *cis*-DDP tissue levels following systemic drug administration [3], the liver was also examined.

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MATERIALS AND METHODS

Animals and treatment groups

Inbred BD IX rats aged 8–12 weeks were kept on a 12 hr light and darkness schedule, fed a standard pellet diet and given water *ad libitum*. The BT_4A tumour was transplanted to the right hind leg as previously described [4]. Two weeks after transplantation at a mean tumour volume of about 300 mm^3, the rats were stratified according to tumour size and randomized into treatment groups, consisting of *cis*-DDP alone, waterbath hyperthermia of the tumour-bearing leg, combined hyperthermia and *cis*-DDP, combined hyperthermia and *cis*-DDP with systemic cooling, and controls.

In a second experiment, rats were randomized to the same treatment groups with addition of one group of rats given combined *cis*-DDP and systemic hyperthermia without leg-heating.

Drug

Cis-diamminedichloroplatinum (Cisplatina, Farmitalia Carlo Erba, Milan, Italy) in vials containing 10 mg active drug, 45 mg NaCl and 300 mg of mannitol was dissolved in sterile water to a 1 mg/ml solution. A dose of 4 mg/kg was given i.p. followed within seconds by a 3% body weight of 0.9% NaCl i.p. to increase diuresis. Hyperthermia and control rats were only given the hydration regimen. Combined *cis*-DDP and hyperthermia-treated rats were given *cis*-DDP and NaCl just prior to start of hyperthermia.

Hyperthermia

The rats were fixed in jigs as previously described [4] and the tumour-bearing foot was immersed for 60 min into a precision controlled, circulating waterbath of 44 ± 0.1° C. In the first experiment a steady rise in body temperature to about 41° C for the second half of the hyperthermia treatment was registered. Cooling by fanning room air towards the rats during waterbath hyperthermia resulted in a mean reduction in rectal temperature of 1° C compared to non-cooled hyperthermia treated rats. Control and *cis*-DDP alone rats were also fixed after injection for 1 hr at room temperature.

In the second experiment, the mean rectal temperature was 0.4° C higher in leg-heated rats due to a higher room temperature. One group of rats was fixed above the waterbath and the rectal temperature profile of leg-heated animals was closely simulated by blowing warm air towards the jigs. By drilling multiple air-holes in the jigs, the cooled rats in this experiment had a mean reduction in rectal temperature of 3° C during the last 30 min of heating, compared to non-cooled rats.

Blood and bone marrow analysis

Two rats in each treatment group were sacrificed 2 days after treatment and five rats in each treatment group at day 3 and 5. The animals were anaesthetized with ether and blood samples from cardiac puncture were drawn into an EDTA-washed syringe before cervical dislocation. The blood was analysed by standard laboratory procedure on haemoglobin, erythrocyte counts, erythrocyte volume fraction, white blood cells with differential counts and creatinine. Alanine amminotransferase was analysed days 2 and 3 after treatment, thrombocytes only at day 3. In the second experiment, creatinine was analysed in a 500 µl blood sample from the femoral vein on day 3 and from the same rats from cardiac blood on day 5.

Rats sacrificed at day 5 of the first experiment had the femur of the non-tumour-bearing leg removed. The bone was flushed through each end by a total of 5 ml culture medium (Dulbecco's modification of Eagle's medium), and the total number of marrow cells was estimated in a Coulter Counter. Marrow smears were dried, fixed in methanol, stained with Giemsa, coded, and differential counts were made under oil immersion microscopy. Erythropoietic and myelopoietic cells were distinguished by standard morphological criteria. The myelopoietic cells were classified as proliferative (myeloblasts, promyelocytes and myelocytes) and non-proliferative cells (metamyelocytes and mature granulocytes). The total counts of each cell compartment for each femur was estimated as described previously [5].

Histopathologic analysis

In the first experiment, two rats in each treatment group were sacrificed at either termination of hyperthermia or 1 hr after drug injection in non-heated rats and 2 days after treatment. At 3 and 5 days five rats in each group were sacrificed. The right kidney was removed and 2 mm sections were made. Slices from the median liver lobe and a 1 cm segment of small intestine from the duodeno-jejeunal junction was removed. All specimens were fixed in 10% buffered formalin, embedded in paraffin, coded and prepared for light microscopy by haematoxylin and eosin staining. The coded specimens were examined by two separate investigators and a joint conclusion was made subsequently.

Statistical analysis

In the first experiment, two ways analysis of variance was used to evaluate differences in blood and bone marrow parameters between controls, hyperthermia, *cis*-DDP and combined treatment groups [6]. Combined treated groups with and without cooling were compared with the Mann–Whitney test [7].

Creatinine levels in the second experiment were tested for normality distribution and compared by two-tailed Student's *t*-test.

RESULTS

Haemoglobin, erythrocyte counts and erythrocyte volume fractions in the treatment group were not significantly different 3 days after treatment, but at 5 days, erythrocyte counts and erythrocyte volume fractions were higher ($p < 0.05$ and < 0.01, respectively) in *cis*-DDP treated groups compared to hyperthermia alone and control groups (Table 1). Total white blood cells (Table 1), differential counts of white blood cells, thrombocytes and alanine amminotransferase (data not shown) were not significantly different from controls in any treatment group.

Analysis of femur marrow is listed in Table 2. The myelopoietic activity was not different in the

Table 1. *Mean haemoglobin, erythrocyte volume fractions, erythrocyte and white blood cell counts in groups of five rats* 3 *and* 5 *days after treatment with* cis-*DDP or hyperthermia alone or in combination (first experiment).* ±, *SEM*

Days after treatment	Haemoglobin g/dl		Erythrocyte count 10^{12}/l		Erythrocyte volume fraction		White blood cells 10^9/l	
	3	5	3	5	3	5	3	5
Controls	13.9 ±0.7	14.0 ±0.8	6.17 ±0.37	7.11 ±0.22	0.40 ±0.02	0.36 ±0.01	9.7 ±1.2	11.9 ±1.5
Hyperthermia 44° C 60 min.	13.0 ±0.9	14.6 ±0.2	5.94 ±0.56	7.36 ±0.10	0.38 ±0.03	0.37 ±0.01	7.3 ±0.5	12.1 ±0.5
Cis-DDP 4 mg/kg	14.1 ±0.8	15.5 ±0.2	5.78 ±0.22	7.81 ±0.11	0.39 ±0.01	0.41 ±0.01	6.8 ±1.1	9.7 ±1.0
Combined *cis*-DDP and hyperthermia	14.7 ±0.9	15.7 ±0.5	5.90 ±0.52	7.86 ±0.30	0.42 ±0.01	0.41 ±0.01	5.9 ±1.4	11.0 ±0.5
Combined treatment with cooling	14.3 ±0.6	14.9 ±0.3	5.81 ±0.28	7.68 ±0.11	0.41 ±0.02	0.38 ±0.01	6.8 ±1.4	11.9 ±1.5

Table 2. *Total numbers of myelopoietic and erythropoietic cells per femur in groups of five rats* 5 *days after treatment. Myeloblasts, promyelocytes and myelocytes were counted as proliferative cells. Means* ± *SEM*

	Myelopoietic cells		Erythropoietic cells 10^6
	Proliferative 10^6	Non-proliferative 10^6	
Controls	21.04 ± 2.55	18.70 ± 2.16	37.22 ± 5.42
Hyperthermia 44° C 60 min	22.91 ± 3.57	20.08 ± 1.80	38.56 ± 5.40
Cis-DDP 4 mg/kg	21.75 ± 3.32	14.86 ± 2.62	15.61 ± 2.87
Combined *cis*-DDP and hyperthermia	19.20 ± 2.78	17.48 ± 2.97	14.05 ± 2.41
Combined treatment with cooling	17.28 ± 1.97	17.27 ± 2.17	21.99 ± 3.29

groups 5 days after treatment. *Cis*-DDP-treated groups had lower numbers of erythropoietic cells than hyperthermia alone or control groups ($p < 0.01$), while there were no significant differences between *cis*-DDP groups with or without hyperthermia or between combined groups with or without systemic cooling.

Creatinine values days three and five in the first experiment are shown in Fig. 1. Hyperthermia alone did not influence creatinine levels. Creatinine was significantly elevated by *cis*-DDP alone at day 3 and 5 ($0.01 < p < 0.05$ and $p < 0.01$, respectively). Creatinine in combined *cis*-DDP and hyperthermia groups with and without systemic cooling were not significantly different from each other, but were higher than in *cis*-DDP alone rats at both days 3 and 5 ($0.01 < p < 0.05$). The variance analysis showed interaction of hyperthermia on the cisplatin effect ($p < 0.01$).

All *cis*-DDP treated rats had higher creatinine levels day 5 than 3 in the second experiment (Fig. 2). At day 5 creatinine was significantly higher in combined *cis*-DDP and non-cooled, leg-heated rats than *cis*-DDP alone rats ($p < 0.01$). *Cis*-DDP and whole body hyperthermia without leg-heating also increased creatinine above *cis*-DDP alone

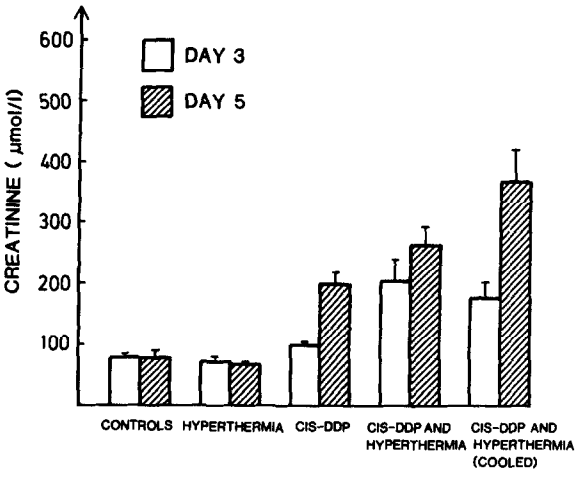


Fig. 1. Mean creatinine levels in groups of five rats sacrificed 3 and 5 days after treatment (first experiment). Vertical bars, SEM.

($p < 0.05$). Creatinine levels in *cis*-DDP and effectively cooled leg-heated rats were statistically not different from rats only given *cis*-DDP.

Sections of small intestine from the duodeno–jejeunal junction were indistinguishable in hyperthermia alone and control rats just after treatment or at 2, 3 and 5 days. All *cis*-DDP and combined treated groups had a similar morphology. At day 2, there were fewer mitotic figures in the crypts, where there was a relative dominance of goblet cells compared to control sections. At day 3 there was an increase in mitotic activity in the crypt epithelium, with both increased nuclear and cytoplasmic size. Five days after treatment there were no consistent differences between treated and control rats.

The liver sinusoids contained more erythrocytes than controls in all hyperthermia treated rats at termination of hyperthermia, while all livers at other timepoints were normal.

All kidneys examined just after hyperthermia or 1 hr after drug injection, as well as all hyperthermia and control rats at all timepoints, were normal. Two days after *cis*-DDP or combined treatment, cytoplasmic vacuolization and cytoplasmic proximal tubular discharges were seen. Occasionally whole necrotic cells were found in the lumina of proximal tubules, mostly in the outer stripe of the outer medulla. At day 3, these findings were more prominent and were found with variations in degree of severity in all parts of the cortex (Fig. 3). In the corticomedullary area, there were vast numbers of necrotic cells in the proximal tubular lumina. Eosinophilic and granular casts were seen, and in some tubule lumina in the outer medulla, calcifications were found. There was a slight degree of tubular dilatation. Animals killed 2 days later had more dilated proximal tubules, with few surviving epithelial lining cells with sparse cytoplasm (Fig. 4). There was nuclear enlargement with prominent nucleoli, indicating regeneration, in some proximal tubule cells. Distal tubule and collecting duct epithelium evidenced slight damage, expressed as nuclear pleomorphism.

There was no qualitative difference in tubular damage between *cis*-DDP alone and combined hyperthermia and *cis*-DDP treated rats. The

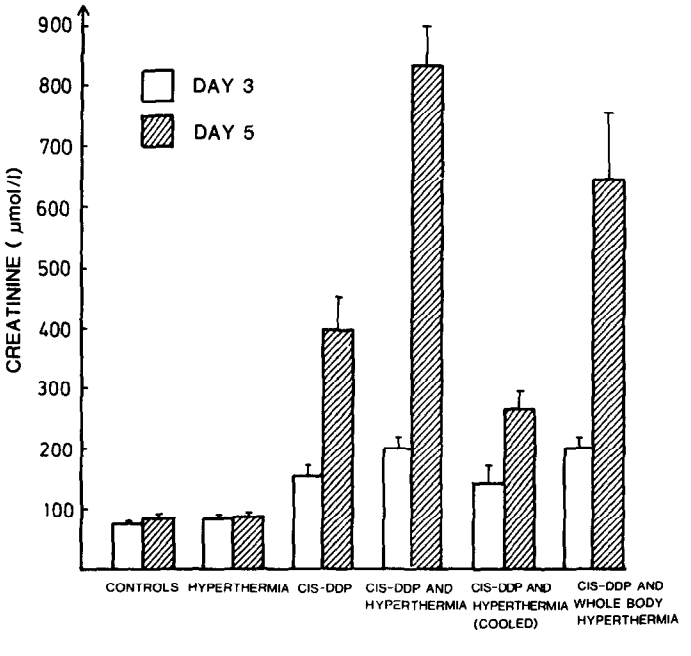
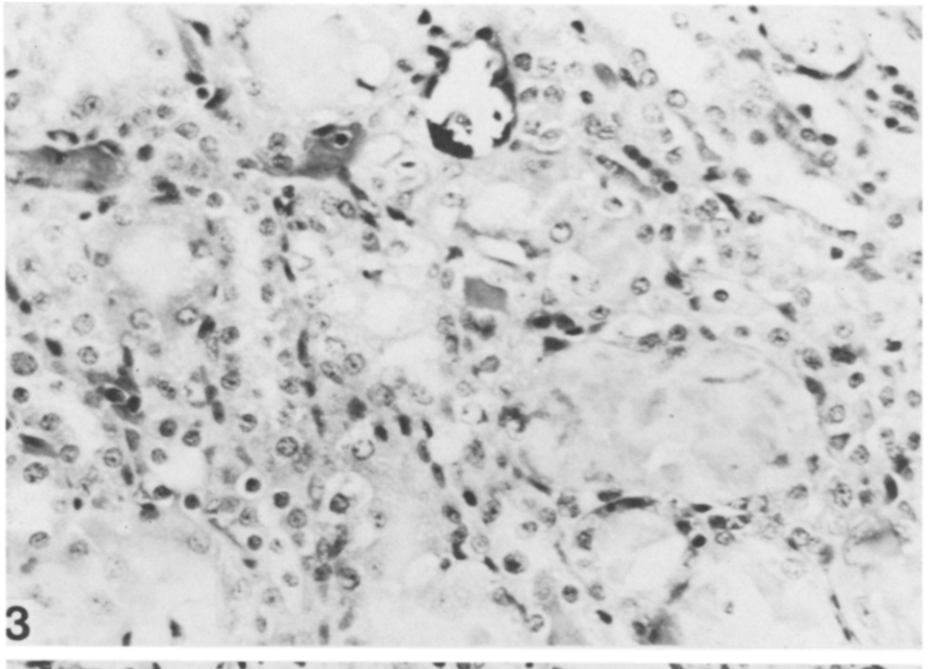


Fig. 2. Mean creatinine levels in groups of five rats treated 3 and 5 days previously (second experiment), with a higher systemic temperature and more effective systemic cooling than in the first experiment. Vertical bars, SEM.



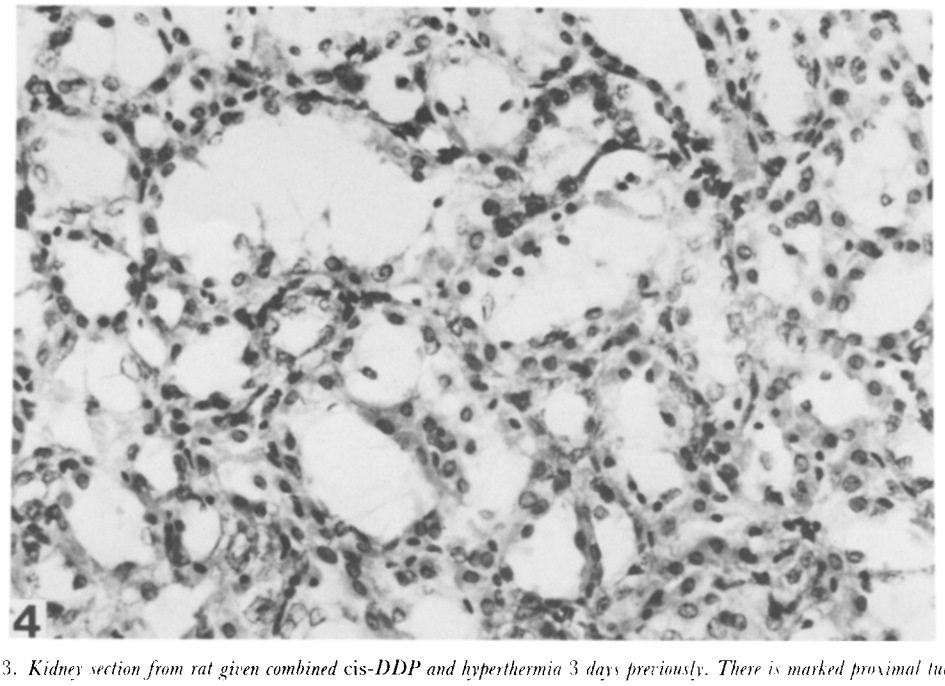


Fig. 3. Kidney section from rat given combined cis-*DDP and hyperthermia 3 days previously. There is marked proximal tubular damage. Haematoxylin and eosin* × 350.

Fig. 4. Kidney from rat 5 days after combined cis-*DDP and hyperthermia. There are more dilated tubules than 3 days after treatment (Fig. 3) and few surviving epithelial cells. Haematoxylin and eosin* × 350.

severity of tubular damage, especially the degree of damage in the cortex, was evaluated from sections of rats killed at day 5. One of five *cis*-DDP alone kidneys, and eight of ten combined-treated kidneys were graded as having severe damage. The other *cis*-DDP exposed kidneys were graded moderately damaged.

The glomeruli in all rats at all timepoints appeared normal.

DISCUSSION

In our previous experiments at *cis*-DDP 4 mg/kg combined with hyperthermia [1], toxic deaths were usually seen at days 7–9. The present experiments demonstrated that bone marrow toxicity was not responsible for the increased mortality rates in combined *cis*-DDP and hyperthermia treated rats. The *cis*-DDP depression of the erythropoietic compartment was not increased by hyperthermia. The effect was probably not solely secondary to the induced uraemia [8]. A haemoconcentration at day 4 has previously been attributed to reduced food and water intake and polyuria after *cis*-DDP exposure [9].

The small intestine damage found was slight and compatible with measurements of DNA synthesis [3] and histopathologic analysis [10] after single dose *cis*-DDP in the normothermic rat. Although *cis*-DDP induced lesions probably are most severe in the ileum [10] and more sensitive methods [11, 12] possibly could detect an increase in intestinal damage in combined-treated rats, our results do not indicate small bowel toxicity as a major contribution to death.

The *cis*-DDP renal toxicity was most severe in proximal tubules in the outer stripe of the outer medulla, which is in accordance with the reported *cis*-DDP toxicity to the straight S_3 segment [8, 13–15]. As Choie *et al.* [16], we found histopathologic changes also in proximal convoluted tubules and to a much lesser degree in distal tubules and collecting ducts. The *cis*-DDP induced histopathologic renal damage increased quantitatively when combined with hyperthermia. Moderate systemic cooling did not reduce the toxicity in the first experiment (Fig. 1). However, the second experiment showed that the renal toxicity was related to the systemic temperature elevation, as effective body cooling during leg-hyperthermia protected from the enhanced *cis*-DDP toxicity (Fig. 2). Thus, burn damage of the leg during local hyperthermia, which theoretically could result in acute tubular necrosis [17], was not responsible.

Our findings seem to contrast the results of Elkon *et al.* [18], who injected *cis*-DDP, 8 mg/kg, 30 min prior to 42.5° C hyperthermia to intact mice kidneys, and concluded that hyperthermia did not increase *cis*-DDP toxicity. Isolated kidney hyperthermia was probably not appropriate to evaluate possible renal effects of systemic hyperthermia and *cis*-DDP. Their finding of necrotic foci in kidneys of solely hyperthermia treated mice and a hyperthermia protection to late *cis*-DDP damage indicate the presence of alterations in tubular function, such as *cis*-DDP secretion, which may be important for *cis*-DDP tubular damage [19, 20]. To our knowledge, there has never been demonstrated *in vitro* hyperthermic protection from *cis*-DDP cytotoxicity.

In a previous study on the effects of timing and sequence of *cis*-DDP and hyperthermia within ± 5 hr in the same model [21], we found a greater weight loss and more toxic deaths in groups of rats with simultaneous timing of the drug and heat. This indicates that the peak blood and glomerular filtrate concentration of *cis*-DDP is important for the kidney damage of the combined modalities.

The reduction in glomerular filtration rate found after normothermic *cis*-DDP treatment has been explained by backleakage of fluid through the damaged straight portion of the proximal tubule [9], increased proximal tubular pressure due to obstruction of the S_3 segment by cellular debris and proteinous casts, or persistent vasoconstriction of the afferent arteriole [14]. Since short term morphological changes in the glomerulus after *cis*-DDP exposure have not been demonstrated, alterations in the glomerular capillary ultrafiltration coefficient seem unlikely. Mercury, a heavy metal close to platinum in the periodic table, results in a mannitol-reversible immediate and prolonged reduction in renal blood flow in dogs [22]. Primary changes in human renal haemodynamics during *cis*-DDP infusion have recently been reported [23]. Altered intrarenal blood flow distribution has been suggested as a primary mechanism for *cis*-DDP nephrotoxicity and the hypothesis is supported by a study showing an angiotensin converting enzyme inhibitor to decrease the initial *cis*-DDP induced reduction in effective renal plasma flow [24].

The glomerular filtration rate measured by ^{51}Cr clearance fell markedly in mice subjected to 41° C whole body hyperthermia [25], but returned to normal after hyperthermia. The most probable explanation was a change in renal blood flow with reduced glomerular ultrafiltration. Also in clinical whole body hyperthermia a shift in total blood flow from the body core, especially the kidneys and liver, to the periphery is seen, resulting in reduced urine production or total anuria for several hours after treatment [26]. Redistribution of or reduction in renal blood flow could result in relative hypoxia in the proximal tubular epithelium, the portion of the nephron known to be especially susceptible to hypoxia [27], which could add to the *cis*-DDP toxicity. A quantitative reduction in

ultrafiltrate could reduce the proximal tubular filtrate flow rate, which results in increased tubule cell exposure time to the drug [28].

In the human kidney, distal tubules are more severely affected by *cis*-DDP than the proximal tubules [29]. Despite this, nephroprotective regimens tested in rats are active in humans [14, 30, 31]. Two pilot studies of whole body hyperthermia combined with *cis*-DDP [32, 33] did not report increased nephrotoxicity when a high urinary output was maintained, but two recent trials demonstrated renal toxicity above that expected from *cis*-DDP alone [34, 35]. Similar *cis*-DDP pharmacokinetics were found during normothermia and later whole body hyperthermia in two patients [34]. Nevertheless, in both *cis*-DDP with whole body hyperthermia resulted in severe renal toxicity judged by creatinine rise. These sparse clinical data are in accordance with findings in our experiments, which demonstrated that systemic hyperthermia markedly enhanced *cis*-DDP renal damage.

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