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Toxicology and Antitumour Activity of Ferrocenylamines and Platinum Derivatives

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Toxicity, antitumour, platinum distribution, hepatotoxicity and histology data are presented for a series of ferrocenylamines: [(η-$C_5H_4(CH_2)_nNH_2$)FeCp] (n = 0,1) (1,2); [(η-$C_5H_4CH_2NHPh$)FeCp] (3); [(η-$C_5H_4CH_2NMe_2$)FeCp] (4); {[η-$C_5H_4CH(Me)NMe_2$]FeCp} (5); [η-$C_5H_4CH_2NMe_2)_2$Fe] (6); {[1,2η-$C_5H_3(CHMeNMe_2)(PPh_2)$] FeCp} (7); {[1,2η-$C_5H_3(CHMeNMe_2)(PPh_2)$]-Fe[η-$C_5H_4PPh_2$]} (8); and their complexes *cis*-$PtCl_2L_2$ (9); *trans* - $Pt(L)(dmso)X_2$ (10); [σ-(L)Pt(dmso)X] (11,12) {σ-(L)[$Pt(dmso)X]_2$} (13); [σ-(L)$PtP(OPh)_3Cl$] (14) (L = ferrocenylamine). The toxicity order is 1–3 $\gg$ 4–8 for the ferrocenylamines; the lower toxicity of tertiary amines may be due to protonation *in vivo*. Pt(II) complexes all show increased toxicity over the ligand. Liver, not kidney, damage is the norm from i.p. injection of 1–14 and detailed platinum distribution, blood serum and histology studies with 9 and 11 show that the platinum distribution does not correlate with liver dysfunction. Complexes 9–14, but not 1–8, were active against P-388 mouse leukaemia tumour and cisplatin-resistant sarcoma, but inactive against L-1210 mouse leukaemia and B-16 melanoma.

Keywords: ferrocenylamines; platinum complexes; toxicity; antitumour

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INTRODUCTION

Metallocenes have had wide application in biological and clinical studies[1] although they are usually more toxic to organisms than their inorganic precursors. This toxicity is the basis for their use in chemotherapy and other biocidal applications. Ferrocene is a useful building block as derivatives are easily synthesized, it is relatively small, and being lipophilic it easily crosses cellular membranes. Ferrocenyl compounds feature as enzyme inhibitors,[2] therapeutic agents,[3–5] metabolic competitors,[6] antimicrobial compounds,[7] radiopharmaceutical[8] and histological agents.[9] Their potential as antitumour agents is well documented.[10–12] The medial lethal dose for ferrocene varies with the method of administration, the major clinical effect being hepatotoxicity.[13,14] Toxicity is dependent on metabolism to water-soluble derivatives via hydroxylation, followed by glucuronide or sulphate conjugation. Hydroxylation *in vivo* is followed by partial degradation liberating Fe^{2+} and subsequent excretion in bile and urine.[15,16] Detoxification primarily occurs inside the liver microsomes.

Although it is expected that ferrocenyl derivatives will demonstrate organ specificity, few detailed toxicity studies other than those for ferrocene itself have been reported. Ferrocene is oxidized to the ferrocenium cation by horseradish peroxidase in the presence of a peroxide source.[17] This cation exhibits weak antitumour properties[10–12] and is a potential radiosensitizing agent[18] but its degradation pathway *in vivo* is unknown. Polyamine derivatives show a decrease in toxicity relative to ferrocene but no antitumour properties[19] and alkyl ferrocenes are relatively inert, whereas acetylferrocene is very toxic and is absorbed through the skin.[20] Compounds in which the *cis*-Pt(II) and ferrocenyl moieties are combined may also be expected to exhibit biological activity.[21] Thus, $(dppf)PtCl_2$, $[(dppf)Pt(\mu\text{-}Cl)]_2^{2+}$, $[(dppf)Pt(\mu\text{-}OH)]_2^{2+}$ and $[(dppf)Pt(DMF)_2]^{2+}$ [DPPF = 1,1-bis(diphenylphosphinoferrocene); DMF = dimethylformamide; lower-case abbreviations are used for legends in complexes (i.e. dppf, dmf)]

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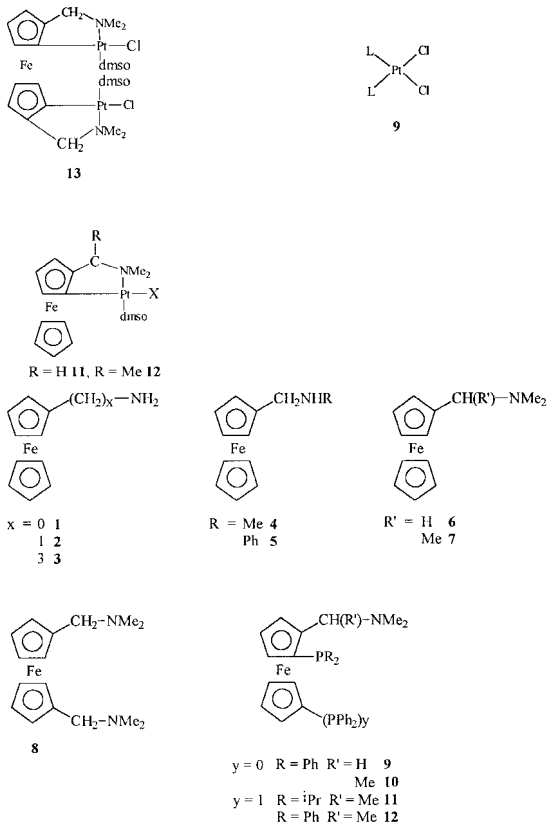

Figure 1

have been shown to exhibit cytostatic activity *in vitro* against the KB cell line.[22]

Ferrocenylamine ligands offer the opportunity to synthesize analogues of the clinically significant cisplatin.[23] The only report of their biological activity concerns 1-dimethylaminomethylferrocene, which prolonged drug-induced paralysis.[6] Recently, a range of ferrocenylamine–platinum (II) complexes with[24–29] or without[30,31] a cyclometallated ferrocenyl ring have been prepared. To establish the parameters which would limit the drug regime for these compounds, we carried out acute toxicity testing, platinum distribution and hepatotoxic studies for a series of ferrocenylamine ligands and their platinum (II) complexes, and a preliminary evaluation of their antitumour properties. The compound numbering scheme is given in Fig. 1; for **9** the specific amine is given in the bracket (e.g. **9**[**1**]) and for **11** the anion (e.g. **11[Cl]**).

Table 1 Selected solubilities in peanut oil[a]

Compound	Solubility (mg/cm^3)	Compound	Solubility (mg/cm^3)
Ferrocene	250	—	
1	>125	**9[1]**	1
4	750	**11[Cl]**	4
5	750	**12**	1
6	>125	**13**	3
3	100	**9[3]**	1
7	250	**9[7]**	1
8	100	**9[8]**	2

[a] The stability of the test compounds in peanut oil was established by NMR. Solutions for injection were freshly prepared immediately before administration. All compounds are insoluble in aqueous solution.

EXPERIMENTAL

The ferrocene derivatives and their Pt(II) complexes were prepared by literature methods: **1**[32,33]; **2, 3** and **4**[34]; **5**[35]; **6**[36]; **7** and **8**[37]. Reaction of these ligands with K_2PtCl_4 gave the cisplatin analogues *cis*-PtL_2Cl_2 (**9**)[30], whereas reaction with *cis*-$Pt(dmso)_2Cl_2$ (DMSO = dimethyl sulphoxide) gave [*trans*-$Pt(L)Cl_2(dmso)$(**10**) and the cycloplatinated complexes **11**–**14**.[24] Peanut oil was chosen as the general vehicle for delivery of the compound in biological testing, as alcohols and DMSO were too variable in their properties and gave unstable solutions. Table 1 gives typical solubility data; NMR was used to check the stability of the solutions.

Acute toxicity

ABS or Swiss random-bred male or female mice (body weights 20–25 g) were obtained from the University of Otago Animal Breeding Station. The animals were allowed free access to food (standard pelleted rodent diet) and tap water throughout the experiment. Groups of mice ($N = 5$) were given a single intraperitoneal (i.p.) injection of the test compound in peanut oil (0.2–0.6 ml vehicle/25 g body weight). The injections were given under light ether anaesthesia. A control group was given the vehicle solution alone. Body weights were measured daily and the animals were observed regularly for signs and symptoms of toxicity over 7 days (ferrocenylamine and platinum derivatives) or for 14 days (ferrocenylamines). At the end of the test period all the animals were examined for evidence of gross organ damage and the liver, kidney, spleen, lung and testes were weighed. Parameters selected

to indicate toxicity were the presence of signs or symptoms of toxicity, a significant reduction in body weight gain in the 24 or 48 h following treatment, a decrease in growth rate over the course of the experiment, evidence of gross organ or tissue damage at autopsy, and an alteration in relative organ weight at the completion of the observation period. The test was repeated using either a higher or lower dose depending on the results of the initial dosing. Typical doses for the ferrocenylamines were 5, 50 and 150 mg kg^{-1}, and for the platinum complexes, doses of 1, 5, 15 and 15 mg kg^{-1}. Body weight measurements for each animal in a group were calculated as a percentage of the body weight at the time of treatment. The group mean values were then determined and the growth rate estimated by linear regression of mean body weight versus time. Relative organ weights were calculated from the ratio of organ weight to total body weight. The different groups were compared statistically by analysis of variance and Duncan's multiple range test using the CLR (Clear Lake Research) ANDVA programme.

Antitumour testing

Ferrocene, the ferrocenylamines and their platinum derivatives were screened in Melbourne for activity against the transplantable P388 mouse leukaemia. The ferrocenyl–platinum compounds found to be active [T/C(%) (defined in Table 8) >130] in the initial screen were tested for activity against mouse L1210 leukaemia and B-16 melanoma. The testing procedures used were essentially in accordance with the protocols of the National Cancer Institute (1972). Additional tests were carried out by Johnson–Matthey, UK.

Platinum distribution and histopathological assays

Groups of mice ($N = 7$) were given a single intraperitoneal injection of **9**[**3**] at 0.98 and 5 mg Pt kg^{-1} and **11**[**Cl**] at 1.77 mg Pt kg^{-1} in peanut oil (0.2 ml vehicle solution/25 g body weight). A control group was given the vehicle solution alone. The animals were killed by cervical dislocation or by exsanguination. The liver and kidneys were excised and weighed, and samples were prepared for flameless AA analyses using a GBC graphite furnace and GBS 907 double-beam atomic absorption spectrometer. Mice were kept as groups in metabolism cages for the collection of urine and faeces for platinum analysis. Standard ashing procedures were employed for solid material, with dry ashing giving better results; urine was analysed directly. For the serum tests each animal in the platinum distribution study was anaesthetised, the thoracic cavity was opened and blood was withdrawn from the heart. The blood was stored in capped 1.5 ml tubes while coagulation took place and after 2–3 h it was centrifuged at 3000 rpm. Serum from each mouse was kept separate and assayed. For effective measurement the serum was used fresh, or frozen for 24 h and used immediately on thawing. For serum γ-glutamyl transpeptidase (GPT)[38], ornithine carbamoyl transferase (OCT)[39] and bilirubin[40] assays, pooled serum, obtained after treatment of groups of seven male mice with 5–25 mg kg^{-1} of the compound, was used. For each assay run, at least three standards were set up for comparison to compensate for differences in concentration between batches of reagents. Separate GPT assays were also run on non-pooled serum, and fresh reagents were used as the sodium pyruvate and pyruvate substrate solution were only stable for a few days. For OCT assays the phenol–nitroprusside and alkaline hypochlorite solutions were mixed quickly to prevent loss of ammonia. For histology the liver and kidneys were removed and weighed immediately to prevent cell disruption. Samples 3–5 mm thick were placed in 10% buffered formalin and fixed for at least three days before processing.

RESULTS AND DISCUSSION

Acute toxicity of ferrocenylamines and their platinum(II) complexes

In order to establish the appropriate dosage regimens for the antitumour screening tests, an acute toxicity test based on the procedure recommended by the British Toxicology Society[41] was carried out on each test compound. The primary aim was to determine the acute toxic effects and the target organs of toxicity, and to estimate the maximum non-lethal and non-toxic doses for each compound. Peanut oil proved to be the most versatile solvent as the compounds were stable in this medium and it had an NMR window in the ferrocenyl region. A wide spectrum of basicity and structure was incorporated in the series of ferrocenylamines and platinum(II) complexes used in the study, including molecules with phosphine substituents on the cyclopentadienyl ring. Ferrocene was also tested

Table 2 Toxicities in male and female ABS random mice[a]

Compound	Maximum non-lethal dose (mg/kg^{-1})		Maximum Non-toxic[b] dose (mg/kg^{-1})		Toxicity classification	p*K*b
	Males	Females	Males	Females		
NH_2	5	5	5	<5	T	8.24
CH(Me)NMe 2	5	5	5	5	Toxic	4.30
Ferrocene	50	50	<50	<50	Harmful	—
4	50	50	50	50	Harmful	5.17
6	150	50	50	<50	Harmful	4.72
7[c]	150	150	150	150	Non-toxic	5.50
8[c]	150	150	150	150	Non-toxic	6.73

[a] Vehicle solution, peanut oil.
[b] Using any of the following criteria: presence of signs and symptoms of toxicity, reduced body weight in the first 24 h after treatment ($P < 0.05$, ANOVA, Duncan's multiple range test), decrease in growth rate over the course of the experiment, evidence of gross organ damage at autopsy, alteration in relative organ weight at autopsy.
[c] Dose of BPPFA and PPFA was limited by solubility in the vehicle solution.

as a reference under the same regime. Our maximum non-toxic dose for ferrocene of 50 mg kg^{-1} can be compared with the reported median lethal dose in mice of 660 mg kg by oral administration[13,14]. In dogs, dosages of ferrocene of 30, 100 and 300 mg kg^{-1} for six months and 1000 mg kg^{-1} for three months[21] by daily oral administration produced haemosiderosis with an unusually high dose-related accumulation of iron.

Ferrocenylamines

Maximum non-toxic and non-lethal dosage data for ferrocenylamines **1**–**8** in male and female mice are shown in Table 2 and their toxic effect in female mice in Table 3 (data for male mice were very similar[24]). With the exception of the pro-chiral representative **5**, the tertiary amine derivatives **4**–**8** were less toxic than ferrocene, with the maximum lethal dose level greater than 50 mg kg^{-1}. In contrast, **1**–**3** and **5** are more toxic than ferrocene, the maximum non-lethal dose being 5 mg kg^{-1}. The addition of a PPh_2 substituent caused a marked decrease in toxicity and there was little difference between **7** and **8**. One general statement can be made: primary and secondary ferrocenylamines are more toxic than tertiary.

Although the addition of an NMe_2 substituent caused a decrease in lethality, the symptoms preceding death were different. Compound **4** caused severe convulsions, apnoea, decreased motor activ-

Table 3 Toxic effects of **1**–**8** in female ABS random mice

Compound	Effect of dose		
	5 mg kg^{-1}	50 mg kg^{-1}	150 mg kg^{-1}
Ferrocene	—	Reduced body weight at day 1 (cf. controls)	—
NH_2	Reduced body weight at day 1 (cf. controls)	5/5 deaths (rapid onset of tremor, decreased motor activity, ataxia, prostration, loss of startle reflex, death within 1 h)	—
4	—[a]	—[a]	3/5 deaths (severe convulsions, apnea, decreased activity, death within 1 h; no effect on survivors
CH(Me)NMe_2	—[a]	4/7 deaths (severe convulsions, apnea, decreased activity, death within 1 h); no effect on survivors	—
6	—[a]	Reduced body weight at days 2, 3 and 4 (cf controls)	1/5 deaths (gradual loss of motor activity, death at ~ 1 h); no effect on survivors
7	—[a]	Reduced body weight at day 1 (cf. controls)	—[a]
8	—[a]	—[a]	—[a]

[a] No effect on any parameter

Table 4 Toxicity data for some platinum (II) complexes in male and female ABS random mice [a]

	Maximum non-lethal dose (mg/kg^{-1})		Maximum non-toxic dose (mg/kg^{-1})[b]	
Compound	Males	Females	Males	Females
Cisplatin	5	5	<5	<5
NH_2	25	25	25	25
[**8**]	25	25	5	5
[**7**]	25	25	5	5
[**3**]	25	25	<25	25
10 [**4**]	15	15	5	15
$CH(Me)NMe_2$	15	15	15	15
[**3**]	15	15	5	5
[**2**]	15	15	<5	<5
[**6**]	15	15	5	5
11 [**Cl**]	25	5	1	<1
[**Br**]	5	5	5	<5
[**I**]	5	5	5	5
[**OAc**]	5	5	<5	<5
12	1	5	1	1
13	1	25	1	5
14	25	25	<5	1

[a] Vehicle solution, peanut oil.
[b] Using any of the following criteria: presence of signs and symptoms of toxicity, reduced body weight in the first 24 h after treatment ($P < 0.05$, ANOVA, Duncan's multiple range test), decrease in growth rate over the course of the experiment, evidence of gross organ damage at autopsy, alteration in relative organ weight at autopsy.

ity and death within one hour, whereas convulsions were not observed with the direct analogue with a substituent on each cyclopentadienyl ring, **6**. Incorporation of an α-CH_3 (**5**) had no effect on the symptoms which preceded death but these occurred at a lower dose. For the primary amines **1** and **2** the symptoms preceding death were again different, with rapid onset of tremor followed by decreased motor activity, ataxia, prostration, loss of startle reflex and death within one hour, but the secondary amine **3** gave responses similar to **6**. Inhalation of acetylferrocene causes similar effects to **4**; rough coat unthrifty appearance, laboured respiration, tremors, decreased body temperature, diarrhoea, nasal discharge, salivation and urogenital staining.[20]

Several factors could be influencing the toxicity of the ferrocenylamines. Non-polar alkyl substituents tend to lower the toxicity of ferrocene compounds,[20] but polarity is unlikely to be a factor here. It is also unlikely that the metabolism is going to be significantly different between ferrocenylamines, although it may be different from that of ferrocene. At a first glance, while there are several orders of magnitude in the range of N-basicity (see Table 2),[30] there does not appear to be a correlation between toxicity and pK_b. However, their chemistry[30] shows that **4**–**8** will exist under most physiological conditions as their salts; that is, the nitrogen lone pair will not be available and the compound is a positively charged ion. This could provide the explanation for the trends in the above data, with the exception of **5**. In the case of **5** the diastereoisomerism is clearly influencing the toxicological response when there is no phosphine ring substituent, but the point in the physiology at which this occur must await details of the mechanism.

Platinum complexes

Summaries of the results of the acute toxicity tests for the ferrocenylamine–platinum(II) complexes **9**–**14** are presented in Tables 4 and 5. No toxicity data or toxicity effects for ferrocenylamine–platinum complexes have been reported previously. Again, toxic but non-lethal responses were indicated for **9**–**14** by a significant reduction in body weight during the first 24 h after the treatment, decrease in growth rate and/or evidence of gross tissue damage at autopsy. The results may be summarized as follows.

(1) In general, coordination of the ferrocenylamine to platinum (II) resulted in a marked increase in toxicity, with the exception of the cisplatin analogues **9** where L is a primary or secondary amine. For example, **9**[**1,3**] was non-toxic at a dose of 25 mg kg^{-1} in both male and female mice, whereas at 5 mg kg^{-1} the corresponding ligands caused a significant reduction in body weight after 24 h.

Table 5 Toxic effects of cycloplatinated ferrocenylamineplatinum(II) complexes in Swiss random mice

Compound	Dose[a] 5 mg/kg^{-1}	Dose[b] 5 mg/kg^{-1}	Dose[b] 25 mg/kg^{-1}
Cisplatin	Reduced body weight on day 1 (cf. controls)	Reduced body weight on day 1 (cf. controls)	–
11[**Cl**]	Reduced body weight at day 1 and rate of growth during recovery, body weights at day 1 lower than in controls, adhesion to peritoneal wall	Reduced body weight at day 1 (cf. controls)	5/5 deaths within 4 h (gradual loss of motor activity)
12	Reduced body weight at day 1 (cf. controls), 1/5 deaths on day 1; adhesions of organs to peritoneal wall; gross liver lesions; yellow deposit around spleen and gastrointestinal tract	Reduced body weight at day 1 (cf. controls)	3/5 deaths on days 1, 2 and 3 (gradual loss of activity)
14	Reduction in overall rate of growth	Reduction in overall rate of growth	Reduced body weight at day 1 (cf. controls)
13	1/5 deaths on day 2; adhesion of organs to peritoneal wall; gross liver lesions	—[c]	Reduced body weight at day 2 (cf. controls)
9[**Br**]		—[c]	1/5 death at day 4; reduction in overall growth rate
9[**1**]		—[c]	5/5 deaths at days 1–2, gradual loss of motor activity;
10[**4**]		—[c]	gross liver damage
CH(Me)NMe$_2$		—[c]	—[c]
10[**3**]		—[c]	Gross liver damage; reduced body weights at days 1 and 2.
10[**2**]		Reduced body weight at days 1–3 (cf. controls)	Gross liver lesions; reduced body weights at days 1 and 2.
10[**6**]		—[c]	Gross liver lesions; reduced body weights at days 1 and 2 (cf. controls)
9[**1,3,8**]		—[c]	—[c]
9[**7**]		—[c]	—[c]

[a] female;
[b] male mice.
[c] No effects on any parameter.

(2) There was a significant trend in toxicity associated with the stereochemistry and co-ordination sphere of the platinum (II), with an increasing non-lethal dose from **9** (non-lethal at 25 mg kg^{-1}) < **10** < cyclometallated complexes, **11**–**14** (lethal at doses from 1 to 5 mg kg^{-1}).
(3) None of **9**–**14** showed evidence of gross kidney damage at 25 mg kg^{-1}.
(4) Most of the cyclometallated compounds **11**–**14** produced adhesion of the tissues to the peritoneal wall and clear evidence of gross liver lesions. It was noted that male mice treated with **9**[**8**] at 25 mg kg^{-1} had relative testes weights significantly lower than the control group, while males treated with **9**[**7**] had higher mean relative spleen weights.
(5) Both **9** and **10** are less toxic than cisplatin, whereas the relative toxicity of the cyclometallated complexes **11** was dependent on the anion and π-acceptor (DMSO or phosphine).

Platinum distribution, hepatotoxicity and histology

Of interest from the post-mortem results was the observation of liver lesions and lack of kidney damage because, for cisplatin, the highest levels of platinum are found[42,43] in the liver and kidneys but normally no liver damage is evident. This sug-

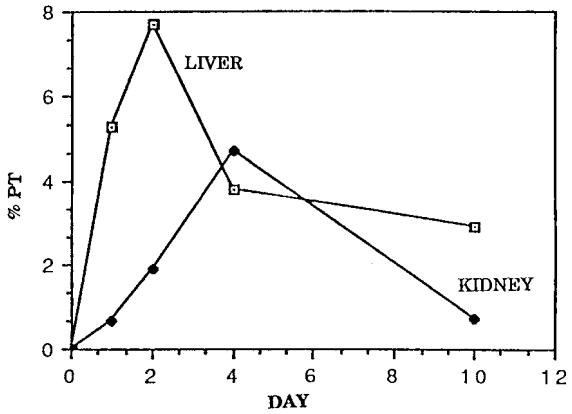

Figure 2 Platinum distribution for **9**[**3**] over 10 days (female mice).

gested that the metabolism of ferrocenylamines and their platinum (II) complexes were different from that of cisplatin, and that an investigation of toxicity at the cellular level was worthwhile.

For further toxicological tests at the cellular level it was necessary to restrict the number of compounds. Visual examination of animal organs seven days after a 5 mg kg^{-1} dose with **11**[**Cl**] showed the liver to be swollen, whereas there was no sign of liver toxicity with **9**[**3**], even at a 25 mg kg^{-1} dose level; these two were the only compounds used in the following cellular studies, in which cellular toxicity was investigated by studying the platinum distribution in the organs and by serum enzyme analyses specific for liver and kidney function, followed by organ histology. Should the function of a cell be disrupted, there will be an elevation of cellular constituents in the blood serum and accompanying histological examination can provide corroborative evidence. With cisplatin, most work[44] has been centred on the measurement of enzyme levels and excretory products associated with kidney, as no clinically significant liver function disturbances have been found.

Platinum distribution

Each test group of mice was dosed with the compound delivered intraperitoneally in peanut oil. The excretion of platinum over a 24 h period following administration of the compound was measured through the collection of urine and faeces. Alternatively, the animals were dosed at 4.24 mg kg^{-1} (0.98 mg Pt kg^{-1}) and the deposition of platinum in the kidney and liver was followed over 10 days. Liver and kidney tissues were taken from the animals in the 10-day platinum time course analysis and also during the acute toxicity and antitumour testing Results from the 24 h platinum distribution study showed that a higher proportion of platinum in the cisplatin analogue **9**[**3**] was deposited in the liver than in the kidney, in contrast to the cyclometallated **11**[**Cl**], where the distribution was virtually equal. At doses of 0.98 mg and 5 mg Pt kg^{-1} of **9**[**3**], the urinary excretion of platinum was respectively 4.8 and 7.5% of the initial dose over a 24 h period. The retention of platinum in other tissues was not explored. In a separate series the platinum was followed through a 10-day time period; typical results for **9**[**3**] at a dose level of 0.98 mg Pt kg^{-1} are shown in Fig. 2. A larger percentage of the platinum accumulated in the liver than in the kidney. The level of platinum in the liver reaches a maximum 1–2 days before the kidney, in contrast to cisplatin where the kidney reaches a higher maximum level than the liver within 24 h[42,43,45].

Hepatotoxicity

Hepatotoxic effects were monitored by assaying for liver constituents in the blood serum. Assays chosen were serum glutamic pyruvic transaminase (SGPT), orthinine carbamoyl transferase (SOCT) and bilirubin because each has a well-known function(s) and site(s) of action[44]. Both GPT and

Table 6 Enzyme assays on pooled blood serum[a]

Assay	Compound	Control	Test	Normal[b]
OCT IU	**9**[**3**]	0.488	0.444	0.070
(IU/100 ml)	**11**[**Cl**]	0.488	0.314	
GPT	**9**[**3**]	0.135	0.190	0.290
(μmol ml^{-1}/15 min)	**11**[**Cl**]	0.135	0.135	
Bilirubin	**9**[**3**]	0.145	0.290	0.400
(mg/100 ml)	**11**[**Cl**]	0.145	0.232	

[a] Obtained from groups of seven male Swiss random mice.
[b] Literature values for control mice.

Table 7 10-Day time course following GPT activity for **11** [**Cl**][a]

Group	Day 1	Day 2	Day 4	Day 10
Treated	0.58 ± 0.13	0.30 ± 0.19	0.24 ± 0.09	0.11 ± 0.09
Control	0.23 ± 0.05	0.32 ± 0.14	0.31 ± 0.11	0.18 ± 0.10

[a] Activity in $\mu mol\ ml^{-1}$/15 min ($\pm$SD); values are averaged; $n = 7$ except days 1 and 10, when $n = 6$.

OCT assays may indicate liver damage (cytotoxicity) and the bilirubin assay assists in the determination of cholestatic injury[39]. In general, the SGPT assay was used as it required lower volumes of serum, and the serum OCT and bilirubin assays used pooled serum.

Results for **9**[**3**] and **11**[**Cl**] from pooled serum are given in Table 6. Compound **9** had more effect on the liver cells and the two-fold increase in GPT activity within 24 h suggests that liver damage had occurred. However, examination of the liver 4–7 days after dosing showed that the liver was swollen with **11**[**Cl**] but not **9**[**3**]. This discrepancy may have been because pooled serum was used and individual SGPT assays should be statistically more reliable. In an SGPT assay, animals dosed with **11**[**Cl**] showed a two-fold elevation in enzyme activity at 24 h, which decreased to normal after 48 h and remained normal for the following eight days (Table 7). In contrast, there was no overall SGPT elevation when animals were dosed with **9**[**3**] at 25 $mg\ kg^{-1}$.

It is pertinent that SGPT assays on mice treated with the cisplatin analogue 'CHIP' found a high level of uptake in the liver and kidney but no effect on SGPT levels, although in this study[46] serum was collected only until day 4.

Histology

Gross examination of livers and kidneys was carried out in conjunction with the acute toxicity tests, platinum analyses and enzyme assays; as already noted, swollen livers were found on all animals receiving a toxic dose, usually after 4–7 days. Liver and kidney tissues from the ten-day trial using **11**[**Cl**] (5 $mg\ kg^{-1}$) were taken for histopathological analysis. The kidney tissue appeared normal throughout. However, on day 4 an inflammatory reaction could be seen in the liver tissue on the surface of several liver sections, possibly due to the build-up of intracellular fluid, with the loops of intestine adhering to their peritoneal surfaces. The inflammatory exudate was clearly present in many sections where several polymorphs and macrophages were evident. In general, this hepatic inflammatory response was localized. Large vacuoles which were seen probably represented fat cells and together with the spindle cells of fibroblast type suggest that the tissue layer on the surface of the liver represents intestinal mesentery or subserosal intestinal tissues. Tissue sections indicated that the points at which the intestine or mesentery adhered corresponded to the areas of inflammatory response. Passage of the platinum complex through the intestine might have led to epithelial cell damage within the intestinal mucosal lining. Once the cells were damaged lumen bacteria would have been able to pass through the mucosal lining, into the muscle and subserosal tissue, and finally to the surface of the liver. At this point the presence of bacteria at the surface of the liver would have caused a peritonitis with the observed inflammatory response. The intestine was not examined in this study.

In a study of toxicological effects caused by cisplatin it was noted that intestinal epithelia were damaged to such a degree in mice and rats that the animals suffered loss of appetite and eventually starved[47]. However, other than a drop in growth rate over the first 24 h, mice treated with **11**[**Cl**] were not affected to this degree over the ten-day study. In biological studies on ferrocene compounds it has been reported[48] that severe intestinal irritation can occur due to the hydrophobic nature of **11**[**Cl**]. Slides from two control animals, one from day 1 and one from day 2, showed a surface reaction due to physical injury rather than a cytotoxic response. A very small area on each had evidence of haemorrhage and a mesothelial cell response. This type of injury correlates well with a needle entering the liver tissue at the time of dosing.

In contrast to the results for **11**[**Cl**], no gross liver damage over a 10-day period was observed with **9**[**3**].

Antitumour Activity

The antitumour activities of ferrocene (reference), ligands and platinum (II) complexes against P-388 mouse leukaemia are shown in Table 8.

Table 8 Antitumour activity of some ferrocenyl derivatives and ferrocenylamine–platinum(II) complexes against P-388 mouse leukaemia tumour in BDF1 mice[a]

Compound	Dose (mg kg/injection)	MST (days)	Range (days)	*T/C* (%)	Survivors, day 30
Ferrocene	160(50)	10.5(21.8)	7–12(20–23)	91.3(189.9)	0/6(0/6)
NH_2	16(50)	13.2(20.7)	12–15(13–27)	114.5(179.7)	0/6(0/6)
6	160(50)	8.5(10.2)	5–16(17–25)	77.3(174.2)	0/6(0/6)
$CH(Me)NMe_2$	160(50)	0(21.8)	5–6(20–23)	0(189.9)	0/6(0/6)
7	160(50)	13.8(20.8)	12–17(13–27)	115.3(173.6)	0/6(0/6)
8	160(50)	6(20.7)	5–6(13–27)	50(172.2)	0/6(0/6)
Cisplatin-NH_2	32(50)	11.5(19.2)	10–13(17–25)	104.6(174.2)	0/6(0/6)
9[**3**]	32(50)	17.3(19)	16–18(17–23)	148.6(162.9)	0/6(0/6)
9[**3**][b]	400(50)	14.7(19)	7–8(17–23)	117.3(162.9)	0/6(0/6)
9[**7**]	32(50)	10.5(18.3)	6–16(17–23)	98.4(171.9)	0/6(0/6)
9[**8**]	32(50)	10.8(18.3)	9–14(17–23)	101.7(171.9)	0/6(0/6)
11[**Cl**]	32(50)	0(18)	5–6(17–19)	0(171.4)	0/6(0/6)
11[**OAc**]	16(50)	14.5(19)	12–16(17–23)	124.3(162.9)	0/6(0/6)
11[**OAc**][b]	32(50)	0(18.2)	5–6(15–26)	0(153.5)	0/6(0/6)
13	32(50)	14.67(18)	13–18(17–19)	139.7(171.4)	0/6(0/6)
13[b]	80(50)	0(18.6)	5–6(16–21)	0(149.3)	0/6(0/6)
L-1210 mouse leukaemia tumour in BDF1 mice					
13	80(50)	8.3(17.2)	5–11(15–24)	60.2(124.3)	0/6(0/6)
9[3][b]	400(17.7)	10.3(17.7)	8–12(15–24)	82.7(141.3)	0/6(0/6)
B-16 melanoma leukaemia tumour in BDF1 mice[c]					
13	80(6)	18.3(51.5)	11–21(42–60)	61.5(172.8)	0/10(2/10)
9[**3**][b]	400(6)	27(51.5)	20–37(42–60)	90.6(172.9)	0/10(2/10)

[a] All data to highest dosage for each compound; data in parentheses are for 5-fluorouracil unless stated otherwise. Mean survival time; vehicle solution = peanut oil unless otherwise stated. Dose schedule QD 159, every four days (QD) (i.e. on days 1,5 and 9 following injection of P-388 cells). *T/C* (%) represents the ratio of the median survival time of the treated animals to that of control animals, expressed as a percentage range for the controls, in sequence: 0, 11–13, 9–17, 100, 0/6.
[b] In Tween/saline
[c] Cisplatin in parentheses.

Ferrocenylamines

For evaluation, an initial percentage *T/C* of 125% is considered necessary to demonstrate activity, and a reproduced value of 125% is worthy of further study. On this basis neither ferrocene nor **1**–**8** can be considered active.

In comparison with these results, Fiorina *et al.*[19] have reported that some ferrocenyl polyamine compounds were active against P-388 lymphocytic leukaemia in mice (taking a *T/C* value of 120% as being required for activity) and 4-ferrocenylamino-glutamic acid derivatives[48,49] caused 30% inhibition of sarcoma-37 at a dose of 40 mg kg^{-1} in mice.

Platinum(II) complexes

Compounds **9**[**1**], **9**[**3**], **9**[**7**], **11**[**Cl**] and **13** were active against the P-388 mouse leukaemia tumour but inactive against the L-1210 mouse leukaemia and against B-16 melanoma. Overall **9**[**3**] has the greatest potential as an antitumour agent against the P-388. This compound showed not only the greatest activity (*T/C* = 148.57%) but this occurred at a dose (16 mg kg^{-1}) which is well below the maximum single non-lethal and non-toxic doses (25 mg kg^{-1}). Compound **9**[**7**] also showed significant activity (*T/C* = 140.63%) at the same dose level (16 mg kg^{-1}) but the maximum non-toxic dose of this compound was only 5 mg kg^{-1}. Similarly, **11**[**Cl**] was active (*T/C* = 130.16%) at a dose of 4 mg kg^{-1}, but the maximum non-toxic and non-lethal doses of this compound were <1 and 5 mg kg^{-1} respectively, and **14** required a dose of 32 mg kg^{-1} to achieve significant activity, whereas the maximum non-lethal and non-toxic doses were 25 and 5 mg kg^{-1} respectively.

None of these complexes was active against the L-1210 leukaemia or the B-16 melanoma; in contrast cisplatin had a *T/C* of 172.82% with 2/10 survivors against B-16 melanoma. The significant result from selective testing against other cell lines was the observation that **11** exhibited strong activity against cisplatin-resistant cells.

Conclusion

This comprehensive study of the toxicology of

ferrocenylamines and their platinum(II) complexes provides an important database. Many of these compounds demonstrate toxicities which could allow them to be incorporated in a clinical regime. In general, ferrocenylamines are less toxic than ferrocenyl compounds with *O*-alkyl side chains; for example, acetylferrocene is highly toxic in rats at 50 mg kg^{-1}, producing over 90% mortality, and for female rats a dose as low at 5 mg kg^{-1} is lethal to all animals.[20] There is a clear distinction in acute toxicity between the ligands **1**–**8** and their platinum(II) complexes **9**–**14**, with the latter being the most toxic. Protonation at the N-terminus is likely to be a significant factor in moderating the activity of the uncoordinated ferrocenylamine in a physiological environment, with tertiary amines being the least toxic. This distinction is blurred once the nitrogen lone pair is involved in coordination to a metal and the steric requirements of a tertiary amine, as well as the absence of N-H functionality, do not encourage biological activity in tertiary ferrocenylamine–metal complexes. Considerable variation in toxicity has also been found within polyamine[19] and glutamic acid[48] derivatives, and the combination of a non-polar tail and a protonated N-terminus lead to the observed toxicity regimes.

Liver, rather than kidney, toxicity as seen for cisplatin is the norm for the ferrocenylamine complexes. Nonetheless, the hepatotoxity results raise the question of the cause of toxicity. The hepatotoxicity of **11**[**Cl**] seen through serum enzyme elevations and pathological examination does not correlate with the equal distribution of platinum between the kidneys and the liver; with **9**[**3**], platinum is deposited to a larger extent in the liver yet it does not cause hepatotoxicity. That is, the deposition of platinum is not solely responsible for the toxicity. Other factors which may be involved include the ease of oxidation to the ferrocenium analogue, the hydrophobic character of the N-substituent and the platinum(II) stereochemistry; one or all may be involved in determining how the compounds interact with the cell. It is also not clear whether all complexes remain intact when passed to the liver and the gross liver damage with cycloplatinated complexes, but not **9**, may be a consequence of the difference in physiology *in vivo*.

Neither the ferrocenylamine nor the platinum (II) complexes show significant cytotoxic properties, except for the activity of cycloplatinated complexes against cisplatin-resistant tumours, but they do have a spectrum of other biological activity and are capable of being radiochemically labelled. A disadvantage of these compounds is their insolubility in water, which leads to the appearance of unwanted side effects (irritation of the intestinal tract etc.). Furthermore, the non-availability of water-soluble compounds has hindered mechanistic work on the action of ferrocenylamines at the cellular level. These difficulties have now been overcome with the synthesis of soluble analogues of the complexes described in this paper (J. Kerr, D. Weston and J. Landells, unpublished work).

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