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The interaction of platinum antitumour drugs with mouse liver mitochondria

M. Rosen*, M. Figliomeni and H. Simpkins
Department of Biology, College of Staten Island of the City University of N.Y. and
**Departments of Pathology, Staten Island University Hospital and State University of New York Health Science Center, New York 11203–2098*

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Summary. A study was undertaken to determine if cis-DDP and its second generation derivatives produced effects on mouse liver mitochondria, and if any of the observed effects could be correlated with the nephrotoxicity of the drugs. Although changes were observed in mitochondrial morphology, enzyme activity, Ca^{2+} influx, terbium binding and surface potential, no specific effect was correlated with nephrotoxicity. cis-DDP produced marked changes in mitochondrial morphology; electron probe analysis showed binding of the drug to the mitochondria. Inhibition of complex I and II activity of the respiratory chain and an ionic-strength-dependent effect on Tb^{3+} (a Ca^{2+} analogue) fluorescence were observed. The non-nephrotoxic derivatives, CHIP and tetraplatin, also produced significant changes in morphology. Treatment with these derivatives also produced decreases in mitochondrial enzyme activity, but the effect on terbium binding had an ionic-strength dependence which was inverse to that observed with cis-DDP. The tetravalent compounds also had a notable effect on mitochondrial surface potential. Carboplatin had an effect on morphology and Ca^{2+} influx and it inhibited the respiratory enzymes, although in a manner different from that observed with cis-DDP. Carboplatin had a minimal effect on terbium binding. It is evident that if the platinum drugs enter a cell to exert their action at the nuclear level, they will also depress mitochondrial function. The observed effects did not correlate with nephrotoxicity but, since all four compounds significantly altered mitochondrial structure and function, they may be related to the cytotoxicity of the drug.

Keywords: platinum antitumour drugs, mitochondrial morphology, enzymes, terbium binding.

cis-Dichlorodiammine platinum (II) (cis-DDP), one of the most widely used antitumour drugs, is used to treat a wide range of solid tumours (Ensley *et al.* 1984, Rosencweig *et al.* 1977). Unfortunately, the therapeutic efficacity of this drug is limited by its severe dose limiting side-effects, the most important being nephrotoxicity (Mijer *et al.* 1983). A series of second generation derivatives have been synthesized in an attempt to

Correspondence: Dr M. Rosen, Department of Pathology, Staten Island University Hospital, 475 Seaview Avenue, Staten Island, NY 10305, USA.

find drugs with less debilitating side-effects than the parent compound. Two of the second generation platinum drugs are cis-diammine-1,1-cyclobutanedicarboxylate platinum (II) (CBDCA (JM-8)) and cis-dichloro-trans-dihydroxybis (isopropylammine) platinum (IV) (CHIP (JM-9)). CBDCA was found to be potent against plasmacytoma ADJ PC6A, human epidermoid P246, and L1210 leukaemia cell lines; CHIP was effective against L1210 and the PC6A cell lines but had little effect on an epidermoid carcinoma line (Harrap *et al.* 1980). The nephrotoxicity of CHIP and CBDCA in animal models was minimal compared with that of the parent compound and in recent clinical trials CBDCA appeared to produce remission rates comparable to those of cis-DDP in advanced ovarian carcinoma (Bramwell *et al.* 1985; Wiltshaw *et al.* 1983). Another report suggests that CBCDA may also exert antitumour activity on breast carcinoma (Curt *et al.* 1983). The prime side-effect of CHIP and CBDCA appears to be myelosuppression rather than nephrotoxicity (Creaven *et al.* 1983).

cis-DDP nephrotoxicity involves both renal tubular dysfunction and depression of the glomerular filtration rate (Choie *et al.* 1981; Chopra *et al.* 1982). The structural changes observed involve acute tubular necrosis, interstitial fibrosis, tubular atrophy and dilatation (Jones *et al.* 1985). Protection may be produced by thiol compounds such as thiourea, methionine and cysteine; however, most of these compounds result in a decreased therapeutic efficacity of cis-DDP in addition to a reduction in nephrotoxicity (Filipski *et al.* 1979). Diethyldithiocarbamate appears to decrease renal toxicity and produces little interference with the ability of cis-DDP to cross-link DNA (Bodenner *et al.* 1986). Kidney tubular epithelial cells contain large numbers of mitochondria, and thus the early toxic effects of cis-DDP may involve a direct interaction with the mitochondrion and effects were observed on mitochondrial respiration (Simmons & Hames 1979). When experiments were performed inducing mild, reversible non-oliguric renal failure in the rat, mitochondrial respiration and calcium accumulation were altered and, at the peak of injury, mitochondrial morphology was affected (Gordon & Guttone 1986). Another recent study by Sugiyama *et al.* (1989) showed that cis-DDP produced changes in rat kidney mitochondrial respiratory function and enzymatic activities as well as glutathione peroxidase levels. A diamminotoluene Pt (II) derivative also produced effects on mitochondrial phosphate transport and Ca^{2+} release (Binet & Volfin 1977). Mitochondria in many carcinoma cells have been shown to exhibit increased uptake of rhodamine 123 and an altered membrane potential (Davis *et al.* 1985; Summerhayes *et al.* 1982) as compared to non-neoplastic control cells, and rhodamine antitumour drugs are now in clinical trial (Herman *et al.* 1988). Recently, *N*-(4-methylphenylsulphonyl)-*N'*-(4-chlorophenyl) urea, a new chemotherapeutic agent urea with high antitumour activity in human adenocarcinoma cells, appeared to be selectively taken up by mitochondria and produces morphologic changes (Houghton *et al.* 1990). Mitochondria with aberrant membrane potentials etc. have also been described in human colon carcinoma cell lines (Modica-Napolitano *et al.* 1989).

Thus the possibility that platinum antitumour drugs may interact at a mitochondrial locus as well as at the nuclear level appeared to be worthy of investigation. In addition, a comparison between any effect produced by cis-DDP and those observed with the non-nephrotoxic but still cytotoxic second generation derivatives appeared to be important.

The investigation involved the use of mouse liver mitochondria, since their preparation and properties are among the best described of any mitochondrial preparation. The studies involved electron microscopy, electron probe analysis, respiratory enzyme analysis and investigation with the fluorescent analogue of Ca^{2+}, terbium. The terbium ion (Tb^{3+}) has been used as a phosphorescent probe of mitochondrial surface potential

and Ca^{2+} binding sites by Hashimoto and Rottenberg (1983), as well as others.

Tb^{3+} is a phosphorescent cation of the lanthanide series and is used as a probe for Ca^{2+} binding sites in many Ca^{2+} binding proteins. The presence of aromatic amino acid residues within close proximity to the binding site allows efficient energy transfer when the residues are excited, e.g. from Tb^{3+} to tryptophan. Since the terbium is positively charged, the extent of terbium binding at low concentration depends on the surface potential near the binding site. The binding can easily be monitored either by the phosphorescence of the protein-bound Tb^{3+} or by chelation of the free Tb^{3+} in the supernatant with an aromatic chelator, such as dipicolinic acid, after the mitochondria have been sedimented. In this investigation, Tb^{3+} was employed to compare the effect of the platinum antitumour drugs on the cardiolipin-rich mitochondrial membrane, as well as the effects of calcium blockers, like verapamil, on the preparations.

Materials and methods

CHIP, CBDCA, tetrachloro-(*d,l*-trans)-1,2-diamminocyclohexane platinum (IV) (tetraplatin) and cis-DDP were kindly provided by the drug synthesis and chemistry branch of The National Cancer Institute, Bethesda, Maryland. The drugs were used without further purification. 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene-*p*-toluene sulphate (TMA-DPH) was purchased from Molecular Probes, Inc. (Eugene, OR).

Preparation of mouse mitochondria and submitochondrial particles

Mitochondria were prepared from the livers of BALB/c mice by homogenization in 0.25 M sucrose, 1 mM *N*-ethylenediaminetetraacetic acid (EDTA), pH 7.4 at 4°C. The livers were trimmed of fat and chopped prior to centrifugation. The preparation was washed by differential centrifugation and filtered through several layers of gauze to remove any remaining pieces. The nuclei were sedimented at 700 *g* for 10 min at 4°C and the mitochondria at 6000 *g* for 20 min. The mitochondrial pellet was washed twice in a medium composed of 0.25 M sucrose, 1 mM MOPS, pH 7.4 to remove the EDTA. *N*-ethylmaleimide (NEM) treatment of the mitochondria was performed by adding 40 nM of NEM/mg protein, incubation for 1 min at room temperature and then washing the treated mitochondria with 0.25 M sucrose (Sottocasa *et al.* 1967).

The purity of the mitochondria was assayed by the specific activity of NADH cytochrome c reductase and succinate-cytochrome c reductase activities on samples prepared in 0.25 M sucrose. The enzyme activities were increased approximately 30–40 times above that of the homogenate (Sottocasa *et al.* 1967). NADPH cytochrome c reductase activity was 5% or less of that in the homogenate, reflecting a low degree of microsomal contamination (Sottocasa *et al.* 1967). Electron microscopy was performed by fixation in 1% glutaraldehyde for 2 hours, 2% buffered OsO_4 for 1 hour, and dehydration in an ethanol series. The sections were stained with uranyl acetate/lead citrate as described by Reynolds (1963).

Submitochondrial particles were prepared by sonication of mitochondria in 0.145 M KCl, 1 mM EDTA, 10 mM Tris HCl pH 8.5 or 0.25 M sucrose, 1 mM EDTA, 10 mM Tris HCl pH 8.5 at 4°C to give particles of low and high cytochrome c content, respectively (approximately two to three-fold difference) (Demant 1983). The sonicated mitochondria were centrifuged at 6000 *g* for 20 min to remove intact mitochondria and then at 120 000 *g* for 1 hour at 4°C to pellet the membrane fragments. The mitochondrial protein concentration was determined according to Lowry *et al.* (1951), employing bovine serum albumin as a standard.

X-ray energy dispersive microprobe analysis

Mitochondrial fractions obtained by ultracentrifugation were rinsed three times with

0.25 M sucrose and spun down into a pellet. Samples of the suspended mitochondria were pipetted onto 200 mesh copper grids coated with Formvar and carbon and permitted to dry in air. Grids bearing air-dried samples of mitochondria were placed in a JEOL-100C transmission electron microscope fitted with an Interface Device (ASID-4) and a Kevex 7000 energy dispersive microprobe analyser. The operating conditions were: accelerating voltage of 80 kV; tilt angle of 30–35°; specimen (probe) current approximately 250 pA. The electron beam was placed over the air-dried sample and X-ray spectra were generated for a period of 200 s with the microscope in the TEM mode. Approximately 55 spectra were generated from the mitochondrial preparations.

Drug treatment

In the enzyme experiments, freshly prepared mitochondria (2 mg) were incubated at 37°C for 20 min with varying drug concentrations (0–200 μM) in 0.25 M sucrose, 1 mM Tris-HCl pH 7.4. Aliquots of mitochondria (1–50 μg) were then assayed for the appropriate enzyme activity. The cytochrome reductase enzymes were assayed according to Sottocasa *et al.* (1967) and the F_0F_1-ATPase was measured on freeze-thawed preparations (−20°C) as described by Modica-Napolitano and Aprille (1987).

Drug treatment prior to the terbium binding experiments was by incubation of the mitochondria or submitochondrial particles in 0.25 M sucrose, 1 mM 3-(*N*-morpholino) propane sulphonic acid (MOPS) pH 7.4 or 0.145 M KCl, 1 mM MOPS pH 7.4 at the specified protein and drug concentrations, for 45 min at 37°C. Terbium was then added at the indicated concentration and incubated at RT for 10 min before fluorescence or phosphorescence measurements were made. No evidence of aggregation of the mitochondrial suspension, which would have been evidenced by changes in light scattering, was observed.

Estimation of terbium uptake into mitochondria

The uptake of terbium into the mitochondria was estimated by measuring the concentration of terbium using dipicolinic acid as a luminescence sensitizer (Barela & Sherry 1976). The mitochondrial suspension (150 μg) was incubated with terbium at concentration of 0–300 μM at 37°C for 45 min, centrifuged at 10000 *g* for 3 min and the supernatant was then reacted with the dipicolinic acid at a final concentration of 400 μM. Fluorescence was measured in a Perkin Elmer 43A fluorescence spectrophotometer at an excitation wavelength of 280 nm and an emission wavelength of 545 nm. (A calibration curve was constructed using a standard $TbCl_3 \cdot 6H_2O$ solution calibrated with EDTA, as described by Gross and Simpkins (1981).)

Terbium fluorescence and phosphorescence decay studies

Increasing concentrations of terbium (0–300 μM) in either sucrose-MOPS buffer or KCl-MOPS buffer were added to the mitochondrial suspension (0–150 μg protein). The concentration of terbium (III) chloride $\cdot 6H_2O$ (Aldrich Chemicals, 99.9% purity) was precisely determined by titration against EDTA (Gross & Simpkins 1981). Mitochondrial suspensions were prepared and washed just prior to use. The fluorescence spectra were measured employing an MPF43A Perkin Elmer fluorescence spectrophotometer with a high pressure xenon lamp. An excitation wavelength of 290 nm and an emission wavelength of 544 nm were employed to maximize energy transfer and minimize intrinsic Tb^{3+} and mitochondrial fluorescence, which is maximal at 270 nm and minimal at 290 nm. Entrance and exit slit widths were kept at 5 nm; this was found to be important to minimize light scattering artefacts. Tryptophan fluorescence of the submitochondrial particles was measured at 330 nm, with an excitation wavelength of 280 nm, in the appropriate buffer following

drug treatment. This was done to determine whether Tb^{3+} fluorescence could be correlated to tryptophan fluorescence.

The labelling of the drug-treated mitochondria with TMA-DPH was performed by making a 5 mM stock solution of the probe in dimethylformamide. This was done to monitor lipid structural order since changes in this parameter have been shown to occur with second generation drugs and mouse thymocytes (Simpkins *et al.* 1988). The solution was diluted to 0.04 mM in the appropriate buffer by vigorous agitation. The diluted probe was then reacted 1:1 with 150 μg mitochondrial protein/ml at room temperature for 15 min, and the fluorescence intensity measured at 37° in a jacketed cuvette holder. The fluorescence was read with the polarization filters at 0–0°, 90–0°, 0–90°, 90–90°. The optimum excitation and emission wavelengths were 358 and 440 nm, respectively. The slit widths were kept constant at 5 nm. The polarization constant (P) was calculated from the formula:

$$P=\frac{V_v-L_v\,(V_H/L_H)}{V_v+L_v\,(V_H\cdot L_H)}$$

where V_v is the fluorescence intensity at 0°/0°, V_H 90°/0°, L_H 90°/90°, L_v 0°/90°.

Phosphorescence decay curves of the submitochondrial particles were obtained with a Perkin-Elmer LS5 luminescence spectrophotometer. Excitation was by repetitive discharge of the lamp with a half-width duration of 10 μs. The phosphorescence decay was measured by varying the delay time 0.02–1.5 ms with a fixed gate time (1 ms).

The experiments employed 100 μg of submitochondrial particles with 10 μM Tb^{3+} to give an adequate signal. All values were corrected for the intrinsic phosphorescence of the submitochondrial suspension.

Results

Electron micrographs showed that cis-DDP treatment (Fig. 1) produced a marked effect on the mitochondrial membrane. There was mitochondrial swelling, loss of cristae, disruption of the outer membrane, deposition of electron-dense material and formation of vesicles. CBDCA showed the least effect and that of CHIP was equal to or greater than that of cis-DDP. Tetraplatin (data not presented) showed an effect similar to or greater than cis-DDP. Electron probe analysis (Fig. 2) shows binding of cis-DDP to the mitochondrion based on detection of a Pt peak. A calcium peak appeared, as did increased phosphorus and sulphur peaks, suggesting influx of Ca^{2+}, PO_4^{3-} and SO_4^{2-} into the mitochondria. The second generation drugs produced no detectable platinum peak, although a small Ca^{2+} peak appeared. Similar but smaller effects were observed when drug concentrations of 100 μM were employed. The copper peaks were due to the grids and the Fe peaks from the electron probe.

The platinum drugs produced significant inhibition of both NADH and succinate cytochrome c reductase systems at pharmacological concentrations (Table 1). The charged compounds cis-DDP and CHIP produced the greatest effects on the NADH (Complex I) as opposed to the succinate (Complex II) system. The lipophilic second generation drug, CBDCA produced its greatest effect on the succinate (Complex II) system, and CHIP, a Pt(IV) drug with a lipophilic side group, produced an effect intermediate between that of cis-DDP and CBDCA. Since the interaction of adriamycin with the mitochondrion is thought to involve cardiolipin, it appears that the platinum drugs may also interact at this site (especially cis-DDP and CHIP) since their pattern of inhibition is similar to that of adriamycin. The report by Modica-Napolitano and Aprille (1987), that the mitochondrial-specific drug rhodamine 123 produced marked inhibition of the F_0F_1-ATPase but little inhibition of respiratory enzymes, prompted an investigation into the effects of these drugs on this inner membrane ATPase. However, all the platinum antitumour drugs and adriamycin produced minimal effect on F_0F_1-ATPase (Table 1). These

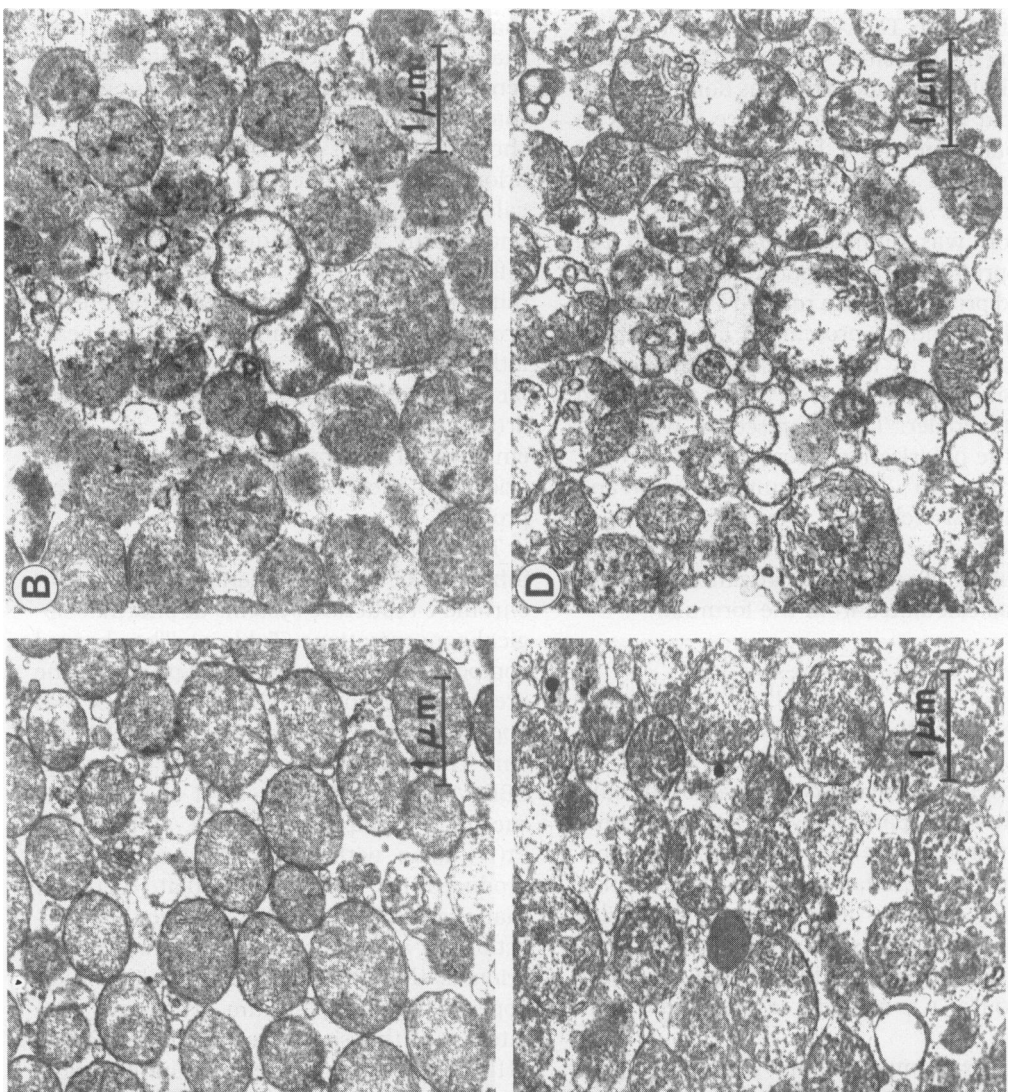

Fig. 1. Electron micrographs of mouse liver mitochondria (2 mg mitochondrial protein) treated with the Pt drugs (100 μM) in 0.44 M sucrose pH 7.4 at 37°C for 45 min. The mitochondria were then washed in 0.1 M phosphate buffer pH 7.4 and then processed as described in the text. The figures show a, control; b, cis-DDP; c, CBDCA; and d, CHIP treated mitochondria. × 22 000.

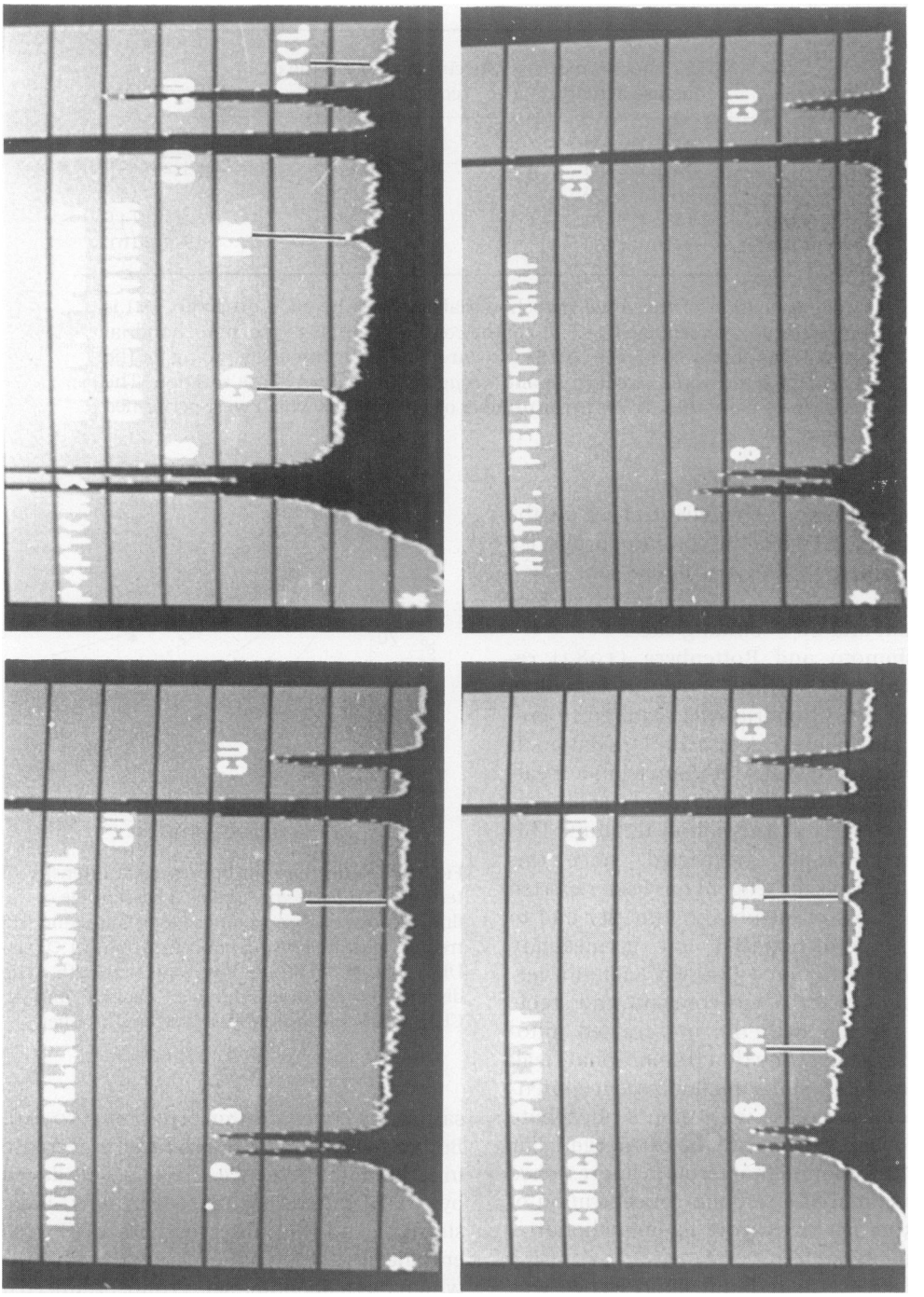

Fig. 2. Electron probe analysis of mouse liver mitochondria treated at 37°C for 45 min. with 200 μM of each drug and mitochondria (2 mg) in 0.25 M sucrose, pH 7.4. The spectra show the individual elements identified following a, no drug treatment; b, cis-DDP; c, CBDCA; and d, CHIP.

Table 1. The effect of platinum antitumour drugs on mouse liver NADH and succinate cytochrome c reductase and F_0F_1-ATPase activities

Drug	NADH cytochrome c reductase activity	Succinate cytochrome c reductase activity	F_0F_1-ATPase
cis-DDP	37±1.9 (7)	75±2.9 (7)	95±2.7 (4)
CHIP	34±1.2 (7)	66±2.5 (7)	94±2.8 (5)
CBDCA	59±1.3 (7)	53±1.4 (7)	94±3.0 (4)
Tetraplatin	17±1.3 (6)	3.1±0.5 (7)	91±4.4 (4)
Adriamycin	32±2.4 (5)	63±2.3 (5)	86±3.2 (6)

The inhibition of the three mitochondrial enzymes by each drug (50 μM) is expressed as a percentage (±s.e.m.) of the control (100%). 3 μg of mitochondrial protein was employed for the NADH cytochrome c reductase assay, 20 μg for the succinate cytochrome c reductase and 50 μg for the F_0F_1-ATPase reaction. The numbers in parentheses refer to the number of experiments which were performed in duplicate.

results, showing a significant effect on the mitochondria by all four platinum drugs, led us to employ Tb^{3+}, a fluorescent Ca^{2+} analogue, to assess mitochondrial surface potential and Ca^{2+} binding sites.

Hashimoto and Rottenberg (1983) reported that *N*-ethylmaleimide treatment of rat liver mitochondria was required to prevent a phosphate leak and Tb^{3+} chelation. It was found here that *N*-ethylmaleimide treatment of mitochondria decreased the fluorescence intensity of the bound terbium. This was not totally unexpected, since this sulphydryl specific reagent has been reported to cause loss of membrane integrity and to increase mitochondrial ion permeability (LeQuoc & LeQuoc 1982). In addition, terbium fluorescence was constant and stable when reacted with the non-treated mitochondria, suggesting that the phosphate leak was negligible, since the fluorescence intensity would decrease with time if phosphate were to leak and chelate the extraorganellar Tb^{3+}. It was found that terbium fluorescence was maximal at a terbium concentration of 200–300 μM with 100 μg mitochondrial protein.

A decrease in terbium fluorescence was observed following reaction of the drug-treated mitochondria (Fig. 3) with a non-

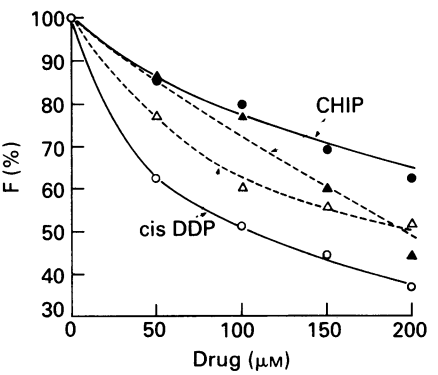

Fig. 3. The decrease in fluorescence intensity of terbium (50 μM) expressed as a percentage of the fluorescence of control mouse liver mitochondria treated with increasing concentrations of ○, cis-DDP and ●, CHIP at low ionic strength. The discontinuous curves show △, cis-DDP and ▲, CHIP treatment in high ionic-strength buffer.

saturating concentration of terbium (50 μM). Both cis-DDP and CHIP produced a decrease in terbium fluorescence. The effect produced by cis-DDP was enhanced at low ionic strength and that observed with CHIP was enhanced at high ionic strength. CBDCA, which has a lipophilic side group, produced a minimal effect (10–15%) on terbium fluorescence intensity. Both cisDDP and CHIP exhi-

Table 2. The effect of Pt drugs on Tb^{3+} uptake into mouse liver mitochondria

Treatment (μM)	0.25 M Sucrose 1 mM MOPS (pH 7.4)	0.145 M KCl 1 mM MOPS (pH 7.4)
Control (300 Tb^{3+})	0.50±0.05 (13)	0.31±0.06 (13)
90 cis-DDP	0.45±0.06 (12)	0.35±0.07 (12)
90 CHIP	0.54±0.05 (12)	0.47±0.07 (12)
200 cis-DDP	0.53±0.08 (12)	0.40±0.05 (12)
200 CHIP	0.58±0.06 (12)	0.48±0.05 (12)
Control (30 Tb^{3+})	0.97±0.04 (13)	0.80±0.10 (13)
90 cis-DDP	0.95±0.03 (12)	0.80±0.12 (12)
90 CHIP	1.00±0.04 (12)	0.85±0.10 (12)
200 cis-DDP	0.95±0.08 (12)	0.85±0.10 (12)
200 CHIP	1.00±0.08 (12)	0.90±0.12 (12)

Tb^{3+} uptake into mitochondria following treatment with platinum drugs expressed as a ratio of Tb^{3+} bound/TB^{3+} total (±s.e.m.). The mitochondria were treated with drugs as described in Material and methods. The free Tb^{3+} in the supernatant was measured with dipicolinic acid as a luminescence sensitizer. The figures in parentheses are the number of determinations.

bited similar behaviour when higher (saturating) concentrations of terbium (300 μM) were employed.

The effect of cis-DDP and CHIP on terbium uptake into the isolated mitochondria was measured (Table 1). It was observed that approximately 30–50% of the terbium was taken up at a saturating terbium concentration (300 μM). This increased to approximately 80–95% at a low Tb^{3+} concentration (30 μM). Increased uptake was also observed in the low ionic-strength buffer. Following drug treatment, there was little change in uptake except for CHIP which produced a small increase, an effect which appeared to be greater at high ionic strength. Verapamil, a calcium channel blocker, produced an effect similar to cis-DDP on terbium fluorescence (see Fig. 4). The effect was most pronounced in a low ionic-strength medium.

Since observations with the electron microscope showed that the drugs produced marked effects on mitochondrial integrity, the experiments were repeated with membrane fragments (submitochondrial particles). The fragments were prepared with

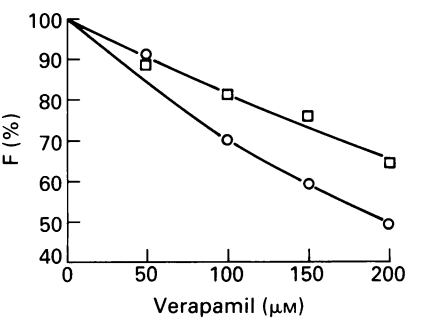

Fig. 4. The effect of increasing concentrations of verapamil on mouse liver mitochondria in 0.25 M sucrose–MOPS at □, high (300 μM) and ○, low (50 μM) Tb^{3+} concentrations.

varying cytochrome c contents in order to determine whether this inner membrane protein was involved in these drug-induced phenomena. In addition, the potential problem of phosphate leak was negated and the effect of the drugs on tryptophan fluorescence could also be investigated directly. Employment of a low Tb^{3+} concentration (10 μM) enabled calculation of the apparent

Table 3. The effect of the Pt drugs on Tb^{3+} and tryptophan fluorescence of sub-mitochondrial particles

Treatment (μM)	0.145 M KCl 1 mM MOPS (pH 7.4)	0.25 M Sucrose 1 mM MOPS (pH 7.4)
Terbium fluorescence		
Control	100	100
100 cis-DPP	85±8 (6)	79±7 (6)
200 cis-DPP	77±10 (8)	56±6 (8)
100 CHIP	73±8 (6)	89±8 (6)
200 CHIP	66±9 (8)	82±7 (8)
100 Tetraplatin	59±5 (5)	80±7 (4)
200 Tetraplatin	35±4 (5)	42±4 (4)
200 CBDCA	102±5 (3)	109±5 (4)
Tryptophan fluorescence		
Control	100	100
100 cis-DDP	93±5 (4)	90±5 (4)
200 cis-DDP	91±4 (4)	80±5 (4)
100 CHIP	92±5 (4)	89±6 (4)
200 CHIP	80±4 (4)	78±3 (4)
100 Tetraplatin	46±4 (4)	46±5 (4)
200 Tetraplatin	25±5 (4)	24±5 (4)
200 CBDCA	102±7 (4)	95±7 (4)

The control value of non-treated mouse liver mitochondrial membrane fragments was arbitrarily given a value of 100%. Inhibition by each drug is expressed as a percentage (±s.e.m.) of the control. Tb^{3+} fluorescence was measured at 544 nm, with excitation at 290 nm. Tryptophan fluorescence was measured at 330 nm with excitation at 280 nm. The numbers in parentheses are the number of determinations.

surface potential of the control and drug-treated membrane preparations.

Treatment of the submitochondrial particles with the platinum derivatives showed that cis-DDP, CHIP and tetraplatin, but not CBDCA, produced a marked decrease in Tb^{3+} fluorescence. The effect of ionic strength was similar to that observed with intact mitochondria. The effects produced by CHIP and tetraplatin were equal to or greater in the high ionic strength medium whereas cis-DDP had the opposite effect (Table 3). The cytochrome c content did not significantly affect the results (data not presented). When the effect of the drugs on tryptophan fluorescence was measured directly, a similar pattern was observed but the magnitude of the change was less with all the drugs except tetraplatin and ionic strength appeared less important.

Phosphorescence decay at a low Tb^{3+} concentration can be analysed assuming two decaying species and an apparent surface potential can be calculated employing the equation

$$\psi = -\frac{1}{3}\frac{RT}{F}\left\{\ln(P/P_0)\right\}$$

where P is the phosphorescence intensity of the slowly decaying species at low ionic strength at zero time and P_0 is the phosphorescence intensity in high ionic strength (i.e. 0.125 M NaCl). This equation is justified because the surface potential becomes vanishingly small in a high salt medium (Robertson & Rottenberg 1983).

Table 4. Surface potential of submitochondrial particles treated with different Pt drugs

Sample (μM)	Surface potential (mV)
Control	-5.0 ± 0.8 (2)
100 cis-DDP	-5.0 ± 0.2 (2)
100 CHIP	-10.7 ± 1.2 (2)
100 Tetraplatin	-11.1 ± 1.2 (2)

The treatment of the mouse liver submitochondrial particles was described in Materials and methods. The surface potential was calculated ($\pm$s.e.m.) from the phosphorescence intensity at zero time in high and low ionic-strength media with 10 μM Tb^{3+} using the Nernst equation. The numbers in parentheses are the number of experiments done in duplicate.

When this calculation was performed with drug-treated submitochondrial particles (Table 4), it was found that cis-DDP had a minimal effect on membrane potential, whereas CHIP and tetraplatin significantly decreased the surface potential. The effects of CHIP and tetraplatin were reproducible, although variations in the absolute value of the surface potential were observed from experiment to experiment.

Discussion

The results show that at pharmacological concentrations platinum antitumour drugs produced significant effects on the integrity of the mitochondrial membrane, that Ca^{2+} influx appeared to occur with some of the drugs, and that platinum binding could actually be observed with cis-DDP. Enzyme activities in the respiratory chain were inhibited. The F_0F_1 ATPase was not affected. The enzyme effects were similar to those observed with adriamycin, which suggests that the negatively charged anionic phospholipid, cardiolipin, may play a role.

The data show that the effect of the drugs on membrane Tb^{3+} binding sites was similar whether whole mitochondria or submitochondrial fragments (fragments of the inner mitochondrial membrane) were employed. The effect of ionic strength was different for the cis-DDP/membrane interaction unlike the prototype Pt(IV) compound, CHIP, and the new Pt(IV) derivative, tetraplatin. The interaction of cis-DDP with mitochondria showed dependence on ionic strength; the effect of the drug was far greater at low ionic strength, whereas the converse was true with Pt(IV) compounds. It is interesting to speculate that the interaction of cis-DDP involves a charge interaction with membrane constituents (cis-DDP binding site?), presumably involving membrane proteins, since no interaction of cis-DDP with membrane lipids has been reported (Simpkins & Pearlman 1986). Another significant finding is that the Pt(IV) compounds produced a marked decrease in the apparent surface potential, an effect which was not observed with cis-DDP. The second generation derivative, CBDCA, produced little effect on terbium sites of either the mitochondrial or thymocyte plasma membrane (Simpkins *et al.* 1988). This is in contradistinction to its effect on plasma membrane lipid structural order (Simpkins & Pearlman 1986).

Terbium fluorescence can be produced by energy exchange between the terbium ion and cytochromes of the respiratory chain in the inner mitochondrial membrane or nearby membrane protein tryptophan residues. However, when membrane fragments of differing cytochrome c content were prepared, no significant differences were observed. This observation suggests that the drugs do not interact directly with the cytochrome c residues in the membrane but rather with neighbouring tryptophan residues, supporting the data showing that most of the drug effects on Tb^{3+} fluorescence can be correlated with their direct effects on tryptophan fluorescence.

The results also show that there are differences in the properties of the terbium sites on the mitochondrial as opposed to the plasma membrane. Verapamil has no effect on mouse thymocyte/plasma membrane

Tb^{3+} sites but it does have a small but significant effect on the mitochondrial membrane. Oxanthrazole, an anthrapyrazole antitumour derivative, has a marked effect on plasma membrane terbium binding sites, as shown by Simpkins *et al.* (1988), but has little effect on mitochondrial binding sites (data not presented). Adriamycin is known to interact directly with the phospholipid, cardiolipin, situated primarily in the inner mitochondrial membrane. Thus, the differences in terbium binding characteristics between the plasma and mitochondrial membrane may be due to association of the latter sites with cardiolipin. cis-DDP produces a significant effect on mitochondrial terbium binding sites; the effect is ionic-strength dependent occurring to the greatest extent at low ionic strength. The non-nephrotoxic drugs, CHIP, tetraplatin and CBDCA either produce effects on Tb^{3+} sites which exhibit a minimal dependence on ionic strength (CHIP < tetraplatin) or little to no effect (CBDCA). Analysis of the results with these second generation drugs is complicated by the possibility that they may react (since they contain lipophilic side groups) with mitochondrial lipid domains as well as membrane proteins. It is intriguing to speculate that CBCDA acts on lipid domains and thus does not perturb the Tb^{3+} sites, whereas the Pt(IV) derivatives act on both the membrane protein (Tb^{3+} site?) and lipid domains.

One interesting conclusion that can be drawn is that these drug effects cannot be correlated with nephrotoxicity, since all the drugs reacted (albeit in different ways) with the mitochondria. In certain systems, CHIP (non-nephrotoxic) produced greater effects than cis-DDP. Significant changes in mitochondrial morphology, respiratory enzyme activity, and Ca^{2+} deposition are also observed with CBDCA, another non-nephrotoxic derivative. Therefore, the possibility that these drugs having entered a cell may exert cytotoxic effects at a mitochondrial locus cannot be ignored. It is possible that since liver but not kidney mitochondria were employed, the different effects might be observed with mitochondria from the latter organ. However, mitochondria from both organs are similar in phospholipid composition (Fleischer *et al.* 1967), and in the effects of anthracyclines and metabolites on Ca^{2+} uptake in contradistinction to heart mitochondria (Revis & Marusic 1979).

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