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LYMPHOID SUPPRESSION BY CIS-PLATINUM(II)AMINES

WHAT ARE THE ACTIVE AGENTS?

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Abstract—Cisplatin and its various hydrolysis products were tested *in vitro* for their effects on the incorporation of [^{3}H]thymidine into lymphocytes isolated from thymuses, spleens and stimulated lymph nodes of rats. Neither cisplatin nor the μ-hydroxo-bridged oligomers formed after hydrolysis significantly inhibited thymidine incorporation at pH 7.4. However, freshly neutralised *cis*-diaquodiammineplatinum(II) was a potent inhibitor of thymidine incorporation by all three lymphocyte populations.

In other experiments, rats were given either cisplatin or one of its hydrolysis products i.p. Cells isolated 17 hr later from the thymuses of all of these animals incorporated much less [^{3}H]thymidine into DNA *in vitro* than thymocytes from saline-injected control animals. None of the platinum species significantly affected either [^{3}H]uridine incorporation or the oxidation of [^{14}C]octanoate to $^{14}CO_2$ by the thymocytes.

Evidence for anation of di- and tri-nuclear μ-hydroxo-bridged platinum(II)amines by chloride has been obtained from spectrophotometric analyses and ^{195}Pt-NMR studies. Thiols also reacted with these platinum complexes at different rates (*cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+} \gg$ derived oligomers > cisplatin).

Various mechanisms for lymphoid suppression by cisplatin and its hydrolysis products are considered.

It is proposed that cisplatin and its μ-hydroxo-bridged derivatives owe their lymphotoxic activity primarily to their *in vivo* transformation to platinum species containing aquo ligands.

Cis-platinum(II)diamines have emerged during the past decade as a new class of potent antitumour/immunosuppressant agents [1–3], the prototype being cisplatin.‡ While structure–activity [4, 5] and structure–toxicity [6, 7] relationships have been investigated, the identity of the active/toxic species has not been clearly established. It is generally believed that the uncharged *cis*-dichlorodiamineplatinum(II) compounds (A; Fig. 1) are stable in the high chloride environment of the blood (103 mM Cl^-) but hydrolyse intracellularly (4 mM Cl^-) to a variety of products (Fig. 1). Calculations based on known equilibrium constants and p*Ka* values (see footnote to Fig. 1) indicate that at pH 7.4 significant amounts of hydrolysis products containing aquo ligands (B, D, E; Fig. 1) may exist inside cells. One of the principal hydrolysis products of cisplatin at neutral pH, *cis*-$[Pt(NH_3)_2(OH_2)(OH)]^+$, olates *in vitro* [10] to form a μ-hydroxo-bridged dimer and trimer (G, H; Fig. 1, amine = NH_3). This is a fundamental difference between the chemistry of *cis*- and *trans*-dichlorodiamineplatinum(II) compounds. Only the *cis* isomer which has antitumour and lymphotoxic activity can form these oligomers.

The contribution of the oligomers and other hydrolysis products to the overall lymphotoxic effects of *cis*-platinum(II) amines *in vivo* has now been examined with particular reference to cisplatin, using both *in vivo* and *in vitro* techniques.

MATERIALS AND METHODS

Chemicals

1,2-Diaminocyclohexane was obtained as 70:30 mixture of *trans*:*cis* isomers from Strem Chemicals (Newburyport, MA). *Cis*-dichlorodiamineplatinum(II) compounds were prepared by the method of Dhara [11]. *Cis*-diaquodiamineplatinum(II) species were prepared from these dichlorides by treatment with silver nitrate (1 Pt:2 Ag) in 50 mM HNO_3 or $HClO_4$ either by stirring overnight or by warming on a steambath (60°, 3 hr). The extent of reaction was monitored by determining the yield of silver chloride precipitate. All reactions were carried out in the dark. The filtrates, containing *cis*-$[Pt(amine)(OH_2)_2]^{2+}$, were stored in the dark at 4° (pH <2) until required. These solutions were neutralised with half volumes of 0.1 M $NaHCO_3$ immediately prior to biological use.

Dinuclear μ-hydroxo-bridged platinum(II)diamines were prepared by a reported method [10]. The trinuclear species $[Pt(NH_3)_2(OH)]_3(NO_3)_3$ was obtained in high yield by first preparing $\{[Pt(NH_3)_2(OH)]_3\}_2(SO_4)_3$ [12]. An aqueous solution of trimer sulphate was then passed down an anion exchange column (Dowex 1-X8, NO_3^- form) and the trimer nitrate was recovered in high yield but only if the solvent was removed at low temperatures

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‡ Cisplatin: *cis*-[Pt(II) $(NH_3)_2Cl_2$].

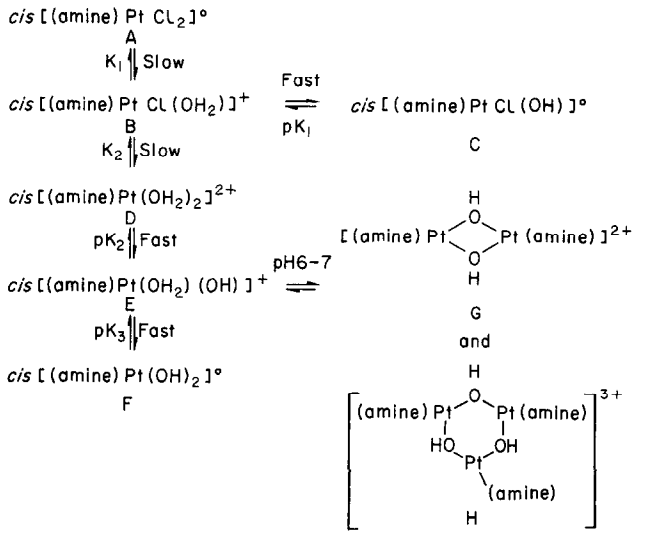

Fig. 1. Hydrolysis of *cis*-dichlorodiamineplatinum(II) complexes. Amine = 2 monodenate or 1 bidentate amine ligands. Amine = $(NH_3)_2$, $pK_1 \approx 7.1$; $pK_2 = 5.6$; $pK_3 = 7.3$ [9]. At 25°, $K_1 = 3.63 \times 10^{-3}$ M; $K_2 = 1.11 \times 10^{-4}$ M [8]. At 35°, $K_1 = 4.37 \times 10^{-3}$ M; $K_2 = 1.88 \times 10^{-4}$ M [8]. By extrapolation expected values at 37°: $K_1 = 4.52 \times 10^{-3}$ M; $K_2 = 2.03 \times 10^{-4}$ M. Amine = $NH_2CH_2CH_2NH_2$ $pK_1 = 7.4$; $pK_2 = 5.8$; $pK_3 = 7.6$ [9]. At 25°, $K_1 = 2.19 \times 10^{-3}$ M; $K_2 = 1.43 \times 10^{-4}$ M [9]. At 35°, $K_1 = 2.76 \times 10^{-3}$ M; $K_2 = 1.38 \times 10^{-4}$ M [9]. By extrapolation expected values at 37°: $K_1 = 2.87 \times 10^{-3}$ M; $K_2 = 1.37 \times 10^{-4}$M.

(≤25°)*. Compounds were characterised by elemental analyses, ^{195}Pt-NMR spectroscopy [13], conductance measurements on 10^{-3} M solutions in water, and X-ray powder diffraction patterns.

In vitro *experiments*

Cell preparations. Rat thymocytes were isolated as described [14]. Stimulated spleen cells and lymph node cells were similarly isolated from 'grafted' PVG × Lew F_1 hybrid rats, five days after inoculating a cell graft of PVG spleen cells into each rear paw [7].

Experimental procedure. Cell suspension (1 ml, 5–10 mg protein) were preincubated with platinum compounds (10–100 μM) in 1 ml Hank's 0.1 M phosphate buffer (1:1 v/v) over pH range 5–8 for 10 min at 37°, then labelled with [6-^{3}H]thymidine for 30 min [14].

In vivo *experiments*

Groups of 2 or 3 male Wistar rats (170–220 g) were injected i.p. with platinum(II)amines dissolved in 5% (w/v) glucose at the dosage 5–30 μmoles Pt/kg. Cyclophosphamide (10, 25 mg/kg) in 5% glucose and 5% glucose only were injected into other groups of rats at the same time to provide positive and negative controls respectively. Animals were sacrificed after various time intervals (3–48 hr). The thymuses, spleens, kidneys and stomachs were weighed and thymocytes isolated as described above.

The capacity of these cells to incorporate [^{3}H]thymidine or [5-^{3}H]uridine was then determined *in vitro*. They were also incubated for 15 min with [1-^{14}C]octanoate (0.1 μCi) in small stoppered flasks with a centre well containing a piece of filter paper soaked in 0.2 ml phenylethylamine to trap $^{14}CO_2$ [7].

Aliquots of cell suspensions were lysed with a Biuret reagent [15] for determining protein content. Isotope incorporation data were normalised with respect to both original thymus weights and the protein content of the thymocyte preparations, for each treated group.

Spectrophotometric analyses

Continuous scans in the 400–300 nm region of the visible spectrum were recorded for 1–3 mM solutions of the di- and tri-nuclear μ-hydroxo-bridged species, $[Pt(NH_3)_2(OH)]_2^{2+}$ and $[Pt(NH_3)_2(OH)]_3^{3+}$, at 37.0 ± 0.1° in 0.1 M Hepes–0.13 M NaCl (adjusted to pH 7.4 with Tris) using a Carey-118 u.v.-visible spectrophotometer with repetitive scan attachment.

^{195}Pt-*NMR spectroscopy*

^{195}Pt-NMR spectra of 0.03–1.5 M solutions of platinum compounds in water were recorded using a JEOL-9OFXQ spectrometer. Measurements were made at 19.2 MHz and chemical shifts were recorded as upfield resonances from an external standard (Na_2PtCl_6 = 19.2623492 MHz). Spectra were obtained using a 10 μsec pulse (90°) and a 15 msec acquisition time.

Platinum–thiol interactions

Aliphatic thiols were incubated with varying proportions of platinum complexes in 0.1 M sodium phosphate solutions (pH 5.9–7.9) at 37°. Residual thiols were measured colorimetrically at 450 nm after adding an Ellman reagent, DTNB† [16].

* Higher temperatures result in formation of significant quantities of dimer [10].

† Abbreviations used: DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); GSH, glutathione; NAC, *N*-acetyl cysteine; MPG, mercaptopropionyl glycine; Dach, 1,2-diaminocyclohexane.

Table 1. Effects of cisplatin, *cis*-diaquodiamineplatinum(II) compounds and their corresponding μ-hydroxo-bridged dimers on [^{3}H]thymidine incorporation by rat thymocytes* *in vitro*

Compound	Pt concn (μM)	% Inhibition at pH 5.8	6.4	6.9	7.4	7.9
Cis-$[Pt(NH_3)_2Cl_2]$	50				0	
	100				7	
Cis-$[Pt(NH_3)_2(OH_2)_2]^{2+}$	50				20	
	100	65	31	27	25	8
	200				77	
Cis-$[Pt(ipr)_2(OH_2)_2]^{2+}$	100	40	25	27	16	
Cis-$[Pt(Dach)(OH_2)_2]^{2+}$	100	98	85	75	63	
$[(H_3N)_2Pt(OH)]_2^{2+}$	50				0	
	100	11	−32	−27	−17	−49
$[(Dach)Pt(OH)]_2^{2+}$	100	−17	−19	−38	−5	−38
$[(H_3N)_2Pt(OH)]_3^{3+}$	50				7	
	100				12	

* Thymocytes derived from Wistar rats.

Results are means ±3%.

Relative incorporation values: controls pH 5.8 = 0.40; 6.4 = 0.68; 6.9 = 0.85; 7.4 = 1.0; 7.9 = 1.17.

pH controlled with 50 mM Na phosphate solutions.

Negative values represent increased thymidine incorporation, compared to controls (confirmed in several experiments).

ipr = isopropylamine.

RESULTS

In vitro *cell metabolism studies*

With Pt *compounds applied* in vitro. At pH 7.4 cisplatin itself does not affect [^{3}H]thymidine incorporation into isolated rat thymocytes (Table 1) but freshly neutralised solutions of *cis*-diaquodiamineplatinum (II) were potent inhibitors in these short term experiments.

Evidence that this was due to a direct reaction of the diaquo species with the cells, rather than an interaction with the thymine moeity [17] of the radioactive thymidine, was obtained as follows. Thymocytes were first incubated with *cis*-diaquoplatinum (II) amines for 10 min at 37°. Unreacted platinum species were then removed either by washing the cells copiously with fresh Hank's medium or by adding a 10-fold excess of *N*-acetyl penicillamine. Thymidine incorporation by these pre-drugged cells was then measured in the absence of platinum compounds. The data (not shown) indicated that removing the residual diaquo species did not restore thymidine incorporation to normal levels.

Cis-diaquodiamineplatinum (II) compounds are however likely to contain a mixture of species (A–H; Fig. 1) since they were exposed to chloride ions (130 mM) and phosphate buffers (100 mM) for 10 min before incubation with cells (15 min). The μ-hydroxo-bridged complexes ('oligomers'), which may not be formed in appreciable quantities from the *cis*-diaquo species either at the low platinum concentrations [9] or over the short duration of these experiments, did not inhibit [^{3}H]thymidine incorporation over the pH range 5.8–7.9 (Table 1). However, all three diaquo species ('monomers') were potent inhibitors of thymidine incorporation, the degree of inhibition decreasing dramatically as the pH of the incubation medium was raised. Thymidine incorporation into immunostimulated spleen and lymph node cells (from grafted rats) was also inhibited by *cis*-diaquodiamineplatinun (II) compounds but not by their derived di-μ-hydroxo-bridged compounds (Table 2). With these lymphoid cells, the effects of adjusting the extracellular pH were similar but less dramatic than those observed with thymocytes.

Table 2. Effects of some *cis*-platinum(II) amines on [^{3}H]thymidine incorporation into stimulated lymph-node and spleen cells from 'grafted' PVG × LEW rats *in vitro*

Compound (100 μM Pt)	% Inhibition: Lymph-node cells pH: 5.9	6.9	7.9	Spleen cells pH: 5.9	6.9	7.9
Cis-$[Pt(NH_3)_2(OH_2)_2]^{2+}$	40	22	21	29	22	20
$[Pt(NH_3)_2(OH)]_2^{2+}$	12	−6	−1	−5	6	16
Cis-$[Pt(Dach)(OH_2)_2]^{2+}$	95	89	93	87	87	84
$[Pt(Dach)(OH)]_2^{2+}$	−1	10	6	13	−12	10

Results are means ±3%.

pH controlled with 50 mM Na phosphate solutions.

Negative values represent increased thymidine incorporation, compared to controls (confirmed in several experiments).

Table 3. *In vivo* administration of platinum compounds to Wistar rats—effects on *in vitro* incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{14}C]octanoate metabolism by thymocytes (pH 7.4)*

Compounds		Σ Pt dose (μmoles/kg)	% Inhibition [^{3}H]thymidine (→DNA)	[^{3}H]uridine (→RNA)	[^{14}C]octanoate (→$^{14}CO_2$)
Glucose (5%)			0	0	0
Cis-$[Pt(NH_3)_2Cl_2]$		30	88	−19	13
		15	49	0	30
Cis-$[Pt(NH_3)_2(OH_2)_2]^{2+}$	—fresh	30	83	21	11
	—aged	30†	48	27	10
$[Pt(NH_3)_2(OH)]_2^{2+}$		30	45	20	20
		20	33	16	15
		30‡	51	−14	17
$[Pt(NH_3)_2(OH)]_3^{3+}$		15§	31	−16	30
Cis-[Pt(Dach) $(OH_2)_2]^{2+}$	—fresh	30	81	19	13
	—fresh	15	47	−9	7
	—aged	30†	48	10	8
$[Pt(Dach)(OH)]_2^{2+}$		45	58	−4	24
Cyclophosphamide		(25 mg/kg)	92	28	15
		(10 mg/kg)	78	33	0

* Cells isolated 17 hr after *in vivo* dosage with Pt.
† Solutions aged for 6 hr (pH 6.4, room temp.) before administration.
‡ These dosages were lethal to 8/18 rats.
§ Higher dosages were always lethal.

In all these brief *in vitro* experiments the μ-hydroxo-bridged species consistently *stimulated* [^{3}H]thymidine incorporation (Tables 1 and 2). The significance of this finding is unknown.

With Pt *compounds applied* in vivo. Both monomeric and oligomeric platinum (II) amines blocked [^{3}H]thymidine incorporation into thymocytes (measured *ex vivo*), when administered to rats 17 hr before isolating the thymocytes for *in vitro* studies (Table 3). This contrasts with the specific inhibition of thymidine incorporation by the diaquo species (D, E; Fig. 1) when these platinum (II) amines were applied *in vitro* (Tables 1 and 2).

The *in vitro* incorporation of [^{3}H]uridine into isolated thymocytes was much less affected by pretreatment of the rats with the same platinum complexes. This concurs with reports that low concentrations of cisplatin *in vitro* affect DNA synthesis to a greater extent than RNA synthesis [18, 19]. Pretreating rats with cycloposphamide likewise preferentially inhibited thymidine incorporation *ex vivo* (Table 3). *Ex vivo* oxidation of [^{14}C]octanoate to $^{14}CO_2$ by thymocytes was used to determine their viability/metabolic capacity in these particular experiments.

These combined *in vivo/in vitro* studies of the effect of cisplatin on rat thymus involution and thymus DNA synthesis showed that there was little effect on either thymus mass or *ex vivo* thymidine incorporation by isolated thymocytes until at least 3 hr after administering cisplatin intraperitoneally. None of the platinum compounds reduced the thymus mass by more than 30% 17 hr after administration.

When the potent diaquo species (containing either NH_3 or Dach) were aged in the dark at 20° in the absence of chloride at pH 6.4 for 6 hr before administering them to rats, their *in vivo* thymotoxic activity (monitored by subsequent [^{3}H]thymidine incorporation *in vitro*) was significantly less than that displayed by fresh solutions (Table 3). Using the relevant pK values given in Fig. 1, calculations indicate that the relative equilibrium percentages of species D, E and F (Fig. 1) formed after adjusting the pH of the diaquo species (stored in acid) to 6.4, would be 12.34, 77.86 and 9.80%, respectively (amine = NH_3). However, upon ageing, species E would have undergone oligomerisation to >80% dimer and trimer (G, H; Fig. 1). The reduced thymotoxicity of the diaquo species aged *in vitro* therefore most likely reflects formation of the much less lymphotoxic μ-hydroxo-bridged species and the inactive bis-hydroxo monomer (F; Fig. 1).

NMR studies

All of these platinum species are evidently transformed *in vivo* to thymotoxic agents. Since each hydrolysis product of cisplatin (Fig. 1) can be identified by the characteristic position of its ^{195}Pt nuclear magnetic resonance signal [13], the stability of various platinum(II) complexes in solution was investigated.

Cis-$[Pt(^{14}NH_3)_2(OH_2)_2](NO_3)_2$ exhibits a single well resolved quintet at +1591 ppm (upfield from the external reference, Na_2PtCl_6) in its ^{195}Pt-NMR spectrum at pH <4. However, at pH 6–7, the signal broadens and broad quintet signals were also observed for $[Pt(^{14}NH_3)_2(OH)]_2^{2+}$ (+1161 ppm) and $[Pt(^{14}NH_3)_2(OH)]_3^{3+}$ (+1518 ppm). At pH ≥7.5 an additional quintet resonance at +1041 ppm was observed and this upfield position is typical of μ-hydroxo-bridged platinum(II) species where considerable 'ring strain' is present [13].

When the di-μ-hydroxo-bridged ^{14}N compound (G; Fig. 1) was reacted with aqueous NaCl (0.5 M) the ^{195}Pt-NMR signal at +1161 ppm was replaced by another signal at +1842 ppm after 24 hr (pH = 7.4, 0.1 M Hepes buffer, 30°, [Pt] = 70 mM). This second

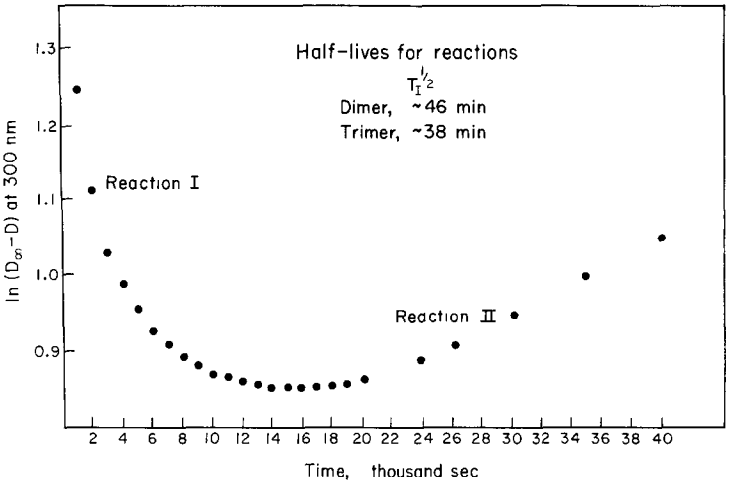

Fig. 2. Change in the absorbance spectrum at 300 nm of $[(H_3N)_2Pt(OH)]_3(NO_3)_3$, (1 mM Pt), in 0.13 M NaCl, pH = 7.4 (0.1 M Hepes buffer), at 37.0° (±0.1°) with time.

resonance corresponds to the position of *cis*-$[Pt(^{14}NH_3)_2(OH_2)Cl](NO_3)$. A similar result has now been confirmed with $[^{15}N]\mu$-hydroxo-bridged species (dimer = 40 mM Pt in 0.15 M NaCl; trimer = 80 mM Pt in 0.4 M NaCl, in either 0.1 M Hepes buffer or without buffer) [13].

Spectrophotometric analyses

Anation of oligomers by chloride. The rate of reaction of the dimer and trimer (amine = NH_3) with chloride at 37° was determined spectrophotometrically. The change in absorbance with time at 300 nm is shown in Fig. 2 for the μ-hydroxo-bridged trimer in excess NaCl (pH 7.4, 37°). The graphical data ($T \geqslant 24 \times 10^3$ sec) represents a pseudo-first order reaction since the plot is linear. A ^{195}Pt-NMR spectrum recorded 24 hr after incubating the dimer with excess NaCl at 30° showed a small signal due to cisplatin, formed as a by-product to *cis*-$[Pt(NH_3)_2Cl(OH)]$. From a large scale reacton, cisplatin (which was identified by its infra red spectrum) precipitated after five days [20]. The second half of the curve in Fig. 2 ($T \geqslant 24 \times 10^3$ sec) is therefore likely to represent the formation of *cis*-$[Pt(NH_3)_2Cl_2]$. The first section of the curve ($T < 24 \times 10^3$ sec) in Fig. 2 must represent the formation of *cis*-$[Pt(NH_3)_2Cl(OH)]$ since this complex was unambiguously identified as the major product of the reaction after 24 hr by ^{195}Pt-NMR spectroscopy. The absorbance change shown in Fig. 2 indicates that the half-life for the anation by one equivalent of Cl^- is less than 1 hr for the trimer.

Reaction of cis*-platinum(II) diamine complexes with thiols.* The decomposition of the dimer and the trimer (amine = NH_3) in the presence of aliphatic thiols at 37° was monitored colorimetrically by thiol consumption using an Ellman reagent, DTNB (Table 4). A 10-fold excess of *cis*-diaquodiamineplatinum(II) reacted rapidly even at room temperature (22°) with various thiols (GSH, MPG, NAC) that are relatively stable in phosphate buffered solutions pH 5.9–7.9, thereby destroying their capacity to react with DTNB. However, this same excess of the diaquo species reacted incompletely with other thiols, e.g. 2-mercaptoethanol and cysteamine at 37°. Cisplatin reacted very slowly at 37° with GSH ($t_{1/10}$ *ca.* 80 min). The oligomers formed from cisplatin were only slightly more reactive ($t_{1/10}$ *ca.* 40 min) with GSH (Table 4).

DISCUSSION

Concerning the dichlorides

Neither cisplatin (Table 1) nor *cis*-$[Pt(Dach)Cl_2]$ (data not shown) are immediately toxic *in vitro* to lymphocytes, but both compounds are lymphotoxic *in vivo* (Table 3). Their mechanism of bio-activation is likely to involve cellular uptake as uncharged

Table 4. Relative reactivity of cisplatin and its hydrolysis products with glutathione (pH 7.4, 37°)

	Percentage residual thiol at various times (min)				
	10	20	40	60	80
No Pt	100	95	86	76	66
Cisplatin	96	92	66	32	02
$[(NH_3)_2Pt(H_2O)_2]^{2+}$	0	0	0	0	0
'Dimer'	77	59	03	0	0
'Trimer'	84	76	26	02	0

[GSH] = 0.4 mM; [Pt complexes] = 4.4 mM in 83 mM Na phosphate, pH 7.4.

complexes via passive rather than active transport [2]. Much of the complex is excreted and the remainder may slowly hydrolyse in the low chloride environment within most cells. The resulting hydrolysis products contain reactive aquo ligands (B, D, E; Fig. 1) and hence could rapidly interact with DNA [21] and other molecules containing sulphur (Table 4), nitrogen or oxygen. They could also undergo intracellular oligomerisation to the dimer and trimer (G, H; Fig. 1). This latter reaction is much slower (for cisplatin, $t_{1/2} \sim 10$ min at 37°) but may become significant in target tissues which concentrate platinum complexes (thymus, kidney, liver). These oligomeric complexes also react slowly with thiols (Table 4) and can apparently also interact with isolated DNA [22].

Concerning the oligomeric hydrolysis products

The μ-hydroxo-bridged platinum(II)amines must be biologically activated in the rat (over 17 hr) since they were lymphotoxic *in vivo* (Table 3) but showed no lymphoid toxicity *in vitro* in short term experiments (Tables 1 and 2).

^{195}Pt-NMR studies indicate that the di- and tri-μ-hydroxo-bridged compounds (G, H; Fig. 1) anate in 0.13 M NaCl at pH 7.4 well within 24 hr to form a monochloroplatinum(II) species. These studies do not distinguish between *cis*-$[Pt(NH_3)_2Cl(OH_2)]^+$ and *cis*-$[Pt(NH_3)_2Cl(OH)]^0$. Calculations using pK 7.1 [23] show that at equilibrium the ratio of these species at pH 7.4 would be approximately 1:2. Therefore, one possible explanation for the lymphotoxic activity of μ-hydroxo-bridged oliogomers (amine = NH_3) is that they slowly break down in the extracellular medium (*in vivo*) to monomeric monochloro-platinum species (B, C; Fig. 1). The *uncharged* monochloromonohydroxo form could then be taken up by target cell and converted to *cis*-$[Pt(NH_3)_2Cl(OH_2)]^+$ and *cis*-$[Pt(NH_3)_2\ (OH_2)\ (OH)]^+$ which are known to react rapidly with isolated DNA [21].

A further possibility is that extracellular oligomers react with ectopic ligands (see below) on the cell surface. Slow interaction of the oligomers with thiols at 37° has been demonstrated (Table 4).

Concerning monomeric hydrolysis products

Uncharged complexes, *cis*-$[Pt(NH_3)_2X]$, where X is one or two anionic ligands which are readily displaced by H_2O (e.g. SO_4^{2-}, NO_3^-), will exhibit similar lymphotoxic effects *in vivo* to *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$.

In vitro studies have shown that *cis*-diaquodiamineplatinum(II) species are lymphotoxic when applied extracellularly (Tables 1 and 2). These experiments involved brief exposure to 0.13 M chloride at 37° and so some reversion to chloride-containing platinum species would occur. However, their lymphotoxic properties cannot be due to extracellular formation of dichlorides since these are not lymphotoxic *in vitro*.

Raising the pH of the extracellular *in vitro* medium apparently reduces the toxicity of *cis*-diaquo species (Tables 1 and 2). Hence *cis*-$[Pt(amine)\ (OH)_2]^0$, which would be more abundant at the higher pHs and as an uncharged complex might be expected to be taken up by cells, is probably not a primary extracellular toxin.

Formation of an extracellular lymphotoxic species from the *cis*-diaquodiamineplatinum(II) complexes by oligomerisation is also unlikely since the oligomers exhibited no lymphotoxic activity *in vitro*.

No evidence was obtained for an interaction between *cis*-diaquodiamineplatinum(II) species and phosphate buffer*. Furthermore, when *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ is reacted in excess NaCl/phosphate buffer pH 6.8, four platinum products can be detected by ^{195}Pt-NMR spectroscopy after 2 hr [13]. Three of these compounds (A, G, H; Fig. 1) are inactive *in vitro* (Table 1) while the fourth product is *cis*-$[Pt(NH_3)_2Cl(OH)]$.

There are seemingly, therefore, only two possible mechanisms whereby *cis*-diaquo species are lymphotoxic *in vitro*: (1) cellular uptake of uncharged *cis*-$[Pt(NH_3)_2Cl(OH)]$ followed by intracellular protonation/hydrolysis to complexes (B, D, E; Fig. 1) containing reactive aquo ligands; and (2) extracellular cationic complexes containing aquo ligands might be lymphotoxic *per se* either after (a) transport (as cations) across cell membranes and subsequent reaction with intracellular DNA, or (b) reaction with ectopic ligands, e.g. DNA associated with surfaces of cells [24–26].

Final comments

All these considerations, together with studies in acellular systems [21, 27] suggest that at least three cationic aquo-complexes, *cis*-$[Pt(amine)\ (OH_2)Cl]^+$, *cis*-$[Pt(amine)\ (OH_2)\ (OH)]^+$ and *cis*-$[Pt(amine)\ (OH_2)_2]^{2+}$ are potential reactive species likely to bind to intracellular DNA. A non-specific cytotoxicity may arise through interactions of aquoplatinum species with intracellular thiols.

Cis-platinum(II)amine complexes containing sulphate and other weak ligands hydrolyse rapidly and their lymphotoxic properties would be expected to resemble those of the diaquo species, i.e. greater toxicity (and antitumour activity) compared with their dichloro analogues. Similarly, compounds containing ligands of similar coordinating ability to Cl^-, e.g. malonate, should exhibit less extracellular reactivity than diaquo species. Thus the non-amine ligand will largely determine the relative intra/extracellular reactivity of *cis*-platinum(II)amines. Variations in intracellular pH should also affect the susceptibility of different cell populations; a lower pH favouring the more reactive platinum species containing aquo ligands (Fig. 1).

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* The only product isolated with excess phosphate (pH 7.4) was the μ-hydroxo-bridged dimer, with phosphate as a counter ion.

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