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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

by

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Abstract

Purpose: Many cisplatin-resistant cell lines have reduced cisplatin accumulation. We postulated that reduced accumulation of diamminedichloroplatinum (II) (DDP), or cisplatin, in resistant cells might be due to decreased intracellular DDP binding, leading to increased passive efflux.

Methods: The *total cellular* ([T-DDP]), *intracellular ultrafilterable* ([F-DDP]) and *precipitable cellular bound* ([B-DDP]) cisplatin concentrations were all compared in the HTB56 human lung adenocarcinoma cell line and its E-8/0.7 variant that has acquired cisplatin resistance. After a 20-minute exposure to 509μM cisplatin, uptake was terminated by spinning cell suspensions in a microcentrifuge at 14000 rpm for 10s. *Ultrafiltration* with a 500 molecular weight cut-off separated cellular free from bound cisplatin. Fragmentation by sonication and micro-centrifugal spinning precipitated cellular bound cisplatin. Flow cytometry was used to measure the intracellular pH (pH_i) of the HTB56 cell line, the E8/0.7 cell lines, the OV2008 cell line and its C13 resistant variant, as well as of the A2780 and its A2780/CP 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 lung cancer cells by exposing them to two pairs of DDP concentrations, i.e., 509 vs 911 μM DDP, and 111 vs 666 μM DDP respectively. After one-hour drug exposure in these equal loading experiments, uptake was terminated and cells were scraped. Platinum was assayed by flameless atomic absorption spectrophotometry.

Results: At time 0 (end of cisplatin exposure), [T-DDP] ($p<0.02$) and [B-DDP] ($p<0.001$) were significantly higher in the sensitive HTB56 parent cell line, whereas [F-DDP] did not differ significantly ($p=0.62$). Two distinct phases of T-DDP efflux were observed. In the first 10s post cisplatin exposure, the rate constant for resistant cells (K_{R1}) was $0.17s^{-1}$, whereas that for sensitive cells (K_{S1}) was $0.14s^{-1}$. From 10s to 50s, however, K_{R2} and K_{S2} became $0.005s^{-1}$ and $0.004s^{-1}$ respectively. [T-DDP] remained lower in resistant cells than in sensitive cells at 10, 30 and 50s (all $p<0.0001$). For 1 hour drug exposure to 509 vs 911 μM 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 ($p=0.18$; $p=0.75$). On the other hand, there were no significant differences found in [F-DDP], [P-DDP] and [DNA-bound DDP] between two cell lines when 111 vs 666 μM DDP was used. Flow cytometry data indicated that the pH_i was significantly higher in the E8/0.7 ($p<0.0186$) and C13 ($p<0.0169$) resistant variants than in the sensitive parent cell lines, whereas pH_i was not significantly different between A2780 and it cisplatin resistant variant A2780/CP ($p=0.0614$).

Conclusions: Cisplatin 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 cell lines with respect to DNA binding may be cisplatin concentration-dependent. We speculate that the reduced early binding and increased early efflux in the resistant line may be

related to the higher pH_i 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 3-fold increase in glucose utilization and lactate production in the cisplatin-resistant variants of the human HTB56 lung and A2780 ovarian 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 activity which would protect cells from intracellular acidification.

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LIST OF ABBREVIATIONS AND CHEMICAL FORMULAE

NaOH	Sodium hydroxide
CO_2	Carbon Dioxide
IMDM	Iscove's Modified Dulbecco's Medium
FCS	Fetal Calf Serum
PBS	Phosphate Buffered Solution
MW	Molecular Weight
RPM	Revolutions Per Minute
Cis-$Pt(Cl)_2(NH_3)_2$	Cis-Diamminedichloroplatinum (II)
dGpdG	Deoxyguanosine-Phosphate-Deoxyguanosine
dApdG	Deoxyadenosine-Phosphate-Deoxyadenosine
dG	Deoxyguanosine
dGpNpdG	Deoxyguanosine-Phosphate-deoxyribonucleoside-Phosphate-Deoxyguanosine
CG	Cytidine Guanosine
HMG	High Mobility Group
LMG	Low Mobility Group
Cis-GG	Cisplatin- Deoxyguanosine Deoxyguanosine
Cis-AG	Cisplatin- Deoxyadenosine Deoxyguanosine
Cis-GTG	Cisplatin- Deoxyguanosine Deoxythymidine Deoxyguanosine
pH_i	Intracellular pH
SNARF-AM	Acetoxymethyl ester of Seminaphthorhodafluors

1 INTRODUCTION

1.1 BRIEF HISTORY OF THE DISCOVERY OF CISPLATIN AS AN ANTITUMOR AGENT

In 1965, Barnett Rosenberg and co-workers published their observations on the induction of filamentous growth in bacterial cells by platinum-amine complexes (Rosenberg et al., 1965). The unexpected biologic activity of platinum complexes was first noted during studies originally designed to test the effects of electric fields on growing cells. An effect was found when Escherichia coli were grown in a continuous culture apparatus containing platinum electrodes. Bacterial growth continued, but cell division was inhibited, and the bacterial rods grew into very long filaments. This effect was found to be due to a small amount (~10 ppm) of an electrolysis product of the platinum electrode formed in the presence of ammonium chloride in the nutrient medium (Rosenberg et al., 1965). Further analysis showed (Rosenberg et al., 1967) that this chemical was the classic Peyrone's Chloride, cis-dichlorodiammineplatinum (II), or its higher oxidation state equivalent, cis-tetrachloro-diammineplatinum (IV) (Figure 1).

Based on this work, Rosenberg then made the intuitive step of testing the complexes on tumor systems, with the argument that the complexes might also inhibit cell division in rapidly growing tumor cells. Malignant cancers are of two major types: (a) solid tumors and (b)disseminated tumors, as exemplified by the leukemias and lymphomas of the blood and lymphatic systems. Activity of cisplatin was found in both a solid tumor system (Sarcoma 180) and in a

leukemia system (L1210 leukemia). The stereospecificity was again confirmed (Rosenberg et al., 1969) and the cis-isomer was found to be active against a wide variety of animal tumor systems (Rosenberg, 1980; Wolpert-DeFilippes, 1980).

1.2 CHEMISTRY OF CISPLATIN

The aqueous chemistry of the platinum amines with respect to biological relevance may be divided into activation, deactivation and toxicity; the interaction with DNA can be considered activation whereas reactions with other cellular components, metabolism and toxicity are related to deactivation.

The chemistry will be initially discussed with respect to dissociation of the chloride ligands. Many of the studies on platinum complexes relate to their interactions with DNA and its constituent bases, but it is relevant here to summarize the aqueous reaction chemistry as it relates to antitumour activity.

Cisplatin reacts primarily by exchange of the labile chlorides for water or hydroxyl ions to produce the monoaqua species (Bruhn et al., 1990). The kinetics of this exchange in water was reported by Reishus and Marin (Reishus and Marin, 1961) and by Cleare et al. (Cleare et al., 1978). This is a sequential reaction with the final diaquo species acting as a weak acid, leading to the acid base equilibrium shown in Figure 17. The rate-limiting step in the biologic reactions of the drug is aquation.

The presence of high chloride ion concentrations (>5meq of Cl^-/liter) suppresses the aquation reaction products (Rosenberg, 1985). Thus, in the presence of the 100 meq/liter of Cl^- in the extracellular fluid, almost all of the cisplatin is in the unreacted form (Rosenberg, 1985). The intracellular Cl^- concentration (approximately 20mM in epithelial cells) is much lower than the extracellular fluid Cl^- concentration (approximately 103mM) for most cells of the body; hence, the aquation products will be formed in high concentrations if the

parent form of cisplatin enters the cell (Rosenberg, 1985). Other species can then arise from subsequent reactions depending on pH and chloride concentration (i.e. monohydroxy, diaqua, etc.), and cisplatin is in dynamic equilibrium with these aquated species (Figure 17). This provides the only known activation process required for cisplatin to react with molecules in the cell. Metabolic activation is not required (Rosenberg, 1985). However, the Cl^- concentrations of cells may vary from the low value of about 4 meq/liter in muscle cells to a high value of about 160 meq/liter in stomach parietal cells.

Use of the rates of hydrolysis and acidity constants, and knowledge that the chloride concentration of blood plasma is 103mM compared to 4mM in the cytoplasm, allow for the relative proportions of species present at the biological pH 7.4 to be calculated for cis-diamminedichloroplatinum(II) (Martin, 1983). Detailed distribution curves have also been plotted (Martin, 1983). The fact that the neutral complex will be essentially undissociated in plasma is one reason for supposing that the mechanism of cellular uptake of cisplatin is passive. In the extracellular fluid, the parent form of the drug should predominate and aquated species should account for no more than a few percent of the total platinum in solution (Martin, 1983; Miller and House, 1991). Analysis of platinum biotransformation products in rat plasma suggests that there is biotransformation of cisplatin in a matter of hours, indicating that a complex mixture of platinum-containing species is probably present in cells (Daley-Yates and McBrien, 1984). The low chloride concentration in the cytoplasm allows the equilibrium of the intact drug with its aquated products to shift towards the aquated species.

The reactive species in cytoplasm will clearly be a combination of species with aqua and hydroxo ligands. The hydroxo ligand is usually considered to be inert but may become involved in reactions via protonation to the labile aqua form (Lim and Martin, 1976). The monoaqua form is about 10 times more reactive with cellular nucleophiles than the parent drug and is believed to be a requisite intermediate for the majority of the platination reactions with cellular constituents (Dedon and Borch, 1987). This reactive intermediate attacks proteins, DNA, RNA, and small nucleophilic molecules such as glutathione and methionine. Little is known about how platinum leaves cell, or whether the form that effluxes is native drug or other biotransformation products.

1.3 BIOCHEMICAL REACTION WITH DNA AND MOLECULAR MECHANISM OF ACTION

The target of cisplatin's cytotoxic action has been widely accepted to be DNA (Bruhn et al., 1990). Approximately 1% of the total cellular platinum ends up bound to DNA (Parker et al., 1991), inducing various types of protein-DNA, interstrand, and intrastrand cross-links (Eastman, 1987; Fichtinger-Schepman et al., 1985). Protein-protein cross-links also occur in the chromatin between low mobility group (LMG) proteins (Scovell et al., 1987). The predominant lesion formed on DNA is cis-$Pt(NH_3)_2$(dGpdG) (Figure 2) where cisplatin has cross-linked adjacent deoxyguanosines at their N(7) positions. This adduct accounts for 40% to 70% of the platinum bound to DNA (Bruhn et al., 1990; Eastman, 1987). Another major lesion is the cis-$Pt(NH_3)_2$(dApdG) adduct, which accounts for approximately 25% of the DNA-bound platinum. The interstrand cross-link is believed to occur between N(7) of opposing dG residues in CG sequences and account for approximately 1% to 5% of the platinum-induced adducts (Eastman, 1987; Jones et al., 1991). The remaining 10% of the lesions are due to trinucleotide adducts of the structure cis-$Pt(NH_3)_2$(dGpNpdG). The protein-DNA cross-links account for a few percent of the total DNA-bound platinum and appear to involve primarily HMG 1 and 2 proteins but not histones (Scovell et al., 1987; Banjar et al., 1984; Hayes and Scovell et al., 1991).

Hayes and Scovell have suggested that the internucleosomal linker region of native chromatin is much more prone to platination than the nucleosome core (Hayes and Scovell, 1991). The cis-GG cis-AG adducts cause a local unwinding

of 13° of the DNA strand and a 35° kink in naked DNA (Bellon et al., 1991). The cis-GTG adduct also kinks DNA by 35°, but causes a local unwinding of 23° (Bellon et al., 1991). This difference in unwinding was hypothesized to account for the enhanced repair of cis-GTG relative to the cis-GG and cis-AD adducts (Bellon et al., 1991). Whether similar effects of the tertiary structure of DNA also occur in native chromatin is uncertain (Hayes and Scovell, 1991). The physiologic consequences of these cross-links and how they bring about cell death is unknown. Cisplatin adducts inhibit replication and transcription, but many cells can repair these lesions and resume normal function. Eastman has suggested that the G2 phase block in the cell cycle caused by this damage triggers programmed cell death (Barry et al., 1990; Eastman, 1990).

1.4 DEVELOPMENT OF RESISTANCE - REDUCED ACCUMULATION

This project focuses on the cellular pharmacology of cisplatin. Although numerous potential mechanisms of acquired resistance to cisplatin have been elucidated in vitro, it is still not known which of these mechanisms contribute to the cisplatin resistance that emerges in tumors during treatment of patients with cisplatin. A high proportion of cisplatin-resistant cell lines has a common phenotype of reduced cisplatin accumulation, suggesting that this may be a particularly important factor in resistance. Waud , Parker, and Bungo have shown that accumulation can be a primary determinant of how much platination of DNA occurs (Waud , 1987; Parker et al., 1991; Bungo et al., 1990). The mechanism, however, by which cisplatin enters cells remains uncertain. It is, therefore, difficult to pinpoint the actual biochemical change responsible for the reduced accumulation. The reasons for impaired cisplatin accumulation could be increased efflux, altered drug binding, decreased influx due to reduced active transport, or decreased influx due to cell membrane lipid changes, etc. Some progress has been made in defining the biochemical change responsible for the transport defects at the influx and efflux steps in resistant cells.

Studies by Mann have suggested that reduced accumulation in 2008 human ovarian carcinoma cells is due in part to *defective drug influx* on the basis that decreased accumulation was detectable just 30 seconds after exposure to cisplatin (Mann et al., 1990). It is controversial whether cisplatin enters cells by passive diffusion, by active uptake (Andrews and Howell, 1990), or through gated ion channels (Gately and Howell, 1993). In favor of passive diffusion of cisplatin

are reports that uptake is not saturable, it is not inhibited by cisplatin analogues, and does not occur against a concentration gradient (Andrews and Howell, 1990; Andrews et al., 1988). In favor of active transport of cisplatin are that cisplatin accumulation in cells is energy and sodium dependent, is stimulated by high cAMP levels, and is inhibited by some metabolic poisons (Andrews and Howell, 1990; Andrews et al., 1988). Interpretation is complicated by the fact that each of these latter factors could exert their influence by means other than altering active cisplatin uptake. Each could also affect drug binding and efflux, and passive diffusion of drug into cells by, for example, altering membrane lipids and fluidity. The role of reduced drug active or passive uptake in cisplatin resistance remains poorly defined.

The role of efflux in cisplatin resistance remains controversial. The multidrug resistance pump, P-glycoprotein, is not a factor in cisplatin resistance (Seeber et al., 1982). The vinca alkaloid vindesine was highly active in cells selected for cisplatin resistance (Ishikawa et al., 1994). Also, cisplatin was curative in anthracycline- and etoposide-resistant cells, and daunorubicin and etoposide were curative in acquired resistance towards cisplatin (Seeber et al., 1982). Cisplatin has never been shown to be a substrate for the MDR-1 gene product, i.e. P-glycoprotein. Cells over-expressing the MDR-1 gene and cross-resistant to a broad range of natural products are not cross-resistant to cisplatin. Cisplatin-resistant cells with accumulation defects do not have increased MDR-1 expression. Overall, P-glycoprotein does not participate in the cellular

acculmulation of cisplatin and is not responsible for the cisplatin accumulation defect in resistant cells.

There are other efflux pumps that might possibly play a role, including the glutathione-conjugate (GS-X) efflux pump (Ishikawa et al., 1994), and a 200-KD membrane glycoprotein expressed by cisplatin resistant mouse lymphoma cells that have reduced cisplatin accumulation (Kawai et al., 1990). The amount of this 200 KD membrane glycoprotein was inversely related to the cisplatin accumulation in these cell lines. Parker has suggested that resistant A2780/CP cells eliminate platinum more rapidly than sensitive cells and thus may have *enhanced drug efflux* (Parker et al., 1991). These results argue against a decrease in the passive permeability of the plasma membrane to cisplatin as the basis of the accumulation defect since this change would affect the flux in both directions. It could be argued that this interpretation is equivocal, however, because we do not know which form of platinum is being effluxed. It is possible that the passive diffusion of cisplatin is indeed decreased, and that there is an enhanced carrier-mediated efflux of biotransformation products in the resistant cells.

The reason why cisplatin becomes relatively less available for efflux in sensitive cells has not been determined. It could be due to relatively more nonspecific binding to cellular constituents, or rapid biotransformation to less effluxable species. This observation emphasizes the caution required in attributing the many reports of decreased accumulation in resistant cells to a transport phenomenon (Andrews and Howell, 1990). The majority of these

reports present accumulation data at times that were often hours after cisplatin exposure, when distribution and binding would be expected to have a considerable impact on overall accumulation. Studies of cisplatin accumulation at times shortly after drug exposure, when transport processes would reasonably be expected to be the dominant feature of accumulation, have remained limited (Andrews and Howell, 1990; Loh et al., 1992; Schmidt and Chaney, 1993).

As noted, increased early efflux could be active or passive. Evidence has been documented for the ATP-dependent transport of the bis-(glutathionato)-platinum (II) (GS-platinum) complex in other reports, e.g. across the plasma membrane of L1210 murine leukemia cells (Ishikawa, 1992). The ATP-dependent transport is inhibited by typical divalent organic anionic substrates of the S-conjugate export pump (GS-X pump), i.e. S-(2,4-dinitrophenyl)-glutathione, glutathione disulfide, and leukotriene C_4, but is not inhibited by doxorubicin, daunorubicin, or verapamil, which are substrates and mutual inhibitors for P-glycoprotein (Ishikawa and Ali-Osman, 1993). The GS-X pump is also found to be over-expressed in cisplatin-resistant human promyelocytic leukemia HL-60 cells, in which the cellular glutathione level is substantially enhanced (Ishikawa et al., 1994).

Even though the exact molecular nature of the GS-X pump is still far from known, progress has been made in identifying several members of the GS-X pump family. One report states that the human multidrug resistance protein (MRP), a 190-kDa member of the ATP-binding cassette transporter (ABC-protein) superfamily, is an ATP-dependent glutathione S-conjugate carrier (GS-X

pump) and is present in membranes of many cells (Muller et al., 1996). Coordinated expression of GS-X pump genes (e.g. MRP1) and gamma-glutamylcysteine synthetase (a rate-limiting enzyme of cellular glutathione biosynthesis) has been observed in both human colorectal and leukemia cell lines (Ishikawa et al., 1996 Aug; 1996 Jun). However, overexpression of MRP is not commonly observed in cisplatin-resistant cell lines (Jain et al., 1996). Transfection of the MRP gene does not confer resistance to cisplatin (Grant et al., 1994), and expression of MRP does not directly correlate with cisplatin resistance in clinical samples (Clifford et al., 1996; Giaccone et al., 1996). Furthermore, in earlier studies from our group, metabolic inhibitors did not appear to alter the rate of cisplatin efflux, suggesting that efflux of cisplatin from our cell lines may be largely passive, rather than via a pump (Stewart et al., submitted)[3]. Such an increase in passive efflux could occur as a result of decreased intracellular binding of cisplatin, and could also occur if there were a decreased rate of intracellular conversion of cisplatin to polar metabolites such as aquated species. The parent drug or hydroxy metabolites (which are non-polar) would both bind less to DNA, etc., and would also be expected to diffuse more readily out of cells than would polar aquated metabolites.

Increased efflux (active or passive or both) could 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 (Teicher et al., 1987), 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. Reduced bound drug content could be due to reduced drug influx or to active efflux.

Altered membrane lipids and fluidity can affect any of active or passive drug uptake or efflux. For example, membrane lipids and fluidity may alter the activity of transport proteins, or, conversely, membrane proteins may alter membrane fluidity and lipid distribution, thereby altering passive diffusion. Cisplatin-resistant 2008 cells have the same membrane fluidity as sensitive cells, although some changes in the cellular phospholipid composition were found (Mann et al., 1988). Only a limited number of studies have examined the role of cell membrane characteristics in cisplatin resistance.

2 OBJECTIVE

The current data paint a confusing picture of cisplatin accumulation in the cell. On the one hand, cisplatin uptake appears to occur by passive diffusion (Gale et al., 1973; Binks and Dobrota , 1990) since it is not saturable (Gale et al., 1973; Hromas et al., 1990; Mann et al., 1990) and is not inhibited by structural analogs (Andrews et al., 1987; Andrews and Albright, 1987). On the other hand, uptake can be modulated both by a variety of pharmacologic agents that do not cause general permeabilisation of the membrane (Andrews et al., 1988; Andrews and Albright, 1987), and by activation of some intracellular signal transduction pathways (Isonishi et al., 1990; Kikuchi et al., 1990; Mann et al., 1991; Jekunen et al., 1992). We, therefore, 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 non-enzymatic 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 intracellular pH in the

OV2008 and A2780 human ovarian carcinoma cell lines and their cisplatin-resistant variants, C13 and A2780/CP, respectively.

3 MATERIAL AND METHODS

3.1 Materials. Cisplatin clinical formulation (1.0mg/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, WI). 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, MA). Acetoxymethyl ester (AM) of carboxy SNARF-1 from a 1.0 mg/ml stock solution in anhydrous dimethylsulfoxide (DMSO) and nigericin were purchased from Molecular Probe (Eugene, OR).

3.2 Cell Lines. The HTB56 human lung adenocarcinoma and the OV2008 human ovarian carcinoma cell lines were obtained from the American Tissue Culture Collection. The cisplatin resistant variant, E-8/0.7, was generated by prolonged exposure of HTB56 and OV2008 cells to 0.7 μg/ml and a high concentration of cisplatin respectively. The OV2008 human ovarian carcinoma cell line and its cisplatin resistant variant, C13, were gifts from Dr. Stephen Howell, San Diego, California. Another human ovarian carcinoma cell line, A2780, and its cisplatin resistant variant, A2780/CP, were kindly provided by Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA). The A2780 line was established from a patient's biopsy prior to initiation of any chemotherapeutic regimen. All 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 in 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 (for non-viable cells) was used.

3.3 Growth Rate Determination. Approximately $5x10^5$ cells were seeded in T25 tissue culture flasks with 5 ml IMDM (supplemented with 5% FCS). Cell number was counted with a hemocytometer, and was plotted against time. Cell population doubling time was calculated from the exponential part of the growth curves.

3.4 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 one hour in the incubator at 37°C, cisplatin-containing medium was aspirated and cells were exposed to 0.05% trypsin for 3 minutes. Harvested cells were resuspended in drug-free medium and counted with a hemocytometer. Three hundred cells were seeded in each 60mm tissue culture dish, and incubations were continued in a humidified air atmosphere containing 5% CO_2 (V/V) at 37°C. On the ninth day, colonies were stained with Giemsa stain after fixing with ethanol (95%). Colonies with > 50 cells were counted, and results were 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.

3.5 Platinum Assay. To assay cells for platinum, cells were homogenized using a Vibra Cell Ultrasonic Processor (Sonics and Materials, CT) for 20 seconds 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 using the Bicinchoninic Acid Protein Assay (Smith et al., 1985). Platinum was assayed using a Varian Atomic Absorption Spectrophotometer AA1475 series and GTA-95 graphite tube atomizer, as previously described (Stewart et al., 1994). The three-stage temperature program consisted of drying at 100 °C for 60 seconds, ashing at 1200 °C for 85 seconds, ramping to 1500 °C in 20 seconds, and atomizing at 2700 °C for 3 seconds at maximum power setting. Argon was used as the inert gas. Homogenate 20µl was injected, and platinum content of the unknown sample was determined from standard curves using known values of platinum. Platinum content was expressed as pmole platinum per mg protein.

3.6 Bicinchoninic Acid Protein Assay. The required amount of protein determination reagent was first prepared by adding 1 part Copper (II) Sulfate Pentahydrate 4% solution to 50 parts Bicinchoninic Acid solution. A standard curve of different protein concentrations was prepared by quantitatively adding the indicated amounts of water, NaOH, and protein standard to Eppendorf tubes. 200µl of each protein concentration of the standard curve and tested samples were added to a 96-well plate. 2.0ml of the protein determination reagent was added to each well, and it was incubated in a water bath at 37°C for 30 min. The plate was then cooled to room temperature and the absorbance at 562nm was determined in a spectrophotometer, using water to zero the instrument. A standard curve was prepared by plating the net absorbance at 562nm vs the known added µg protein standard. The standard curve created should determine the amount of protein in the unknown samples provided that the net absorbance at 562nm fell within the range of the standard curve.

3.7 Total and Cellular Bound Cisplatin Measurements. HTB56 (sensitive) and E-8/0.7 (resistant) cells were grown on 60mm 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 cells were at their post-log phase, cells were dislodged off dishes by pipetting 1500µl of 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 850µl was transferred to Eppendorf tubes and the cell solution was pre-warmed in a water bath at 37°C. Cisplatin solution 150µl was added to the cell suspension. Cells were exposed to cisplatin at a final concentration of 509µM for 20 minutes with constant shaking in a water bath at 37°C. This duration of exposure is clinically relevant since the half-life of free cisplatin in humans after intravenous administration is approximately 20-30 minutes. Moreover, since uptake, efflux, and binding were all occurring simultaneously, we felt that it would be preferable to use the shortest incubation time that was practical. Incubation times of less than 20 minutes would not have been useful since the platinum concentrations present after such short incubation times would have been below the detection limit of atomic absorption spectrophotometry. Cisplatin exposure was terminated by spinning the cell solution in a microcentrifuge at 14,000 rpm for approximately 10 seconds.

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 1000µl of ice cold PBS. The pellet was resuspended in 1000µl of ice cold

PBS and was sonicated briefly at the 40% output level using a Vibra Cell Sonicator.

Cell solution 200µl 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 800µl sonicated cell solution at 14,000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed twice with 1000µl of ice cold PBS. The pellet was resuspended in 800µl of 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 seconds after termination of cisplatin exposure, cells were exposed to cisplatin for 20 minutes, uptake was terminated by spinning, the supernatant was discarded, and the pellet was washed twice with 1000µl of ice cold PBS. The pellet was resuspended in 1000µl of FCS-free IMDM at 37°C for 10, 30 or 50 seconds. The efflux for different times was stopped immediately by spinning the cell solution at 14,000 rpm for approximately 10 seconds. The supernatant was discarded and the pellet was washed twice with 1000µl of ice cold PBS. The pellet was resuspended in 500µl of ice cold PBS and was sonicated for 20 seconds at 40% output level. Cell solution 200µl was removed for both total protein and total cisplatin measurements. The cellular-bound fraction was precipitated as a pellet by spinning down the remaining 300µl sonicated cell solution at 14,000rpm for 5 minutes. The supernatant was discarded as it might contain some free drugs, and the pellet was washed twice with 1000µl of ice cold PBS. The pellet was

resuspended in 300µl of ice cold PBS and was homogenized briefly by sonication for both cellular-bound cisplatin and protein measurements.

3.8 Ultrafilterable Intracellular Cisplatin Measurements. For the assessment of ultrafilterable intracellular cisplatin, cells were exposed to cisplatin as in studies of total cellular cisplatin content. HTB56 (sensitive) and E-8/0.7 (resistant) cells were grown on 60mm 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 cells were at their post-log phase, cells were dislodged off dishes by pipetting 1500µl of 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 850µl was transferred to Eppendorf tubes and the cell solution was pre-warmed in a water bath at 37°C. Cisplatin solution 150µl was added to the cell suspension. Cells were exposed to cisplatin at a final concentration of 509µM with constant shaking for 20 minutes in a water bath at 37°C. Cisplatin exposure was terminated by spinning the cell solution in a microcentrifuge at 14,000 rpm for approximately 10 seconds.

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 1000µl of ice cold PBS. The pellet was resuspended in 1000µl of ice cold PBS and was sonicated briefly at the 40% output level using a Vibra Cell Sonicator.

Following sonication, 200µl of the cell solution was removed for both total cellular protein and total cellular cisplatin measurements. Cell solution 300µl was

transferred to the sample reservoir of an Amicon Micropartition System (Figure 3) that contained a Diaflo ultrafiltration membrane with 500 MW cut-off. The filled devices were placed in a fixed-angle centrifuge rotor and were spun at 4000 rpm for 30 minutes at 5 °C. The ultrafiltrate was then analyzed for cisplatin.

For assessment of disappearance on ultrafilterable cisplatin by 10, 30 and 50 seconds, cells were exposed to cisplatin for 20 minutes, uptake was terminated by spinning, the supernatant was discarded, and the pellet was washed twice with 1000µl of ice cold PBS. The pellet was resuspended in 1000µl of FCS-free IMDM at 37°C for 10, 30 or 50 seccnds. The efflux and binding of ultrafilterable cisplatin for different times were stopped immediately by spinning the cell solution at 14,000 rpm for approximately 10 seconds. The supernatant was discarded and the pellet was washed twice with 1000µl of ice cold PBS. The pellet was resuspended in 500µl of ice cold PBS and was sonicated for 20 seconds at 40% output level. The sonicated cell solution 200µl was removed for both total protein and total cisplatin measurements. The remaining sonicated cell solution 300µl was transferred to the sample reservoir of an Amicon Micropartition System for ultrafiltration. The ultrafiltrate was then analyzed for cisplatin.

3.9 Equal Loading Experiments. HTB56 and E-8/0.7 cells were exposed to various cisplatin concentrations and cisplatin content was assessed. Cisplatin doses required for both sensitive and resistant cells to accumulate similar intracellular cisplatin concentrations were determined to be 509 vs 911 μM, respectively, in one set of experiments, and 111 vs 666 μM, respectively, in another set of experiments. With 1-hour exposures to these relative cisplatin doses, 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 1.0ml PBS. Of this, 200μl was used for total cellular cisplatin and cellular protein measurements, and 300μl for ultrafilterable intracellular cisplatin measurement. The remaining 500μl was used for DNA-Bound cisplatin and precipitated Protein-Bound cisplatin measurements.

3.10 DNA-Bound and Protein-Bound Cisplatin Measurements. A Genomic DNA Purification Kit was used in this experiment, utilizing the manufacturer's standard protocol. Cell solution 500µl from the equal loading experiment was transferred to a 1.5ml microcentrifuge tube, and was centrifuged for 10s at 16,000 x g to pellet the cells. The supernatant was removed, 600µl of Nuclei Lysis Solution was added, and the sample was pipetted until no visible cell clumps remained. RNase Solution 3.0µl was then added to the nuclear lysate and the sample was mixed by inverting the tube 25 times. The mixture was incubated for 15-30 minutes at 37°C. The sample was allowed to cool to room temperature for 5 minutes before proceeding further. Protein Precipitation Solution (Promega, WI) 200µl was then added to the RNase-treated cells. The sample was vortexed vigorously at high speed for 20 seconds and was centrifuged for 3 minutes at 16,000 x g. The precipitated protein formed a tight white pellet which was redissolved in 1000µl PBS and tested for both protein and cisplatin content. The supernatant containing the DNA was carefully removed, transferred to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol, and gently mixed by inversion until the white thread-like strands of DNA formed a visible mass. The DNA was visible as a small white pellet after the isopropanol solution was centrifuged for 1 minute at 16,000 x g at room temperature. The supernatant was carefully decanted, 600µl of room temperature 70% ethanol was added to the DNA pellet, the tube was inverted several times to wash the DNA, and was centrifuged for 1 minute at 16,000 x g at

room temperature. The ethanol was carefully aspirated using a Pasteur pipet. The tube was inverted on a clean absorbent paper and the pellet was air dried for 10-15 minutes. DNA Rehydration Solution (Promega, WI) 100µl was added to rehydrate the DNA by incubating the solution overnight at room temperature. DNA was stored at 2-8 °C until tested for DNA purity and cisplatin content.

3.11 Measurement of Intracellular pH using Flow Cytometry with Carboxy-SNARF-1. HTB56 (sensitive) and E-8/0.7 (resistant) cells were grown in T75 tissue culture flasks at 37°C in a humidified incubator containing 5% CO_2 in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) fetal calf serum (FCS). On day 4, cells were trypsinized and were resuspended in 1.0ml FCS-free IMDM (Gibco) in 1.5ml Eppendorf tubes at a concentration of approximately $5x10^5$ cells/ml. We then added 3.4µl of the acetomethyl ester of SNARF (SNARF-AM) from a 1.0 mg/ml stock solution in anhydrous dimethylsulfoxide (DMSO) (to protect it from hydrolysis), to give a final concentration of 5.0 µM. Cells were incubated at 37 °C for 30 min to allow the cleavage of the AM ester. SNARF-AM is a nonfluorescent molecule that enters cells easily. Once within the cytoplasm the AM groups are cleaved by the action of nonspecific esterases, yielding the highly fluorescent molecule SNARF. We then centrifuged the cell solution, discarded the supernatant and resuspended the pellets in 1.0ml PBS.

For calibration samples, the pellets were resuspended in high [K^+] buffer (containing 140 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5 mM glucose, 20 mM Tris) (Chow et al., 1996) of varying pH values. The H^+/K^+ ionophore, nigericin (2.0µl, 1.0mg/ml in absolute ethanol), was added to equilibrate the intracellular / extracellular pH five minutes before the measurements of intracellular pH (pH_i).

Nigericin is a linear molecule with heterocyclic oxygen-containing rings together with a hydroxyl group (David Nicholls et al., 1992). In the membrane,

the molecule cyclizes to form a structure similar to that of valinomycin, with the oxygen atoms forming a hydrophobic interior. Unlike valinomycin, nigericin loses a proton when it binds a cation, forming a neutral complex which can then diffuse across the membrane as a mobile carrier. Nigericin is also mobile in its protonated non-complexed form, with the result that the ionophore can catalyse the overall electroneutral exchange of K^+ for H^+ ions.

Calibration procedures basically consist of recording fluorescence signals corresponding to a series of precisely manipulated extracellular hydrogen ion concentrations. For calibration based on nigericin, cells are exposed to nigericin in a solution containing a high concentration of potassium. In the presence of nigericin, it is predicted that the ratio of intracellular to extracellular hydrogen ion concentration will be equal to the ratio of intracellular to extracellular potassium ion concentration. If $[K^+]_i$ and $[K^+]_e$ were equal, then $[H^+]_i$ would be equal to $[H^+]_e$, and hence pH_i could be estimated simply by measuring pH_e. The pH_i of a sample was extrapolated from a reference curve that was derived from cells in high K^+ ($[K^+]_e$) solutions buffered to known pH_e.

The accuracy of this method of calibration depended on the equality of intra and extracellular potassium concentrations which typically were not determined experimentally but were assumed to be of the order of 130-140 mM. This was a valid assumption for most cell types, and the method gave values of pH_i which were close to those obtained using other techniques. At least four high $[K^+]$ calibration solutions were prepared with pH increasing in 0.2-0.3 pH unit increments.

All flow cytometry was done with a Coulter EPICS V (Hialeah, FL) by the method of Hedley (Hedley and Boyer, 1994). Excitation of SNARF was provided by the 488-nm line of an argon laser. The emitted light was passed through a 625 dichroic filter, and the resultant beams were narrowed by passage through 620-nm band pass and 575-nm band pass. Following excitation at 480nm, the emission intensity of SNARF at 575nm was pH dependent with greater intensity at higher pH (Figure 4). In order to make measurement of pH_i, a ratio was taken between a pH-dependent emission intensity (e.g. 575nm) and a pH independent emission intensity (e.g. 640nm). A number of fluorescence measurement artifacts were eliminated with this ratiometric method, including photobleaching, cell thickness, instrument instability and leakage and non-uniform loading of the indicator.

The ratio of 620/575nm fluorescence was measured, and this ratio increased with increasing pH. The intracellular measurement for OV2008, A2780, and their variants C13 and A2780/CP respectively, were treated in the same manner.

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

4 RESULTS

Figure 5 shows that HTB56 cells (sensitive) had a higher growth rate, as their cell doubling time at log phase was 18 hours, compared to 29 hours in E-8/0.7 cells (resistant). The maximum cell density attained at 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) (Figure 5). 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 (Figure 6).

Figure 7 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 ($p=0.01$) (Figure 7). Total cellular cisplatin remained lower in resistant than in sensitive cells at 10, 30 and 50s following cessation of cisplatin exposure during the early efflux stage (all $p<0.0001$). The rate constants of efflux for two phases, 0-10s and 10-50s, for total cellular cisplatin in each cell line are shown in Table 2. The term "rate constant" is not correct for the time from 0-10s, since only 2 time points were available for calculating this value. However, for simplicity, this term will be used throughout the remainder of the thesis. It was not possible to obtain data between 0 and 10s because it was not possible technically to prepare samples with a shorter duration of efflux.

The simple passive flux of molecules down a concentration gradient is given by Fick's Law of diffusion:

FLUX (MOLECULES PER UNIT TIME)=(C1 - C2)X(AREA X PERMEABILITY COEFFICIENT)/THICKNESS

Where C1 is the higher concentration, C2 is the lower concentration, area is the area across which diffusion is occurring, permeability coefficient is a measure of the mobility of the drug molecules in the medium of the diffusion path, and the thickness is the thickness (length) of the diffusion path (Katzung, 1995). These three parameters constitute the concept of a rate constant, K. If first-order molecule kinetics are assumed, the rate at any given time is proportional to the concentration (C) at that time (t) only, i.e. $-dC/dt = KC$. In practice a more convenient form of this equation is obtained by using logarithms to the base 10, i.e. $\log_{10}C = \log_{10}C_0 - (K \cdot t) / 2.303$.

Three important parameters may account for the change or the disappearance in ultrafiltered intracellular cisplatin during the 50s time course, and they are the amount of free cisplatin left to efflux, the amount of intracellular cisplatin binding, and the proportion of cisplatin that is in a neutral, non-polar form (which could passively diffuse across a cell membrane).

Efflux rate constant was higher in the resistant line than in the sensitive line in the first 10 seconds, but was comparable between the two cell lines at later times (as shown in Table 2; Figure 7). 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 ultrafilterable "free" intracellular cisplatin did not differ significantly between the two cell lines at time 0 ($p=0.95$) (Figure 8). As with total cisplatin content, two distinct phases of free intracellular cisplatin disappearance were observed (Figure 8). In the first 10s of free

intracellular cisplatin disappearance, the rate constant for resistant cells (K_{R1}) was 0.29s^{-1}, whereas that for sensitive cells (K_{S1}) was 0.21s^{-1} (Figure 8). From 10s to 50s of free intracellular cisplatin disappearance, however, K_{R2} and K_{S2} became 0.0018 and 0.0200s^{-1} respectively (Figure 8). Hence, the decline of ultrafilterable cisplatin in resistant cells was faster than in sensitive cells in the first 10s phase, but was slower for the subsequent 10 to 50s. 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.

Figure 9 shows that precipitable cellular bound cisplatin was significantly lower in resistant cells than in sensitive cells from 0 to 10s after termination of exposure to cisplatin ($p=0.00030$ at time 0; $p=0.028$ at 10s). The change in concentration of precipitable cellular bound cisplatin during the post-exposure phase was presumably related to the exchange of cisplatin between this precipitable cellular bound component, free cisplatin, and non-precipitable cellular bound cisplatin (e.g. glutathione-cisplatin complexes, DNA-cisplatin complexes, etc). There was a general trend to a decrease in precipitable cellular bound cisplatin in sensitive cells from 0 to 50s, and this drop was especially prominent from 0 to 10s (Figure 9). On the contrary, there was a gradual increase in precipitable cellular bound cisplatin in resistant cells from 0 to 50s (Figure 9).

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 DDP, the concentrations of both ultrafilterable intracellular cisplatin and precipitated protein-bound cisplatin were similar when the two cell lines were compared (Figure 10). However, the concentration of DNA-bound cisplatin was significantly lower in the resistant cell line ($p<0.0001$) (Figure 11). In another series of studies using 111 vs 666 μM DDP, concentrations of both ultrafilterable intracellular cisplatin and precipitated protein-bound cisplatin were once again comparable in the two cell lines (Figure 12). Unlike our earlier experiments, concentration of DNA-bound cisplatin was also comparable between the two cell lines ($p=0.4624$) (Figure 13). This suggests that cisplatin-DNA binding may be relatively 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 pH 7.65 ± 0.06 in E8/0.7 cells (Figure 14) and pH 7.38 ± 0.13 in C13 cells (Figure 15), slightly but significantly more alkaline than sensitive cells, 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. There was no significant difference in intracellular pH between A2780 and A2780/CP ($p=0.0614$), although the pH was again slightly higher in the resistant line (Figure 16).

5 DISCUSSION

Several processes may play a role in cisplatin resistance in cancer cell lines (Richon et al., 1987). Resistance may be due to the combined effects of decreased intracellular drug accumulation (Bungo et al., 1990; Mistry et al., 1992), differential DNA damage (Easter and Schulte, 1988; Parker et al., 1991), and enhanced inactivation by intracellular detoxification systems (Richon et al., 1987; De Graeff et al., 1988). 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. Some studies suggested that the plasma membranes function as a barrier against efflux as well as influx (Melvik et al., 1992). Electropermeabilisation after cisplatin exposure protected NHIK 3025 cells from cisplatin toxicity, presumably by increasing cisplatin efflux from cells (Melvik et al., 1992). Another group measured the efflux of cisplatin from OV2008 ovarian carcinoma cells and found that efflux was biphasic, with a very rapid initial phase followed by a much slower terminal phase (Mann et al., 1990). They also found that the initial efflux was more rapid in the cisplatin-resistant variant of OV2008 cells. These results were consistent with what we found in our cell lines.

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 (eg, 1.4 fold difference in DNA-cisplatin binding) (Figure 11) and the differences in efflux occur at very early time periods (Figure 8). 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 leading to the 4.6 fold difference in cytotoxicity observed between the two cell lines (Figure 6).

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.

Early efflux (in the first 10 seconds) was increased in our resistant line, while later efflux between 10 and 50 seconds occurred at roughly the same rate constant as in the sensitive parent line. When we just looked at ultrafilterable platinum (free drug plus platinum bound to small soluble molecules), it was

comparable in the two cell lines at the termination of a 20-minute drug exposure. The filter membrane used in our ultrafiltration studies had a cut-off of 500 molecular weight (MW) and cisplatin is only 300 MW. Hence, some low molecular weight bound species might also have passed through the filter.

Even though the non-specific binding of the filter membrane to cisplatin, if there is any, has not been determined, it was assumed that this binding would have had the same effect on results for both cell lines; hence, it should not have affected the results of relative comparisons of the cell lines.

Ultrafilterable drug disappeared more rapidly from the resistant line than from the sensitive parent line in the first 10 seconds after termination of drug exposure, but disappeared less rapidly between 10 and 50 seconds. This suggested 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 constant for ultrafilterable intracellular cisplatin in resistant cells than in sensitive cells could result from either increased active efflux, increased passive efflux, increased binding, or all of them together. The higher K_{S1} and K_{R1} for ultrafilterable intracellular cisplatin ($K_{S1}=0.21s^{-1}$, $K_{R1}=0.29s^{-1}$, Figure 8) than for total cellular cisplatin ($K_{S1}=0.14s^{-1}$, $K_{R1}=0.17s^{-1}$, Figure 7) indicates that there was probably binding in addition to efflux of ultrafilterable intracellular cisplatin very early after cisplatin entry into the cell. According to our kinetic studies method, at least part of this binding was probably to non-precipitable 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 non-precipitable 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 10s (Figure 9), at a time when the rate constant of disappearance of ultrafilterable intracellular cisplatin (Figure 8) exceeded the rate constant of cisplatin efflux (Figure 7). The later (10-50 second) comparability of total drug efflux but reduced disappearance of free drug in the resistant variant compared to the sensitive variant suggests reduced later 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 (Figure 9). However, the amount of precipitable platinum then decreased in the sensitive parent line while gradually increasing in the resistant variant (Figure 9), at the same time that the disappearance of ultrafilterable platinum in the resistant line slowed to a rate constant that was less than in the sensitive parent line (Figure 8). These observations suggest that platinum was binding to non-filterable, non-precipitable intracellular macromolecules at a higher rate in the sensitive cell line than in the resistant variant. The extent of cisplatin binding to non-precipitable molecules could not be determined, as technical factors precluded direct measurement of those components. We were unable to isolate the non-filterable, non-precipitable fraction from the other cellular fractions. The idea of a higher "non-precipitable" intracellular binding ability in sensitive cells is supported by the observation that the total cellular cisplatin was always higher in

sensitive cells (Figure 7), despite ultrafilterable (Figure 8) and precipitable platinum (Figure 9) being comparable in sensitive and resistant cells by 50s post exposure. It is, therefore, presumed that curves for non-precipitable 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 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 these initial studies, we could not assess the amount of platinum bound to DNA. The platinum content of the DNA was too low to permit quantitation with the very small amounts of DNA that could be extracted.

Hence, we increased the duration of cisplatin exposure to 1 hour (from 20 minutes), and also varied the cisplatin dose to achieve comparable total cellular platinum content between the two cell lines. With this approach, we found that ultrafilterable 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 (Figures 8 and 10). Based on our 20-minute exposure studies, we had anticipated that we would find higher ultrafilterable platinum content and 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-minute cisplatin exposures. DNA-bound cisplatin content was found to be significantly lower in the resistant line than in the sensitive line when higher cisplatin equal loading doses were used (Figure 11), 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 equal loading experiment shown in Figure 12 was a repeat of the first equal loading experiment shown in Figure 10, except the fact that the loading cisplatin concentrations for both cell lines were lower. Again, there was no significant difference between the 2 cell lines in ultrafiltered or precipitable protein-bound cisplatin when total cisplatin content was equalized by using different doses in the 2 lines. The lack of a significant difference in DNA-bound cisplatin content when lower cisplatin doses were used (Figure 13) 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.

Taken together, these data suggest that at least part of the reduction in net accumulation of cisplatin in our resistant cell line is due to increased early efflux. The increased early efflux, in turn, may be at least partly due to reduced early intracellular drug binding, with a higher proportion of the intracellular drug being available for efflux. It is uncertain whether efflux is active or is passive.

Some investigators believe that the glutathione-X-conjugate pump may play a role in cisplatin efflux (Ishikawa et al., 1994; Ishikawa, 1992; Ishikawa and Ali-Osman, 1993). However, in earlier studies, members of our group found no evidence of an effect of various metabolic inhibitors on cisplatin efflux from our cell lines (Stewart et al., submitted)[3], suggesting that efflux was passive, rather than active. Recent in-vitro findings from other laboratories have suggested that overexpression ofmultidrug resistance-associated protein (MRP) does not induce cisplatin resistance (Grant et al., 1994). It has been postulated that the newly discovered member of the ATP-binding cassette (ABC) transporter superfamily, canalicular multispecific organic anion transporter (cMOAT) (Taniguchi etal., 1996), may participate in platinum drug transport. However, the steady-state cMOAT mRNA level has been found to have no association with platinum drug exposure (Oguri et al., 1998), suggesting that it does not play a major role in cisplatin transport and/or resistance. Overall, we think our observations would best be explained by reduced early drug binding making increased amounts of cisplatin available for passive efflux.

We found a small but statistically significant increase in intracellular pH in our resistant lung cancer cell line compared to its sensitive parent (Figure 14). We noted similar changes in intracellular pH in a resistant variant of the OV2008 ovarian carcinoma cell line (Figure 15) that also has reduced net accumulation of cisplatin (Mann et al., 1990). The fact that similar pH changes were found in the resistant variants of two completely unrelated cell lines suggests that the pH change is an integral component of the resistant phenotype. In a third line

(A2780), there was a difference in pH between sensitive and resistant variants, but it did not quite reach statistical significance (p=0.0614) (Figure 16). 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 (Miller and House, 1990). 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 Figure 17, 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 the parent molecule does. 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 (Jennerwein and Andrews, 1995). 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 (Jennerwein and Andrews, 1995) 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. A lower pH, on the other hand, would favor the production of charged aquated species (which

would bind avidly to DNA and which would not diffuse readily back out across the cell membrane). Whether this small incremental increase in intracellular pH of resistant cells explains our observed difference in drug efflux, binding ability and cytotoxicity of cisplatin remains to be confirmed.

The mechanism of increased intracellular pH in the resistant lines is unknown. We have previously documented a 3-fold increase in glucose utilization and lactate production in the cisplatin-resistant variants of the human HTB56 lung, A2780 ovarian, and U373MG glioma cell lines (Stewart et al., submitted)[4], and this increased lactate production should have reduced intracellular pH instead of raising it. The augmented pH in the resistant lines occurs despite increased lactate production. 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 (Wakabayshi et al., 1997). Such a high pH_i is maintained by several plasma membrane H^+ extrusion systems including a Na^+/H^+ exchanger, Na^+-dependent and –independent Cl^-/HCO_3^- exchangers, and an ATP-dependent H^+ pump (Wakabayshi et al., 1997). Na^+/H^+ exchangers are ubiquitous proteins present in virtually all cell types (Sardet et al., 1989). Apart from involvement in cell volume regulation (Wakabayshi et al., 1997), they mainly function to remove intracellular H^+ for extracellular Na^+ (Wakabayshi et al., 1997; Dibrov and Fligel, 1998). Na^+/H^+ exchangers, therefore, protect cells from intracellular acidification (Dibrov and Fligel, 1998). 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 (Ober and Pardee, 1987; Gilles et al., 1990), it may be inferred that our alkaline resistant cells also have a higher Na^+/H^+ exchange activity as compared with sensitive cells. We are investigating this further in our cell lines.

In our experiments, we used cisplatin concentrations much higher than those that are achievable clinically. Such high concentrations were used since it was only at these concentrations that the intracellular cisplatin accumulation could be accurately quantitated by atomic absorption spectrophotometry. However, we felt that results obtained using these cisplatin concentrations would be relevant since cisplatin net accumulation in both our HTB56 and E-8/0.7 cells (data not shown) and other cell lines (Andrews and Howell, 1990; Andrews et al., 1988) is proportional to cisplatin dose over all doses tested.

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.

6 REFERENCES

1. Andrews P.A. and Howell S.B. Cellular Pharmacology of cisplatin: Perspectives on mechanisms of acquired resistance. Cancer Cells *2*: 35-43, 1990.
2. Andrews P.A., Velury S., Mann S.C. and Howell S.C. cis-Diamminedichloroplatinum (II) accumulation in sensitive and resistant human ovarian carcinoma cells. Cancer Res. *48*: 68-73, 1988.
3. Andrews P.A., Mann S.C., Velury S. and Howell S.B. Cisplatin uptake mediated cisplatin-resistance in human ovarian carcinoma cells. *In:* Nicolini M. (ed.), Platinum and other metal coordination compounds in cancer chemotherapy, pp. 248-254. Padua, Italy: Martinus Nijhoff Publishing, 1987.
4. Andrews P.A. and Albright K.A. Role of membrane ion transport in cisplatin accumulation. *In*: Howell SB (ed.), Platinum and other metal coordination compounds in cancer chemotherapy, pp. 151-159. New York, NY: Plenum Press, 1987.
5. Banjar Z.M., Hnilica L.S., Briggs R.C., Stein J. and Stein G. cis- and trans-Diammindichloroplatinum (II)-mediated cross-linking of chromosomal non-histone proteins to DNA in HeLa cells. Biochemistry 23: 1921-1926, 1984.
6. Barry M.A., Behnke C.A. and Eastman A. Activation of a programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem. Pharmacol. 40: 2353-2362, 1990.

7. Bellon S.F., Coleman J.H., and Lippard S.J. DNA unwinding by site-specific intrastrand cross-link of the antitumor drug cis-diamminedichloroplatinum (II). Biochemistry 30: 8026-0835, 1991.
8. Binks S.P. and Dobrota M. Kinetics and mechanics of uptake of platinum-based pharmaceuticals by the rat small intestine. Biochem. Pharmacol. *40*: 1329-1336, 1990.
9. Bruhn S.L., Toney J.H. and Lippard S.J. Biological processing of DNA modified by platinum compounds. Prog. Inorg. Chem. Bioinorg. Chem. 38: 477-516, 1990.
10. Bungo M., Fujiwara Y., Kasahara K., Nakagawa K., Ohe Y., Sasaki Y., Irino S., Saijo N. Decreased accumulation as a mechanism of resistance to cis-diamminedichloroplatinum (II) in human non-small cell lung cancer cell lines: relation to DNA damage and repair. Cancer Res. *50*: 2549, 1990.
11. Chow S., Hedley D. and Tannock I. Flow cytometric calibration of intracellular pH measurements in viable cells using mixtures of weak acids and bases. Cytometry *24*: 360-367, 1996.
12. Cleare MJ, Hydes PC, Malerbe BW and Watkins DW. Antitumor platinum complexes: Relationships between chemical properties and activity. Biochimie 60:835, 1978.
13. Clifford S.C., Neal D.E. and Lunec J. Alternations in expression of the multidrug resistance-associated protein (MRP) gene in high-grade transitional cell carcinoma of the bladder. Br. J. Cancer *73:* 659-666, 1996.

14. Daley-Yates P.T. and McBrien D.C.H. Cisplatin metabolites in plasma, a study of their pharmacokinetics and importance in the nephrotoxic and antitumour activity of cisplatin. Biochem. Pharmacol. 33: 3063-3070, 1984.

15. De Graeff A., Slebos R.J.C., Rodenhuis S. Resistance to cisplatin and analogues: mechanisms and potential clinical implications. Cancer Chemother. Pharmacol. *22(4):* 325-32, 1988.

16. Dedon P.C. and Borch R.F. Characterization of the reactions of platinum antitumor agents with biologic and nonbiologic sulfur-containing nucleophiles. Biochem. Pharmacol. 36: 1955-1964, 1987.

17. Dibrov. P and Fligel L. Comparative molecular analysis of Na+/H+ exchangers: a unified model for Na+/H+ antiport? FEBS *424*: 1-5, 1998.

18. Eastman A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells 2: 275-280, 1990.

19. Eastman A., Schulte N. Enhanced DNA repair as a mechanism of resistance to cis-diamminedichloroplatinum (II). Biochemistry *27(13)*: 4730-4, 1988.

20. Eastman A. The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. Pharmacol. Ther. 34: 155-166,1987.

21. Fichtinger-Schepman A.M.J., van der Veer J.L., den Hartog J.H.J., Lohman P.H.M., and Reedijk J. Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formatin, identification, and quantitation. Biochemistry 24: 707-713, 1985.

22. Gale G.R., Morris C.R., Atkins L.M. and Smith A.B. Binding of antitumor platinum compound to cells as influenced by physical factors and pharmacologically active agents. Cancer Res. *33*: 813-818, 1973.

23. Gately D.P. and Howell S.B. Cellular accumulation of the anticancer agent cisplatin: A review. Br. J. Cancer *67*: 1171-1176, 1993.

24. Giaccone G., van Ark-otte J., Rubio G.J., Gazdar A.F., Braxtermann H.J., Fleus M.J., Scheper R.J. and Pinedo H.M. MRP is frequently expressed in human lung-cancer cell lines, in non-small-cell lung cancer and in normal lungs. Int. J. Cancer *66:* 760-767, 1996.

25. Gilles R.J., Martinez-Zaguilan R., Martinez G.M., Serrano R. and Perona R. Tumorigenic 3T3 cells maintain an alkaline intracellular pH under physiological conditions. Proc. Natl. Acad. Sci. USA *87*: 7414-7418, 1990.

26. Grant C.E., Valdimarsson G., Hipfner D.R., Almquist K.C., Cole S.P. and Deeley R.G. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. Cancer Res. *54:* 357-361, 1994.

27. Hayes J.J. and Scovell W.M. cis-Diammindichloroplatinum (II) modified chromatin and nucleosomal core particle probed with DNase I. Biochim. Biohys. Acta 1088: 413-418, 1991.

28. Hayes J.J. and Scovell W.M. cis-Diammindichloroplatinum (II) modified chromatin and nucleosomal core particle probed with DNase I. Biochim. Biohys. Acta 1089: 377-385, 1991.

29. Hedley D.W. and Boyer M.J. Measurement of intracellular pH. Methods in Cell. Bio. *41*: 135-148, 1994.

30. Hromas R.A., North J.A. and Burns C.P. Decreased cisplatin uptake by resistant L1210 leukemia cells. Cancer Letters *36*: 197-201, 1987.

31. Ishikawa T., Bao J.J., Curley S.A., Ikeguchi M., Johnston D.A and Kuo M.T. Frequent coordinated overexpression of the MRP/GS-X pump and gamma-glutamylcysteine synthetase genes in human colorectal cancers. Cancer Res. *56(16):* 3642-4, 1996 Aug.

32. Ishikawa T., Bao J.J., Yamane Y., Akimaru K., Frindrich K., Wright C.D. and Kuo M.T. Coordinated induction of MRP/GS-X pump and gamma-glutamylcysteine synthetase by heavy metals in human leukemia cells. J. Biol. Chem. *271(25):* 14981-8, 1996 Jun.

33. Ishikawa T. The ATP-dependent glutathione S-conjugate export pump. Trend Biochem Sci. *17:* 463-468, 1992.

34. Ishikawa T. and Ali-Osman F. Glutathione-associated cis-diamminedichloroplatinum (II) metabolism and ATP-dependent efflux from leukemia cells. J. of Biol. Chem. *268:* 20116-20125, 1993.

35. Ishikawa T., Wright C.D. and Ishizuka H. GS-X pump is functionally overexpressed in cis-diamminedichloroplatinum (II)-resistant human leukemia HL-60 cells and down-regulated by cell differentiation. J. Biol. Chem. *269*: 29085-29093, 1994.

36. Isonishi S., Andrews P.A. and Howell S.B. Increased sensitivity to cis-diamminedichloroplatinum (II) in human ovarian carinoma cells in response to treatment with 12-O-tetradecanoylphorbol 13-acetate. J. Biol. Chem. *265*: 3623-3627, 1990.

37. Jain N., Lam Y.M., Pym J. and Campling B.G. Mechanisms of resistance of human small cell lung cancer lines selected in VP-16 and cisplatin. Cancer (Phila.) *77:* 1797-1808, 1996.

38. Jekunen A.J., Vick J., Sanga R., Chan T.C.K. and Howell S.B. Synergism between dipyridamole and cisplatin in human ovarian carcinoma cells in vitro. Cancer Res. *52*: 3566-3571, 1992.

39. Jennerwein M. and Andrews PA. Effect of intracellular chloride on the cellular pharmacodynamics of cis-diamminedichloroplatinum(II). Drug Metab Dispos *23*:178-184, 1995.

40. Jones J.C., Zhen W., Reed E., Parker R.J., Sancar A. and Bohr V.A. Genespecific formation and repair of cisplatin intrastrand adducts and interstrand cross-links in Chinese hamster ovary cells. J. Biol. Chem. 266: 7010-7107, 1991.

41. Katzung, B.G. In: Katzung, B.G. (ed) Basic & Clinical Pharmacology. Appleton & Lange, Norwalk, Connecticut, p.5 (1995).

42. Kawai K., Kamatani N., Georges E. and Ling V. Identification of a membrane glycoprotein over-expressed in murine lymphoma sublines resistant to cis-diamminedichloroplatinum (II). J. Biol. Chem. *265*: 13137-13142, 1990.

43. Kikuchi Y., Iwano I., Miyauchi M., Sasa H., Nagata I. and Kuki E. Restorative effects of calmodulin antagonists on reduced cisplatin uptake by cisplatin-resistant human ovarian carcinoma cells. Gynecol. Oncol. *39*: 199-203, 1990.

44. Lim M.C. and Martin R.B. Coordination of uridine and adenosine to PD(II) and PT(II) Complexes. J. Inorganic and Nuclear Chemistry *38 (10)*: 1915-1919, 1976.

45. Loh S.Y., Mistry P., Kelland L.R., Abel G. and Harrap R.P. Reduced drug accumulation as a major mechanism of acquired resistance to cisplatin in a human ovarian carcinoma cell line: Circumvention studies using novel platinum (II) and (IV) ammine/amine complexes. Br. J. Cancer 66: 1109-1115, 1992.

46. Mann S.C., Andrews P.A. and Howell S.B. Modulation of cis-diamminedichloroplatinum (II) accumulation and sensitivity by forskolin and 3-isobutyl-1-methylxanthine in sensitive and resistant human ovarian carcinoma cells. Int. J. Cancer *48*: 866-872, 1991.

47. Mann S.C., Andrews P.A. and Howell S.B. Short term cis-diamminedichloroplatinum (II) accumulation in sensitive and resistant human ovarian carcinoma cells. Cancer Chemother. Pharmacol *25*: 236-240, 1990.

48. Mann S.C., Andrews P.A. and Howell S.B. Comparison of lipid content, surface membrane fluidity, and temperature dependence of cis-diamminedicloroplatinum (II) accumulation in sensitive and resistant human ovarian carcinoma cells. Anticancer Res. 8: 1211-1215, 1988.

49. Martin R.B. Hydrolytic equilibria and N7 versus N1 binding in purine nucleosides of cis-diamminedichloroplatinum (II): palladium (II) as a guide to platinum (II) reactions at equilibrium. In platinum, Gold, and Other Metal Chemotherapeutic Agents, edited by S.J. Lippard American Chemical Society: Washington DC, pp. 231-244, 1983.

50. Martin Reishus JW and Marin DS. Cis-dichlorodiammineplatinum (II): Acid hydrolysis and isotopic exchange of the chloride ligands. JACS. 83: 2457, 1961.

51. Miller S.E. and House D.A. The hydrolysis products of cis-diamminedichloroplatinum (II) 5. The anation kinetics of cis-Pt(X)(NH3)2(OH2)+ (X=Cl, OH) with glycine, monohydrogen malonate and chloride. Inorg. Chim. Acta 187: 125-132, 1991.

52. Melvik J.E., Domish J.M., and Pettersen E.O. The binding of cis-diamminedichloroplatinum (II) to extracellular and intracellular compounds in relation to drug uptake and cytotoxicity in vitro. Br. J. Cancer *66:* 260-265, 1992.

53. Miller S.E. and House D.A. The hydrolysis products of cis-dichlorodiammineplatinum (II) 3. Hydrolysis kinetics at physiological pH. Inorganica Chimica Acta *173*: 53-60, 1990.

54. Mistry P., Kelland L.R., Loh S.Y., Abel G., Murrer B.A. and Harrap K.R. Comparison of cellular accumulation and cytotoxicity of cisplatin with that of tetraplatin and amminedibutyratodichloro(cyclohexylamine)-platinum(IV) (JM221) in human ovarian carcinoma cell lines. Cancer Res. *52*: 6188, 1992.

55. Muller M., de Vries E.G. and Jansen P.L. Role of multidrug resistance protein (MRP) in glutathione S-conjugate transport in mammalian cells. J. Hepatology *24 Suppl 1:* 100-108, 1996.

56. Nicholls D.G. and Ferguson S.J. Ion Transport Across Energy-Conserving Membranes. In Bioenergetics 2, Chapter 2, P.23-37. Academic Press, London, (1992).

57. Ober S.S. and Pardee A.B. Intracellular pH is increased after transformation of Chinese hamster embryo fibroblasts. Proc. Natl. Acad. Sci. USA *84*: 2466-2770, 1987.

58. Oguri T., Fujiwara Y., Isobe T., Katoh O., Watanabe, H. and Yamakido M. Expression of γ-glutamylcysteine synthetase (γ-GCS) and multidrug resistance-associated protein (MRP), but not human canalicular multispecific organic anion transporter (sMOAT), genes correlates with exposure of human lung cancers to platinum drugs. Br. J. Cancer. *77 (7)*: 1089-1096, 1998.

59. Parker R.J., Eastman A., Bostik-Bruton F. and Reed E. Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. J. Clin. Invest. *87(3):* 772-7, 1991.

60. Richon V.M., Schulte N. and Eastman A. Multiple mechanisms of resistance to cis-diamminedichloroplatinum (II) in murine leukemia L1210 cells. Cancer Res. *47(8)*: 2056-61, 1987.

61. Rosenberg B., Van Camp L. and Krigas T. Nature (London) 205: 698, 1965.

62. Rosenberg B., Van Camp L., Grimley E.B. and Thomson A.J. The inhibition of growth or cell division in Escherichia coli by different ionic species of platinum complexes. J. Biol. Chem. 242(6):1347-52, 1967 Mar 25.

63. Rosenberg B., Van Camp L., Trosko J.E. and Mansour H.V. Platinum compounds: a new class of potent antitumour agents. Nature. 222(191):385-6, 1969 Apr 26.

64. Rosenberg B: Cisplatin, Current Status and New Developments (Eds. A.W. Prestayko, S.T. Crooke, and S.K. Carter), p.9. Academic Press, London (1980).

65. Rosenberg B. Fundamental studies with cisplatin. Cancer 55: 2303-2316, 1985.

66. Sardet, C., Franchi, A., and Pouyssegur, J. Molecular cloning, primary structure expression in the human growth factor-activatable Na+/H+ antiporter. Cell, *56*: 271-280, 1989.

67. Schmidt W. and Chaney S.G. Role of carrier ligand in platinum resistance of human carcinoma ell lines. Cancer Res. 53: 799-805, 1993.

68. Scovell W.M., Muirhead N., and Kroos L.R. Cis-diamminedichloroplatinum(II) selectively cross-links high mobility group proteins 1 and 2 to DNA in micrococcal nucleae accessible regions of chromatin. Biochem. Biophys. Res. Commun. 142: 826-835, 1987.

69. Seeber S., Osieka R., Schmidt C., Acherrath W. and Crook S. In vivo resistance towards anthracyclines, etoposide, and cis-diamminedichloroplatinum. Cancer Res. *42*: 4719-4725, 1982.

70. Sherman S.E., Gibson D., Wang A.H.J., and Lippard S.J. X-ray structure of the major adduct of the anticancer drug cisplatin with DNA: cis-. Science. *230 (4724):* 412-7, 1985 Oct.

71. Smith P.K., Krohn R.I., Hermanson G.T., Mallia A.K., Gartner F.H., Provenzano M.D., Fujimoto E.K., Goeke N.M., Olson B.J. and Klenk D.C. Measurement of protein using bicinchoninic acid Anal. Biochem. *150(1)*: 76-85, 1985 Oct.

72. [3]Stewart D.J., Grewaal D., Raaphorst G.P., Montpetit V.A.J., Goel R. Effects of metabolic inhibitors on cisplatin net uptake into and efflux from the HTB56 human lung adenocarcinoma cell line and its E-8/0.7 cisplatin-resistant variant. Submitted.

73. [4]Stewart DJ, Grewaal D, Popovic P, Mohamed Ali, Kates M, Raaphorst GP, Cybulski S, Dong P, Goel R.: Characteristics of the HTB56 human lung adenocarcinoma cell line and its E-8/0.7 cisplatin-resistant variant. Submitted.

74. Stewart D.J., Dulberg C., Molepo J.M., Mikhael N.Z., Montpetit V.A.J., Redmond M.D., Goel R. Factors affecting human autopsy kidney cortex and kidney medulla platinum concentrations after cisplatin. Cancer Chemother. Pharmacol. *34*: 14-22, 1994.

75. Taniguchi K., Wada M., Kohno K., Nakamura T., Kawabe T., Kawakami M., Kagotani K., Okumura K., Akiyama S. and Kuwano M. A human canalicular multispecific organic anion transporter (sMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. Cancer Res. *56*: 4124-4129, 1996.

76. Teicher B., Holden S., Kelley M., Shea T., Cucchi C., Rosowsky A., Henner W.D., Frei III E. Characterization of a human squamous carcinoma cell line resistant to cis-diamminedichloroplatinum II. Cancer Res. *47*: 388-393, 1987.

77. Waud W.R. Differential uptake of cis-diamminedicholorplatinum (II) by sensitive and resistant murine L1210 leukemia cells. Cancer Res. 47: 6549-6555, 1987.

78. Wakabayshi S., Shigekawa M. and Pouyssegur J. Molecular physiology of vertebrate Na+/H+ exchangers. Physiol. Reviews. *77*: 51-74, 1997.

79. Wolpert-DeFilippes M.K.: Cisplatin, Current Status and New Developments (Eds. A.W. Prestayko, S.T. Crooke, and S.K. Carter), p.183. Academic Press, London (1980).

Table 1 summarizes differences between the HTB56 cell line and its E-8/0.7 cisplatin-resistant variant based on both previous results and the author's own data. All studies were done in exponentially growing cells.

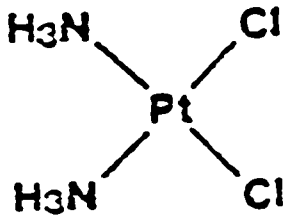
Characteristics	HTB56 (sensitive)	E-8/0.7 (resistant)
Plating Efficiency	11% Higher	Lower
Cell Doubling Time at Log Phase	18 hours	29 hours
Max. Cell Density at Plateau Phase(cells/ml)	app. 3.2 millions	app. 1.3millions
Intracellular pH	7.513±0.055	7.647±0.062
Cytotoxicity by Cisplatin (IC_{50})	11.7 μM	53.3 μM
Glucose Utilization [a]	Lower	Higher
Lactate Production [a]	Lower	Higher

[a] Stewart et al., unpublished data[4].
[b] Each value is the mean ± SD of three separate experiments, performed in triplicate.

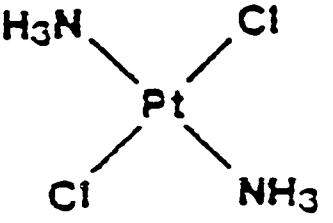
Table 2 compares rate constants (s^{-1}) of two efflux phases in and between cell lines found in curves from Figures 7 and 8.

	K_{S1}	K_{R1}	K_{S2}	K_{R2}
Early Efflux of Total Cellular Cisplatin from Figure 7	0.140	0.170	0.004	0.005
Early Disappearance of Ultrafilterable Intracellular Cisplatin from Figure 8	0.210	0.290	0.020	0.002

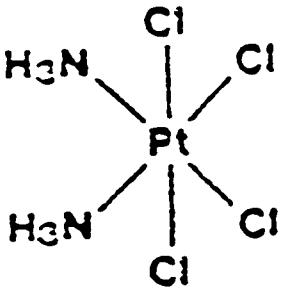
Where K_{S1} and K_{R1} are efflux rate constants of slopes of cisplatin concentration from 0 to 10s during the efflux phase in sensitive and resistant cells respectively, and K_{S2} and K_{R2} are efflux rate constants of slopes of cisplatin concentration from 10 to 50s during the efflux phase in sensitive and resistant cells respectively.



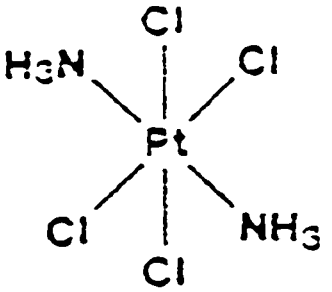
cis-$PtCl_2(NH_3)_2$



trans-$PtCl_2(NH_3)_2$



cis-$PtCl_4(NH_3)_2$



trans-$PtCl_4(NH_3)_2$

Figure 1. Structures of original platinum complexes studied for antibacterial and anti-tumor activity.

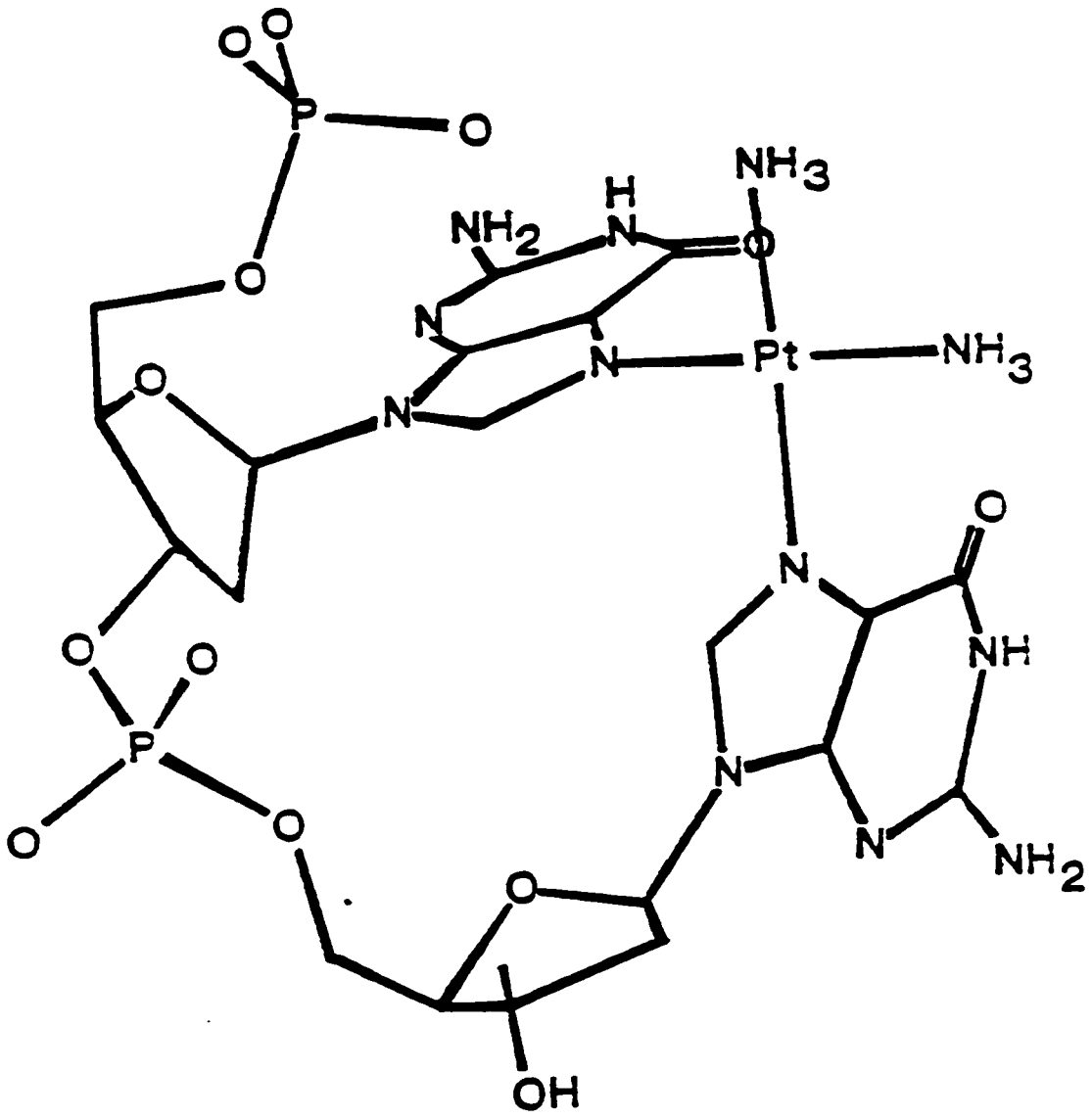


Figure 2. Schematic representation of the structure of the adduct of cis-$[Pt(NH_3)_2Cl_2]$ with the dinucleotide d(pGpG) (Sherman SE et al., 1985).

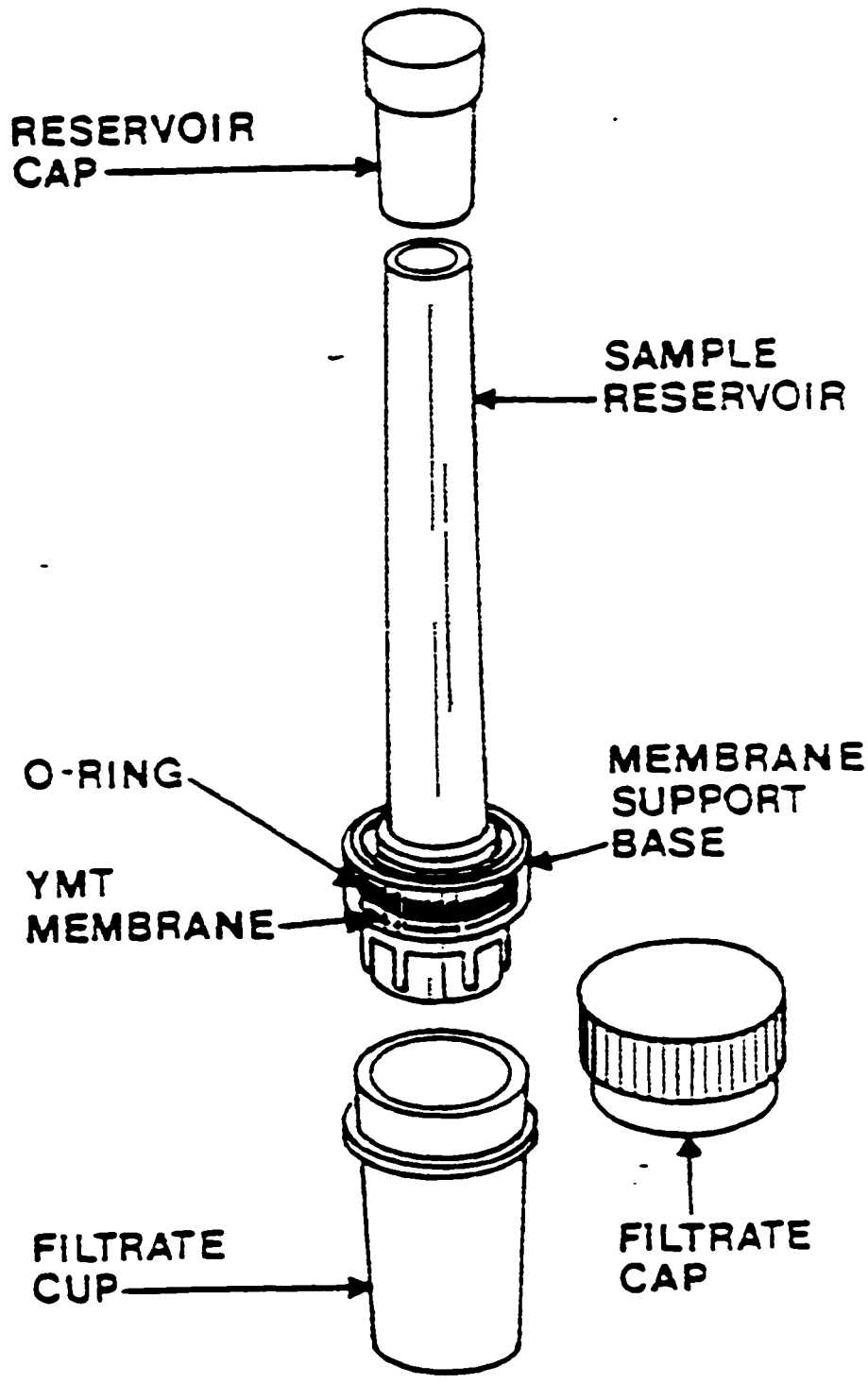


Figure 3. Component parts of the Amicon Micropartition System for the separation of free from protein-bound cisplatin.

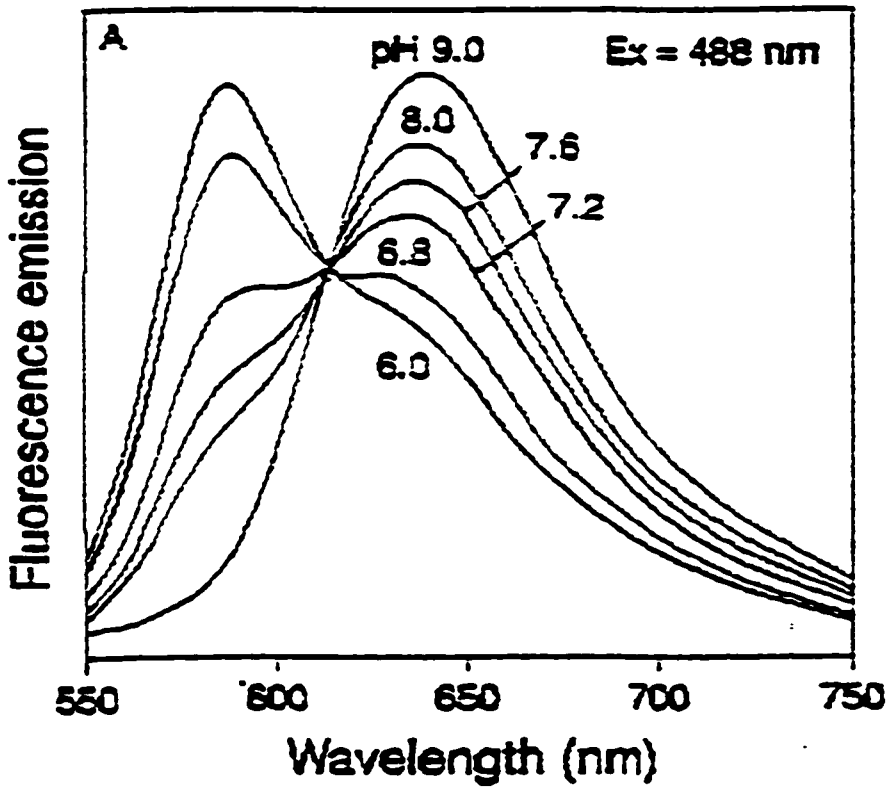


Figure 4. Emission spectra of carboxy SNARF-1 in 50mM potassium phosphate buffers at various pH values. Samples were excited at 488nm.

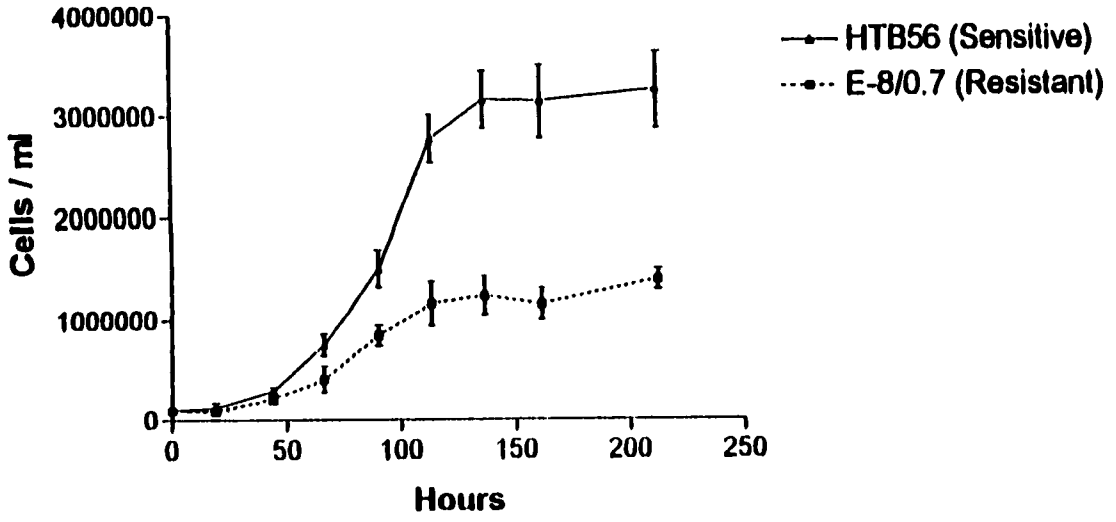


Figure 5. Growth rate determination by plotting cell number per ml versus time. Points are mean values from 3 independent experiments, performed in triplicate; bars are SD.

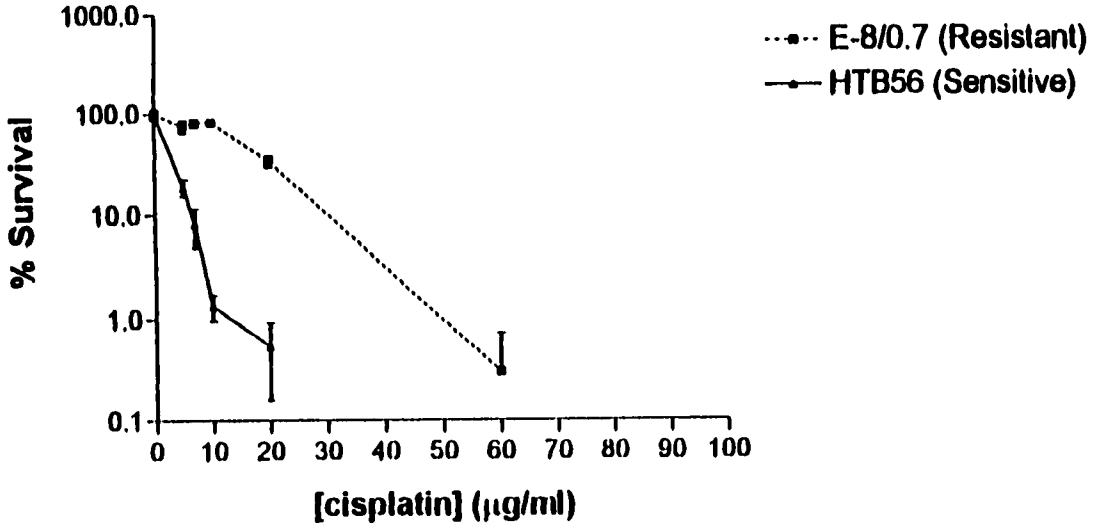


Figure 6. Sensitivity of HTB56 (Sensitive) cells and E-8/0.7 (Resistant) cells to cisplatin. Cells were exposed to appropriate concentrations of drug for 1 hour at 37°C and cell survival was measured in terms of number of colonies formed on Day 9. Points are mean values from 3 independent experiments, performed in triplicate; bars are SD.

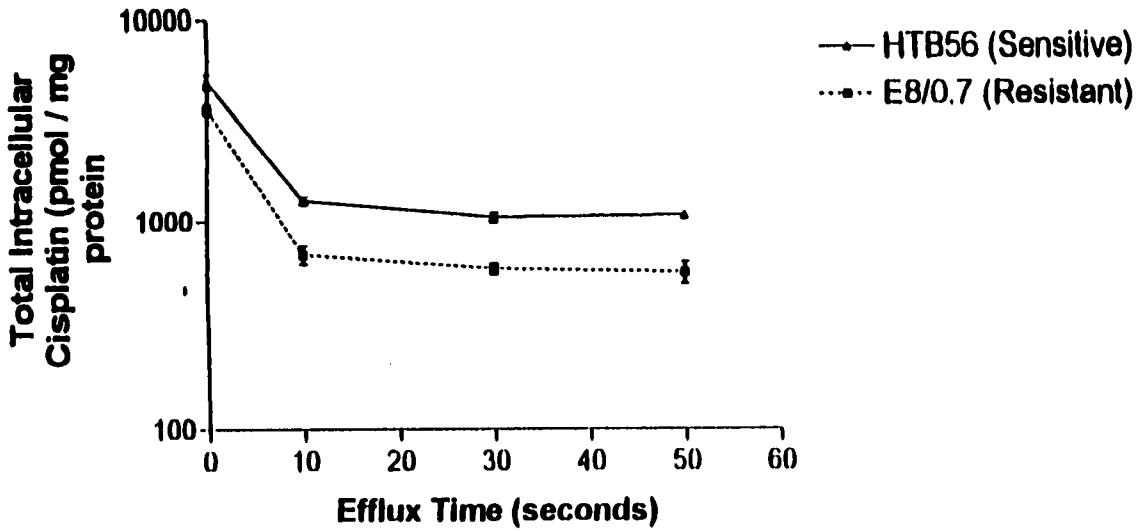


Figure 7. Early efflux of total cellular cisplatin from the HTB56 human lung adenocarcinoma cell line and its cisplatin resistant variant, E-8/0.7. Data are means ± SEM of three independent experiments, performed in triplicate. Legend symbols are bigger than error bars in the sensitive cell line.

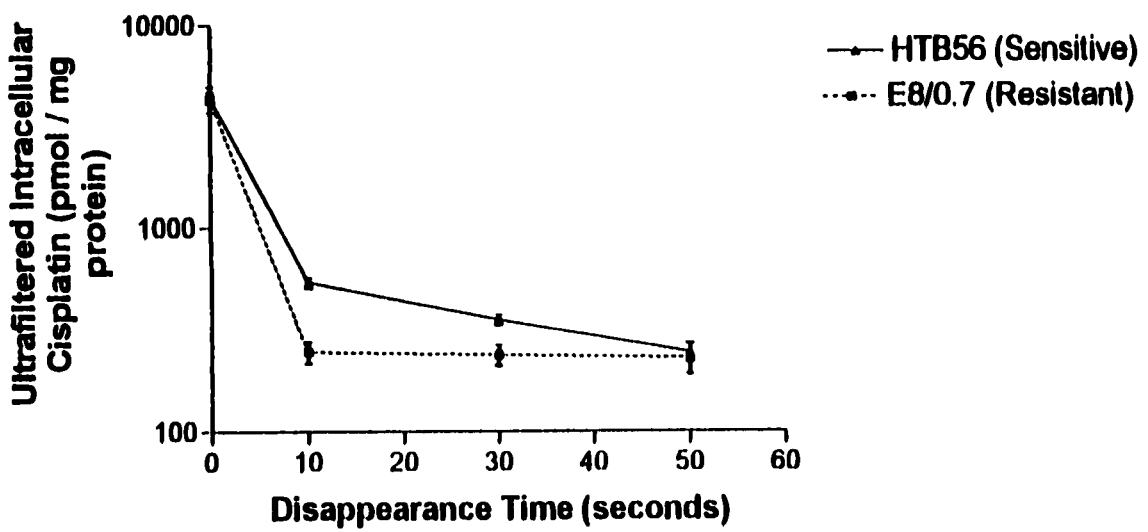


Figure 8. Early disappearance of ultrafilterable intracellular cisplatin from the HTB56 human lung adenocarcinoma cell line and its cisplatin resistant variant, E-8/0.7. Data are means ± SEM of three independent experiments, performed in triplicate.

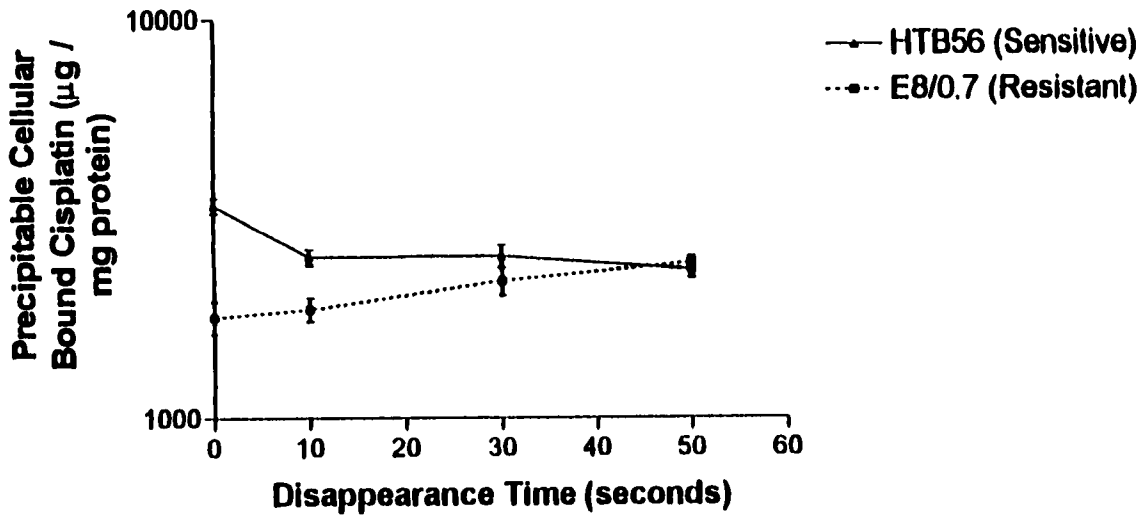


Figure 9. The change in precipitable cellular bound cisplatin during early disappearance for the HTB56 human lung adenocarcinoma cell line and its cisplatin resistant variant, E-8/0.7. Data are means ± SEM of three independent experiments, performed in triplicate.

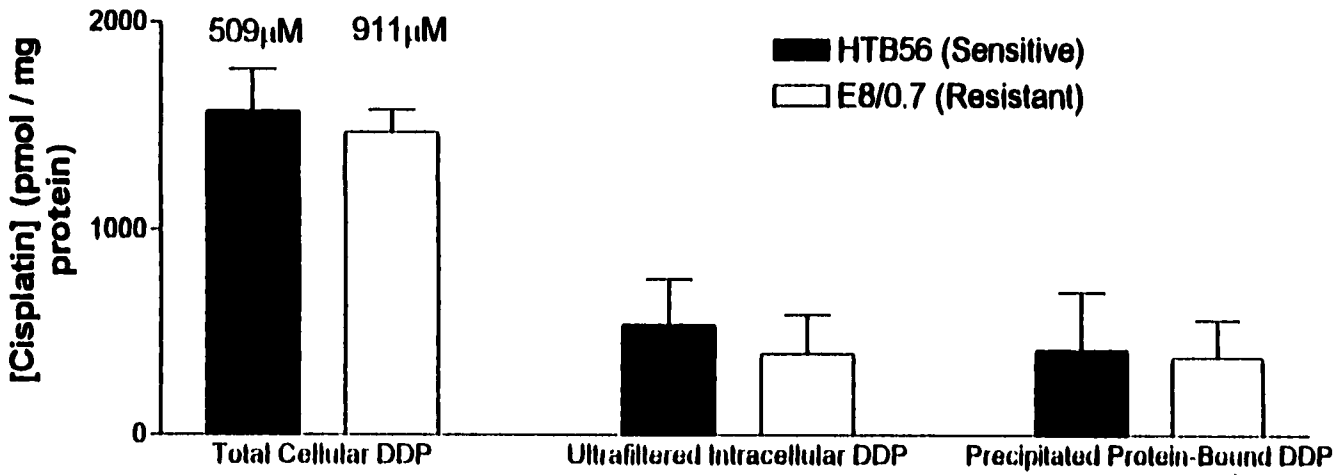


Figure 10. Equal loading experiments, in which HTB56 and E-8/0.7 cell lines were treated with different cisplatin doses (509 vs 911μM) designed to give comparable platinum concentrations in the 2 cell lines. Ultrafilterable intracellular cisplatin (DDP) and precipitable protein-bound cisplatin were compared after the total cellular cisplatin had been set to be similar between the two cell lines. Bars are SD from three independent experiments, performed in triplicate.

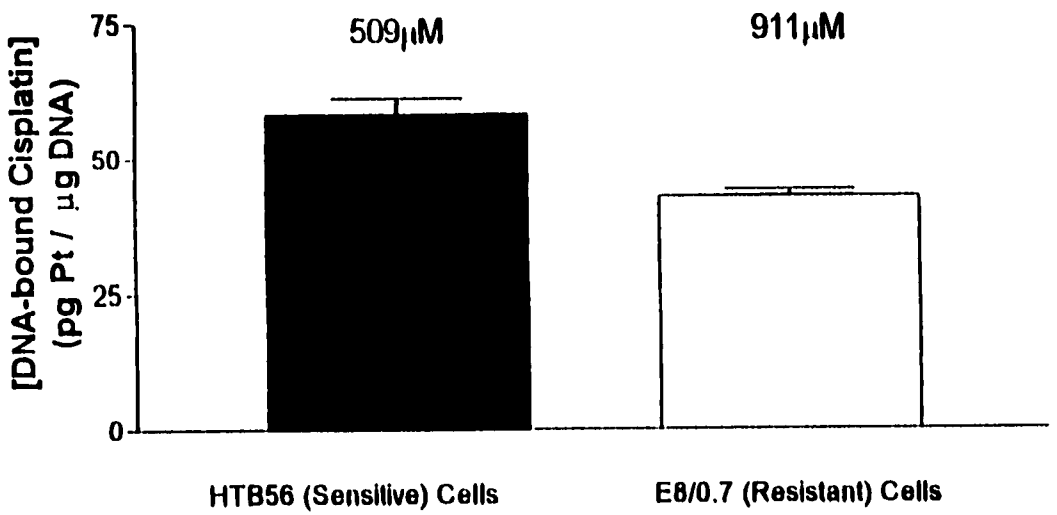


Figure 11. The DNA-bound platinum was compared in the equal loading experiments in which the total cellular cisplatin had been set to be similar between the two cell lines by exposing sensitive cells to cisplatin 509 μM and resistant cells to cisplatin 911μM. Bars are SEM from three independent experiments, performed in triplicate.

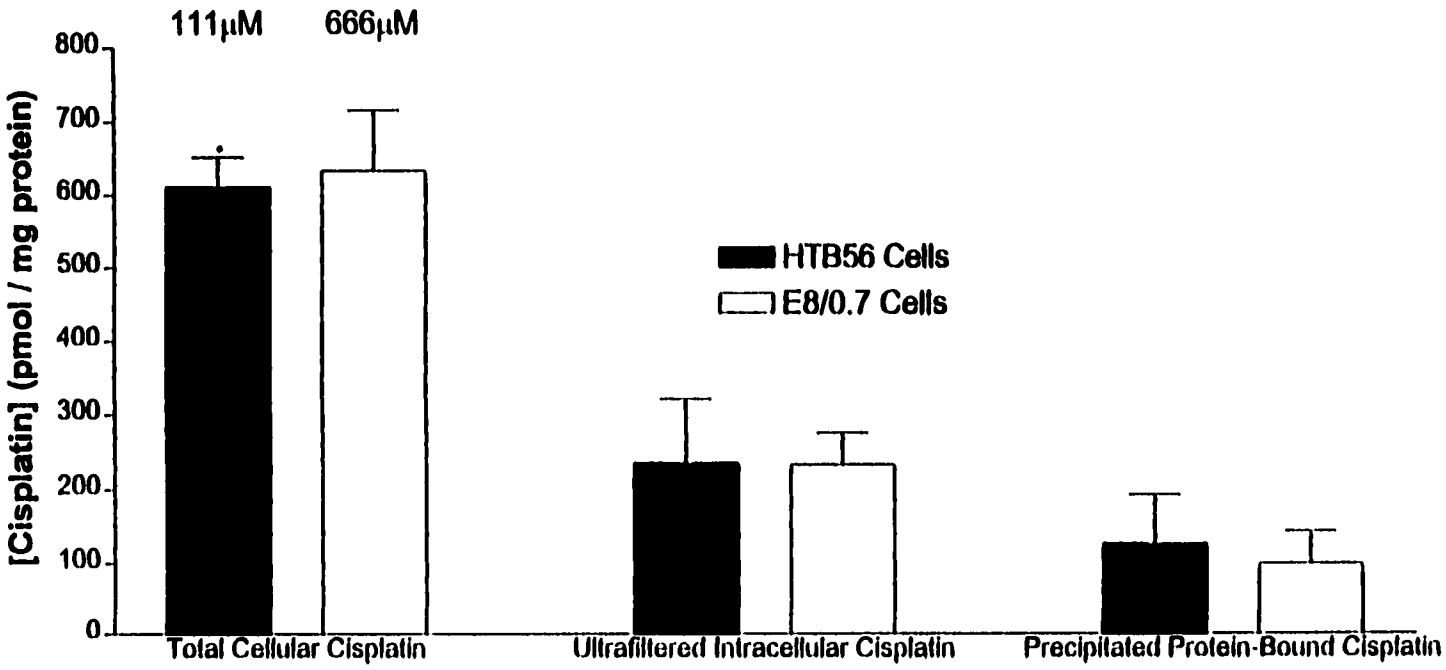


Figure 12. Equal loading experiments, in which HTB56 and E-8/0.7 cell lines were treated with different cisplatin doses (111 vs 666 μM) designed to give comparable platinum concentrations in the 2 cell lines. Ultrafilterable intracellular cisplatin (DDP) and precipitable protein-bound cisplatin were compared after the total cellular cisplatin had been set to be similar between the two cell lines. Bars are SD from three independent experiments, performed in triplicate.

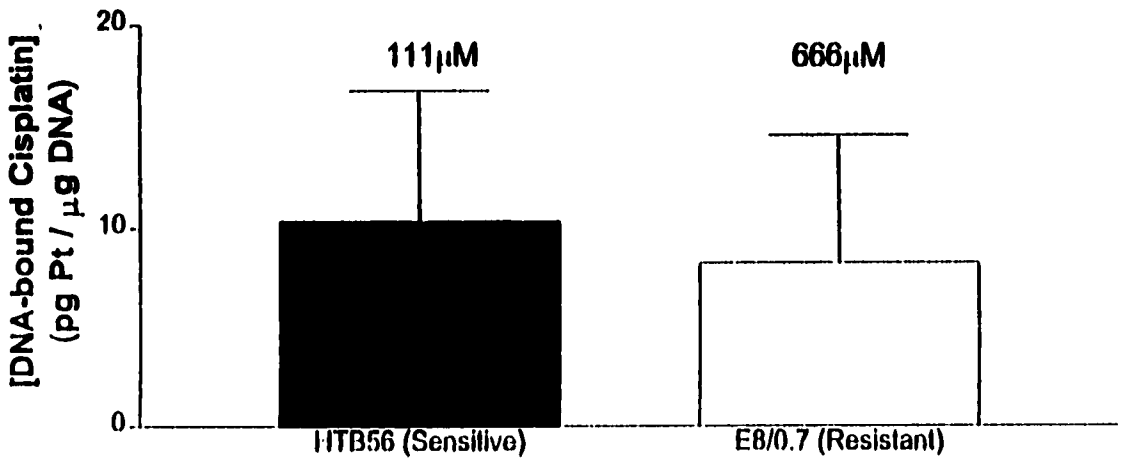


Figure 13. The DNA-bound platinum was compared in the equal loading experiments in which the total cellular cisplatin had been set to be similar between the two cell lines by exposing sensitive cells to cisplatin 111 µM and resistant cells to cisplatin 666 µM. Bars are SD from three independent experiments, performed in triplicate.

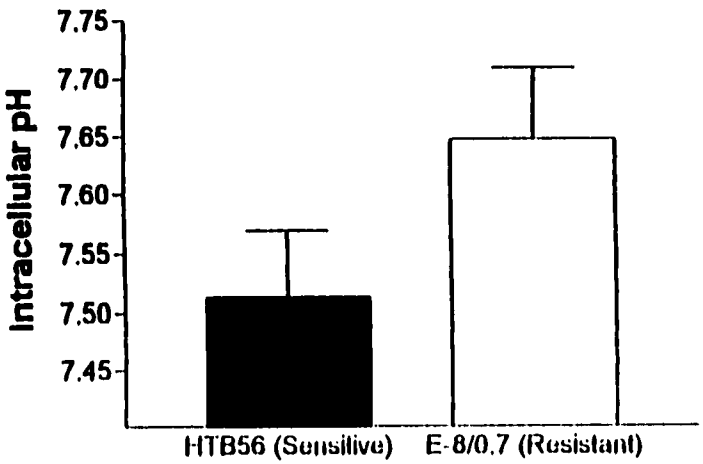


Figure 14. Intracellular pH studies in HTB56 and E-8/0.7 human lung cancer cell lines using flow cytometry and carboxy-SNARF-1. Bars are SD from at least three independent experiments, performed in triplicate.

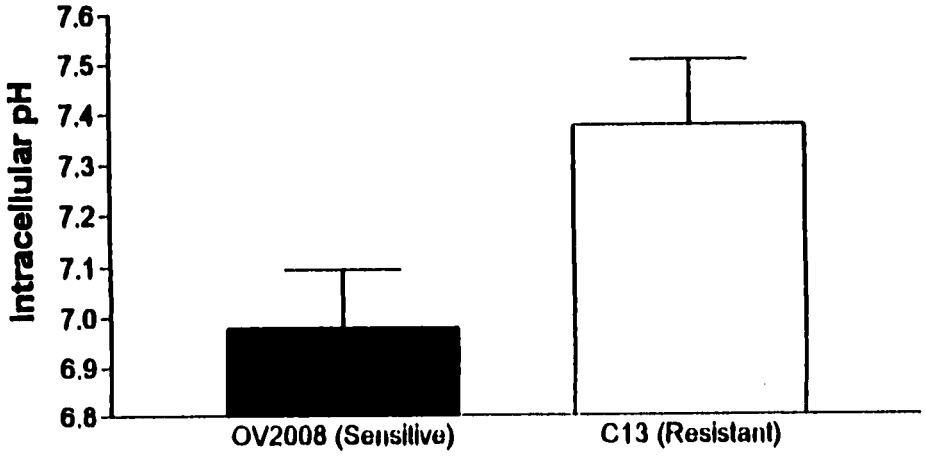


Figure 15. Intracellular pH studies in OV2008 and C13 human ovarian cancer cell lines using flow cytometry and carboxy-SNARF-1. Bars are SD from at least three independent experiments, performed in triplicate.

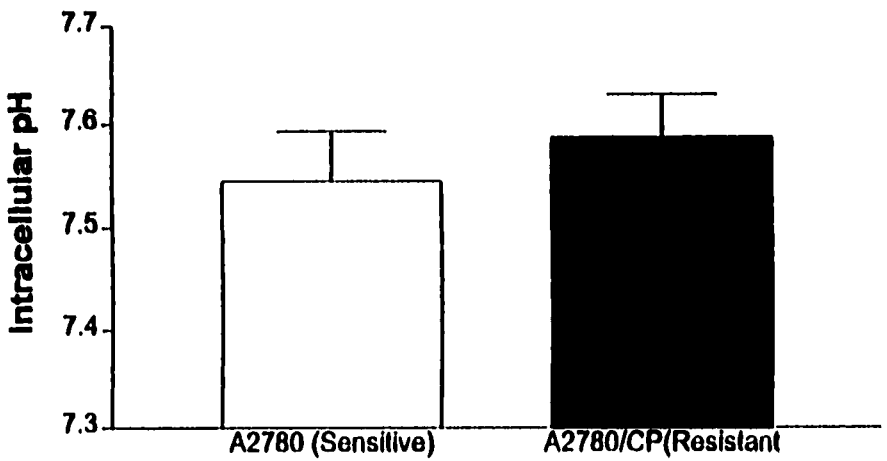


Figure 16. Intracellular pH studies in A2780 and A2780/CP human ovarian cancer cell lines using flow cytometry and carboxy-SNARF-1. Bars are SD from at least three independent experiments, performed in triplicate.

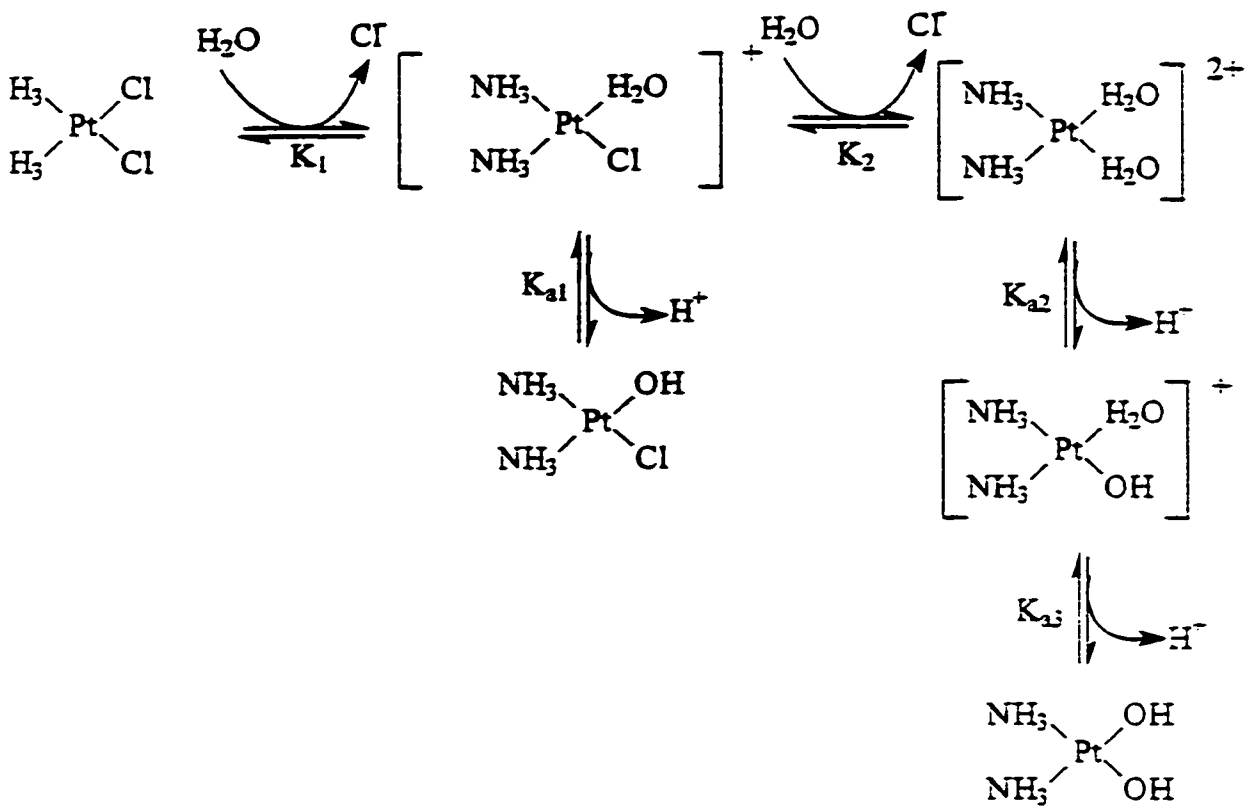


Figure 17. Structures and equilibria of cisplatin and the species found in aqueous solution (adapted from Jennerwein M. and Andrews P.A., 1995)