Intracellular Localization and Trafficking of Fluorescein-Labeled Cisplatin in Human Ovarian Carcinoma Cells

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ABSTRACT

Purpose: We sought to identify the subcellular compartments in which cisplatin [cis-diamminedichloroplatinum (DDP)] accumulates in human ovarian carcinoma cells and define its export pathways.

Experimental Design: Deconvoluting digital microscopy was used to identify the subcellular localization of fluorescein-labeled DDP (F-DDP) in 2008 ovarian carcinoma cells stained with organelle-specific markers. Drugs that block vesicle movement were used to map the traffic pattern.

Results: F-DDP accumulated in vesicles and were not detectable in the cytoplasm. F-DDP accumulated in the Golgi, in vesicles belonging to the secretory export pathway, and in lysosomes but not in early endosomes. F-DDP extensively colocalized with vesicles expressing the copper efflux protein, ATP7A, whose expression modulates the cellular pharmacology of DDP. Inhibition of vesicle trafficking with brefeldin A, wortmannin, or H89 increased the F-DDP content of vesicles associated with the pre-Golgi compartments and blocked the loading of F-DDP into vesicles of the secretory pathway. The importance of the secretory pathway was confirmed by showing that wortmannin and H89 increased whole cell accumulation of native DDP.

Conclusions: F-DDP is extensively sequestered into vesicular structures of the lysosomal, Golgi, and secretory compartments. Much of the distribution to other compartments occurs via vesicle trafficking. F-DDP detection in the vesicles of the secretory pathway is consistent with a major role for this pathway in the efflux of F-DDP and DDP from the cell.

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INTRODUCTION

The mechanisms that control the cellular accumulation of cisplatin [cis-diamminedichloroplatinum (DDP)] are poorly understood. Studies of the cellular pharmacology of DDP have suggested that specialized membrane-bound proteins mediate the majority of DDP uptake and efflux, and nearly all cell lines selected for resistance to DDP exhibit alterations in drug accumulation (1, 2). Recent studies have identified the copper importer CTR1 (3, 4), and the copper efflux transporters ATP7A (5), and ATP7B (6, 7), as being important to the cellular pharmacokinetics and cytotoxicity of DDP.

Identification of the conduits along which DDP moves between subcellular compartments after it enters the cell, and the sites in which it accumulates, has been thus far accomplished only indirectly by noting the damage caused by DDP in various organelles or by low-resolution microscopy with energy-dispersive X-ray microanalysis (8–10) and electronic probes (11). Electron microscopy (12) can provide the needed resolution for identification of subcellular sites of DDP accumulation, but its capability is limited by the relative solubility of DDP. Recently, Molenaar et al. (13) showed that fluorescein-tagged DDP, abbreviated here as F-DDP, could be used to obtain high-resolution images of the subcellular distribution of DDP. They found that DDP was not uniformly distributed in cells but could be detected in many cytoplasmic vesicles as well as in the nucleus.

In the current study, we used digital deconvoluting microscopy in combination with organelle-specific markers to examine the intracellular distribution of F-DDP, and employed pathway inhibitory drugs to identify major conduits through which F-DDP traffics as it enters and eventually effluxes from cultured human ovarian carcinoma cells.

MATERIALS AND METHODS

Reagents. Brefeldin A was obtained from ICN Biochemicals, Inc. (Aurora, OH), wortmannin from Sigma, Co. (St. Louis, MO), H89 from EMD Biosciences (San Diego, CA), and media and sera were from Invitrogen (Carlsbad, CA). Antibodies to TGN38 and ATP7A were purchased from BD Transduction Laboratories (San Diego, CA), antibodies to early endosomal antigen 1 (EEA1), rab4, and rab11 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the antibody to cMOAT protein 2 (MRP2) was from Alexis Biochemicals (San Diego, CA). The antibody to golgin97 and specific dyes Alexa Fluor 647 phalloidin, Hoechst 33342, and LysoTracker Red were obtained from Molecular Probes (Eugene, OR). Texas red–conjugated secondary antibodies against mouse, rabbit, and goat antigens were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). F-DDP was synthesized according to a procedure described elsewhere (14). DDP was a gift from Bristol-Myers Squibb, Co. (Princeton, NJ).

Cell Culture and Histochemistry. Ovarian carcinoma 2008 cells (15) and the DDP-resistant subline 2008/C13*5.25...
then stained with PBS for 15 minutes, washed three times, 15 minutes each, and PBS. Samples were permeabilized with 0.3% Triton X-100 in PBS by treatment for 15 minutes with 3.7% formaldehyde in PBS. All samples were fixed after three quick rinses with OptiMEM containing 1 μg/mL of LysoTracker Red for 30 minutes. All samples were fixed after three quick rinses with PBS by treatment for 15 minutes with 3.7% formaldehyde in PBS. Samples were permeabilized with 0.3% Triton X-100 in PBS for 15 minutes, washed three times, 15 minutes each, 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. Cells were then washed three times, 15 minutes each, with PBS and incubated for 1 hour with secondary antibodies in PBS and 0.3% bovine serum albumin, followed by three 3, 15-minute washes in PBS and mounting on glass slides. Mounting medium contained 24 g polyvinyl alcohol, 60 g glycerol, 60 mL H2O, 120 mL 0.2 mol/L Tris-HCl (pH 8.5).

Microscopy was done at the University of California, 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 μ sections by 100× and 40× lenses and SoftWorx software (Applied Precision) was used for deconvoluting data. Image quantification was done with Data Inspector program in SoftWorx or by NearCount software.

Measurement of Platinum Accumulation. One million cells were seeded in 35 mm dishes and grown until they were 75% to 80% confluent. The medium was replaced by 1 mL of fresh medium containing 2 μmol/L DDP or DDP plus inhibitory drugs, and the cells were incubated at 37°C. At the requisite time points, the medium was poured off and the dishes were quickly rinsed thrice with ice-cold PBS and then 215 μL 70% nitric acid was added to each plate. The cell lysates were then transferred into 15 mL centrifuge tubes, capped and placed at 65°C for 2 hours, after which the samples were diluted with an indium (Acros Organics, Tustin, CA) solution to a final concentration of 5% acid and 1 ppb of indium. Platinum was measured with a Thermo Finnigan inductively coupled plasma mass spectrometer (model element 2) at the Analytical Facility at the Scripps Institute of Oceanography. Platinum values were normalized to the protein content measured by the Bradford method using the Bio-Rad kit (Hercules, CA).

RESULTS

Synthesis and Characterization of F-DDP and CHMA-F. F-DDP, consisting of DDP to which a fluorescein was linked, was synthesized using a modification of the method described by Molenaar et al. (13). Briefly N3-Boc-1,2,3-propanetriamine was prepared from 3-amino-1,2-propanediol and then reacted with potassium tetrachloroplatinate to obtain 1-(Boc-aminomethyl)-1,2-ethylenediamine dichloroplatinum(II). The Boc group was removed with 0.1 N HCl at 70°C and the resulting platinated salt was neutralized with 2 N NaOH and then reacted with 5(6)-carboxyfluorescein preactivated by treatment with ethyldimethylaminopropyl carbodiimide and N-hydroxysuccinimide in dimethyl formamide. After centrifugation and successive washes with water, ethanol, and ether, the final product was characterized for DDP and fluorescein contents by 1H-nuclear magnetic resonance and mass spectrometry. The chemical structure of F-DDP is presented in Fig. 1 along with the structure of cyclohexylmethylamido fluorescein (CHMA-F), which consists of fluorescein coupled to the linker in the absence of DDP.

F-DDP can become attached to proteins at sites where it is sequestered intracellularly by two different mechanisms. In addition to direct reaction of DDP with nucleophilic sites on intracellular molecules, in the presence of formaldehyde, F-DDP can also become cross-linked to immediately adjacent proteins via the CHMA-F moiety. To show that F-DDP does in fact become firmly bound by one or the other of these mechanisms to proteins or other molecules at sites where it is concentrated, 2008 cells were incubated with 2 μmol/L F-DDP for 1 hour, fixed with 3.7% formaldehyde in PBS and treated with increasing concentrations of Triton X-100 for 15 minutes prior to being stained with antibodies to specific structural markers. As shown in Fig. 2, F-DDP remained associated with the subcellular structures into which it was initially sequestered even after treatment with 1% Triton X-100, demonstrating that F-DDP binds cellular components very firmly under these fixation conditions. Examination of a large number of cells indicated that the differences in vesicle size and distribution apparent in Fig. 2 reflected cell-to-cell heterogeneity rather than an effect of increasing Triton X concentration. The heterogeneity in staining pattern is likely to be related to differences in the flux of vesicular trafficking which may be associated with such factors as cell cycle phase.

To determine the extent to which F-DDP mimics the behavior of DDP, F-DDP, CHMA-F, and DDP were compared with regard to their cytotoxicity, cross-resistance, and accumulation levels using the 2008 DDP-sensitive parental human ovarian carcinoma cell line and the 2008/C13*5.25 DDP-resistant subline. Both DDP and F-DDP showed concentration-dependent cytotoxicity that was greater for 2008 than for the
2008/C13*5.25 cells, whereas CHMA-F was not toxic even at a concentration of 500 μmol/L. The ratio of the IC50 values for the 2008/C13*5.25 and 2008 cells, determined by clonogenic assay, was similar for both compounds, being 5.3 μmole/L for DDP and 5.7 μmole/L for F-DDP. As previously documented for DDP (14), the accumulation of F-DDP was reduced in the 2008/C13*5.25 cells to 26% of that in the 2008 cells at 1 hour. Thus, F-DDP is subject to the same mechanisms that render cells resistant to DDP.

Additional evidence that F-DDP mimics the behavior of DDP was provided by analyses of the subcellular distribution of F-DDP and CHMA-F in 2008 cells. Figure 3 shows that although CHMA-F was distributed in structures that extensively colocalized with filamentous actin in 2008 cells (A), the distribution of F-DDP was quite different (C and E). F-DDP was localized to discrete perinuclear vesicular structures and the nucleus. This indicates that the subcellular distribution of F-DDP is driven predominantly by the DDP moiety rather than the CHMA-F fluorophore in the F-DDP complex. Furthermore, as shown in Fig. 3B, D, and F, the distribution of CHMA-F was also markedly different from that of F-DDP in the DDP-resistant 2008/C13*5.25 cells. It is also clear that the compartmental distribution of F-DDP in the DDP-sensitive 2008 and DDP-resistant 2008/C13*5.25 is quite different. The basis for this is not currently known but is under active investigation.

Subcellular Distribution of F-DDP. As shown in Fig. 3, incubation of cells with 2 μmol/L F-DDP for 1 hour produced readily detectable fluorescence in a variety of cytoplasmic structures and less intense fluorescence in the nucleus (Fig. 3E and F). Although there was substantial variability in the intensity of F-DDP fluorescence between cells of a given field, a 1-hour exposure was sufficient to produce detectable fluorescence in the majority of cells in single preparation. Consistent with the observations made by Molenaar et al. (13), F-DDP in the nucleus exhibited a finely granular pattern, whereas elsewhere in the cell, F-DDP was distributed in discrete vesicular structures rather than being diffusely localized throughout the cytoplasm (Fig. 3E). A serial analysis of the change in intracellular distribution of F-DDP over the first 30 minutes of exposure is presented in Fig. 4. When F-DDP was applied to the cells and then immediately washed away (time 0) there was intense fluorescence at the cell surface but little activity elsewhere in the cell. By 2 to 5 minutes, fluorescence appeared in cytoplasmic organelles, and then over the ensuing 10 to 30 minutes it appeared in nuclear structures.

Colocalization of F-DDP with Endosomal/Lysosomal Markers. Antibodies to the EEA1, rab4, and rab11 were used to identify the early (17), fast, and slow recycling (18) endosomes, respectively. LysoTracker Red was used to identify the acidic lysosomal structures. Figure 5A shows representative images from studies in which 2008 cells were exposed to F-DDP and then stained with anti-EEA1. There was very little colocalization of F-DDP with EEA1 at any point up to 60 minutes of exposure to F-DDP. Likewise, as shown in Fig. 5B, there was no detectable colocalization of F-DDP with rab4. Staining of F-DDP-labeled cells with antibodies against...
the small GTPase Rab11, which is a marker for the slowly recycling endosomal compartment (18), showed limited and weak colocalization between the two fluorescent signals as shown in Fig. 5C. Thus, there seems to be little transit of F-DDP through the early endosomal compartment during accumulation in the cell. F-DDP did not enter endosomes that recycle rapidly back from the early endosome to the cell surface and entered slowly recycling endosomes only to a minimal degree.

In contrast to the early and recycling endosomal compartments, F-DDP accumulated extensively in lysosomal vesicles identified on the basis of their accumulation of LysoTracker Red as shown in Fig. 5D. Strong colocalization between F-DDP and LysoTracker Red was especially pronounced in regions near the plasma membrane, suggesting that these vesicles may be part of the lysosomal vesicular secretory pathway.

**Association of F-DDP with the Golgi and Trans - Golgi Network.** To examine the involvement of the Golgi apparatus in F-DDP trafficking, 2008 cells were exposed to 2 μmol/L F-DDP for 1 hour and then stained with a monoclonal antibody against the grip domain Golgi protein, golgin97 (19). Golgin97 localizes to the trans side of the Golgi apparatus and is believed to participate in the trafficking of vesicles between the Golgi stacks and the trans-Golgi network (20). As shown in Fig. 6A, a subset of the golgin97-positive vesicles accumulated F-DDP and these were scattered throughout the cytoplasm. In contrast, as shown in Fig. 6B, there was no detectable colocalization of F-DDP with the trans-Golgi network marker.

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**Fig. 3** Distribution of F-DDP and CHMA-F in 2008 ovarian carcinoma cells. Cells were incubated with 2 μmol/L of either F-DDP or CHMA-F for 1 hour in parallel, and then stained for filamentous actin (red) with Alexa Fluor 647 phalloidin and with Hoechst 33342 for nuclei (blue) prior to being imaged for F-DDP (green) accumulation. A, distribution of CHMA-F in DDP-sensitive 2008 cells; B, distribution of CHMA-F in DDP-resistant 2008/C13*5.25 cells; C, distribution of F-DDP in DDP-sensitive 2008 cells; D, distribution of F-DDP in DDP-resistant 2008/C13*5.25 cells; E, distribution of F-DDP in a single 2008 cell; F, distribution of F-DDP in a single 2008/C13*5.25 cell.
TGN38, a protein found predominantly in the tubulovesicular subcompartment of the trans-Golgi network (21). These results show the involvement of golgin97-positive Golgi-derived vesicles in the trafficking of F-DDP, and indicate that F-DDP is directed in a selective manner to downstream compartments other than the TGN38-positive subcompartment of the trans-Golgi network. It is of interest that F-DDP colocalized with golgin97-positive vesicles found not just in the region of the Golgi and trans-Golgi network but also out at the periphery of the cell. This suggests that either F-DDP is sequestered into the Golgi first and then carried over long distances, or that F-DDP enters these vesicles after they have departed the Golgi and moved toward the periphery of the cell.

**Colocalization with ATP7A and MRP2.** Antibodies against two markers of vesicles belonging to the secretory pathway, MRP2 and ATP7A, both of which have been implicated in the transport of DDP (22, 23), showed extensive colocalization with F-DDP. As shown in Fig. 6B, vesicles exhibiting colocalization of F-DDP with ATP7A were found predominantly in a perinuclear distribution, although some vesicles were dispersed throughout the cell. As shown in Fig. 6C, vesicles exhibiting colocalization of F-DDP and MRP2 were located mainly in a perinuclear distribution. NearCount software determined that 64 ± 2% (mean ± SE) of total cytoplasmic F-DDP colocalized with ATP7A and 64 ± 2% (mean ± SE) with MRP2 in 2008 cells. The extensive

![Fig. 4 Variation in the distribution of F-DDP as a function of time in 2008 cells. Cells were exposed to 2 μmol/L F-DDP for 0, 2, 5, 10, and 30 minutes, fixed and costained with Alexa Flour 647 phalloidin to localize the actin filaments (artificially colored red). Yellow, overlapping of the F-DDP signal with that for actin.](image-url)
association of F-DDP with vesicles of the export pathway suggests that ATP7A and MRP2, or closely associated proteins, are involved in the efflux of this drug.

**Effect of Pathway Inhibitory Drugs on F-DDP Localization.** To further define the role of each vesicular compartment in the trafficking of F-DDP, 2008 cells were treated with F-DDP and agents that selectively block various trafficking pathways. Brefeldin A was used to inhibit initial steps of the vesicular secretory pathway and wortmannin was used to inhibit PI3 kinase-mediated fusion of vesicles along the pathway from endosomes to lysosomes (24). The protein kinase A inhibitor H89 was used to block post-Golgi secretory events (25).

Blockade of vesicle movement from the endoplasmic reticulum to Golgi stacks with brefeldin A (26) is known to cause intermixing of Golgi components with those of the ER as well as fusion of endosomes, lysosomes, and the trans-Golgi network (27). As expected, treatment of 2008 cells with brefeldin A caused Golgi fragmentation and collapse of golgin97-positive structures toward the perinuclear region (Fig. 7A). However, the golgin97-positive vesicles in brefeldin A–treated cells were almost completely devoid of F-DDP, indicating that the blockade of ER to Golgi movement also blocked the loading of F-DDP into these vesicles, presumably due to incorrect localization of those proteins normally responsible for sequestering F-DDP. This conclusion was confirmed by the results of an experiment in which brefeldin A and F-DDP-treated cells were stained with antibodies against MRP2 and ATP7A. As shown in Fig. 7B and C, the ATP7A- and

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**Fig. 5** Colocalization of F-DDP (green) with specific compartmental markers (red) in 2008 cells incubated with 2 μmol/L F-DDP for 1 hour. **A,** colocalization with EEA1-positive early endosomal vesicles; **B,** colocalization of F-DDP with rab4-positive recycling endosomes; **C,** colocalization of F-DDP with rab11-positive recycling endosomes; **D,** colocalization of F-DDP with lysosomal marker LysoTracker Red. Yellow, structure is positive for both F-DDP and organelle markers. Nuclei are stained with Hoechst 33342 (blue).

**Fig. 6** Colocalization of F-DDP (green) with vesicles expressing specific markers (red) for (A) golgin97, (B) ATP7A, and (C) MRP2. Yellow, structure is positive for both F-DDP and organelle markers. Nuclei are stained with Hoechst 33342 (blue).
Fig. 7  Effect of brefeldin A, wortmannin, and H89 on the subcellular distribution of F-DDP. A, effect on colocalization of F-DDP (green) with golgin97 (red). B, effect on colocalization of F-DDP with vesicles expressing ATP7A (red).
**Fig. 7 (cont’d)**  
*C,* effect on colocalization of F-DDP with vesicles expressing MRP2 (red).  
*D,* effect on colocalization of F-DDP with vesicles expressing TGN38 (red). Yellow: the structure is positive for both the F-DDP and organelle markers.
MRP2-expressing vesicles were also completely devoid of F-DDP in brefeldin A–treated cells. Thus, failure to load F-DDP into upstream vesicles associated with the trans-Golgi network was accompanied by a lack of F-DDP in the more downstream vesicles of the vesicle secretory pathway. Interestingly, treatment of cells with brefeldin A resulted in some degree of colocalization between F-DDP and the TGN38 marker (Fig. 7D) presumably due to the dissolution of TGN38-containing vesicles into the endosomal/lysosomal compartment (27). This indicated that the Golgi and trans-Golgi compartments are downstream of the endosomal compartments in the intracellular transport of F-DDP.

At 200 nmol/L wortmannin is reported to inhibit the late stages of the endosomal fusion and the maturation of endosomes into lysosomes (24). At this concentration, wortmannin is relatively specific for inhibition of the PI3 kinases involved in vesicular trafficking (28). Treatment of 2008 cells with wortmannin increased the overall uptake of F-DDP: quantification of raw digital images with Softworks software indicated that the mean F-DDP fluorescence per cell was increased by $27 \pm 1\%$ (SE; $P = 5.62 \times 10^{-8}$). However, wortmannin prevented the sequestration of F-DDP into golgin97-expressing vesicles of the Golgi or vesicles of the secretory pathway expressing MRP2 or ATP7A (Fig. 7A–C). In the presence of wortmannin, the structures that contained golgin97 were not only devoid of F-DDP but also localized to a small corner near the nucleus (Fig. 7A). Interestingly, as wortmannin blocked the flux of F-DDP into the vesicles of the secretory pathway, it caused F-DDP to back up into the more upstream TGN38-positive vesicles that in the absence of wortmannin did not accumulate F-DDP at all (Fig. 7D). These data indicate that the endosomal/lysosomal system is involved in directing F-DDP into the vesicle of the secretory pathway.

The isoquinolinesulfonamide protein kinase inhibitor H89 is known to block steps downstream of the Golgi vesicles within the secretory pathway (29). By blocking COPII recruitment and export from the endoplasmic reticulum, H89 causes the collapse of the endoplasmic reticulum–associated Golgi compartment (30, 31) allowing proteins resident in the Golgi to remain juxtanuclear (31, 32). Treatment of 2008 cells with H89 produced the expected juxtanuclear localization of the golgin97 protein and increased total cellular F-DDP fluorescence (Fig. 7A), but that the same time blocked the accumulation of F-DDP in vesicles expressing ATP7A or MRP2 (Fig. 7B and C). As was observed with wortmannin, H89 caused the backing-up of F-DDP into TGN38-positive vesicles that were found in a well-defined ring around the nucleus (Fig. 7D). These results are consistent with a role for a protein kinase in the loading of F-DDP into vesicles destined for subsequent export from 2008 cells.

**Effect of Pathway Inhibitory Drugs on Whole Cell Accumulation of DDP.** The studies with digital deconvoluting microscopy showed that wortmannin and H89 increased whole cell accumulation of F-DDP and caused excessive accumulation in vesicles at the upper end of the pathway, at the same time reducing the amount of F-DDP founding vesicles at the lower end of the vesicle secretory pathway. To confirm that these drugs produced the same effect on the cellular pharmacology of native DDP, 2008 cells were treated for 1 hour with 2 μmol/L DDP instead of F-DDP and the effect of wortmannin and H89 on whole cell platinum accumulation was determined by inductively coupled plasma mass spectroscopy. At a concentration of 200 nmol/L wortmannin, increased whole cell uptake by 6.0 ± 0.2-fold (mean ± SE; $P < 0.001$). H89 produced a similar effect and increased whole cell DDP uptake by 7.5 ± 0.1-fold (mean ± SE; $P < 0.001$). Thus, wortmannin and H89 produced similar effects on the accumulation of F-DDP and native DDP.

**DISCUSSION**

In this study a fluorescent form of DDP was used to identify the subcellular compartments into which the drug is sequestered and through which it traffics. The results confirm and extend our previous work showing that F-DDP mimics DDP with respect to cytotoxicity, accumulation pattern, and colocalization with the DDP export protein ATP7B (14). The finding that the majority of the intracellular F-DDP was associated with vesicular structures confirms the observation of Molenaar et al. (13) that, instead of diffusing freely through the cytoplasm, F-DDP is sequestered into specific families of vesicles presumably by specialized membrane-bound proteins that mediate the transport of DDP.

Several lines of evidence indicate that labeling of DDP with fluorescein does not change those components of the cellular pharmacology of the drug that are central to the DDP-resistant phenotype. First, the resistant cells accumulate less F-DDP in parallel to their failure to accumulate DDP. Second, despite the fact that F-DDP is less potent than DDP, it retains substantial cytotoxicity and the DDP-resistant cells are as resistant to F-DDP as they are to DDP. Thus, mechanisms essential to the DDP-resistant phenotype are operative on F-DDP as well as DDP. Third, the subcellular distribution of F-DDP is markedly different from that of the fluorophore, CHMA-F, indicating that the presence of the DDP component in F-DDP predominates over the effect of the CHMA-F moiet. Finally, F-DDP showed localization in intracellular structures known to accumulate DDP on the basis of direct measurements of platinum content, including the mitochondria (33).

F-DDP initially becomes sequestered into the lysosomes and is subsequently found in vesicles derived from the Golgi that participate in the vesicle of the secretory pathway. Endosomes that express EEA1 protein constitute the first intracellular station for endocytic vesicles derived from the plasma membrane (34). The near absence of F-DDP from these early endosomes indicates that the uptake of F-DDP is not mediated by the endocytic pathways that deliver their cargo to this station. Similarly, vesicles expressing rab4 do not seem to function in the flux of F-DDP in 2008 cells. However, a role for recycling vesicles expressing rab11 cannot be completely ruled out because there was a small degree of colocalization between F-DDP and antibodies to rab11. The rab11 GTPase has been localized to recycling endosomes and the trans-Golgi network and is required for transport of recycling endosomes back to the plasma membrane (35–37).
It is possible that some of the F-DDP that reaches the vesicle of the secretory pathway is delivered by rab11-positive vesicles.

Accumulation of F-DDP in lysosomes is consistent with the reported role of this compartment in the regulation of tumor cell sensitivity to DDP (38, 39). Lysosomes are at the junction of both biosynthetic and the degradative pathways. They receive their enzymes and polysaccharide membrane proteins from the trans-Golgi network mostly via multivesicular bodies which also sort the endocytosed proteins for recycling or degradation by lysosomes (reviewed by Katzmann et al. 40). It is possible that some of the F-DDP found in this compartment became associated with proteins elsewhere, and which were then directed to lysosomes for degradation. Whether any of the F-DDP or DDP that becomes sequestered in lysosomes finds its way to the cytoplasm remains to be determined. However, lysosomes do possess metal transporters such as NRAMP2, which functions to export iron from lysosomes (41). It is thus likely that transporters capable of exporting DDP from lysosomes would also be present in this compartment.

One step in the trafficking of F-DDP seems to involve the trans side of the Golgi, marked by the grip domain protein golgin97. This protein is proposed to function in tethering of docking of vesicles (19). Golgi-derived vesicles containing F-DDP seem to traffic towards the cell surface, at the same time retaining the golgin97 protein. The Golgi is also reported to be the site of origin for vesicles destined for export from the cell via the secretory pathway that express MDR1 (42) and MRP2 (42). Thus, the finding of F-DDP in both golgin97 and MRP2-positive vesicles is consistent with the proposal F-DDP passes through the Golgi and is then directed to vesicles belonging to the secretory pathway. Further evidence is provided by the observation that F-DDP becomes associated with vesicles expressing ATP7A, another marker of vesicles belonging to the secretory pathway (43).

The association of F-DDP with MRP2 is consistent with a previous study suggesting that MRP2 plays a role in the efflux of DDP (44). It is noteworthy that the majority of MRP2-expressing vesicles that contained F-DDP were located deep in the interior of the cells and not at the plasma membrane. This suggests that, in these carcinoma cells that lack polarity, MRP2 functions to sequester F-DDP, or its glutathione conjugates, into intracellular vesicles that are subsequently exported rather than pumping F-DDP directly out of the cell across the plasma membrane.

The substantial degree of colocalization between F-DDP and the copper efflux transporter ATP7A is of particular interest given that ATP7A has now been shown to control the cellular pharmacology of DDP and sensitivity to the cytotoxic effect of this drug (23, 45). Many malignant cells also express the other copper efflux transporter ATP7B, and ATP7B has also been shown to modulate the cellular pharmacology of DDP and sensitivity to its cytotoxic effect (2, 6). Recently ATP7B has been shown to colocalize with F-DDP in vesicles of the secretory pathway (14).

Figure 8 presents a schematic diagram of the trafficking of F-DDP in human ovarian carcinoma cells and summarizes the available information from this and prior studies (6, 13, 14, 23, 38, 45). F-DDP becomes associated with lysosomes and golgin97-expressing vesicles of the Golgi and is also found in vesicles that express markers identifying them as belonging to the secretory pathway that eventually exports vesicles from the cell. Disruption of the secretory pathway with brefeldin A, wortmannin, or H89 produced effects consistent with the hypothesis that F-DDP first enters an upstream compartment and is then transferred to the vesicle secretory pathway and hence out of the cell. Whether this upstream compartment consists solely of components of the Golgi, the lysosomes, or both cannot be determined with the available data, although the fact that wortmannin prevented loading of F-DDP into golgin97-positive vesicles suggests that the flux of F-DDP is predominantly from the lysosomal compartment toward the Golgi. These drugs increased total cellular F-DDP, increased the F-DDP content of vesicles associated with the lysosomes at the upstream end of the pathway, and decreased the flux of F-DDP into vesicles at the downstream end of the secretory pathway. The accumulation of F-DDP in vesicles expressing TGN38 in the brefeldin A and wortmannin-treated cells suggests that inhibition of the delivery of F-DDP from the prelysosomal pathway to downstream compartments caused the backup of F-DDP into an upstream compartment which also receives input from the trans-Golgi network. The concept that a substantial fraction of the efflux of DDP is mediated by sequestration into vesicles that are then exported from the cell is further supported by the fact that wortmannin and H89 increased whole cell accumulation of both F-DDP and native DDP.

The finding of high levels of F-DDP in lysosomes is of particular interest given the observation that cells selected for DDP resistance have major defects in lysosomal function (38, 46). The fact that F-DDP did not colocalize with EEA1-expressing precursor early endosomes suggests that F-DDP enters the lysosomes via another uptake pathway, perhaps via a pinocytotic mechanism (47). Further work is required to determine the exact sequence of drug sequestration into the lysosomes and the mode of its delivery to the secretory compartment.
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REFERENCES

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