cis-Diaminedichloroplatinum(II) Accumulation in Sensitive and Resistant Human Ovarian Carcinoma Cells

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INTRODUCTION

DDP4 is an effective agent in the treatment of human ovarian, testicular, bladder, and head and neck cancers (1). Despite its usefulness in these diseases, the appearance of DDP resistance is a frequent occurrence in these tumors (2, 3). Several potential biochemical mechanisms of DDP resistance have begun to be elucidated. Some DDP-resistant cells have elevated glutathione (4, 5); however, this is not a universal mechanism and it is not known if this is secondary to other alterations in resistant cells (6, 7). Elevated metallothioneins can be one mechanism of DDP resistance, but its relevance to clinical resistance is unclear (8, 9). Increased DNA repair has also been implicated as a factor in resistant cells (10–12).

Much research effort has been directed at the study of the interaction of DDP with the presumed major target, DNA (13). Less attention, however, has been given to the study of the possible interactions that may occur as DDP crosses biological membranes on its way to the nucleus. The mechanism(s) by which DDP enters cells has not been well defined. It has been assumed for many years that DDP accumulates into cells by a concentration gradient, suggesting that DDP uptake does not involve primary active transport. The metabolic inhibitors, dinitrophenol and NaF, did not decrease DDP accumulation; iodoacetate had a stimulatory effect. Dinitrophenol, however, in combination with NaF or iodoacetate decreased DDP accumulation. A 30-min exposure to 0.2 mM ouabain also decreased DDP accumulation in both parent and resistant cells. A component of DDP accumulation thus appears to be energy dependent. These studies have identified decreased DDP accumulation as an important mechanism of resistance that is expressed early in the acquisition of DDP resistance in human ovarian carcinoma cells.

MATERIALS AND METHODS

Drugs and Chemicals. DDP (clinical formulation) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). A mixture of [195Pt]DDP and [197Pt]DDP (33) was obtained from Oak Ridge National Laboratories (Oak Ridge, TN). 3-O-[methyl-3H]-D-glucose (79 Ci/mmol) was obtained from NEN Research Products (Boston, MA). Iodoacetate acid, dinitrophenol, and ouabain octahydrate were obtained from Sigma Chemical Co. (St. Louis, MO). NaF was from Mallinkrodt, Inc. (Paris, KY).

Cell Lines. The 2008 cell line, established from a patient with serous cystadenocarcinoma of the ovary, was used in these studies (7, 8). The DDP-resistant 2008/DDP cell line was generated as previously described by monthly selection with 1 μM DDP. These cells are designated 2008/DDP (7). The 2008/DDP cells were grown in monolayers in RPMI Medium 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM freshly added glutamine, and 1% Fungi-bact (Irvine Scientific, Santa Ana, CA). Cultures were equilibrated with humidified 5% CO2 in air at 37°C. Cells were routinely tested at 6-wk intervals with a Gen-probe mycoplasma detect-
CISPLATIN ACCUMULATION

DDP Accumulation. For DDP accumulation studies, parental and resistant cells were seeded into 6-well tissue culture plates or 100-mm tissue culture dishes depending on the specific activity of the $[^{195mPt}]$DDP. After 3 to 4 days, when the plates approached confluency, the medium was aspirated and replaced with 37°C RPMI Medium 1640 containing the indicated concentrations of $[^{195mPt}]$DDP. The dishes were immediately returned to the incubator. At appropriate time points the plates were removed from the incubator, the medium was aspirated, and the cells were washed 4 times with 4°C PBS (Oxoid, Columbia, MD). For 100-mm plates, 3 ml of 1 N NaOH was added and the cells were allowed to digest overnight. An aliquot was removed for the determination of protein content by the method of Bradford (34), and 2.5 ml were either placed in test tubes and counted with a Trak 1191 gamma counter (Tracer Analytic, Elk Grove Village, IL) or mixed with 8 ml 3a70B scintillation fluid (RPI Corp., Mount Prospect, IL) and placed in 2.5 ml scintillation vials (Beckman, Fullerton, CA) with the channel windows set to wide open.

When $[^{195mPt}]$DDP was not available, accumulation studies were conducted with unlabeled DDP. Cells were seeded into 100-mm tissue culture plates and when the cells approached confluency the medium was replaced with 5 ml RPMI 1640 containing 100 μM DDP. After a 1-h incubation, the medium was aspirated and the monolayers were washed once with 4°C 0.05% trypsin/EDTA and then trypsinized with 5 ml of 37°C 0.05% trypsin/EDTA for 6 min. The trypsinized cells were added to 10 ml of 4°C complete medium and centrifuged 3 min at 500 × g. The cell pellet was resuspended in 10 ml 4°C PBS and then centrifuged again. The cell pellet was then resuspended in 1.0 ml 4°C 0.9% saline and sonicated for 30 sec at a power setting of 3 and a 30% duty cycle (5 watts pulsed) (Sonifier 450; Branson, Danbury, CT). One hundred μl were removed and mixed with 10 μl 1 N NaOH for protein determination. The remaining cell lysate was then analyzed for platinum by atomic absorption spectrophotometry using a Perkin Elmer 373 atomic absorption spectrophotometer equipped with a 2200 graphite furnace (Perkin Elmer, Norwalk, CT). The following heating program was used: 90°C for 50 sec, ramp to 1300°C in 10 sec and hold for 30 sec, 2500°C under maximum power for 7 sec. No differences in the results were noted between accumulation studies conducted with radiolabeled DDP versus unlabeled DDP.

To determine the amount of total platinum in 2008 cells that was ultrafilterable at 1 h, cells were treated as just described with 100 μM DDP. An aliquot of the cell lysate was placed into a Centriffree micropartition device (Amicon, Lexington, MA) and centrifuged at 2000 × g at 4°C for 45 min. Platinum content was determined in the ultrafiltrate and compared directly to the total cellular platinum that was ultrafilterable. We found that the effects of various metabolic poisons on DDP accumulation were assessed by incubating the cells in RPMI Medium 1640 containing the poisons for 30 min prior to DDP exposure and then for the additional h of DDP exposure. To determine the effect of these treatments on cell viability, cells seeded in 6-well tissue culture plates were treated identically as for an uptake experiment. At the end of the DDP exposure, the medium was aspirated and 0.3 ml of trypan blue-RPMI 1640 (1:1) were added. The cells were then observed qualitatively by microscope over the course of 1 h for the ability to exclude trypan blue.

Cell Volume Determinations. The intracellular water was determined in parent and resistant cells with 3-O-[methyl-3H]-D-glucose by the method of Kletzien et al. (35).

Metabolite Analysis. For the analysis of intracellular metabolites in parent and resistant cells, subconfluent 150-mm tissue culture dishes of 2008 cells were treated with 100 μM DDP in RPMI Media 1640 at 37°C. Following incubation with the drug, the cells were washed and trypsinized as described for accumulation studies with unlabeled DDP except that an aliquot was removed and counted for cell number after trypsinization. The pellet was resuspended in 2 ml water and then sonicated with three 20-sec pulses at 5 watts. An aliquot of the cell lysate was removed for the determination of total platinum and the remainder divided equally into two Centriffree micropartition devices (Amicon) and centrifuged at 2000 × g at 4°C for 45 min. The collected ultrafiltrate for each cell type was injected onto the high-performance liquid chromatography column and chromatographed exactly as described by Daley-Yates and McBrien (36). We used a 25 cm x 4.6 mm Spherisorb ODS-1 column (5 μm particle size) (Phase Separations, Norwalk, CT) and a linear gradient from 5 mM sodium dodecyl sulfate (solvent A) to 90% acetonitrile/10% solvent A in 60 min following a 10-min delay. The flow rate was 0.5 ml/min. Collected 0.5-ml (1-min) fractions were analyzed for platinum by atomic absorption spectrometry. The picogram of platinum in each fraction was plotted versus fraction number. Peaks were then cut and weighed, and the weight was normalized to cell number.

RESULTS

The DDP-resistant cells had been selected 3 times with 1 μM DDP and were 3.3-fold resistant at the time that these accumulation studies were conducted. The same degree of resistance was found whether cells were exposed to DDP continuously or for 1 h in clonogenic assays. The 50% inhibitory concentrations for DDP in parent 2008 cells were 3.5 ± 0.8 μM (n = 9), 0.29 ± 0.10 μM (n = 12), and 0.24 ± 0.05 μM (n = 13) for 1 h, 24 h, and continuous exposure, respectively. Cell volumes were assessed by determining intracellular water space with 3-O-[methyl-3H]-D-glucose in parent and resistant cells. Cell volumes were not significantly different; parent 2008 cells had 2.94 ± 0.40 μl/mg protein (n = 4) and 2008/DDP cells had 3.03 ± 0.52 μl/mg protein (n = 4). Differences in DDP accumulation could therefore not be attributed to changes in cell size.

We chose to determine DDP accumulation in RPMI Media 1640 so as to present the cells with the drug in an environment to which they have naturally adapted. In addition, since cells are exposed to DDP in RPMI Media 1640 in clonogenic assays, this allowed comparison of uptake data directly to cytotoxicity data. DDP accumulation was linear with concentration over the range 0.25 to 100 μM in both parent and DDP-resistant cells (Fig. 1). The equations for the linear regression lines for these points were $y = 9.1x - 3.75$ (r = 0.9999) for 2008 cells, and $y = 5.05x + 8.89$ (r = 0.9992) for 2008/DDP cells. The slope of the accumulation versus concentration line for 2008/DDP cells was thus 55.4% of the 2008 cells.

The DDP uptake versus time in these cells is shown in Fig. 2. Net uptake of 1 μM DDP was linear for approximately 3 h in both cell lines at which time the net uptake began to slow. The accumulation continued to rise for 24 h and never showed clear signs of reaching equilibrium. The average accumulation for 2008/DDP cells was 59.4 ± 6% of parent cells for the five time points determined. Using the intracellular water volume stated above, it can be calculated that the intracellular concentration of total platinum reaches and then surpasses the extracellular concentration of 1 μM DDP at 20 min for parent cells and 35 min for the resistant cells. At 24 h the parent cells had accumulated only 0.5% of the available platinum in the media.

Cell lysates were fractionated to determine the amount of the total cellular platinum that was ultrafilterable. We found that after 1 h of exposure to 100 μM DDP, 28.4% ± 5.6% (n = 7) of the platinum in the 2008 cells lysates was ultrafilterable and 25.6 ± 7.9% (n = 7) was ultrafilterable in the 2008/DDP cell lysates.
We next determined what effect the decrease in DDP accumulation had on the distribution of DDP metabolites in resistant cells. All of the platinum species that were present in parent cells were also present in resistant cells but in what appeared to be decreased amounts (Fig. 3). The large variability inherent in these determinations, however, precluded an assignment of statistical significance at the 95% confidence level to any of these decreases except for metabolite D. The mean levels for the 7 metabolites detected in DDP-resistant cells averaged 35.8 ± 15.1% of the mean levels detected in parent cells. The relative amount of intact DDP in cells at 1 h was similar in both cell types. The percentage of ultrafilterable platinum that was accounted for by intact DDP was 37 and 42% in parent and resistant cells, respectively.

The amount of cell-associated platinum that was available for exodus is shown in Fig. 4. No reproducible difference between the cell types was observed. After a 1-h exposure to DDP and then an additional 1 h in drug-free media, 2008 cells lost 23% of their accumulated platinum compared to 27% for 2008/DDP cells. The amount of platinum that escaped was independent of whether the cells were loaded with 2, 5, or 10 μM [195Pt]DDP.

The effects of various metabolic poisons on DDP accumulation were examined in both parental and resistant cells (Table 1). Dinitrophenol and NaF did not affect DDP accumulation. Iodoacetate produced a stimulatory effect on DDP uptake suggesting that DDP efflux might be an energy-dependent process. However, when the inhibitors were combined so that ATP production by both oxidative phosphorylation and glycolysis was prevented, DDP uptake was reduced (Table 1). Iodoacetate and dinitrophenol were more effective (55% inhibition) than NaF and dinitrophenol (20 to 30% inhibition). A 30-min exposure to 0.2 mM ouabain, a specific Na⁺,K⁺-ATPase inhibitor, produced a 23% decrease in DDP uptake in parent cells.
cells and a 27% decrease in 2008/DDP cells. The immediate
effect of these poisons on cell viability either alone or combined
with 10 or 100 μM DDP was determined by the ability of the
cells to exclude trypan blue added immediately after drug
exposure. Less than 1% of the control cells was permeable to
trypan blue after 90 min in RPMI 1640 alone. Treated cells
were indistinguishable from untreated cells when exposed
either immediately or 1 h after trypan blue addition with the
exception of treatments containing iodoacetate. These cells
were also initially identical to control cells, but after approxi-
mately 10 min trypan blue permeable cells began to appear.
After 1 h, 20 to 30% of the cells were stained blue.

DISCUSSION

Numerous studies are beginning to appear that identify de-
creased accumulation of DDP as a factor in the in vitro acquisi-
tion of resistance to this antitumor compound (6, 26–32).
Reports are also appearing that show that certain compounds
such as ouabain, anguidine (37), dipyridamole (38), and alde-
hydes (25) can modulate DDP accumulation. Although the
mechanisms of DDP uptake have not yet been defined, these
reports suggest that a carrier is involved. The presence of a
DDP transporter that is diminished in resistant cells and inhib-
ited by these compounds would be an attractive explanation
for these observations. Both sets of observations, however, could
be explained by nonspecific effects on plasma membrane fluidity
caused either by these compounds or by inherited changes in
membrane lipid composition in the resistant cells. Indeed, there
is compelling evidence that DDP uptake does not involve
carrier-mediated transport. DDP accumulation was not satu-
rable up to 100 μM DDP in our studies and does not appear to
be saturable up to 3.3 mM5, although this does not necessarily
rule out a carrier-mediated uptake (15, 16). We also have
preliminary evidence that the structural analogues carboplatin
and cis-diaminedichloropalladium(II) do not competitively
inhibit DDP accumulation. Other evidence that would indicate
that DDP uptake is carrier mediated, such as dependence on
cotransport or competitive exchange, have not as yet been
reported.

The intracellular concentration of platinum rapidly surpasses
the extracellular concentration and this would normally indi-
cate the involvement of active transport. However, DDP is an
electrophile and once inside the cell it aquates and rapidly
platinates many cellular constituents. It is not yet known what
percentage of the cell-associated platinum is actually free drug
at any given time in our studies except at 1 h. At 1 h we found
that approximately 27% of the total cellular platinum was
ultrafilterable. Of this ultrafilterable platinum, the metabolite
profile shows that 37% was intact DDP in parent cells and 42%
was intact DDP in resistant cells. This means that only 10 to
11% of the total platinum is intact DDP in either cell type at 1 h.
If the assumption is made that these percentages do not change
with time or DDP concentration, then this translates into an intracellular concentration of 0.33 μM and 0.19 μM
DDP for parent and resistant cells respectively at 1 h when
cells are exposed to 1 μM DDP (Fig. 2). One can also calculate
that the extracellular concentration of 1 μM until 4 h for parent cells
and 16 h for DDP-resistant cells. These data thus provide strong
evidence that DDP is not transported uphill against a concen-
tration gradient by primary active transport; or, that if it is,
then the efflux of DDP must be similar in value to its influx.

Dinitrophenol, an inhibitor of oxidative phosphorylation,
and NaF and iodoacetate, inhibitors of glycolysis, did not cause a
decrease in DDP accumulation. The combination of these
poisons, however, did decrease accumulation. These results
indicate that approximately 50% of DDP transport is in some
way energy dependent. This presents the paradox that a com-
ponent of DDP transport is energy dependent despite evidence
that argues against a carrier (accumulation is not saturable and
not competitively inhibited). This situation could be explained
by pinocytosis, but its role in DDP accumulation remains to be
explored. Iodoacetate alone had a stimulatory effect on DDP
accumulation. A plausible explanation may be attributed to
iodoacetate’s propensity to react with thiols. Carboxymethyl-
lation of critical thiols by iodoacetate may lead to a disruption
of membrane integrity and an increase in the permeability to DDP.
This increase may be overridden by the effect of ATP loss when
iodoacetate is combined with dinitrophenol. The increased
permeability to trypan blue observed after iodoacetate treatment
supports this explanation. Studies on the effect of other meta-
bolic inhibitors on DDP accumulation and on the ATP levels
in our cells are being pursued.

The inhibition of DDP accumulation by ouabain, a specific
inhibitor of Na+,K+-ATPase (EC 3.6.1.3), is an intriguing ob-
ervation. DDP is known to inhibit Na+,K+-ATPase albeit at
relatively high concentrations (39–42). Several studies have
shown that DDP affects the movement of single ions such as
calcium, sodium, and chloride (43, 44). DDP-resistant L1210
cells have also been shown to have altered calcium channels
(45). The inhibition of DDP accumulation by ouabain indicates
that either DDP accumulation is dependent upon the electro-
chemical gradient across plasma membranes or that DDP is
transported by the Na+,K+-ATPase which ouabain inhibits. It
also suggests that the observed inhibition of DDP accumulation
by metabolic poisons may be a direct result of loss of the
electrochemical gradient due to starvation of the Na+,K+-
ATPase for ATP. Our studies and others (43–45) thus raise the
question of whether alterations in the homeostasis of anions
and cations in cells may be a key component of the resistant
phenotype and a direct cause of the altered transport of DDP.

The observation that 25% of the intracellular platinum is
available for exodus yet only 10% of the intracellular platinum
is intact DDP may be explained by two possibilities. One is
that other metabolites of DDP may be available for escape from
the cell. The form(s) of the intracellular platinum that is re-
turned to the media from the cells is not presently known. The
second explanation is that the determination of the intact DDP

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Footnote:
in cells is artificially low and is higher than 10%. To make these determinations, approximately 90 min are required to take the cells from DDP-loaded monolayers to an ultrafiltrate that can be injected on the high-performance liquid chromatograph. Although these manipulations are done at 4°C, there is certainly ample time for the DDP to continue to react with intracellular constituents. The actual concentration of DDP in the cytoplasm after 1 h and available for exodus may thus be higher than what was determined after an additional 90 min of manipulations.

Acquired alterations in plasma membranes can protect resistant cells against DDP (6,26–32). There is also a variety of evidence that DDP directly affects a number of properties of cellular membranes. Studies have shown that DDP inhibits amino acid uptake in L1210 cells and that perturbations in amino acid transport systems arise in DDP-resistant L1210 cells and K562 cells (46–48). DDP inactivates several plasma membrane-situated phosphatases (49) and interacts with the rat liver mitochondrial phosphate carrier (50). Simpkins and Pearlman have reported that DDP, carboplatin, and iroproplatin produce fluidity changes in plasma membranes of thymocytes as detected with fluorescent probes (51). An investigation with a spin-labeled probe also indicates that DDP induces fluidity changes in cell membranes (52). These studies all suggest that pharmacologically significant events are caused by DDP at membrane interfaces. It is not surprising, then, that cells selected with DDP work to alter their membranes to minimize DDP damage and/or DDP permeability. Although the definitive mechanisms of DDP transport and how they have changed in resistant cells are not yet understood, our studies have shown that human ovarian carcinoma cells selected 3 times with DDP and possessing 3- to 4-fold resistance have a 50% decrease in DDP accumulation. This change thus represents a very early adaptation in the development of the DDP-resistant phenotype.

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REFERENCES


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