The cytotoxic action of four ammine/amine platinum(IV) dicarboxylates: a flow cytometric study

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Summary We have used flow cytometry to study the mechanism of cytotoxic action of a series of ammine/amine Pt(IV) dicarboxylates [ammine diacetatodichloro(cyclohexylamine) platinum(IV), JM216; ammine dibutyrytadichloro(cyclohexylamine) platinum(IV), JM223; ammine dibenzoatodichloro(propylamine) platinum(IV), JM234]. JM216 has been shown to have clinical potential and has recently entered phase II trials. All the compounds caused a slowdown in S-phase transit followed by a block in G2. Cells died either through apoptosis (largely during S-phase) or by failing to overcome the G2 block (some days after treatment). In G2, the cells either divided or enlarged and died. At equitoxic doses, JM216 showed the most apoptotic cells and had the most platinum bound to DNA. JM223 showed the fewest apoptotic cells and had the least platinum bound to DNA. We suggest that whether apoptosis was triggered or not was governed by the total amount of Pt bound to the DNA; the type of lesion was more important in determining whether a cell became blocked in G2.

Keywords: Pt(IV) dicarboxylates; apoptosis; flow cytometry; cell cycle

Cis-dichlorodiamineplatinum(II) (cisplatin) is of major importance in the treatment of cancer, particularly ovarian and testicular carcinomas (Loecher and Einhorn, 1984; Wiltshaw and Carr, 1984). Unfortunately, its value is limited by its toxicity and the frequency with which tumours develop resistance. While the side-effects of cisplatin have been circumvented by the development of carboplatin, both drugs show a similar pattern of resistance in a wide range of tumours (Harrap, 1985; Gore et al., 1989; Mangioni et al. 1989). The search for a third-generation platinum compound, which does not show cross-reactivity with cisplatin and carboplatin, has led to the development of a series of platinum(IV) ammine/ammine dicarboxylates of general structure, c, t, c-[PtCl2(OCOR)2NH2(RNH2)] (Harrap et al., 1991a,b; Kelland et al., 1992a). One of these new compounds [ammine diacetatodichloro (cyclohexylamine)-platinum(IV), JM216], which has been shown to have clinical potential (Kelland et al., 1993), has recently entered phase II trials.

In human ovarian cell lines, it has been shown that the cytotoxicity could be increased by increasing the number of carbons in the dicarboxylate ligand (R); cytotoxicity was also increased by placing an alicyclic group in the amine ligand (Kelland et al., 1992b). Two of the dicarboxylates [ammine dibutyryatodichloro(cyclohexylamine)platinum(IV), JM232; and ammine dibenzoatodichloro(propylamine)platinum(IV), JM244] were capable of overcoming acquired cisplatin resistance, which is caused by decreased intracellular accumulation but were not able to overcome resistance at the level of DNA platination and removal (Kelland et al., 1992b). The possible value of this class of compound in overcoming cisplatin resistance has been supported by a recent study of 17 ammine/ammine Pt(IV) dicarboxylates using the murine leukaemia cell line, L1210, and a cisplatin-resistant subline in which it was found that a wide range of these compounds overcame resistance to cisplatin (Orr et al., 1994).

It is important to learn more about the mechanism of action of the new compounds, particularly in relation to cisplatin. Since attention has recently been focused on the role of apoptosis in drug-induced cytotoxicity (Barry et al., 1990; Dive and Hickman, 1991; Hickman, 1992), we have investigated the contribution of apoptosis to cell killing by cisplatin. In a human ovarian carcinoma cell line, CH1, cisplatin induced apoptosis over the whole dose range studied (Ormerod et al., 1994b). In L1210 cells, the data were consistent with a dual mechanism of cell death—higher doses of drug led to rapid death through apoptosis; lower doses led to death at later times resulting from a failure to overcome a block in G2 of the cell cycle (Ormerod et al., 1994a).

As part of our programme of drug development, using the murine leukaemia cell line, L1210, we have undertaken a comparative study of the mechanism of toxic action of four platinum(IV) ammine/ammine dicarboxylates, namely, JM216, JM223 and JM244 (structures, see Table I). These compounds were chosen as representative of the range of compounds of this class, in that the amine contained either an alicyclic (JM216 and JM223) or an alkyl (JM223 and JM244) group and the axial ligand either an aryl (JM244) or an alkyl substituent (JM216, JM223 and JM244). Flow cytometry was used to follow changes in the cell cycle parameters after incubation with drug and to measure the induction of apoptosis.

Materials and methods

Chemicals

The Pt(IV) dicarboxylates were supplied by the Johnson Matthey Technology Centre (Reading, Berks, UK) and the Johnson Matthey Biomedical Research Center (West Chester, PA, USA) (Giandomenico et al., 1991). Cell culture medium and serum were purchased from ICN Flow (High Wycombe, Bucks, UK), agar noble from Difco Laboratories (Detroit, MI, USA) and all other reagents from Sigma (Poole, Dorset, UK).

Cells and drug treatment

The L1210 murine leukaemia cell line was grown as a suspension culture in RPMI-1640 medium supplemented with 10% horse serum, 2 mM L-glutamine and antibiotics (100 U ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin) (cell doubling time = 14 h).
The Pt(IV) dicarboxylates were dissolved in ethanol, with the exception of JM216 which was dissolved in unsupplemented medium. The final concentrations of ethanol (0.5%) in the cultures did not inhibit growth over 48 h. All experiments were initiated at a cell density of $2 \times 10^5$ ml$^{-1}$. Following exposure to drug at different concentrations for 2 h, cells were centrifuged at 800 $g$ for 5 min, washed once with medium and resuspended in fresh medium.

In cell survival assays, triplicate cultures of cells were exposed to drug for 2 h, washed, resuspended in fresh medium and serially diluted to $2 \times 10^5$ cells ml$^{-1}$. Duplicate aliquots (2 ml) were added to polystyrene tubes containing 3 ml of medium supplemented with 20% horse serum and 0.2% agar at 42°C. Tubes were plunged into iced water to set the agar, incubated at 37°C for 7 days and colonies counted. Plating efficiency of control cells was 78%. The concentration of drug required to reduce colony formation by 50% (IC$_{50}$) was recorded.

### Determination of platinum associated with whole cells and with DNA

The Pt content of cell sonicates of extracted DNA was measured by flameless atomic absorption spectroscopy as has been fully described by Orr et al. (1994) and Nicholson et al. (1992). The results were expressed as nmol Pt g$^{-1}$ protein or nmol Pt g$^{-1}$ DNA.

### Flow cytometry

Flow cytometric measurements were made either on an Ortho Cytofluorograf 50H or a Coulter Elite ESP, both instruments using Spectra-Physics argon-ion lasers tuned to produce either 200 mW at 488 nm or 100 mW in the UV. On the Cytofluorograf, data, normally from $2 \times 10^6$ cells, were acquired and analysed on an Ortho 2150 computer system. Univariate and bivariate histograms (the latter referred to as cytograms) were transferred to an IBM compatible PC and figures prepared using our own software (written by MGO). For the figures, the frequency scale was adjusted to optimise the display of the data. On the Elite, data were acquired on an IBM-PC compatible computer. Figures were prepared using the WINMDI program supplied by Dr Joe Trotter, Salk Institute, USA.

On the Ortho Cytofluorograf, five detectors were available recording, in a forward direction, scattered light and, orthogonally, blue (488 nm, scattered light; or 460 nm, fluorescence), and green (520 nm), orange (570 nm) and red (>630 nm) fluorescence. A similar optical arrangement was used on the Coulter. If the red fluorescence was measuring DNA, then both the peak and the integrated area of the fluorescent signal were recorded and pulse shape analysis was performed to eliminate any cell clumps (Ormerod, 1994).

For cell cycle analysis, approximately $10^5$ cells were fixed in ice-cold 70% ethanol and stored at 4°C. After washing, cells were resuspended in 800 ml phosphate-buffered saline (PBS) and 100 ml propidium iodide (PI) solution (100 $\mu$g ml$^{-1}$) and 100 ml RNase solution (1 mg ml$^{-1}$) added before incubation for 2 h at 37°C. The flow cytometer was operated at 488 nm and, after pulse shape analysis and gating on a cytogram of orthogonal vs forward light scatter, either a histogram of cell number against red (DNA-PI) fluorescence or a cytogram of light scatter vs DNA was recorded. Cell cycle analysis was carried out using either our own program (data recorded on the Cytofluorograf; Ormerod et al., 1987) or the MultiCycle program (Phoenix Flow Systems, San Diego, CA, USA) (data recorded on the Elite).

The fraction of apoptotic cells was estimated from the 'sub-G1' peak in the DNA histogram. If apoptotic cells undergo internucleosomal degradation, on fixation, the cells lose low molecular weight DNA and give a peak in the DNA histogram of lower fluorescence than cells in G$_1$ of the cell cycle (see, for example, Nicoletti et al., 1991; Ormerod et al., 1992; Darnyzkiewicz et al., 1993). We have shown previously that apoptotic L1210 cells undergo internucleosomal degradation and produce such a peak in the DNA histogram (Ormerod et al., 1994a). The fixed apoptotic L1210 cells also had smaller light scatter and the analysis could be improved by setting a region on a cytogram of right angle light scatter vs DNA.

To measure cell cycle progression, 50 $\mu$m bromodeoxyuridine (BrdUrd) was added to the cultures. Samples were taken at different times, the cells centrifuged and resuspended in ice-cold 100 mM Tris-HCl, 154 mM sodium chloride, 1 mM calcium chloride, 0.5 mM magnesium chloride, 0.1% (v/v) Nonidet-P40, 0.2% (w/v) bovine serum albumin, 1.2 $\mu$g ml$^{-1}$ Hoechst 33258, pH 7.4; PI was added to a final concentration of 2 $\mu$g ml$^{-1}$ (Poot and Ormerod, 1994). UV radiation was used for the flow cytometric analysis, which was performed on the Ortho Cytofluorograf. After gating on a cytogram of peak vs area of the red fluorescent (PI-DNA) signal, a cytogram of red vs blue (Hoechst-DNA) fluorescence was recorded.

### Results

#### Cell survival

The survival of L1210 cells, as measured in a soft agar colony assay, after incubation with the four compounds for 2 h is shown in Figure 1. The values of the IC$_{50}$ together with the structures of the compounds are given in Table I. The concentration of drug needed to achieve the same level of cytotoxicity varied by a factor of 100–JM216 requiring the most drug, JM244 the least.

#### Platinum uptake and platination of DNA

Table II shows the amount of platinum associated with DNA and with the cells after a 2 h incubation with the four drugs at $10 \times$ IC$_{50}$. There was significantly more Pt bound to DNA after treatment with JM216 compared with JM244 but the difference, which was reflected in the amount of intracellular Pt, was less than 3-fold. There was a smaller, but also significant, difference between JM216 and JM221.

#### Cell cycle analysis of fixed cells

After a 2 h incubation with the four compounds, cells were collected at different times, fixed in ethanol, stained with PI and their DNA histograms analysed (Figure 2). At $10 \times$ IC$_{50}$ by 6 h after treatment with JM216, there was substantial apoptosis, the apoptotic cells giving a cluster with less DNA ('sub-G1' peak) and lower light scatter. Although apoptosis

### Table I Structures of four amine/ammine Pt(IV) dicarboxylates

| Compound | $R_1$     | $R_2$     | IC$_{50}$ ($\mu$M) |
|----------|-----------|-----------|------------------|
| JM216    | Methyl    | Cyclohexylamine | 7.4              |
| JM221    | n-Propyl  | Cyclohexylamine | 0.44             |
| JM223    | n-Propyl  | Isobutylamine | 0.44             |
| JM244    | Phenyl    | Propylamine  | 0.065            |

IC$_{50}$ for cisplatin = 2.1 $\mu$M
could be detected after incubation with 10 × IC₅₀ of other drugs, there were significantly less apoptotic cells present (Figure 3). For all four drugs, at 3 × IC₅₀, no apoptosis could be detected (Figure 3 for JM216, data not shown for the other drugs). The cells that did not die during the first 24 h underwent a G₂ delay, as is demonstrated in a plot of the fraction in each phase of the cell cycle vs time (Figure 4). By 24 h, the arrested cells showed increased light scatter, presumably caused by an increase in cell size, as has been observed after incubation with cisplatin (Sorenson et al., 1990; Ormerod et al., 1994a). By 48 h, cycling cells of normal light scatter were again evident. The cell cycle effects after incubation with the drugs at 3 × IC₅₀ were similar, but less marked, to those at 10 × IC₅₀ (data not shown).

Table II The amount of Pt associated with the cells and the amount bound to DNA after a 2 h incubation of L1210 cells with four ammine/ammine Pt(IV) dicarboxylates

| Drug  | nmol Pt g⁻¹ protein | nmol Pt g⁻¹ DNA |
|-------|---------------------|-----------------|
| JM216 | 840 ± 65            | 65 ± 16         |
| JM221 | 378 ± 40            | 31 ± 8          |
| JM223 | 333 ± 40            | 37 ± 27         |
| JM244 | 237 ± 30            | 24 ± 9          |

The dose of drug was 10 × IC₅₀ in each case. The results of two measurements are shown for Pt associated with the cells. Standard deviations are shown for Pt bound to DNA (n = 3). The amount of platinum bound to DNA after incubation with JM216 was significantly higher than that bound after incubation with JM244 (P < 0.05). The amount of platinum associated with the cells after incubation with JM216 was significantly higher than that associated after incubation with either JM221 or JM244 (P < 0.05). All other comparisons showed no significant difference (P > 0.05).

Figure 1 Cell survival of L1210 cells following a 2 h exposure to JM216 (△), JM221 (○), JM223 (■) or JM244 (●). Survival was measured by a soft agar colony assay and the determinations were performed in triplicate. The error bars represent ± the standard deviation and are shown when they are larger than the symbols.

Figure 2 Flow cytometric cytograms (RALS against PI/DNA red fluorescence) of L1210 cells at different times after a 2 h incubation with Pt(IV) compounds at 10 × IC₅₀. The numbers on the top of the Figure represent the time in h. The positions of cells in G₁, G₂ and apoptotic cells (Ap) are marked on the DNA axis. The cells were fixed in 70% ethanol and, after rehydration, stained with PI. Coulter Elite, 488 nm excitation.
Although there was some variation between experiments in the percentage of apoptotic cells observed after a given dose of drug, it was consistently observed that, at equitoxic doses, the percentage of apoptotic cells followed the progression JM216 > JM221 ≈ JM223 > JM244.

**Cell cycle progression**

Progression of cells through the cycle was followed by incubating the cells continuously in BrdUrd after a 2 h incubation with drug. Permeabilised cells were stained with the DNA-binding dyes, Hoechst 33342 and PI. The red fluorescence (DNA-PI) identified the cell cycle compartment, while the blue fluorescence (DNA-Hoechst) was quenched by BrdUrd and identified those cells which had taken up the thymidine analogue (Rabinovitch et al., 1988). A detailed description of the application of this method to asynchronous cells has been given by Ormerod and Kubbies (1992), Poot and Ormerod (1994) and Ormerod (1994).

Figure 5 shows cytograms obtained from untreated cells.
At time 0, G1-, S- and G2/M-phases of the cell cycle could be identified from both the red (PI) and blue (Hoechst) fluorescence. After 3 h in BrdUrd, cells originally in G2/M had divided and moved into G1 (unlabelled). Cells in S-phase showed increasing red fluorescence with cell cycle progression, but no increase in blue fluorescence (quenched by the BrdUrd) and reached the region labelled G2*. At 6 h, all the cells now in S had been in G1 at time 0 h (S1 on Figure 5); some had progressed as far as G2/M (G2f) and divided again (marked G2f). Cells which had begun the experiment in S-phase and had now reached G1 are labelled G1*. At 9 h, there were few cells which had not left G1. Cells which had completed one cycle (G1) were clearly visible.

The effects of incubation with drug are illustrated at three time points for JM244 at 10 × IC50 and 3 × IC50 (Figure 6). Three hours after incubation with both doses of drug, the major effect observed was a slowdown in movement of cells through S-phase, while most of the cells in G2/M at the time of treatment had divided. At 6 h, after 3 × IC50, some cells from late S-phase had progressed back to G1 (9% of the total); cells treated in mid and early S-phase were held up in late S/G2. By 9 h, most of the cells which had been in G1 and G2/M at time 0 had progressed into S (S1), some had reached G2 and become blocked there (G2f). Most cells treated in mid and late S-phase had overcome any G2 block and divided. (Cells treated in S-phase, which had become blocked in G2, would have been to the right of the position marked G2f in Figure 6). After 10 × IC50, the same pattern was observed, but the general slowdown in cell cycle progression was even more marked. After 9 h, cells from G1 and early S had still to reach G2.

Incubation with the other drugs at 3 × and 10 × IC50 gave similar results, except that apoptotic cells were evident after the higher concentration of drug (Figure 7). The number of apoptotic cells observed was greatest with JM216 and least with JM244.

The BrdUrd-Hoechst/PI method was also used to explore the fate of cells, which became blocked in G2 (Figure 8). Cells were incubated with 10 × IC50 JM221 for 2 h, washed, and either BrdUrd was added immediately or they were incubated for a further 24 h, when BrdUrd was added. Incubation with BrdUrd for 24 h showed that the large majority of the cells in G1 were cells that had been exposed to the drug in G1 or G2/M of the cell cycle (G2f in Figure 8). Most of the cells in G1 had divided during the previous 24 h (G2f). Addition of BrdUrd 24 h after incubation with drug showed that there were two populations of cells, one cycling normally, the other blocked in G2 and disappearing from the culture.

![Figure 5](image-url) L1210 cells incubated continuously with BrdUrd. Cytograms of red (PI-DNA) vs blue (Hoechst-DNA) fluorescence after staining permeabilised cells with Hoechst 33352 and PI. The cell cycle phases are marked. G2f marks those cells in G2/M which were in S-phase at the time of addition of BrdUrd; S1 cells which were initially in G1 and had moved into S-phase; G1* cells which were initially in S-phase and had divided and were in G1, G1* cells which were initially in G1 and had cycled through to G1 after addition of BrdUrd and S and G2f are cells in their second cycle. The numbers on the cytograms give the time in h after addition of BrdUrd.
normally cycling cells are in the compartment marked $G_1$, $G_2$, $G_2'$ and $G_2^*$ in the two bottom panels in Figure 8. After a total of 48 h (24 h with BrdUrd), the cells blocked in $G_2$ had almost completely disappeared from the culture. If they had divided, there would have been cells in the compartment, $G_1$, so it can be concluded that these cells had died.

**Figure 6** L1210 cells incubated continuously with BrdUrd after a 2 h incubation with JM244 at $10 \times IC_{50}$. Cytograms of red (PI-DNA) vs blue (Hoechst-DNA) fluorescence after staining permeabilised cells with Hoechst 33352 and PI. The cell cycle phases are marked according to the description given in Figure 5. The numbers on the cytograms give the time in h after addition of BrdUrd.
A major feature of the survival data in Figure 1 was the large differences in the concentration of drug needed to achieve the same level of cytotoxicity. JM216 was used at 100 times the concentration of JM244 with JM221 and JM223 lying in between. A large part of the differences would have been caused by differential uptake of the drugs by cells and by their intracellular metabolism. When the reaction of the drug with the cell, as measured by the amount of intracellular platinum or the platinum bound to DNA, is measured at equitoxic doses, the difference is only 3-fold. At equitoxic doses, the platinum bound to the cell had the same rank order as the concentration of drug added (JM216 > JM221 ≈ JM223 > JM244). The ratio of the amount of Pt g⁻¹ protein to Pt g⁻¹ DNA was approximately

Figure 7 L1210 cells were incubated with JM216, 221 or 223 at a concentration equivalent to either 3 × IC₅₀ or 10 × IC₅₀ for 2h, washed and then incubated with BrdUrd for 6h. Details as in Figure 5. Ap marks apoptotic nuclei.
the same for the four drugs (about 10), so that the rank order held whichever measurement was used.

At doses of drug of 3 × IC₅₀ for all four drugs or at 10 × IC₅₀ for JM221, JM223 or JM244, the effects on the cell cycle were similar to those observed with cisplatin (Sorenson and Eastman, 1988a,b; Sorenson et al., 1990; Demarq et al., 1992; Fujikane et al., 1994a,b). Initially, there was a slowdown in transit through S-phase followed by a G₂ block. This conclusion can be drawn from both the DNA histograms and the data acquired after incubation with BrdUrd (Figure 6). The cells blocked in G₂ enlarged (as evidenced by their increased light scatter, Figure 2) before eventually dying.

From our previous work, we have concluded that there are two mechanisms whereby cisplatin kills cells (Ormerod et al., 1994a,b, 1996). The earlier mechanism is apoptosis, which is probably triggered while the cells are held up in S-phase. If cells complete S-phase and if they fail to overcome the block in G₂, they die in the blocked G₂ stage of the cell cycle, probably by a non-apoptotic mechanism. In human ovarian carcinoma cells, apoptosis predominated at doses of drug 3 × IC₅₀ (Ormerod et al., 1996), whereas, in L1210 cells, apoptosis was only observed at doses of drug 15 × IC₅₀ (Ormerod et al., 1994a).

The same mechanisms were observed in this study. With a sufficiently high dose of any of the four drugs, apoptosis was observed after 4–12 h. However, at equitoxic doses, the number of apoptotic cells observed was ranked JM216 > JM221 > JM223 > JM244; this was the same ranking as the amount of Pt bound to the DNA. The induction of apoptosis seemed to be related to the amount of damage to DNA. The inability to overcome a G₂ block might be more closely related to the type of lesion.

While there have been several studies of the lesions caused by the reaction of cisplatin with DNA (for example, see Roberts and Friedlos, 1987; Eastman, 1987), little is known about the reaction of the Pt(IV) dicarboxylates with the DNA in cells. Indeed, it is probable that the metabolites of these drugs react with the DNA rather than the parent compound (Kelland et al., 1992). For example, it appears that iroplatin, cis-dichloro-trans-dihydroxy-cis-bis(isopropylamine)Pt(IV), undergoes reduction to a Pt(II) metabolite before reacting with DNA in cells (Pendyala et al., 1990). In cultured human ovarian carcinoma cells, metabolites of JM216 include JM118--cis-ammine dichloro (cyclohexylamine) Pt (II), JM338 - bis-acetato ammine (cyclohexylamine) dihydroxy Pt (IV)–and a glutathione adduct (Raynaud et al., 1996).

From our data, it would appear that JM244 is more effective than JM216 in creating lesions on the DNA, which block the cell in G₂ of the cell cycle. At doses of drug < 15 × IC₅₀, this effect predominated. In contrast, because the lesions created by JM216 were less effective at blocking the cells in G₂, at an equitoxic dose, sufficient DNA damage accumulated to trigger apoptosis.

It would be interesting to study the effect of these drugs on the human ovarian cell line, CH1. Cisplatin triggers apoptosis in these cells at doses ≥ 3 × IC₅₀. A comparative study of JM216 and JM244 might reveal whether these cells are more sensitive to the induction of apoptosis than L1210 cells or whether they are more resistant to a G₂ block.

Recently, attention has been focused on the role of apoptosis in drug-induced cytotoxicity. The data presented in this paper, taken together with our earlier data (Ormerod et al., 1994a,b, 1996), suggest that, when mechanisms of resistance are studied, other cytotoxic mechanisms should also be taken into account.
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