Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells

Roohangiz Safaei, Barrett J. Larson, Timothy C. Cheng, Michael A. Gibson, Shinji Otani, Wiltrud Naerdemann, and Stephen B. Howell

Rebecca and John Moore University of California at San Diego Cancer Center, University of California at San Diego, La Jolla, California

Abstract
Previous work has shown that cisplatin (CDDP) becomes concentrated in lysosomes, and that acquired resistance to CDDP is associated with abnormalities of protein trafficking and secretion. The lysosomal compartment in CDDP-sensitive 2008 human ovarian carcinoma cells was compared with that in CDDP-resistant 2008/C13*5.25 subline using deconvoluting imaging and specific dyes and antibodies. The lysosomal compartment in CDDP-resistant cells was reduced to just 40% of that in the parental CDDP-sensitive cells (P < 0.002). This was accompanied by a reduced expression of the lysosome-associated proteins 1 and 2 (LAMP1 and LAMP2) as determined by both microscopy and Western blot analysis. The CDDP-resistant cells released more protein as exosomes and Western blot analysis revealed that these exosomes contained substantially more LAMP1 than those released by the CDDP-sensitive cells. Following loading of the whole cell with CDDP, the exosomes released from 2008/C13*5.25 cells contained 2.6-fold more platinum than those released from sensitive cells. Enhanced exosomal export was accompanied by higher exosomal levels of the putative CDDP export transporters MRP2, ATP7A, and ATP7B. Expression profiling identified significant increases in the expression of several genes whose products function in membrane fusion and vesicle trafficking. This study shows that the lysosomal compartment of human ovarian carcinoma cells selected for stable resistance to CDDP is markedly reduced in size, and that these cells abnormally sort some lysosomal proteins and the putative CDDP transporters into an exosomal pathway that also exports CDDP. [Mol Cancer Ther 2005; 4(10):1595–604]

Introduction
Repeated exposure of tumor cells to cisplatin (CDDP) results in rapid development of resistance, which eventually contributes to therapeutic failure (1). Resistance to CDDP is often accompanied by resistance to metalloids and is associated with alterations in DNA repair (2), thiol content (3), and drug accumulation (4). Recent studies have linked the expression of several transporters, particularly the copper export proteins ATP7A and ATP7B, with CDDP resistance and suggest that they play a direct role in the efflux of CDDP (5).

In tumor cells, CDDP accumulates in a variety of vesicular structures, including lysosomes (6). Studies of the cellular pharmacology of CDDP in cells overexpressing ATP7A and ATP7B indicate that CDDP is sequestered into vesicles belonging to the secretory pathway as well as lysosomes (7, 8). Microscopic analyses with a fluorescein-conjugated CDDP have confirmed that CDDP accumulates in vesicles that contain ATP7A or ATP7B (9, 10). Additional confocal microscopic analysis of fluorescein-conjugated CDDP in cells treated with drugs that inhibit trafficking pathways suggests that the accumulation of CDDP in the secretory compartment may require lysosomal function (10).

Lysosomes are an essential part of the vesicular compartment and connect the outside medium with many classes of cellular targets in the cytosol, nucleus, mitochondria, endoplasmic reticulum, and Golgi (reviewed in ref. 11). In particular, lysosomes seem to play an important role in the detoxification of heavy metals. Studies in insect cells have shown lysosomal storage of metal conjugates (12), and some heavy metals can cause destabilization and rupture of lysosomal membranes resulting in cell death (13). Lysosomal abnormalities are often found in drug-resistant cells; such alterations include aberrant morphology and secretory phenotypes (14, 15). CDDP-resistant cells release unusually large amounts of lysosomal enzymes and membrane proteins into their environment (15, 16). Aberrant function and expression of the lysosomal H+-pump has also been noted in such CDDP-resistant cells (17).

Although a systematic survey of lysosomal function in CDDP-resistant cells is lacking, several lines of evidence indicate that the development of CDDP resistance is associated with mis-sorting of lysosomal proteins. Lysosomes...
and plasma membrane proteins involved in CDDP efflux originate from trans-Golgi network and are often routed to multivesicular bodies that also receive endocytosed plasma membrane proteins and receptors. The positioning of a protein in either the internal or external membrane of the multivesicular bodies determines whether it recycles back to the plasma membrane, is destroyed in lysosomes, or is secreted to the extracellular environment via 50- to 200-nm vesicles known as exosomes (reviewed in ref. 18). A link between abnormal sorting of proteins at multivesicular bodies and tumorigenesis has been established in transgenic mice (reviewed by ref. 19), and several studies have shown that the protein composition of exosomes from tumor cells is different from that of nonmalignant cells (18, 20). Mislocalization of lysosomal and plasma membrane proteins has been noted in CDDP-resistant cells (17, 21). In addition, disruption of lysosomes with bafilomycin A1 (17, 22) and chloroquine (23) has been reported (17, 21). In addition, disruption of lysosomes with bafilomycin A1 (17, 22) and chloroquine (23) has been reported (17, 21).

We report here on further studies of the lysosomal compartment in human ovarian carcinoma cells that were selected for stable acquired resistance. This work shows that the lysosomal compartment of such cells is markedly reduced in size and that these cells release protein as exosomes in larger amounts and export CDDP via this route at increased levels. The resistant cells also abnormally sort some lysosomal proteins, including putative CDDP transporters, into the exosomal pathway that also exports CDDP.

**Materials and Methods**

**Reagents**

Cell culture media and sera were purchased from Invitrogen (Carlsbad, CA). Antibodies to lysosome-associated proteins 1 and 2 (LAMP1 and LAMP2), β-actin, and tubulin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and those for MRP2 were from Alexis Biochemicals (San Diego, CA). The specific dyes Alexa Flour 647 phalloidin, Hoechst 33342, and Lysotracker Red were purchased from Molecular Probes (Eugene, OR). Fluorescein-conjugated secondary antibodies against mouse, rabbit, and goat immunoglobulin were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Other chemicals were purchased from Sigma Co. (St. Louis, MO) and Fisher Scientific Co. (Tustin, CA). CDDP (PLATINOL-AQ) was a gift of Bristol Laboratories (Princeton, NJ).

**Cell Culture and Histochemistry**

Ovarian carcinoma 2008 cells and the 2008/C13*5.25 subline selected for stable acquired CDDP resistance were used for all studies (28). Cells were cultured in RPMI with 10% fetal bovine serum in humidified air with 5% CO2. Histochemistry was done on cells 1 day after seeding them on coverslips. Labeling with Lysotracker Red was done in Opti-MEM containing 1 μg of the dye/mL at 37°C for 30 minutes. The cells were rinsed quickly thrice with PBS and fixed by treatment for 15 minutes with 3.7% formaldehyde in PBS, permeabilized with 0.3% Triton X-100 in PBS for 15 minutes, washed thrice for 15 minutes each with PBS, and then stained with 1 μg/mL Hoechst 33342 and 0.4 μg/mL Alexa Flour 647 phalloidin, both dissolved in PBS, for 45 minutes and then mounted on glass slides or processed for immunostaining. Immunostaining was done after a 1-hour incubation of fixed and permeabilized samples with 0.3% bovine serum albumin. Primary antibodies were diluted in PBS containing 0.3% bovine serum albumin and then overlaid on cells for 1 hour. Cells were then washed thrice for 15 minutes, each with PBS, and incubated for 1 hour with secondary antibodies in PBS and 0.3% bovine serum albumin followed by three washes in PBS for 15 minutes each and mounting on glass slides. Mounting medium contained 24 g polyvinyl alcohol, 60 g glycerol, 60 mL H2O, and 120 mL of 0.2 mol/L Tris-HCl (pH 8.5).

Deconvoluting microscopy was done at the University of California at San Diego Cancer Center Digital Imaging Shared Resource using a DeltaVision deconvoluting microscope system (Applied Precision, Inc., Issaquah, WA.). Images were captured from 0.2-μm sections by ×100, ×60, and ×40 lenses; SoftWorx software (Applied Precision) was used for deconvoluting data. Image quantification was done with Data Inspector program in SoftWorx or by NearCount software.

**Exosome Harvest**

Exosomes were isolated from 2008 and 2008/C13*5.25 cells according to the method described by Escola et al. (29) with minor modifications. Cells were cultured in 100-mm dishes until 80% confluent. They were then rinsed with serum-free RPMI 1640 to remove proteins and vesicular structures already released from the cells or present in the fetal bovine serum. In parallel, one set of cells received fresh serum-free RPMI 1640 alone, whereas another set was incubated with RPMI 1640 plus 2 μmol/L CDDP for 1 hour in a 37°C humidified tissue culture incubator. The supernatants were then removed and the plates rinsed thrice with warm RPMI 1640; and then 12 mL of fresh serum-free RPMI 1640 was layered over cells in each plate; and cells were incubated for 1 hour at 37°C. The supernatant from each plate was then transferred to a 15-mL conical tube and subjected to two successive rounds of centrifugation at 10,000 × g, each for 15 minutes, to remove loose cells and cellular debris. Each supernatant was then gently transferred to a new SW41 ultracentrifuge tube and centrifuged at 70,000 × g for 60 minutes. The pellet was resuspended in 100 μL of lysis buffer containing 100 mmol/L Tris (pH 7.0), 150 mmol/L NaCl, 5 mmol/L EDTA, and 0.25% NP40, mixed thoroughly, and an aliquot of 25 μL was taken for measurement of protein concentration using the Bradford kit from BIO-RAD (Hercules, CA). The remainder of the exosomal suspension in each experimental group was pooled for polyacrylamide SDS gel analyses.
Uptake of PKH67-Labeled Exosomes

Exosomes from 2008 and 2008/C13*5.25 were labeled with the lipid dye PKH67 Green (Sigma, St. Louis, MO) according to a method previously described (30). Briefly, an aliquot of exosomes containing 100 μg of protein was mixed with 0.5 μmol/L PKH67 Green and incubated at 37°C for 10 minutes. The mixture was diluted with ice-cold PBS and centrifuged at 70,000 × g for 1 hour. Vesicles were washed twice by being resuspended in fresh PBS and sedimented again by centrifugation at 70,000 × g for 1 hour. Experiments to determine whether exosomes were taken up by 2008 and 2008/C13*5.25 cells were done by incubating PKH67 Green–labeled exosomes (10 μg of total protein) with 1 × 10^6 cells in complete medium for 5 minutes. Cells were then rinsed with PBS thrice and fixed and processed for confocal microscopy. Negative controls were prepared of samples of 2008 and 2008/C13*5.25 cells in the absence of staining for PKH67 to correct for autofluorescence.

Measurements of Platinum Content

Whole cell platinum (Pt) content following a 1-hour period of incubation with CDDP and a subsequent 1-hour period of efflux was assayed as previously described (10). Exosomal content of Pt was measured by rapidly mixing 215 μL of 70% analytic grade nitric acid with 75 μL of resuspended exosomes and then incubating the mixture in a 65°C water bath overnight. Samples were then diluted with an indium (Acros Organics, Tustin, CA) solution to a final concentration of 5% acid and 1 ppb indium. Indium content was used to correct for flow variation in the inductivity-coupled plasma mass spectrometry. A Thermo Finnigan inductivity-coupled plasma mass spectrometry (model Element 2) from the Analytical Facility at the Scripps Institute of Oceanography was used for measurement of Pt concentration. Pt values were normalized to the protein content of the exosome preparation.

Western Blotting

Cells were rinsed twice with PBS, scraped with a rubber policeman, suspended in 10 mL PBS, and centrifuged for 10 minutes at 2,500 rpm. Cells were lysed in 0.25% NP40 in 100 mmol/L Tris-HCl (pH 8.0) supplemented with protease inhibitor tablets (complete from Roche Applied Sciences, Penzberg, Germany) at 4°C and for 30 minutes. Post-nuclear fractions were obtained by centrifugation of cell lysates for 10 minutes at 600 × g. Samples containing 50 to 100 μg of protein were electrophoresed on 4% to 10% SDS polyacrylamide gels. Gels were blotted onto nitrocellulose filters using a Bio-Rad Mini Transblot apparatus (Bio-Rad, Hercules, CA) and incubated for 1 hour with 5% fat free dry milk in TBS at room temperature and then overnight in primary antibody mixed with 5% milk in TBS at 4°C. Blots were washed thrice for 15 minutes each at room temperature with 0.025% Tween 20 in TBS. The secondary antibody was diluted in 5% fat-free dry milk in TBS and added to blots for 1 hour at room temperature. Blots were washed again at room temperature thrice for 15 minutes each in 0.025% Tween 20 in TBS. The extent of specific staining was quantified by chemiluminescence using the Enhanced Chemiluminescence kit from Amersham Life Science (Piscataway, NJ). A ChemilImage 4400 instrument (Alpha Inotech, San Leandro, CA) was used for determining the density of protein bands.

Expression Profiling

RNA was harvested and cDNA prepared, labeled, and hybridized to cDNA microarrays obtained from the Stanford Functional Genomics Facility (http://www.microarray.org) that contained 43,200 elements representing ~29,593 genes as estimated by UniGene clusters exactly as recently reported from this laboratory in detail (31). Significance analysis of microarrays (http://www-stat.stanford.edu/~tibs/SAM/index.html) was used to determine which genes were significantly differentially expressed based on the D score (31).

Results

Reduced Lysosomal Compartment in CDDP-Resistant Cells

The CDDP-resistant ovarian carcinoma 2008/C13*5.25 cells were selected for acquired resistance by repeated cycles of exposure to CDDP. These cells were developed 17 years ago and have maintained a stable level of resistance while in continuous culture in the absence of additional CDDP exposure. The vital dye Lysotracker Red DN-99 was chosen for its capacity to remain in acidic compartments of cells following formaldehyde fixation. The 2008 and 2008/C13*5.25 cells were exposed to 1 μg/mL Lysotracker Red in parallel cultures and processed together throughout the experiment. Figure 1 shows images of these cells in which the filamentous-actin filaments were also stained with Alexa Flour 647 phalloidin. CDDP-resistant 2008/C13*5.25 cells were found to contain markedly fewer lysosomes than the CDDP-sensitive parental 2008 cells. Quantification of raw images with NearCount software showed that resistant cells had 2.11 ± 0.03-fold (mean ± SE) fewer acidic vesicles than their sensitive counterparts (P ≤ 0.002).

To document that the differences in the numbers of acidic vesicles detected by Lysotracker Red DN-99 were in fact due to differences in the number of lysosomes in the two types of cells, parallel preparations of 2008 and 2008/C13*5.25 cells were stained with monoclonal antibodies against LAMP1 and LAMP2. These two proteins are both well-validated markers of lysosomal vesicles (32). The images presented in Fig. 2 confirm reduction in the lysosomal compartment in the CDDP-resistant cells. The number of LAMP1-containing vesicles was markedly reduced in the 2008/C13*5.25 cells. The number of vesicles staining positively for LAMP2 was also reduced in the 2008/C13*5.25 cells but to a lesser extent than LAMP1. To confirm the results of the immunohistochemical studies, LAMP1 and LAMP2 levels were measured by Western blot analysis of the 2008 and 2008/C13*5.25 cells using post-nuclear lysates. Figure 2 (bottom) shows that although LAMP1 was expressed in the 2008 cells, it was only very faintly detectable in the 2008/C13*5.25 cells. It also shows that LAMP2 levels were reduced by >3.2-fold in the 2008/
C13*5.25 cells. Thus, three different analytic approaches indicated a marked reduction in number of visible lysosomes and the content of lysosomal proteins in the cells selected for CDDP resistance.

**Analysis of Exosomes Released from CDDP-Sensitive and CDDP-Resistant Cells**

Because expression profiling of the CDDP-sensitive and CDDP-resistant cell pairs suggested altered levels of mRNA for some of the genes involved in exosome formation and release (see below), an analysis was undertaken of the exosomes released by 2008 and 2008/C13*5.25 cells. A multistep centrifugation procedure was used to produce high quality exosomes released from living cells for subsequent analysis (30). This technique excludes the possibility that the exosomes originate from serum in the tissue culture medium or from dead cells or cell fragments as these are removed before exosome collection. To show that the exosomes released by the CDDP-sensitive and CDDP-resistant cells were functional, they were labeled with PKH67 Green and then incubated with their cell of origin. Figure 3 shows that exosomes from both types of cells were able to reassociate with their cell of origin, although when exposed to exosomes containing equal levels of protein, the 2008/C13*5.25 cells accumulated less PKH67 Green than the 2008 cells. This is consistent with previous observations that exosomes can reenter the cell of origin (33–35). The amount of exosomal protein released into serum-free medium during a 1-hour period was used as an estimate of the differential ability of the 2008 and 2008/C13*5.25 cells to export exosomes. Figure 4 (top) shows that relative to the total cellular protein in the culture, the CDDP-resistant cells released significantly more exosomal protein over this time period ($P = 2.3 \times 10^{-5}$, $n = 20$).

Western blot analysis of the exosomes from 2008 and 2008/C13*5.25 cells showed that the CDDP-resistant cells contained a substantial amount of LAMP1, whereas this protein was undetectable in the exosomes released from the CDDP-sensitive cells (Fig. 4, bottom). However, the level of LAMP2 was only 1.1-fold higher in the exosomes from the resistant than the sensitive cells. Actin and tubulin were present at similar levels in the exosomes released by both CDDP-sensitive and CDDP-resistant cells. Thus, lysosomal proteins were found in exosomes, and the level of at least one such protein, LAMP1, was much higher in exosomes released by the DPP-resistant cells.

Because exosomal export can potentially serve as an alternative route for cells to discard proteins when lysosomal degradation is impaired, the exosomes released from the 2008 and 2008/C13*5.25 cells were subjected to Western blot analysis to measure the level of three other proteins that are believed to play a role in the detoxification of CDDP via sequestration into intracellular vesicles. Figure 4 (bottom) shows that MRP2 (ABCB2) and the copper efflux transporters ATP7A and ATP7B were all found at much higher levels in the exosomes from the CDDP-resistant cells. Quantitative analysis of the Western blots showed that the exosomes from CDDP-resistant cells contained, respectively, 3.4-, 2.6-, and 2.6-fold higher levels MRP2, ATP7A, and ATP7B relative to the levels of these proteins in exosomes released by the CDDP-sensitive cells. The finding that the exosomes from 2008 cells contain ATP7B was quite surprising, because this protein is not detectable in whole cell lysates prepared from this cell line, suggesting that ATP7B is concentrated in the exosomes. The finding that the exosomes from the CDDP-resistant cells contain much more ATP7B is consistent with our
earlier observation that the expression of this protein is markedly increased in 2008/C13*5.25 cells (36).

Pt Content of Exosomes Released from CDDP-Sensitive and CDDP-Resistant Cells

Recent studies suggest that one mechanism by which CDDP is exported from the cell is via sequestration into vesicles of the secretory pathway (9). Because exosomes represent one of the outputs of this pathway, it was of interest to determine whether exosomes released by the CDDP-resistant cells contain more CDDP than those from the sensitive cells following loading of the cells with CDDP. The 2008 and 2008/C13*5.25 cells were exposed to 2 μmol/L CDDP for 1 hour to load them with CDDP. They were then washed to remove all extracellular-free drug and the exosomes released from the cells over the next 1 hour were collected. The Pt content of the cells at the end of the loading period and at the end of the subsequent 1 hour period of exosome release and the Pt content of the released exosomes were measured and normalized to protein content. Figure 5 shows that the amount of Pt/mg exosomal protein was 2.6 ± 0.8-fold higher in the exosomes released from the CDDP-resistant cells ($P = 3.8 \times 10^{-5}$). Taking into account that the 2008/C13*5.25 cells released 1.9-fold more exosomal protein over this time period, the total exosomal Pt export was 4.9-fold higher from the resistant cells, despite the fact that the resistant cells accumulated only 41% as much CDDP during the 1-hour loading incubation as the sensitive cells as shown in Fig. 5, bottom (see also ref. 36). However, the $0.0037 \pm 0.00009$ pmol of Pt/mg exosomal protein found in the exosomes

![Figure 2](https://example.com/figure2.jpg)

**Figure 2.** Comparison of the levels of the lysosomal proteins LAMP1 and LAMP2 in 2008 and 2008/C13*5.25 cells. **Top,** deconvoluted confocal images of cells stained with monoclonal antibodies against LAMP1 and LAMP2 (green). Actin was labeled with Alexa Flour 647 phalloidin (artificially colored red). Nuclei were stained with Hoechst 33342 (blue). **Bottom,** Western blot analysis of whole cell lysates of 2008 and 2008/C13*5.25 cells probed with antibodies against LAMP1, LAMP2, and actin. Each lane was loaded with 25 μg of protein.
released over the first hour from CDDP-sensitive cells represented only 0.037% of the total of 10.08 ± 0.87 pmol of Pt lost from the cells that produced these exosomes during this period of time. In the case of the CDDP-resistant cells, the 0.01818 ± 0.00033 pmol of Pt in the exosomes represented only 0.075% of the 24.08 ± 1.27 pmol lost from the cells. Thus, although CDDP-resistant cells export more Pt via the exosomal pathway than CDDP-sensitive cells, this represents only a very small fraction of all the Pt loss from the cell during the first hour after the end of drug exposure.

**Expression Pattern of Genes Whose Products Function in Lysosome Formation**

RNA was harvested from log phase 2008 and 2008/C13*5.25 cells grown under identical conditions and used to determine the expression profile by hybridizing the labeled cDNA derived from reverse transcription of RNA against each other using a cDNA microarray containing sequences corresponding to 29,593 genes. Each experiment was repeated six times using independently isolated RNA samples. Genes that were significantly differentially expressed were determined using the "statistical analysis of microarrays" software (http://www-stat.stanford.edu/~tibs/SAM/index.html). With variables set such that the number of genes falsely discovered is expected to be ≤1, a total of 411 genes were found to be significantly differentially expressed between the 2008 and 2008/C13*5.25 cells. As shown in Table 1, among these were nine genes belonging to families whose products are known to be involved in the formation of lysosomes and multivesicular bodies. All nine genes were expressed at a higher level in the CDDP-resistant cells.

**Discussion**

We have previously reported that CDDP becomes localized in lysosomes in CDDP-sensitive parental human ovarian carcinoma 2008 cell line (10). In the current study, we examined the lysosomal compartment in the 2008/C13*5.25 subline whose degree of CDDP resistance has been stable for >17 years of continuous culture in the absence of additional drug selection. We confirmed reports from other laboratories that the lysosomal compartment is markedly abnormal in cells selected for stable acquired CDDP resistance (17, 37). In addition, we discovered that CDDP enters the exosomal pathway, and that CDDP-resistant cells export both more CDDP and more putative CDDP transporters via this pathway.

Lysosomes are a key station along the path by which nutrients, pathogens, and cytotoxic agents traffic within cells. In the trafficking of metals, they seem to serve as an
intermediary compartment that receives metals and metalloids from influx pathways and either stores them or distributes them to efflux systems or other destinations in the cell (38, 39). CDDP is known to be concentrated by the copper efflux transporter ATP7A into vesicles of the microsomal fraction (7), and it is likely that ATP7B also concentrates CDDP into such vesicles (9). Fluorescently labeled CDDP colocalizes with lysosomal markers in 2008 cells (40). By virtue of their high content of catabolic enzymes, lysosomes also participate in apoptotic (41), necrotic (42), and autophagic (43) cell death pathways, often in collaboration with stress management organelles, such as the nucleus, mitochondria, endoplasmic reticulum, and Golgi apparatus (11). There is evidence that metalloids can activate these cell death pathways as a direct result of the damage that they do to the lysosomes in which they become concentrated (15, 44).

We found major alterations of the lysosome compartment in CDDP-resistant 2008/C13*5.25 cells. The number of visible lysosomes was reduced when imaged with either Lysotracker Red DN-99 or antibodies to LAMP1 or LAMP2 and the level of expression of LAMP1 and LAMP2 was reduced when examined by Western blot analysis. In addition, the trafficking of proteins through this compartment was abnormal as reflected by both the larger amount of exosomal protein released by the resistant cells and the altered distribution of the proteins that were found in the exosomes. We have documented similar changes in two other CDDP-resistant ovarian carcinoma cell lines, A2780/CP and IGROV-1/CP (45). It is noteworthy that, despite the fact that both LAMP1 and LAMP2 levels were decreased in the CDDP-resistant cells, only LAMP1 was found in higher levels in the exosomes. Quantitatively, it is unlikely that increased export of either protein via the exosomal pathway accounts for the marked decrease in their level in the whole cell. Instead, we interpret the fact that LAMP1 levels were increased in exosomes released by the resistant cells, whereas LAMP2 levels were not as evidence of an abnormality of protein trafficking or exosome formation in the lysosomal compartment. It is important to note that the reduction in the size of the lysosomal compartment found in this study is a stable characteristic of the acquired CDDP-resistant phenotype as the 2008/C13*5.25 cells have been grown in the absence of CDDP for many years. The finding that a number of genes coding for proteins involved with the formation of lysosomes and multivesicular bodies were overexpressed at the mRNA level in the CDDP-resistant cells suggests a secondary compensatory response to some primary abnormality that prevents correct assembly of lysosomes or enhances their destruction. It was of particular interest to find that several transporters believed to be involved in the movement of CDDP across vesicle membranes were found in exosomes, and that the level of ATP7A, ATP7B, and MRP2 (ABCB2) was higher in the exosomes released from the CDDP-resistant cells than those released from sensitive cells. This may reflect a major abnormality in intracellular protein trafficking such as has been described in other types of CDDP-resistant cells (17, 37). We speculate that the altered distribution of the
types of proteins found in exosomes may report on the CDDP-resistant phenotype. Because exosomes are found in the systemic circulation, this offers a potential route to early detection of the emergence of drug resistance during treatment.

There is now a substantial body of evidence that CDDP is sequestered into intracellular vesicles, some of which belong to the secretory pathway (7, 9, 10). Presumably, many of these vesicles travel directly to, and fuse with, the plasma membrane releasing CDDP to the outside of the cell in a form no longer encapsulated in a lipid membrane. The released Pt may be either free drug, a conjugate, or a complex with cellular proteins to which it has become bound. The results reported in this article indicate that CDDP is also exported via exosomes and thus suggest a function for multivesicular bodies, from which these vesicles originate, in the routing of intracellular CDDP. How CDDP enters the exosomal pathway is not entirely clear. Possibly, cytoplasmic CDDP is entrapped in exosomes as they form by invagination of the limiting membranes of the endosomes that are the precursors of mature multivesicular bodies. CDDP would then be expected to exit the cell when the multivesicular bodies fuses with the plasma membrane to release its content of exosomes. In the CDDP-resistant 2008/C13*5.25 cells, a larger fraction of cellular protein was released as exosomes per hour and the exosomes contained a larger amount of Pt. Taken together with the fact that the CDDP-resistant cells start out with a smaller amount of Pt in the whole cell following incubation with CDDP, and the fact that exosomes are recycled by these cells, it is apparent that a larger fraction of the Pt in the cell enters the exosomal pathway in CDDP-resistant cells. This is consistent with the finding of increased amounts of the putative CDDP or CDDP-conjugate transporters ATP7A, ATP7B, and MRP2 (ABCB2) in the exosomes released by the resistant cells. At what step along the pathway CDDP loading occurs remains unknown, but ATP7B has been identified as being resident in late endosomes (46) and is thus positioned to play a role. Although export via exosomes does not account

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<th>Gene Expression ratio*</th>
<th>Function</th>
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<tr>
<td>Annexin A10 (ANXA10) 1.9</td>
<td>Membrane bending, invagination of MVB</td>
</tr>
<tr>
<td>Decay-accelerating factor for complement (CD55, DAF) 1.9</td>
<td>GPI-anchored tetraspanin, exported in exosomes</td>
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<tr>
<td>Transmembrane 4 superfamily member 2 (TM4SF2) 1.7</td>
<td>Member of the family of tetraspanin proteins, which are proposed to function in membrane bending, fusion, invagination of MVB</td>
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<tr>
<td>Baculoviral IAP repeat–containing 6 (apollon; BIRC6) 1.9</td>
<td>Involved in membrane bending, fusion, invagination of MVB</td>
</tr>
<tr>
<td>Integrin β1 (fibronectin receptor, β polypeptide, antigen CD29 includes MDF2, MSK12; ITGB1) 1.7</td>
<td>Exported in exosomes</td>
</tr>
<tr>
<td>MHC class I C (HLA-C) 1.8</td>
<td>Exported in exosomes</td>
</tr>
<tr>
<td>Sorting nexin 5 (SNX5) 2.0</td>
<td>Early endosomal recycling; binds clathrin; binds to Fanconi anemia complementation group A (FANCA) protein (FANCA is expressed in response to stress)</td>
</tr>
<tr>
<td>RAB4A, member RAS oncogene family (RAB4A) 1.5</td>
<td>Regulates receptor recycling from early endosomes</td>
</tr>
<tr>
<td>Rab3 GTPase-activating protein, noncatalytic subunit (150 kDa; RAB3-GAP150) 1.6</td>
<td>Implicated in exocytic release of neurotransmitters and hormones; mutation causes Warburg Micro syndrome</td>
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Abbreviation: MVB, multivesicular bodies.
*Ratio of expression in 2008/C13*5.25 cells relative to that in 2008 cells.
†All d scores were statistically significant.
for a very large fraction of the Pt loss from either CDDP-sensitive or CDDP-resistant cells over the first hour after the end of drug exposure and cannot account for the differences in whole cell CDDP accumulation in the sensitive and resistant cells, the difference in the amount of Pt exported via this pathway in the two cell types indicates that, as for the lysosomal compartment, the resistant phenotype is associated with a major alteration in the function of the exosomal pathway that perhaps simply reflects upstream abnormalities in the lysosome compartment.

How the alterations in the lysosomal and exosomal pathways are linked to the CDDP-resistant phenotype is not currently apparent. One possibility it that the sequestration of CDDP into lysosomes, rather than serving as a detoxification pathway, is actual a mechanism by which CDDP triggers apoptosis due to damage to the lysosome and release of lysosomal contents into the cytoplasm. Thus, reduction in the size of the lysosomal compartment in CDDP-resistant cells may by itself facilitate the survival following CDDP exposure. Another is that the changes in the lysosomal and exosomal pathways observed in the CDDP-resistant cells reflect primary abnormalities of protein trafficking that also affect the delivery of CDDP to the nucleus where it can attack DNA. It will be of interest to determine whether the increased exosomal export of CDDP from the resistant cells is due to dysfunction of a pathway that normally traffics CDDP to some other destination in the cell.

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References


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