Mitochondrial Defects in cis-Diamminedichloroplatinum(II)-resistant Human Ovarian Carcinoma Cells

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ABSTRACT

We have been studying the membranes of cisplatin (DDP)-resistant 2008 human ovarian carcinoma cells (C13* cells) for alterations that may account for their decreased DDP accumulation. We now report that C13* cells have significant changes in their mitochondrial and plasma membrane potentials and in their mitochondrial morphology. C13* cells accumulated 2.0 ± 0.1-fold more of the membrane potential marker [3H]tetraphenylphosphonium cation (TPP+) than sensitive cells. In high K+ medium, which depolarizes the plasma membrane but not the mitochondrial membrane, [3H]TPP+ accumulation was still 2.3 ± 0.1-fold greater in resistant cells, indicating that the mitochondrial membrane potential was higher. In the presence of carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone, which depolarizes the mitochondrial membrane but not the plasma membrane, [3H]TPP+ accumulation demonstrated that the plasma membrane potential in C13* cells was elevated as well. These elevations were also present in C8 cells with low-level DDP resistance. After ouabain treatment, exposure to nigericin stimulated [3H]TPP+ accumulation 3-fold in sensitive cells but had no effect in C13* cells, indicating either that: (a) the mitochondrial pH gradient was minimal; or (b) the mitochondrial electric potential was already at a maximal level in C13* cells. Fluorescence microscopy of living cells stained with the mitochondria-specific dye rhodamine 123 revealed that resistant cells had significant changes in their mitochondrial morphology. Electron microscopy also revealed major alterations in the cristae structure. The C13* cells, which were approximately 15-fold resistant to DDP, were 5-fold hypersensitive to the mitochondrial poison rhodamine 123. We conclude that these DDP-resistant 2008 cells have an elevated plasma membrane potential and alterations in their mitochondria as indicated by their membrane potential, morphology, and sensitivity to mitochondrial poisons. These results imply that mitochondria play an important role in the cellular pharmacology of DDP.

INTRODUCTION

Ovarian carcinoma is a major cause of cancer deaths in women (1). The addition of DDP to the chemotherapeutic attack of this disease has dramatically improved response rates (2). Nonetheless, up to 80% of all ovarian carcinoma patients will succumb to their disease within 5 years (1–3). The major factor contributing to this somber statistic is believed to be the emergence of drug resistant tumor populations. Although little is known about the mechanisms of DDP resistance in patients, mechanistic studies of resistance in a plethora of in vitro and in vivo model systems have identified 3 major themes. These are decreased drug accumulation, increased drug inactivation by elevation of intracellular nucleophiles (glutathione and metallothionein), and enhanced removal of the DDP-induced lesions in DNA (4).

Our studies on the mechanisms of resistance in 2008 human ovarian carcinoma cells have focused on the decreased DDP accumulation in resistant cells (5–9). We have recently reported that DDP accumulation is partially energy dependent, partially Na+ dependent, and partially ouabain inhibitible (5, 9). Changes were identified in the Na+,K+-ATPase in the DDP-resistant 2008 cells (designated C13*), but these changes had no effect on the Na+ gradient. Since the Na+,K+-ATPase is the primary generator of the membrane potential, we asked whether the altered Na+,K+-ATPase may be affecting the membrane potential in C13* cells. We report here that C13* cells have not only an elevated plasma membrane potential, but also, surprisingly, an elevated mitochondrial membrane potential. The mitochondria of these C13* cells are morphologically deranged and the cells have become hypersensitive to lipophilic cations.

MATERIALS AND METHODS

Drugs and Chemicals. DDP (clinical formulation) was obtained from Bristol-Myers Oncology Division (Syracuse, NY). [3H]TPP+ bromide (39.5 Ci/mmol) was obtained from New England Nuclear Research Products (Boston, MA). Rhodamine 123, nigericin octahydrate, FCCP, and valinomycin were from Sigma Chemical Co. (St. Louis, MO). TPP+ bromide and sodium tetraphenylboron were obtained from Aldrich Chemical Co. (Milwaukee, WI). RPMI 1640 powder medium without glutamine, glucose, NaHCO3, NaCl, and KCl was obtained from Flow Laboritories, Inc. (McLean, VA).

Cell Lines. The 2008 cell line, established from a patient with serous cystadenocarcinoma of the ovary, was used in these studies (10). The DDP-resistant cells were generated as previously described by monthly selection with DDP (10). Cells that were selected 8 times were designated C8 and were approximately 3-fold resistant as defined by the ratio of DDP concentrations causing 50% inhibition of colony formation in a continuous exposure clonogenic assay. After 13 monthly selections, the cells were exposed chronically to DDP at 0.25 µM and incrementally increased to 5.25 µM. These cells are designated C13* and were approximately 15-fold resistant. Cells were grown as monolayers in RPMI 1640 supplemented with 5% heat-inactivated bovine calf serum supplemented with iron (Hyclone, Ogden, UT), 2 mM freshly added glutamine, and 50 µg/ml gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). Cultures were equilibrated with humidified 5% CO2 in air at 37°C. Cells were routinely tested at 6-week intervals with a Mycoplasma detection kit (Gen-Probe, San Diego, CA). All studies were done with Mycoplasma negative cells. Clonogenic assays on plastic were conducted as described previously (10).

Membrane Potential. Relative membrane potential was determined with the indicator [3H]TPP+ (11–13). Cells were seeded into 60-mm tissue culture dishes. When the cells were subconfluent, the medium was aspirated and replaced with 1 ml of 37°C RPMI 1640 (normal K+ medium) containing 5 µM [3H]TPP+ (0.2 µCi/ml). Some plates received RPMI 1640 in which the NaCl had been replaced by equimolar KCl (high K+ medium) in order to depolarize the plasma membrane. The plates were returned to the incubator, and at appropriate times they were removed, the medium aspirated, and the wells rapidly washed 4 times with 4°C phosphate buffered saline consisting of, per liter: 8 g NaCl, 0.2 g KCl, 1.15 g Na2HPO4, and 0.2 g KH2PO4. The cells were...
digested overnight in 2 ml 1N NaOH. A 1.6-ml aliquot was mixed with Ecolite(+) scintillation fluid (ICN Biomedical, Inc., Costa Mesa, CA) acidified with 12 ml glacial acetic acid per liter, and radioactivity measured on an LS 1801 liquid scintillation counter (Beckman). Protein was determined on 50-μl aliquots (14). In some experiments, 5 μg/ml nigericin was included with the [3H]TPP+ to hyperpolarize the cells. Nigericin was added from a 5 mg/ml stock in ethanol kept at −20°C. Some plates were preincubated with 0.2 mM ouabain for 1 h in RPMI 1640 before [3H]TPP+ addition. In other experiments, 5 μM FCCP or 2 μM valinomycin were included to depolarize the mitochondria. FCCP was kept as a 5 mM stock in dimethyl sulfoxide at −20°C and nigericin (± TPP+) than controls or other treatments, but the number was well below 1% of the total cells. Plasma membrane potential was estimated from the equation (13):

$$\Delta \psi_{\text{plasma}} = -61.5 \text{ mV} \times \log [\text{TPP}^+]_{\text{FC-cp}}/[\text{TPP}^+]_{\text{out}}$$

where [TPP+]_{FC-cp} was the concentration of [3H]TPP+ inside cells exposed to FCCP, calculated using a value of 5 μM/mg protein for cell volume in both cell types.

Fluorescence Microscopy. Cells were grown to subconfluence on square glass coverslips in 100-mm tissue culture dishes. The medium was aspirated and replaced with 10 mM Hepes buffer, pH 7.4, containing 12.5 to 50 μM rhodamine 123 and returned to the incubator (15, 16). The Hepes buffer also contained 123 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM MgCl2, and 1 mM CaCl2. After 10 min, the medium was again aspirated, and the cells were washed with Hepes buffer and then incubated in Hepes buffer until microscopic examination 10–20 min later. Coverslips were inverted onto microscope slides containing 2 drops of Hepes buffer. Stained cells were observed by epifluorescent photomicroscope using the Planapochromat ×63 objective lens. Cells were photographed with Kodak TMAX ASA 400 black and white film pushed one stop and using the −2 automatic exposure adjustment.

Electron Microscopy. Electron micrographs were provided by the service of Dr. Cheng-ming Chang of the Scripps Clinic and Research Foundation (La Jolla, CA). Subconfluent cultures were fixed for 1 h at 4°C in modified Karnovsky’s fixative consisting of 1.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2. The cells were then postfixed in 1% OsO4 in 0.1 M cacodylate buffer for 1 h at room temperature, en-block stained in half-strength saturated aqueous uranyl acetate for 1 h, dehydrated through graded ethanol, and embedded in TAAB 812 (TAAB Laboratory Equipment, Ltd., Reading, UK). Silver to light gold sections were cut parallel to the culture dish surface of Dr. Cheng-ming Chang of the Scripps Clinic and Research Foundation (La Jolla, CA). Subconfluent cultures were fixed for 1 h at 4°C in modified Karnovsky’s fixative consisting of 1.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2. The cells were then postfixed in 1% OsO4 in 0.1 M cacodylate buffer for 1 h at room temperature, en-block stained in half-strength saturated aqueous uranyl acetate for 1 h, dehydrated through graded ethanol, and embedded in TAAB 812 (TAAB Laboratory Equipment, Ltd., Reading, UK). Silver to light gold sections were cut parallel to the culture dish surface.

RESULTS

[3H]TPP+ is a lipophilic cation that accumulates in proportion to the membrane potential (11–13). Fig. 1 shows the time course of [3H]TPP+ accumulation in 2008 and C13* cells. As found with most carcinoma cells (12, 16), [3H]TPP+ continued to accumulate and levels did not plateau up to 2 h after exposure in either cell line. Neither addition of 5 μM sodium tetrabenzylboron [the tetrabenzylboron anion has been reported to accelerate membrane penetration by phosphonium cations by forming an ion pair (17)] to the medium nor placing the cells in suspension prior to [3H]TPP+ exposure eliminated this steady rise in [3H]TPP+ levels (data not shown). The C13* cells accumulated more [3H]TPP+ than 2008 cells at all time points (Fig. 1). Using the last 3 time points, the C13* to 2008 [3H]TPP+ accumulation ratio was 2.0 ± 0.1 (n = 3). Cells were also incubated in medium containing high concentrations of K+ to depolarize the plasma membrane potential; the remaining [3H]TPP+ accumulation in high K+ medium was thus due to the mitochondrial membrane potential. High K+ conditions cause minimal changes in cell volume in these cells (data not shown). Mitochondrial accumulation accounted for 57 ± 4% (n = 5) and 53 ± 4% (n = 5) of the total [3H]TPP+ accumulation in 2008 and C13* cells, respectively. The ratio of total (normal K+) to mitochondrial (high K+) [3H]TPP+ accumulation was 1.9 ± 0.1 and 2.1 ± 0.1 for the last 3 time points in 2008 and C13* cells, respectively; this difference was not statistically significant. The elevation in [3H]TPP+ levels in C13* cells compared to 2008 cells was maintained in high K+ medium, indicating that the mitochondrial membrane potential was higher in these cells. Using the 30-, 60-, and 120-min time points, the C13* to 2008 mitochondrial [3H]TPP+ accumulation ratio was 2.3 ± 0.1 (n = 3).

Cells were treated with the ionophore valinomycin to confirm that the [3H]TPP+ accumulation in high K+ medium was mitochondrial and was accurately reflecting the membrane potential. Valinomycin is a K+ ionophore that slightly hyperpolarizes the plasma membrane but depolarizes the mitochondrial membrane potential (12, 15, 18). When 2 μM valinomycin was added to 2008 cells that had been loaded 1 h with [3H]TPP+, there was an immediate egress of [3H]TPP+. The [3H]TPP+ dropped to 47 ± 3% (n = 2, ± range) of controls after 5 min (from 952 to 454 pmol/mg protein). If cells were exposed to 2 μM valinomycin in high K+ medium, and 53 ± 4% (n = 5) of the total [3H]TPP+ accumulation in 2008 and C13* cells, respectively; this difference was not statistically significant. The elevation in [3H]TPP+ levels in C13* cells compared to 2008 cells was maintained in high K+ medium, indicating that the mitochondrial membrane potential was higher in these cells. Using the 30-, 60-, and 120-min time points, the C13* to 2008 mitochondrial [3H]TPP+ accumulation ratio was 2.3 ± 0.1 (n = 3).

![Fig. 1.](image-url) [3H]TPP+ accumulation in 2008 and C13* cells. A, time course in normal K+ medium. •, 2008 cells; ○, C13* cells. B, time course in high K+ medium. •, 2008 cells; ○, C13* cells. Points, mean values from 3 experiments; bars, SE.

* This assumes that the plasma membrane potential was primarily a K+ diffusion potential in these cells. Contributions from other ions such as Cl− to the membrane potential may also need to be considered.
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Fig. 2. [3H]TPP* accumulation at 1 h in 2008 and C8 cells. •, normal K*; O, high K* medium. Columns, mean values of either 3 (2008) or 4 experiments (C8); bars, SE.

Fig. 3. Effect of nigericin on 1 h [3H]TPP* accumulation in 2008 and C13* cells. Cells were exposed to 5 μg/ml of nigericin at the time of [3H]TPP* exposure. Where indicated, ouabain (0.2 mM) was present 1 h before and during [3H]TPP* exposure. •, control; O, with nigericin added. Columns, mean values of 3 experiments; bars, SE.

FCCP reduced [3H]TPP* accumulation at 1 h to 164 ± 8 pmol/mg (n = 3) in 2008 cells and to 315 ± 27 pmol/mg (n = 3) in C13* cells. These values should reflect accumulation only due to the plasma membrane potential with a small correction for background binding (13, 19). The C13* cells thus accumulate 1.9-fold more [3H]TPP* due to an elevated plasma membrane potential, and this difference was statistically significant (P < 0.01). Although final equilibrium may not have been reached, entry of these values into the Nernst equation delivers values of 50 and 67 mV for the plasma membrane potential of 2008 and C13* cells, respectively.

To determine whether elevation of the membrane potential was an early change in the development of DDP resistance or arose later, i.e., when cells had been treated chronically with DDP, we determined [3H]TPP* accumulation in C8 cells that were approximately 3-fold resistant to DDP (Fig. 2). The total and mitochondrial [3H]TPP* accumulation was also elevated in this cell population.

Nigericin is a K*,H* ionophore that hyperpolarizes cells (12, 15). When 5 μg/ml nigericin was added to the medium, the total accumulation of [3H]TPP* at 1 h increased 5.3 ± 0.4 (n = 3)- and 2.9 ± 0.4 (n = 3)-fold in 2008 and C13* cells, respectively (Fig. 3). The greater hyperpolarization in 2008 cells was statistically significant (P < 0.05). To block the hyperpolarizing effects of nigericin on the plasma membrane, the cells were exposed to 0.2 mM ouabain for 1 h prior to the experiment. Nigericin exposure then can only affect the mitochondrial membrane potential. Under these conditions, nigericin stimulated mitochondrial [3H]TPP* accumulation 3.0 ± 0.3-fold (n = 2, ± range, P < 0.01) in 2008 cells, but only 1.2 ± 0.1-fold (n = 3, not significant) in C13* cells pretreated with ouabain (Fig. 3).

Since the C13* cells had a major elevation in their mitochondrial membrane potential, we examined these cells by fluorescence microscopy after staining with the mitochondria-specific dye rhodamine 123 to determine whether there were any changes in their mitochondrial morphology (Fig. 4). The [3H]TPP* data implicated that C13* cells would take up approximately twice as much rhodamine 123 as 2008 cells. The C13* cells were therefore treated with one-half the concentration of rhodamine 123 as 2008 cells so that micrographs of approximately equal fluorescence intensity could be compared. The mitochondria of 2008 cells were primarily perinuclear and had a normal tubular structure (15, 16). The mitochondria of C13* cells, however, were dispersed throughout the cell and had a disordered appearance. The cells were then examined by electron microscopy to view the morphological alterations in more detail (Fig. 5). The mitochondrial membranes of C13* cells were less electron dense than 2008 cells. The cristae in C13* mitochondria were thicker and more irregular, and were absent in many mitochondria. Prominent, multiple nucleoli were also noted in the resistant cells.

The elevated mitochondrial membrane potential suggested that the C13* cells might be more susceptible to killing by lipophilic cations than the parent 2008 cells. Fig. 6 demonstrates that the C13* cells were indeed 5.0 ± 0.5 (n = 4)-fold hypersensitive to rhodamine 123.

DISCUSSION

Our studies on the mechanisms of the accumulation defect in DDP-resistant 2008 human ovarian carcinoma cells have

Fig. 4. Fluorescence micrographs of living cells stained with rhodamine 123. Each field was photographed under identical conditions. a, 2008 cells stained with 25 μM rhodamine 123; b, C13* cells stained with 12.5 μM rhodamine 123.
previously led us to the observation that these cells have alterations in their Na⁺,K⁺-ATPase (9). Since the Na⁺,K⁺-ATPase is the major electrogenic pump of the plasma membrane, we asked whether these DDP-resistant cells might also have changes in their membrane potential. Our present work documents that C13* cells accumulate approximately twice as much of the membrane potential marker [³H]TPP⁺ as sensitive 2008 cells. This change was accounted for by elevations (i.e., more negative values) in both the plasma and mitochondrial membrane potentials. While the elevated [³H]TPP⁺ accumulation could be due to other factors besides membrane potential, our data appear to rule out several possibilities. (a) The addition of the ionophore valinomycin to [³H]TPP⁺ loaded cells caused an immediate loss (5 min) of approximately 50% of the accumulated [³H]TPP⁺. The [³H]TPP⁺ thus rapidly equilibrated across membranes and appears to be a true marker of membrane potential changes in these cells (13). (b) Accumulation in the presence of high K⁺ and valinomycin, which should collapse both the plasma and the mitochondrial membrane potentials, reduced [³H]TPP⁺ levels to 11 and 14% of the normal K⁺ controls. The elevated [³H]TPP⁺ accumulation thus was not due to potential-independent background binding or accumulation into other organelles besides mitochondria. (c) Another issue is mitochondrial volume. Although this would not affect the conclusion that the plasma membrane potential was elevated as assessed after FCCP treatment, a doubling of mitochondrial volume could explain the 2-fold increase in [³H]TPP⁺ levels under high K⁺ conditions. A formal morphometric analysis of multiple electron micrographs was not undertaken to assess mitochondrial volume, but qualitative analysis of available photographs did not suggest that there were more mitochondria in C13* cells. The [³H]TPP⁺ accumulation data thus
The marked hypersensitivity to lipophilic cations, however, possibly provides an exploitable approach for overcoming this DDP-resistant phenotype.

It is clearly difficult to understand why the mitochondrial defects described should be associated with the DDP-resistant phenotype. Several hypotheses present themselves. One possibility could be that the elevated membrane potential and structural abnormalities are irrelevant changes in these DDP-resistant cells. For example, the changes may have arisen from DDP-induced mutations to the mitochondrial DNA and have no impact on DDP sensitivity. However, the next article (20) suggests that the elevated mitochondrial membrane potential is a central facet of the DDP-resistant phenotype in these cells. A second hypothesis is that there is a critical target for DDP in the mitochondria that the cells have striven to protect. Again, it is difficult to envision why a more negative potential should protect the mitochondria from the neutral DDP molecule or its positively charged aquated metabolites. On the other hand, the elevated potential may merely reflect a secondary effect of the protection scheme. A third hypothesis, which is the converse of the preceding conjecture, is that the mitochondria are noncritical targets for DDP. The elevated mitochondrial membrane potential in C13* cells may serve to electropherotically scavenge the cationic, aquated-DDP species from the cytoplasm into the mitochondria and so prevent the platination of more critical targets such as nuclear DNA. A final possibility is that the membrane potential plays a central role in a signal transduction pathway that can modulate DDP sensitivity. Recent reports indicate that a variety of signal transduction pathways can affect DDP sensitivity. For example, the intracellular Ca²⁺ signals may be particularly important for events such as apoptosis, the cell death program apparently triggered by DDP (31, 32). Mitochondrial Ca²⁺ signals may play an important role in Ca²⁺ signal transduction, and this role is intimately connected to the membrane potential (33, 34). Determining the validity of these hypotheses will stimulate our future experimentation.

Although numerous reports document the effects of DDP on renal mitochondria (35–39), little is known about the impact...
of DDP on tumor cell mitochondria. A number of agents reported to potentiate DDP cytotoxicity, however, are known to have significant effects on mitochondria and these include: (a) cyclosporin A, which interferes with mitochondrial Ca\(^{2+}\) transport (40, 41); (b) lonidamine, which is believed to inhibit mitochondrial hexokinase (42); (c) dequalinium, a lipophilic cation believed to poison the F\(_{0}\)F\(_{1}\)-ATPase (43); (d) novobiocin, which is a significant mitochondrial poison in addition to a nifedipine, a calcium channel blocker that can also affect mitochondrial Ca\(^{2+}\) channels (47, 48); and, finally, (g) cytariabine, which has been recently reported to stimulate mitochondrial respiration (49, 50). In conjunction with our data, this evidence suggests that mitochondria may hold important keys for modulating DDP cytotoxicity in tumor cells.

In summary, we have shown that DDP-resistant 2008 cells have an elevated plasma membrane potential and have mitochondrial defects as indicated by physiological, morphological, and pharmacological criteria. The elevated plasma membrane potential may be linked to the DDP accumulation defect in these cells and the previously reported alterations in the Na\(^{+}\),K\(^{-}\)-ATPase (9). Our data suggest that mitochondria may play an important role in the cellular pharmacology of DDP either directly as targets or sinks, or indirectly by modulating the “signal” that leads to cell death following DDP exposure. The data in this cell line suggest that combinations of DDP with mitochondrial poisons may be a useful therapeutic strategy for attacking drug-resistant tumors.

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