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Short-term *cis*-diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells*

Stephen C. Mann, Paul A. Andrews, and Stephen B. Howell

Cancer Center, University of California, San Diego, La Jolla, CA 92093, USA

Summary. We examined the short-term accumulation of cisplatin (DDP) in sensitive 2008 human ovarian carcinoma cells and in a 2- to 3-fold DDP-resistant and accumulation-deficient variant. During the 1st min of exposure to 500 μ*M* DDP, sensitive cells accumulated platinum at a rate of 187 ± 63 pmol/mg protein per min, whereas resistant cells accumulated platinum at 123 ± 85 pmol/mg protein per min, a rate that was 66% that of sensitive cells. From 2–10 min of exposure, sensitive and resistant cells accumulated the drug at rates of 51.4 ± 21.5 and 34.0 ± 9.70 pmol/mg protein per min, respectively. In resistant cells, this rate again represented 66% that of sensitive cells. For each cell line, the DDP accumulation was 3.6 times faster during the 1st min than it was over 2–10 min. Initial DDP accumulation was linear with drug concentration in each cell line. Efflux measurements were made over a 50-min period after a 10-min load in 500 μ*M* DDP. The loss of platinum was biphasic in each cell line, with an initial, rapidly effluxing component being lost within 10 min in each cell line. The rate constant for loss of platinum from this rapidly effluxing pool, measured after a 10-min loading period in 500 μ*M* DDP, was 0.67 ± 0.09 s^{-1} in sensitive cells and 1.03 ± 0.15 s^{-1} (a 53% increase) in resistant cells. Between 5 and 50 min of an accumulation time course in 500 μ*M* DDP, the size of the rapidly effluxing platinum pool remained relatively constant in each cell line, with the major contribution to the increase in total platinum over time coming from growth of the slowly effluxing platinum pool. We conclude that diminished retention of platinum in the rapidly effluxing pool of resistant cells is a major determinant of decreased DDP accumulation in these cells.

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Abbreviations: DDP, cis-diamminedichloroplatinum(II); DEP, *cis*-dichloro(ethylenediamine)platinum(II); RPMI, Roswell Park Memorial Institute; PBS, phosphate-buffered saline, consisting of (per liter) 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4

Offprint requests to: Cancer Center, T-012, University of California, San Diego, La Jolla, CA 92093, USA

Introduction

Investigators have observed a positive correlation between the accumulation and cytotoxicity of DDP in cancer cell lines from different tissues and organisms [5] as well as in comparisons between cells and their derivatives selected for DDP resistance [2, 12–14, 18, 20, 21]. We selected a DDP-resistant variant of the 2008 human ovarian carcinoma cell line by repeated in vitro exposure to the drug. DDP accumulation in the DDP-resistant cells was approximately 50% that of sensitive cells when measurements were made over several hours at 37° C [2]. The objective of the present study was to make short-term DDP accumulation and efflux measurements to determine whether an altered DDP transport process (influx or efflux) could account for this DDP accumulation deficiency in resistant cells. These experiments were carried out in 500 μ*M* DDP and the cell-associated platinum was assayed by atomic absorption spectrophotometry. Tumor nodules are routinely exposed to DDP concentrations of this magnitude during intraperitoneal chemotherapy for ovarian carcinoma [11]. The DDP exposure periods used in this study (30 s to several minutes) were similar to those previously used by other investigators to study the transport of DDP and other anticancer agents [7–9, 19].

Methods

Drugs and chemicals. Pure DDP powder was obtained from Alfa Products (Danver, Mass); PBS and 0.85% NaCl were products of Oxoid, Inc. (Columbia, Md); and 0.4% trypan blue was obtained from Gibco Labs (Chagrin Falls, Ohio).

Cell lines. Human ovarian carcinoma cell line 2008 [4] was used in all of these experiments. A resistant subline, designated 2008/DDP, was obtained by monthly selections with 1 μ*M* DDP [1]. Resistant cells were maintained in drug-free medium for several months before these experiments were begun. At the time of these studies, the 2008/DDP cells were 2–3 times more resistant to DDP than were the parental 2008 cells, as determined by the 50% inhibitory concentration for colony formation during a 10-day continuous exposure to the drug. Sensitive and resistant cells were grown on tissue-culture dishes in a humidified incubator and a 5% CO_2 atmosphere at 37° C. They were maintained in medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf

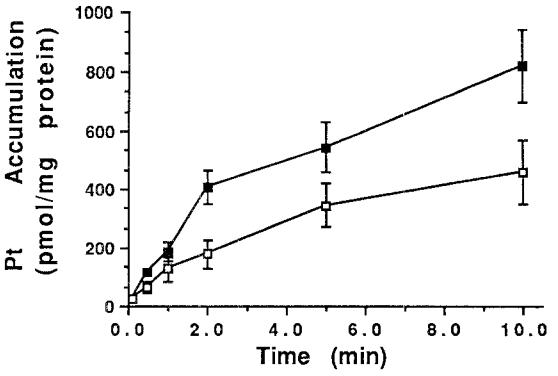

Fig. 1. The 10-min accumulation time course of 500 μ*M* DDP in 2008 and 2008/DDP cells. Incubations were terminated by quickly washing the plates in excess PBS at 4°C. Cells were then scraped from the plates and assayed for Pt and protein. *Points* represent the mean of five determinations; *bars*, the SE. Where absent, the SE bars were smaller than the symbol. ■, 2008 cells; □, 2008/DDP cells

serum, 2 m*M* glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, Calif.).

DDP accumulation (10-min time course). Duplicate 60-mm tissue-culture dishes were seeded with 10^6 2008 or 2008/DDP cells and incubated in medium for 2 days. Accumulation experiments were then initiated by rinsing dishes twice with RPMI 1640 and adding 2 ml RPMI 1640 containing 500 μ*M* DDP. Incubations were conducted by placing dishes in a 37°C water bath and were continued for 5 s, 30 s, and 1, 2, 5, and 10 min. Incubations were terminated by quickly aspirating the medium and submerging the dishes successively into each of two beakers containing 1 l PBS at 4°C. This procedure required about 15 s. A separate experiment in which accumulated platinum was allowed to efflux into drug-free PBS at 4°C indicated that <5% of the accumulated platinum was lost during this termination procedure. In all, >99% of 2008 and 2008/DDP cells exposed to 500 μ*M* DDP for up to 50 min retained the capacity to exclude trypan blue, indicating that the conditions of the experiment did not lead to gross changes in membrane permeability.

Cells were harvested by scraping the dishes with a rubber policeman into 0.15–0.20 ml 0.85% NaCl. Cell suspensions were homogenized with a Branson 450 Sonifier using 10–20, 0.1-s bursts at the lowest output setting (Branson Sonic Power Company, Danbury, Conn). Aliquots of the sonicated suspension were then assayed for protein and platinum. Protein content was determined by the method of Bradford [3] after the assay samples had been digested overnight in 1 N NaOH. Platinum was determined using a Perkin Elmer 373 atomic absorption spectrophotometer with a graphite furnace accessory and an HGA 2200 temperature controller (Perkin Elmer, Norwalk, Conn). The temperature program was: dry – 100°C for 60–120 s, depending on injection volume; char – a 10-s ramp to 1,500°C, then 30 s at 1,500°C; atomize – 2,500°C for 7 s at the Maximum Power setting. Platinum in unknown samples was determined by comparison with a plot of absorbance peak heights vs the injection volume of standard DDP. Accumulated platinum was expressed as pmol/mg protein.

Concentration dependence of initial DDP accumulation. These experiments were conducted in the same way as the 10-min accumulation time course, except that all incubations were 30 s in duration and the concentration of DDP in the medium ranged from 200 μ*M* to 3 m*M*.

DDP efflux. For the determination of the initial efflux time course, dishes were incubated for 10 min in 500 μ*M* DDP using the same procedure as for the accumulation time course. At the end of this drug-loading period, monolayers were rinsed five times with 10 ml drug-free RPMI 1640; this rinse required 30 s. Drug-free medium was added back to the dishes and they were returned to a 37°C water bath for the indicated efflux time, measured from the time of addition of the first 10 ml drug-free medium. Drug effluxes were continued for 30 s, 1 min, and 1.5 min. For establishment of the more extended efflux time courses, effluxes were ended after periods of up to 50 min. In one set of experiments, the DDP loading time was varied from 5 to 50 min and the efflux time was fixed at 10 min. For DDP loading or efflux periods of >10 min, dishes were returned to the incubator. Effluxes were terminated and monolayers were assayed for protein and platinum as described above.

Results

Figure 1 shows the 10-min time course for DDP accumulation in sensitive 2008 and resistant 2008/DDP cells during an incubation in 500 μ*M* DDP. Accumulation rates pertaining to these graphs are presented in Table 1. DDP ac-

Table 1. The 10-min time course of DDP accumulation in 2008 and 2008/DDP cells

	Accumulation rate (pmol/mg protein per min)[a]:		Accumulation (pmol/mg protein):
	0–1 min	2–10 min	10 min
2008	187±63.2*,**	51.4±21.5	819±279*
2008/DDP	123±85.2 (65.8)***	34.0± 9.70 (66.1)[b]	460±180 (56.2)

[a] Values represent the mean ±SD of five separate experiments
[b] Values in parentheses indicate the percentage of the 2008 value
* Significantly different from the corresponding value for 2008/DDP at the $P<0.05$ level, as determined by *t*-test on paired data
** Significantly different from the corresponding 2- to 10-min value at the $P<0.01$ level, as determined by the *t*-test on grouped data
*** Significantly different from the corresponding 2- to 10-min value at the $P<0.05$ level, as determined by the *t*-test on grouped data

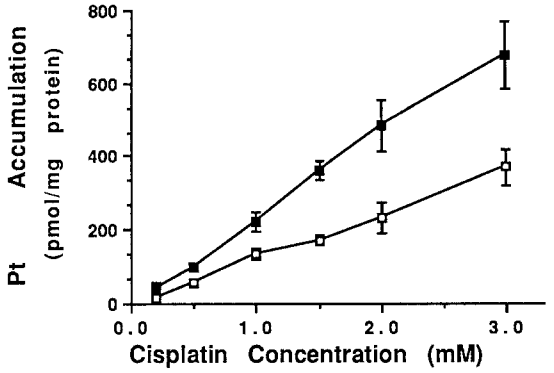

Fig. 2. Concentration dependence of the 30-s DDP accumulation. Incubations at the indicated DDP concentrations were terminated by quickly washing the plates in excess PBS at 4°C. Cells were then scraped from the plates and assayed for Pt and protein. *Points* represent the mean of four determinations; *bars*, the SE. Where absent, the SE bars were smaller than the symbol. ■, 2008 cells; □, 2008/DDP cells

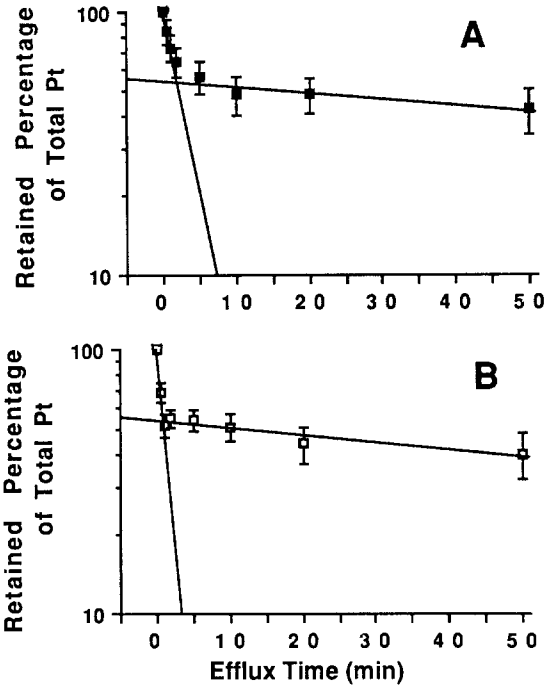

Fig. 3. Loss of Pt into drug-free medium from cells preloaded for 10 min in 500 μ*M* DDP. At indicated times, incubations were terminated by quickly washing the dishes in excess PBS at 4°C. Cells were then scraped from the dishes and assayed for Pt and protein. Data is plotted on a semilogarithmic scale. **A** Efflux time course for total preloaded Pt in 2008 cells. *Lines* represent least-squares fits to portions of the curve as indicated in the text; *points*, the mean of four determinations; *bars*, the SE. The mean Pt present at 0 time was 1,230 pmol/mg protein. **B** Efflux time course for total preloaded Pt in 2008/DDP cells. *Lines* represent least-squares fits to portions of the curve as indicated in the text; *points*, the mean of four determinations; *bars*, the SE. The mean Pt present at 0 time was 484 pmol/mg protein. ■, 2008 cells; □, 2008/DDP cells

cumulation in 2008/DDP cells was less than that in 2008 cells throughout the 10-min period, beginning at the earliest measurable point, which occurred at 30 s. No measurable platinum was associated with cells from which DDP was removed immediately after the start of exposure (approximately 5 s). The accumulation rate over the 1st min was significantly greater in 2008 than in 2008/DDP cells, as was the total accumulation after a 10-min incubation (Table 1). In each cell line the accumulation rate during the 1st min was significantly greater than that over 2 – 10 min. In sensitive cells, the latter rate was 27% that of the initial rate, whereas in resistant cells it was 28% of the initial rate. The reduced platinum content in 2008/DDP cells after the 10-min accumulation resulted from both the decreased accumulation rates as described and the fact that the initial rapid accumulation rate in these cells was short-lived in comparison with that in sensitive cells, where rapid accumulation persisted for 2 min. Figure 2 shows that the 30-s DDP accumulation in sensitive and resistant cells was a linear function of DDP concentration in the medium up to the limit of drug solubility.

The time course for platinum efflux into drug-free medium was determined in 2008 and 2008/DDP cells following a 10-min loading period in 500 μ*M* DDP. The efflux time course, expressed as the percentage of loaded platinum retained at efflux times from 30 s to 50 min, is depicted in Fig. 3. The efflux was at least biphasic in each cell line. The more rapid part of the efflux was completed after approximately 1 min in 2008/DDP cells but continued for up to 5 min in 2008 cells. The biphasic nature of the efflux plots is confirmed by the fact that the slope of a straight line fit to the initial (0 – 1 min) part of either curve is significantly greater than that of a line fit to the final portion of the curve (5 – 50 min). The slope of the initial rapid portion of the efflux curve in 2008 cells was -0.145 ± 0.053, whereas that of the slower portion was -0.0038 ± 0.0016, the difference being statistically significant at the $P < 0.05$ level using the *t*-test on grouped data. The corresponding slopes for 2008/DDP cells were -0.294 ± 0.039 and -0.0031 ± 0.0015, the difference being

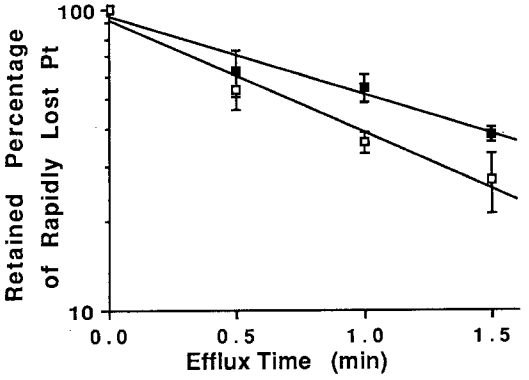

Fig. 4. Initial efflux time course for the rapidly effluxing Pt pool after a 10-min loading period in 500 μ*M* DDP. The rapidly effluxing Pt pool was defined as the Pt that was lost in the 1st 10 min of efflux. Least-squares fits to the semilogarithmic plot are indicated. *Points* represent the mean of seven determinations; *bars*, the SE. The size of the mean, rapidly effluxing Pt pool was 695 pmol/mg protein in 2008 cells and 352 pmol/mg protein in 2008/DDP cells. ■, 2008 cells; □, 2008/DDP cells

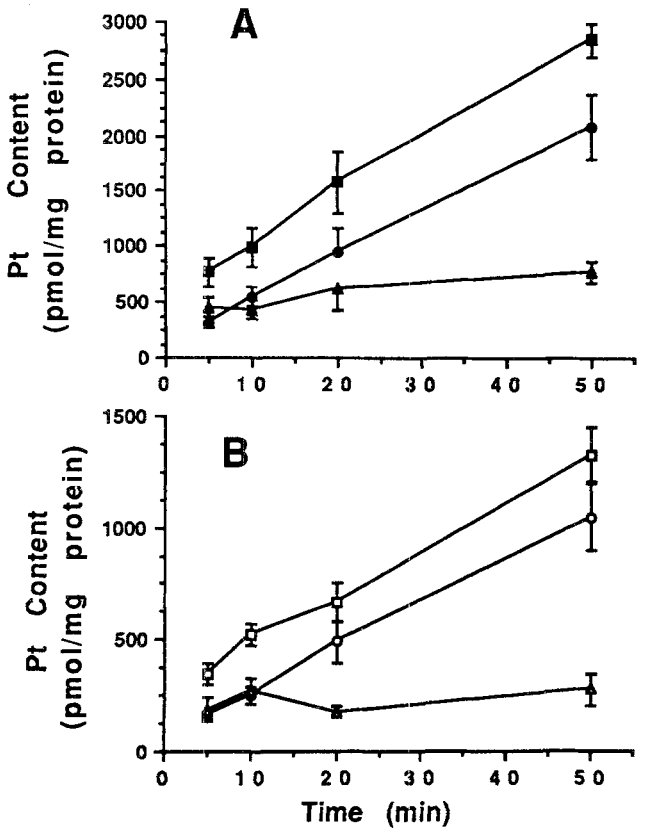

Fig. 5. The 50-min accumulation time course of total, slowly effluxing, and rapidly effluxing Pt. Rapidly effluxing Pt was defined as the Pt that was lost during a 10-min efflux after each accumulation time point. *Points* represent the mean of four determinations; *bars*, SE. **A** 2008 cells. **B** 22008/DDP cells. ■, □, total Pt; ●, ○, slowly effluxing Pt; ▲, △, rapidly effluxing Pt

statistically significant at the $P < 0.01$ level. When the efflux time course following a 50-min DDP-loading period was examined, a biphasic pattern was again obtained (data not shown), but the percentage of the total loaded platinum lost during the rapid efflux phase was diminished. We designated the platinum lost during, the 1st 10 min of efflux as the rapidly effluxing platinum and the remainder, as the slowly effluxing platinum, since, as Fig. 3 shows, the rapid phase of efflux in each cell line was clearly complete by the 10th min in drug-free medium.

Additional experiments were conducted to determine the initial rate of platinum loss from the rapidly effluxing pool in 2008 and 2008/DDP cells following a 10-min loading period. The results, presented in Fig. 4, were fit to first-order decay curves, yielding correlation coefficients of -0.98 for 2008 and -0.99 for 2008/DDP cells. The calculated first-order efflux-rate constants were $0.67 \pm 0.09\ s^{-1}$ for 2008 cells and $1.03 \pm 0.15\ s^{-1}$ for 2008/DDP cells. The efflux rate constant for resistant cells was 53% greater than that for sensitive cells. This difference was significant at the $P < 0.05$ level using the Mann-Whitney *U*-test.

Figure 5 shows results from a set of experiments where the 50-min time course of total platinum accumulation was subdivided into rapidly and slowly effluxing parts by measuring for each point both the total platinum and the platinum remaining after a 10-min efflux period. For both 2008 and 2008/DDP cells, the amount of rapidly effluxing platinum was relatively constant throughout the time course and the growth in slowly effluxing platinum paralleled the increase in total platinum.

Discussion

The present studies show that during the 1st min, DDP-resistant 2008 human ovarian carcinoma cells accumulated platinum only 66% as rapidly as did sensitive cells. Since 2008/DDP cells have been shown to accumulate 50% as much platinum as 2008 cells over the course of several hours [2], much of the long-term accumulation deficiency of the resistant cells is determined very soon after the drug comes in contact with the cells. We found very little platinum associated with 2008 or 2008/DDP cells when DDP was removed 5 s after its addition to the dishes, indicating little immediate surface binding of the drug to these cells [16]. The significant decline in the platinum accumulation rate seen after 1 or 2 min in our cell lines could have been caused by the development of significant drug efflux from an intracellular pool that built up during the rapid initial accumulation phase.

The DDP accumulation experiments were carried out in 500 μM DDP. This concentration approximates that previously used for intraperitoneal treatment of ovarian carcinoma, where levels in excess of 600 μM have been given [11]. The present studies are likely to be valid for other DDP concentrations as well, since results presented both here and elsewhere have consistently shown a linear relationship between platinum accumulation and concentration. Features of DDP accumulation seen in an earlier study of longer-term incubations of 2008 and 2008/DDP cells in 0.25–100 μM DDP [2] are consistent with the present results: the 50% reduction in DDP accumulation by resistant cells and the similarity in the accumulation rate divided by DDP concentration in each cell line from the 2nd min to the 4th h of drug exposure.

The observation that DDP accumulation is linear with concentration has now been made in a variety of cell and tissue types, at time intervals ranging from 30 s to 18 h and at concentrations of DDP or analogs ranging from 0.25 μM to 3 mM [2, 5, 6, 10, 12]. This finding implies that over a variety of incubation periods, DDP accumulation in cells is a first-order process, that is, it is not limited by the saturation of a binding site. It should be pointed out, however, that the upper limit of 3 mM used in the present saturation study was set by the solubility limit of the drug. DDP may have interacted with a relatively low affinity at a specific binding site that would require much higher concentrations to achieve saturation.

Our results show that the loss of platinum from 2008 and 2008/DDP cells into drug-free medium was biphasic. The existence of a portion of accumulated DDP that is not readily effluxable has been noted by other investigators [9, 17, 21]. The initial rate of loss of platinum from the rapidly effluxing pool following a 10-min DDP-loading period was 53% greater in resistant cells. We conclude that this decrease in the ability of resistant cells to retain platinum is a significant contributor to the reduced early accumulation of DDP in these cells. The increased platinum efflux rate from resistant cells could be due to a platinum efflux process located at the plasma membrane of these cells. Alternatively, platinum might undergo a very rapid and rela-

tively loose association with intracellular components in sensitive cells that somewhat retards its diffusion back out of the cell. The current studies do not rule out the possibility that a decreased unidirectional influx rate in resistant cells could contribute to the observed decrease in early DDP accumulation. More definitive determination of influx rates will require exposures to DDP that are shorter than those used in the present study, to ensure zero-*trans* conditions [15, 16].

We showed in this study that most of the increase in cellular platinum between the 5th and 50th min of DDP exposure was contributed by the slowly effluxing platinum. This observation is consistent with the idea that intracellular platinum initially present in the rapidly effluxing pool is converted over time to the slowly effluxing pool. By such a process, differences in the initial platinum accumulation into the rapidly effluxing pool could result in long-term accumulation differences that are cytotoxically relevant.

Among the other investigators who have been working with DDP-sensitive and accumulation-deficient, resistant cell lines, Hromas et al. [12] and Waud [21] have found that their resistant murine L1210 leukemia cells accumulate less DDP or DEP than the sensitive counterparts within the 1st 10 min exposure to the drug, indicating that the present results could be generalizable to other DDP-resistant cell lines. However, Waud did not find a difference in the initial efflux of DEP between his sensitive and resistant cells [21]. Other investigators who have been working with DDP accumulation-deficient, resistant lines have not examined accumulation or efflux data over the initial 10 min [13, 18, 20]. The current work represents the most thorough documentation to date of an early decline in DDP accumulation in an accumulation-deficient, DDP-resistant cell line. It is also the first report of results from short-term efflux experiments that implicate decreased platinum retention in the early decline of DDP accumulation in resistant cells.

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