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Serine Protease Inhibition and Mitochondrial Dysfunction Associated with Cisplatin Resistance in Human Tumor Cell Lines: Targets for Therapy

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ABSTRACT. Indicators of mitochondrial function were studied in two different cell culture models of *cis*-diamminedichloroplatinum-II (CDDP) resistance: the intrinsically resistant human ovarian cancer cell line CI-80-13S, and resistant clones (HeLa-S1a and HeLa-S1b) generated by stable expression of the serine protease inhibitor—plasminogen activator inhibitor type-2 (PAI-2), in the human cervical cancer cell line HeLa. In both models, CDDP resistance was associated with sensitivity to killing by adriamycin, etoposide, auranofin, bis[1,2-bis(diphenylphosphino)ethane]gold(I) chloride {[Au(DPPE)$_2$]Cl}, $CdCl_2$ and the mitochondrial inhibitors rhodamine-123 (Rh123), dequalinium chloride (DeCH), tetraphenylphosphonium (TPP), and ethidium bromide (EtBr) and with lower constitutive levels of ATP. Unlike the HeLa clones, CI-80-13S cells were additionally sensitive to chloramphenicol, 1-methyl-4-phenylpyridinium ion (MPP^+), rotenone, thenoyltrifluoroacetone (TTFA), and antimycin A, and showed poor reduction of 1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), suggesting a deficiency in NADH dehydrogenase and/or succinate dehydrogenase activities. Total platinum uptake and DNA-bound platinum were slightly lower in CI-80-13S than in sensitive cells. The HeLa-S1a and HeLa-S1b clones, on the other hand, showed poor reduction of triphenyltetrazolium chloride (TTC), indicative of low cytochrome c oxidase activity. Total platinum uptake by HeLa-S1a was similar to HeLa, but DNA-bound platinum was much lower than for the parent cell line. The mitochondria of CI-80-13S and HeLa-S1a showed altered morphology and were fewer in number than those of JAM and HeLa. In both models, CDDP resistance was associated with less platinum accumulation and with mitochondrial and membrane defects, brought about one case with expression of a protease inhibitor which is implicated in tumor progression. Such markers may identify tumors suitable for treatment with gold phosphine complexes or other mitochondrial inhibitors. BIOCHEM PHARMACOL 53;11:1673–1682, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. ovarian cancer; cisplatin resistance; type-2 plasminogen activator inhibitor; mitochondria; platinum accumulation

CDDP resistance is an important obstacle in the treatment of human tumors, including ovarian cancer. Acquired CDDP resistance appears in most patients after they receive several courses of chemotherapy, and is believed to be one of the major causes contributing to the high death rate from ovarian cancer. Factors that have been identified with drug resistance include reduced drug accumulation [1–3], elevated glutathione and metallothioneins [4–6], reduced DNA damage, increased DNA repair and tolerance of DNA damage [7, 8]. CDDP accumulation was found to be a partially energy-dependent process where Na^+,K^+-ATPase plays a pivotal role [9, 10]. In addition, mitochondrial dysfunction has been found to be associated with CDDP resistance and reduced drug accumulation [11–13]. More specific knowledge of the mitochondrial defects may allow alternative forms of therapy to be devised.

CDDP-resistant human tumor cell lines have been derived by *in vitro* treatment of sensitive cells [11, 14, 15, 16]. Some of these cell lines showed decreased platinum accumulation, high sensitivity to rhodamine-123 (Rh123), increased Rh123 uptake, increased plasma membrane potential and increased mitochondrial membrane potential [11,

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Abbreviations: [Au(DPPE)$_2$]Cl, bis[1,2-bis(diphenylphosphino)ethane]-gold(I) chloride; CDDP, *cis*-diamminedichloroplatinum-II; DeCH, dequalinium chloride; EtBr, ethidium bromide; MPP^+, 1-methyl-4-phenylpyridinium ion; *mt*, mitochondrion; MTT, (1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PAI-2, type-2 plasminogen activator inhibitor; PBS, phosphate buffered saline, pH 7.2; Rh123, rhodamine-123; TPP, tetraphenylphosphonium; TTC, triphenyltetrazolium chloride; TTFA, thenoyltrifluoroacetone; and ICP-MS, inductively coupled plasma-mass spectroscopy.

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13]. A CDDP-resistant subclone of murine P388 leukemia was not cross-resistant to the gold(I) phosphine complexes bis[1,2-bis(diphenylphosphino)ethane]gold(I) chloride {[Au(DPPE]$_2$)Cl} and auranofin, but a subclone resistant to [Au(DPPE)$_2$]Cl was cross-resistant to several mitochondrial inhibitors including tetraphenylphosphonium (TPP) and Rh123 [17]. Exposure to Au[(DPPE)$_2$]$^+$ resulted in depletion of cellular ATP, which occurred more rapidly in the sensitive than in the resistant cells [18]. Hoke *et al.* showed that [Au(DPPE)$_2$]Cl uncoupled oxidative phosphorylation by increasing the permeability of the inner mitochondrial membrane to cations and protons [19, 20].

The potentials of the plasma membrane and mitochondria create an inside negative gradient, which allows the uptake and retention of positively charged lipophilic cations, such as [Au(DPPE)$_2$]$^+$, Rh123, dequalinium chloride (DeCH), TPP and 1-methyl-4-phenylpyridinium ion (MPP$^+$) [19–24]. The multiple drug resistance pathway does not appear to be responsible for reduced uptake [25]. Once inside the cells, these drugs may exhibit different mechanisms of toxicity. Rh123 was shown to inhibit mitochondrial ATPase [26] and DeCH inhibits ATP production [22]. MPP$^+$ causes a selective blockage of NADH-linked respiration [27]. Ethidium bromide (EtBr) was described as specific inhibitor of mitochondrial DNA replication [28, 29], and chloramphenicol blocks mitochondrial protein synthesis [30]. Rotenone, triphenyltetrazolium chloride (TTFA) and antimycin A inhibit complex I, complex II, and complex III in the mitochondrial respiratory chain respectively [31]. The antiarthritic gold(I) phosphine complex auranofin is cytotoxic to a variety of cancer cells *in vitro*, perhaps by inhibition of respiratory complex III [32] after being transported through a sulfhydryl exchange process involving proteins of the plasma membrane [33].

The aim of the present study was to more closely define the molecular nature of the defects in CDDP-resistant tumor cell lines with apparent mitochondrial dysfunction. Two human models of CDDP resistance derived without CDDP selection are described: the intrinsically resistant ovarian cancer cell line (CI-80-13S), which was compared with other ovarian tumor cell lines; and clones of HeLa (HeLa-S1a and HeLa-S1b) derived by stable expression of plasminogen activator inhibitor (PAI-2). The latter were found to be CDDP-resistant, during a study of phenotypic changes induced by PAI-2. Both CI-80-13S and HeLa-S1a were found to have reduced numbers of mitochondria and exhibited markers of mitochondrial dysfunction including sensitivity to agents that could be considered for therapeutic use, such as Rh123 [21, 34, 35], DeCH [22, 36], TPP [37] and gold(I) phosphine complexes [17].

MATERIALS AND METHODS

Drugs and Chemicals

[Au(DPPE)$_2$]Cl were synthesized as previously described [17]. Hoechst 33258 was purchased from Boerhinger Mannheim (Germany). All other drugs were purchased from Sigma Chemical Company (St. Louis, USA).

Cell Lines and Culture Conditions

The human ovarian tumor cell lines CI-80-13S [38], JAM [39], and GG [40] were used in this study. Construction of PAI-2 expression vectors and characterization of PAI-2 expressing HeLa subclones has been described [41]. Essentially the subclones were derived by stably transfecting the human cervical cancer cell line, HeLa, with PAI-2 cDNA in the sense orientation (HeLa-S1a, HeLa-S1b) and the antisense orientation (HeLa-A2/17) under the control of the cytomegalovirus (CMV) promoter. The ASM1 subclone contains sense PAI-2 cDNA wherein the active site Arg [380] has been mutated to Ala [380], thereby abolishing the inhibitory activity of PAI-2 for urokinase-type plasminogen activator. Each of the stably transfected subclones was isolated following G418 selection and expression of the PAI-2 transgene characterized by immunoblot analysis and quantitated by ELISA. Cells were grown as monolayers in Roswell Park Memorial Institute (RPMI) medium 1640 (Flow Laboratories, Sydney, Australia) supplemented with 1 mM pyruvate and 200 μM nicotinamide. Cultures were maintained at 37°C in a humidified incubator with a atmosphere of 95% air and 5% CO_2. Cells were routinely tested at 4-weekly intervals with the Hoechst 33258 assay for *Mycoplasma* [42].

Cell Survival

Cell survival was determined by ^{3}H-thymidine incorporation in colonies 4–7 days after treatment [43]. Cells were seeded in 96-well plates (2–4 replicates) at a density of 2–3 × 10^3 cells/100 μl per well and incubated overnight to allow attachment, followed by addition of drug. After 4–7 days, the cells were incubated for 4–6 hr in medium containing 5 μCi/ml [methyl-^{3}H]-thymidine, washed in PBS (phosphate-buffer saline, pH 7.2), detached (0.02% trypsin and 0.1 mM EDTA in PBS) and washed onto glass fibre disks (Whatman GF/A) for liquid scintillation counting. Cell survival was calculated for 5 doses spanning the measurable range of toxicity (1–100% survival), as a percentage of cpm in control (untreated) cells. The D_{37} for each drug (concentration giving 37% survival, and which on average inactivates one cell) was calculated by interpolation from a plot of log % survival vs drug concentration. Stock solutions of drugs were freshly prepared in culture medium for each experiment.

Glutathione Determination

Glutathione levels of JAM, CI-80-13S, HeLa and HeLa-S1a were assayed as described by Godwin [4] and Hissin [44]. Briefly, 2–5 × 10^6 of 60% confluent cells were lysed by sonication in PBS for 30 sec, and then centrifuged at 10000 rpm for 10 min at 4°C. To precipitate the protein 12%

5-sulfosalicylic acid (SSA) was added (1 vol of SSA to 3 vol of sample). The samples were centrifuged (10000 rpm for 10 min at 4°C) after standing on ice for 1–2 hr. SSA extract (100 μl) was assayed with 0.5 units of glutathione reductase per well in the microtitre plate. Absorbance at 410 nm was measured by an ELISA scanner.

ATP Assay

The method was modified from Ahmann *et al.* [45]. Cells were seeded in black microtitre plates at a density of 2×10^4 cells/100 μl per well 24 hr previously, and then treated with CDDP or Rh123 for 1 hr, washed with PBS, 100 μl of hot water added and the bottom of the plate exposed to steam from a boiling water bath for 2 min to inactivate ATPases. After cooling, 10 μl of luciferase solution (5 mg/mL Sigma FLE-50 firefly lantern extract, 1 mg/ml acetyl CoA, 20 mM $MgSO_4$ and 50 mM potassium arsenate, pH 7.4) was added to each well and the luminescence read immediately on a Packard Top Plate counter.

Platinum Accumulation in Cellular Protein and Genomic DNA

Cells were harvested after treatment with 3.3 μM and 15.5 μM CDDP for 1 hr at 37°C and three washes in PBS, and then pelleted by centrifugation. The pellets were resuspended in PBS and sonicated for 30 sec at 4°C. Bicinchoninic acid reagent (Pierce Chemical Co., Illinois, USA) was used for protein estimation. Genomic DNA was isolated using the salt precipitation method [46] after 1 hr exposure of cells to 3.3 μM CDDP, and measured at 260 nm for DNA estimation. The suspensions were digested in 1% hydrochloric acid and then analysed for platinum concentration on an inductively coupled plasma-mass spectrometer (ICP-MS, Plasma Quad, Fisons, Middlewich, UK) [47]. The platinum content of the samples were determined by comparing with a standard, and an internal control was used.

Respiratory Chain Enzyme Assays

Enriched mitochondrial fractions were prepared from cultured cells by a method described for fibroblast mitochondria [48]. Respiratory chain complexes I (rotenone-sensitive NADH:coenzyme Q_1 oxidoreductase), II (succinate: coenzyme Q_1 oxidoreductase), II + III (succinate:cytochrome c oxidoreductase), III (decylbenzylquinol:cytochrome c oxidoreductase), IV (cytochrome c oxidase) and the mitochondrial marker enzyme citrate synthase were assayed spectrphotometrically in duplicate in these preparations by modifications of published methods [48–51], as described in detail elsewhere [52].

MTT and TTC Assays

Modified MTT and TTC assays were done essentially as described by Musser *et al.* [53] and Mosmann *et al.* [54]. The MTT and TTC were dissolved at 5 mg/mL and 10 mg/mL respectively in PBS, filter sterilized using a 0.22 μm disposable filter (Sartorius) and stored at 4°C. Cells (1×10^4 in 0.1 mL medium) were placed into individual wells of a flat bottomed 96-well microtiter-plate and incubated at 37°C overnight. MTT or TTC stock solution (10 μl) was added and incubated at 37°C for 4 hr to allow mitochondria to form a colored, insoluble formazan product. The supernatant was carefully aspirated and 100 μl/well of dimethyl sulphoxide was added to solubilize the formazan crystals. The microplate was agitated for 1 hr at room temperature on a mechanical shaker (Quantum Scientific) to solubilize crystals completely. The absorbance at 570 nm (MTT) and 490 nm (TTC) were measured by a Bio-Rad 3350 microplate reader.

Electron Microscopy

Cultures of JAM, CI-80-13S, HeLa and HeLa-S1a were trypsinized, washed twice in ice-cold PBS and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. The pellet was washed and post-fixed with 1% OsO_4 prior to embedding in epoxy resin. Sections were examined with a JOEL 12 EX transmission microscope. The numbers of mitochondria were counted in thirty cells, selecting sections that showed a complete nucleus. Statistical significance was determined by *t* test.

RESULTS

Sensitivity of CDDP-resistant Cells to Anticancer and Other Agents

As found previously [55], the CI-80-13S cell line was much more resistant to CDDP than JAM (Fig. 1A) but not to other antitumor agents including melphalan, adriamycin and etoposide (Table 1). On the other hand, compared with JAM, the CI-80-13S line was highly sensitive to $CdCl_2$ (Table 1), Rh123 (Fig. 1B), and a wide range of other mitochondrial inhibitors (Table 2), with the exception of the complex II inhibitor TTFA. The PAI-2 positive clones were resistant to CDDP (Fig. 2A) and melphalan (Table 1), but sensitive to adriamycin, etoposide and $CdCl_2$ compared with the parent HeLa line and nonexpressing clones HeLa-A2/17 and ASM1 (Table 1). Compared with the parent line, HeLa-S1a and S1b cells were sensitive to Rh123 (Table 2, Fig. 2B), and some mitochondrial inhibitors (EtBr, TPP, auranofin, and $[Au(DPPE)_2]Cl$), but were not particularly sensitive to chloramphenicol, DeCH, MPP^+, rotenone, TTFA and antimycin A (Table 2).

The above sensitivities to mitochondrial inhibitors may have arisen from a defect in glycolysis, limiting the supply of substrates for the respiratory chain. However, supplementation of the culture medium with pyruvate or malate up to 5 mM did not relieve the toxicity of Rh123 in CI-80-13S cells (data not shown). There were no major differences in the doubling times or sizes of the cell lines tested.

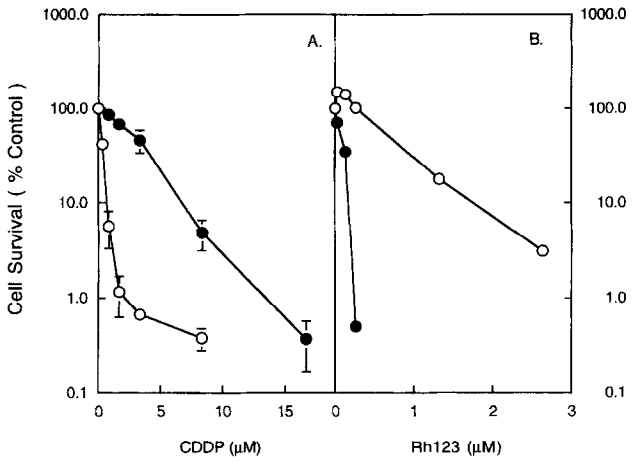

FIG. 1. Drug concentration response for survival of JAM (○) and CI-80-13S (●) treated continuously with CDDP (panel A) or Rh123 (panel B). Points, mean ± SE (N = 3–6 separate experiments).

Platinum Accumulation in Cellular Protein and Genomic DNA

Cellular platinum accumulation and DNA-bound platinum were examined in four cell lines. Table 4 demonstrates that CI-80-13S cells had 30–50% lower platinum levels in cellular protein and DNA compared with JAM. For the PAI-2 model, while the total cellular level of platinum in the resistant cells (HeLa-S1a) was slightly higher than the sensitive cells, the level of DNA platination in the HeLa-S1a cells was extremely low (Table 3). The platination levels are lower than a previous report [56], presumably because the fetal calf serum, included here to maintain conditions comparable to the cell survival assays, bound a significant proportion of CDDP.

Respiratory Chain Enzyme Activities in Disrupted Mitochondria

Enzymes were assayed in the mitochondrial fraction, to obtain more specific information about mitochondrial properties that might be relevant to the cell sensitivities noted above. The specific activities of respiratory chain complexes I to IV in mitochondrial fractions of CI-80-13S and JAM cells were not markedly different (Table 4). Activity of the mitochondrial matrix enzyme citrate synthase, however, was substantially higher in CI-80-13S cells, suggesting a possible increase in total mitochondrial volume. Elevated citrate synthase activity is a common finding in biopsies or cell lines from patients with respiratory chain dysfunction, who often have mitochondrial proliferation. This compensatory response to a respiratory chain defect can mask the enzyme defect, unless enzyme activities are expressed relative to another mitochondrial enzyme rather than to total cellular protein. It is common practice to correct for this by expressing respiratory chain enzyme activities relative to citrate synthase (CS ratios). The CS ratios for CI-80-13S cells ranged from 30% to 70% of the corresponding values for JAM, and were mostly at the lower end or below the observed ranges found in mitochondria from other cultured cells such as EBV-transformed lymphoblasts and primary skin fibroblasts. The data are thus suggestive of a global respiratory chain defect, particularly affecting complexes IV and II, whose residual activities were 30–40% of JAM.

Mitochondrial Reduction of Tetrazolium Dyes in Intact Cells

Modified MTT [53, 54] and TTC assays [53] were used to compare functional enzyme activities in the mitochondrial respiratory chain of intact cells. MTT is a tetrazolium salt whose main electron donor is ubiquinol (i.e. reduced ubiquinone formed by complexes I and II) and which can also accept electrons less efficiently directly from complexes I and II and from another site which appears to be either reduced cytochrome c or complex III [53, 54, 57]. This assay has been used not only to assess metabolic function and cell viability, but also mitochondrial damage in cells [53, 58]. TTC reduction occurs at the terminal end of the respiratory chain where it accepts electrons directly from complex IV (cytochrome c oxidase) [53].

TABLE 1. Relationship between sensitivity to anticancer drugs and glutathione levels

Cell Lines	D^*_{37} CDDP (μM)	Melphalan (μM)	Adriamycin (nM)	Etoposide (nM)	$CdCl_2$ (μM)	GSH Level nmol/10^6 cells
JAM	0.83 ± 0.3 (6)	2.98 ± 0.45 (3)†	>40 (3)	240 ± 50.0 (3)	30 ± 4.40 (2)	212.9
CI-80-13S	4.17 ± 0.5 (6)	3.93 ± 0.53 (3)	13.6 ± 4.60 (3)	14 ± 3.0 (3)	2.19 ± 0.44 (2)	94.7
GG	1.50 ± 0.33 (3)	4.85 ± 0.82 (3)	>40 (3)	64 ± 1.0 (3)	5.26 ± 0.88 (2)	74.6
HeLa	0.287 ± 0.04 (3)	2.46 ± 0.43 (3)	21 ± 3.20 (3)	40 ± 5.0 (3)	38.6 ± 5.26 (2)	36.6
HeLa-S1a	0.617 ± 0.083 (3)	4.66 ± 0.95 (3)	6 ± 1.10 (3)	9.5 ± 1.0 (3)	8.11 ± 1.10 (2)	42.4
HeLa-S1b	0.587 ± 0.047 (3)	7.56 ± 1.02 (3)	7.4 ± 2.60 (3)	11.9 ± 3.0 (3)	7.89 ± 0.44 (2)	NT‡
HeLa-A2/17	0.317 ± 0.03 (3)	3.11 ± 0.43 (3)	14 ± 2.20 (3)	22.6 ± 2.0 (3)	37.7 ± 13.4 (2)	NT
HeLa-ASM1	0.320 ± 0.017 (3)	2.72 ± 0.43 (3)	24 ± 4.5 (3)	40 ± 5.6 (3)	39.9 ± 4.4 (2)	NT

* Concentration required to reduce survival to 37% determined by cell survival assay.
† Mean ± SE. Number of independent experiments in parenthesis.
‡ NT: not tested.

TABLE 2. Sensitivity of human cell lines to mitochondrial inhibitors

Mitochondrial Inhibitor	Site of Action	Ref. No	D^*_{37} (μM) JAM	CI-80-13S	HeLa	HeLa-S1a	HeLa-S1b	HeLa-A2/17	HeLa-ASM1	GG
Chloramphenicol	mt Protein Synthesis	[30]	710 ± 7.1 (3)†	92.9 ± 17.3 (3)	838.7 ± 86.0 (3)	1220.1 ± 92.9 (3)	NT‡	NT	NT	NT
EtBr	mtDNA Synthesis	[28] [29]	2.1 ± 0.28 (4)	0.17 ± 0.07 (4)	1.78 ± 0.05 (4)	0.59 ± 0.17 (4)	NT	NT	NT	NT
Rotenone	Complex I	[31]	0.38 ± 0.07 (3)	0.023 ± 0.002 (3)	0.247 ± 0.005 (2)	0.176 ± 0.008 (2)	NT	NT	NT	NT
MPP^+	membrane Complex I	[24] [27]	1.37 ± 0.07 (3)	0.27 ± 0.02 (3)	1.13 ± 0.13 (3)	1.01 ± 0.02 (3)	1.28 ± 0.17 (3)	1.04 ± 0.03 (3)	NT	0.95 ± 0.03 (3)
TTFA	Complex II	[29]	40.5 ± 8.8 (2)	31.5 ± 3.6 (2)	63.1 ± 2.3 (2)	43.24 ± 6.8 (2)	NT	NT	NT	NT
Antimycin A	Complex III	[31]	20.9 ± 2.2 (2)	0.15 ± 0.03 (2)	25.5 ± 2.7 (2)	46.4 ± 2.9 (2)	NT	NT	NT	NT
TPP	Membrane	[23]	33.3 ± 5.0 (3)	8.2 ± 0.050 (3)	40.4 ± 3.7 (3)	16.7 ± 2.1 (3)	22.8 ± 3.5 (3)	40.5 ± 4.2 (3)	33.3 ± 5.5 (3)	21.4 ± 4.1 (3)
Rh123	Membrane	[21]	0.90 ± 0.03 (3)	0.105 ± 0.026 (3)	2.73 ± 0.28 (4)	0.58 ± 0.08 (3)	0.77 ± 0.11 (2)	2.63 ± 0.32 (2)	3.11 ± 0.25 (3)	1.16 ± 0.34 (3)
DeCH	Membrane	[22]	5.27 ± 1.06 (4)	0.72 ± 0.076 (4)	1.58 ± 0.27 (3)	1.08 ± 0.06 (3)	NT	NT	NT	NT
Auranofin	Membrane? Complex III?	[32] [33]	1.4 ± 0.02 (3)	0.27 ± 0.03 (3)	0.32 ± 0.03 (3)	0.11 ± 0.02 (3)	0.12 ± 0.02 (3)	0.67 ± 0.12 (3)	0.89 ± 0.15 (3)	2.50 ± 0.40 (4)
$[Au(DPPE)_2]Cl$	Membrane?	[17]	0.73 ± 0.15 (3)	0.20 ± 0.03 (3)	0.95 ± 0.13 (3)	0.073 ± 0.005 (3)	0.06 ± 0.013 (3)	0.96 ± 0.15 (3)	0.86 ± 10.16 (3)	NT

* Concentration required to reduce survival to 37% determined by cell survival assay.
† Mean ± SE. Number of independent experiments in parenthesis.
‡ NT, not tested.

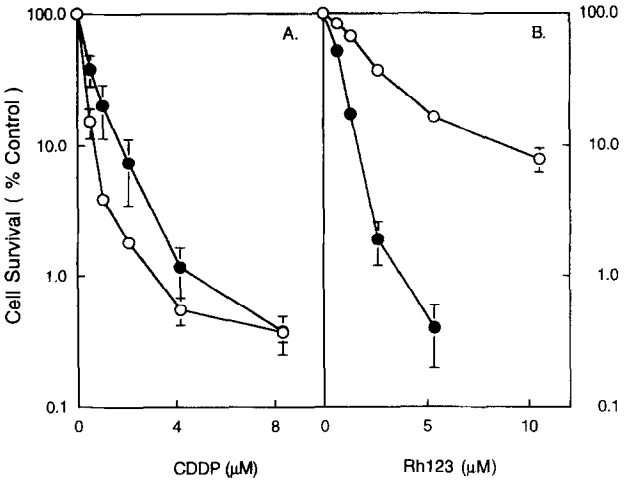

FIG. 2. Drug concentration response for survival of HeLa (○) and HeLa-S1b (●), treated continuously with CDDP (panel A) or Rh123 (panel B). Points, mean ± SE (N = 3).

The levels of MTT and TTC reduction in CDDP-resistant cells relative to other cell lines were taken to indicate the possible sites of defects in the respiratory chain upstream of Complex IV. CI-80-13S showed depleted MTT reduction compared with the other ovarian lines and HeLa (Fig. 3). MTT reduction was inhibited by $[Au(DPPE)_2]Cl$ in JAM, HeLa, HeLa-S1a and HeLa-S1b, to the same level as in untreated CI-80-13S cells. Reduction of TTC by HeLa-S1a and S1b cells was much less effective than in their PAI-2 nonexpressing counterparts (Fig. 4). TTC reduction in ovarian tumor cell lines did not correlate with CDDP resistance.

Mitochondrial Morphology

JAM, CI-80-13S, HeLa and HeLa-S1a cells were examined by electron microscopy to determine the morphology of their mitochondria (Fig. 5). Both CI-80-13S (25.8 ± 13.4 mitochondria per cell section) and HeLa-S1a (25.2 ± 10.4) showed approximately half the mitochondrial number of JAM (44.6 ± 19.5) and HeLa (40.7 ± 13.2) ($P < 0.001$). The mitochondria in CI-80-13S cells were larger, with condensed matrix and enlarged spaces within cristae, and the cristae were thicker and absent in some mitochondria, compared with JAM cells. The mitochondria of HeLa-S1a tended to be more spherical than those of HeLa cells.

Constitutive ATP Levels and Depletion by Drug Treatment

The ATP content of cells was measured before and after treatment with CDDP or a mitochondrial inhibitor (Rh123), to determine whether ATP levels were associated with cell survival. Preliminary experiments (not shown) demonstrated that the luciferase assay for ATP was highly sensitive and gave a linear response with cell number, as found previously [45], and that a drug incubation time of 1

TABLE 3. Platinum accumulation in total cellular protein and genomic DNA

Cell line	Treatment CDDP (μM)	Platinum accumulation* (pg Pt/mg protein)	DNA platination* (pg Pt/μg DNA)
JAM	0	0.0 ± 0.0†	0.0 ± 0.0
	3.3	260.31 ± 39.01	0.129 ± 0.04
	16.7	1494.20 ± 142.20	1.54 ± 1.15
	33.3	4221.60 ± 240.20	2.93
CI-80-13S	0	0.0 ± 0.0	0.0 ± 0.0
	3.3	188.81 ± 37.20	0.102 ± 0.03
	16.7	774.24 ± 26.22	0.699 ± 0.10
	33.3	2362.30 ± 420.10	1.44
HeLa	0	0.0 ± 0.0	0.0 ± 0.0
	3.3	129.53 ± 15.09	1.55 ± 0.33
	16.7	1329.64 ± 170.90	3.11 ± 0.4
	33.3	NT‡	7.64
HeLa-S1a	0	0.0 ± 0.0	0.0 ± 0.0
	3.3	192.14 ± 7.80	0.1716 ± 0.0002
	16.7	1499.90 ± 60.59	0.7737 ± 0.12
	33.3	NT	0.9120

* Cellular protein and DNA were isolated as described in the text; platinum was determined by ICP-MS.
† Mean ± SD were from three experiments (N = 3) or one typical experiments with triplicate (SD < 20%).
‡ NT, not tested.

hr led to maximum loss of ATP without loss of cells from the culture.

The results from repeated experiments (Fig. 6) showed that the CDDP-resistant, Rh123-sensitive cell lines CI-80-13S and HeLa-1a had approximately 30% less ATP than JAM or HeLa cells. Drug treatment reduced the ATP content by up to 50% in all 4 cell lines, with no major difference in the concentration response being found in the Rh123-sensitive cells.

DISCUSSION

It is critical to understand the mechanisms of CDDP resistance so that clinically-relevant approaches can be formulated to overcome, or to exploit the CDDP-resistant phenotype. This study gives new information about the nature of the mitochondrial defects associated with CDDP resistance, the contrasting biological effects of other metal complexes, and the potential role of PAI-2 in drug sensitivity, and identified agents that may find use in treating tumors that are spontaneously resistant to CDDP. The targets for the drugs used in this study and the proposed defects in the two cell models are represented in Fig. 7 (modified from ref. [54]).

Multiple mechanisms of cellular resistance to CDDP require consideration in order to explain the CDDP-resistant phenotypes in the above models. Elevated metallothionein levels would appear unlikely in the CDDP-resistant lines because these cells were sensitive to cadmium chloride. No significantly increased GSH level was found in either model (Table 1). It also appears unlikely that tolerance or increased repair of DNA damage is involved in CDDP resistance because no major difference in CDDP-treated adenovirus replication was found between JAM and

TABLE 4. Respiratory chain enzyme activities in mitochondria isolated from ovarian cancer cell lines

Cell Lines	Complex I	Complex II	Complex II + III	Complex III	Complex IV	Citrate Synthase
			Enzyme Activities*			
JAM	133	162	104	8.4	7.8	364
CI-80-13S	128	113	117	8.5	4.0	600
			CS Ratios†			
JAM	364	445	286	23	22	—
CI-80-13S	214	118	196	14	7	—
Lymphoblasts‡	275–410	170–510	205–450	30–62	10–17	—
Fibroblasts‡	180–520	153–390	135–510	25–112	12–46	—

* Enzyme activities are specific activities for complexes I, II, II + III and citrate synthase (nmol/min/mg of protein) and first order rate constants for complexes III and IV (/min/mg of protein). Activities were determined in duplicate and duplicate values agreed within ± 10%.
† CS ratios are enzyme activities divided by the citrate synthase activity.
‡ Observed normal ranges for enzymes in comparable mitochondrial preparations from 6 normal Epstein Barr virus-transformed lymphoblast cell lines and 35 normal primary skin fibroblast cell lines.

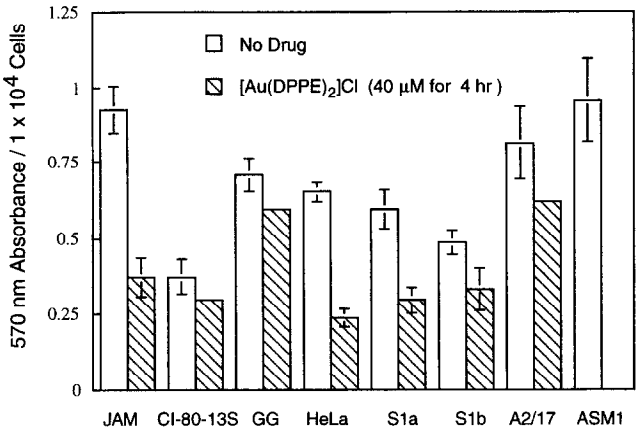

FIG. 3. Reduction of MTT by cells in culture. Cells were treated with $[Au(DPPE)_2]Cl$ for 1 hr, then 500 μg/mL MTT added for a further 3 hr. Bars, mean ± SE of 3–5 independent experiments.

CI-80-13S cells [55]. The inverse relationship between resistance to CDDP and mitochondrial inhibitors may therefore point to ATP deficiency being an advantage in limiting the accumulation of CDDP [1, 9, 10]. Such an association was found in this study although with different intracellular outcomes in each model. CI-80-13S cells demonstrated less platinum associated with cellular protein and with DNA, consistent with the CDDP resistance of these cells although perhaps not low enough to explain the relatively high level of resistance. In PAI-2 expressing cells, however, protection from platination occurred exclusively at the DNA level, indicating that transfer of CDDP to the nucleus or binding of CDDP to DNA itself may be severely limited. Given the role of PAI-2 as a serine protease inhibitor, expression of PAI-2 may affect proteolytic mechanisms regulating processes involving nuclear transport of CDDP and mitochondrial function.

Mitochondrial uptake of $[Au(DPPE)_2]Cl$, Rh123, DeCH, TPP and MPP^+ is driven by high membrane potential [18, 21–24], which may explain the sensitivity of some cell lines to these agents. CI-80-13S and HeLa-S1a cells, however, did not differ greatly from other cell lines in Rh123 uptake

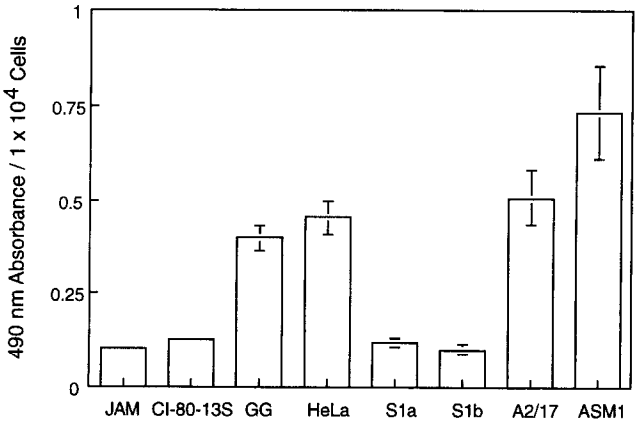

FIG. 4. Reduction of 1 mg/mL TTC by cells during 4 hr in culture. Bars, mean ± SE of 3–4 independent experiments.

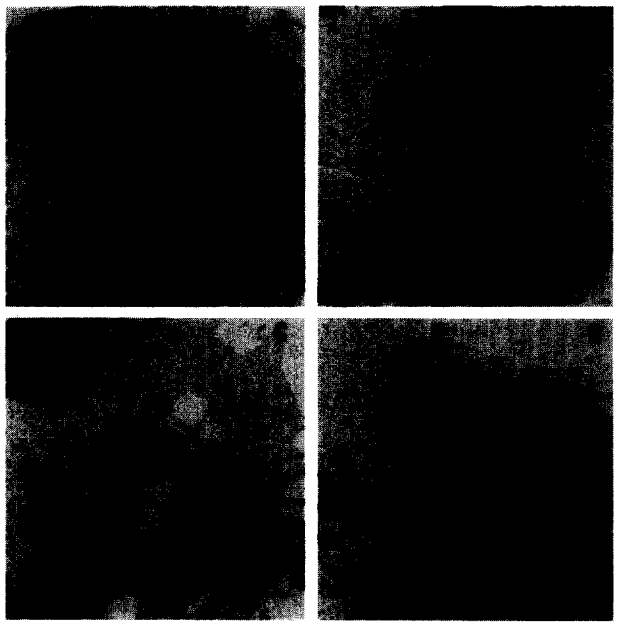

FIG. 5. Electron micrographs showing mitochondria in JAM (a), CI-80-13S (b), HeLa (c) and HeLa-S1a (d). Original magnification was ×10000. Bar: 500 nm.

when analysed by flow cytometry (data not shown). It should also be noted that drug uptake may be regulated by total mitochondrial volume as well as by membrane potentials. In addition, CDDP-resistant CI-80-13S cells were found to be sensitive to mitochondrial inhibitors that are not lipophilic cations, rotenone and chloramphenicol. Thus specific mitochondrial defects are more likely to explain the drug sensitivities of the present models than a change that results in selective uptake of lipophilic cations.

The CI-80-13S cell line and the PAI-2 HeLa subclones appear to have different and possibly multiple deficiencies in the mitochondrial respiratory chain, associated with 30% lower ATP levels. Most of the individual respiratory chain enzyme complexes in disrupted mitochondria of CI-80-13S had lower activity than those of JAM when expressed relative to the mitochondrial marker enzyme, citrate synthase. The measured *in vitro* enzyme activities do not necessarily reflect the primary site of respiratory chain damage, so reduction of the tetrazolium dyes MTT and TTC by intact cells was therefore used to assess respiratory chain function. MTT reduction by CI-80-13S was found to be much lower than the other ovarian cancer cell lines. MTT accepts electrons most readily from ubiquinol formed by either complex I or complex II [53, 54]. CI-80-13S cells were also sensitive to MPP^+, which is taken up into mitochondria as a result of the membrane potential [24] and preferentially inhibits NADH dehydrogenase (complex I) (Fig. 7) [27]. These findings taken together suggest that the CI-80-13S cell line has lower functional activity of complex I (NADH dehydrogenase) or of both complex II (succinate dehydrogenase) and complex I. Such reduced activity may result in insufficient ion transfer and consequent swelling of the mitochondria, and additional stress

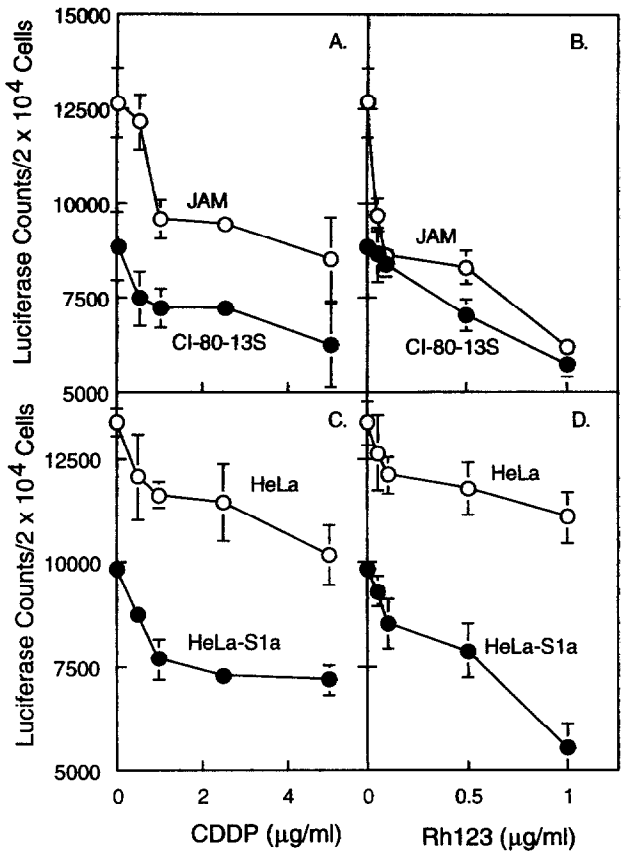

FIG. 6. ATP levels in cells treated with CDDP or Rh123 for 1 hr. Points, mean ± SE of 4 experiments.

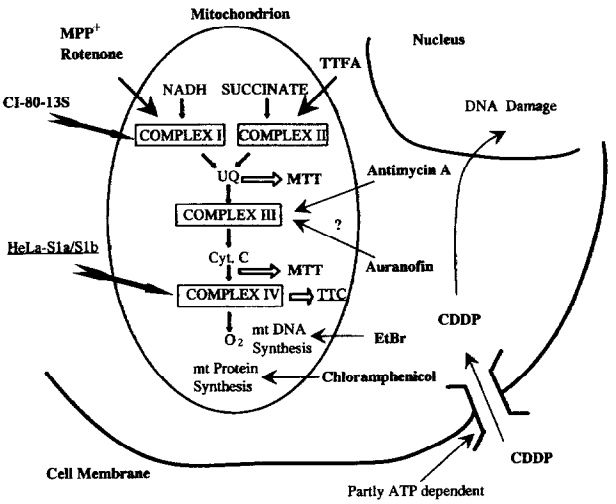

FIG. 7. Mitochondrial reduction of tetrazolium salts and proposed sites of action of mitochondrial agents (shown as bold type for agents selectively toxic to CI-80-13S and underlined for agents selective for HeLa-S1a/S1b).

from any mitochondrial inhibitor may be sufficient to affect cell viability. Such defects may give rise to lower Na^+,K^+-ATPase activity and higher membrane potentials. Hela-S1a and S1b cells might have lower complex IV (cytochrome c oxidase) activity since reduction of TTC was lower in these cells and the cells were not sensitive to inhibitors of complexes I to III in the respiratory chain (MPP^+, rotenone, and antimycin A). Thus, defects in particular respiratory chain complexes may explain the different drug sensitivities of the cells examined in this study.

The two CDDP-resistant models were highly sensitive to a range of mitochondrial inhibitors. Further evaluation will be needed to determine whether gold complexes are more suitable for therapeutic use than DeCH or other lipophilic cations [37]. If such selectivity can be confirmed using other cell types *in vitro*, and is not associated with side effects such as cardiac toxicity *in vivo*, some of these agents may be appropriate for intensive, second line therapy in patients where the tumor can be identified as having mitochondrial defects. This might be possible using some of the markers described above, or by assaying tumors for PAI-2 like activity. None of the ovarian tumor cell lines examined here expressed PAI-2. However, it is possible that CI-80-13S cells express some other protease inhibitor or have lost a protease activity during transformation, resulting in a growth advantage. Such clones may be present in other tumors and become dominant following CDDP therapy.

Platinum was assayed with the help of Matthew Downing, Chemistry Department, Queensland University of Technology. Electron micrographs were produced by Clay Winterford, Pathology Department, University of Queensland. Respiratory chain enzymes were assayed by Denise Kirby, the Murdoch Institute, Royal Children's Hospital, Melbourne. This work was supported by the Australian International Development Assistance Bureau, the Queensland Cancer Fund and the National Health and Medical Research Council.

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