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Genotoxicity and cell cycle effects of platinum-based imaging agents in cisplatin-resistant human tumour cells

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Summary. A series of *cis*-dichloroplatinum(II) 2,3-diaminopropionamide complexes synthesised as potential imaging agents was tested for activity against a human ovarian tumour cell line (CI-80-13S) with high natural resistance to cisplatin and carboplatin as compared with other human cells. The most potent compound, the dimethyl ester of dichloro-[4-(methyleneiminodiacetic acid)phenyl (2',3'-diamino-propionamide)]platinum(II) (complex III), exhibited toxicity towards CI-80-13S cells similar to that observed in other cell lines, an effect that was not shown by the ligand alone or by *cis*-dichloroplatinum(II) 2,3-diaminopropionamide. However, complex III ester reproduced the genotoxic effects of cisplatin as judged by differential inactivation of two strains of adenovirus and by inhibition of cellular DNA and RNA synthesis; no major differences in these properties were observed between CI-80-13S and cisplatin-sensitive cells. Substantial inhibition of DNA and RNA synthesis was found within 2 h of treatment, much earlier than the effect of cisplatin. Complex III ester, which was 30- to 100-fold less potent than cisplatin, inhibited cell cycle progression in a similar way to equitoxic cisplatin, with cells accumulating in G_2 at a dose of low toxicity and being arrested in all stages at higher levels. The latter in combination with colcemid caused extensive fragmentation of CI-80-13S cells. These results suggest that the mechanism of toxicity of such complexes involves factors, in addition to DNA damage, which rapidly inhibit nucleic acid synthesis and overcome natural resistance to cisplatin in the CI-80-13S cell line.

Introduction

Drug resistance is a major obstacle to achieving successful chemotherapy of solid tumours such as ovarian cancer, where a significant proportion of patients respond to DNA cross-linking agents, including cisplatin, but often become resistant to most available drugs [4, 5, 14]. Of the many cisplatin analogues tested for tumour activity [3, 12, 20], carboplatin appears to be one of the more useful owing to fewer side effects [12]. Its utility for treating resistant tumours, however, has not yet been demonstrated.

Three new platinum complexes (I–III, Fig. 1) were recently synthesised and shown to have potential as tumour scintigraphic agents in the form of ^{99m}Tc complexes [1]. These compounds contain both an iminodiacetic acid group for chelating ^{99m}Tc and the 2,3-diaminopropionamide group for complexing platinum dichloride in the *cis* configuration. The presence of the latter moiety, not previously tested as a ligand for anticancer effects, and its selective uptake by the EMT6 murine sarcoma [1] prompted a study of the chemical toxicity and selectivity of these compounds (I–III) in human tumour cell lines in vitro as compared with cisplatin and carboplatin. Although less potent overall than cisplatin, some of these compounds were found to be active against human tumour cells naturally resistant to cisplatin.

Materials and methods

Complexes I [dichloro[4-hydroxy-3-(methyleneiminodiacetic acid) phenyl (2', 3'-diaminopropionamide)]platinum(II)], II [dichloro[3-(methylene-iminodiacetic acid)phenyl (2',3'-diaminopropionamide)] platinum (II)], III [dichloro-4(methyleneiminodiacetic acid) phenyl (2',3'-diaminopropionamide)]platinum (III)] and their derivatives were synthesised as previously described [1].

The origins of HeLa-S_3 and the human melanoma cell line MM96L have been described elsewhere [6, 18]. The MM474F and MM489F strains of normal fibroblasts were established from human melanoma biopsies. The human ovarian tumour cell line CI-80-13S [2], kindly provided by Dr. R. Bradley (Cancer Institute, Melbourne), was derived from a 71-year-old woman with stage IV metastatic ovarian carcinoma and no history of chemotherapy. The human ovarian tumour lines GG [16] and JAM [17] were obtained from Dr. B. Ward and Dr. I. Hayward, University of Queensland.

Cells were cultured in 5% CO_2/air at 37°C in Roswell Park Memorial Institute (RPMI) medium 1640 (Flow Laboratories, Sydney,

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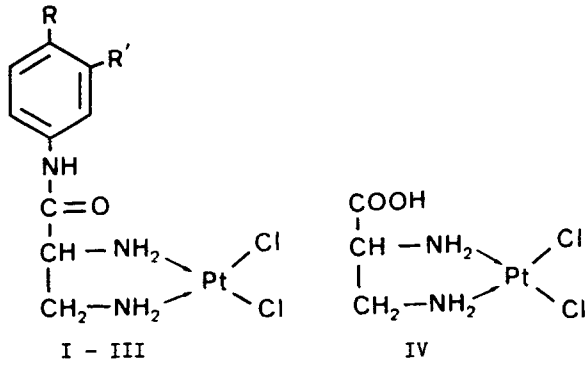

Fig. 1. Structures of potential tumour scintigraphic agents. Complex I: R = OH; R^1 = $CH_2N(CH_2CO_2H)_2$. Complex II: R = H; R^1 = $CH_2N(CH_2CO_2H)_2$. Complex III: R = $CH_2N(CH_2CO_2H)_2$; R^1 = H

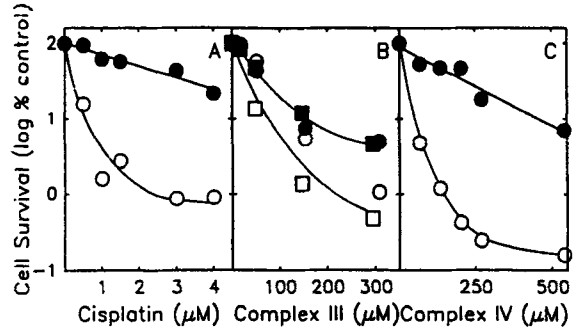

Fig. 2. Toxicity of platinum complexes in human tumour cells determined by pulse-labelling cultures with [^{3}H]-thymidine 5–7 days after commencement of treatment. *Panels A–C:* ●, CI-80-13S; ○, HeLa. *Panel B:* ■, CI-80-13S and complex III dimethyl ester; □; HeLa and complex III dimethyl ester

Table 1. Toxicity of platinum complexes in human cells

Cell	D_{37}(μM)							
	Cisplatin	Carbo-platin	I	II	III	III ester	2–3 diamino-propionic acid complex (IV)	III ester without Pt
CI-80-13S	2.5 ±0.6[a]	12 ±1.4	387±190	172±56	81±20	61±27	213±30	410
GG	0.82±0.36					52	73	
JAM	0.52±0.15					55	73	
HeLa	0.2 ±0.07	2.0±0.2	153± 10	23	53±11	31± 4	19	135
MM96L	0.65±0.22		347	51			70	
MM489F	0.33				149	77		
D_{37} ratio:								
CI-80-13S/HeLa	12	6	2.5	7.5	1.5	2.0	11	3.0

[a] Mean ± SE (n = 2–6)

Australia) supplemented with 1 m*M* pyruvate, 200 μM nicotinamide, 100 IU/ml penicillin, 100 μg/ml streptomycin, 3 m*M* 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid and 10% fetal calf serum. Assays for *Mycoplasma* by culture on agar were negative.

Because the CI-80-13S line did not form discrete colonies readily countable by eye, cell survival was determined by a modified colony assay. This method, which gives results similar to those attained by visual counting of colonies for a variety of agents [6], involved the addition of drug to duplicate cultures (2×10^3 cells/16-mm well) seeded 24 h previously and, after 5–7 days of continuous exposure, labelling the cultures with [^{3}H]-thymidine for 2–4 h. Cells were detached [0.02% trypsin and 0.1 m*M* EDTA in phosphate-buffered saline (PBS)], lysed with water and harvested onto glass-fibre disks for liquid scintillation counting. The D_{37} (dose required to give 37% survival), D_0 (dose giving a reduction of 0.37 on the linear portion of the survival curve), and D_q (size of the shoulder) were calculated from dose-response curves obtained using five doses.

In the virus experiments, partially purified adenovirus 5 was treated with drug in PBS for 20 h at 37° C, after 100-fold dilution to give 4 m*M* NaCl. Duplicate cultures (5×10^3/6-mm well) were infected with 10-fold dilutions of virus for 1 h and then washed once with medium. Viral replication at 32° C was determined after 2 days by counting the number of virus-infected, immunoperoxidase-labelled cells [9]. One ID was defined as the amount of virus required to produce one infected cell. Dose responses of virus replication were obtained as described for cell survival. Virus (1/10 dilution) was treated with the agent in PBS, dialysed overnight against PBS and then diluted further as described above for replication in cells. The virus strains were Ad5wt (wild type) and Ad5ts125, a temperature-sensitive mutant replicating at 32° C but not at 39.5° C due to a mutation in its DNA binding protein [19].

Cellular DNA and RNA synthesis was measured in cultures (5×10^4 cells/16-mm well) given drug treatment for the periods described above and then pulse-labelled for 45 min with [2-^{14}C]-thymidine (0.005 μCi/ml; 20 Ci/mol) and [5-^{3}H]-uridine (5 μg Ci/ml; 40 Ci/mmol). The cells were detached with trypsin, lysed and harvested onto glass-fibre discs with H_2O and solubilised in Soluene 350 (Packard Instruments, Zurich, Switzerland) prior to liquid scintillation counting.

To determine the effect of drugs on cell cycle progression, cells (3×10^5/60-mm plate) were treated for 48 h, detached with trypsin and stained with a mixture of propidium iodide (50 μg/ml), RNase (100 μg/ml) and Triton X-100 (1%) in PBS. DNA content was analysed with a FACS IV flow cytometer (Becton Dickinson, Sunnyvale, Calif) operated at 488 nm. The proportion of cells in each phase of the cell cycle was determined from the relevant area of the DNA histogram.

Results

Cell survival

As previously found [10], the CI-80-13S ovarian tumour line, which had a near diploid DNA content and a doubling time of 30 h, was highly resistant to cisplatin compared with other human cells, including normal fibroblasts and

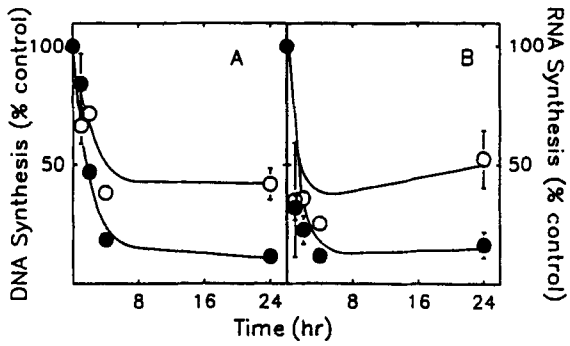

Fig. 3. Temporal response of DNA synthesis (*A*) and RNA synthesis (*B*) to treatment with 160 μM complex III ester. ●, CI-80-13s; ○, HeLa

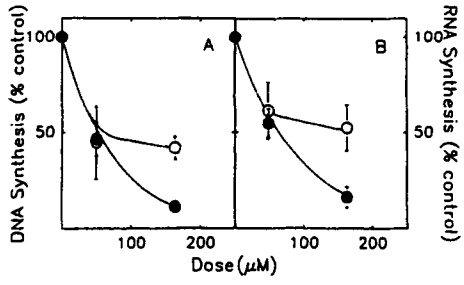

Fig. 4. Dose response of DNA synthesis (*A*) and RNA synthesis (*B*) to 24 h treatment with complex III ester. ●, CI-80-13S; ○, HeLa

Table 2. Effect of platinum complexes on cell cycle progression

Treatment[a]	CI-80-13S:			HeLa:		
	G_1	S	G_2	G_1	S	G_2
Control	72[b]	8	20	61	12	27
Colcemid (2.5 μM)	11	7	82	0	0	100
Cisplatin (5 μM)	44	10	46	59	17	24
Cisplatin (5 μM) + colcemid (2.5 μM)	13	9	78	55	27	18
Complex III ester (50 μM)	71	9	20	23	5	72
Complex III ester (50 μM) + colcemid (2.5 μM)	14	10	76	14	6	80
Complex III ester (160 μM)	58	11	31	38	12	50
Complex III ester (160 μM) + colcemid (2.5 μM)	11[c]	11[c]	78[c]	46	8	46

[a] 48 h for platinum complexes, last 24 h for colcemid

[b] Percentage of total cells in each phase of the cell cycle

[c] 60% of the DNA histogram consisted of cell fragments (<G1 DNA content)

tumour cell lines such as HeLa and MM96L, which have a similar doubling time (Fig. 2A, Table 1). All of the new complexes studied were less toxic than cisplatin and several (complexes II and IV) offered no advantage over cisplatin in killing the highly resistant CI-80-13S line, relative to sensitive cell lines such as HeLa (Fig. 1, Table 1). However, complex III and its dimethyl ester, although less potent than cisplatin, exhibited a level of toxicity to CI-80-13S close to that for HeLa cells (Fig. 2B), as did the hydroxy derivative (complex I). This property did not arise solely from the ligand itself because the platinum-free compound was less potent overall and relatively less toxic to CI-80-13S cells. Complex III ester was chosen for further study because it was generally the most potent and achieved the best killing of the cisplatin-resistant cell line, relative to HeLa cells.

Inhibition of DNA and RNA synthesis

The temporal response of nucleic acid synthesis to treatment by complex III ester in HeLa and CI-80-13S cells showed inhibition during the first 4 h of treatment (Fig. 3). Significant inhibition of RNA synthesis was also found, occurring slightly earlier than DNA synthesis; the effects in each case were slightly more pronounced in the CI-80-13S line at 24 h. A dose-response study involving 24-h treatment showed that inhibition of nucleic acid synthesis occurred at ester doses of minimal toxicity (Fig. 4), the enhanced inhibition in CI-80-13S being observed only at a dose of 160 μM.

Effect of complex III ester on cell cycle progression

For determining the influence of platinum complexes on the cell cycle, cells were treated with the drug for 48 h, a period that would enable maximal effects to be demonstrated in cells having a doubling time of 24–30 h. The mitotic inhibitor colcemid was added to some cultures during the last 24 h to determine whether the cells were still cycling. Used alone, colcemid caused both cell lines to accumulate in G_2 (Table 1).

At a supratoxic level, cisplatin caused CI-80-13S to accumulate in G_2 but had little apparent effect on HeLa (Table 2). In the latter case, however, the DNA distribution was insensitive to colcemid, indicating that HeLa cells were "frozen" in all stages of the cycle. At 50 and 160 μM, the complex III ester had little effect on the cycling of CI-80-13S cells except that some accumulation in G_2 was observed at the higher dose. In combination with colcemid, it caused extensive fragmentation of G_1 cells, as indicated by 60% of the histogram showing a DNA content less than that of G_1; G2 cells formed the majority of the remainder. HeLa cells were blocked in the G_2 phase at both drug levels, the higher dose showing some evidence for freezing in the other stages when colcemid was used.

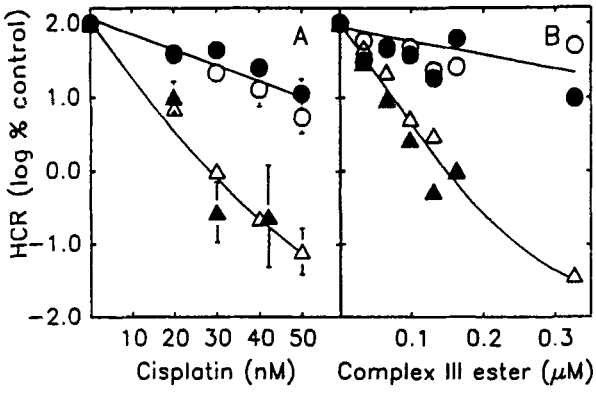

Fig. 5. HCR of drug-treated adenoviruses Ad5wt (○, ●) and Ad5ts125 (△, ▲) in CI-80-13S (●, ▲) and HeLa (○, △)

Inhibition of adenovirus replication

Host cell reactivation (HCR) of cisplatin-treated SV-40 in human cells has been used to demonstrate genotoxicity of the drug and deficient DNA repair [11]. Adenovirus is a double-stranded DNA virus that replicates readily in human cells and has the potential to be used similarly. Previous studies (submitted for publication) have shown that at the low chloride concentration corresponding to intracellular levels [20], cisplatin was highly toxic to wild-type adenovirus (Ad5wt) and even more so to a ts mutant (Ad5ts125). Carboplatin was less potent than cisplatin but exhibited a parallel selectively (Table 3). Similar results were obtained with the complex III ester (Table 3), its D_{37} for virus replication being lower than that for cell survival and overall less potent than that of cisplatin. No difference in HCR of treated virus was found between the two cell lines (Fig. 5).

Discussion

The present study showed that a new type of platinum complex inactivates the human and adenovirus genomes in a manner similar to that observed for cisplatin yet has the important distinction of being effective against cisplatin-resistant human tumour cells. Although only a limited number of structural types were studied, the latter phenomenon appears to depend on the presence of an aromatic ring substituted in a precise manner, because the diaminoproprionic acid complex IV and the *meta*-substituted complex II resembled cisplatin rather than complex III in selectivity.

Complex III ester, chosen as a prototype structure for further study, reproduced the action of cisplatin in that it inhibited DNA and RNA synthesis and was selectively toxic to the Ad5ts125 strain of adenovirus 5. Considering previous reports of inhibition of DNA synthesis and virus replication by cisplatin [11, 13, 15], these effects are consistent with a mode of action dependent on DNA damage. However, it is of interest that DNA synthesis was inhibited within 2 h by complex III ester vs >4 h by cisplatin [13]. In addition, the CI-80-13S line, which was slightly more resistant than HeLa in cell survival, was slightly more susceptible to inhibition of nucleic acid synthesis by this drug. It therefore seems likely that complex III ester kills cells by a combination of two different mechanisms, one involving DNA cross-linking as for cisplatin and the second associated with rapid inhibition of DNA and RNA synthesis. The CI-80-13S line would appear to be susceptible to the second mechanism. The fact that cisplatin "froze" HeLa cell cycle progression and caused only a partial accumulation in G_2 in the much more resistant CI-80-13S cells was consistent with the known dose-dependent effects of such agents on the cell cycle, presumably resulting from various degrees of template damage [13]. The extensive cell fragmentation induced by the combination of colcemid and complex III ester in CI-80-13S cells at a toxic dose (160 μM) that blocked HeLa cells in G_2 is further evidence that the drug has a different mechanism of toxicity in the former cell line.

Cellular damage caused by complex III ester either is not recognised by the cisplatin resistance mechanism in CI-80-13S or kills cells in a different way than does cisplatin. The mechanism of cisplatin resistance in CI-80-13S cells does not involve a DNA repair difference detectable by a viral probe, as no difference in the HCR of drug-treated virus was found, and is unlikely to involve inhibited transport because the cells were resistant to other cross-linking agents of different structures as well as to topoisomerase II inhibitors [10]. This leaves intracellular SH [7, 8] or DNA inaccessibity to be considered as resistance mechanisms bypassed by the complex III ester. The latter seems the more likely, due to steric constraints expected for any agent binding to DNA in chromatin vs viral DNA. Alternatively, it is possible that DNA binding is an insignificant cause of complex III toxicity and that the critical target is a metalloprotein, with binding of the platinum to an SH group and that of the iminodiacetic acid moiety to a metal ion. The iminodiacetic acid group would be expected to be available from hydrolysis of the ester within the cell by esterases.

Table 3. Inhibition of the replication of adenovirus strains by platinum complexes[a]

Cell line	D_{37} (μM):					
	Cisplatin		Carboplatin		Complex III ester	
	Ad5wt	Ad5ts125	Ad5wt	Ad5ts125	Ad5wt	Ad5ts125
CI-80-13S	0.024	0.007	>2	0.24	0.11	0.026
HeLa	0.018	0.007	>2	0.31	0.16	0.040

[a] Virus was treated with the drug and then used to infect untreated cells at 32° C as described in Materials and methods

Low potency in cell toxicity may be a limitation to direct use of the present series of complexes in in vivo models. The complex III ester was 10-fold less effective than cisplatin for inactivating adenovirus and 24-fold lower in cellular toxicity, presumably due to less efficient access to cellular DNA. Further study of structure/activity relationships may enable potency to be increased, perhaps by complexing a radioactive isotope with the iminodiacetic acid moiety while retaining activity against the cisplatin-resistant phenotype.

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