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ORIGINAL ARTICLE

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Cisplatin efflux, binding and intracellular pH in the HTB56 human lung adenocarcinoma cell line and the E-8/0.7 cisplatin-resistant variant

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Abstract *Purpose*: Many cell lines resistant to cisplatin (DDP) have reduced DDP accumulation. We postulated that reduced accumulation of DDP in resistant cells might be due to decreased intracellular DDP binding, leading to increased passive efflux. *Methods*: The total cellular ([T-DDP]), intracellular ultrafiltrable ([F-DDP]) and precipitable cellular bound ([B-DDP]) DDP concentrations were all compared in the HTB56 human lung adenocarcinoma cell line and its E-8/0.7 variant that has acquired DDP resistance. Cells were exposed to 509 μ*M* DDP for 20 min. Ultrafiltration with a 500 molecular weight cut-off separated cellular free from bound cisplatin. Fragmentation by sonication and microcentrifugal spinning precipitated cellular bound cisplatin. Flow cytometry was used to measure the intracellular pH (pH_i) of the HTB56 cell line, the E-8/0.7 cell line, as well as of the OV2008 cell line and its C13 resistant variant. The DNA-bound DDP and protein-bound DDP ([P-DDP]) were also compared when equal [T-DDP] was achieved for both sensitive and resistant cells by exposing them for 1 h to two pairs of DDP concentrations, i.e. 509 vs 911 μ*M* DDP, and 111 vs 666 μ*M* DDP, respectively. Platinum was assayed by flameless atomic absorption spectrophotometry. *Results*: At time 0 (end of cisplatin exposure), [T-DDP] and [B-DDP] were significantly higher in the sensitive HTB56 parent cell line ($P < 0.02$ and $P < 0.001$, respectively), whereas [F-DDP] did not differ significantly ($P = 0.62$). Two distinct phases of T-DDP efflux were observed. In the first 10 s after DDP exposure, the rate constant for resistant cells (K_{R1}) was 0.17 s^{-1}, whereas that for sensitive cells (K_{S1}) was 0.14 s^{-1}. From 10 s to 50 s, however, K_{R2} and K_{S2} became 0.005 s^{-1} and 0.004 s, respectively. [T-DDP] remained lower in resistant cells than in sensitive cells at 10, 30 and 50 s (all $P < 0.0001$). For 1 h drug exposure to 509 vs 911 μ*M* cisplatin concentrations designed to give comparable [T-DDP] in the sensitive and resistant cell lines, only [DNA-bound DDP] was found to be significantly higher in sensitive cells ($P = 0.002$), whereas both [F-DDP] and [P-DDP] did not differ significantly ($P = 0.18$, $P = 0.75$, respectively). On the other hand, there were no significant differences found in [F-DDP], [P-DDP] and [DNA-bound DDP] between the two cell lines when 111 vs 666 μ*M* DDP was used. Flow cytometry data indicated that the pH_i was significantly higher in the E-8/0.7 ($P < 0.0186$) and C13 ($P < 0.0169$) resistant variants than in the sensitive parent cell lines. *Conclusions*: DDP binds more slowly in resistant than in sensitive lung cancer cells, despite comparable amounts of free drug. Early efflux is higher in the resistant variant. Differences between the lines with respect to DNA binding may be DDP concentration-dependent. We speculate that the reduced early binding and increased early efflux in the resistant line may be related to the higher pH in this line. A higher pH is supposed to favor production of neutral hydroxyl metabolites rather than charged aquated metabolites, and these neutral metabolites would be expected to react less readily with intracellular molecules and to efflux more readily across cell membranes. Since we have previously documented a threefold increase in glucose utilization and lactate production in the DDP-resistant variants of the human HTB56 lung cancer cell lines, and this increased lactate production would have been expected to reduce the intracellular pH instead of raising it, it is possible that our alkaline-resistant cells have a higher Na^+/H^+ exchanger

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activity which would protect them from intracellular acidification.

Key words Cisplatin · Lung cancer · Resistance · pH · Intracellular binding · Efflux

Introduction

Although numerous potential mechanisms of acquired resistance to cisplatin have been elucidated in vitro, it is still not known which of these mechanisms contributes to the cisplatin resistance that emerges in tumors during treatment of patients with cisplatin. The role of efflux in cisplatin resistance remains controversial. While the multidrug-resistance pump, P-glycoprotein, is not a factor in cisplatin resistance [1], there are other efflux pumps that might possibly play a role. These include the glutathione-conjugate (GS-X) efflux pump [2] and a 200-kDa membrane glycoprotein expressed by cisplatin-resistant mouse lymphoma cells that have reduced cisplatin accumulation [3].

Increased efflux (active or passive or both) could also occur if there were reduced intracellular drug binding, such that there was increased free drug available for efflux. While the amount of cisplatin bound to negatively charged cellular constituents within resistant cells has been found to be low in some instances [4], there are no data to indicate whether the reduction in bound drug is the result of or the cause of reduced intracellular platinum content in resistant cells.

We hypothesized that the reduced accumulation of cisplatin found in resistant cells may be due predominantly to decreased cellular drug binding (with resultant increased passive efflux), rather than being due to any changes in active or passive uptake. We also postulated that changes in intracellular conditions might alter cisplatin reactivity by altering its nonenzymatic conversion to the aquated species. The effect of intracellular pH might be of importance in affecting cisplatin intracellular accumulation and, therefore, its cytotoxicity. In order to test this hypothesis, we looked for differences in total, ultrafiltered, protein-bound and DNA-bound cisplatin, and intracellular pH between the HTB56 human lung adenocarcinoma cell line and its cisplatin-resistant variant, E-8/0.7. The resistant cell line has many cellular, morphological and biochemical differences compared to the sensitive parent. Some of the characteristics are shown in Table 1. We also examined the intracellular pH in the OV2008 human ovarian carcinoma cell line and its cisplatin-resistant variant, C13.

Table 1 Summary of differences between the HTB56 cell line and its E-8/0.7 cisplatin-resistant variant based on both previous and the present results. All studies were done in exponentially growing cells. Each value is the mean of three separate experiments, performed in triplicate

Characteristic	HTB56 (sensitive)	E-8/0.7 (resistant)
Plating efficiency	11% Higher	Lower
Cell doubling time in log phase	18 h	29 h
Maximum cell density in plateau phase (cells/ml, approximate)	3.2 million	1.3 million
Intracellular pH	7.513 ± 0.055	7.647 ± 0.062
Cisplatin cytotoxicity (IC_{50})	11.7 μ*M*	53.3 μ*M*
Glucose utilization[a]	Lower	Higher
Lactate production[a]	Lower	Higher

[a] Stewart et al., unpublished data

Material and methods

Materials

Cisplatin clinical formulation (1.0 mg/ml) was supplied by Bristol-Myers Squibb (Montreal, QC). Iscove's modified Dulbecco's medium (IMDM), fetal calf serum (FCS) and trypan blue stain (0.4%) were purchased from Gibco (Burlington, Ontario). Both the bicinchoninic acid protein assay kit and propidium iodide (PI) were obtained from Sigma (St. Louis, Mo.). The genomic DNA purification kit was from Promega (Madison, Wis.). Both the Amicon micropartition system kit (MPS-1) and Diaflo ultrafiltration membranes, YC05, with 500 molecular weight (MW) cut-off were obtained from Millipore (Bedford, Mass). Acetoxymethyl (AM) ester of carboxy SNARF-1 from a 1.0 mg/ml stock solution in anhydrous dimethylsulfoxide (DMSO) and nigercin were purchased from Molecular Probe (Eugene, Ore.).

Cell lines

The HTB56 human lung adenocarcinoma cell line was obtained from the American Tissue Culture Collection. The E-8/0.7 resistant cell line was generated by prolonged exposure of HTB56 cells to 0.7 μg/ml cisplatin. The OV2008 cell line and its C13 cisplatin-resistant variants were gifts from Dr. Stephen Howell, San Diego, California. The cell lines were cultured in IMDM containing 5% FCS. Incubation was performed at 37 °C in an atmosphere of air containing 5% CO_2. All studies were done on exponentially growing cells. Cell viability was confirmed by trypan blue staining in most sets of experiments with the exception of the pH measurement by flow cytometry, in which PI was used.

Growth rate determination

Approximately 5×10^5 cells were seeded in T25 tissue culture flasks with 5 ml IMDM (supplemented with 5% FCS). Cells were counted with a hemocytometer, and plotted against time. Cell population doubling time was calculated from the exponential part of the growth curves.

Clonogenic assays, dose-response relationships and plating efficiency determinations

HTB56 and E-8/0.7 cells cultured in T25 tissue flasks on day 4 were exposed to different concentrations of cisplatin contained in 5 ml IMDM without FCS. After 1 h in the incubator at 37 °C, the cisplatin-containing medium was aspirated and the cells were exposed to 0.05% trypsin for 3 min. Harvested cells were resuspended in drug-free medium and, after counting with a hemocytometer, 300 cells were seeded in each 60-mm tissue culture dish, and incubations were continued in a humidified air atmosphere containing 5% CO_2 (v/v) at 37 °C. On the 9th day, colonies were stained with Giemsa stain after fixing with ethanol (95%).

Colonies with more than 50 cells were counted, and the results expressed as the percentage surviving fraction compared to the untreated controls. The surviving fraction of colonies formed in the untreated controls revealed the plating efficiencies of the cell lines.

Platinum and protein assays

To assay cells for platinum, cells were homogenized using a Vibra cell ultrasonic processor (Sonics and Materials, Ct.) for 20 s at 40% output. Cellular protein was solubilized overnight at room temperature by mixing aliquots of homogenized cells with equal volumes of 1 *M* NaOH, and was assayed by the method of Smith et al. [5] using the bicinchoninic acid protein assay. Platinum was assayed using a Varian atomic absorption spectrophotometer AA1475 series and GTA-95 graphite tube atomizer, as previously described [6]. Platinum content was expressed as picomoles platinum per milligram protein.

Total and cellular bound cisplatin measurements

HTB56 (sensitive) and E-8/0.7 (resistant) cells were grown on 60-mm tissue culture dishes at 37 °C in a humidified incubator containing 5% CO_2 in IMDM supplemented with 10% (v/v) FCS. On day 4, when the cells were in their post-log phase, cells were dislodged off the dishes by pipetting 1500 µl FCS-free IMDM (Gibco) onto them. Trypsin was not used since it was felt that it might affect cisplatin transporters, if there were any. Cell suspension (850 µl) was transferred to Eppendorf tubes and the cell solution was prewarmed in a water bath at 37 °C. Cisplatin solution (150 µl) was added to the cell suspension. Cells were exposed to cisplatin at a final concentration of 509 µ*M* with constant shaking for 20 min in a waterbath at 37 °C. Cisplatin exposure was terminated by spinning the cell solution in a microcentrifuge at 14 000 rpm for approximately 10 s.

To measure cisplatin at the end of cisplatin exposure, and prior to the efflux phase, the supernatant was discarded and the pellet was washed twice with 1000 µl ice-cold phosphate-buffered saline (PBS). The pellet was resuspended in 1000 µl ice-cold PBS and sonicated briefly at the 40% output level using a Vibra cell sonicator.

Cell solution (200 µl) was removed for both total cellular protein and total cellular cisplatin measurements. The cellular-bound fraction was precipitated as a pellet by spinning down the remaining 800 µl sonicated cell solution at 14 000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with 1000 µl ice-cold PBS. The pellet was resuspended in 800 µl ice-cold PBS and was homogenized briefly by sonication for both cellular-bound cisplatin and protein measurements.

To determine the degree of efflux by 10, 30 and 50 s after termination of cisplatin exposure, cells were exposed to cisplatin for 20 min, uptake was terminated by spinning, the supernatant was discarded, and the pellet was washed twice with 1000 µl ice-cold PBS. The pellet was resuspended in 1000 µl FCS-free IMDM at 37 °C for 10, 30 or 50 s. The efflux for different times was stopped immediately by spinning the cell solution at 14 000 rpm for approximately 10 s. The supernatant was discarded and the pellet was washed twice with 1000 µl ice-cold PBS. The pellet was resuspended in 500 µl ice-cold PBS and was sonicated for 20 s at 40% output level. Cell solution (200 µl) was removed for both total protein and total cisplatin measurements. The cellular-bound fraction was precipitated as a pellet by spinning down the remaining 300 µl sonicated cell solution at 14 000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with 1000 µl ice-cold PBS. The pellet was resuspended in 300 µl ice-cold PBS and was homogenized briefly by sonication for both cellular-bound cisplatin and protein measurements.

Ultrafiltrable intracellular cisplatin measurements

For the determination of ultrafiltrable intracellular cisplatin, cells were exposed to cisplatin as in the studies of total cellular cisplatin content. Following sonication, 200 µl of the cell solution was removed for both total cellular protein and total cellular cisplatin measurements. Cell solution (300 µl) was transferred to the sample reservoir of an Amicon micropartition system that contained a Diaflo ultrafiltration membrane with a 500 MW cut-off. The filled devices were placed in a fixed-angle centrifuge rotor and were spun at 4000 rpm for 30 min at 5 °C. The ultrafiltrate was then analyzed for cisplatin.

For 10-, 30- and 50-s efflux studies, the total cellular cisplatin, total cellular protein and ultrafiltrable intracellular cisplatin were determined after the cell solution had been prepared, and the pellet resuspended in 1000 µl FCS-free IMDM at 37 °C for 10, 30 or 50 s and was processed by ultrafiltration.

Equal loading experiments

HTB56 and E-8/0.7 cells were exposed to various cisplatin concentrations and the cisplatin content was determined. Cisplatin doses required for both sensitive and resistant cells to accumulate similar intracellular cisplatin concentrations were determined to be 509 vs 911 µ*M*, respectively, in one set of experiments, and 111 vs 666 µ*M*, respectively, in another set of experiments. With 1-h exposures to these relative cisplatin doses, the total cellular cisplatin content was similar in the two cell lines. Cells grown on 60-mm tissue culture dishes were exposed to cisplatin, and then were scraped and resuspended in 1.0 ml PBS. Of this suspension , 500 µl was used for total cellular and ultrafiltrable intracellular cisplatin measurements, and cellular protein measurement. The remaining 500 µl was used for DNA-bound cisplatin and precipitable protein-bound cisplatin measurements.

DNA-bound and protein-bound cisplatin measurements

A genomic DNA purification kit was used in this experiment, following the manufacturer's standard protocol. The precipitated protein formed in the process was redissolved in 1000 µl PBS and tested for both protein and cisplatin content.

Measurement of intracellular pH using flow cytometry with carboxy-SNARF-1

Cells were grown in T75 tissue culture flasks at 37 °C in a humidified incubator containing 5% CO_2 in IMDM supplemented with 10% (v/v) FCS. On day 4, cells were trypsinized and were resuspended in 1.0 ml FCS-free IMDM (Gibco) in 1.5-ml Eppendorf tubes at a concentration of approximately 5×10^5 cells/ml. A 3.4-µl aliquot of the AM ester of SNARF (SNARF-AM) was then added from a 1.0 mg/ml stock solution in anhydrous DMSO to give a final concentration of 5.0 µ*M*. Cells were incubated at 37 °C for 30 min to allow the cleavage of the AM ester. For calibration samples, the pellets were resuspended in high $[K^+]$ buffer (containing 140 m*M* KCl, 1.0 m*M* $MgSO_4$, 2.0 m*M* $CaCl_2$, 5 m*M* glucose, 20 m*M* Tris) [7] of various pH values. All flow cytometry was done with a Coulter EPICS V (Hialeah, Fl.) by the method of Hedley and Boyer [8]. Excitation of SNARF was provided by the 488-nm line of an argon laser. The emitted light was passed through a 625-nm dichroic filter, and the resultant beams were narrowed by passage through 620-nm band pass and a 575-nm band pass. The ratio of 620 nm to 575 nm fluorescence was measured, and this ratio increased with increasing pH.

Statistical analysis

Student's *t*-test was used, and $P < 0.05$ was applied as the significance level.

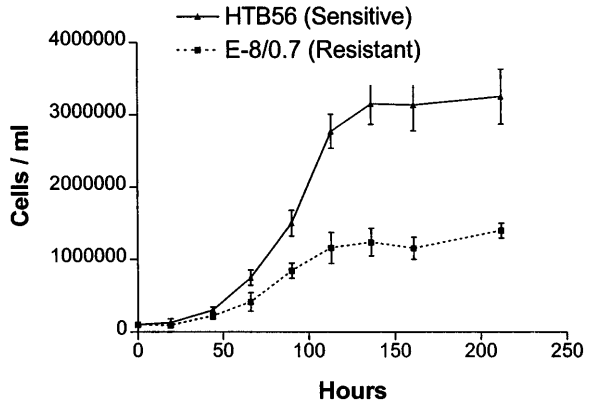

Fig. 1 Growth rate determination by plotting cell number per milliliter versus time. Points are mean values from three independent experiments, performed in triplicate; *bars* are SD

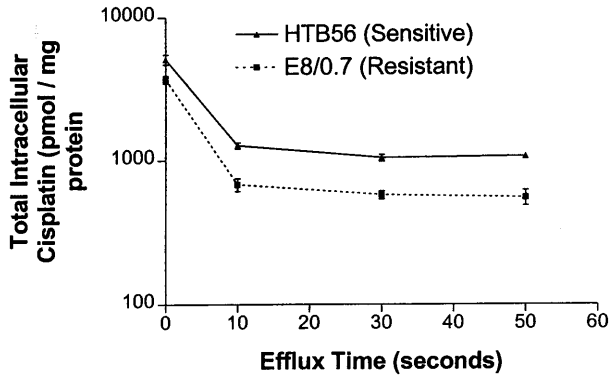

Fig. 3 Early efflux of total cellular cisplatin from the HTB56 human lung adenocarcinoma cell line and its cisplatin resistant variant, E-8/0.7. Points are means ± SEM from three independent experiments, performed in triplicate. Where no error bars are shown the symbol is larger than the error

Results

Figure 1 shows that HTB56 (sensitive) cells had a higher growth rate, as their cell doubling time in the log phase was 18 h, than E-8/0.7 (resistant) cells (29 h). The maximum cell density attained in the plateau phase in sensitive cells was approximately 3.2 million cells/ml, about 2.5-fold higher than in resistant cells (1.3 million cells/ml) (Fig. 1). Plating efficiency was 11% lower in resistant cells, as determined by comparisons of colonies formed after 9 days at zero drug concentration. Moreover, the IC_{50} was 3.5 μg/ml of cisplatin in sensitive cells and 16.0 μg/ml in resistant cells, indicating that E-8/0.7 cells were about 4.6-fold more resistant than HTB56 cells (Fig. 2).

Figure 3 is an efflux kinetics graph. At time 0 (i.e. the end of 20-min cisplatin exposure), total cellular cisplatin in HTB56 cells was significantly higher than in E-8/0.7 cells ($P = 0.01$; Fig. 3). Total cellular cisplatin remained lower in resistant than in sensitive cells at 10, 30 and 50 s following cessation of cisplatin exposure during the early efflux stage (all $P < 0.0001$). The rate constants of efflux for two phases, 0–10 s and 10–50 s, for total cellular cisplatin in each cell line are shown in Table 2.

We found that total platinum content was lower in our resistant lung cancer variant than in the sensitive parent at the end of the period of drug uptake and at all later times tested. This could have resulted from decreased influx of drug or from increased efflux. Any measure of uptake reflects the net effect of influx minus efflux, and at least passive diffusional efflux would be expected to begin almost as soon as influx began. Because there are no practical assays of platinum or cisplatin that are sufficiently sensitive to measure very small quantities, it was not possible to measure very early influx, and it is not possible to selectively block efflux in such a way that platinum content would reflect only influx.

The efflux rate of the total cellular cisplatin was higher in the resistant line than in the sensitive line in the first 10 s, but was comparable between the two cell lines at later times (as shown in Table 2 and Fig. 3). While total cellular cisplatin content differed significantly between the two cell lines by the end of the drug uptake phase and through the early efflux phase, the ultrafiltrable "free" intracellular cisplatin did not differ significantly between the two cell lines at time 0 ($P = 0.95$; Fig. 4). As with total cisplatin content, two distinct phases of free intracellular cisplatin disappearance were observed (Fig. 4). In the first 10 s of free intracellular cisplatin disappearance, the rate constant for resistant cells (K_{R1}) was 0.29 s^{-1}, whereas that for sensitive cells (K_{S1}) was 0.21 s^{-1} (Fig. 4). From 10 s to 50 s of free intracellular cisplatin disappearance, however, K_{R2} and K_{S2} became 0.0018 s^{-1} and 0.0200 s^{-1}, respectively (Fig. 4). Hence, the decline of ultrafiltrable cisplatin in resistant cells was faster than in sensitive cells in the first 10 s phase, but was slower during the subsequent 10 to

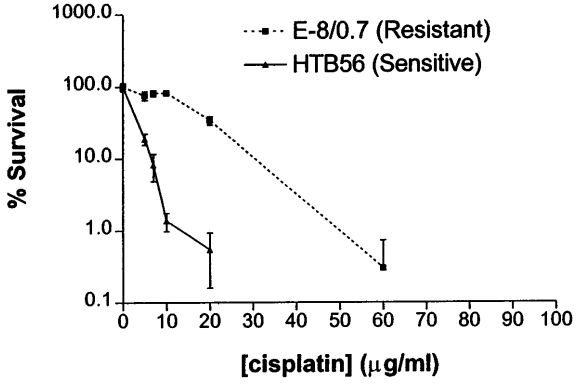

Fig. 2 Sensitivity of HTB56 (Sensitive) cells and E-8/0.7 (Resistant) cells to cisplatin. Cells were exposed to appropriate concentrations of drug for 1 h at 37 °C and cell survival was measured in terms of number of colonies formed on day 9. Points are mean values from three independent experiments, performed in triplicate; *bars* are SD

Table 2 Comparison of rate constants of two efflux phases in and between cell lines found in curves shown in Figs. 3 and 4 (K_{S1} and K_{R1} are rate constants of the slopes of cisplatin concentration from 0 to 10 s during the efflux phase in sensitive and resistant cells, respectively, and K_{S2} and K_{R2} are rate constants of the slopes from 10 to 50 s during the efflux phase in sensitive and resistant cells respectively)

	K_{S1}	K_{R1}	K_{S2}	K_{R2}
Early efflux of total cellular cisplatin from Fig. 3	0.140	0.170	0.004	0.005
Early disappearance of ultrafiltrable intracellular cisplatin from Fig. 4	0.210	0.290	0.020	0.002

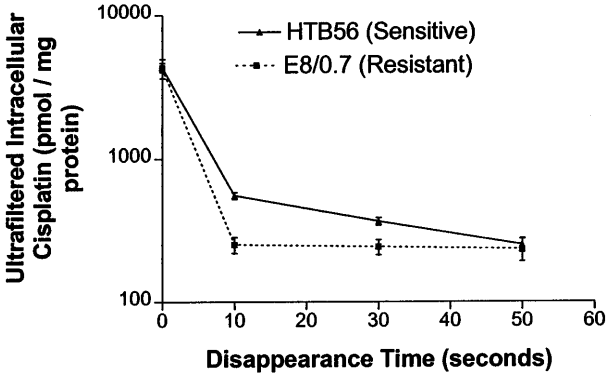

Fig. 4 Early disappearance of ultrafiltrable intracellular cisplatin from the HTB56 human lung adenocarcinoma cell line and its cisplatin-resistant variant, E-8/0.7. Points are means ± SEM from three independent experiments, performed in triplicate

50 s. Note that free intracellular cisplatin could decrease either due to efflux of drug, or to binding of drug, or to a combination of these two processes. Also, the filter membrane used in our ultrafiltration studies had a cut-off of 500 MW and cisplatin is only 300 MW. Hence, some low molecular weight bound species might also have passed through the filter.

Figure 5 shows that precipitable cellular bound cisplatin was significantly lower in resistant cells than in sensitive cells from 0 to 10 s after termination of exposure to cisplatin ($P = 0.00030$ at time 0; $P = 0.028$ at 10 s). There was a general trend to a decrease in precipitable cellular bound cisplatin in sensitive cells from 0 to 50 s. On the contrary, there was a gradual increase in precipitable cellular bound cisplatin in resistant cells from 0 to 50 s.

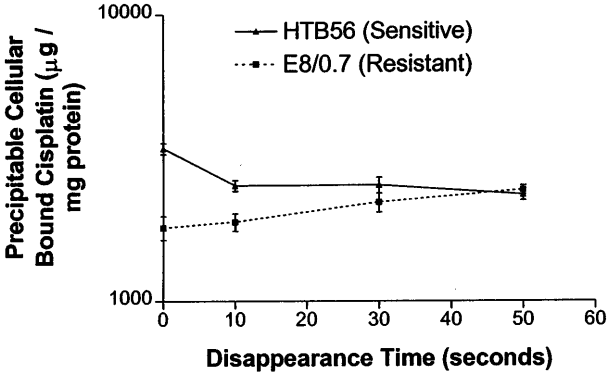

Fig. 5 The change in precipitable cellular bound cisplatin during early disappearance for the HTB56 human lung adenocarcinoma cell line and its cisplatin-resistant variant, E-8/0.7. Points are means ± SEM from three independent experiments, performed in triplicate

In equal loading experiments, in which resistant cells were exposed to a higher cisplatin dose than sensitive cells in order to achieve comparable total cellular cisplatin concentrations between the two cell lines, the precipitated protein was isolated during the DNA purification process. In one series of studies using 509 vs 911 μM cisplatin, the concentrations of both ultrafiltrable intracellular cisplatin and precipitated protein-bound cisplatin were similar when the two cell lines were compared (Fig. 6). However, the concentration of DNA-bound cisplatin was significantly lower in the resistant cell line ($P < 0.0001$; Fig. 7). In another series of studies using 111 vs 666 μM cisplatin, concentrations of both ultrafiltrable intracellular cisplatin and precipitated protein-bound cisplatin were once again comparable in the two cell lines (Fig. 8). Unlike in our earlier experiments, the concentration of DNA-bound cisplatin was also comparable between the two cell lines ($P = 0.4624$; Fig. 9). This suggests that cisplatin-DNA binding may be more saturable at higher cisplatin doses in the resistant variant than in the sensitive parent.

In studies of intracellular pH using flow cytometry and carboxy-SNARF-1, the intracellular pH of resistant cells was found to be 7.65 ± 0.06 in E-8/0.7 cells (Fig. 10) and 7.38 ± 0.13 in C13 cells (Fig. 11), slightly but significantly more alkaline than their sensitive parent cell lines, which had intracellular pH values of 7.51 ± 0.06 in HTB56 cells ($P < 0.0186$) and 6.98 ± 0.11 ($P < 0.0169$) in OV2008 cells.

Discussion

Although there have been many studies on the uptake and accumulation of cisplatin, there has been relatively little work reported on the efflux of cisplatin from cells. Based on the results we observed in our lung cancer cell lines, we feel that cisplatin resistance may be related to decreased cisplatin binding and an associated more rapid efflux of free drug. The difference in these parameters between sensitive and resistant cells is small (1.4-fold difference in DNA-cisplatin binding) (Fig. 7) and the differences in efflux occur during very early time periods (Fig. 4). These changes probably play a major role in the decreased intracellular accumulation of cisplatin in resistant cells. This decreased intracellular accumulation, in turn, is probably one of the factors

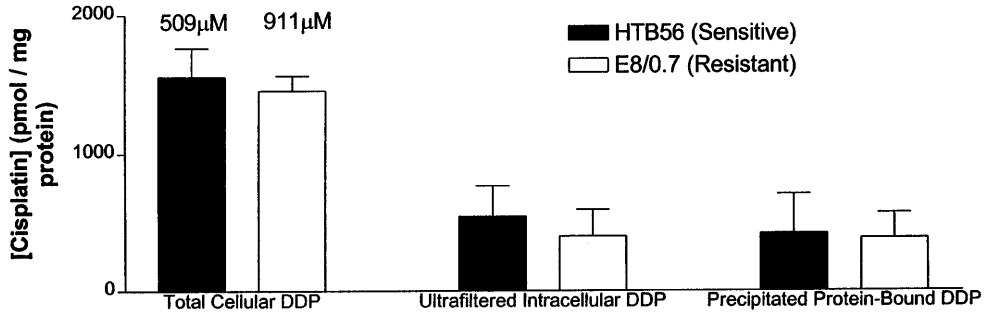

Fig. 6 Equal loading experiments, in which HTB56 and E-8/0.7 cell lines were treated with different cisplatin doses (509 vs 911 μ*M*) designed to give comparable platinum concentrations in the two cell lines. Ultrafiltrable intracellular cisplatin (*DDP*) and precipitable protein-bound cisplatin were compared after the total cellular cisplatin had been set to be similar between the two cell lines. *Bars* are means + SD from three independent experiments, performed in triplicate

leading to the 4.6-fold difference in cytotoxicity observed between the two cell lines (Fig. 2).

Ultrafiltrable drug disappeared more rapidly from the resistant line than from the sensitive parent line in the first 10 s after termination of drug exposure, but disappeared less rapidly between 10 and 50 s. This suggests that the comparability of free drug content in the two lines at the termination of drug exposure was the result of two different offsetting processes. The higher early disappearance rate for ultrafiltrable intracellular cisplatin in resistant cells than in sensitive cells could result from increased active efflux, increased passive efflux, increased binding, or all of them together. The higher K_{S1} and K_{R1} for ultrafiltrable intracellular cisplatin (K_{S1} = 0.21 s^{-1}, K_{R1} = 0.29 s^{-1}; Fig. 4) than for total cellular cisplatin (K_{S1} = 0.14 s^{-1}, K_{R1} = 0.17 s^{-1}; Fig. 3) indicates that there was probably binding in addition to efflux of ultrafiltrable intracellular cisplatin very early after cisplatin entry into the cell. At least part of this binding was probably to nonprecipitable molecules such as glutathione and DNA in addition to binding to precipitable molecules such as cell membrane and protein. We conclude that there was binding to nonprecipitable molecules since there was a modest fall in precipitable bound cisplatin in sensitive cells and only a minimal increase in precipitable bound cisplatin in resistant cells in the first 10 s (Fig. 5), at a time when the rate of disappearance of ultrafiltrable intracellular cisplatin (Fig. 4) exceeded the rate of cisplatin efflux (Fig. 3). The later (10–50 s) comparability of total drug efflux rate but reduced disappearance rate of free drug in the resistant variant compared to the sensitive variant suggests reduced later (10–50 s) binding of free drug in the resistant variant.

When we examined the amount of precipitable platinum (presumably bound to insoluble macromolecules such as protein) in the two lines, we found lower precipitable platinum in the resistant variant than in the sensitive parent line (Fig. 5). However, the amount of precipitable platinum then decreased in the sensitive parent line while gradually increasing in the resistant variant (Fig. 5), at the same time that the disappearance of ultrafiltrable platinum in the resistant line slowed to a rate that was less than in the sensitive parent line (Fig. 4). These observations suggest that platinum was binding to nonfiltrable, nonprecipitable intracellular macromolecules at a higher rate in the sensitive cell line than in the resistant variant. The extent of cisplatin binding to nonprecipitable molecules could not be determined, as technical factors precluded direct measurement of those components. We were unable to isolate the nonfiltrable, nonprecipitable fraction from the other cellular fractions. The idea of a higher "nonprecipitable" intracellular binding ability in sensitive cells is supported by the observation that the total cellular cisplatin was always higher in sensitive cells (Fig. 3), despite ultrafiltrable (Fig. 4) and precipitable platinum (Fig. 5) being comparable in sensitive and resistant cells by 50 s after exposure. It is, therefore, presumed that curves for nonprecipitable intracellular bound cisplatin would diverge between the two cell lines, if we were able to assess this compartment. Overall, our results suggest that cisplatin initially binds more rapidly but more reversibly to precipitable intracellular macromolecules in the sensitive parent than in the resistant

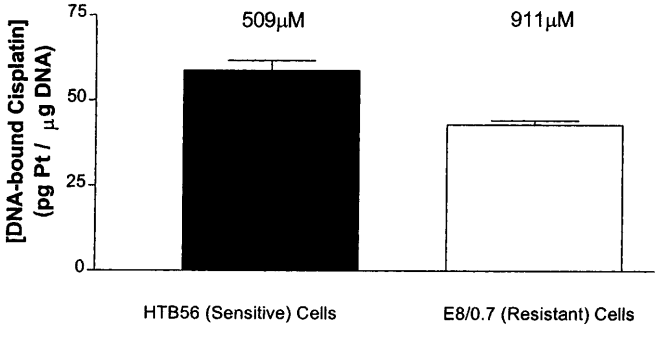

Fig. 7 The DNA-bound platinum was compared in the equal loading experiments in which the total cellular cisplatin had been set to be similar between the two cell lines by exposing sensitive cells to cisplatin 509 μ*M* and resistant cells to cisplatin 911 μ*M*. *Bars* are means + SEM from three independent experiments, performed in triplicate

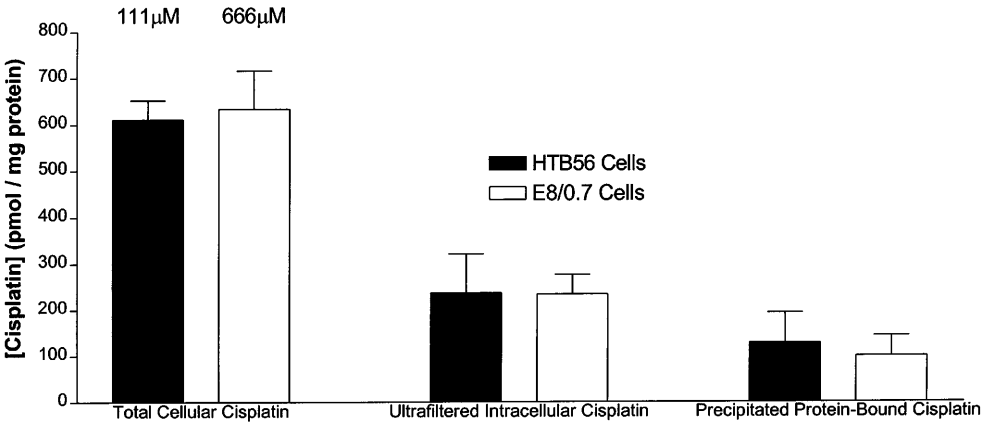

Fig. 8 Equal loading experiments, in which HTB56 and E-8/0.7 cell lines were treated with different cisplatin doses (111 vs 666 μM) designed to give comparable platinum concentrations in the two cell lines. Ultrafiltrable intracellular cisplatin and precipitable protein-bound cisplatin were compared after the total cellular cisplatin had been set to be similar between the two cell lines. *Bars* are means + SD from three independent experiments, performed in triplicate

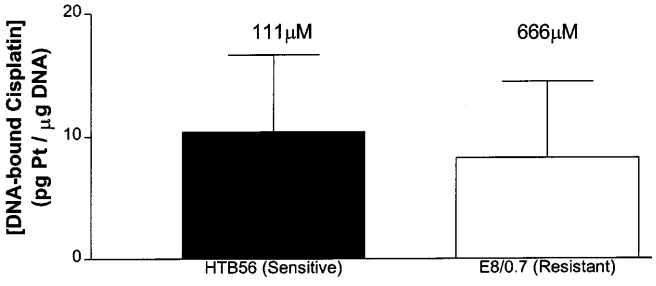

Fig. 9 The DNA-bound platinum was compared in the equal loading experiments in which the total cellular cisplatin had been set to be similar between the two cell lines by exposing sensitive cells to cisplatin 111 μM and resistant cells to cisplatin 666 μM. *Bars* are means + SD from three independent experiments, performed in triplicate

variant. In the sensitive parent, cisplatin may then shift to other intracellular targets while continuing to gradually bind to proteins in the resistant variant.

In the equal loading experiments, we found that ultrafiltrable and precipitable bound platinum content was the same in the two cell lines when cisplatin dose was set to give comparable total cellular platinum content (Figs. 6 and 8). Based on our 20-min exposure studies, we had anticipated that we would have found a higher ultrafiltrable platinum content and a slightly lower precipitable bound platinum content in the resistant line than in the sensitive parent line in these equal loading studies. The fact that we did not find this would suggest that there might have been a shift from free to bound platinum over the longer cisplatin exposure times used in these equal loading studies. This explanation is in keeping with our observation of increased late protein binding of cisplatin in the resistant cell line in our studies using 20-min cisplatin exposures. DNA-bound cisplatin content was found to be significantly lower in the resistant line than in the sensitive line when higher cisplatin doses were used (Fig. 7), suggesting that the decreased cytotoxic activity of cisplatin in resistant cells might be related at least in part to its reduced ability to induce DNA adduct formation. The lack of a significant difference in DNA-bound cisplatin content when lower cisplatin doses were used (Fig. 9) may have been due to the fact that DNA-bound cisplatin content was at the lower limit of detection in experiments using lower cisplatin doses. Alternatively, it is possible that relative DNA binding is concentration dependent, with less efficient DNA binding in resistant cells treated with higher cisplatin doses.

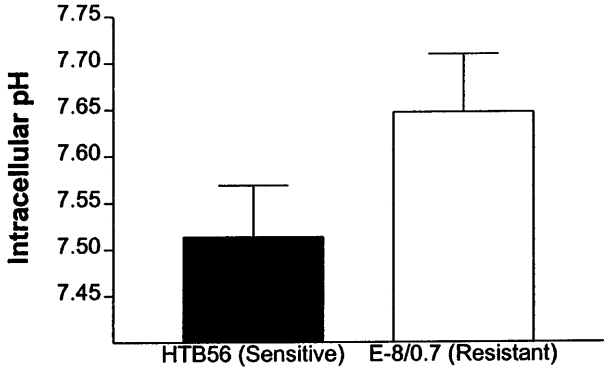

Fig. 10 Intracellular pH studies in HTB56 and E-8/0.7 human lung cancer cell lines using flow cytometry and carboxy-SNARF-1. *Bars* are means + SD from at least three independent experiments, performed in triplicate

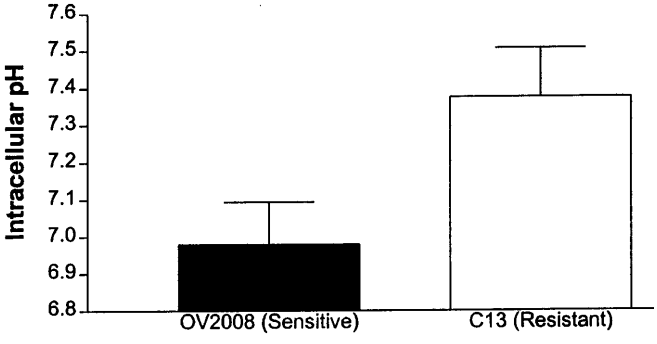

Fig. 11 Intracellular pH studies in OV2008 and C13 human ovarian cancer cell lines using flow cytometry and carboxy-SNARF-1. *Bars* are means + SD from at least three independent experiments, performed in triplicate

It is uncertain whether efflux is active or passive. Some investigators believe that the GS-X conjugate pump may play a role in cisplatin efflux [2, 9, 10]. However, in previous studies, members of our group have found no evidence of an effect of various metabolic inhibitors on cisplatin efflux from our cell lines (Stewart et al., submitted for publication), suggesting that efflux is passive, rather than active. Recent in vitro findings from other laboratories have suggested that overexpression of multidrug resistance-associated protein (MRP) does not induce cisplatin resistance [11]. It has been postulated that the newly discovered member of the ATP-binding cassette (ABC) transporter superfamily, canalicular multispecific organic anion transporter (sMOAT) [12], may participate in platinum drug transport. However, the steady-state cMOAT mRNA level has been found to have no association with platinum drug exposure [13], suggesting that it does not play a major role in cisplatin transport and/or resistance.

The fact that intracellular pH was significantly higher in the resistant variant than in the sensitive parent of two completely unrelated cell lines suggests that the pH change may be an integral component of the resistant phenotype. At physiological pH and chloride concentration, cisplatin is hydrolyzed and equilibrium is maintained between cisplatin and the charged (chloro)(aqua) species and the neutral (chloro)(hydroxo) species [14]. Hydrolysis inside the cell produces cationic complexes that diffuse to DNA, itself a polyanion, where they bind to form cytotoxic lesions. The hydrolysis reactions of cisplatin are an important aspect of its biological activity. As shown in Fig. 12, the chloride ions are displaced in a stepwise manner to form aqua and hydroxo species. These hydrolyzed forms of the drug react more rapidly with DNA than does the parent molecule. One report has suggested that cisplatin is *predominantly* in the electroneutral form, i.e. parent drug cisplatin and the (chloro)(hydroxo) species in extracellular fluids [15]. These electroneutral species would be capable of diffusing into the cell. A new equilibrium between species is re-set inside the cell, and the low concentration of charged aquated species causes intracellular cisplatin accumulation in cells, probably due to ion trapping [15] and intracellular binding. Hence, a higher pH would be expected to result in a higher proportion of neutral hydroxy species. In our studies, the higher intracellular pH in resistant cells would favor the further shift in equilibrium to the right of the hydrolysis equation towards the neutral (chloro)(hydroxo) species, resulting in a faster diffusion back out across the cell membrane and less DNA binding.

The mechanism of increased intracellular pH in the resistant lines is unknown. We have previously documented a threefold increase in glucose utilization and

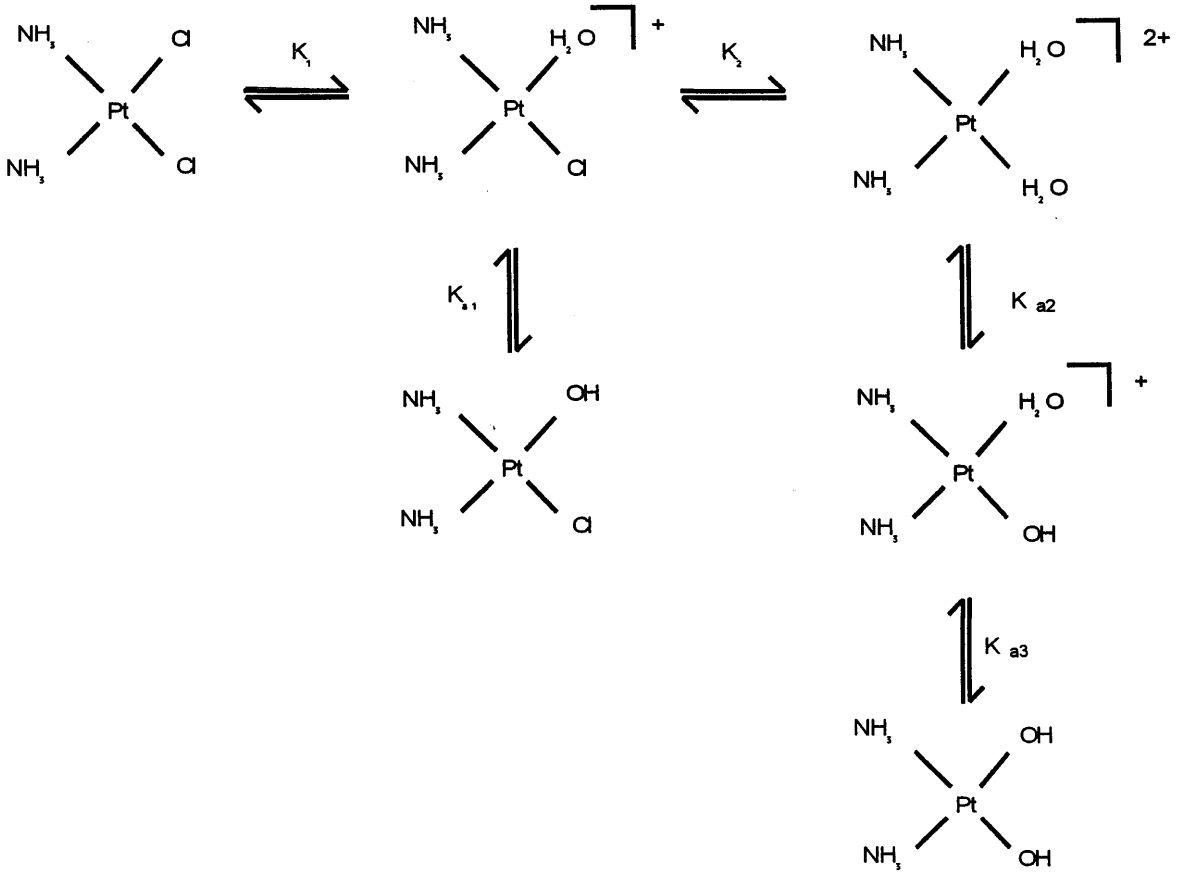

Fig. 12 Structures and equilibria of cisplatin and the species found in aqueous solution (adapted from reference 15)

lactate production in the cisplatin-resistant variants of the human HTB56 lung, A2780 ovarian, and U373MG glioma cell lines (Stewart et al., submitted for publication), and this increased lactate production should have reduced intracellular pH instead of raising it. The intracellular pH is maintained at a neutral range under physiological conditions, which is much higher than the pH (~6.2) calculated by assuming that intra- and extracellular H^+ distribution follows purely its electrochemical gradient [16]. Such a high pH_i is maintained by several plasma membrane H^+ extrusion systems including a Na^+/H^+ exchanger, Na^+-dependent and Na^+-independent Cl^-/HCO_3 exchangers, and an ATP-dependent H^+ pump [16]. Na^+/H^+ exchangers are ubiquitous proteins present in virtually all cell types [17]. Apart from involvement in cell volume regulation [16], they mainly function to exchange intracellular H^+ for extracellular Na^+ [16, 18]. Na^+/H^+ exchangers, therefore, protect cells from intracellular acidification [18]. Since it has been documented that transformed cells have a higher Na^+/H^+ exchange activity or a higher pH_i as compared with untransformed cells [19, 20], it may be inferred that our alkaline-resistant cells also have a higher Na^+/H^+ exchange activity as compared with sensitive cells. Since its regulation can be affected by calcium/calmodulin [21–23], protein kinase C [24], ATP depletion [25], etc, and this profile is similar to agents that modulate cisplatin accumulation [26], we are also investigating its potential for cisplatin transport in our cell lines.

In conclusion, the lower accumulation of cisplatin in resistant cells during cisplatin exposure may be due to increased efflux. The increased efflux, in turn, may be due to decreased intracellular binding of the cisplatin, as well as being due to a higher proportion of the intracellular cisplatin being present in a nonpolar, electroneutral state. The higher intracellular pH in the resistant variant would be expected to favor the production of such electroneutral species, which should bind much less avidly to DNA than the charged aquated species. The reason for an increased intracellular pH in the resistant cell lines is unknown. However, it occurs despite higher glucose utilization and lactate production. Further work is underway to try to explain these pH changes and to confirm their importance in cisplatin resistance.

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