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Effects of Cisplatin on Mitochondrial Function in Jurkat Cells

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In this work, we measured the effects of pharmacological concentrations of cisplatin (*cis*-diamminedichloroplatinum II) on mitochondrial function, cell viability, and DNA fragmentation in Jurkat cells. The exposure of cells to 0–25 μ M cisplatin for 3 h had no immediate effect on cellular mitochondrial oxygen consumption, measured using a palladium–porphyrin oxygen sensing phosphor. Similarly, the cell viability as measured by trypan blue staining was unchanged immediately following exposure to the drug, and no small DNA fragments, characteristic of drug-induced apoptosis, appeared. At 24 h after exposure to cisplatin, cellular respiration and viability decreased relative to controls and the amount of small DNA fragments, measured using quantitative agarose gel electrophoresis, was proportional to the concentration of cisplatin present during the drug exposure period. The small DNA fragments showed the banding pattern (with a spacing of \sim 300 bp) characteristic of drug-induced cell death by apoptosis. The changes in respiration and DNA fragmentation correlated linearly with the amount of platinum bound to DNA, determined by atomic absorption spectroscopy immediately following drug exposure. The oxygen consumption by beef heart mitochondria was not affected 0–24 h after exposure to 25 μ M cisplatin or to solutions containing the monoaquated form of the drug, suggesting that the drug does not attack the mitochondrial respiratory chain directly. Cells exposed to the peptide benzyloxycarbonyl-val-ala-aspartate-fluoromethyl ketone, which blocks apoptosis by the caspase pathway, showed a decrease in cisplatin-induced DNA fragmentation but not in the impairment of cellular respiration. Thus, although apoptosis is caspase-dependent, the impairment of cellular respiration is independent of the caspase system. Collectively, these results suggest that alteration in mitochondrial function is a secondary effect of cisplatin cytotoxicity in Jurkat cells.

Introduction

The mitochondria are important for cellular energy metabolism and apoptosis (1, 2). These vital organelles generate over 90% of the cellular ATP via oxidative phosphorylation, using energy derived from oxidations in the respiratory chain. This process is driven by the consumption of molecular oxygen (1). Moreover, mitochondria are known effectors of two major apoptotic pathways, which result in the release of cytochrome *c* into the cytosol (2–4). The release of cytochrome *c* causes an impairment of mitochondrial respiratory function. A direct inhibition of the respiratory chain by other toxins (including some drugs), on the other hand, rapidly depletes cellular ATP, promoting nonapoptotic (necrotic) cell death (5, 6). Thus, because of their critical role in cell survival, mitochondria serve as targets for cellular toxins and chemotherapeutic agents (7).

Cisplatin¹ is widely used for the treatment of many cancers. Clinically, the drug is administered intra-

venously at \sim 20–120 mg/m² over 30–60 min, producing a maximum serum concentration of \sim 5–25 μ M, with a half-life ($t_{1/2}$) of \sim 30 min (8). In the plasma, where the chloride concentration is relatively high (\sim 100 mM), cisplatin exists mainly in the dichloro neutral form, cisplatin. However, if the chloride concentration is low, as is the case in the cytoplasm where it is \sim 4 mM, the drug aquates producing the monoquo and diaquo complexes, *cis*-[Pt(NH₃)₂(H₂O)Cl]⁺ and *cis*-[Pt(NH₃)₂(H₂O)₂]²⁺. These compounds can react with DNA, GSH, metallothionein, and other cellular components (9–11). It was originally thought that cisplatin entered the cell by passive diffusion (12). However, there is growing evidence suggesting that a more sophisticated transport mechanism (13, 14) plays a role. In the nucleus, cisplatin reacts with nitrogen atoms on the bases of DNA, forming uni-

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¹ Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II) or *cis*-Pt(NH₃)₂Cl₂; [O₂], oxygen concentration; [cisplatin], cisplatin concentration in the incubation medium in micromolar; AAS, atomic absorption spectroscopy; Pd phosphor, palladium(II) complex of *meso*-tetra(4-sulfonatophenyl)tetrabenzoporphyrin; RT, room temperature; BSA, bovine serum albumin; dH₂O, distilled deionized water; FBS, fetal bovine serum; Pt, platinum; *k*, zero-order rate constant for cellular respiration; TBE, Tris–borate–EDTA; bp, base pair; kb, kilo base pair; zVAD-fmk, benzyloxycarbonyl-val-ala-aspartate-fluoromethyl ketone; BHM, beef heart mitochondria.

and bidentate adducts (15). Some of the Pt-DNA adducts can be removed by the nucleotide excision repair (NER) mechanism, but others cannot be removed (16) because they are protected by the structure specific recognition protein. Pt lesions that remain on DNA impair template function and ultimately promote cell death through apoptotic pathways (15, 17).

A major factor limiting the treatment with cisplatin is acute, dose-dependent, and cumulative nephrotoxicity, which seems to be associated with mitochondrial injury (18, 19). In human proximal-derived renal tubule cells, cisplatin-induced cell death has been shown to be dependent on intact ATP-producing mitochondria (20). However, in porcine proximal renal tubule cells, cisplatin concentrations of 50–500 μM have been shown to immediately inhibit (within 20 min) mitochondrial oxygen consumption, suggesting that cisplatin concentrations less than 50 μM promote cell death by apoptosis, while higher concentrations produce necrosis in renal cells (21–23).

Mitochondria are also thought to be a major target for cisplatin in cancer cells (24–28). Cisplatin binding results in a significant decrease in the mitochondrial function of melanoma cells (28), and alterations in mitochondrial function have been implicated in cancer cell resistance to chemotherapeutic agents (29). Determining whether mitochondria are primary or secondary targets of cisplatin chemotherapy is important for understanding the drug's mechanism of action. Previous reports indicated that cisplatin may affect mitochondrial function by directly attacking mitochondrial DNA (mtDNA) (27, 28). The depletion of mtDNA was shown to enhance cisplatin-induced apoptosis in U937 lymphoma cells (27). Moreover, in studies with human malignant melanoma cells, cisplatin binding to mtDNA is 50 times greater than to chromosomal DNA. We recently reported that cisplatin (0–99 μM) had little to no immediate (within 1–3 h) effect on mitochondrial function in various cancer cell lines, tumor cells, and beef heart submitochondrial particles as measured by oxygen consumption (30). However, the long-term impact of cisplatin on mitochondrial function remains unclear and warrants further investigation.

In the present study, we quantitate the long-term effects (after many hours) of clinically relevant concentrations of cisplatin on mitochondrial function in Jurkat cells and isolated BHM. We also describe the relations between drug exposure, cellular viability, and DNA fragmentation. We show that cisplatin produces an indirect, delayed, and dose-dependent impairment of mitochondrial function, which is independent of the caspase system. We also show that the amount of Pt bound to DNA, measured immediately after incubation with the drug, correlates linearly with the loss of mitochondrial function and the amount of DNA fragmentation, both observed 24 h after exposure to the drug. These studies provide a quantitative basis for understanding the interrelationships between DNA platination, mitochondrial function, DNA fragmentation, and cellular viability in a well-characterized cancer cell line.

Materials and Methods

Chemicals. Cisplatin (1 mg/mL or ~ 3.3 mM) was obtained from American Pharmaceutical Partners (Los Angeles, CA); the Pd(II) complex of Pd phosphor (sodium salt, MW ~ 1300) was

purchased from Porphyrin Products, Inc. (Logan, UT); proteinase k, ribonuclease A (DNase-free from bovine pancreas; contained ~ 80 units/mg), rotenone, trifluorocarboxylcyanide phenylhydrazone (FCCP), ADP, bromophenol blue, xylene cyanol FF, and fatty acid free BSA were purchased from Sigma-Aldrich (St. Louis, MO); nonyl phenyl-poly(ethylene glycol) (Nonidet NP-40) was purchased from Fluka (Ronkonkoma, NY); agarose (molecular biology grade) was purchased from Promega (Madison, WI); Dulbecco's PBS (without calcium or magnesium), FBS, and RPMI-1640 medium with L-glutamine (pH 7.15) were purchased from Mediatech (Herndon, VA); zVAD-fmk (MW 468, 2 mM solution) was purchased from Biovision (Mountain View, CA); DNA Hyper-Ladder I (200–10 000 bp) was purchased from Bioline USA Inc. (Randolph, MA); and Complete Protease Inhibitor Cocktail Tablets were purchased from Roche Applied Science (Indianapolis, IN).

Solutions. "Aged" cisplatin was prepared by diluting the stock drug solution (3.3 mM in 154 mM NaCl) to 33 μM in cisplatin with dH_2O (final NaCl concentration, 1.54 mM), followed by incubation at RT in the dark for at least 24 h prior to use. Under these conditions, the drug aquates, losing chloride and producing a mixture of cisplatin (13%), the monoaquo species (73%), and the diaquo species (14%) (31). At a pH near 7, about 75% of the monoaquo species is in the monohydroxy form (neutral) and 25% is in the monoaquo form (cation); the diaquo form has a very small concentration, and the aquo-hydroxy (monocation) and dihydroxy (neutral) species have approximately equal concentrations. Because the rate constants for formation of the monoaquo and diaquo species are ~ 0.2 h^{-1} , the 24 h period represents about seven half-lives, so our aged cisplatin represents an equilibrium mixture of the forms. Pd phosphor was dissolved in dH_2O (2.5 mg/mL or ~ 2.0 mM) and stored in the refrigerator for less than 2 weeks. Rotenone (1.0 mM) was freshly dissolved in absolute ethanol. Phosphate-citrate buffer consisted of 200 mM Na_2HPO_4 , with the pH adjusted to 7.8 with 0.1 M citric acid. Proteinase k (20 mg/mL) and ribonuclease A (10 mg/mL) solutions were made in dH_2O and stored at -20 °C. The NP-40 0.25% (v/v) solution was made in dH_2O and stored at RT. The gel loading buffer contained (v/v) 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in dH_2O . The TBE buffer contained 40 mM Tris, boric acid, and 2 mM EDTA (pH 8.3). The Tris-sucrose buffer contained 10 mM Tris-Cl and 250 mM sucrose (pH 7.5). The chloride-free Tris-sucrose buffer contained 10 mM Tris- NO_3 and 250 mM sucrose (pH 7.5). The complete protease inhibitor cocktail was prepared by dissolving two tablets in 0.5 mL of dH_2O and was stored at -70 °C. The cocktail was added at a final concentration of 5 $\mu\text{L}/\text{mL}$ buffer.

The Pd phosphor solution contained 2 μM Pd phosphor, 2% (w/v) BSA, and 5 mM ADP in RPMI (containing ~ 6 mM Na_2HPO_4 and 10 mM glucose); the final pH was ~ 7.5 . The solution was freshly made in a 30 mL quartz tube and continuously stirred for at least 90 min prior to use.

Cells. The human T-cell lymphoma cell line, Jurkat, was a gift from Dr. Edward Barker. The cells were maintained in a suspension culture under a fully humidified atmosphere containing 5% CO_2 at 37 °C. The medium was RPMI-1640 supplemented with 10% (v/v) FBS, 100 $\mu\text{g}/\text{mL}$ streptomycin, 100 IU/mL penicillin, and 2.0 mM L-glutamine. The number of cells in the population (cell count) and the percentage of viable cells in the population (viability) were determined immediately prior to experimental measurements by light microscopy, using a hemacytometer under standard trypan blue staining conditions (32).

Incubation with Cisplatin. The incubations were carried out in RPMI medium plus 10% FBS at 37 °C. Cells in logarithmic growth ($\sim 10^7$ /condition) were exposed to cisplatin (0–25 μM) for 3 h. At the end of the incubation periods, the cells were collected by centrifugation and analyzed immediately or maintained in culture (drug-free medium) and analyzed 24 or 48 h later.

BHM Treatments. BHM were used to investigate the effects of cisplatin and an aged solution of the drug on mitochondrial function. The BHM were prepared as previously described (33, 34) and underwent multiple freeze–thaw cycles, making them NADH permeable. As a consequence, 1 h of incubation at 37 °C with 20 μM FCCP did not significantly increase the respiration as compared to an untreated control, indicating that the mitochondrial respiratory chain was not tightly coupled (data not shown). For experiments with cisplatin, BHM (~ 10 mg per condition) were suspended in 2.0 mL of 10 mM Tris-Cl (pH, 7.5) containing 250 mM sucrose. For experiments with aged cisplatin, BHM were suspended in the buffer, 10 mM Tris-NO₃ (pH, 7.5) containing 250 mM sucrose. The use of nitrate instead of chloride ion in the buffer ensured that the distribution of complexes in the aged solution would not greatly change during the time course of the experiments (35). The mixtures were incubated at 37 °C for 3 h with and without the drug. At the end of the incubation period, the mitochondria were collected by centrifugation (23000g at 4 °C for 30 min), washed, and suspended (with three passages of the pestle through a glass–Teflon homogenizer) in 10 mL of the same buffer. The suspensions were incubated at 37 °C for 0, 2, 4, and 24 h. The samples assayed 24 h after drug exposure were incubated in the presence and absence of 5 μL of complete protease inhibitor cocktail per mL of buffer. At the end of the incubation periods, the mitochondria were collected, washed in the same buffer, and suspended in Pd phosphor solution as described above. Approximately 0.5–1.0 mg of each suspension was diluted to 0.7 mL with the Pd phosphor solution. Following the addition of 50 mM NADH, mitochondrial oxygen consumption was assayed as described below. Each experiment included a measurement in the presence of 50 μM rotenone (which inhibits complex I of the respiratory chain) as a positive control.

Incubation with zVAD-fmk. The pan-caspase inhibitor, zVAD-fmk, was used to investigate the role of caspases in cisplatin-induced cytotoxicity (36). Cells ($\sim 10^7$ per condition) were pretreated with 2 μM zVAD-fmk for 2 h at 37 °C. At the end of the incubation period, 25 μM cisplatin was added and the suspensions were incubated at 37 °C for 3 h. The cells were then maintained in culture (in drug-free medium plus 10% FBS with and without 2 μM zVAD-fmk) for ~ 24 h. At the end of the incubation period, the cells were collected and washed with PBS (pH 7.4). The cellular mitochondrial oxygen consumption and DNA fragmentation were assayed as described below.

Cellular Oxygen Consumption. Respiration was measured at RT in sealed vials containing cells suspended in 0.7 mL of the Pd phosphor solution. The substrate for the cells was glucose, and for the BHM, the substrate was NADH. The concentration of oxygen [O₂] in the solution was measured as a function of time using the phosphorescence probe Pd(II) meso-tetra-(4-sulfonatophenyl)tetrabenzoporphyrin as described (30, 37). This method is based on oxygen quenching of the phosphorescence of the Pd phosphor (37).

The rate of cellular mitochondrial oxygen consumption ($\mu\text{M O}_2 \text{ min}^{-1}$) was calculated as the negative slope of the linear portion ([O₂] $\geq 150 \mu\text{M}$) of the [O₂] vs the time curves. As expected, the oxygen consumption rates were linear with the number of viable cells up to 1.5×10^7 ($R > 0.99$). However, the slope of the curve decreased by $\sim 12\%$ at a cell count of 2.0×10^7 ($R > 0.97$, overall, data not shown). The value of k was set equal to the negative slope of each curve divided by the total number of cells ($\times 10^6$) in each sample. Each experiment included a measurement in the presence of rotenone. The rate of oxygen consumption for the Pd phosphor solution without cells was (mean \pm SD) $0.28 \pm 0.05 \mu\text{M O}_2 \text{ min}^{-1}$, and for $\sim 10^7$ cells incubated with rotenone (50 μM at 37 °C for 1 h), it was $0.36 \pm 0.16 \mu\text{M O}_2 \text{ min}^{-1}$.

Extraction of DNA Fragments. The DNA fragments were extracted as described (38) with some modifications. The cells were washed with PBS and suspended in 1.0 mL of ice-cold PBS. The cells ($\sim 3 \times 10^6$ /condition) were fixed in 8.0 mL of ice-cold 70% ethanol. The suspension was incubated at -20 °C for 24 h

Table 1. Time-Dependent Effects of Cisplatin on Mitochondrial Function as Measured by Oxygen Consumption^a

| postdrug exposure (h) | untreated cells k ($\mu\text{M O}_2 \text{ min}^{-1}/10^6$ cells) | treated cells k ($\mu\text{M O}_2 \text{ min}^{-1}/10^6$ cells) |
|-----------------------|--|--|
| 0 | 0.46 ± 0.18 | 0.36 ± 0.15 |
| 24 | 0.42 ± 0.16 | 0.17 ± 0.06 |
| 48 | 0.20 ± 0.05 | 0.07 ± 0.04 |

^a Cells ($\sim 10^7$ per condition) were incubated at 37 °C for 3 h without (untreated cells) and with (treated cells) 25 μM cisplatin. At the end of the incubation period, the cells were maintained in drug-free medium for 0, 24, or 48 h at 37 °C. A small volume of each cell suspension was then removed to determine the number of cells and viability. The remaining cells were suspended in the Pd phosphor solution and placed in the instrument. The values of k were calculated as the negative slopes of the linear parts of the curves normalized to 10^6 cells analyzed. The numbers are means \pm SD of three experiments.

and then centrifuged (1000g for 5 min at 4 °C). The supernatant was discarded, and the ethanol was allowed to thoroughly evaporate at RT. The pellet was suspended in 50 μL of phosphate-citrate buffer and incubated at 20 °C for 90 min. The suspension was centrifuged (as above), and the supernatant was transferred to a fresh tube. The supernatant was recentrifuged as above, and the supernatant was lyophilized in the Speed Vac (model SC 110, equipped with refrigerated vapor trap, model RVT 100; Farmingdale, NY). The lyophilized pellet was suspended in 6 μL of NP-40 (0.25% solution), 6 μL of SDS (10%), 6 μL of ribonuclease A (10 mg/mL solution), and 2 μL of ribonuclease T₁. After overnight incubation at 37 °C, 6 μL of proteinase k (20 mg/mL solution) was added and the mixture was incubated overnight. Twenty-four microliters of the gel-loading buffer was added (final volume $\sim 50 \mu\text{L}$).

Agarose Gel Electrophoresis. Approximately 22 μL of the above extract was loaded on 4 mm thick, 1.0% agarose gels and electrophoresed at 25 V and 11 mA for ~ 15 h in TBE buffer. Each extract was loaded on two separated gels. The gels were stained in 300 mL of 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide for 30 min in the dark at RT. After destaining for 15 min in dH₂O, the gels were stored at 4 °C in 300 mL of TBE. The images were captured using a Gel Doc digital camera system with Quantity One software (Bio-Rad, Richmond, CA). The individual lanes were scanned (Sigma Scan, version 2.0, SPSS Inc.) from the loading well to the low molecular weight end of the streaks. The measured intensity at each position was the average intensity across a broad line (17 pixels wide). The background of each lane was calculated as the minimum average intensity in each lane. Net intensity vs position plots were exported to Peak-Fit software (version 4.1, SPSS Inc.). The position–size (bp) calibration curve was derived from the plot of the Hyper-Ladder (200–10 000 bp) lane and was used to determine the approximate size and spacing of the low molecular weight DNA fragments. Intensity vs size plots were fitted either to a sum of Gaussians with width increasing with size or to a sum of Gaussians plus a linear function of size.

Pt-DNA Adducts. Cellular (genomic) DNA was extracted immediately following the 3 h incubation with cisplatin. The Pt-DNA adducts were determined by AAS as previously described (11).

Results

Cisplatin-Induced Impairments of Cellular Respiration. The time and dose-dependent effects of cisplatin (0–25 μM at 37 °C for 3 h) on cellular mitochondrial oxygen consumption (measured 24 and/or 48 h after drug exposure) are shown in Tables 1 and 2 and Figures 1 and 2. In the untreated cells, the value of k at 0 and 24 h after exposure to drug was unchanged, but at 48 h, it was about half of its original value; the latter was

Table 2. Effects of Cisplatin on Mitochondrial Dysfunction and DNA Fragmentation^a

| [cisplatin] (μM) | k ($\mu\text{M O}_2 \text{ min}^{-1}/10^6 \text{ cells}$) | integrated DNA fragment intensity (arbitrary units) |
|-------------------------------|---|---|
| 0 | 0.31 ± 0.05 | 5.4 ± 0.9 |
| 5 | 0.29 ± 0.05 | 8.3 ± 2.2 |
| 10 | 0.22 ± 0.10 | 17.3 ± 7.8 |
| 15 | 0.19 ± 0.08 | 19.8 ± 5.2 |
| 20 | 0.17 ± 0.05 | 20.4 ± 1.4 |
| 25 | 0.16 ± 0.05 | 29.4 ± 0.5 |

^a Cells ($\sim 10^7$ per condition) were incubated at 37°C for 3 h with and without the indicated [cisplatin]. At the end of the incubation period, the cells were maintained in drug-free medium for ~ 24 h. A small volume of each cell suspension was then removed to determine the number of cells and viability. The remaining cells were then suspended in the Pd phosphor solution and placed in the instrument. The values of k were calculated from the negative slopes linear to the parts of the curves (Figure 4A–C) divided by the number of cells in each suspension. The numbers are means \pm SD of three experiments.

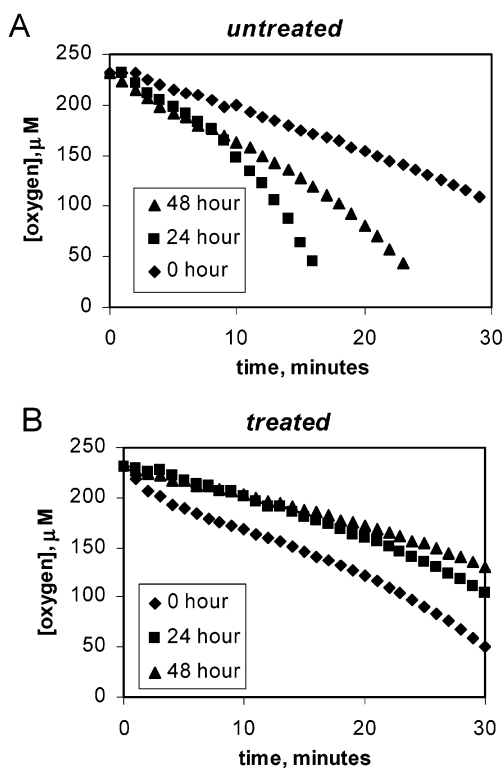


Figure 1. Time dependence of cisplatin-induced mitochondrial dysfunction as measured by decreased cellular oxygen consumption. The results of one of three experiments are shown. Cells ($\sim 10^7$ per condition) were incubated at 37°C for 3 h without (A) and with (B) $25 \mu\text{M}$ cisplatin. The cells were then maintained in drug-free medium for 0, 24, or 48 h at 37°C . At the end of the incubation periods, a small volume of each cell suspension was removed to determine the number of cells and viability. The remaining cells were washed with PBS, suspended in the Pd phosphor solution, and placed in the instrument at hour 0 (circles), hour 24 (triangles), or hour 48 (squares). The average values of k , calculated from the negative slopes of the linear parts of the curves, are shown in Table 1.

attributed to a high cell population density (Figure 1A and Table 1). In the treated cells, the value of k at 24 h was $\sim 50\%$ of the value at 0 h, and at 48 h, it was $\sim 20\%$ of the value at 0 h (Figure 1B and Table 1). This is less than half of the value for untreated cells at the same time. Because the respiration rate for the cells in the absence of drug was essentially unchanged at 0 and 24

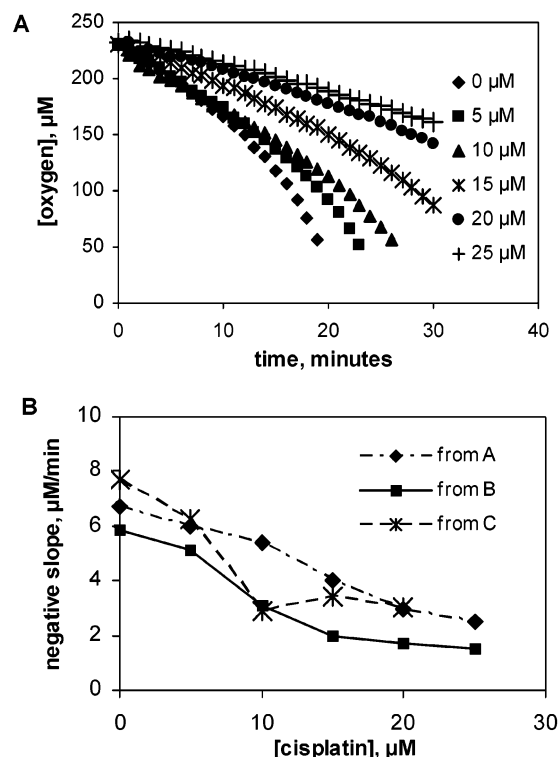


Figure 2. Concentration dependence of cisplatin-induced mitochondrial dysfunction as measured by decreased cellular oxygen consumption. The results of one of three experiments are shown (A). The cells were incubated at 37°C for 3 h with and without the indicated cisplatin concentration. After the incubation period, the cells were maintained in drug-free medium and placed in the instrument 24 h after exposure to cisplatin. The negative slopes of the linear parts of the curves are plotted as a function of [cisplatin] (B). The values of k are shown in Table 2.

h (but decreased significantly at 48 h, Table 1), the measurements to establish the concentration dependence of cisplatin's effect on mitochondrial function were done 24 h after exposure to the drug. The results of these experiments are shown in Figure 2A and Table 2. The rate of respiration decreased with increased [cisplatin] in the range 0– $25 \mu\text{M}$ (Figure 2B).

Effect of Cisplatin on Isolated BHM Oxygen Consumption. The ability of BHM to respire in the presence of NADH after treatment with cisplatin and aged solutions of cisplatin was measured 0, 2, 4, and 24 h after exposure to $25 \mu\text{M}$ drug at 37°C for 3 h. The value of respiration rate, k , without cisplatin treatment from 0 to 4 h after the initial incubation was $307 \pm 99 \mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ (mean \pm SD, $n = 3$), and with exposure to cisplatin, the rate was $266 \pm 90 \mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$. Over the same time period, the value of k for BHM in the Tris buffer medium containing nitrate ion (mean \pm SD, $n = 3$) was $295 \pm 27 \mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ and the value of k for BHM treated with aged solutions of cisplatin in the nitrate buffer medium was $233 \pm 50 \mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$. Incubation with $50 \mu\text{M}$ rotenone decreased BHM oxygen consumption by $\sim 98\%$ (mean \pm SD, $n = 2$) to $5.5 \pm 1.0 \mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$.

The value of k without cisplatin treatment and in the Tris buffer containing nitrate ion decreased by a factor of ~ 40 to 8.5 ± 1.4 and $4.4 \pm 0.75 \mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ (mean \pm SD, $n = 6$) 24 h after the initial incubation period in the presence and absence of protease inhibitors, respectively. The same decrease in respiration was

observed 24 h after BHM were treated with cisplatin and aged solutions of the drug. The value of k for BHM treated with cisplatin and its aquated derivative was 7.5 ± 1.9 and $4.35 \pm 0.78 \mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ (mean \pm SD, $n = 6$), respectively. BHM remained sensitive to rotenone over the extended incubation period. The value of k for BHM incubated with $50 \mu\text{M}$ rotenone (37°C for 1 h after the extended incubation period) was $1.46 \pm 0.14 \mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ (mean \pm SD, $n = 6$), which is $\sim 75\%$ less than the value of k for BHM analyzed 24 h after a 3 h incubation in the presence and absence of cisplatin or aged cisplatin. Although the extended incubation period caused a significant decrease in BHM respiration, neither cisplatin nor aged solutions of the drug had an effect on BHM respiration when compared to a positive control.

Effect of Cisplatin on Cell Number and Viability.

The effects of cisplatin ($25 \mu\text{M}$ at 37°C for 3 h) on cell number and viability were measured 0, 24, and 48 h after drug exposure. The average viability for the untreated cells at 0, 24, and 48 h was essentially the same as for the treated cells at 0 h for all experiments: $91 \pm 2.7\%$ (mean \pm SD). The viability for treated cells decreased at 24 and 48 h to 55 ± 7 and $23 \pm 4\%$, respectively. Over the same time period, the number of untreated cells increased by a factor of 2.5 ± 0.3 at 24 h and a factor of 3.6 ± 0.9 at 48 h. In 48 h, however, the number of cells in the treated population increased by a factor of $\sim 2.7 \pm 0.5$, as compared to the cell number at 0 h, indicating that treatment with cisplatin slightly affected cell replication during this period. The total number of cells and viability measured 24 h after exposure to 0– $25 \mu\text{M}$ cisplatin (3 h, 37°C) for all experiments is shown in Figure 3A,B, respectively. Both the total number of cells and the viability cells decreased exponentially with increased [cisplatin].

The decrease in respiration (value of k), normalized to the total number of cells, mirrors the decrease in viability. However, even if normalized to the number of viable cells in the population, k values still decreased as compared to the treated cells at hour 0. Note that during the second 24 h period after exposure to the drug, the cell population went through one or two replications, but the total number of cells decreased by $\sim 30\%$, indicating that some cells underwent a lethal mitosis (15). This is consistent with an "evaluation period" during which the cells are accessing the commitment to apoptosis.

Cisplatin-Induced DNA Fragmentation. The effect of cisplatin ($25 \mu\text{M}$ at 37°C for 3 h) on inducing DNA fragmentation was measured 0, 24, and 48 h after drug exposure. The average integrated fragment intensities for the untreated cells at 0, 24, and 48 h and for the treated cells at 0 h were essentially the same: 8.2 ± 1.5 (mean \pm SD, arbitrary units). The integrated fragment intensity for treated cells increased at 24 h by a factor of 2.1, to 17.4 ± 6.2 (arbitrary units). However, the amount of DNA fragments increased only slightly, if at all, between 24 and 48 h (to 19.6 ± 5.8 arbitrary units).

The dose-dependent effect of cisplatin (0– $25 \mu\text{M}$ at 37°C for 3 h) on DNA fragmentation, measured 24 h after drug exposure, is shown in Figure 4A–C. The set of intensity vs position plots for the lanes in Figure 4A is shown in Figure 4B. The integrated intensity under each curve, representing the amount of fragmented DNA, was calculated as described in the Materials and Methods. The amount of fragmented DNA increased almost linearly ($R > 0.96$) as a function of [cisplatin] (Figure 4C).

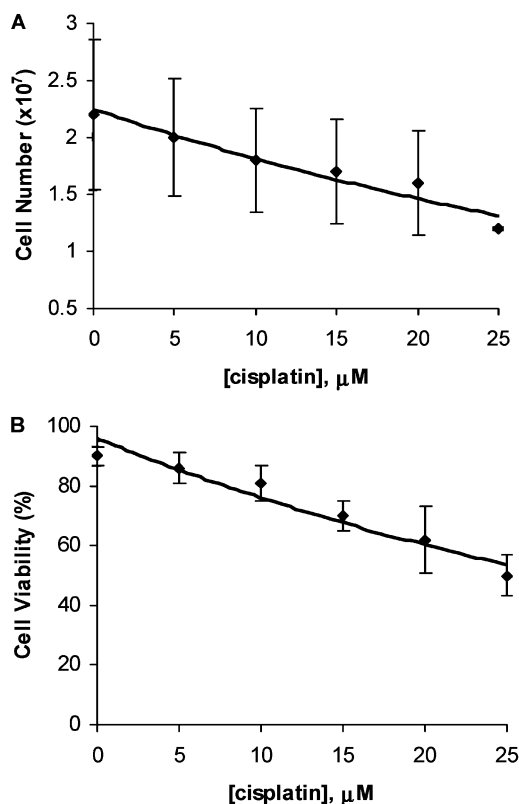


Figure 3. Concentration dependence of cisplatin cytotoxicity as measured by cell growth inhibition and loss of cell viability. The cells were exposed to cisplatin at 0– $25 \mu\text{M}$ for 3 h at 37°C . The number of cells and viability were measured 24 h after exposure. (A) Cell growth inhibition results (error bars are the SD of 2–3 measurements in each case) with best-fit exponential. (B) Cell viability results (error bars are the SD of 2–3 measurements in each case) with best-fit exponential.

The intensity vs position plot of the Hyper-Ladder was used to convert the x -axis from position to length (bp). Each plot of intensity vs size (length in bp), like those shown in Figure 4B, was fitted to a sum of Gaussians to locate the maxima. The widths of the Gaussians increased with fragment size, as should be the case for apoptotic cleavage. The average spacing between the nucleosomal fragments was obtained by differencing the first six peak positions (1–6, Figure 4B) in the plots for the four highest cisplatin concentrations. The average value of the differences was 305.9, with a population SD of 32.4. Although the fit to a sum of variable width Gaussians was good, it could be improved by adding a linear function of size (which would be indicative of necrosis). Thus, the presence of a background due to necrosis cannot be ruled out.

Effect of Cisplatin on Cellular Respiration, DNA Fragmentation, and Viability in the Presence of zVAD-fmk. The effect of zVAD-fmk on cisplatin-induced impairment of cellular respiration (measured 24 h after drug exposure) is shown in Table 3. The presence of zVAD-fmk alone in the incubation medium ($2 \mu\text{M}$ at 37°C for 29 h) had no significant effect on cellular respiration, viability, or DNA fragmentation, when compared to the untreated control. Incubation with cisplatin alone ($25 \mu\text{M}$ at 37°C for 3 h) decreased the viability and the value of k by factors of ~ 1.5 and ~ 2.8 respectively, and increased the amount of DNA fragments by a factor of ~ 4 , when compared to the untreated control (Figure 5 and Table 3). For a shorter incubation with zVAD-fmk

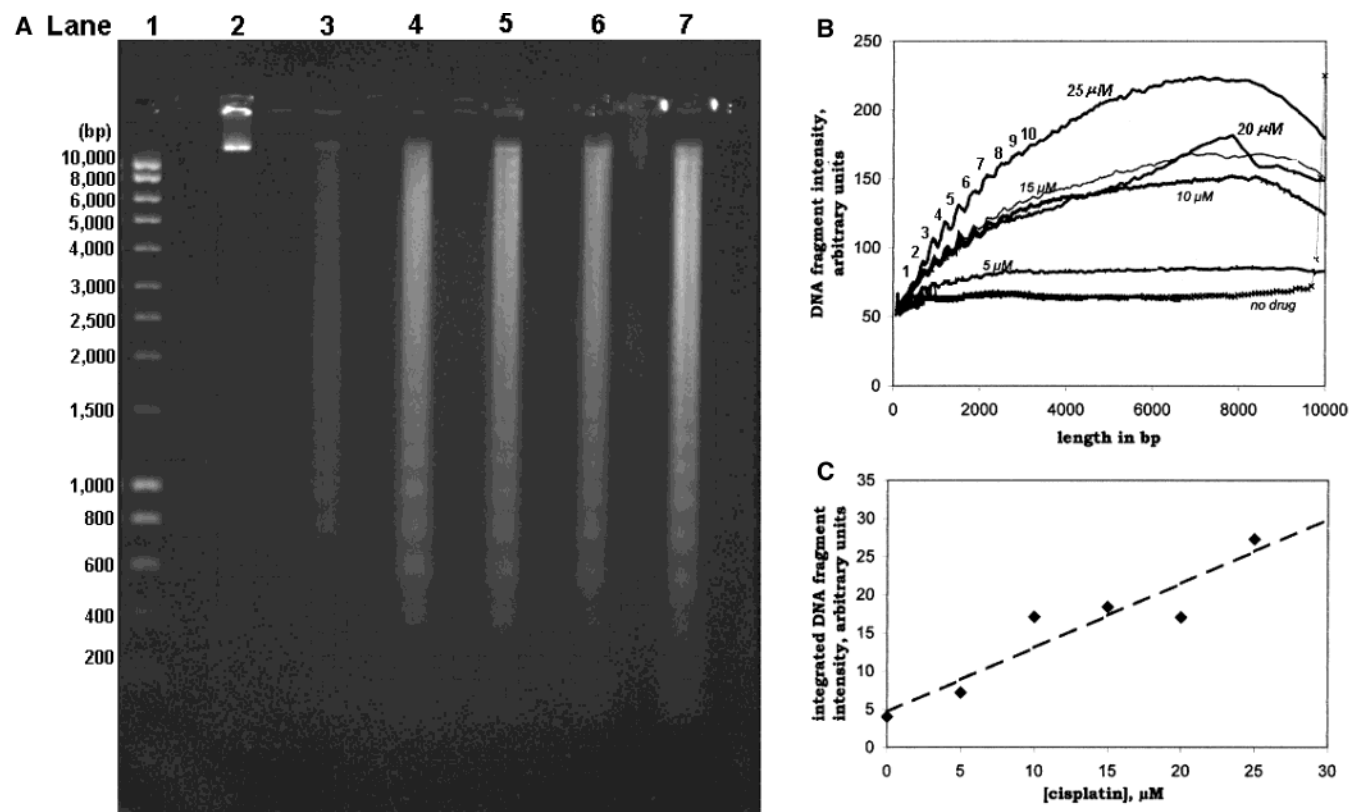


Figure 4. DNA fragmentation in the presence of cisplatin. The experimental conditions were as described in the legend to Table 2. Three independent experiments were done, and their results (mean \pm SD) are shown in Table 2. A representative gel is shown in panel A. Lane 2 (from left), signal of DNA from cells incubated with no addition; lane 3, 5 μ M cisplatin; lane 4, 10 μ M cisplatin; lane 5, 15 μ M cisplatin; lane 6, 20 μ M cisplatin; and lane 7, 25 μ M cisplatin. Lane 1 is a DNA Hyper-Ladder I (200–10 000 bp). (B) The set of intensity vs position plots for these lanes. The intensity vs position plot of the Hyper-Ladder was used to render a position vs bp length calibration curve. The average spacing between peaks 1 and 6 of the four highest cisplatin concentrations in panel B was 305.9 bp, with a population standard deviation of 32.4. (C) The integrated intensities (area below the curves in panel B) as a function of [cisplatin], with the best linear fit.

Table 3. Effects of the Pan-Caspase Inhibitor zVAD-fmk on Cisplatin-Induced Loss of Cell Viability, Mitochondrial Dysfunction, and DNA Fragmentation^a

| additions | cell viability (%) | k (μ M O ₂ min ⁻¹ /10 ⁶ cells) | DNA fragment intensity (arbitrary units) |
|-----------------------------------|--------------------|--|--|
| none ^b | 96 \pm 1.5 | 0.31 \pm 0.06 | 4.4 \pm 1.5 |
| cisplatin ^c | 63 \pm 7.0 | 0.11 \pm 0.01 | 19.4 \pm 4.3 |
| zVAD-fmk ^d | 95 \pm 4.2 | 0.29 \pm 0.13 | 4.5 \pm 1.0 |
| zVAD-fmk + cisplatin ^e | 91 \pm 5.5 | 0.09 \pm 0.02 | 16.7 \pm 3.3 |
| zVAD-fmk + cisplatin ^f | 94 \pm 2.0 | 0.04 \pm 0.05 | 7.2 \pm 0.4 |

^a All samples were incubated for a total of 29 h. Cells ($\sim 10^7$ per condition) were incubated for 2 h at 37 °C without^{b,c} and with^{d-f} 2 μ M zVAD-fmk. Twenty-five micromolar cisplatin was then added,^{c,e,f} and the incubations were continued for 3 h. At the end of the incubation period, the cells were maintained in cisplatin-free medium without^{b,c,e} and with^{d,f} 2 μ M zVAD-fmk for 24 h. The number of cells, viability, k , and DNA fragment intensity were determined as described in the Materials and Methods. The numbers are means \pm SD of three experiments.

plus cisplatin (5 h total, pretreatment with 2 μ M zVAD-fmk for 2 h followed by an additional 3 h incubation in the presence of 25 μ M cisplatin; the cells were then suspended in a zVAD-fmk-free medium for 24 h), the cellular respiration decreased by a factor of ~ 1.2 , the viability increased by a factor of ~ 1.5 , and the amount of DNA fragments decreased by a factor of ~ 1.2 as compared to cells treated with cisplatin alone. Longer incubations with zVAD-fmk plus cisplatin (29 h total, 2

μ M zVAD-fmk for 2 h followed by additional 3 h in the presence of 25 μ M cisplatin; the cells were then suspended in medium containing 2 μ M zVAD-fmk for 24 h) were also carried out. The result was that the viability increased by a factor of ~ 1.5 , the value of k decreased by a factor of ~ 2.8 , and the amount of DNA fragments decreased by a factor of ~ 2.7 , as compared to cells treated with cisplatin alone (Table 3 and Figure 5).

Pt-DNA Adducts. Pt-DNA adducts were measured at the end of a 3 h incubation (at 37 °C) with 0–25 μ M cisplatin. The number of adducts was approximately linear with [cisplatin] ($R > 0.93$, Figure 6A). During the incubation period with cisplatin, Pt-DNA adducts are continuously formed and removed (11). Inhibition of the NER mechanism has been shown to increase adduct levels ~ 2 -fold (39). Thus, the numbers of Pt-DNA adducts measured were net values, representing the difference between the number of adducts formed and the number of adducts removed.

The amount of Pt-DNA adducts correlated with the decrease in the value of k ($R > 0.93$, Figure 6B) and with the increase in DNA fragment intensities ($R > 0.88$, Figure 6C). In addition, the decrease in the value of k correlated approximately linearly ($R > 0.95$) with the increased DNA fragment intensities (Figure 6D). These observations are consistent with Pt-DNA adducts producing proportional apoptotic stimuli as is generally believed (15, 17).

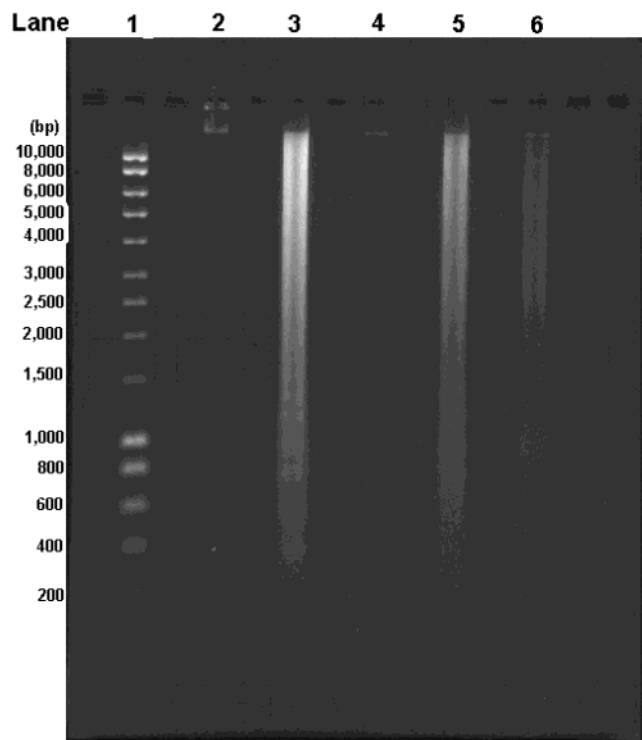


Figure 5. Effect of zVAD-fmk on cisplatin-induced DNA fragmentation. The experimental conditions were as described in the legend to Table 3. Lane 2 (from left) shows the DNA from Jurkat cells incubated without cisplatin. Lane 3 shows the DNA from cells incubated with 25 μM cisplatin. Lane 4 shows the signal of the DNA from cells incubated with zVAD-fmk alone for ~ 24 h. Lane 5 shows the DNA from cells incubated with zVAD-fmk for 2 h followed by 25 μM cisplatin for 3 h; the cells were then maintained in the medium with no addition for ~ 24 h. Lane 6 shows the DNA from cells incubated with zVAD-fmk for 2 h followed by 25 μM cisplatin for 3 h; the cells were then maintained in the medium plus zVAD-fmk for ~ 24 h. Lane 1 is a DNA Hyper-Ladder I (200–10 000 bp).

Discussion

In this work, we studied the effects of cisplatin on Jurkat cells by measuring the rate of cellular respiration, the amount of drug-induced DNA fragmentation, and the amount of cell death using the viability assay. An interesting finding in the study is that the rate of oxygen consumption by the cells immediately following exposure to the drug is normal, but it decreases at later times (24 and 48 h), indicating that cell death is occurring. By contrast, pharmacological concentrations of alkylating agents and anthracyclines produce immediate impairment of mitochondrial function in Jurkat cells (30). The decrease in respiration 24 h after exposure is proportional to the concentration of cisplatin (0–25 μM) and is linearly correlated with the increase in the amount of small DNA fragments induced by cisplatin damage to genomic DNA (Figure 6D). Moreover, both the changes in respiration and the amount of small DNA fragments are proportional to the amount of Pt bound to DNA, as measured immediately after exposure to the drug (Figure 6B,C). If DNA is isolated at later times after exposure to the drug, repair is found to have removed most of the Pt from the DNA, leaving concentrations of bound Pt, which are too low to be reliably measured with AAS (11). As is evident from Table 2, the increase in the amount of DNA fragments with cisplatin concentration correlates with the amount of cell death as measured by a staining (viability) assay. These results are consistent with several

studies demonstrating the time- and dose-dependent cytotoxicity of cisplatin and other Pt drugs in various cancer cell lines and tumor cells (40–44).

While it is well-known that cisplatin can modify the template activity of DNA, thereby initiating events that ultimately lead to cell death, less is known about the mechanism by which the drug blocks mitochondrial function. In this study, we measure the effect of treatment of BHM with cisplatin. Inside the cell, where the chloride concentration is low, more aquated cisplatin species are produced. These species are more reactive chemically and, hence, more cytotoxic than the parent drug (45, 46). The concentration of the diaquated forms in the cell is likely to be small (31), but this form has been shown to increase oxygen consumption (characteristic of an uncoupling of oxidative phosphorylation) in isolated rat kidney mitochondria (47). Neither fresh cisplatin nor aged cisplatin (which is about three-fourths monoaquated) affects the ability of BHM to consume oxygen. Because aging increases the amount of the monoquo form, it seems that neither this species nor the dichloro form directly attacks the mitochondrial respiratory chain in Jurkat cells. While the drug is known to platininate mtDNA (27, 28), the correlation observed between the impairment of respiration and the DNA-Pt adduct formation (Figure 6B) suggests that the signal to terminate respiration involves a pathway that probably has platinination of genomic DNA as its initiating step.

To determine if there is a link between the cascade of events that produce apoptosis and those that cause injury to the mitochondria, we added the apoptotic inhibitor zVAD-fmk to incubation media containing Jurkat cells and cisplatin. This compound inhibits all caspases, thus blocking the cell's commitment to apoptosis (36, 48). Each experiment with Jurkat cells and z-VAD-fmk was 29 h in length, with viability, respiration, and DNA fragmentation measured at the end. Exposure of the cells to 25 μM cisplatin from hour 2 to hour 5 of the experiment, with no inhibitor present, resulted in a loss of cell viability and cellular respiration and an increase in DNA fragmentation, as compared to the control. This shows that exposure to the drug causes a substantial population of the cells to undergo apoptosis. However, if 2 μM VAD-fmk is present for the duration of the experiment, with drug present from hour 2 to hour 5, viability and DNA fragment intensities are essentially the same as for the control. Thus, 2 μM inhibitor blocks the normal functioning of the caspase system responsible for DNA fragmentation and loss of viability. Interestingly, it does not block the ability of the drug to decrease cellular respiration, since, as shown in Table 3, respiration is greatly reduced when drug is present, with or without inhibitor. Table 3 also shows that if 2 μM inhibitor is present for the first 5 h of the experiment and drug is present during the period 2–5 h, apoptosis takes place, as evidenced by DNA fragmentation. Respiration is decreased, but viability remains high, $91 \pm 5.5\%$, as is also the case for extended exposure to the inhibitor (29 h). This shows that the cell has an intact membrane (as determined by trypan blue staining) even though the DNA inside is fragmented. This observation is consistent with other reports showing that DNA fragmentation precedes the loss of membrane integrity (49, 50) for cells treated with anticancer drugs.

The small DNA fragments analyzed in the experiments are the result of cleavage of genomic DNA by nucleases as the cell dies. Because we observe the end of this

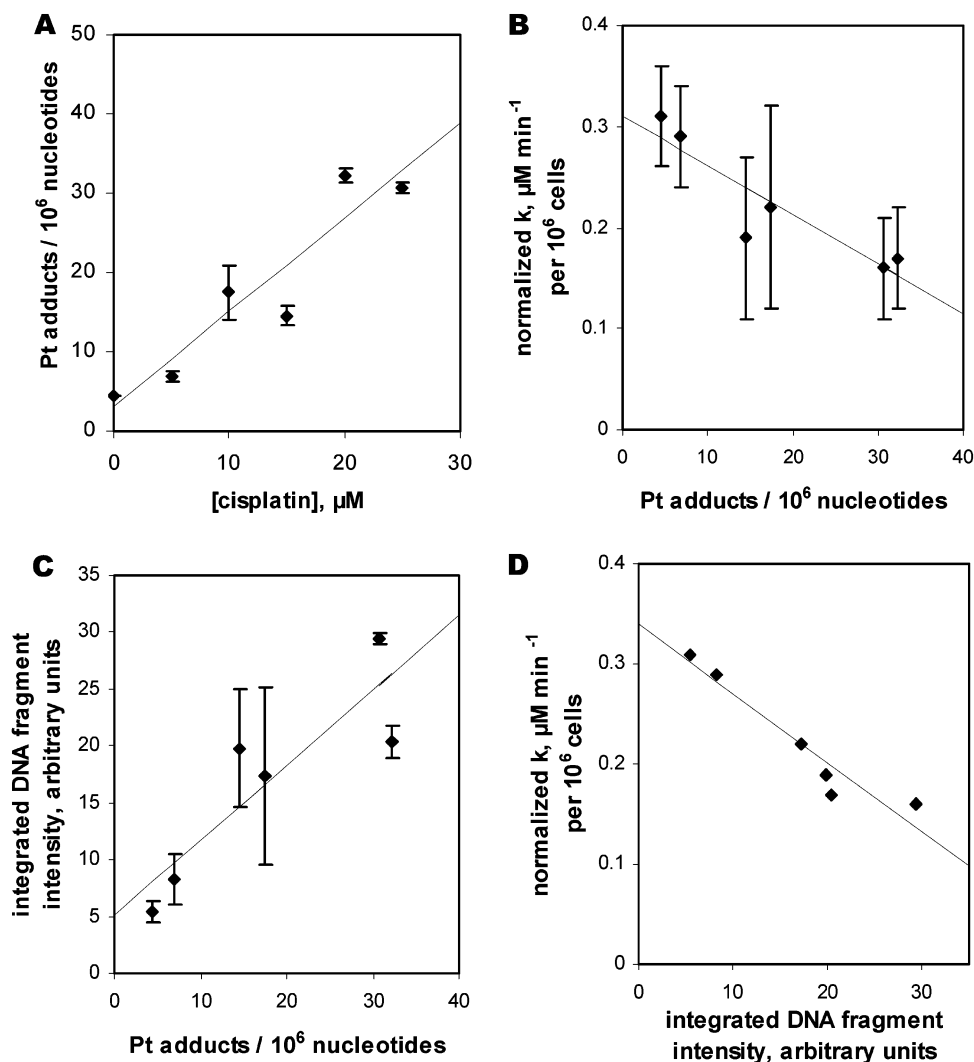


Figure 6. Correlations among DNA platination levels, mitochondrial dysfunction, and apoptotic DNA fragmentation. Cells ($\sim 10^7$ cells per condition) were incubated at 37°C for 3 h with 0–25 μM cisplatin. At the end of the incubation period, the DNA was immediately extracted and the Pt-DNA adducts were determined on the AAS. (A) The amount of adducts vs [cisplatin] with the best linear fit. (B) The values of k , measured 24 h postcisplatin exposure, as a function of Pt-DNA adducts with a linear fit. (C) The intensities of DNA fragmentation, measured after 24 h postcisplatin exposure, as a function of Pt-DNA adducts with a linear fit. (D) The relationship between the values of k and the intensities of DNA fragmentation with a linear fit.

cutting process, the peaks (bands) observed in the gel should correspond to the spacing between the nucleosomes of fragmenting nuclear DNA. The estimated spacing between the nucleosomes (bands) as determined by Peak Fit analysis of the intensity vs position plots (Figure 4B) was 306 ± 32 bp, which is somewhat larger than that reported for the “DNA ladder” (~ 200 bp) obtained by cleavage between nucleosomes in apoptosis (51). The entire fragmentation pattern could be fitted to a series of Gaussian bands, with widths increasing with molecular weight, consistent with all of the fragments being produced by cutting of structured genomic DNA, as in apoptosis. Because the addition of a smooth background improves the fit slightly, the presence of a background of randomly cleaved DNA (due to necrosis) cannot be entirely ruled out. In fact, it has been previously reported that apoptosis and necrosis may take place simultaneously within the same population of cisplatin-treated cells (17).

The DNA fragmentation pattern observed in all gel lanes (Figures 4 and 5) decreased in cells treated with zVAD-fmk (Table 3 and Figure 5). While the observed

DNA fragmentation pattern could be due to a combination of apoptosis on a background of random cutting from necrosis, the fact that the caspase inhibitor blocks most of the DNA cleavage strongly suggests that the observed pattern is mainly due to cisplatin-induced apoptosis and suggests that the contribution of necrosis to the observed DNA fragmentation pattern is minimal. However, as with the analysis of the DNA fragmentation pattern in cells treated with cisplatin alone, the presence of a small amount of cisplatin-induced necrosis could not be completely ruled out.

The data from the present investigation are used, in part, to generate a hypothetical model for cisplatin cytotoxicity in Jurkat cells (Figure 7). We measured cisplatin-induced platination of genomic DNA, mitochondrial dysfunction, and DNA fragmentation. Platination of DNA correlates with a loss of mitochondrial function (Figure 6B) and fragmentation of DNA (Figure 6C). In addition, DNA fragmentation correlates with decreased cellular respiration (Figure 6D and Figure 7). It is conceivable that adduct formation with RNA and proteins may contribute to the observed cisplatin-induced cyto-

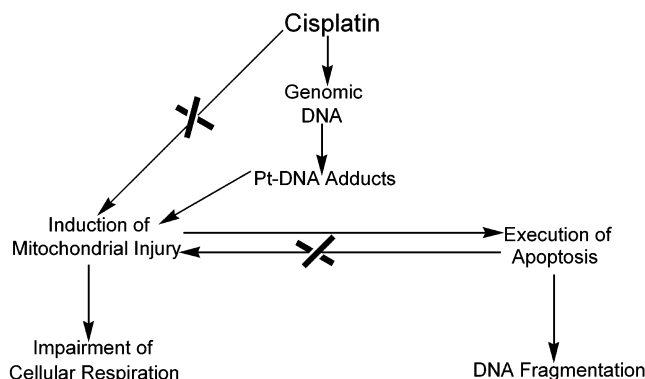


Figure 7. Hypothetical model for cisplatin-induced cell death in Jurkat cells, based partly on the results of the present investigation. Pathways with an "x" appear to be nonfunctional with cisplatin.

toxicity (17). However, our results are in accord with previous studies in cisplatin sensitive and resistant cells showing that cytotoxicity is proportional to DNA platination (40–42).

Incubations with BHM demonstrate that cisplatin's action on mitochondrial respiratory function is indirect. The presence of zVAD-fmk did not prevent drug-induced reduction in respiration but did prevent drug-induced DNA fragmentation. Platination of genomic DNA is probably the initiating step for the signal to terminate respiration, and the decrease in cellular respiration likely occurs prior to caspase activation (Table 3). The loss of mitochondrial respiratory function has been shown to follow the release of cytochrome *c* into the cytosol during apoptosis, which has been shown to be caspase-independent in Jurkat cells (6, 52). Thus, the mitochondrial dysfunction that we observe 24 h after cisplatin exposure is likely preceded by cytochrome *c* release. Our model is consistent with the observations that under pharmacological conditions, cisplatin-induced cell death accompanying mitochondrial dysfunction occurs after Pt-DNA adduct formation (3, 15, 17, 43, 51, 53).

While the mechanisms by which the execution phase of cisplatin-induced apoptosis occurs are well-known (15, 17), the pathway(s) leading from Pt-DNA adducts to apoptotic induction (schematic in Figure 7) has not been fully defined (54). Several mechanisms have been proposed including activation of the c-jun N-terminal kinase/stress-activated pathway, JNK/SAPK (52). Other studies have suggested that the proapoptotic protein Bak mediates the release of cytochrome *c* during cisplatin-induced apoptosis in Jurkat cells (55). By contrast, overexpression of the Bcl-2 protein has been associated with a ~3-fold increase in cellular GSH and resistance to cisplatin (56, 57). However, the number of Pt-DNA adducts was not affected by the increased GSH concentration suggesting that GSH may contribute to resistance by blocking the cells commitment to apoptosis (56).

In summary, we quantitatively studied the long-term impact of cisplatin on cellular respiration and how it relates to cisplatin-induced cell death in Jurkat cells, a well-characterized cancer cell line. We showed that while platination of DNA is immediate, effects of the drug on respiration, DNA fragmentation, and viability are delayed. Because cisplatin and aged solutions of the drug have no effect on the respiration of BHM, the drug-induced, caspase-independent decrease in respiration observed in Jurkat cells is likely not due to direct attack

of the drug on the mitochondrial respiratory chain. While the precise mechanism(s) initiated by the drug and responsible for terminating respiration remains unknown, it is likely preceded by cisplatin-induced release of cytochrome *c* (6, 7, 24). However, cisplatin-induced inhibition of key metabolic genes responsible for maintaining oxidative phosphorylation cannot be ruled out (58). The results obtained should provide the basis for more quantitative work with other cells, including sensitive and drug resistant tumors. The results of these studies should help in constructing a comprehensive model for cisplatin cytotoxicity that will shed light on the molecular mechanisms responsible for cisplatin-induced impairments of mitochondrial function. In turn, the model will aid in the design of novel therapeutic strategies for enhancing cisplatin efficacy, by inhibiting mitochondrial respiration (59).

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