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AN IN VITRO NEPHROTOXICITY SCREENING SYSTEM FOR PLATINUM COORDINATION COMPLEXES: A CYTOCHEMICAL APPROACH. presented by

Mark Andrew Batzer

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Master of Science degree in Zoology

S. K. Aggarwal

Major professor

S. K. Aggarwal

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AN IN VITRO NEPHROTOXICITY SCREENING SYSTEM FOR PLATINUM COORDINATION COMPLEXES: A CYTOCHEMICAL APPROACH.

By

Mark Andrew Batzer

A THESIS

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Dedicated to my parents
who constantly supported my work.

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Appreciation is due also to Dr. R.N. Band and Dr. R.R. Brubaker, who served as guidence committee members and advisors during my years at Michigan State University.

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ABSTRACT

AN IN VITRO NEPHROTOXICITY SCREENING SYSTEM FOR PLATINUM COORDINATION COMPLEXES: A CYTOCHEMICAL APPROACH.

By

Mark Andrew Batzer

Currently many animals and labor hours are required to screen drugs (platinum coordination complexes). To decrease the time required for screening an in vitro screen system is proposed and tested. Isolated rat kidney tubules were cultured for 8 hours with various drugs. Aliquots were taken at 0, 1, 2, 3, 4, 5, 6 and 8 hours, frozen and analyzed for the amount of Na^+/K^+ ATPase, Ca^{2+} ATPase, alkaline phosphatase, and acid phosphatase. Culture media was also analyzed biochemically for the amount of alkaline phosphatase present. The amount of alkaline phosphatase in the culture media increased by varying amounts, over time, in a drug dependent manner and equated with cytochemical analysis of tubular enzymes. In vivo studies comparing urine analysis to analysis of kidney cross-sections yielded similar results, but over a longer period of time, in a strain dependant manner. This leads to the following relation: 1 hour in vitro is equivalent to 1 day in vivo. Since these enzymes are responsible for transport in the kidney, their presence/absence is probably responsible for nephrotoxicity.

INTRODUCTION

Heavy metal platinum coordination complexes are attracting considerable attention as potential chemotherapeutic agents against a number of tumors (53). Of these cis-dichlorodiammineplatinum II (cisplatin, DDP) is currently used in the treatment of ovarian and testicular cancers (49). The drug is, however, not without certain toxic side effects in the kidney (proteinuria, morphological damage) (8, 11, 24, 57, 66), intestine (diarrhea and anorexia) (57), and lymphatic system (spleenic atrophy) (4, 57). Nephrotoxicity is the most important side effect, as it is the limiting factor in the chemotherapeutic uses of the drug (24, 25, 29, 66, 67). Currently efforts are underway to synthesize new, less toxic compounds which retain their chemotherapeutic potential (14, 29, 54).

Toxic effects of cisplatin have been extensively studied (4, 5, 6, 7, 18, 24, 25, 31, 35, 57, 66, 67), although the mechanism of action of the drug is not clearly understood (50, 53). Cisplatin has been shown to inhibit DNA replication in mammalian cells (47), as well as in prokaryotes (12, 13, 38, 42). It's mechanism of action seems to be through both inter- and intra-strand crosslinking of the DNA (46, 48). Protein synthesis and RNA synthesis may also be inhibited by the drug (26). Cisplatin has also been shown to inhibit mammalian cytokinesis (1, 3), and cause a decrease in surface associated transport enzymes (6, 11, 68), and cause embryotoxicity (31). Cisplatin also has drastic effects on metamorphosis in anurans (41). The exact mechanism of cisplatin's action with regard to its toxicities or tumor regression is not completely understood.

Second-generation analogs of cisplatin are currently being synthesized and tested (29, 49). As new compounds are developed the question of the mechanism of action resurfaces. The new compounds may act in a manner similar to CDDP, or

they may have their own, unique modes of action. Since the chemotherapeutic value of the platinum complexes is of primary concern, the mechanism of action is of equal importance.

As new platinum analogs are synthesized there is an increasing need for a fast, efficient system to evaluate the chemotherapeutic potential and nephrotoxicity of these drugs. Currently many laboratory animals and man hours are required to test each drug. This process takes a long period of time and tends to hinder the chemotherapeutic application of these new analogs. In this study an in vitro screen system is proposed and tested. To test the nephrotoxicity isolated kidney tubules are cultured the duration of each experiment. Since the main functions of the kidney are transport related (23, 65), membrane transport enzymes (Ca^{2+}-activated ATPase, Na^+/K^+-activated ATPase, Alkaline Phosphatase, and 5' Nucleotidase) were demonstrated and quantitated cytochemically, then used as indicators of normal and impaired renal function (11, 64). Similar studies were repeated in rats and results were compared to those in in vitro studies for uniformity. Attempts were also made to delineate the mechanisms of action of various platinum coordination complexes.

MATERIALS AND METHODS

In Vivo Studies

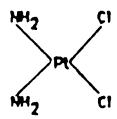
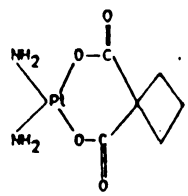
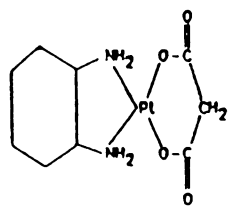
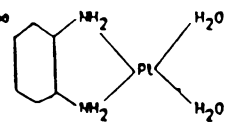
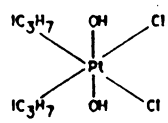
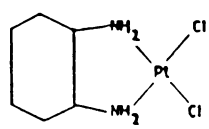
Inbred male Swiss Wistar and Long Evans (Hooded) rats (Charles River lab.), weighing 150-300 grams, were injected (intra peritoneally) i.p. with cis-diammine-1,1-cyclobutane dicarboxylate platinum II (CBDCA, JM 8) 50 mg/kg, or cis-dichloro diammine platinum II (DDP, Cisplatin) 5.0 mg/kg (Johnson Matthey Research Laboratories) injection vehicles are shown in Table 1. The day of the injection was taken as day 0. Sampling intervals were 0, 3, 5, 10 and 20 days post injection. Four animals were killed by cervical dislocation at each sampling interval. The kidneys from the left half were removed and mounted on cryostubs (I. E. C.) in OCT mounting medium , frozen, and kept until use. Sections 10 uM in thickness were cut using IEC cryostat microtome for enzymatic analysis.

Kidneys from the right half were removed, fixed in 100 % ethyl alcohol (-20°C), embedded in paraffin. Sections 5 µM in thickness were cut using a rotary microtome (American Optical). The sections were processed for the cytochemical localization of thiol (-SH) groups.

In Vitro Studies

Three inbred Wistar rats weighing 150-300 grams were killed, and their kidneys were removed. Kidney tubules were isolated following a modified version of the procedure developed by Nagata and Rasmussen (43). The medulla of each kidney was excised, and the remaining cortices were then mechanically minced and placed in Hank's solution (Grand Island Biological Supply Co.). The minced

Table 1. Platinum coordination complexes tested.

Drug	Structure	Abbreviation	Dosage	Vehicle	JM Number
cis dichloro diammine Pt		Cisplatin, CDDP	5.0 mg/kg	.15 M Saline	——
cis diammine 1,1-cyclobutane dicarboxylate Pt		CBDCA	50 mg/kg	5% Glucose	8
Malonato-1,2 diammine cyclohexane Pt		Malonato	80 mg/kg	DMF 1.5% $NaHCO_3$	74
Aquosulfato 1,2 diammine cyclohexane Pt		Sulfato	12 mg/kg	5% Glucose	20
cis diisopropylammine trans dihydroxy di chloro Pt IV		CHIP	40 mg/kg	.15 M Saline	9
dichloro 1,2 diammine cyclohexane Pt		DACH-Cl_2	20 mg/kg	.15 M Saline	—

cortices were then enzymatically digested in 100 mL of Hank's solution which also contained 40 mg collagenase (Worthington Biochemical), 100 mg hyaluronidase (Sigma T I, Sigma Chemical Co.), 25 mg streptomycin sulfate and 180 mg glucose. The tubules were then washed and mechanically dispersed using a dispo pipet. The sample was then divided into 13 equal portions and incubated in minimal essential media (Grand Island Biological Supply Co.). One part of the sample served as control, while platinum coordination complexes were added to the rest at chemotherapeutic dosages as shown in Table 1. Samples were taken at 1, 2, 3, 4, 5, 6 and 8 hours, and fixed in 1 % glutaraldehyde in .05 M cacodylate buffer (pH 7.4), washed in 4.5 % sucrose-cacodylate buffer (ph 7.4), mixed with Tissue Tek II embedding media (Lab Tek division of Miles Laboratories) and frozen until use. Sections 10 μM in thickness were cut using a CTD international Harris cryostat (International Equipment Company), and placed on dry gelatine (2.5 %) coated coverslips, placed breifly in buffered glutaraldehyde (to firmly attach the sections to the slip), and processed for cytochemical studies described below.

Cytochemical Studies

Both kidney cross-sections and isolated kidney tubules sections were tested for the following phosphatases.

Ca^{2+}-activated ATPase (Ca^{2+}-ATPase) was detected by incubation in 0.1 M Tris-maleate buffer (pH 7.3), adenosine 5' triphosphate (ATP) 10 mM, 3 % lead nitrate, 1 mM calcium chloride, 0.2 mM magnesium chloride and distilled water (17, 22). Incubation lasted 45 minutes at 37°C. The control incubation omitted the substrate or additionally contained 15 ug/ml quercetin(an inhibitor of Ca-ATPase activity (21))

Na^{+}-K^{+}-activated ATPase (Na^{+}-K^{+}-ATPase) was determined by incubation in media composed of Tris-maleate buffer 0.1M (pH 7.3), 10mM ATP, 3 % lead nitrate, 100 mM sodium chloride, 10 mM magnesium sulfate, 5 % sucrose and distilled water

(37). Control media omitted ATP,Na, or additionally contained 0.7 mg/ml ouabain, an inhibitor of Na/K ATPase activity (23, 33). Incubations were carried out for 45 minutes at 37°C

Alkaline Phosphatase (AP) activity was visualized by incubation in media containing 0.2 M Tris-maleate buffer (pH 8.2), sodium ß-glycerophosphate (1.2 %), 1 % lead nitrate, 0.2 mM magnesium chloride and distilled water for 45 minutes at 37°C according to the method of Hugon and Borgers (28). Control incubation contained 50 mM L-phenylalanine, an inhibitor of AP activity (15).

5' Nucleotidase (5' N). For visualization of 5' N activity incubation in media consisting of 0.1 M Tris-maleate buffer (pH 7.3), 1.4 mM adenosine 5'-monophosphate (AMP), 1 % lead nitrate, 10 mM magnesium sulfate and 5 % sucrose was carried out at 37°C for 45 minutes according to the procedure developed by Uusitalo and Karnovsky (62). Control media omitted the substrate (AMP).

Acid Phosphatase (Acid P) activity was visualized by incubation in medium containing .2 M Tris-maleate buffer (pH 5.2), sodium ß-glycerophosphate (1.2 %), 1 % lead nitrate, 0.2 mM magnesium chloride and distilled water for 45 minutes at 37°C (45).

Sections (10 µM) of control and drug treated tissue after incubation in the appropriate incubation medium were treated with 1 % ammonium sulfide and mounted in glycerine jelly. Slides were photographed using a Zeiss Photomicroscope II, loaded with Kodak Plus X film. The reaction product (rp) was quantitated on these negatives by scanning microdensitometry using a Joyce Loebl MK III C double beam recording microdensitometer (JL and Company LTD, Electron House, princesway Team Valley Gateshead -on-Tyne II England), and by direct microscopic visualization.

Thiol Groups Thiols were localized according to the method of Engel and Zerlotti (20). The method involves treatment of the sections with 95 % ethanol, followed by staining with the azomercurial reagent 4-(p-dimethylamino benzene azo) phenyl mercuric acetate, and retreatment with 95 % ethanol. A control is prepared with preincubation in N-ethyl maleimide (75mM) in phosphate buffer (pH 7.0) at 37°C for 2 hours (9), blocking the thiol groups, or by controlled trypsin (protease) digestion demonstrating that the thiol groups are protein bound. The sections were then mounted using Permount (Fisher Chemical Company), and observed with monochromatic blue (L= 458 nM) light.

Determination of Tubule Viability.

Tubule viability was determined by removal of control and drug treated tubules at each sampling interval. These samples were then treated with 0.2 % trypan blue solution, and counts made of the viable kidney tubules (viable tubules do not take up the stain) (19).

Biochemical Assays

Spectrophotometric assays were performed on culture media from each sampling interval during the in vitro screening process, to determine the amount of alkaline phosphatase activity present in the media (34). AP activity was detected by adding 0.1 ml of culture media to a mixture of 19 mM disodium p nitrophenyl phosphate and 0.1 M carbonate bicarbonate buffer (pH 10.0) which had been warmed for 10 minutes at 30°C. The mixture was then placed in a 5 ml cell with a 1 cm path length, and checked for absorbance at 400 nm using a Beckman 25 spectrophotometer. Then, using the absorbence, the number of units of alkaline phosphatase/ml of culture media was determined using Beer's law.

Rat urine was collected from 5 pairs of male Wistar and Long Evans rats at 0,1,3,5,7,10 days post injection with CBDCA or CDDP. The urine was analyzed either immediately or frozen until use. It was spun in an IEC clinical centrifuge for 8

minutes at high speed, and dialyzed for 3 hours using Spectrapore membrane tubing (VWR scientific Biochemistry Stores) and distilled water 4°C. The urine was then assayed for the presence of both alkaline phosphatase and acid phosphatase (34). Acid phosphatase activity was determined by incubation in medium consisting of .1 M acetate buffer pH. 4.5, .15 M substrate Na-B-glycerophosphate (Sigma 104-0 dissolved in acetate buffer), 20 % TCA (tri-chloro acetic acid) was added after 10 minutes of incubation. Alkaline phosphatase activity was determined, as described above, for the culture media. The media was spectrophotometrically assayed and the amount of acid phosphatase and alkaline phosphatase present in the urine calculated similar to that described for culture medium analysis, with the addition of a correction for the amount of urine excreted.

Data Analysis

All data analysis was performed on a Macintosh microcomputer using a Number Cruncher Statpak. Prior to the analysis of variance, data was checked for equality and normality of variance using both the F max test, and Bartlett's homogeneity test.

RESULTS

Cytochemical localization of alkaline phosphatase in kidney cross-sections is depicted in Figure 1A, and 5 days post CDDP treatment in Figure 1B. In vitro localization in isolated kidney tubules is shown in Figure 1C, and 5 hours post CDDP treatment in Figure 1D. The enzymatic reaction shows that alkaline phosphatase is mainly located on the lumen side of kidney tubule cells in the microvilli. A lack of reaction product is seen in both the in vivo and in vitro sections after CDDP treatment, although CBDCA does not cause a large decrease in alkaline phosphatase activity. A similar lack of reaction product is seen in negative control sections which were incubated with either L-phenylalanine or leavamisole. Sodium ATPase and calcium ATPase show a distribution similar to that of alkaline phosphatase with the addition of strong intensity on the cells of the basal lateral border of kidney tubules. Following either CDDP or CBDCA treatment both calcium and sodium ATPase are affected in the same way as alkaline phosphatase. Acid phosphatase is present in lysosomes throughout the kidney, and increases substantially in quantity after CDDP treatment, but shows only a slight increase after CBDCA treatment. The activity of 5'-nucleotidase shows a distribution similar to that of alkaline phosphatase, and is affected in a similar manner after drug treatment. The decreases correlate for both in vivo and in vitro studies.

Results of reaction densities as viewed through the light microscope are shown for in vivo studies in Figure 2, and for in vitro studies with CDDP and CBDCA in Figure 5. These results were further analyzed and quantified using a Joyce Loebl scanning microdensitometer. The results of alkaline phosphatase scans are shown for in vivo studies in Figures 3 and 4, and for in vitro studies in Figures 6 and 7. Enzyme levels decreased in both an analog and time dependent manner.

Figure 1 A Cross-section of a male wistar rat kidney showing alkaline phosphatase reaction product (r.p.) (arrows) in a normal animal. Original magnification X 250. Bar = 100 μM.

B Cross-section of male wistar rat kidney 5 days post cisplatin (5 mg/kg) treatment. Showing only patches of alkaline phosphatase activity. Original Magnification X 250. Bar = 100 μM.

C Isolated rat kidney tubules showing alkaline phosphatase r.p. (arrows) in normal tubules. Original magnification X 950. Bar = 25 μM.

D Isolated rat kidney tubules 5 hours post CDDP (5 mg/kg) treatment showing no alkaline phosphatase reaction product. Original magnification X 950. Bar = 25 μM.

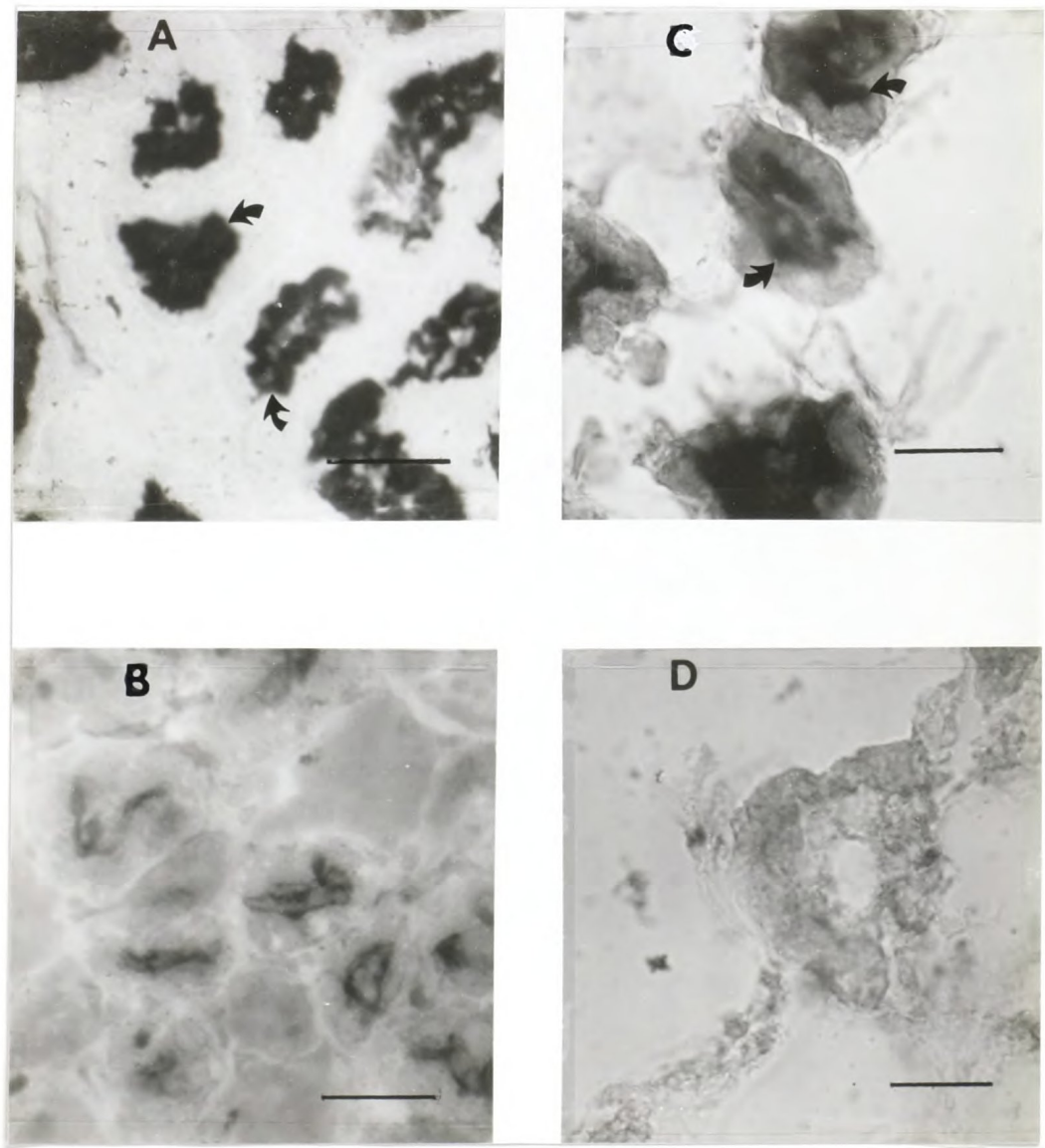

A
C
B
D

Figure 2 In vivo alkaline phosphatase, Ca^{2+} ATPase and Na^+/K^+ ATPase reaction product density in control, CBDCA (50 mg/kg) treated and CDDP (5 mg/kg) treated male Wistar rats.

In Vivo Transport Enzyme Activity [12]

	Alkaline Phosphatase		Ca^{2+} ATPase		Na^{+}/K^{+} ATPase	
Treatment	CDDP	CBDCA	CDDP	CBDCA	CDDP	CBDCA
0	++++	++++	++++	++++	++++	++++
1	++	++++	++	++++	++	++++
3	±	+++	±	+++	±	+++
5	±	+++	±	+++	±	+++
10	+	++++	+	++++	+	++++

Days post injection

[1] After treatment with CDDP 5.0 mg/kg or CBDCA 50 mg/kg

[2] ++++ very dense reaction; +++ dense reaction; ++ average reaction; + poor reaction

± less than 10 % reaction; - no reaction

Figure 3 In vivo scanning densitometry curves of alkaline phosphatase reaction product for A control, B 3 days after (5mg/ kg) cisplatin treatment, and C 5 days post (5 mg/kg) CDDP treatment.

Figure 4 <u>In vivo</u> relative alkaline phosphatase reaction product density in male Wistar rat kidney cross-sections for control —◇—, CDDP (5 mg/kg) —▲— CBDCA (50mg/kg) —●— treatment.

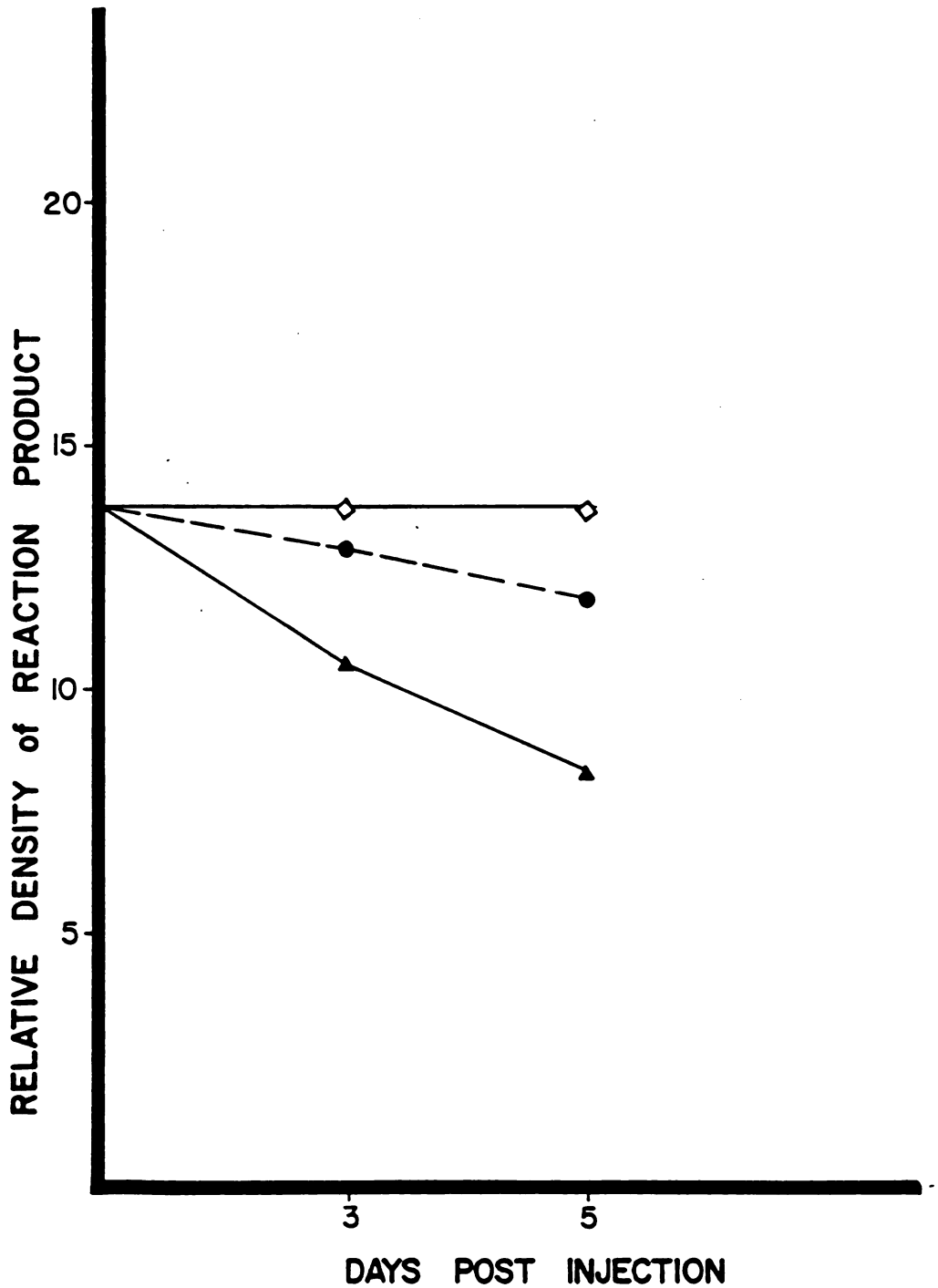

20
15
10
5
RELATIVE DENSITY of REACTION PRODUCT
3
5
DAYS POST INJECTION

Figure 5 *In vitro* alkaline phosphatase, Ca^{2+} ATPase and Na^{+}/K^{+} ATPase reaction product density in control, CBDCA (50 mg/kg) treated and CDDP (5 mg/kg) treated male Wistar rats.

In Vitro Transport Enzyme Activity [12]

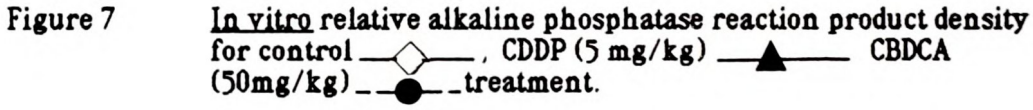
	Alkaline Phosphatase		Ca^{2+} ATPase		Na^{+}/K^{+} ATPase	
Hours post incubation / Treatment	CDDP	CBDCA	CDDP	CBDCA	CDDP	CBDCA
0	++++	++++	++++	++++	++++	++++
1	++	++++	++	++++	++	++++
3	±	+++	±	+++	±	+++
5	±	+++	±	+++	±	+++
8	-	++	-	++	-	++

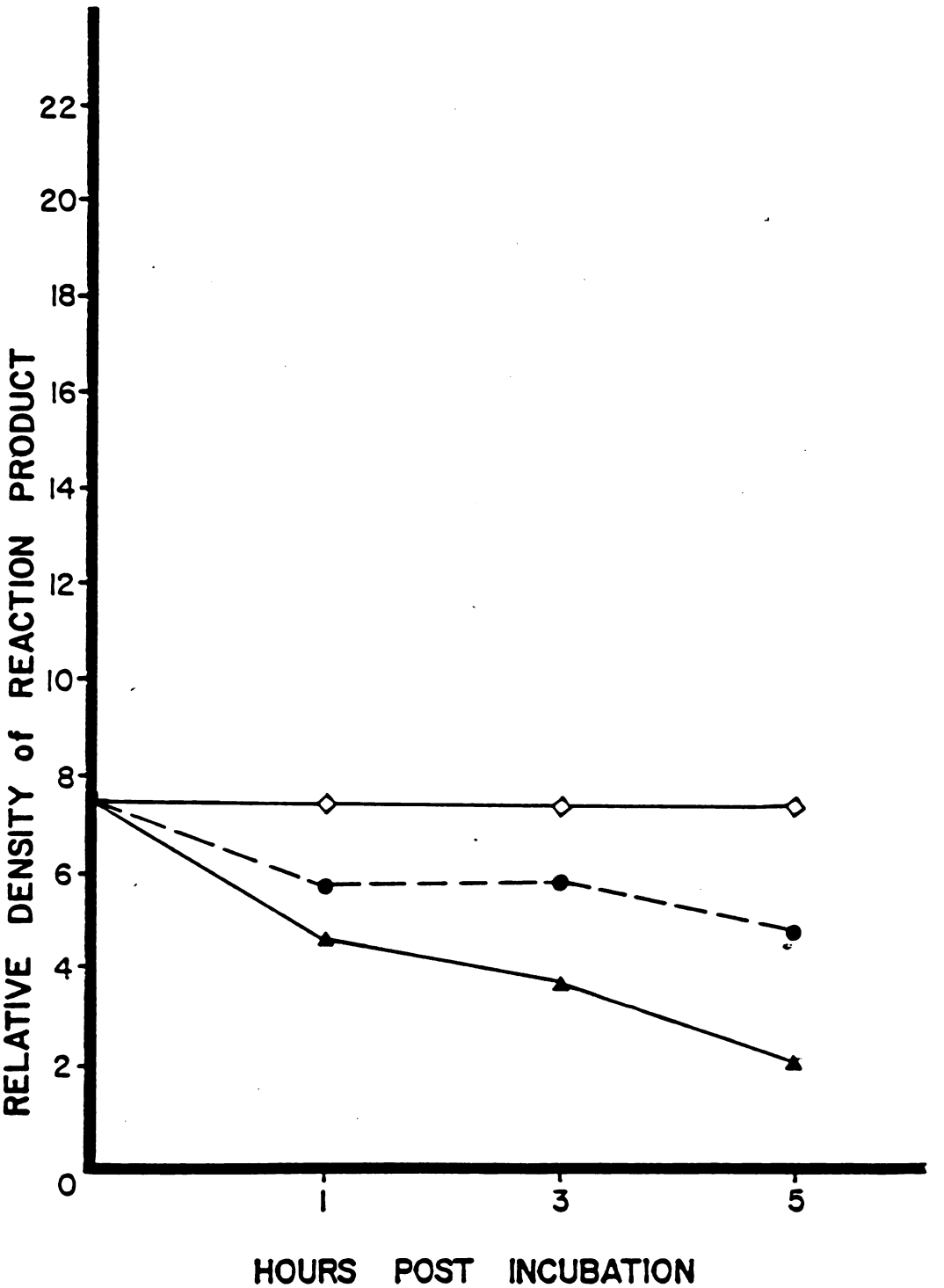
[1] After treatment with CDDP 5.0 mg/kg or CBDCA 50 mg/kg

[2] ++++ very dense reaction; +++ dense reaction; ++ average reaction; + poor reaction

± less than 10 % reaction; - no reaction

Figure 6 In vitro scanning densitometry curves of alkaline phosphatase reaction product for A control, B 1 hour after (5mg/ kg) cisplatin treatment, C 3 hours post (5 mg/kg) CDDP treatment, and D 5 hours after 5 mg/kg CDDP treatment.

Figure 7 
In vitro relative alkaline phosphatase reaction product density
for control , CDDP (5 mg/kg) CBDCA
(50mg/kg) treatment.


RELATIVE DENSITY of REACTION PRODUCT
22
20
18
16
14
12
10
8
6
4
2
0
1
3
5
HOURS POST INCUBATION

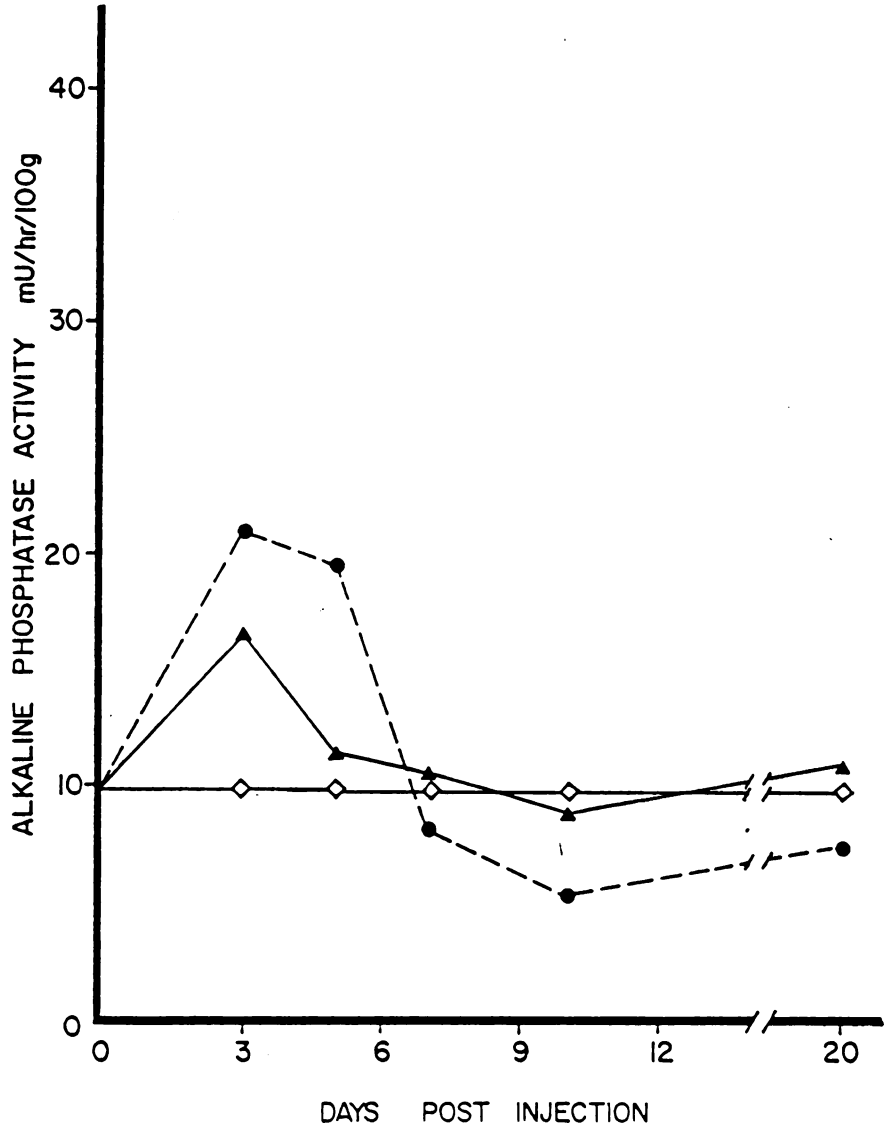
Further conformation of the in vivo studies was provided by analyzing rat urine enzymes for alkaline phosphatase and acid phosphatase data for male Wistar rats (Figures 8 and 9 respectively), and for male Long Evans rats (Figures 10 and 11 respectively). The urine analysis indicates an increase in alkaline phosphatase activity after injection with either CDDP or CBDCA, but that the magnitude of increase after CBDCA treatment is much less than that following CDDP treatment. Acid phosphatase levels seem to increase 6 days post injection, followed by a subsequent decrease, to normal levels. Acid phosphatase increased significantly more after CDDP treatment than after CBDCA treatment.

The rat urine volumes were also monitored post injection results shown in Figure 12 for Wistar rats, and Figure 13 for Long Evans rats. A decrease in urine volume is evident following CBDCA injection , although an increase is found after CDDP injection. Weight gain in control, CDDP, and CBDCA treated rats is compared in Table 2. These studies indicate a weight loss after CDDP treatment, but a slight, insignificant decrease in weight following CBDCA treatment relative to control animals.

In vitro studies were similarly confirmed using both culture media assayed for alkaline phosphatase activity (Figures 14 and 15), and tubules assayed for viability (Figures 16 and 17). In vitro studies show that the various analogs tested may be ranked from most to least toxic relative to membrane enzyme damage (Table 3).

Pictures depicting in vivo thiol group localization are shown for control (Figure 18A), 5 days post CDDP treatment (Figure 18B) and for 5 days post CBDCA treatment (Figure 18C). The thiol groups appear to be localized on the brush and basal lateral borders of kidney tubule cells. The photomicrographs indicate a decrease in thiol groups following CDDP treatment, but show little decrease after CBDCA treatment, similar to alkaline phosphatase.

Figure 8 Alkaline phosphatase activity (mU/hr/100 g) in male Wistar rat urine for control ——◇—— , CDDP (5 mg/kg) — ● — — and CBDCA (50 mg/kg) ——▲—— treatment.


ALKALINE PHOSPHATASE ACTIVITY mU/hr/100g
40
30
20
10
0
0
3
6
9
12
20
DAYS POST INJECTION

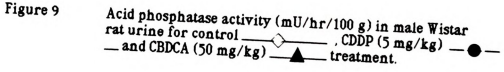


Figure 9 Acid phosphatase activity (mU/hr/100 g) in male Wistar rat urine for control ——◇—— , CDDP (5 mg/kg) —●— and CBDCA (50 mg/kg) ——▲—— treatment.

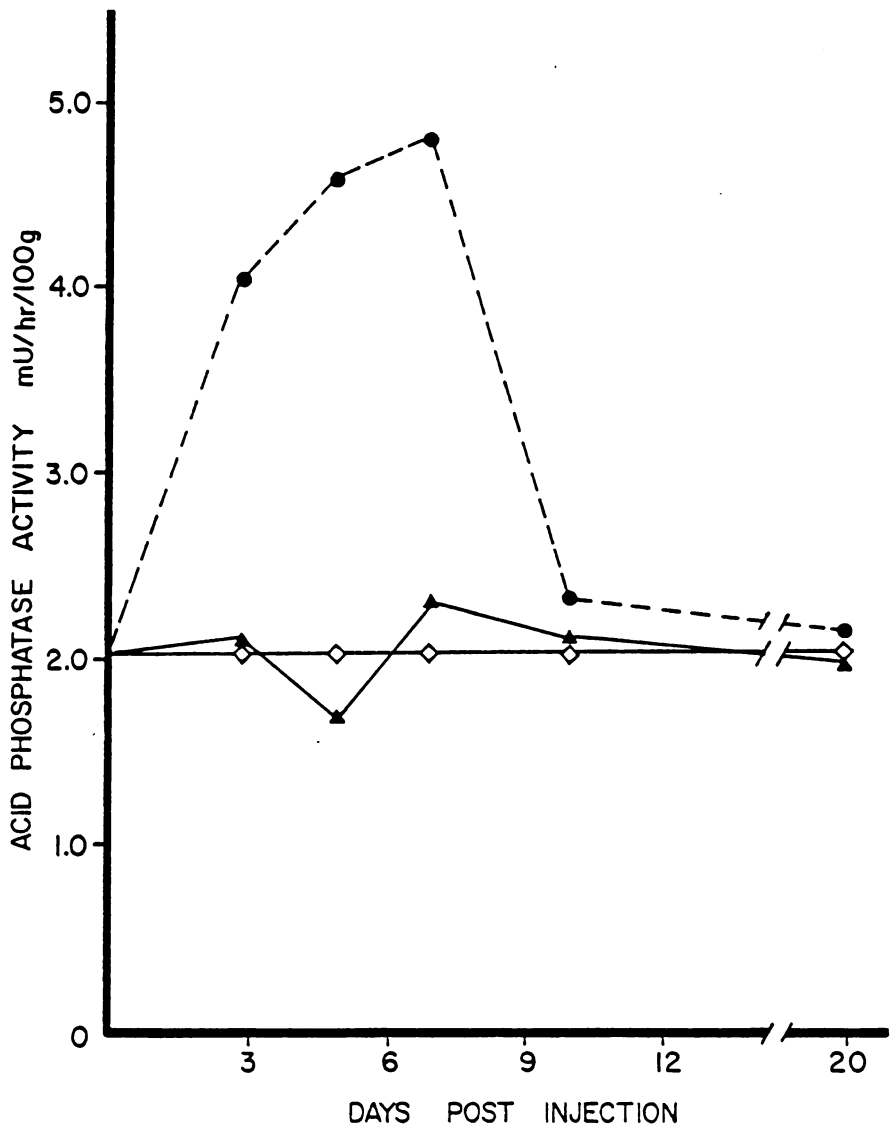

ACID PHOSPHATASE ACTIVITY mU/hr/100g
5.0
4.0
3.0
2.0
1.0
0
3
6
9
12
20
DAYS POST INJECTION

Figure 10 Alkaline phosphatase activity (mU/hr/100 g) in male Long Evans rat urine for control —◇—, CDDP (5 mg/kg) —●— and CBDCA (50 mg/kg) —▲— treatment.

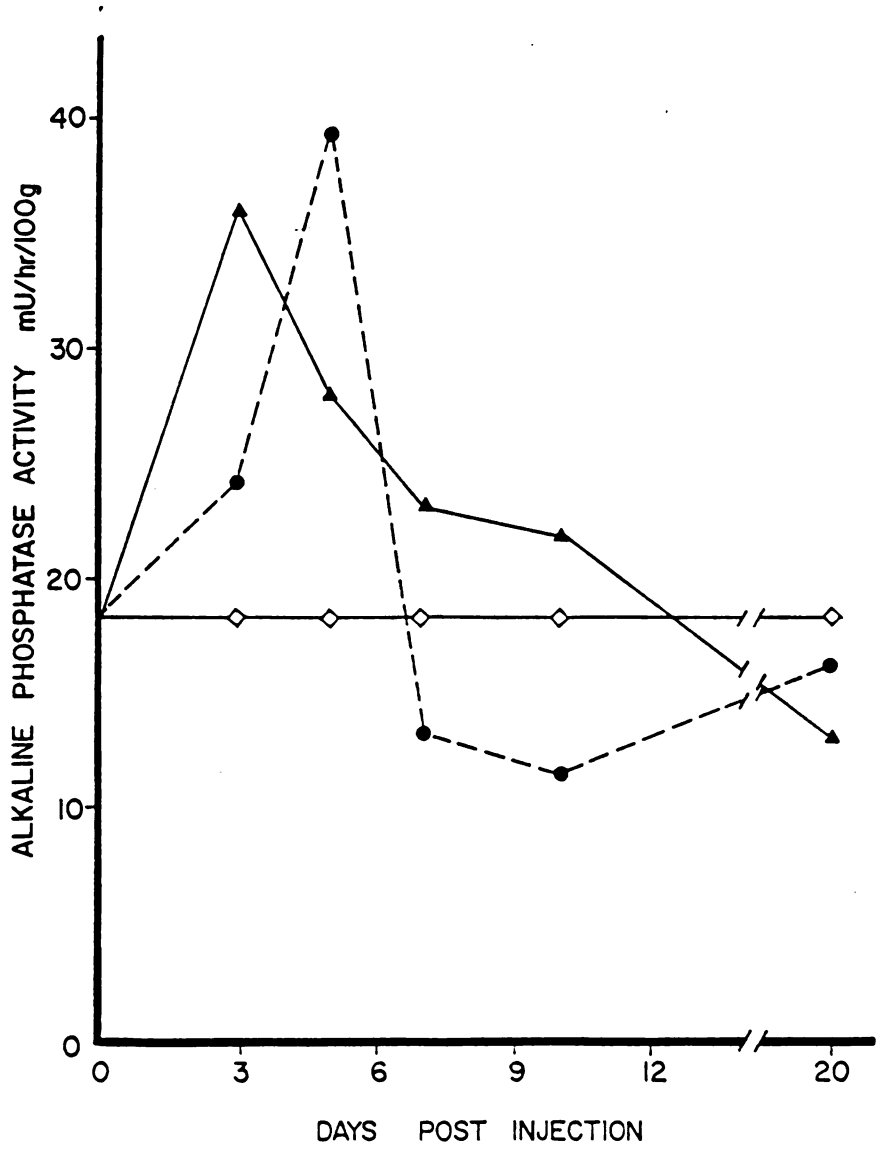
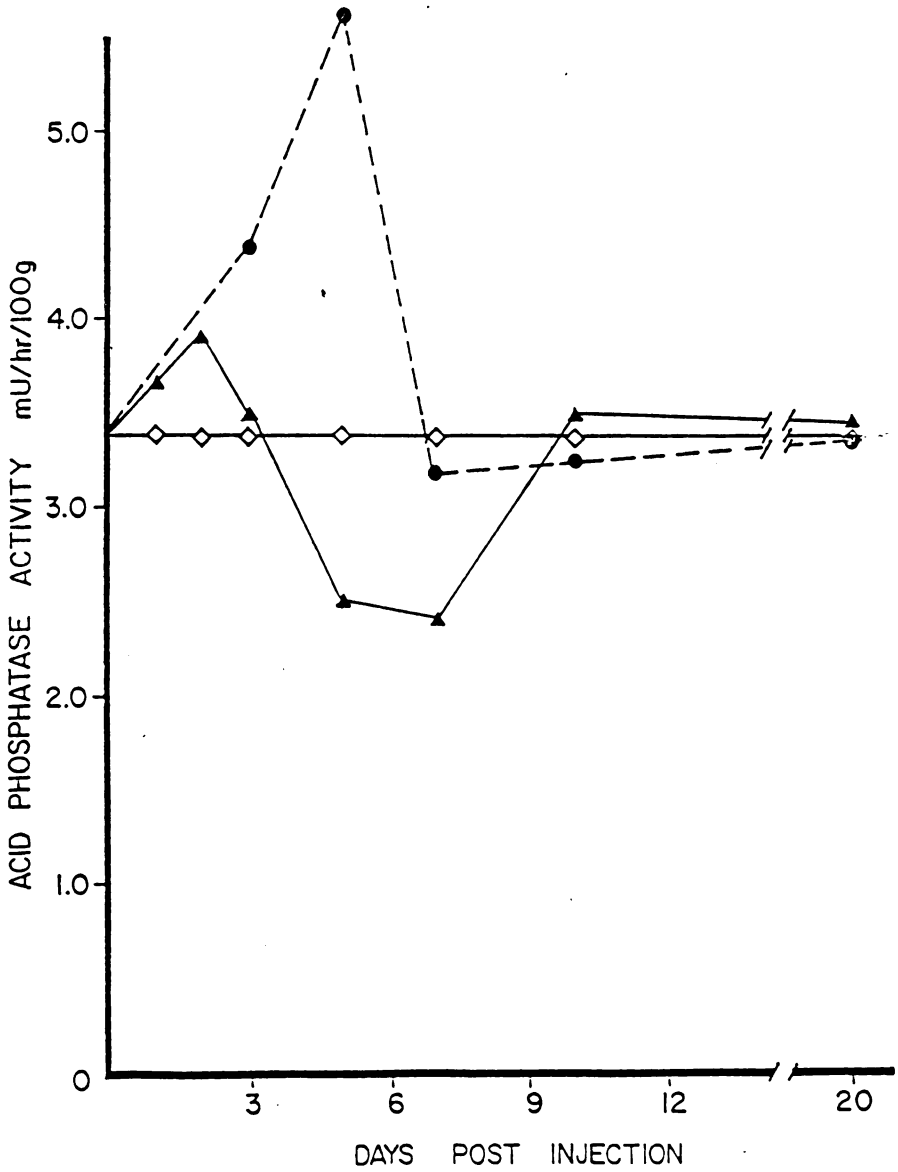

ALKALINE PHOSPHATASE ACTIVITY mU/hr/100g
40
30
20
10
0
0
3
6
9
12
20
DAYS POST INJECTION

Figure 11 Acid phosphatase activity (mU/hr/100 g) in male Long Evans rat urine for control —◇—, CDDP (5 mg/kg) —●— and CBDCA (50 mg/kg) —▲— treatment.


ACID PHOSPHATASE ACTIVITY mU/hr/100g
5.0
4.0
3.0
2.0
1.0
0
3
6
9
12
20
DAYS POST INJECTION

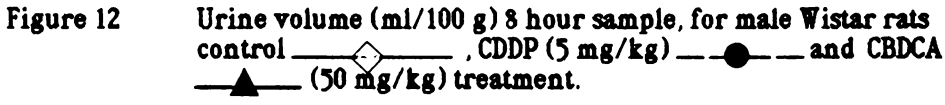


Figure 12 Urine volume (ml/100 g) 8 hour sample, for male Wistar rats control ——◇—— , CDDP (5 mg/kg) — —●— — and CBDCA —▲— (50 mg/kg) treatment.

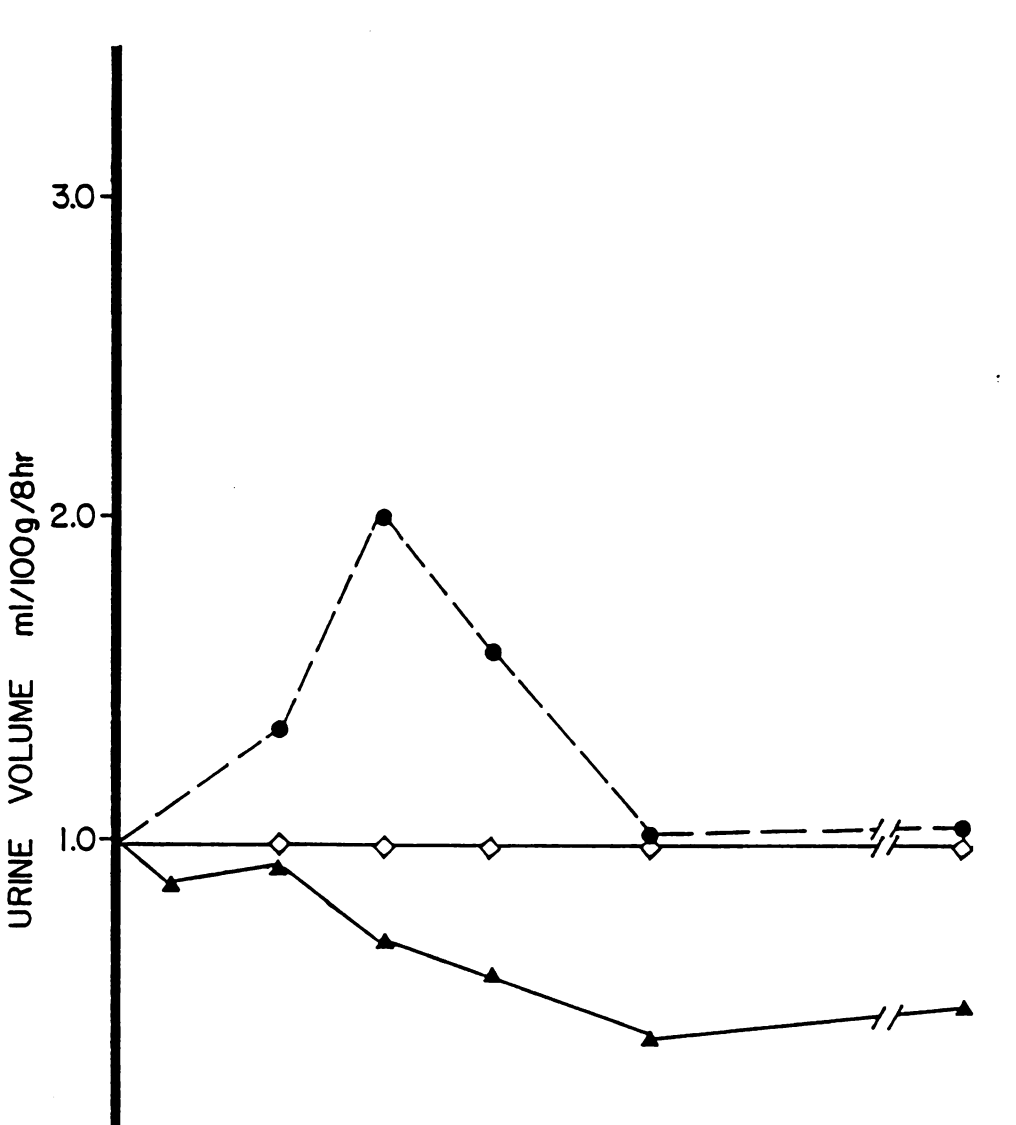

3.0
2.0
1.0
0
URINE VOLUME ml/100g/8hr
3
6
9
12
20
DAYS POST INJECTION

Figure 13 Urine volume (ml/100 g) 8 hour sample, for male Long Evans rats control ——◇—— , CDDP (5 mg/kg) — —●— — and CBDCA (50 mg/kg) —▲— treatment.

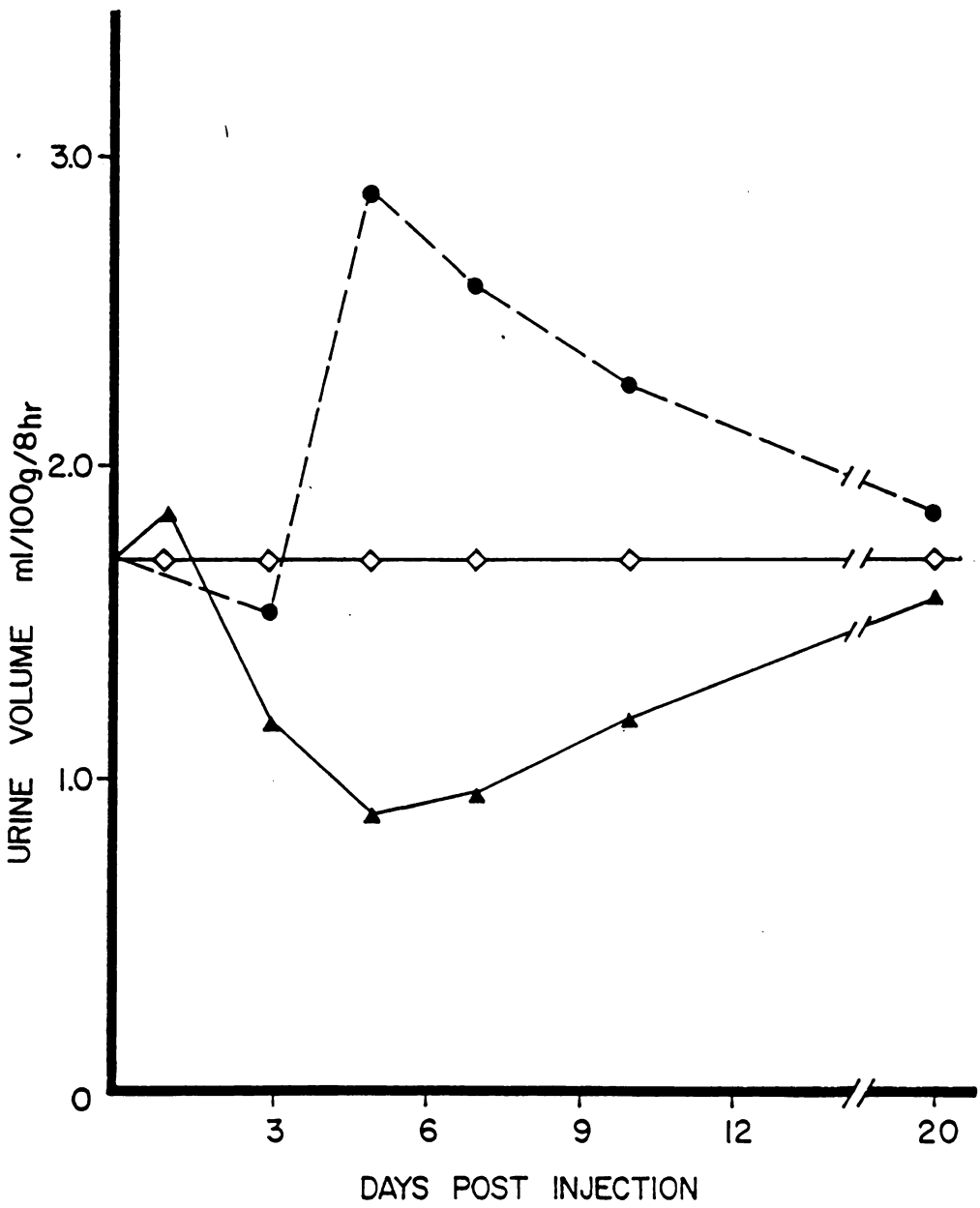

3.0
2.0
1.0
0
URINE VOLUME ml/100g/8hr
3
6
9
12
20
DAYS POST INJECTION

Table 2. Weight gain/loss with and without drug treatment [1,2]

Rat	Treatment	Average weight (gain/loss)/day
Long Evans	Control	6.7 ± 0.9 gm/day
	CDDP	-2.97 ± 1.6 gm/day
	CBDCA	6.2 ± 2.2 gm/day
Wistar	Control	4.2 ± 1.1 gm/day
	CDDP	-2.73 ± 1.14 gm/day
	CBDCA	3.83 ± 2.08 gm/day

[1]-6 replicates of each treatment

[2]-Based on average weight gain/loss 5 days after injection.

Figure 14 In vitro alkaline phosphatase in units/dl of culture media. For control ___ . _ . ___ , CDDP 5.0 mg/kg . . . . . , CBDCA 50 mg/kg _ _ _ _ , CDDP 50 mg/kg _____ and CBDCA 500 mg/kg _ _ _ _ _ treated samples.

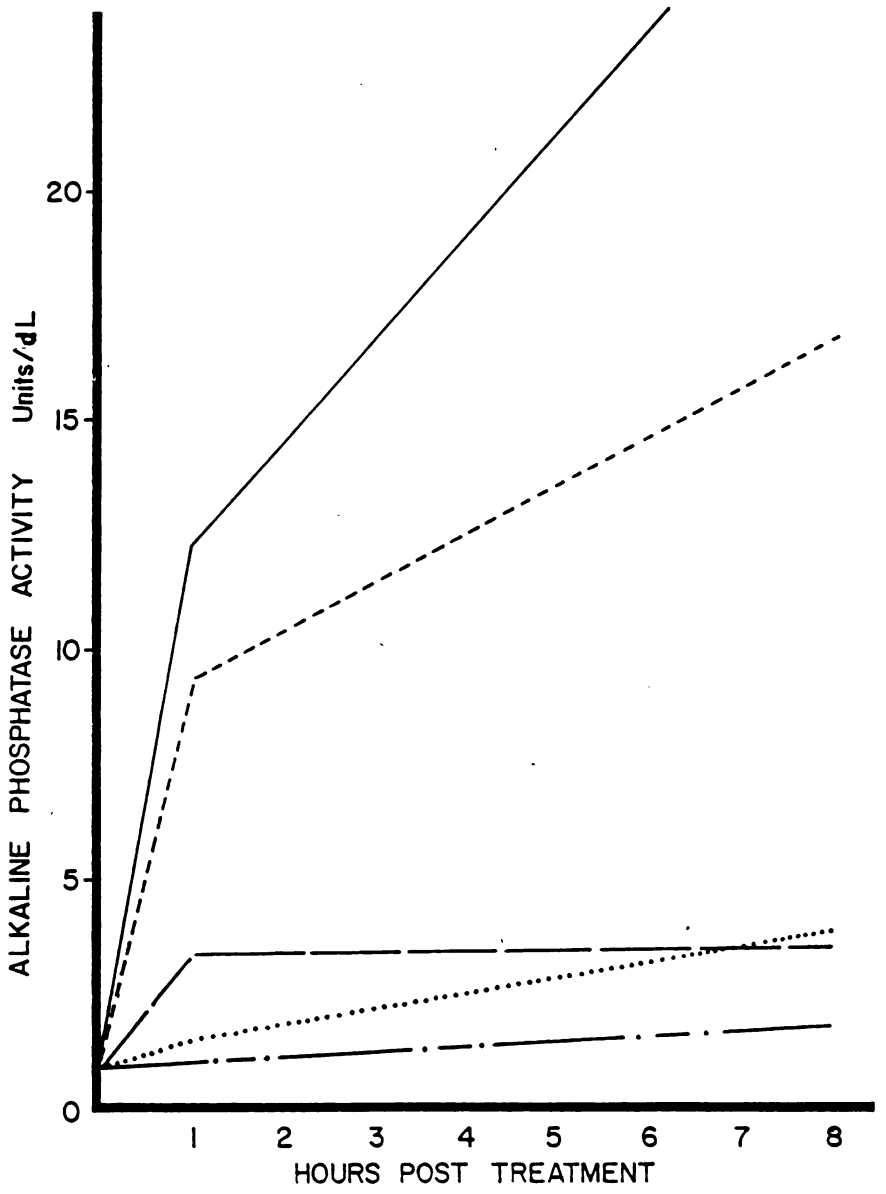

ALKALINE PHOSPHATASE ACTIVITY Units/dL
0
5
10
15
20
1
2
3
4
5
6
7
8
HOURS POST TREATMENT

Figure 15 In vitro alkaline phosphatase activity in units/dl of culture media. For Control _ _ _ _, CHIP (40 mg/kg) ____ _ ____, Dach-Cl (20 mg/kg) __ . . . . _, Malanato (80 mg/kg) . . . . . , Sulfato (12 mg/ kg)_____ and Dead __ __ __ tubules.

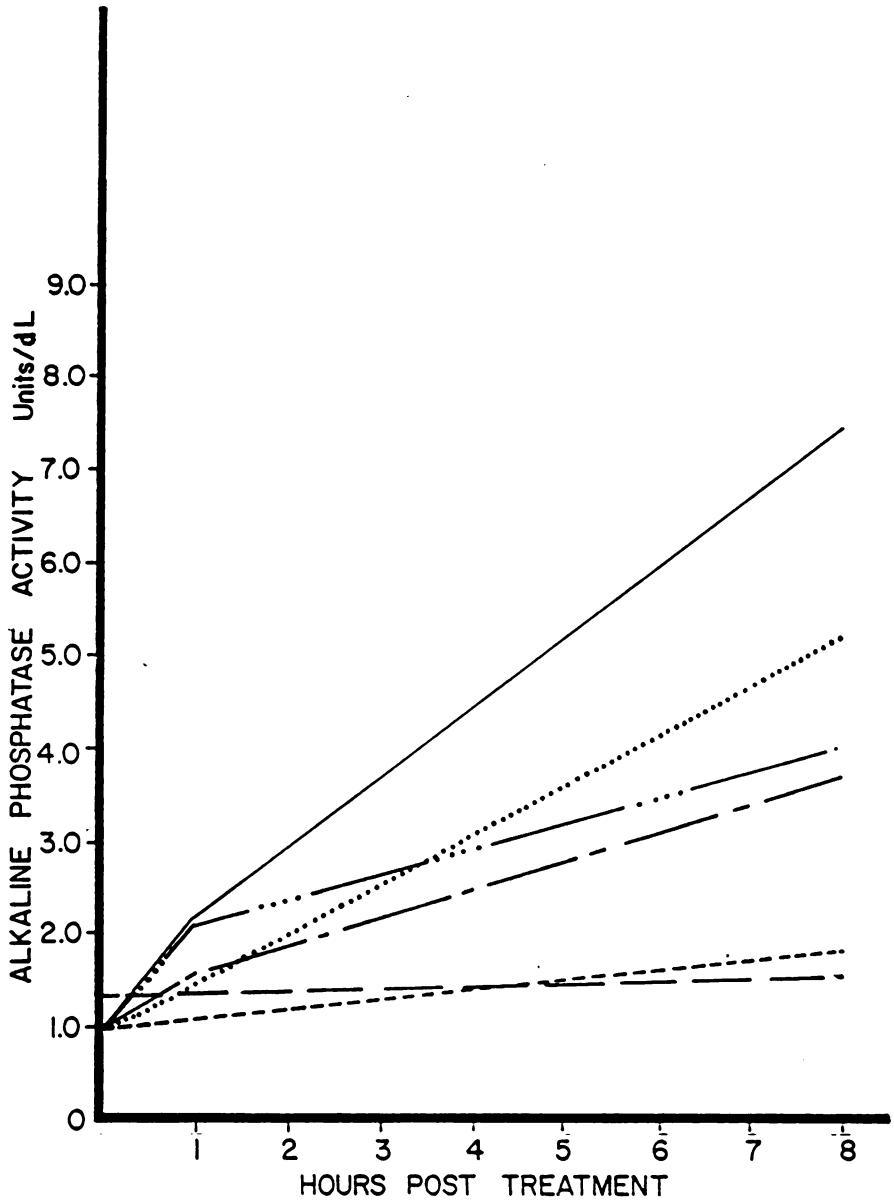

ALKALINE PHOSPHATASE ACTIVITY Units/dL
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1
2
3
4
5
6
7
8
HOURS POST TREATMENT

Figure 16 In vitro tubule viability measured by .2 % trypan blue exclusion, plotted as percent viable, for control —·●·—, CBDCA 50 mg/kg —○—, CDDP 5.0 mg/kg —··□··—, CBDCA 500 mg/kg —■— and CDDP 50 mg/kg ··✪·· treated tubules.

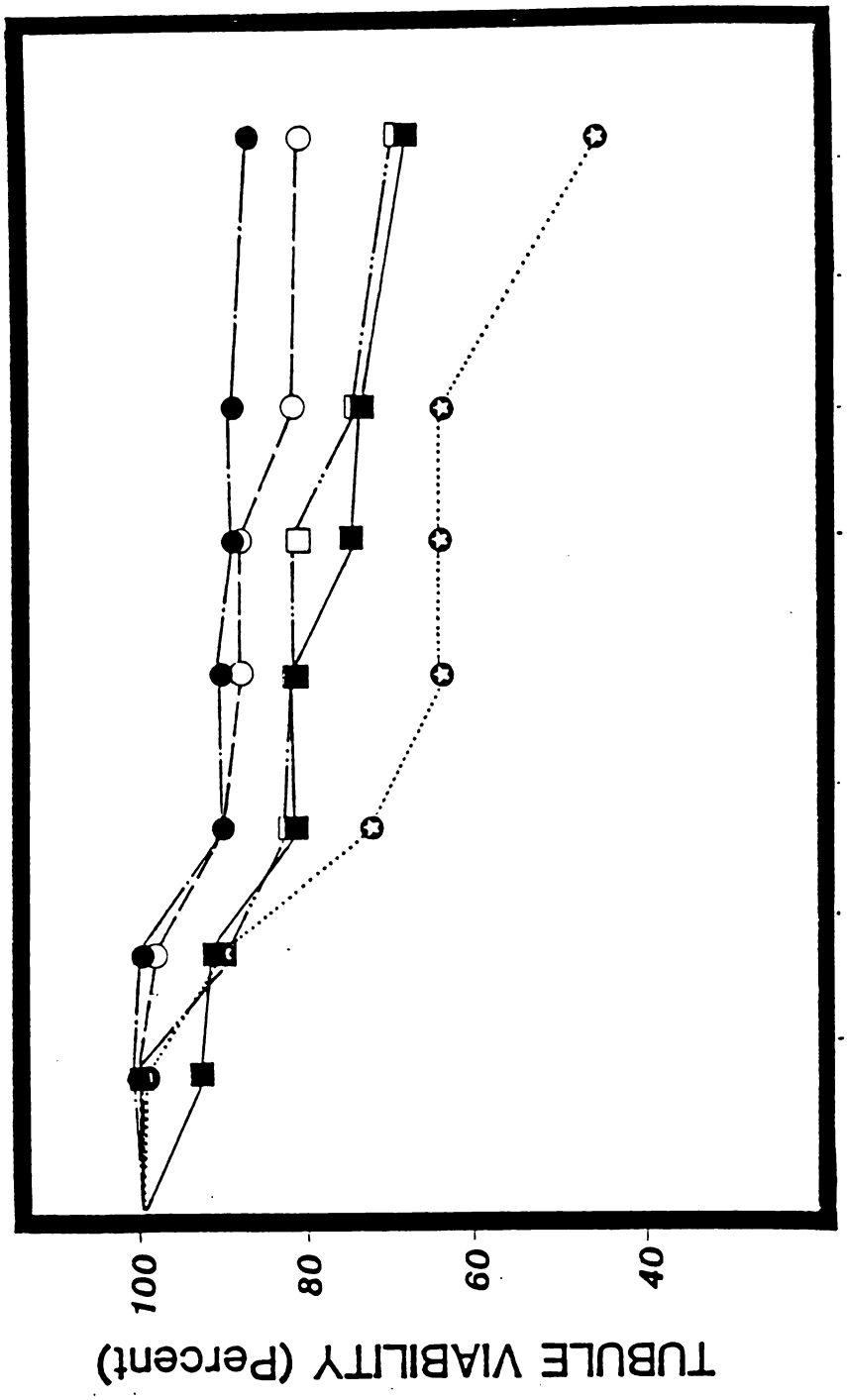

TUBULE VIABILITY (Percent)
100
80
60
40
1
2
3
4
5
6
7
8
TIME (Hours)

Figure 17 In vitro tubule viability measured by .2 % trypan blue exclusion. Plotted as percent viable, for Sulfato (12 mg/kg) . . . ☆ ., Malanato (80 mg/kg) —▲— , CHIP (40 mg/kg) —.. ★.. — — and Dach-Cl (20 mg/kg) —◇— treated tubules.

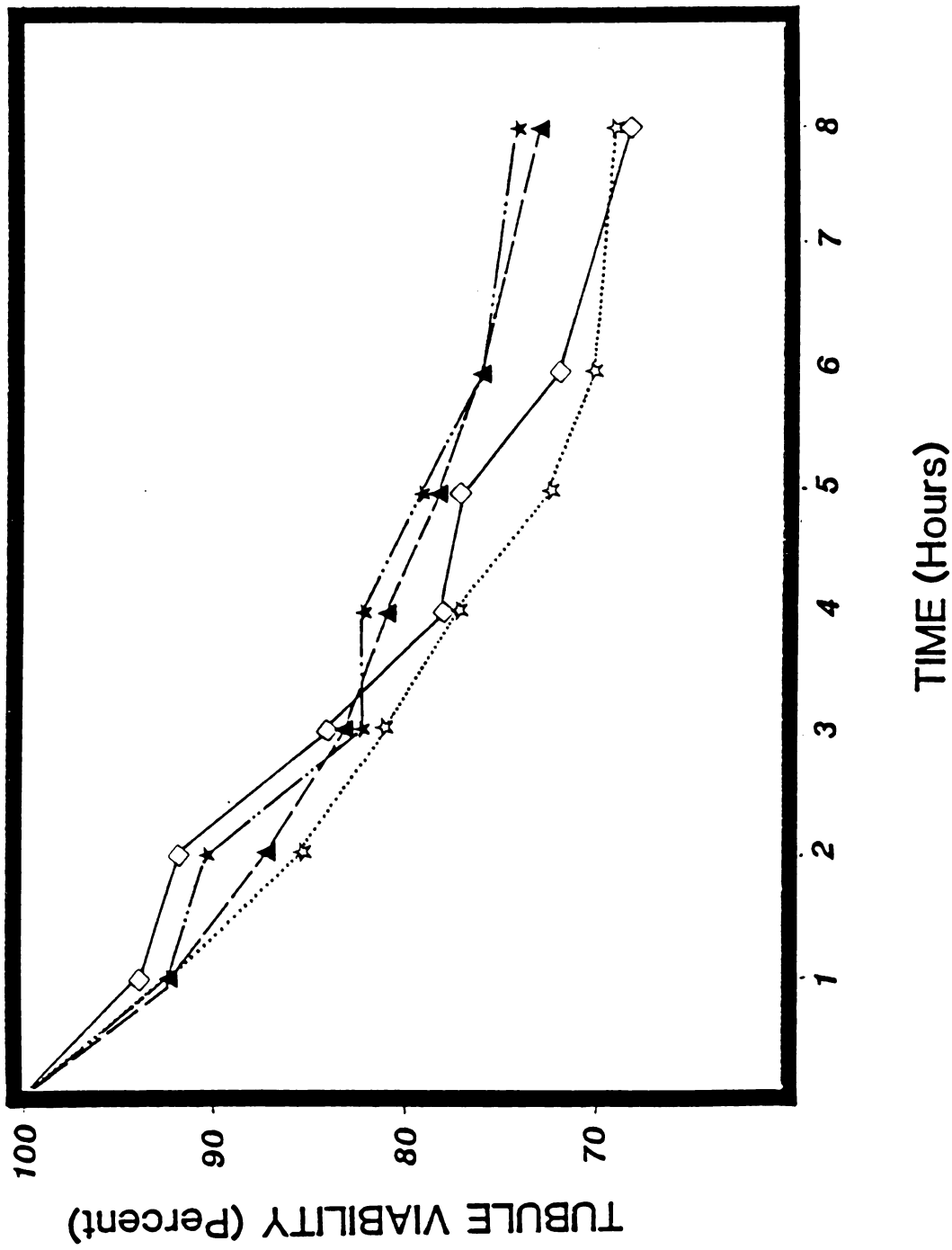

TUBULE VIABILITY (Percent)
100
90
80
70
1
2
3
4
5
6
7
8
TIME (Hours)

Table 3 Relative Nephrotoxicity of Platinum Complexes as Determined by In Vitro Screening System [1]

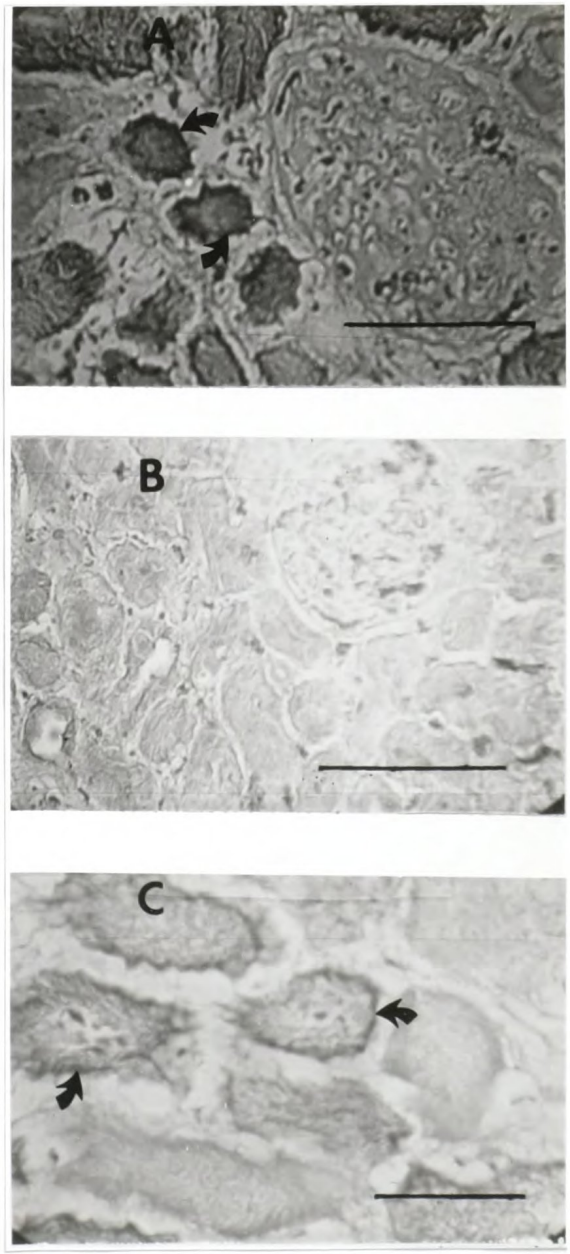
1	CDDP 50 mg/kg	+++++
2	DACH-Cl_2 20 mg/kg	++++
3	SULFATO 12 mg/kg	++++
4	CBDCA 500 mg/kg	++++
5	CDDP 5.0 mg/kg	+++
6	Malanato 80 mg/kg	++
7	CHIP 40 mg/kg	++
9	CBDCA 50 mg/kg	+

[1] Ranking from 1. (+++++) most- 9. (+) least toxic.

Figure 18 A Cross-section of a male Wistar rat kidney showing the distribution of thiols (arrows) in a normal section. Original magnification X 250. Bar = 100 µM.

B Cross-section of a male Wistar rat kidney showing the lack of thiols 5 days post CDDP 5 mg/kg treatment. Original magnification X 250. Bar = 100 µM.

C Cross-section of a male Wistar rat kidney showing the distribution of thiols 5 days post CBDCA 50 mg/kg treatment. Original magnification X 400. Bar = 50 µM.


A
B
C

DISCUSSION

This study addresses itself to three main issues. First and foremost is the proposal and testing of an in vitro nephrotoxicity assay system. This is particularly appropriate since it has been established that nephrotoxicity is one the most severe side effects of CDDP chemotherapy (24). Second, through the in vivo screening procedures an attempt is made to determine whether there exists strain-specific differences in both the normal quantity of rat urine enzymes and the physiologic response to drug compromise. Third, the study compares cisplatin's mechanism of action to a proposed mechanism for a second-generation platinum coordination complex, which could account for both anti-metastastic ability and organismal toxicity (50, 53).

The idea of establishing an in vitro correlate to in vivo toxicty systems is not new . However, the use of functional organ systems (such as isolated kidney tubules, etc.) is more recent. This type of toxicity assay promises to save both many laboratory animals, and many labor hours, which are of importance ethically and financially.

Transport enzymes are of major biological importance within the kidneys (23, 65) and elsewhere throughout the organism. They are responsible for metabolite exchange (32, 40) and have been implicated in the determination of normal and metastastic phenotypic states (32, 36, 40). The transport enzymes have also been implicated in the control of ion balance (Ca ATPase, Na/K ATPase) , and indirectly implicated, through ionic imbalance, with interference in mitotic apparatus assembly and disassembly thus interfering with cytokinesis (1, 3). Inactivation of these enzymes would tend to alter both the function and viability of kidney tubules.

The mechanism of interaction/inactivation of membrane enzymes may be many fold. Depending on the strength of membrane attachment (ATPases are integral (33,

39), Alkaline phosphatase is peripheral (40), and acid phophatase is lysosomal (45)) the enzymes can be removed yet remain functional ; inactivated, but not removed (ATPases); or be removed and inactivated; or stimulated to increase in quantity (acid phosphatase). Inactivation of membrane ATPases would tend to lead to an ionic imbalance (60), which is probably responsible for cellular mortality (60).

In the case of acid phosphatase, lysosomal build-up occurs until the cells lyse, releasing their enzymes into the urine in a functional form. This is indicated by the delayed peaks of urine acid phosphatase activity, as compared to that of alkaline phosphatase. The disappearance of alkaline phosphatase occurs 3-5 days post drug treatment in vivo, and corresponds with increases in urinary alkaline phosphatase during the same time interval . This increase in discharge of enzyme was also compared to the increase in enzymes found in in vitro cultured cells showing that a similar situation occurs in the in vitro model.

It may also be shown that cells must be alive, post isolation to be affected by the drugs in a significant manner, showing that the drugs must be metabolized in order to affect the cells. Comparisons of enzymatic quantity after incubation must be continually referenced to a parallel run base line due to the fact that the cells are continually metabolizing the enzymes, and due to any damage that is suffered during the isolation procedure . Such damage is reflected in minor differences in enzyme activity. A comparison of in vivo and in vitro models leads to the approximate relation that 1 day in vivo is equivalent to 1 hour in vitro . Taking into account the results presented, the in vitro model seems to be a very reliable and easily applicable to nephrotoxicity screening of the platinum coordination complexes.

A naturally occuring difference in the quantity of various rat urinary enzymes is seen in Long Evans and Wistar rats. This is not suprising in that, currently, differences in gene family functional products (isozymes) between species are being used to construct genetically determined phylogenies in many insects, as well as other

organisms, and to even show genetic variation within a species . The discovery of strain-specific baselines for alkaline phosphatase activity, acid phosphatase activity, urine volume, and daily weight gain are clearly shown. These findings indicate that such differences must be taken into account when choosing animals for toxicological study, a specific animal's sensitivity should be considered in experimental design and also should be accounted for in the presentation of data. Results indicate a higher base line in all categories for the Long Evans rats when compared to Wistar rats. Since CDDP has a significant effect on rats post injection (relative to weight , acid phosphatase activity, and alkaline phosphatase activity) the question of strain response difference (Long Evans vs. Wistar) is also of importance. Alkaline phosphatase activity after CDDP treatment shows a clear ($P< 0.01$) difference at 5, 7, 10 and 20 days following injection. Acid phosphatase activity also indicates a species response difference at 10 days ($P< 0.05$) and at 20 days ($P< 0.001$) after injection. Urine volume data also show a significant ($P< 0.05$) species difference in response at 7, 10 and 20 days after receiving the drug. This data clearly supports the contention that there exist strain and response differences between Long Evans and Wistar rats, and that the differences are relevant to toxicological studies.

The mechanism of action of CDDP has puzzled the scientific community since the discovery of its anti-metastastic and nephrotoxic properties. CDDP tends to show significant decreases of transport enzyme activity within the cell as well as corresponding increases in similar urinary enzyme activity. A significant increase occurs at 3, 5 ($P< 0.01$), and 7 days ($P< 0.05$) post injection in acid phosphatase activity. Similar increases are seen at 3 and 5 days ($P< 0.01$) post injection in urine alkaline phosphatase activity . Treatment with a second-generation analog, CBDCA, does not show nearly the same effect at 3 days post injection ($P< 0.01$) and 7 days post injection ($P< 0.05$) in urine alkaline phosphatase activity , although there is a slight, statistically insignificant increase in acid phosphatase activity after CBDCA injection.

The increase in acid phosphatase activity following injection is not surprising since it is a lysosomal enzyme, and lysosomes respond to a foreign substance (such as either drug) penetrating the body. The increase in alkaline phosphatase activity in the urine however, does indicate that while both drugs have a nephrotoxic effect, CBDCA has much less of an effect. This is documented by a comparison to both in vitro and in vivo enzyme reaction product densities.

CBDCA has been shown to have anti-metastastic activity similar to CDDP although the chemotherapeutic dosage is 10 times that of CDDP (27, 30). CBDCA has also been shown to have little effect on blood pressure up to 20 days post injection, while CDDP does (2). CBDCA has additionally been shown to cause a significant increase in red blood cell hemolysis (58), probably through the creation of an artificial osmotic gradient, while CDDP does not have as great an effect on red blood cell hemolysis (58).

Results of thiol cytochemistry indicate a decrease in membrane-bound thiols after CDDP treatment, although post CBDCA treatment there is not as drastic a reduction in thiol levels . This is important in that thiols located within proteins have been shown to be responsible for secondary structure and inter-chain attachments (58) (insulin and immunoglobulin G, both similar in size to alkaline phosphatase) and may be implicated in the linkage of enzymes such as alkaline phosphatase to the membrane. Thiols have also been implicated as key structural units in the binding sites of some enzymes (10, 16, 56, 59).

The observations noted above lead to the conclusion that although both drugs are anti-metastastic their mechanisms appear to be distinct. As such, a theoretical model for CBDCA's mechanism of action can be established. CBDCA binds to the surface of both tumor and red blood cells selectively and remains there causing an osmotic imbalance. The one unique component which is found in characteristically high amounts in both these cells is sialic acid residues (55, 61). These residues have been implicated in the transplantability of various tumors, and their absence with a decrease in the tumors'

transplantability potential (55). This binding, an osmotic mechanism, may also account for the increase in dosage needed to achieve therapeutic dose levels with CBDCA. This seems to be a new type of mechanism with regard to that of the parent compound, CDDP. CDDP has been shown to not only be more nephrotoxic than CBDCA, but also to inhibit cytokinesis and DNA replication, decrease transcription rates through its ability to form adducts at the DNA level, and cause changes in cellular calcium levels.

The results in this study indicate that CBDCA is a promising second-generation platinum analog with respect to decreased toxicity without substantial loss of anti-metastastic potential, although not all of the second-generation analogs are as promising (63).

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APPENDIX

APPENDIX

DATA AND STATISTICS

Table A1 In vivo urine alkaline phosphatase means and standard deviation.

Table A2 In vivo urine alkaline phosphatase ANOVA.

Urine Alkaline Phosphatase Activity Data.[1, 2]

Rat	Treatment	Days post treatment 0	3	5	7	10	20
Long Evans	Control	18 1 ± 4.2	—	—	—	—	—
	CDDP	—	24 ± 9 1	39 4 ± 17	13 4 ± [illegible]	11 8 ± 4	17 [illegible] ± 3 [illegible]
	CBDCA	—	3[illegible] ± 7 4	28 ± 5 2	22 ± 7	21 ± 5 3	13 ± 4 5
Wistar	Control	9 58 ± 1 6	—	—	—	—	—
	CDDP	—	20 71 ± 6 2	19 46 ± 5	8 ± 1 [illegible]	5.2 ± 1 1	8.7 ± 2.3
	CBDCA	—	16 86 ± 5 2	11 4 ± 3 7	10 2 ± 3 3	9.2 ± 2 1	10 3 ± 2.9

[1] Mean ± Standard Deviation Shown

[2] Reported in mU/hr/100 [illegible]

5 replicates at each point

[illegible]

Source of variation	Df[2]	Days post treatment 3	5	7	10	20
Treatment[1]	5	[illegible]**	[illegible]***	[illegible]***	[illegible]***	[illegible]***
Across species Control vs CBDCA & CDDP	1	[illegible]	[illegible]**	[illegible]**	[illegible]***	[illegible]
Across species Control vs CBDCA	1	[illegible]**	[illegible]	[illegible]*	[illegible]	[illegible]
Species response Control vs CDDP	1	[illegible]	[illegible]**	[illegible]***	[illegible]***	344 [illegible]***
Error	24	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

* Mean square values are presented in the table with significance as noted

[illegible]

[1] Treatment denotes Control, CBDCA or CDDP treated

[2] df denotes degrees of freedom

Table A3 In vivo urine acid phosphatase means and standard deviation.

Table A4 In vivo urine acid phosphatase ANOVA.

Urine Acid Phosphatase Activity Data. [1] [2]

Rat	Treatment	Days post treatment 0	3	5	7	10	20
Long Evans	Control	3.4 ± .62	—	—	—	—	—
	CBDCA	—	2.7 ± .85	2.46 ± 1.4	2.42 ± 1.3	3.42 ± 1.1	3.38 ± 1.9
	CDDP	—	4.37 ± 1.5	5.62 ± 2.5	3.19 ± 1.4	3.23 ± .9	3.2 ± 1.2
Wistar	Control	2.04 ± .7	—	—	—	—	—
	CBDCA	—	2.1 ± .2	1.68 ± .7	2.31 ± .35	2.05 ± .86	1.96 ± .97
	CDDP	—	4.05 ± 1.6	4.6 ± 1.5	4.9 ± 1.3	2.3 ± 1.1	2.1 ± .89

[1] Mean ± Standard Deviation Shown

[2] Reported in mU/hr/100g

5 replicates at each point.

Analyses of Variance for Urine Acid Phosphatase Activity [*]

Source of variation	Df [2]	Day post injection 3	5	7	10	20
Treatment [1]	5	4.75*	12.07*	5.05*	1.62	2.70***
Across species Control + CBDCA vs CDDP	1	17.92**	49.17***	13.70*	.31	.06
Across species Control vs CBDCA	1	.351	2.15	.395	.484	.04
Species response Control vs CDDP	1	3.55	7.24	.09	6.37*	9.15***
Error	24	1.63	3.63	1.86	.89	.45

[*] Mean square values are presented in the table with significance as noted.

* $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$

[1] Treatment denotes Control, CBDCA or CDDP treated.

[2] df denotes degrees of freedom

Table A5 In vivo 8 hour urine volume means and standard deviation.

Table A6 In vivo 8 hour urine volume ANOVA.

Rat Urine Volume Data. [1] [2]

Rat	Treatment	Days post treatment 0	3	5	7	10	20
Long Evans	Control	1.7 ± .25	—	—	—	—	—
	CDDP	—	1.53 ± .74	2.37 ± 1.2	2.57 ± 1.3	2.26 ± 1.1	1.93 ± .49
	CBDCA	—	1.17 ± .34	.85 ± .26	.91 ± .26	1.16 ± .31	.73 ± .25
Wistar	Control	.966 ± .17	—	—	—	—	—
	CDDP	—	1.33 ± .52	2 ± 1.1	1.58 ± .57	1 ± .68	1.05 ± .6
	CBDCA	—	.95 ± .3	.67 ± .2	.58 ± .21	.39 ± .24	.45 ± .23

[1] Mean ± Standard Deviation Shown

[2] Reported in mL/100g/8 hr

5 replicates at each data point.

Analyses of Variance for 8 Hour Urine Volume Data *

Source of variation	Df [1]	Day post injection 3	5	7	10	20
Treatment [1]	5	.40	2.82*	2.57**	2.05**	1.5***
Across species Control + CBDCA vs CDDP	1	.27	10.1**	7.07**	2.17*	.47
Across species Control vs CBDCA	1	.45	1.93	1.77	1.89*	.41
Species response Control vs CDDP	1	.87	1.98	3.63*	4.35**	2.56***
Error	24	.27	.84	.56	.35	.15

* Mean square values are presented in the table with significance as noted.

* $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$

[1] Treatment denotes Control, CBDCA or CDDP treated.

[2] df denotes degrees of freedom

Weight gain/loss with and without drug treatment [12]

Rat	Treatment	Average weight (gain/loss)/day
Long Evans	Control	6.7 ± 0.9 gm/day
	CDDP	-2.97 ± 1.6 gm/day
	CBDCA	6.2 ± 2.2 gm/day
Wistar	Control	4.2 ± 1.1 gm/day
	CDDP	-2.73 ± 1.14 gm/day
	CBDCA	3.83 ± 2.08 gm/day

[1]-6 replicates of each treatment

[2]-Based on average weight gain/loss 5 days after injection.

Analyses of variance for average daily weight gain *

Source of variation	df[2]	
Treatment[1]	5	112.22***
Across species control + CBDCA vs CDDP	1	530.65***
Across species control vs CBDCA	1	1.03
Species response control vs CDDP	1	7.54
Error	54	6.35

* mean square values are presented in this table with significance as noted.

* $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; *** $p < 0.001$

[1] treatment denotes control CBDCA, or CDDP treated.

[2] df denotes degrees of freedom

Table A9 In vitro culture media alkaline phosphatase activity means and standard deviation.

Table A10 In vitro culture media alkaline phosphatase activity ANOVA and Fisher LSD.

In vitro culture media alkaline phosphatase activity statistics [1] [2]

Drug	Time post incubation (hours) 0	1	2	3	4	5	6	8
CBDCA 50 mg/kg	—	3.0 ± .7	3.0 ± .7	3.2 ± 1.2	3.4 ± .4	3.6 ± .4	3.2 ± .8	2.6 ± 1
CDDP 5 mg/kg	—	3.0 ± 1.3	2.2 ± 1.1	3.0 ± 1.4	2.2 ± 1.2	3.2 ± 1.1	3.6 ± .7	3.8 ± .7
CBDCA 500 mg/kg	—	8.5 ± 1.8	7.8 ± .6	13.4 ± 1.9	13.8 ± 3	14.2 ± 3.1	17.2 ± 3	13.8 ± 2
CDDP 50 mg/kg	—	8.4 ± .7	16 ± .7	18.2 ± 1.2	20 ± 4	21 ± 4.4	23.4 ± 4	24 ± 1.6
SULFATO	—	2.8 ± .8	2.7 ± .4	3.6 ± 1	3.5 ± 6	5.6 ± .9	6.2 ± 1	7.2 ± 1.2
MALANATO	—	1.6 ± 6	2.0 ± .4	3.2 ± 1.1	2.6 ± .6	2.8 ± .8	3.6 ± .7	5.9 ± .9
CHIP	—	1.52 ± .6	1.66 ± .7	3.0 ± .9	2.3 ± .3	2.0 ± 6	3.2 ± 1.2	3.9 ± 1
DACH-CL	—	2.2 ± 1.2	2.1 ± .9	3.1 ± 1	2.5 ± 1	3.2 ± .7	3.6 ± .5	4.0 ± .4
CONTROL	1.0 ± .3	1.0 ± .5	1.12 ± .3	1.4 ± .4	1.4 ± .3	1.64 ± .5	1.64 ± .3	1.64 ± .2
DEAD 0.8	1.36 ± .5	—	—	—	—	—	—	1.52 ± .2

[1] - 5 replicates at each point

[2] - Data reported in units/dL (culture media)

Anova Table

Source	D.F.	Sum of Squares	Mean Square	F Ratio	Probability
Treatments	9	1915.423	212.8247	54.89	.000
Error	40	155.1011	3.877528		
Adj Total	49	2070.524			

Columns used in this analysis:
Column 1 : Control
Column 2 : CBDCA 50
Column 3 : CDDP 5
Column 4 : CBDCA 500
Column 5 : CDDP 50
Column 6 : sulfato
Column 7 : malanato
Column 8 : CHIP
Column 9 : DACH-Cl
Column 10 : DEAD

Fisher's Least Significant Difference Test $\alpha = .01$

Columns	LSD	Difference	Test Result
1 2	3.367525	-.4720001	NOT SIGNIFICANT
1 3	3.367525	-1.536	NOT SIGNIFICANT
1 4	3.367525	-11.548	SIGNIFICANT
1 5	3.367525	-20.248	SIGNIFICANT
1 6	3.367525	-5.328	SIGNIFICANT
1 7	3.367525	-3.832	SIGNIFICANT
1 8	3.367525	-2.012	NOT SIGNIFICANT
1 9	3.367525	-1.8	NOT SIGNIFICANT
1 10	3.367525	.568	NOT SIGNIFICANT

Table A11 In vitro tubule viability means and standard deviation.

Table A12 In vitro tubule viability ANOVA and Fisher LSD.

In vitro tubule viability statistics [1] [2]

Drug	Time post incubation (hours)							
	0	1	2	3	4	5	6	8
CBDCA 50 mg/kg	—	98 ± 13	96 ± 10	92 ± 13	87 ± 13	87 ± 10	81 ± 10	79 ± 10
CDDP 5 mg/kg	—	93 ± 13	88 ± 14	81 ± 11	80 ± 13	76 ± 18	73 ± 13	69 ± 13
CBDCA 500 mg/kg	—	100 ± 0	93 ± 12	83 ± 12	81 ± 11	80 ± 0	76 ± 20	73 ± 10
CDDP 50 mg/kg	—	98 ± 20	88 ± 14	70 ± 0	63 ± 20	65 ± 0	63 ± 13	43 ± 18
SULFATO	—	92 ± 11	85 ± 17	81 ± 10	77 ± 19	72 ± 20	70 ± 16	69 ± 21
MALANATO	—	92 ± 17	87 ± 15	83 ± 15	81 ± 12	78 ± 11	76 ± 13	73 ± 15
CHIP	—	92 ± 20	90 ± 16	82 ± 13	82 ± 11	79 ± 10	76 ± 10	74 ± 10
DACH-Cl	—	94 ± 18	92 ± 13	84 ± 23	78 ± 20	77 ± 22	72 ± 20	68 ± 18
CONTROL	100 ± 0	100 ± 0	98 ± 08	92 ± 10	92 ± 11	90 ± 10	90 ± 14	88 ± 13

1.- 5 replications at each data point

2.- Data reported in percent viability

Anova Table

Source	D.F	Sum of Squares	Mean Square	F Ratio	Probability
Treatments	8	6314.4	789.3	55.17	0.000
Error	36	534.4	14.84444		
Adj Total	44	6848.8			

Columns used in this analysis:

Column 1 : control	Column 2 : CBDCA 50	Column 3 : CDDP 5.0
Column 4 : CBDCA 500	Column 5 : CDDP 50	Column 6 : sulfato
Column 7 : malanato	Column 8 : CHIP	Column 9 : DACH-Cl

Fisher's Least Significant Difference Test

Columns	LSD	Difference	Test Result
1 2	6.625747	8.199997	SIGNIFICANT
1 3	6.625747	20.2	SIGNIFICANT
1 4	6.625747	16	SIGNIFICANT
1 5	6.625747	46.4	SIGNIFICANT
1 6	6.625747	20.2	SIGNIFICANT
1 7	6.625747	15.6	SIGNIFICANT
1 8	6.625747	15.39999	SIGNIFICANT
1 9	6.625747	21.2	SIGNIFICANT

Table A13 In vivo scanning densitometry data.

Table A14 In vivo scanning densitometry statistics.

In vivo scanning densitometry data [1]

Treatment	Days post injection		
	0	3	5
Control	13.8 ± 2.1	—	—
CDDP	—	10.6 ± 1.4	8.4 ± 2
CBDCA	—	12.9 ± 1.4	11.9 ± 2.1

[1] Mean ± Standard Deviation Shown

10 replicates at each point.

Analyses of Variance for in vivo scanning densitometry *

Source of variation	Df[2]	Day post injection 3	5
Treatment[1]	2	34.73***	73.56***
Control + CBDCA vs CDDP	1	59.8***	129.05**
Control vs CBDCA	1	9.67	18.05*
Error	27	2.32	4.2

* Mean square values are presented in the table with significance as noted.

* $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$

[1] treatment denotes control, CBDCA or CDDP treated.

[2] df denotes degrees of freedom

Table A15 In vitro scanning densitometry data.

Table A16 In vitro scanning densitometry statistics.

In vitro scanning densitometry data [1]

Treatment	Hours post incubation			
	0	1	3	5
Control	7.5 ± 1.3	—	—	—
CDDP	—	4.7 ± 1	3.8 ± 1.1	2.13 ± 0.8
CBDCA	—	5.7 ± 1.2	5.8 ± .7	4.8 ± 1.1

[1] $\bar{x}_{mean}$ ± Standard Deviation Shown

10 replicates at each point.

Analyses of variance for in vitro scanning densitometry *

Source of variation	df[2]	Hours post incubation		
		1	3	5
Treatment[1]	2	15.24***	23.31***	48.94***
Control + CBDCA vs CDDP	1	14.40**	37.34***	70.72***
Control vs CBDCA	1	16.07**	9.41*	24.02**
Error	18	1.07	0.97	1.13

* mean square values are presented in this table with significance as noted.

* $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; *** $p < 0.001$

[1] treatment denotes control CBDCA, or CDDP treated.

[2] df denotes degrees of freedom