Apopotic and non-apoptotic cell death induced by cis and trans analogues of a novel ammine(cyclohexylamine)dihydroxodichloroplatinum(IV) complex

CF O'Neill, MG Ormerod, D Robertson, JC Tittley, Y Cumber-Walsweer and LR Kelland
Cancer Research Campaign Centre for Cancer Therapeutics, The Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG, UK.

Summary It has been previously demonstrated that cisplatin induces apoptosis in the CH1 human ovarian carcinoma cell line. This study demonstrates that two novel platinum (Pt) analogues JM149 and JM335, which are the cis and trans geometry respectively of ammine(cyclohexylamine)dihydroxodichloroplatinum(IV), initiate apoptosis in this cell line at physiologically relevant concentrations (IC50 values 2 h drug exposure were 35.3 μM for JM149 and 18.7 μM for JM335). While at equimolar drug concentrations there was a 2-fold higher level of total platinum–DNA adducts following exposure to JM335 vs JM149, at equitoxic concentrations, the levels were similar (80 vs 70 pmol Pt mg−1 DNA respectively). Following a 2 h incubation with 2 × IC50 of both drugs, cells rounded up and detached in a time-dependent manner but with the kinetics of apoptosis being more rapid for JM335. The majority of detached cells exhibited morphology associated with apoptosis which was further supported by the presence of a 50 kb fragment detected in DNA lysates prepared from these cells. JM149 induced apoptosis across a range of concentrations (2 ×, 5 × and 10 × IC50) with a 50 kb DNA fragment being detected at all concentrations. However, in marked contrast to this, JM335 failed to cause apoptosis at 10 × IC50, the detached cells neither displaying apoptotic morphology nor a detectable 50 kb DNA fragment. Moreover, these detached cells showed evidence of extensive vesiculation while the DNA remained normal in appearance and thus appeared to have died by a non-apoptotic mode. Apoptosis also appeared to be induced to a lesser extent at 5 × IC50 of JM335 as demonstrated by a less intense 50 kb fragment compared with that seen at 2 × IC50. The main cell cycle effect of these drugs (at 2 × IC50) was a slowdown in S-phase traverse during which most but not all of the apoptosis appeared to occur. However, at 5 × IC50 of JM335 cells appeared frozen in all phases of the cell cycle with little progress from G1 to S accompanied by a build-up of cells in G2 indicative of a G2/M block. This difference in cell cycle effect may account for the reduced level of apoptosis at this concentration and a failure to engage apoptosis at higher concentrations. These data suggest that the nature of the platinum drug (and consequently, the nature of resultant DNA damage) may have important implications in determining the rate and mechanism of cell death in this cell line. The cell death effects observed with the trans complex JM335 may correlate with the induction of DNA single-strand breaks in this cell line.

Keywords: apoptosis; cis/trans platinum analogues

Apoposis is a biological mechanism by which cells undergo programmed cell death. Many chemotherapeutic agents of disparate mechanisms of action engage this conserved cellular response, including etoposide (Liu, 1989), camptothecin (Kaufmann, 1989), dexamethasone (Cohen et al., 1992; Wood et al., 1994) and taxol (Milas et al., 1995). It is thought that apoptosis may be the major mechanism through which drugs mediate their cytotoxic effects (Hickman, 1992; Kerr et al., 1994). Furthermore, it has been proposed that tumour sensitivity and resistance to such drugs (and thus clinical outcome) may, at least in part, be attributable to the degree of activation of a genetic programme for cell death (Dive and Hickman, 1991).

Cisplatin has been shown to induce apoptosis in a number of different cell lines including Chinese hamster ovarian cells, immature rat thymocytes, rat hepatoma cells and L1210 murine leukaemic cells (Barry et al., 1990; Sorenson et al., 1990; Evans and Dive, 1993; Ormerod et al., 1994a). In recent studies, the kinetics of cisplatin-induced apoptosis have been measured in vivo in mammary and ovarian adenocarcinomas in rodents (Meyn et al., 1995). We have demonstrated that cisplatin induced apoptosis in three human ovarian carcinoma cell lines, the CH1, the acquired resistant CH1cisR and the intrinsically resistant SKOV-3 without concomitant internucleosomal cleavage (Ormerod et al., 1994b; Ormerod et al., 1996). The resistance factors for CH1cisR and SKOV-3 following a 2 h exposure to cisplatin were 3- and 13-fold respectively (O’Neill et al., 1995) with equitoxic concentrations of drug required to induce similar levels of apoptosis (Ormerod et al., 1996). Furthermore, we observed that apoptosis was the major mode of cell death, occurring at concentrations ranging from the physiologically relevant IC50 (the dose required to give 50% growth inhibition) to 10 times this dose. In the data presented here we have extended these studies to new platinum drugs. The work centres on two novel platinum compounds, JM149 and JM335, which are cis and trans analogues respectively of ammine(cyclohexylamine)dihydroxodichloroplatinum(IV). These drugs have been synthesised as part of a drug discovery programme aimed at identifying more effective platinum-based anti-cancer drugs. Recent studies have shown that JM335 exhibited a different pattern of cross-resistance from JM149 in an in vitro panel of sensitive and resistant cell lines (Kelland et al., 1994). Furthermore, preliminary DNA binding studies in the SKOV-3 cell line demonstrated that, at equimolar concentrations, more Pt was bound to DNA with JM335 than with JM149. Moreover, JM335 like cisplatin also formed interstrand cross-links (ISC) which are thought to be the most cytotoxic of Pt/DNA lesions formed (Zwelling et al., 1981; Hansson et al., 1988), while JM149 did not. In contrast to this, in the CH1 cell line, while JM149 initiated very low levels of ISC formation, these were undetectable following equimolar concentrations of JM335, which instead caused DNA strand breaks (Mellish et al., 1995). We have assessed
this Pt IV cis/trans pair of Pt analogues for their ability to induce apoptosis and their effects on the cell cycle in CH1 cells.

Materials and methods

JM149 and JM335 (Figure 1) were synthesised by and obtained from The Johnson Matthey Technology Centre (Reading, Berkshire, UK). Dulbecco’s modified Eagle medium (DME), trypsin, phenol and cell culture supplements were purchased from Gibco/BRL (Uxbridge, Middlesex, UK). Agarose (ultra pure), fluorescein diacetate (FDA), propidium iodide (PI), pulse field molecular weight markers and all other reagent chemicals were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, UK).

Cell culture and cytotoxicity

The CH1 human ovarian carcinoma was cultured as a monolayer in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), MEM non-essential amino acids, 2 mM glutamine, 5 μg ml⁻¹ insulin, 0.4 μg ml⁻¹ hydrocortisone, 2.5 μg ml⁻¹ amphotericin and 50 ng ml⁻¹ gentamicin. JM149 and JM335 were dissolved in sterile 0.9% sodium chloride solution and IC₅₀ values determined at 96 h following a 2 h exposure to drug by the sulphorhodamine B assay (SRB) as previously described (Kelland et al., 1992). The rate of cellular detachment was measured over a 72 h period following a 2 h incubation with 2x and 5x the IC₅₀ of both drugs.

Measurement of platinum bound to DNA

Following a 2 h incubation with equimolar concentrations of JM149 and JM335, DNA was extracted using the phenol method as previously described (O’Neill et al., 1995). Dried DNA pellets were dissolved overnight at 37°C in 0.2% nitric acid and platinum content measured by flameless atomic absorption spectroscopy (FAAS) using a Perkin Elmer 1100B/HGA 700. DNA content was measured using the Burton assay, a colorimetric method which quantitates 2'-deoxyribose units (Burton, 1956). Results were expressed as pmoles of platinum (Pt) per mg of DNA. The DNA extraction efficiency was an average of 90 μg ± 10 per 10⁶ cells.

Microscopy

Cells were exposed to 2x and 10x IC₅₀ concentrations of JM149 and JM335 for 2 h and collected 24 h later. Detached cells were harvested from the culture medium by centrifugation with attached cells being removed by incubation with 0.2% trypsin for 5 min before collection. Cell pellets were washed in phosphate-buffered saline (PBS) and fixed in 2% glutaraldehyde in 0.05 M PBS, 0.05 M sucrose, pH 7.3, for 2 h at room temperature. Following this, pellets were post-fixed in 1% osmium tetroxide, dehydrated through a graded series of ethanol, infiltrated and embedded in epon. For light microscopy, 1 μm sections were cut and stained with toluidine blue. For electron microscopy, 0.1 μm sections were picked up on copper grids and double stained with uranyl acetate and lead citrate and viewed with a Philips CM10 using 80 kV accelerating voltage.

Flow cytometry

For cell cycle analysis, cultures were exposed to 2x and 5x IC₅₀ concentrations of JM149 and JM335 for 2 h and attached cells washed, incubated with trypsin as above and collected by centrifugation at 0, 2, 5, 16 and 24 h following removal of drug. Cell pellets were resuspended in 200 μl ice-cold PBS and fixed for at least 30 min in 1.8 ml ice-cold 70% ethanol. The fixed cells were centrifuged and the pellets resuspended in 800 μl PBS, 100 μl (1 mg ml⁻¹) RNAase and 100 μl of PI (200 μg ml⁻¹) and left to incubate at 37°C for 30 min. Flow cytometry was carried out on a Coulter Elite equipped with a Spectra-physics argon-ion laser with an output of 200 mW at 488 nm. Data from 2x10⁶ cells were analysed with forward and orthogonally scattered light and red fluorescence (peak and integrated area) recorded. Pulse shape analysis was performed to eliminate any cell clumps and data were gated on light scatter before recording a histogram of red (PI-DNA) fluorescence (Ormerod et al., 1994a).

Viability studies

Cell viability was estimated by flow cytometry using the fluorescein diacetate/propidium iodide (FDA/PI) method (Ormerod, 1994); the cells were incubated in medium at room temperature for 10 min with 50 ng ml⁻¹ FDA. PI at 5 μg ml⁻¹ was added and green and red fluorescence recorded in a flow cytometer using an argon-ion laser tuned to 488 nm. Green-positive, red-negative cells were scored as viable, green-negative, red-positive cells as dead.

DNA gel electrophoresis

Cells were incubated with 2x, 5x and 10x IC₅₀ of JM149 and JM335 with attached and detached cells collected 24 h later by centrifugation. Cell pellets (approximately 5x10⁵ per 50 μl) were incubated for 1 h at 37°C in a lysis buffer [200 mM Tris, 100 mM EDTA, 2% sodium dodecyl sulphate (SDS)] containing 1 mg ml⁻¹ proteinase K final concentration. An aliquot of 10 μl of a 1 mg ml⁻¹ solution of RNAase per 50 μl of sample was added and incubation continued for a further hour. Aliquots of cell lysate were added directly to the gel, the wells being sealed with 1% low melting point agarose. Field inversion gel electrophoresis (FIGE) was performed with 1x TAE (40 mM Tris, 20 mM sodium acetate and 1 mM EDTA) using a Bio Rad FIGE Mapper. Horizontal gels were run for 20 h in 1x TAE buffer with a forward voltage of 10 V cm⁻¹ and reverse of 7 V cm⁻¹ with linear ramping T1 = 1 s to T2 = 12 s. The temperature of the TAE buffer was controlled to 14°C using a Bio Rad 1000 mini-chiller. Sigma Pulse Marker λ fragments 0.1–200 kb and S. cerevisiae fragments 225–2200 kb were run with the samples.

Figure 1 The structures of JM149 and JM335.
Results

Cytotoxicity

The cytotoxicity of both drugs was measured using the SRB assay 96 h following a 2 h exposure to drug (Figure 2). The IC_{50}'s were 35.3±1.5 μM for JM149 and 18.7±8.4 μM for JM335; this difference just attained statistical significance (P = 0.03). This gave an approximately 1.9-fold difference in activity, similar to that seen in previous studies following continuous exposure to drug for 96 h (Kelland et al., 1994).

Platinum bound to DNA

The amount of Pt bound to DNA was measured following a 2 h incubation with 25, 50 and 100 μM JM149 and JM335 (Figure 3). There was approximately twice as much Pt bound to DNA following incubation with JM335 compared with JM149. For example, incubation with 50 μM drug resulted in 50±2 pmol mg^{-1} DNA for JM149 and 110.5±35.5 pmol mg^{-1} DNA for JM335 (P-value 0.04). However, at equitoxic concentrations (2×IC_{50}) total platinum levels on DNA (i.e. inclusive of monofunctional and bifunctional adducts) were similar for the two isomers; JM149 at 70 μM, 70 pmol mg^{-1} DNA, JM335 at 37 μM, 80 pmol mg^{-1} DNA.

Cell death induced by JM149 and JM335

The detachment of cells was measured after a 2 h incubation with 2× and 5×IC_{50} of JM149 and JM335, both attached and detached cells being counted at 0, 5, 16, 24, 48, and 72 h following the removal of drug. The number of attached cells remaining at each time point was calculated as a percentage of cell number at time 0 (Figure 4a). Following exposure to JM149 numbers of attached cells continued to increase for up to 24 h and 16 h after 2× and 5× the IC_{50} respectively, while no increase in the number of the attached cell population was observed after incubation with either concentration of JM335.

The number of detached cells was calculated as a percentage of the total number of cells present at each time point so as to take into account any increase in cell number following removal of drug (Figure 4b). At equitoxic concentrations of drug, JM335 initiated cellular detachment at a much faster rate than JM149 which appeared to exhibit a time lag for up to 15 h following removal of drug. By 24 h following removal of drug, approximately 77% and 82%
Figure 5  Light microscopy of CH1 cells harvested 24 h following exposure to 2 × and 10 × the IC₅₀ of drug. (a) Control untreated attached cells. (b) Attached cells at 2 × JM149. (c) Attached cells at 2 × JM335. (d and e) Detached cells at 2 × JM149 and JM335. (f and g) Detached cells at 10 × JM149 and JM335. Original magnification ×600, bar = 25 μm.
respectively of cells had detached following 2x and 5xIC50 JM335 compared with only 17% and 43% for JM149. The majority of JM149-treated cells had detached by 72 h. The combined total of attached and detached cells 24 h after exposure to either drug at either concentration was similar e.g. JM149 (2xIC50) 2.8x10^6 cells, (5xIC50) 2.3x10^6 cells; JM335 (2xIC50) 2.4x10^6 cells, (5xIC50) 2.6x10^6 cells. Thus, although cell numbers increased with JM335, these cells appeared to be dying and detaching immediately following cell division whereas the majority of the JM149-treated cells remained attached.

Morphology and viability of attached and detached cells

The morphology of attached and detached cells was examined by light and electron microscopy 24 h following a 2 h incubation with 2x and 10xIC50 of JM149 and JM335. The attached cells remaining after incubation with 2xIC50 of JM149 showed no evidence of chromatin condensation associated with apoptosis, with the chromatin remaining similar in appearance to that of control untreated cells (Figure 5a and b). On the other hand while the majority of the attached cells remaining after 2xIC50 of JM335 exhibited normal morphology, there was evidence that some apoptosis had taken place in that there were a small number of cells showing evidence of chromatin condensation (arrowed on Figure 5c). At 2xIC50 of both drugs the majority of detached cells exhibited the morphology consistent with apoptosis, displaying the characteristic condensation and fragmentation pattern of chromatin around the periphery of the nucleus (Figure 5d and e). At 10xIC50 of JM149, the detached cells were again apoptotic in appearance (Figure 5f and Figure 6a), however, in contrast to this, the detached cells induced by the trans complex, JM335, at this concentration did not exhibit morphology associated with apoptosis (Figure 5g and Figure 6b). The chromatin was not condensed and resembled that of the attached cells. Moreover, there was evidence of extensive vesiculation in these cells which could be seen more clearly upon closer examination by electron microscopy (Figure 6b). Cell viability was estimated by measuring the number of cells with intact plasma membranes by flow cytometry 24 h following a 2 h incubation with both drugs. Across the range of concentrations of both drugs the average percentage of viable cells was 91% for the attached cells and between 35% and 64% for the detached cells (Table I).

DNA gel electrophoresis

FIGE was carried out on cell lysates prepared from attached and detached cells harvested 24 h after a 2 h exposure to 2x, 5x and 10xIC50 of JM149 and JM335. A fragment approximately 50 kb in size, was detected in the detached cells obtained with 2x, 5x and 10xIC50 of JM149. This fragment was readily observable in the detached cells following 2xIC50 of JM335, was less apparent at 5xIC50 but was not detectable at 10xIC50 JM335 (Figure 7). This fragment was also absent from the attached cells at all concentrations of both drugs. Furthermore, internucleosomal cleavage could not be detected in the cells undergoing apoptosis (data not shown).

Cell cycle analysis

Flow cytometric analysis of the effects of 2x and 5xIC50 of JM149 and JM335 on the progression of cells through the cell cycle was carried out on attached cells only. The DNA histograms indicated that the main effect of both concentrations of JM149 and 2xIC50 of JM335 was a slowdown of passage through S-phase (Figure 8) as evidenced by the increase in the percentage of cells in this phase of the cell cycle by 16 h and 24 h following removal of drug (Figure 9). By 24 h, cells exposed to 2xIC50 of JM335 and 5xIC50 of JM149 had not progressed beyond early S-phase, while those exposed to 2xIC50 JM149 had progressed to late S-phase (Figure 8). However, at 5xIC50 of JM335 cells seemed almost frozen in the cell cycle with little movement from G1 into S and this was accompanied by a build-up of cells in G2 representative of a G2/M block (Figure 9).

Table I Percentage viability of attached and detached cells measured 24 h following a 2 h incubation with JM149 and JM335

| IC50 | Percentage viability of attached cells | Percentage viability of detached cells |
|------|--------------------------------------|---------------------------------------|
| JM149 | x2 | 99±0.0 | 41.2±1.4 |
| | x5 | 88.5±10.4 | 43.3±6.2 |
| | x10 | 84.8±18.4 | 39.6±11.8 |
| JM335 | x2 | 91.4±6.2 | 35.2±4.7 |
| | x5 | 94.5±1.7 | 41.6±26 |
| | x10 | 90.1±3.8 | 64±2.1 |
Discussion

We have demonstrated the potential of two novel isomeric platinum analogues JM149 (cis) and JM335 (trans) ammine (cyclohexylamine) dihydroxodichloroplatinum (IV) (Kelland et al., 1994) to induce apoptosis in the CH1 human ovarian carcinoma cell line. This cell line has previously been shown to be relatively sensitive to cisplatin (e.g. Kelland et al., 1994) and to undergo apoptosis following exposure to physiologically relevant concentrations of cisplatin (Ormerod et al., 1994b, 1996). Following a 2 h exposure to various concentrations of cisplatin, cells rounded up and detached from the monolayer in a time- and dose-dependent manner with the majority of cells detaching at between 24 and 48 h following removal of drug. Electron and light microscopy revealed that the detached cells from both CH1 cell lines displayed typical morphological features of apoptosis with the DNA degraded into fragments of 30-50 kb in size. The major cell cycle effect appeared to be S-phase slowdown with a comparatively small G2 block. Studies have shown that CH1 cells possess a wild-type p53 gene sequence, which is induced approximately 5-fold following 5 Gy gamma-irradiation (Walton et al., 1996) and express Bcl-2 protein (Beale et al., 1996).

Both platinum-based isomers induced apoptosis in this cell line at 2 x IC50, a concentration which is roughly equivalent to a 95% inhibition of cell growth. The detached cells elicited by the two drugs exhibited fragmentation and condensation of chromatin around the periphery of the nuclear membrane (Figure 5d and e) consistent with the morphology of apoptosis (Wyllie, 1980; Arends et al., 1990; Arends and Wyllie 1991). That apoptosis had indeed occurred was further substantiated by the presence of a 50 kb DNA fragment (Walker et al., 1991; Oberhammer et al., 1993; Brown et al., 1993; Ormerod et al., 1994b and 1996) observed in the

![Figure 7](image_url) FIGE of attached and detached cells collected 24 h following a 2 h exposure to 2 x, 5 x and 10 x IC50 of JM149 and JM335. Lanes: 1, S. cerevisiae standards 225 - 2200 kb; 2 and 15, λ fragments 0.1 - 200 kb; 3 - 5, JM149 attached cells; 6 - 8, JM335 attached cells; 9 - 11, JM149 detached cells; 12 - 14, JM335 detached cells.

![Figure 8](image_url) DNA histograms showing the cell cycle changes with time (h) following a 2 h exposure to 2 x and 5 x IC50 of JM149 and JM335.
detached cells induced by both drugs on FIGE (Figure 7). In many cases the DNA of apoptotic cells is ultimately cleaved at internucleosomal sites into fragments of 180 bp and multiples thereof (Arends et al., 1990; Compton, 1992) giving a DNA ladder effect on gel electrophoresis. In common with our studies with cisplatin, internucleosomal degradation was not observed in the apoptotic cells induced by either JM149 or JM335 in the CH1 cell line. There was no morphological evidence of apoptosis in the attached cells remaining following JM149 (Figure 5b), but a small number of apoptotic-like cells were identified in the attached cell population following exposure to JM335 (Figure 5c). However, none of the attached cells exhibited the presence of a 50 kb fragment on FIGE, not even at the highest concentrations of both drugs. Thus the level of apoptosis seen in the attached cells following JM335 was insufficient under these experimental conditions to yield a detectable 50 kb fragment. Interestingly, cell viability studies showed that around 40% of the detached cells retained intact plasma membranes at 24 h which is comparatively lower than that seen with cisplatin, in which over 60% of detached apoptotic cells maintained plasma membrane integrity (Ormerod et al., 1994b).

The cis analogue JM149 induced apoptosis across the wide range of concentrations used (2 ×, 5 × and 10 × IC<sub>50</sub>) as evidenced by either change in morphology or the presence of a 50 kb fragment, with features of apoptosis being observed only in the detached cell population. In striking contrast to this, the trans isomer JM335 did not induce apoptosis at 10 × IC<sub>50</sub>. The chromatin of the detached cells remained normal in appearance with no morphological evidence of apoptosis having taken place (Figure 5g). Moreover, no 50 kb DNA fragment or fragments of DNA of signaling length were detected on FIGE (Figure 7). Notably, the detached cells displayed extensive vesiculation of the cytoplasm at this concentration which was not observed in cells undergoing apoptosis following the lower dose of this drug. The reason for this is unclear but may have been due to loss of osmotic control and subsequent water imbalance. In certain respects the vesiculation observed with JM335 resembles structures observed in cells undergoing necrosis following induction of temperatures of between 42 and 47°C (Harmon et al., 1990), but in the case of JM335, vesiculation occurred on a greater scale occupying in some cases almost 50% of cell volume. Furthermore, very few of these cells displayed the irregular fragmentation pattern of condensed chromatin associated with cells undergoing necrosis (Harmon et al., 1990; Collins et al., 1992) and intriguingly a higher proportion of these detached cells (64%) had intact cell membranes at 24 h compared with the apoptotic cells. This does not, however, eliminate necrosis as the mode of cell death in these cells.

It can be seen in the detached cell population that while there was a strong 50 kb band at 2 × IC<sub>50</sub> of JM335, this was less intense at 5 × IC<sub>50</sub> and undetectable at 10 × IC<sub>50</sub>. Great care was exercised to ensure that equal numbers of cells were loaded into each well in order to reduce variance in nuclear material between samples; thus it can be reasonably assumed that the difference in band intensity seen between 2 × and 5 × IC<sub>50</sub> of this drug was entirely due to a smaller number of cells undergoing apoptosis at the higher concentration. We conclude, therefore, that the complete absence of 50 kb fragment and lack of apoptotic morphology indicates that cell death did not occur through apoptosis at 10 × IC<sub>50</sub> of JM335, with cells dying by an alternative process.

Another striking difference in activity between the two drugs was that at both 2 × and 5 × IC<sub>50</sub> of JM149, cells appear to have experienced a lag phase (approximately 15 h) to the onset of apoptosis, whereas at 2 × IC<sub>50</sub> of JM335 the rate of induction of apoptosis is almost linear (Figure 4b). Moreover, while at 2 × IC<sub>50</sub> of JM149 there was a measurable increase in the number of attached cells up to 24 h, no increase in the attached cell population was observed with either concentration of JM335 (Figure 4a). However, at 24 h after drug incubation for attached and detached cells were similar for each concentration of both JM149 and JM335. Thus it is possible that following exposure to JM335, cells underwent aberrant division and the dividing cells detached immediately thereafter. Other studies have shown that drugs of disparate structures and mechanisms of action display a differential time lag to the induction of apoptosis and that there are differences in the way various agents ultimately induce apoptosis (Wood et al., 1995). However, JM149 and JM335 differ only in the orientation of a chlorine and amine group around a central Pt. Nonetheless, JM335 induced apoptosis at a much greater rate than JM149, such that at 2 × IC<sub>50</sub> of both drugs over four times as many cells had undergone apoptosis by 24 h with the trans compound. In addition, this concentration of JM335 induced apoptosis at a faster rate than 5 × IC<sub>50</sub> of JM335 DNA fragments.

Interestingly, the more rapid kinetics of apoptosis seen with JM335 does not appear to be related to the amount of total Pt binding to DNA. While at equimolar concentrations this is 2-fold higher for JM335 compared with JM149 (approximately 110 and 50 pmol mg<sup>-1</sup> DNA respectively at 50 μM), at equitoxic concentrations of both drugs the levels of Pt bound to DNA are similar (80 vs 70 pmol Pt mg<sup>-1</sup> DNA at 2 × IC<sub>50</sub>). Thus the more rapid induction of apoptosis by JM335 may relate to differences in the nature of DNA damage induced by the two isomers in this cell line. Notably, JM335, but not JM149, was shown to induce DNA.

---

**Figure 9** Percentage number of cells in each phase of the cell cycle measured over a 24 h period following a 2 exposure to JM149 and JM335. Open symbols, 2 × IC<sub>50</sub> and closed symbols, 5 × IC<sub>50</sub> (G<sub>1</sub>, □); (S, △); (G<sub>2</sub>, ●).
strand breaks in the CH1 cells following 2–4 h drug exposure to 25 and 100 µM (Mellish et al., 1995). In contrast, Pt–DNA interstrand cross-links were measurable in CH1 cells following exposure to JM149 (25 or 100 µM × 4 h), but not with JM335. Moreover, the nature of intrastrand cross-links induced by the two drugs appear to differ; a monoclonal antibody raised against cisplatin-treated DNA (and known to recognize the major 1,2 G–G intrastrand adduct induced by cisplatin) recognised DNA adducts in CH1 cells produced by JM149 exposure but not by JM335 (Mellish et al., 1995). These comparative DNA-binding properties suggest that the slower rate of induction of apoptosis with the cis complex JM149, may relate to a requirement for the conversion of cross-links to strand breaks in order to generate an apoptotic response. This may be at least partially addressed through investigations of p53 protein induction in this cell line by these two drugs.

Other observations made in freshly isolated rat thymocytes showed that, in contrast to agents such as etoposide which directly induce DNA strand breaks and which readily induced apoptosis, cisplatin-induced apoptosis may need to be coupled to a cell cycle-mediated event (Evans and Dive, 1993). Thus this may also apply for JM149 but not for JM335 (which induces strand breaks directly in this line). An important detail was that B-cell human lymphoma cells which underwent apoptosis at comparatively low concentrations of idarubicin and doxorubicin failed to do so at higher concentrations of these drugs (Smith et al., 1994). It was proposed that, at high concentrations of drug, a failure to traverse S-phase was associated with the failure of these cells to engage the process of apoptosis. Previous studies from our group have shown that the main cell cycle effect of cisplatin on CH1 cells was a slowdown in S-phase transit with apoptosis occurring predominantly, although not exclusively, from this phase of the cell cycle (Ormrod et al., 1996). Other investigations have shown that cisplatin induces apoptosis in all phases of the cell cycle in some cell types (HL-60 cells) (Gorczyca et al., 1993). Our observations show that the cell cycle effects of JM149 and JM335 were similar to those observed with cisplatin in that the main feature was a slowdown in the passage of cells through S-phase. By 24 h cells were detained in either early S-phase (2 × 10^6 and 5 × 10^5 of JM335 and JM149 respectively) or late S-phase (2 × 10^5 JM149) (Figure 8). There was no evidence of a G2/M block at 24 h at these concentrations and therefore it is likely that apoptosis occurred mainly from cells in S-phase. On the other hand, following exposure of the CH1 cell line to 5 × 10^5 of JM335, cells appeared almost frozen in the cell cycle with evidence of a build-up of cells in G2/M indicative of a G2/M block. The DNA histograms in Figure 8 give the impression that the majority of cells were prevented from cycling and were dying from all phases of the cell cycle. Thus the inability of these cells to progress effectively from one phase of the cell cycle to another at this concentration of JM335 may be the reason for their failure to engage apoptosis.

In conclusion we have shown that JM149 and JM335 induced apoptosis in the CH1 cell line at equitoxic and physiologically relevant concentrations of drug and that the kinetics of apoptosis was more rapid with the trans compound JM335. This may relate to differences in DNA adduct formation by these two isomers. However, at high concentrations (10 × 10^5) of JM335, CH1 cells fail to undergo apoptosis and cell death occurs by an alternative method.

Acknowledgement

This work has been supported by the Cancer Research Campaign.

References

AREND S M J AND W Y L L I E A H. (1991). Apoptosis, mechanisms, role in pathology. Int. Rev. Exp. Pathol. 32, 223–254.
AREND S M J, MORRIS R G AND W Y L L I E A H. (1990). Apoptosis: the role of the endonucleases. Am. J. Pathol., 136, 593–607.
BARRY MA, BEHNKE CA AND EASTMAN A. (1990). Activation of a programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hypothermia. Biochem. Pharmacol., 40, 2353–2362.
BEALE P, SHARP SY, WALTON MI AND KELLAND LR. (1996). Bel-2 and chemosensitivity in human ovarian carcinoma cell lines (abstract). Proc. Am. Assoc. Cancer Res., 37, 210.
BROWN DG, SUN XM AND COHEN GM. (1993). Dexamethasone induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. J. Biol. Chem., 268, 3037–3039.
BURTON K. (1956). A study of the condition and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonuclease. Biochem. J., 62, 315–323.
COHEN GM, SUN XM, SNOWDEN RT, DINSDALE D AND SKILLETEN DR. (1992). Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem. J., 286, 331–334.
COLLINS RJ, HARMON BV, GOBE GC AND KERR JF R. (1992). Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. Int. J. Radiat. Biol., 61, 451–453.
COPHOM A. (1992). A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. Cancer Metastasis Rev., 11, 105–119.
DIVE C AND HICKMAN JA. (1991). Drug-target interactions: only the first step to the commitment to a programmed cell death? Br. J. Cancer, 64, 192–196.
EVANS DL AND DIVE C. (1993). Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and non proliferating immature thymocytes. Cancer Res., 53, 2133–2139.
GOZCZA W, GONG J, ARDELT M, TRAGANOS F AND DARZYN-KIEWICZ. (1993). The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. Cancer Res., 53, 3186–3192.
HANSSON J, LWEWINSON R AND RINGBORG U. (1988). Cis-diaminedichloroplatinum(II) toxicity in human melanoma cells and lymphocytes as related to cellular platinum accumulation, DNA cross-linking and inhibition of DNA synthesis. Acta. Oncol., 27, 383–392.
HARMON BV, CORDER AM, COLLINS RJ, GOBE GC, ALLEN J, ALLAND AND KERR JR. (1990). Cell death induced in a murine mastocytoma by 42–47°C heating in vitro: evidence that the form of death changes from apoptosis to necrosis above critical heat load. Int. J. Radiat. Biol., 58, 845–853.
HICKMAN JA. (1992). Apoptosis induced by anticancer drugs. Cancer Metastasis Rev., 11, 121–139.
KAUFMANN SH. (1989). Induction of endonucleolytic DNA cleavage in human acute myelogenous leukaemic cells by etoposide, camptothecin and other cytotoxic anticancer drugs: a cautionary note. Cancer Res., 49, 5870–5878.
KELLAND LR, MISTRY P, ABEL G, LOH SY, O'NEILL CF, MURRER BA AND HARRAP KR. (1992). Mechanism-related circumscription of acquired cis-diaminedichloroplatinum(II) resistance using two pairs of human ovarian carcinoma cell lines by ammine/amine platinum(IV) disaccharates. Cancer Res., 52, 3857–3864.
KELLAND LR, BARNARD CJF, MELLISH KJ, JONES M, GODDARD PM, VALENTI M, BRYANT A, MURRER BA AND HARRAP KR. (1994). A novel trans-platinum coordination complex possessing in vitro and in vivo antitumour activity. Cancer Res., 54, 5618–5622.
KERR JF R, WINIFORD CM AND HARMON BV. (1994). Apoptosis – its significance in cancer and cancer therapy. Cancer, 73, 2013–2026.
LIF'S. (1989). DNA topoisomerase poisons as anti-tumour drugs. Ann. Rev. Biochem., 58, 351–371.
MELLISH KJ, BARNARD CJF, MURRER BA AND KELLAND LR. (1995). DNA-binding properties of novel cis and trans platinum-based anticancer agents in 2 human ovarian carcinoma cell lines. Int. J. Cancer, 62, 711–723.
MEYN, RE, CLIFTON STEPHENS, L, HUNTER, NR, AND MILAS, L. (1995). Kinetics of cisplatin-induced apoptosis in murine mammary and ovarian adenocarcinomas. *Int. J. Cancer*, 60, 725–729.

MILAS, L, HUNTER, NR, KURDOGLU, B, MASON, KA, MEYN, RE, STEPHENS, LC, AND Lester, PJ. (1995). Kinetics of mitotic arrest and apoptosis in murine mammary and ovarian tumors treated with taxol. *Cancer Chemother. Pharmacol.*, 35, 297–303.

OBERHAMMER, F, WILSON, JW, DIVE, C, MORRIS, ID, HICKMAN, JA, WAKELING, AE, WALKER, PR, AND SIKORSKA, M. (1993). Apoptotic death in epithelial cells, cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.*, 12, 3679–3684.

O'NEILL, CF, ORR, RM, KELLAND, LR, AND HARRAP, KR. (1995). Comparison of platinum binding to DNA and removal of total platinum adducts and interstrand cross-links in three human ovarian carcinoma cell lines sensitive and resistant to cisplatin. *Cellular Pharmacol.*, 2, 1–7.

ORMEROD, MG. (1994). Further applications to cell biology *In Flow Cytometry: a Practical Approach*, Ormerod MG. (ed.) pp. 261–273. IRL Press at Oxford University Press: Oxford.

ORMEROD, MG, ORR, RM, AND PEACOCK, JH. (1994a). The role of apoptosis in cell killing by cisplatin: a flow cytometric study. *Br. J. Cancer*, 69, 93–100.

ORMEROD, MG, O'NEILL, CF, ROBERTSON, D, AND HARRAP, KR. (1994b). Cisplatin induces apoptosis in a human ovarian carcinoma cell line without concomitant internucleosomal degradation of DNA. *Exp. Cell Res.*, 211, 231–237.

ORMEROD, M, O'NEILL, C, ROBERTSON, D, KELLAND, LR, AND HARRAP, KR. (1996). Cis-diaminedichloroplatinum(II)-induced cell death through apoptosis in sensitive and resistant human ovarian carcinoma cell lines. *Cancer Chemother. Pharmacol.*, 37, 463–471.

SMITH, PJ, RACKSTRAW, C, AND COTTER, F. (1994). DNA fragmentation as a consequence of cell cycle traverse in doxorubicin- and idarubicin-treated human lymphoma cells. *Ann. Hematol.*, 69, S7–S11.

SORENSON, CM, BARRY, MA, AND EASTMAN, A. (1990). Analysis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin. *J. Natl Cancer Inst.*, 82, 749–755.

WALKER, PR, SMITH, C, YOUDALE, T, LEBLANC, J, WHITFIELD, JF, AND SIKORSKA, M. (1991). Topoisomerase II-reactive chemotherapeutical drugs induce apoptosis in thymocytes. *Cancer Res.*, 51, 1078–1085.

WALTON, MI, WU, F, SHARP, SY, AND KELLAND, LR. (1996). p53 status and CDDP sensitivity in a panel of ovarian carcinoma cell lines (abstract). *Proc. Am. Assoc. Cancer Res.*, 37, 2747.

WOOD, AC, WATERS, CM, GARNER, A, AND HICKMAN, JA. (1994). Changes in c-myc expression and the kinetics of dexamethasone-induced programmed cell death (apoptosis) in human lymphoid cells. *Br. J. Cancer*, 69, 663–669.

WOOD, AC, ELVIN, P, AND HICKMAN, JA. (1995). Induction of apoptosis by anti-cancer drugs with disparate modes of action: kinetics of cell death and changes in c-myc expression. *Br. J. Cancer*, 71, 973–941.

WYLIE, AH. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endonuclease activation. *Nature*, 284, 555–556.

ZWELLING, AL, MICHAELS, S, SCHWARTZ, H, DOBSON, PP, AND KOHN, KW. (1981). DNA cross-linking as an indicator of sensitivity and resistance of mouse L1210 leukemia to cis-diaminedichloroplatinum(II) and L-phenylalanine mustard. *Cancer Res.*, 41, 640–649.