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Platinum Drugs: Combined Anti-lymphoproliferative and Nephrotoxicity Assay in Rats

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Summary. *A 4-day drug schedule was used to explore the efficacy and simultaneous toxicity of cisplatin and 30 other platinum (II) amines given IP to PVG×Lew F_1 hybrid rats at cumulative doses of 10–300 μmol/kg. Toxic effects monitored were stomach enlargement, kidney hypertrophy with tubular necrosis and proteinuria, evident visceral mucin, and lymphoid involution (thymus, spleen). Immunosuppressive effects were monitored as inhibition of the lymph node hypertrophy induced by grafting PVG spleen cells into each paw of F_1 hybrids. No significant activity/toxicity was observed with 'platinum-(pyrimidine) blues'. N-alkyl derivatives of cisplatin were less active/toxic and some had no immunosuppressant effect, though they are reported as effective antitumour agents (in mice). μ-Hydroxobridged aminoplatinum (II) dimers were highly toxic, effective immunosuppressants and their toxicity profiles were distinct from the dihalo or diaquo diaminoplatinum species.*

1,2-Diaminocyclohexane platinum derivatives showed a wide range of potency, all being much less nephrotoxic than cisplatin.

Introduction

Am(m)inoplatinum (II) complexes have attracted considerable attention as potential anti-tumour agents [14, 19, 21, 23]. Initial screening for chemotherapeutic activity has been usually carried out in mice with L1210 or sarcoma 180 tumour burdens. Inevitably, compounds are selected that not only may be potential anti-leukemic agents but might also compromise protective lymphoproliferation, e.g., that associated with a tumour-directed immune response. In another context, it would be useful to know whether a lymphodepressant drug selected by these mouse screens can be sufficiently specific to be considered as a potential immunosuppressive drug, e.g., to treat auto-intolerance, rheumatoid arthritis.

We have explored the possible utility of conducting preliminary bioassays in F_1 hybrid rats, in which a drug-sensitive (local) graft-versus-host response (GvHR) is readily induced [4] to measure the following activities simultaneously:

A) Anti-lymphoproliferative, i.e., suppression of the induced GvHR (= lymph node and splenic hypertrophy);

B) Lymphodepressant, i.e., causing thymus (and splenic) involution;

C) Nephrotoxicity, i.e., proteinuria and shedding of renal (tubular) enzymes into the urine, together with morphological changes in fixed kidney sections;

D) Gross toxicity reflected in weight loss, alopecia, diarrhoea or mortality.

The results show that it is possible to (i) rapidly identify compounds with unacceptable toxicity, particularly towards the kidneys; (ii) explore a structure-activity, *and* a structure-toxicity, relationship in one and the same series of experiments, and (iii) determine whether or not an adjunct therapy, given to counteract the nephrotoxicity, also interferes with the cytostatic (potential therapeutic) effects of a given platinum drug.

Special emphasis was given to studying the in vivo properties of 1,2-diaminocyclohexanplatinum (II) and platinum-pyrimidine complexes. The latter so-called platinum blues are highly water-soluble derivatives of Pt(II), formed by removing the

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Abbreviations used in this paper: Cisplatin or *cis*-DDP, *cis*-diamminodichloroplatinum (II); BSS, balanced salt solution; DACH, 1,2-diaminocyclohexane; GvHR, local graft-versus-host reaction; en, ethylenediamine; SDS, sodium dodecylsulphate; ND, not determined

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chlorine from *cis*-$Pt(NH_3)_2Cl_2$ (DDP, cisplatin) and reacting the (hydrolysis) product with various pyrimidines (or amides) to obtain oligomeric products. Some of these are excellent stains for detecting nucleic acids under electron microscopy [1–3], but their in vivo properties have not been well characterized.

Materials and Methods

Platinum Chemicals

Platinum blues were supplied by Drs. A. Sargeson (Research School of Chemistry, The Australian National University) and M. J. Cleare (Johnson Matthey Research Laboratories, Sonning, Commons, Reading, UK). Hydrolysis products of various dichlorodiamineplatinum complexes were prepared by overnight treatment (20 ± 2° C) with the stoichiometric amounts of $AgClO_4$ or $AgNO_3$ in 50-m*M* solutions of $HClO_4$ or HNO_3, with magnetic stirring. The reactions were allowed to proceed until the theoretical yield of AgCl was precipitated. The products were characterized by their conductivities and stored in the dark at 4° C until tested. Immediately before use these solutions were neutralized with 100 m*M* Na_2HPO_4 or 100 m*M* $NaHCO_3$ and diluted with 0.3 *M* glucose or 0.15 *M* NaCl. Hydroxobridged platinum (II) complexes were prepared by the general procedure used with DDP [8] and characterized by IR spectroscopy, elemental analysis, proton-NMR spectroscopy and X-ray powder diffraction patterns (Fairlie and Broomhead, to be published).

Cyclohexane-1,2, diamine (Strem Chemicals, Newbury Port, MA 01950) was used as a 70 : 30 *trans : cis* mixture and reacted with K_2PtCl_4 in water to form *trans : cis* 1,2 diaminocyclohexanePtCl_2. Other cyclohexane-1,2 diamine complexes of platinum were kindly donated by Drs. A. Khan (Dallas) and G. Gale (Charleston, SC). Further platinum compounds were obtained from Drs. I. A. Roos (Adelaide) and S. E. Livingstone (Sydney).

All compounds in solution were protected from light.

Animals

All rats were bred in the SPF facility, Animal Breeding Establishment, John Curtin School of Medical Research.

Preliminary toxicity studies (to establish tolerated doses) were carried out in both Wistar-derived (Canberra outbred) and PVG × Lew F_1 rats. The GvHR was established in F_1 PVG × Lew rats with spleen cells from the parental PVG (Hooded) strain.

Procedures

Spleen cells ($1-5 \times 10^7$ leucocytes) in 0.1–0.2 ml Hank's medium were injected into front and rear paws of F_1 hybrids on day 0 [4]. Cells from male donors were injected to both male and female recipients. Cells from female donors were only given to female recipients.

In a few experiments, the direct cytotoxic/lymphosuppressant activity of test compounds towards the grafted cells in vitro was determined by incubating an aliquot of the spleen cells in Hank's medium: 0.1 *M* Hepes buffer, pH 7.4 (4 : 1 v/v) at 37° C for 15–25 min with the test compound (< 100 μ*M*) with occasional gentle shaking. Spleen cells were then re-isolated, washed once with Hank's medium, resuspended in final volume of Hank's medium (< 1 ml) for injection into the right paws of at least three F_1 hybrid (host) rats. An equal number of incubated, but undrugged, spleen cells was injected into the corresponding left paws of the same animals. Cell preparations with viability < 85% (trypan blue exclusion) were discarded.

The local GvHR was determined by measuring the hypertrophy of the draining lymph nodes (popliteal plus lumbar/para-aortic for each rear paw; elbow node only for front paw). Within each animal it was possible to compare the separate response to two graft populations (i.e., one having been drug-treated in vitro, the other being undrugged).

Drugs and test compounds were administered IP to animals as solutions prepared directly in 0.15 *M* NaCl or 0.3 *M* glucose or first dissolved in DMF, then diluted with 99 vol 0.1% Tween 20 in saline or glucose. Usually the first dose was given on the first day after initiation of the GvHR (day +1) and once each day thereafter for a total of four daily doses. Controls received injections of vehicle only. Animals were weighed on days +1, +3, and +5 and sacrificed by decapitation (day +5). The following organs from each rat were then weighed: kidneys (× 2), thymus, spleen, each popliteal, lumbar (para-aortic), and elbow lymph node. The kidneys were immediately sliced in two (saggital section) and fixed in 2% freshly prepared glutaraldehyde solution. Routine histological sections were examined after staining with haematoxylin-eosin. Electron microscopy studies were conducted with sections fixed first in glutaraldehyde then in 1% OsO_4, and if necessary further stained with uranyl acetate and lead hydroxide.

Urine collections were made by placing each animal individually in a metabolism cage with free access to water but without food. Urine samples were collected overnight at room temperature, then acidified with an equal volume of 10% (w/v) trichloracetic acid (TCA). The precipitate was washed once with 5% TCA, dissolved in 2.5 ml 0.5 *N* NaOH and suitable aliquots (0.1–0.5 ml) taken in duplicate to determine total protein with 1.5 ml biuret reagent [10]. (Corrections for turbidity, etc., were made by taking a similar aliquot of the alkaline protein solution and diluting it with 1.5 ml biuret reagent minus copper.)

Results

Diaminoplatinum (II) Dihalides

Table 1 compares the effects of the reference platinum drug *cis*-DDP as an immunosuppressant/antilymphoproliferative agent when given IP at various times and in single or divided dosages. The most satisfactory dose regimen was found to be a cumulative dosage of 9 mg/kg (30 μmol/kg) given in four divided doses, beginning on the first day after grafting (group E). There was a high ratio of survivors (18/22) to day 10, when observations ceased. The criteria for accepting this dose regimen were low morbidity (weight loss, incidence of diarrhoea) and highly effective suppression of the lymphoid hypertrophy (nodes, spleen) associated with the GvHR. This same dose regimen was used in all further experiments.

DDP had no activity when given orally. However, effective IP doses of DDP invariably induced significant thymus involution ($P < 0.01$), kidney swelling

Table 1. Effect of *cis*-DDP[a], given at various times, on (i) a local GvHR and (ii) kidney and proteinuria in F_1 hybrid rats

Group	Σ Dose[b] (mg/kg)	Day[c]	Δ Body[d] weight (g)	Thymus[e] (%)	Spleen[f]/ body weight	Kidney (2)[g]/ body weight	Kidney[h] damage	Urinary[i] protein	Lymph node hypertrophy[j]	
									L + P	E
A	0	–	−11 ± 2	100	4.0 ± 0.3	7.5 ± 0.2	0	0	100	100
	6	−3 only	−50 ± 2	13	2.5 ± 0.2	11.9 ± 0.3	2+	2+	23	15
B	9	−3, −2, −1	−44 ± 3	14	2.4 ± 0.1	13.1 ± 0.7	2+	+	15	9
	6		−17 ± 2	27	3.2 ± 0.1	10.1 ± 0.7	2+	0	58	37
	3		−12 ± 2	86	3.8 ± 0.2	6.8 ± 0.2	±	ND	87	75
C	6	−1 only	−37 ± 2	16	2.4 ± 0.3	12.8 ± 0.5	+	+	27	14
D	9	+1 only	−23 ± 3	22	1.4 ± 0.1	11.5 ± 0.7	2+	2+	11	6
	9	+3 only	−17 ± 1	61	2.6 ± 0.1	9.7 ± 0.2	3+	0	67	62
E	9	+1, 2, 3, 4	−23 ± 3	26	2.0 ± 0.1	10.7 ± 0.1	3+	3+	37	26
	6		−18 ± 3	32	2.4 ± 0.1	9.5 ± 0.2	2+	+	61	53
	3		−14 ± 2	68	3.0 ± 0.2	8.8 ± 0.2	+	ND	78	69

[a] DDP was administered IP in 0.15 *M* NaCl on days indicated. Controls received NaCl only. Mininum number of animals per group, 3. Data are means (± SE) of parameters measured on the 5th day after grafting of parental spleen cells into all four paws (Day 0)
[b] Accumulated dosage
[c] Equal divided doses on days specified. Day 0, day of grafting
[d] From first day of treatment to day +5; includes effect of overnight fasting (day 4/5) during period of urine collection
[e] Thymus weights, as % of controls ($n \geq 3$), varied considerably (0.6–3.0 mg/g body weight) according to age in each experiment. In each experiment all animals had a range of birthdates ≤ 6 days and were > 100 days old
[f] $\times 10^{-3}$, i.e., mg/100 g. Spleen weights of the GvH controls were significantly higher (approx. 85%) than in nongrafted, undrugged, controls
[g] Kidney weights (2) of ungrafted controls were > 90, < 100% those of grafted animals (extreme range was 6.5–8.5 mg/kg body weight, $n = 90$; weights $\times 10^{-3}$ mg/100 g)
[h] Damage to kidney tubules, determined from histological preparations; 3+ = 50%–60%; 2+ = 30%–40%; 1+ = 10%–25% cellular lysis in proximal tubules
[i] From urine collection (16 h) immediately before sacrifice on day 5. Score 0 ≤ 3 mg, + = 3–10 mg, 2+ = 10–20 mg, 3+ = > 20 mg protein shed per rat by at least 4/6 rats in the group
[j] Compared with controls (n = L). L, lumbar; P, popliteal; E, elbow nodes, measured individually from each animal (i.e., 2/animal). Lymph node hypertrophy in control (undrugged) animals with GvH reaction was approximately 10-fold, i.e., L + P nodes weighed in excess of 180 mg, as against 15–20 mg in littermates not receiving graft

($P < 0.02$), and shedding of protein into the urine ($P < 0.05$; Student's *t*-test with Bessel's corrections for small samples [16]). This nephrotoxicity was minimised (> 80%) when particle-free extracts of rat kidney (i.e., cytosol) were co-injected with *cis*-DDP, but the lymphosuppressive activity was considerably impaired (data not shown).

Another side effect of DDP observed frequently was the non-expulsion of food from the stomach into the small intestine (Fig. 1). Consequently, many of the animals may have lost weight due to inanition, even though their stomachs were packed with food.

Table 2A shows the specificity of *cis*-DDP as an inhibitor of the GvHR and in causing renal damage (hypertrophy, proteinuria). The *trans*-isomer (compound 3), the *cis*-iodide (compound 5), the platinum II tetrammine (compound 4) and the platinum IV compound $(NH_4)_2PtCl_6$ (data not shown) were all inactive. Some activity/toxicity was shown by K_2PtCl_4 (compound 1). Substitution of primary alkyl amines for the ammonia in DDP while retaining the chloro groups (compounds 6, 7) reduced both the lymphoid and renal toxicity. Replacing the ammine ligands in DDP with the sulphur and nitrogen of L-methionine (compound 8) abolished both the lymphosuppression and the nephrotoxicity (Table 2A).

Immediate Hydrolysis Products of Diaminoplatinum (II) Dihalides (Table 2B)

A series of diaminodiaquoplatinum (II) cations (compounds 9–14, monomers) were prepared by reacting diaminoplatinum (II) dihalides with $AgNO_3$. They were administered to rats in a chloride-free medium to avoid reversion in vitro. The cations prepared from *trans*-DDP and Met.$PtCl_2$ were inactive. The hydrolysis products prepared from *cis*-diamines were much more soluble and rather more toxic/potent than their parent dihalides.

All hydrolysis products tested induced food retention in the stomach (as in Fig. 1). The diaquo

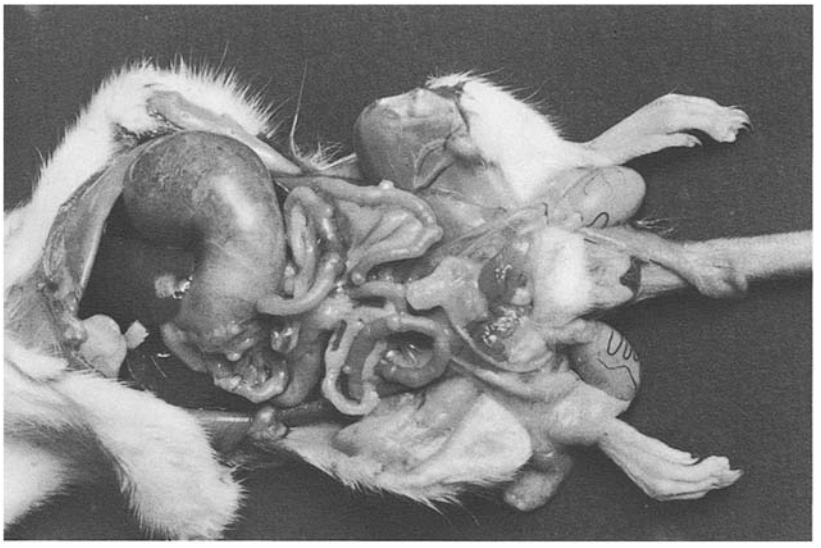

Fig. 1. Dissected rat previously treated with *cis*-diaminodiaquoplatinum (II) showing the bloated stomach and empty intestine

Table 2. Effect of (A) some inorganic Pt compounds and (B) some $(RNH_2)_2PtCl_2$ compounds and their hydrolysis products on GvHR, kidney size, and proteinuria in F_1 hybrid rats[a]

Compound	Dose Pt[b] (μ*M*/kg)	Body weight (g)	Thymus %	Spleen/ body weight	Kidneys (2)/ body weight	Kidney damage	Urinary protein	% Lymph node hypertrophy	
								L+P	E
A									
0 None	–	−05 ± 1	100	3.6 ± 0.2	7.5 ± 0.2	0	0	100	100
1 K_2PtCl_4	60	−10 ± 1	62	3.3 ± 0.1	10.1 ± 0.1	2+	2+	61	ND
2 *cis*-$[(NH_3)_2PtCl_2]$	30	−23 ± 3	26	2.0 ± 0.1	10.7 ± 0.1	3+	3+	37	26
3 *trans*-$[(NH_3)_2PtCl_2]$	30	−10 ± 4	109	3.1 ± 0.1	7.5 ± 0.4	0+	0+	84	82
4 $[(NH_3)_4Pt(II)]Cl_2$	27	−09 ± 2	87	3.7 ± 0.1	8.2 ± 0.2	0+	0+	83	81
5[c] *cis*-$[(NH_3)_2PtI_2]$	30	−07 ± 1	100	3.5 ± 0.2	7.3 ± 0.1	0	0	86	ND
6 $[(i.C_3H_7NH_2)_2PtCl_2]$	100	−13 ± 1	76	3.0 ± 0.2	7.5 ± 0.1	0	0	97	84
7 $[en.PtCl_2]$	30	−12 ± 2	67	3.1 ± 0.1	8.0 ± 0.6	0	0	84	96
$[en.PtCl_2]$	100	−23 ± 2	32	1.7 ± 0.1	10.0 ± 0.1	2+	2+	30	ND
8 $[Met.PtCl_2]$	100	−11 ± 2	93	3.6 ± 0.1	7.2 ± 0.1	0	0	90	ND
B[d, e]									
9 *cis*-$[(NH_3)_2PtCl(H_2O)]^+$	30	−23 ± 3	38	1.5 ± 0.2	10.5 ± 0.3	2+	2+	39	ND
10 *cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$	30	−22 ± 2	20	2.1 ± 0.2	10.8 ± 0.3	3+	3+	35	18
11 *trans*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$	30	−10 ± 1	95	3.3 ± 0.3	7.8 ± 0.2	0+	0+	96	69
12 *cis*-$[(i.C_3H_7NH_2)_2Pt(H_2O)_2]^{2+}$	100	−19 ± 2	30	2.2 ± 0.1	8.0 ± 0.1	0+	0+	75	ND
13 $[en.Pt(H_2O)_2]^{2+}$	30	−24 ± 1	40	3.7 ± 0.3	10.5 ± 0.3	2+	2+	70	ND
14 $[Met.Pt(H_2O)_2]^{2+}$	100	−07 ± 1	101	3.7 ± 0.3	7.5 ± 0.1	0	0	87	ND
15 Dimer of 10	30[f]	−16 ± 1	20	1.5 ± 0.1	10.5 ± 0.2	3+	3+	20	ND
	20	−11 ± 1	55	2.2 ± 0.2	9.4 ± 0.1	3+	3+	54	ND
	10	−10 ± 2	74	2.8 ± 0.2	9.3 ± 0.5	2+	3+	84	ND
16 Dimer of 12	30	−10 ± 1	40	2.8 ± 0.2	7.9 ± 0.1	0+	0	80	ND
17 Dimer of 13	30	−21 ± 1	28	1.6 ± 0.1	10.8 ± 0.5	3+	3+	43	ND
18[g] *N*-acetyl-L-cysteine + 10	30	−09 ± 1	49	3.0 ± 0.2	7.3 ± 0.1	0+	0+	77	53

[a] All compounds given in four divided doses IP on days 1–4 inclusive (day 0, day of graft). See Table 1 for footnotes and other details. Values given are means ± SE

[b] Total Pt. This represents 2 × μmol dimer/kg

[c] Administered in 0.15 NaI. This compound was inactive when given orally (60 μmol/kg)

[d] Tested as nitrates and administered in 0.3 *M*glucose. Similar results were obtained with perchlorates. Almost identical data were obtained when these cations were administered in 0.15 *M* NaCl

[e] Higher doses caused significant mortality

[f] This dosage was lethal in 5/12 rats. Data are from survivors. Dead animals exhibited delayed *rigor mortis*

[g] 30 μmol $[(NH_3)_2Pt(H_2O)_2](NO_3)_2$/kg together with 600 μmol *N*-acetyl cysteine/kg in four divided (daily) doses

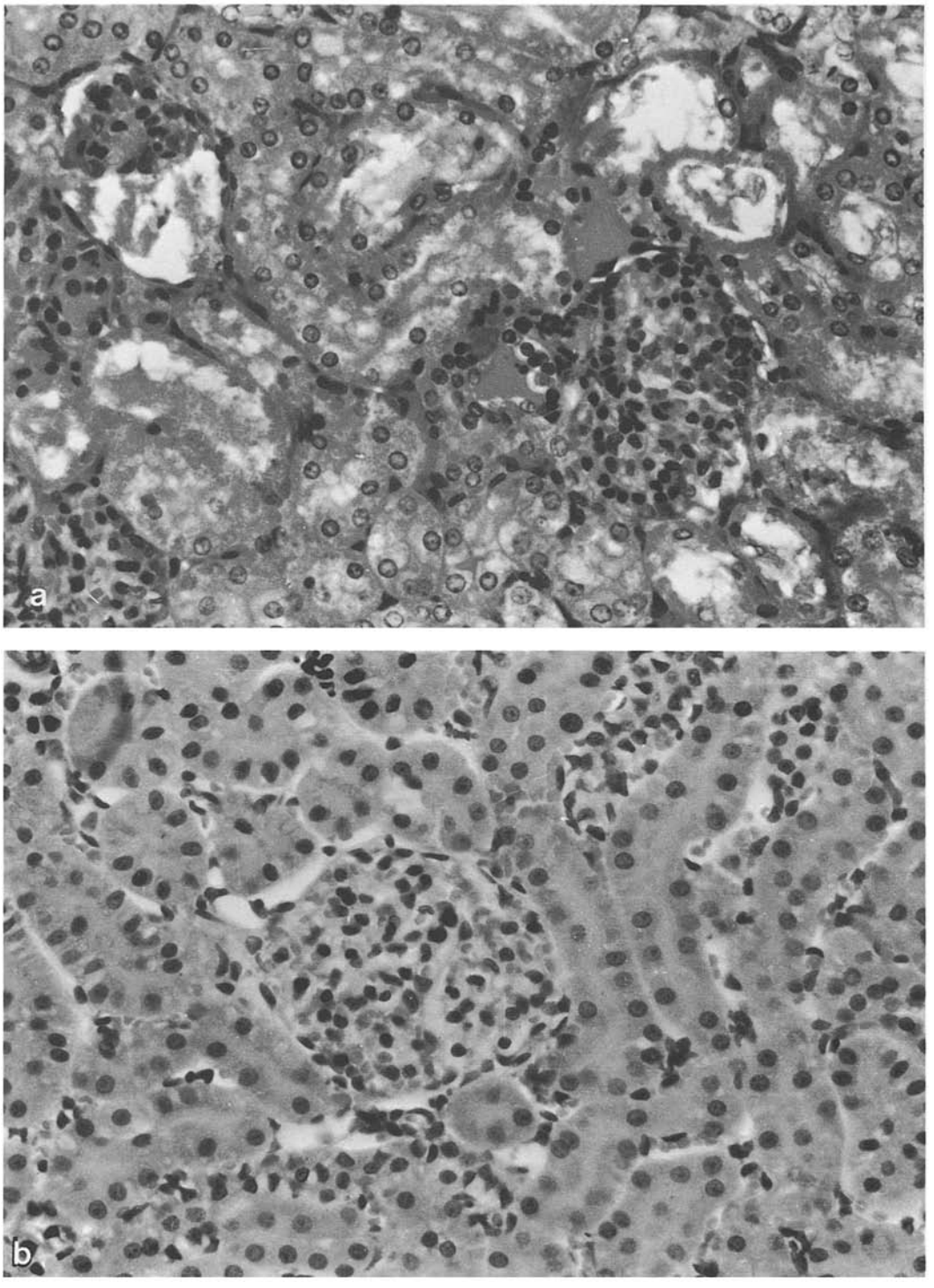

Fig. 2a and b. Photomicrographs showing the damage to the kidney tubules after *cis*-diaminodiaquoplatinum (II) administration (**a**); however, damage could be prevented by the co-injection of *N*-acetyl-L-cysteine (**b**). × 1250

species also caused some degree of peritoneal irritation, most notably the presence of a very slimy mucin in the visceral cavity. This was not analysed but may be of bacterial origin.

Co-injection of *N*-acetylcysteine (20 equivalents) with *cis*$[(NH_3)_2Pt(H_2O)_2]^{2+}$ reduced its nephrotoxicity (Fig. 2), prevented mortality, and abolished both the stomach bloat and production of visceral mucin.

However, the immunosuppressive effects of this cation on the lymph node proliferation and the thymus mass were reduced differentially (by the acetylcysteine).

Oligomeric Products Derived from Diaminodiaquoplatinum (II) Cations

The μ-hydroxobridged dimer $[(NH_3)_2Pt(OH)_2Pt(NH_3)_2]^{2+}$, compound 15, was more toxic than the monomer $[(NH_3)_2Pt(H_2O)_2]^{2+}$, compound 10, after both repeated low dosage (Table 2B) and single doses (Table 3). After single high doses, animals injected with this dimer died within 24 h; whereas animals given the same dose of either diaquo- or dichloro-diamminoplatinum (II) survived at least 3 days (when observations were terminated). Animals killed by the dimer showed remarkable flaccidity suggesting impaired *rigor mortis*.

This dimer (compound 15) caused considerably more nephrotoxicty (renal hypertrophy, proteinuria)

Table 3. Comparison of toxicities of DDP and its hydrolysis products after both single and repeated dosage in F_1 (nongrafted) female PVG × Lew rats starved for 24 h after final dose

Compound[a]		Dose Pt (μ*M*/kg)	Thymus (%)	Spleen/ body weight	Kidneys (2)/ body weight	Kidney damage	Urinary protein	Stomach[b] (%)
A[c]	None	–	100	2.0 ± 0.1	6.9 ± 0.1	0	0	100
	(2) *cis*-$[(NH_3)_2PtCl_2]$	7.5 × 1	115	2.0 ± 0.1	7.2 ± 0.3	+	+	ND
		× 2	88	1.9 ± 0.1	7.8 ± 0.1	2+	+	159
		× 3	62	1.6 ± 0.1	7.8 ± 0.1	3+	2+	225
	(15) $[(NH_3)_2Pt(OH)_2Pt(NH_3)_2]^{2+}$	7.5 × 1	95	1.9 ± 0.1	7.9 ± 0.3	+	+	ND
		× 2	103	1.9 ± 0.1	7.9 ± 0.2	+	+	133
		× 3	73	1.5 ± 0.1	9.0 ± 0.2	3+	2+	156
B	(2) *cis*-$[(NH_3)_2PtCl_2]$	30.0 × 1	40	1.7 ± 0.1	8.1 ± 0.1	3+	+	533
	(10) *cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$	22.5 × 1	39	1.5 ± 0.1	8.3 ± 0.1	3+	2+	417
		30.0 × 1	26	1.6 ± 0.1	8.2 ± 0.1	3+	2+	399
	(15) $[(NH_3)_2Pt(OH)_2Pt(NH_3)_2]^{2+}$	15.0 × 1	72	1.8 ± 0.1	8.6 ± 0.1	3+	+	158
		22.5 × 1[d]	105	2.0 ± 0.1	8.7 ± 0.1	3+	Anuric	125
		30.0 × 1[d]	86	1.8 ± 0.1	9.5 ± 0.2	3+	Anuric	101

[a] Compounds (numbered in parentheses, as in Table 2). Those in Group A were given as single or repeat daily doses, as indicated. DDP was administered in 0.15 *M* NaCl; other compounds in 0.3 *M* glucose

[b] Undrugged animals = 100%; higher values indicate food retention/gastric distension

[c] This Table compares two compounds given in daily doses. All rats were sacrificed 24 h after the final dose

[d] These doses were lethal within 24 h and data are from rats, post mortem. Other data in Group B are from survivors sacrificed 3 days after their single dose of Pt

Table 4. Effects of some platinum blues on GvHR, kidney size, and proteinuria in F_1 hybrid rats[a]

Preparation[b]	Body weight (g)	Thymus mass (%)	Spleen/ body weight	Kidneys (2)/ body weight	Kidney damage	Urinary protein	% Lymph node hypertrophy		VM[c]
							L + P	E	
–	– 02 ± 6	100	3.3 ± 0.3	7.2 ± 0.1	0	0	100	100	0
Pt-Uracil blue 75 h	– 01 ± 5	90	3.3 ± 0.2	8.6 ± 0.2	±	ND	100	90	++
140 h	– 03 ± 3	80	3.5 ± 0.3	7.7 ± 0.1	±	ND	100	83	+
200 h	– 06 ± 7	100	3.1 ± 0.3	7.7 ± 0.4	±	ND	98	66	±
–	– 05 ± 3	100	3.1 ± 0.1	7.8 ± 0.2	0	0	100	100	0
Pt-Thymine blue 190 h	– 12 ± 2	68	2.7 ± 0.1	7.9 ± 0.2	+	++	76	ND	+
300 h	– 11 ± 1	78	3.8 ± 0.1	8.9 ± 0.1	+	+	83	89	0
400 h	– 14 ± 2	70	3.0 ± 0.2	8.1 ± 0.2	+	+	78	ND	0
Pt-Acetamide blue	– 09 ± 3	95	3.5 ± 0.1	7.6 ± 0.1	±	+	110	102	+

[a] See Table 1 for footnotes and other details

[b] The times noted are the duration of reaction between $[(NH_3)_2Pt(H_2O)_2]^{++}$ and the pyrimidine at 37° C in each case. Compounds were tested at 300 μmol/kg (approx. 100 mg/kg), cumulative dose, given IP in four equal doses on days 1–4

[c] VM, visceral mucin coating internal organs

but much less stomach bloat than DDP (compound 2) at the same doses (Table 2B and 3). In contrast to $[(NH_3)_2Pt(H_2O)_2]^{2+}$ (compound 10), the dimer did not elicit appreciable visceral mucin in animals surviving to autopsy. Corresponding dimers containing ethylene-diamine, di(isopropylamine) (compounds 16, 17) and a number of other alkylamines (Fairlie and Broomhead, work in progress) also caused (a) greater nephrotoxicity, (b) less stomach bloat, and (c) more rapid mortality than the corresponding *cis*-diaquo- and dichloro-platinum (II) diamines.

The dimers $[A_2Pt(OH)_2PtA_2]^{2+}$ (A = NH_3 or $NH_2CH_2CH_2NH_2$; 30 μmol/kg Pt, A = $Me_2NCH_2CH_2NMe_2$, 30 μmol/kg Pt) caused convulsions after giving the fourth quarter dose. No convulsions were observed after administration of $[PtA_2(H_2O)_2]^{2+}$ or $[PtA_2Cl_2]$ at the same dosages.

Platinum Blues (Table 4)

Platinum blues are condensation products of $[(NH_3)_2Pt(H_2O)_2]^{2+}$ with various amides.

None of the preparations tested exhibited significant activity in vivo at doses equal to 10 times the effective dose of *cis*-DDP. Some proteinuria and visceral mucin production was observed with those preparations that had been reacted with uracil/thymine for the shortest time. These were likely to contain the most unreacted diaquodiamminoplatinum (II), which is known to induce visceral mucin. The high water solubility of these blues probably precludes effective biodistribution and retention by target lymphoid tissues. The low nephrotoxicity of these blues also probably reflects their low retention by the kidney.

1,2-Diaminocyclohexane (DACH) Platinum (II) Compounds (Table 5)

DACH compounds were certainly less nephrotoxic than DDP and its derivatives, but higher doses still caused damage to the distal tubules. The following side effects were very evident: severe diarrhoea (with rectal bleeding) with some compounds; considerable visceral mucin even with the dihalide (30 μmol/kg); pronounced stomach bloat with the dichloro and diaquo compounds but not with the dimer. These DACH compounds were useful immunosuppressant drugs, showing a range of potencies according to the

Table 5. Effects of some DACH platinum (II) complexes on (local) GvHR in rats[a]

Group	Compound	Σ dose Pt (μ*M*/kg)	Body weight (g)	Diarrhoea[b]	Thymus (%)	Spleen/ body weight	Kidneys (2)/ body weight	Kidney damage	Urinary protein	% Lymph node hypertrophy L + P
A	–	–	− 05 ± 1	0	100	3.6 ± 0.3	8.5 ± 0.1	0	0	100
	$[(DACH)PtCl_2]$	30	− 10 ± 2	0	54	2.0 ± 0.3	9.2 ± 0.2	+	+	69
		60	− 18 ± 3	+	25	1.0 ± 0.2	9.9 ± 0.3	2+	2+	30
		90	− 22± 3	+	7	0.6 ± 0.3	11.5 ± 1.0	2+	ND	21
	$[(DACH)Pt(H_2O)_2]^{2+}$	30	− 18 ± 1	+	35	1.4 ± 0.4	9.6 ± 0.3	2+	2+	47
		60	− 20 ± 1	+	19	1.0 ± 0.1	10.6 ± 0.1	2+	2+	26
	$[(DACH)Pt(OH)_2Pt(DACH)]^{2+}$	20	− 10 ± 1	0	95	2.9 ± 0.2	9.2 ± 0.1	+	+	79
		45	− 10 ± 2	0	81	2.4 ± 0.3	9.8 ± 0.3	+	+	73
	cis-$[(NH_3)_2PtCl_2]$	30	− 14 ± 3	0	39	1.7 ± 0.2	10.5 ± 0.5	3+	3+	52
B[c]	–	–	− 05 ± 1	0	100	3.8 ± 0.1	7.7 ± 0.2	0	0	100
	trans(−)(RR)[(DACH)$PtSO_4$] (SHP)	100	− 15 ± 2	+	24	1.6 ± 0.2	8.2 ± 0.2	ND	0	57
	[(DACH)Ptmalonate]	100	− 06 ± 2	0	75	3.4 ± 0.2	7.7 ± 0.2	ND	0	85
	(NSC-224964)	300	− 15 ± 5	0	32	2.2 ± 0.1	8.0 ± 0.1	ND	+	47
	[(DACH)Pt(4-Carboxy-phthalate)]	100	− 14 ± 2	0	54	2.8 ± 0.1	7.9 ± 0.1	ND	0	54
	(NSC-271674)	300[d]	− 32 ± 2	+	16	0.9 ± 0.1	11.2 ± 0.5	ND	2+	23
	cis-$[(NH_3)_2PtCl_2]$	30	− 12 ± 3	0	25	1.6 ± 0.2	11.2 ± 0.5	3+	3+	35

[a] See Table 1 for details. Values given are means ± SE

[b] Diarrhoea, observed on day 5, was not prevented by intensive oral antibiotic therapy (Tetracycline and Kanamycin)

[c] These compounds were supplied by Drs Khan and Gale and may contain differing proportions of *cis* and *trans* isomers. They did not cause stomach bloat

[d] Very pale livers were observed at autopsy

(non-nitrogen) ligand employed; the diaquo cation was definitely more potent than the dichloride.

Studies in vitro (Table 6)

The direct effect of some platinum compounds upon the splenic graft cells was determined by pre-incubation of the cells to be grafted with or without the addition of the test compound for 15–25 min at 37° C in a phosphate-buffered saline medium (Hank's BSS). Thus the drugs were tested in the presence of excess chloride ion (120 m*M*). These studies show that the diaquo species were more toxic to the cells than the dichloride (Table 4). The platinum blues were almost devoid of activity in vitro in this assay. In control experiments the graft reaction was abrogated by treating the cells with a nitrogen mustard (mechlorethamine), with retention of viability, or by destroying their viability (brief heating at 56° C).

Table 6. Effect of some platinum compounds applied in vitro on the ability of parental spleen cells to mount a subsequent GvH response in F_1 hybrid rats

Treatment (spleen cells)	Concentration (m*M*)	Viability[a] (%)	GvHR[b] (%)
No drug	–	90	100
Pt-Thymine blue[c]	3.0	90	96
Pt-Uracil blue[d]	1.0	85	78
Pt-Acetamide blue	1.0	90	100
cis-$[(NH_3)_2PtCl_2]$	0.5	90	38
	0.1	95	56
$[(NH_3)_2Pt(H_2O)_2](NO_3)_2$	0.5	95	50
$[(C_3H_7NH_2)_2PtCl_2]$	0.5	90	25
$[(C_3H_7NH)_2Pt(H_2O)_2](NO_3)_2$	0.5	90	25
Mechlorethamine HCl	0.002	>80	13
Heated at 56°, 10 min	–	< 5	3

[a] Viability of cells (Trypan blue exclusion) at time of grafting
[b] Data are means from four observations in each case and represent hypertrophy of local draining lymph node(s) after inoculation with treated cells, compared with hypertrophy of contralateral nodes in the same animal after inoculating the same number of cells incubated without drug into the contralateral paws (see Materials and Methods)
[c] 190-h sample (see footnote, Table 3)
[d] 75-h sample (see Table 3)

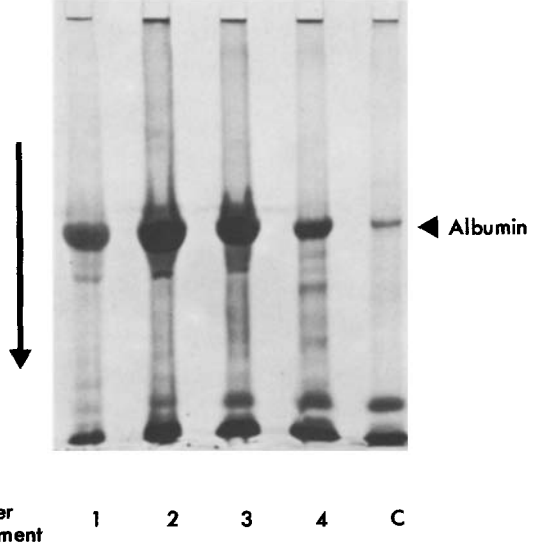

Fig. 3. Gel electrophoresis at pH 8.3 (Tris-glycine with SDS) on 8% acrylamide gel, indicating significant albuminuria but no shedding of high-molecular-weight proteins after DDP. C, urine from control rat (no DDP). *Numbers* refer to times of urine collection on 1st, 2nd, 3rd, and 4th days after administration of the first dose (= day 0) of 2.25 mg/kg and again on days 1–3 inclusive. *Arrow* indicates direction migration

Analysis of Renal Toxicity

Electrophoretic analysis of proteinuria after DDP indicated tubular toxicity with preferential shedding of albumin and low-molecular-weight proteins (Fig. 3). Glomerular injury would be revealed by presence of proteins with higher mol. wt. than albumin [17]. Detection of ligandin, by enzymatic assay [11], in the urines of DDP-treated rats indicates likely restriction of proximal tubular damage to the *pars recta* [19].

Discussion

The anti-lymphoproliferative and the major side effects of DDP and its congeners at sublethal doses were readily monitored simultaneously, in minimally burdened rats. Using rats, in preference to mice, it was no problem to obtain adequate (and continuous) urine collections, by which to monitor renal toxicity with proteinuria before death. Changes in normal weight gain of young male rats afforded an early warning of gross toxicity.

Potential Immunosuppressant Activity of DDP

These studies confirm and amplify previous reports of the effects of *cis*-DDP on GvHR of rodents [12, 18]. Effective suppression of a systemic GvHR (splenic hypertrophy) in mice was reported with an accumulative dose of 5–10 mg *cis*-DDP/kg, given either singly on day 0 or in four divided dosed [12]. In another study *cis*-DDP was administered to the parental donor rats without affecting the ability of their spleen cells to mount a subsequent local GvHR in the F_1 hybrid [18]. In this present study the graft was established for 24 h before the host was treated, to ensure that the host's response that was being suppressed, rather than the graft cells being killed in vivo immediately after inoculation (possible if the drugs are given on day 0).

The powerful anti-lymphoproliferative effect of DDP may be an undesirable feature for an oncolytic drug used to treat non-lymphoid tumours. However, the feeble anti-lymphoid activity of *cis*-diaquo- and -dichloro-di(isopropylamino)-platinum II may be a commendable property in view of the powerful anti-tumour activity reported for this dichloro complex [6]. In our assays the platinum blues appear to have no significant activity, confirming earlier studies using the L1210 tumour system [7]. 1,2-Diaminocyclohexane platinum (II) compounds at first sight appeared to offer advantages over DDP as less nephrotoxic immunosuppressants, but exhibited other toxicities (see below).

The consensus is that both the anti-tumour effects and the side effects of DDP are due to the products formed after hydrolysis, which at neutral pH would be $[(NH_3)_2Pt(OH)(H_2O)]^+$ and the μ-hydroxo-bridged dimer formed therefrom [5, 15, 20]. Rosenberg [20] suggested that the dimer (from DDP) was not an active anti-tumour agent at low doses, in contrast to the diaquo species ('monomer'). Our data indicate that this dimer was always as potent as the diaquo species in causing thymus and splenic involution or suppressing lymph node hypertrophy. It is unlikely that the dimer owed all its lymphotoxic effects to adrenal stimulation.

The dimer is believed to be a stable species, only reverting to the monomer at low pH (in kidney, perhaps) or being decomposed by powerful nucleophiles (e.g., thiols). It could exhibit extrarenal toxicity by interacting with lymphoid receptors that are not abundant in/on the experimental tumours used by Rosenberg. Alternatively the poor anti-tumour activity could reflect its powerful lymphotoxic activity, ablating a tumour-depressing immune response.

Side Effects and Toxicity

Both DDP and $[(NH_3)_2Pt(H_2O)_2]^{2+}$ caused nephrotoxicity and gross stomach bloat. The dimer, $[(NH_3)_2Pt(OH)_2Pt(NH_3)_2]^{2+}$, was more lethal and nephrotoxic and caused convulsions but had little effect on the stomach mass.

Single doses (10 mg/kg) or diaquoplatinum (II) diamines at neutral pH were convulsant and rapidly lethal in mice [5]. These data probably reflect a property of the dimers, which are rapidly formed at neutral pH [20]. Rosenberg reported [20] that the dimer formed from DDP caused prompt mortality but his animals (mice?) took 3 days to die from lethal doses of DDP or diaquo species – a finding we fully confirmed in rats. This delayed death could be the result either of superinfection after immunosuppression or of slow formation of the dimer in vivo.

1,2-Diaminocyclohexaneplatinum (II) compounds that show less nephrotoxicity than DDP in rodents [13, 22, 24] also showed much less nephrotoxicity in our rats. However, they did cause severe diarrhoea and elicited copious visceral mucin. Remarkably, even low doses of the dichloro complex elicited this visceral mucin production, whereas no other dichloro complexes (out of > 20 tested) caused this peculiar response (at doses less than 100 mg/kg).

Two other side effects, namely the bloated stomach (with several diaquo species) and the delayed *rigor mortis* (with the dimer from DDP) might indicate paralysis of muscle contraction. Preliminary experiments have shown that *cis*-DDP and its hydrolysis products can inhibit rabbit myosin ATPase activity in vitro. However, measurements of the contractility of smooth muscle strips prepared from the stomachs of animals predosed with *cis*-DDP showed normal (or hyperactive) contractile responses (Aggarwal and Kennedy, unpublished data). The defect in stomach emptying may therefore lie in the control of the pylorus (being indicative of neurotoxicity?).

In in vitro assays (Whitehouse and Aggarwal, in preparation) we have found that the cytotoxic effects of *cis*-DDP are selectively inhibited by L-methionine, whereas corresponding cytotoxic effects of diaminodiaquoplatinum (II) are abolished by *N*-acetyl-L-cysteine and other thiols that are stable at physiological pH. It was of interest to find that *cis*-[L-methionine$PtCl_2$] was totally devoid of in vivo activity (therapeutic/toxic).

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