The Role of Cellular Accumulation in Determining Sensitivity to Platinum-Based Chemotherapy*

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cisplatin, carboplatin, resistance, cellular accumulation, drug uptake

Abstract

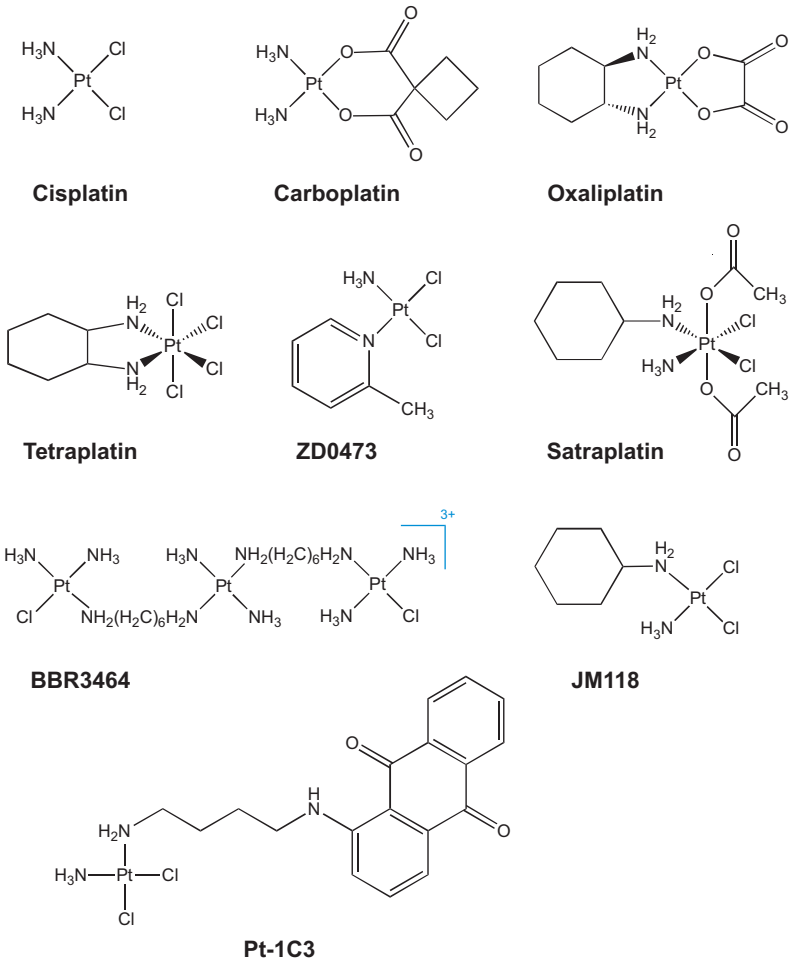
The platinum (Pt) drugs cisplatin and carboplatin are heavily employed in chemotherapy regimens; however, similar to other classes of drugs, a number of intrinsic and acquired resistance mechanisms hamper their effectiveness. The method by which Pt drugs enter cells has traditionally been attributed to simple passive diffusion. However, recent evidence suggests a number of active uptake and efflux mechanisms are at play, and altered regulation of these transporters is responsible for the reduced accumulation of drug in resistant cells. This review suggests a model that helps reconcile the disparate literature by describing multiple pathways for Pt-containing drugs into and out of the cell.

INTRODUCTION

The development of resistance to platinum (Pt)-based chemotherapy in the clinic is a major challenge for cancer chemotherapy. Although the phenomenon of multidrug resistance against natural product drugs exemplified by the ATP-dependent efflux pump P-glycoprotein and other transporters is well characterized (1), the cellular responses that confer resistance to cisplatin (DDP, *cis*-[$PtCl_2(NH_3)_2$], **Figure 1**) are multifactorial and less well understood (2, 3). This is particularly so for the reduced drug accumulation commonly reported in Pt drug-resistant cell lines (4). The generally accepted intracellular mechanisms by which cells acquire resistance to cisplatin and its congeners are (*a*) increased detoxification of drugs by the thiols glutathione and metallothionein; (*b*) improved repair of, and tolerance to, nuclear lesions, leading to a concomitant reduction in apoptosis; and (*c*) diminished accumulation of cisplatin (3, 5).

Figure 1

Platinum complexes described in the text.



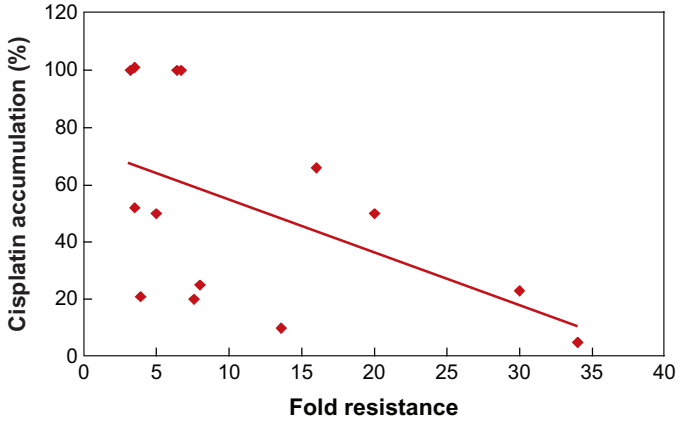


Figure 2

Plot of cisplatin-resistant human-derived cell lines showing the correlation between the fold-resistance of cell lines and the percent decrease in cisplatin accumulation compared with the parental cell line. Adapted from data in References 8 and 9, representing nine unique parental cell lines and fourteen selected cell lines.

Studies reported over the past 30 years have assessed the ability of Pt-containing drugs to accumulate in cancer cell lines and measured the ability of new compounds to accumulate in resistant cells (thereby circumventing resistance), and they have consistently demonstrated that accumulation of drug is a determinant of cellular sensitivity (6, 7). In a survey of human- and rodent-derived cisplatin-resistant cell lines reported by Andrews & Howell, most cell lines demonstrated reduced cisplatin accumulation relative to their parental strains (adapted with new data for **Figure 2**), and accumulation defects were more common than increased glutathione expression (8). Johnson et al. reported a strong correlation ($r = 0.98$) between cisplatin accumulation and relative cisplatin resistance for a series of increasingly resistant lines derived from the BEL7404 human hepatoma cell line (9). Koga et al. examined seven primary bladder cancer cell lines derived from untreated transitional cell cancer of the urinary bladder (10) and found a positive correlation between cisplatin accumulation and sensitivity ($r = -0.778$) among the intrinsically resistant cell lines, but no correlation with GSH levels or expression of a range of proteins (10). **Figure 2** demonstrates this general trend for a range of resistant cell lines.

It is important to note that the diminished accumulation[1] of drug with increasing resistance is not unique to cisplatin, and it has been observed for the clinical analogs carboplatin ([Pt(CBDCA)$(NH_3)_2$]) (11) and oxaliplatin ([Pt(oxalato)(*R*,*R*-chxn)], L-OHP) (12) and compounds in clinical trials such as tetraplatin ([$PtCl_4$(chxn)]) (13) and ZD0473 (*cis*-[$PtCl_2(NH_3)$(2-methylpyridine)]) (14). In addition, cross-resistance among Pt complexes was observed at an early stage in Pt drug research (15), and in

[1]There is some variability in the literature in the terms used to describe drug flux into and out of cells, and total intracellular drug levels. Accumulation is used here to avoid the possible ambiguity of the term uptake. The term uptake can be interpreted to reflect only the passage of drug into cells irrespective of the mechanism of cell entry, whereas the net total drug levels within a cell at the end of a given period of exposure is the accumulation of drug. The exit of drug from cells is termed efflux. Uptake and efflux of a molecule can be by passive diffusion down the concentration gradient, by facilitated transport down a concentration gradient, or by active (energy-dependent) transport insensitive to the concentration gradient. Reduced uptake versus increased efflux is not as easy to demonstrate as might be supposed because total accumulation is most often used as a parameter in the literature and efflux can be rapid enough to mimic reduced uptake.

some cases reflects reduced accumulation of these compounds (although there are exceptions to this, see below) (11).

There are a number of complicating factors associated with examining cisplatin-accumulation defects; a wide range of cell lines from different tissue types have been utilized in studies, with resistant cell lines usually selected by chronic exposure to physiologically unrealistic levels of Pt drug to elicit a measurable alteration in cisplatin accumulation. Further, the relative importance of each of the resistance mechanisms described in vitro has not yet been demonstrated to correlate with intrinsic or acquired resistance mechanisms in clinical samples. Stewart et al. examined Pt concentrations in human autopsy samples and demonstrated that patients whose tumors responded to Pt-containing therapies had higher tumor Pt concentrations than those that failed to respond, seeming to indicate that Pt accumulation is an important factor for clinical efficacy (16).

The major questions relating to Pt drug accumulation are:

1. How does cisplatin enter cells?
2. Is cisplatin actively extruded from cells, and if so how?
3. What are the cellular changes in acquired resistance associated with reduced cisplatin accumulation?

The diverse and often disparate literature on the mechanisms by which cisplatin and its congeners enter and leave cells has not been assessed in detail since an excellent review by Gately & Howell published in 1993 (6) [more recent results have been summarized in part by Andrews (17)]. The conclusions of this survey were that cisplatin enters the cell by passive diffusion and through gated channels (6). Early studies noted that cellular uptake of cisplatin was linear, concentration dependent, and nonsaturable—all hallmarks of passive (rather than actively mediated) drug entry into the cell. Barnett Rosenberg, the discoverer of cisplatin, stated that, "the drug is passively transported across the cellular membrane—no active transport is necessary," and this paradigm seems to have ruled drug design approaches (18). This review explores more current data in the literature and from our own laboratory, and we conclude that accumulation of cisplatin occurs by a variety of mechanisms, including passive diffusion and facilitated transport by multiple transport systems. To reduce drug accumulation to a significant extent, or to confer cross-resistance to multiple cytotoxic Pt-containing drugs, cells must simultaneously inactivate more than one of these transport systems, and this pleiotropic response occurs in cells selected for cisplatin resistance in vitro. These uptake systems vary depending on the species of Pt drugs under consideration, so we begin with a detailed consideration of the chemistry of Pt species [see below and **Supplemental Section** on Speciation of Platinum Drugs (follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**)].

SPECIATION OF PLATINUM DRUGS

The question of how Pt drugs enter cells is inextricably tied to an understanding of what specie(s) enter cells. One of the main assumptions of studies into cellular uptake,

and associated cisplatin resistance mediated through diminished drug accumulation, is that the neutral, intact drug is the species that traverses the lipid bilayer by passive diffusion. Conventional wisdom has been that neutral cisplatin is prevented from aquation/hydrolysis outside the cell owing to the high chloride levels present in plasma and media (19). Recent results question the need for a neutral Pt complex for efficacy (20). Farrell and colleagues have reported a series of "rule-breaking" multinuclear complexes that show increasing accumulation with increasing positive charge (21), and a number of transporters have been implicated in Pt drug accumulation defects that do not necessarily transport intact drug. It may be that one or more biotransformation products of Pt drugs contribute to the pool of drug that enters cells by a number of different pathways (see below).

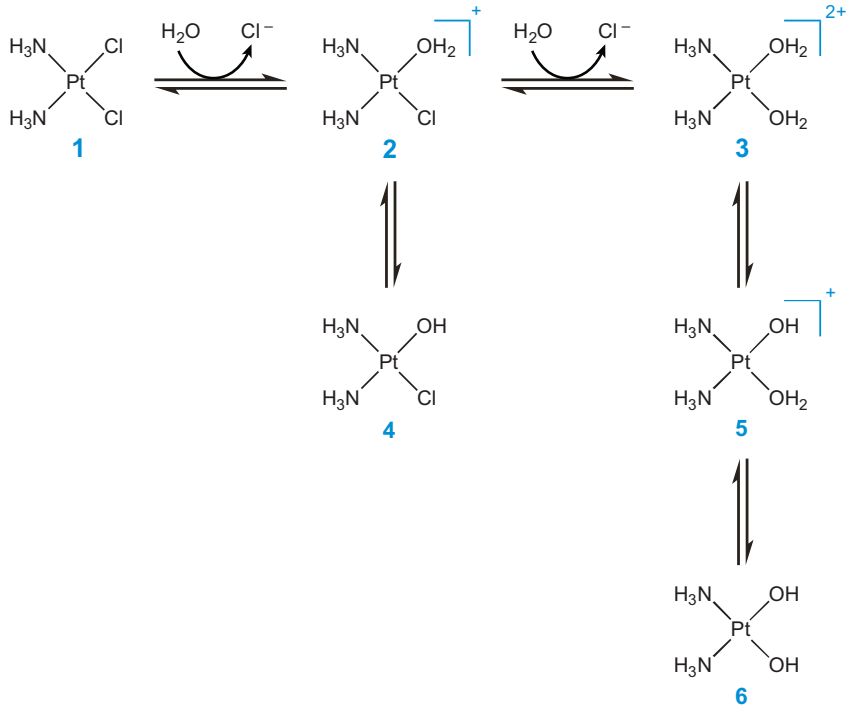
The key mechanistic step in the activation of cisplatin is the first aquation step; the replacement of a chloride leaving group by water (H_2O) gives a singly positively charged aquachloro species (**2** in **Figure 3**) that can then be hydrolyzed to a hydroxo ligand (OH) yielding the neutral chlorohydroxo complex (**4** in **Figure 3**). Note that **2** is neutral, and could also potentially diffuse across the lipid bilayer. The proportion of aquachloro (**2**) to chlorohydroxo (**4**) species is dependent on the pK_a of the hydroxo ligand, and hence the pH of the solution (22). It is the aquated species (**2**) that is more reactive (labile) and can subsequently bind to the N7 of guanine and adenine on DNA in the cell, the generally accepted cytotoxic target of cisplatin. Speciation studies using a range of techniques, particularly those pioneered by Appleton and Berners-Price/Sadler using isotopically labeled ^{15}N cisplatin (see Reference 23 for an excellent review of these techniques and experimental examples), have demonstrated that the aquation of cisplatin is suppressed (but not prevented) in a high-chloride environment (~104 mM) of blood plasma and equivalent media, compared with the low (4 mM) chloride concentration in the cytoplasm. Jennerwein & Andrews have examined the intracellular concentration of chloride in a range of human carcinoma cell lines, normally stated to be 4 mM, and shown it to range from 20–55 mM (24).

A number of studies have examined cisplatin aquation at varying pHs, concentrations of chloride, and temperatures (reviewed in References 25, 26), incorporating equilibria and acidity constants determined primarily by House and coworkers (22). These have been used to determine the relative proportions of the aquation species present at high and low chloride concentrations (though the dynamic nature of plasma means a true equilibrium never exists). **Figure 3** includes two sets of data from the literature showing the percent of each of the aquation products of cisplatin present at pH 7.4.

The two sets of values cited in **Figure 3** for high chloride concentration are in good accord with one another and reveal that at a high chloride concentration, the two dominant species are cisplatin (**1**) and the chlorohydroxo species (**4**), both of which are neutral and relatively unreactive. The only other significant species is the reactive aquachloro (**2**) complex that would be expected to undergo rapid reaction and deactivation in biological media. Given the pK_a equilibrium that exists between **2** and **4** in solution, experimental determination of the lipophilicity of **4** is difficult. However, using experimentally verified theoretical prediction (27), it has been calculated that the lipophilicity of cisplatin (**1** in **Figure 3**, $\log P_{oct} = -2.4$) is actually similar to

Figure 3

Schematic showing the stepwise aquation and hydrolysis of cisplatin in aqueous solution. The percentage of each species calculated to exist at equilibrium (pH 7.4) in high- (100 mM and 104 mM) and low-chloride (4 mM) concentrations is also shown in the table below.



Compound	104 mM (26)	100 mM (22)	4 mM* (26)
1	67	68	3
2	4	7	5
3	<1	<1	<1
4	26	24	30
5	<1	<1	28
6	1	<1	35

*Note that reported fractions total to >100%.

that of the hydroxo species *cis*-[PtCl(OH)(NH_3)$_2$] (**4**, log $P_{oct} = -2.7$), and as such both species have a similar potential for diffusing across a lipid bilayer (28).[2]

The assumption that cisplatin diffuses into the cell carries the implicit expectation that charged species could not enter the cell by such means. While the doubly aquated species **3**, **5**, and **6** are observed in simple aquation studies, their formation in biological media is doubtful given the rapid reaction of the singly aquated

[2]Although it may seem initially surprising that displacement of chloro with hydroxo gives a similarly lipophilic complex, Lewis acidity barely affects lipophilicity. For example, ethanol and dimethylether have similar log P_{oct} values.

species with biomolecules in the extracellular medium and cell membrane. Hambley and coworkers observed only the monoaquated species when using HSQC NMR to monitor the reaction of cisplatin with DNA, as this reacted with nucleobases at a rate greater than that which would lead to formation of the diaqua species (29).

Given the near ubiquitous use of cell lines for studies of Pt uptake, it is important to consider that the concentration of chloride in blood plasma is usually reported as around 104 mM, and the chloride concentration in RPMI growth medium is 108 mM and phosphate buffered saline (PBS) is 137 mM. PBS is usually employed for short-term exposure of drug by researchers who want to minimize aquation and side reactions with components of media. The levels of phosphate in PBS (~9 mM) do not appear to interfere with the primary aquation products of cisplatin, although Davies et al. report that the formation of phosphate products with the diaqua species of cisplatin occurs after 3 h in NMR spectra (30). The commercial formulation of cisplatin (Platinol in the United States) is supplied in 154 mM NaCl solution (31).

Significantly, Dabrowiak and coworkers examined the aquation of cisplatin in RPMI growth medium and observed that the major long-lived metabolite of cisplatin is, in fact, a carbonato complex, *cis*-[PtCl(OCO_2)(NH_3)$_3$] (**7** in **Figure 4**) (31). In carbonate buffer (but not growth medium), the bicarbonato complex (**8**) is also detected. Given that the concentration of carbonate in growth media and blood plasma is ~24 mM, and the propensity of carbonate as a ligand for metal ions (32), it is surprising that these species were not considered sooner. When Jurkat T-lymphocyte cells were added to the growth media, the signal corresponding to **7** disappeared rapidly, suggesting that the negatively charged complex may be rapidly accumulated by the cells.

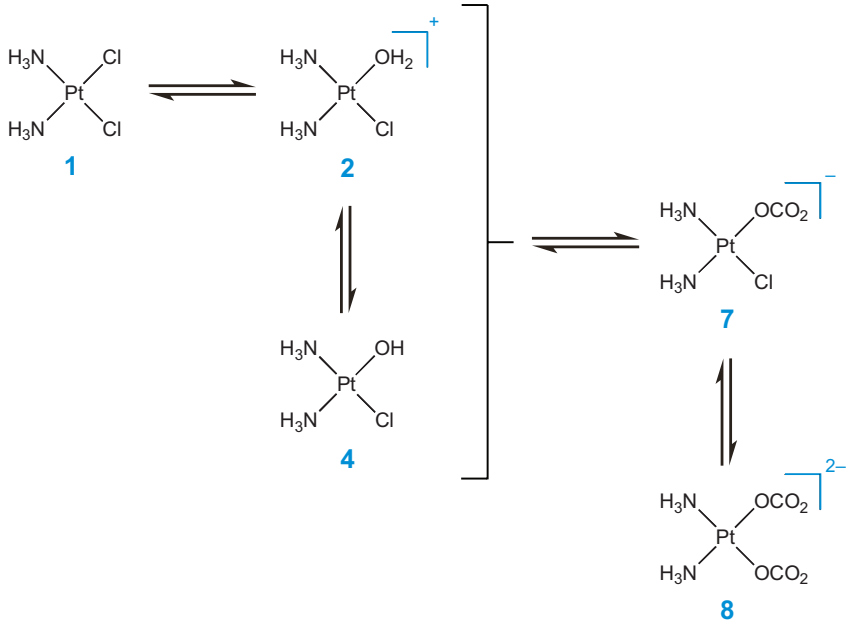


Figure 4

The formation of carbonato species. (**7**) *cis*-[PtCl(OCO_2)(NH_3)$_3$] can form in both growth medium and carbonate buffer; however, the dicarbonato (**8**) is only observed in carbonate buffer. Adapted from Reference 31.

Similar considerations come into play with carboplatin and other Pt compounds (see **Supplemental Section** on Speciation of Platinum Drugs and References 33–38).

As well as exchange reactions with electrolytes and small molecules, cisplatin can bind covalently and noncovalently (reversibly) with proteins present in cell growth media. It is generally reported that protein binding renders Pt drugs inactive (39), and cisplatin preincubated with serum protein is not cytotoxic in vitro (40) or active in vivo (41). Preincubation in cell culture medium also diminishes cisplatin cytotoxicity to a lesser extent (42). Hambley and coworkers have shown that after 6 h in RPMI medium supplemented with FCS at 37°C (standard cell line growth media conditions), 63% of the cisplatin analogue *cis*-[$PtCl_2$(en)] was free (passed through a 30 kDa filter), and 25% was free after 24 h (43). The ultrafilterable portion has been shown to be cytotoxic (44). Although reactions with proteins occur in media, a considerable portion of the drug is free to enter cells during short (0–4 h) exposures. Pt binding to human plasma proteins is also to some extent reversible (45), although Melvik et al. have shown that protein-bound Pt cannot enter cells (40).

That metabolites of cisplatin can enter the cell is supported by a number of studies (46–49). Cisplatin accumulates in cells more rapidly at pH 6 than at pH 7.4 (extracellular tumor pH is often lower than 7.4), suggesting that a species other than cisplatin (which does not have a pK_a in the pH range 4–8) can be taken into cells (50), and resistant cells show less pH dependence on uptake than sensitive cells (51). Species that cannot hydrolyze, such as $[Pt(en)_2]^{2+}$, are not detectable in cells in short-term experiments (47). Jennerwein & Andrews found that aquated cisplatin accumulated in the OV 2008 human ovarian carcinoma cell line at the same rate as intact cisplatin, and that 1.9 times more DNA binding was found in cells treated with aquated cisplatin. Using the same cell line, Shirazi et al. prepared a range of cisplatin metabolites, aquated cisplatin, mono- and bis-methionine products, and ultrafiltered human plasma incubated with cisplatin, and examined their activity alongside cisplatin (48). The aquated cisplatin (created by dissolving cisplatin in water) was three times more active than cisplatin after 1 h exposure, and the mono-methionine and ultrafilterable cisplatin preparations showed similar potency to cisplatin (the bis-methionine preparation was inactive). Again, the accumulation of the metabolites after 1 h correlated with their activity ($r = -0.997$), and the aquated cisplatin showed a threefold increase in accumulation compared with cisplatin, suggesting that an aquated species can be preferentially accumulated (48). That the aquated, mono-methionine and plasma ultrafiltered preparation all showed greater or equipotent activity to cisplatin demonstrates the complicated nature of the mechanisms of cisplatin accumulation and activation. These observations are supported by Ehrsson and coworkers, who isolated pure mono-aquated cisplatin (**2**) and showed it to be more active against the U-1285 small-cell lung cancer cell line than cisplatin (49).

An understanding of the specific cisplatin metabolites that form and the nature of those that can enter cells is essential. Indeed, given the dynamic nature of aquation and the compartmentalization of drug into proteins, it is possible that a number of species enter the cell by different routes with varying kinetics, and determining the routes of entry of active drug into the cell may enable improved drug design.

ARGUMENTS FOR PASSIVE DIFFUSION

As noted above, early experiments incubating cells with increasing concentrations of cisplatin and analogues revealed linear uptake that was not saturable against either time or drug concentration (up to 3 mM), consistent with passive diffusion down a concentration gradient (52–56). In one of the first papers on uptake, using tritiated *cis*-[$PtCl_2(py)_2$] (py = pyridine), Gale et al. showed linear uptake in Ehrlich ascites tumor cells was limited only by solubility, which was confirmed by a double-reciprocal plot of concentration against uptake that intersected with the origin (54). Gale (54) and Ogawa (57) found little difference in accumulation and potency at 4°C and 37°C and increased uptake at 60°C, also suggestive of passive entry into cells. Similar studies with everted rat intestine, L1210 mouse leukemia cells, and OV2008 human ovarian carcinoma cells supported these conclusions (52, 56, 58, 59).

Gale and Andrews also showed that other compounds, including Pt compounds, could not lower accumulation by competitive inhibition as would be expected if a unique active transporter were at play, whereas compounds that compromised membrane integrity increased accumulation (51, 54). Other compounds believed to enter the cell by passive diffusion alone, such as mannitol, show similar uptake profiles in cisplatin-sensitive and -resistant cells (60), and long-term exposure to Pt drugs correlates well with drug log P_{oct} values, probably because it allows for equilibration via passive diffusion (27, 61); however, in vivo peak plasma drug concentrations persist for a short period of time, and uptake kinetics during short-term exposure are more likely to be relevant to drug efficacy. Results showing nonsaturability of uptake of cisplatin are consistent either with a major component of passive diffusion at high cisplatin concentrations or uptake via other nonsaturable systems, such as fluid-phase endocytosis. The latter process, which allows compounds to enter cells via membrane-lined invaginations, would also be expected to be energy dependent, as has been demonstrated (see below).

CHANGES IN PLASMA MEMBRANE COMPOSITION AND BIOPHYSICS

If plasma diffusion is the major mechanism of cisplatin entry into cells, then cell lines that show reduced uptake (or accumulation without increased efflux) should have alterations in the biophysical properties of the plasma membrane. There have been a number of studies into the interactions of cisplatin, primarily with erythrocyte membranes, showing that cisplatin perturbs membranes (reviewed partially in Reference 62 and summarized in the **Supplemental Section** on Changes in Plasma Membrane Composition and Biophysics), although few focus on the nature of cisplatin's passage across the cell membrane. Few or no changes have been seen in biophysical studies of plasma membrane function in cisplatin or carboplatin-resistant cell lines (see **Supplemental Section** on Changes in Plasma Membrane Composition and Biophysics and References 63–65). Collectively, there is evidence that a drug can enter a cell by passive diffusion; however, given that resistant cells demonstrate only small changes (if any) in their membrane composition and biophysics, the lowered

accumulation must be due to other alterations. The relative amount of drug entering a cell by passive diffusion is dependent on the concentration of drug: at low drug concentrations, active transporter(s) may mediate the uptake of the majority of drug, but at high concentrations most uptake would be via passive diffusion.

The ability of drugs to enter cells by passive diffusion led to the design of lipophilic Pt complexes, such as satraplatin, that are capable of circumventing cisplatin resistance through increased accumulation in resistant cells, reducing the need for any active component of uptake (66). It may be that Pt(IV) complexes can only enter cells by passive diffusion. Hall et al. showed that a series of relatively hydrophilic Pt(IV) complexes demonstrated lower rates (versus concentration and time) of accumulation than cisplatin in A2780 cells; however, although cisplatin accumulation is halved in the A2780 cisR line, the Pt(IV) accumulation is marginally reduced, suggesting that removing the active transport does not affect Pt(IV) uptake (67). This effect is also observed in the CH1/CH1cisR pair, where the accumulation of *cis*- and *trans*-Pt(IV) complexes and satraplatin [a Pt(IV) drug] was unaffected. Resistant lines generated from the 41M and CH1 human ovarian carcinoma cell lines showed a range of cellular alterations (increased MT and DNA repair), but accumulation was unaffected in both resistant lines, suggesting that no alterations in membrane composition occur in these cell lines to reduce Pt(IV) drug uptake (66).

A number of membrane-disrupting agents, such as the detergent gemcitidine and the antifungal drug amphotericin B, have been shown to enhance Pt drug accumulation in vitro (68, 69) and in vivo (70) by facilitating passage of a drug across the cell membrane. Other compounds, such as spermidine, have been shown to increase cellular accumulation by an as-yet-unidentified mechanism (the agents are detailed in the **Supplemental Section** on Membrane Disrupting Agents).

EARLY EXPERIMENTS ON CARRIER-MEDIATED UPTAKE

The first postulation that carrier-dependent processes were involved in cisplatin uptake was made by Byfield & Calabro-Jones in 1981, who observed that proliferating T-lymphocytes were more sensitive to cisplatin than resting cells, as were other drugs known to be carrier-mediated, such as melphalan (amino acid carriers) and nitrogen mustard (choline carrier) (71). This was in contrast to carrier-independent drugs, such as mitomycin C, which showed identical survival curves for resting and proliferating cells. The authors argued this effect was due to enhanced import of drugs in cycling cells. However, the authors did not consider cell phase-specific events, the actual accumulation of drug was not measured, and as cisplatin was prepared in double-distilled water, a significant amount of drug would have been aquated (**Figure 3**), which may have exaggerated any transport effect.

Early experiments into the accumulation of cisplatin employed compounds that nonspecifically disrupted membrane protein function. Dornish et al. conducted a series of early experiments with cisplatin and found that the protein reactive agent benzaldehyde inhibited cisplatin's activity in the NHIK 3025 human cervical carcinoma line (72), and cellular accumulation was shown to be halved in the presence of the aldehydes benzaldehyde, pyridoxal, and pyridoxal 5′-phosphate (73, 74). When

cells were electropermeabilized, benzaldehyde no longer affected accumulation, and structural analogs not containing a reactive aldehyde functional group did not affect accumulation (73). Aldehydes form Schiff base imine bonds with protein amine groups (75), and given that the cell-impermeable aldehyde pyridoxal 5′-phosphate also lowered drug accumulation, it is likely that it deactivates transporters and prevents active accumulation, although no controls (other drug classes or endogenous substrates) were employed to confirm this general effect (74).

The implication that transporters can mediate a portion of cisplatin accumulation in cells was reinforced by observations that the metabolic inhibitors dinitrophenol or sodium fluoride alone did not affect accumulation, but a combination with iodoacetate did reduce accumulation by almost half in 2008 cells, but to a lesser degree in resistant 2008/DDP cells, suggesting the active component of uptake is already lost in the resistant cells (52). Shen et al. also showed that carboplatin uptake in 7404 cells was temperature dependent, and ATP depletion by antimycin A or oligomycin reduced uptake in 7404 cells to levels observed in 7404-CP20-resistant cells (11).

Andrews et al. noted that preincubation with the Na^+,K^+-ATPase-specific inhibitor ouabain reduced cisplatin accumulation in both the sensitive and resistant 2008 cells by up to 50% (52, 76). Na^+,K^+-ATPase maintains the sodium gradient across the cell membrane (pumps sodium out and potassium into the cell), and a number of facilitated transporters are dependent on this gradient for function. Na^+,K^+-ATPase also regulates cell volume, although cell volume was not affected at the concentrations used (that would artificially alter Pt accumulation values) (76). Short-term accumulation experiments showed that drug accumulation was immediately reduced on exposure, suggesting Na^+,K^+-ATPase inhibition affects influx; replacement of sodium with choline in cell growth media reduced cisplatin uptake by a similar amount as ouabain; and cisplatin uptake increases with extracellular sodium concentration, suggesting it is the sodium gradient dissipation rather than Na^+,K^+-ATPase inhibition that lowers cisplatin uptake (76). Andrews did not find a difference in Na^+,K^+-ATPase expression or function between 2008 and 2008/DDP cell lines, but Fujimara did observe lowered function in PC-9/CDDP cells (77).

Interestingly, tissues subject to cisplatin toxicity, such as kidney and the inner ear, do express high levels of Na^+,K^+-ATPase (76, 78, 79). How the observation that decreased osmolarity enhances cisplatin accumulation relates to the requirement for a high sodium gradient remains to be determined (80). D-methionine reverses cisplatin toxicities associated with Na^+,K^+-ATPase owing to its antioxidant properties (e.g., 81), although in actuality, this is probably due to methionine chelation of cisplatin and subsequent inactivation, as a number of sulfur-containing amino acid analogs have been shown to be capable of inhibiting nephrotoxicity (82). This may be related to the observation that cisplatin inhibits tetraethylammonium uptake by organic base transporters [see Organic Cation Transporters (The SLC22 Family) below] that are highly expressed in kidney tissues, but cisplatin-methione complexes do not (83). Perhaps SLC transporters present in tissues require Na^+ cotransport, or a high Na^+ gradient for cisplatin transport.

Sharp et al. showed in two human ovarian carcinoma cell lines (41M and CH1) that low temperature or ouabain reduced accumulation. However, accumulation was

not affected in the resistant lines (41McisR6 and CH1cisR6, respectively), suggesting active/facilitated transport was lost in these selected lines that probably required a Na^+ gradient for optimal function (84). Others have subsequently shown a greater decrease in accumulation with ouabain or ATP-depleting conditions in a range of parental cells compared with resistant lines (85–87). Kishimoto found that expression of the Na^+,K^+-ATPase A1 subunit was reduced in H4-II-E/CDDP-resistant cells. Bando et al. demonstrated that the expression of Na^+,K^+-ATPase alone is not sufficient for increased cisplatin accumulation because, even though Na^+,K^+-ATPase was expressed in SCLC and NSCLC cell lines, ouabain inhibition lowered accumulation in three NSCLC lines, but not in three SCLC lines (88).

Experimental evidence indicates that, in part, Pt drug uptake can be mediated by active or facilitated influx. Nonspecific aldehydes prevent membrane transporters from functioning and energy-depleting agents lower accumulation either directly or indirectly. Cisplatin is not a substrate for Na^+,K^+-ATPase (89), and the inhibition of Na^+,K^+-ATPase by ouabain reduces the Na^+ gradient across the cell membrane that drives active/facilitated transport of cisplatin, although the specific transporters that rely on this sodium gradient have not been identified. The observations of Bando et al. suggest that the mechanisms of drug uptake are tissue-specific (88) and are dependent on the endogenous expression of transporters. An understanding of tissue-specific transporter expression may allow for tailored therapies that enhance cisplatin uptake and efficacy.

THE SEARCH FOR TRANSPORTERS

If a single transporter is at play in the accumulation of cisplatin, then either saturation of accumulation kinetics and/or substrate specificity would be expected. Although saturable kinetics has not been generally observed in native or transfected cells (discussed above), this could be attributed to a number of transporters, with various affinities for cisplatin, operating collectively to accumulate drug. Adequately designed experiments provide evidence of influx transport kinetics. When synthetic diaquated cisplatin (**6** in **Figure 3**) was incubated with cells, the initial rate of uptake was 40-fold higher than that for cisplatin (but equal in both cell lines), greater at pH 6 than pH 7.4, but most importantly, tended to be saturable, suggesting the active transport of aquated cisplatin species can occur (47). Analysis of uptake kinetics yielded a k_M = 1.9 mM indicative of a low-affinity transporter, which certainly accounts for the lack of saturable uptake in studies that limit concentrations to no higher than 500 μM.

Gately and Howell proposed that cisplatin accumulation could be facilitated via a gated channel, but cisplatin's minimum cross-section (3.97 Å by 6.92 Å) is greater than that of most channel pores (17), with the possible exception of aquaporin 9 (AQP9), recently reported (by two-dimensional crystallography) to have a pore size of approximately 7 Å by 12 Å (90). AQP9 has been shown to permit the passage of neutral molecules, such as glycerol, urea, purines, and pyrimidines (91), and its expression correlates with As_2O_3 (arsenite) accumulation in myeloid and lymphoid leukemia lines (92). Although the ability for the aquaporins to transport Pt drugs has not been directly demonstrated, we note here (**Supplemental Figure 3**) that

Pt-resistant lines have reduced expression of AQP2 and AQP9, presenting a potential new Pt drug transport family.

Influx selectivity of Pt drugs has been demonstrated: Helleman et al. showed that the A2780-Pt ovarian carcinoma cell line displayed reduced accumulation of the *cis* Pt drugs cisplatin, carboplatin, tetraplatin, and oxaliplatin, but transplatin accumulation was unaffected (93). This loss of *cis*-specific drug uptake in resistant cells is probably not uncommon, but *trans* complexes are examined less often owing to the (erroneous) assumption that *trans* complexes are inactive (94). The unchanged transplatin accumulation is similar to the effect described above for Pt(IV) complexes, and suggests that the A2780-Pt cells are lacking a transporter or transporters on the cell surface that the parental line possesses.

More recently, proteomic and mRNA microarray studies comparing cisplatin-sensitive and -resistant cell lines have rarely identified altered gene or protein expression associated with cellular accumulation (either influx- or efflux-related), and there is a lack of significant cross-over in hits between studies (93, 95–103). It should be noted that this feature is not unique to transport proteins, and no dominant biochemical pathway emerges from analyses, although mRNA and proteomic screens do not take account of alterations in protein localization (discussed below) (95). The only statistically significant alterations in transporter expression reported in these publications are an increase in SLC27A2 and SLC2A3 and the general decrease in the mRNA levels of five transport proteins (none of which are implicated in drug resistance) between the Tca8113- and Tca/cisplatin-resistant human oral squamous cell carcinoma cell lines (102), and a small change in ABCA13 and SLC22A2 expression in the IGROV-1/CP ovarian cancer cell line (101). SLC22A2 is discussed below.

CTR1 COPPER INFLUX TRANSPORTER

CTR1 (copper transporter 1, *SLC31A1*) is an evolutionarily conserved copper influx transporter present in plants, yeast, and mammals, and is the main copper importer in mammalian cells. The human version, hCTR1, is expressed in all tissues and is a key player in the exquisite homeostatic regulation of intracellular copper levels to ensure that nutritional delivery of copper to enzymes such as cytosolic Cu,Zn-superoxide dismutase and mitochondrial cytochrome oxidase is maintained while surplus copper, which can cause toxicity, is avoided (104). Human hCTR1 is a 197–amino acid protein containing three transmembrane domains residing mainly in the plasma membrane, transporting Cu in a temperature-, pH-, and K^+-dependent (but Na^+-independent) manner with saturable kinetics that are not energy-dependent (105). The transporter can trimerize, forming a channel-like pore that facilitates substrate transport (106). An extracellular amino terminus of CTR1 containing a methionine- and histidine-rich domain has been implicated in initial Cu binding (note that both Met and His side groups are good Pt ligands), followed by Cu internalization by a little-understood mechanism that is believed to result in lysosomal Cu sequestration prior to cytosolic trafficking.

The role of CTR1 in cisplatin accumulation has been reviewed, and only the key observations pertaining to Pt drug influx are described here (107, 108). Deletion of the

Saccharomyces cerevisiae CTR1 gene lowers accumulation of, and increases resistance to, cisplatin, and coadministration with copper inhibits cisplatin uptake in wild-type (Ctr1p expressing) strains (109, 110). Yeast Ctr1p was also demonstrated to transport oxaliplatin, carboplatin, and ZD0473 (110), and mouse mCtr1 knockout strains also show reduced cisplatin accumulation (109). Although transfection of hCtr1 cDNA into SCLC cell lines conferred sensitivity, Song et al. showed that of five resistant small-cell lung cancer (SCLC) cell lines, only one (SR2) showed a slight reduction in hCtr1 protein expression, along with a reduction in the accumulation of cisplatin, carboplatin, and oxaliplatin (111). Alternatively, Zisowsky et al. reported a reduction in hCtr1 mRNA levels in A2780cis-resistant ovarian and HeLaCK cisplatin-resistant cell lines (112). Collectively, this suggests that the contributions of CTR1 to Pt drug accumulation and the development of resistance are tissue-specific phenomena.

Although CTR1 has been shown to be capable of transporting a number of Pt analogs, there are clear exceptions that suggest some structural discrimination is exercised by the transporter. Howell and coworkers have shown that while cisplatin and carboplatin accumulate with high affinity in mouse embryonic CTR+/+ cells (compared with CTR−/− cells), oxaliplatin accumulation appears not to be as dependent on CTR1 for uptake into cells, although CTR−/− cells still accumulate drug, albeit at a lower rate (113). It has also been shown that the Pt(II) analog of satraplain, JM118 [*cis*-ammine(aminocyclohexyl)dichloroplatinum(II)] is not a CTR1 substrate (114).

CTR1 can transport metal ions other than Cu but with much lower affinity, and the nature of the metal ion speciation is unclear. It may be that a hydrated metal ion passes through the trimeric pore, although given the requirement for the Cu(I)-selective extracellular methionine residues (copper-sensing domain) for transport (115, 116), it seems likely that the Cu is chelated by methionine residues before internalization of copper. Is copper transported by CTR1 in the same fashion as Pt drugs? Their coordination chemistry is remarkably different; Cu has two readily accessible oxidation states under physiological conditions (+I and +II), whereas Pt(II) drugs remain in their +II oxidation states; their geometries are different; and Pt complexes are more inert to ligand exchange. Cisplatin treatment promotes a stable homotrimeric form of CTR1, probably owing to intrameric cross-linking by Pt, especially given that Pt(II) also has a high affinity for the thioether sulfur donor contained in methionine residues (117).

Given that Pt drugs may be binding to methionine residues as part of CTR1-mediated transport, one must question whether the Pt pool imported is cytotoxic, or if it loses the ligands required for activity. Cells with increased CTR1 expression demonstrate increased Pt accumulation (109, 113), and in most instances increased sensitivity to cisplatin (109), yet DNA platination did not increase in A2780 transfected to express 20-fold more hCTR1 than parental cells (118). It seems that Pt is transported into cells, but perhaps the am(m)ine ligands are displaced in binding to the extracellular methionine or histidine residues prior to internalization, rendering the Pt center inactive and unable to effectively bind to DNA. Howell argues that this observation may be due to internalization by macropinocytosis of hCTR1 on exposure to cisplatin, the Pt later being released from hCTR1 within the cell (118). However, the nature of the Pt species is not probed. While CTR1 is capable of internalizing cisplatin in cells, it seems that this Pt is not necessarily contributing to the cytotoxic

pool of Pt in cells, or that yeast and mouse CTR1, but not human CTR1, are capable of transporting Pt drugs.

What of the lowered CTR1 expression of the influx transporter in resistant cells then? How can it be that lowered CTR1 expression correlates with reduced cellular accumulation and DNA binding in resistant cell lines (112), yet transfecting A431/Pt cervical squamous cell carcinoma-resistant lines with CTR1 did not recover the accumulation defect (but did recover copper accumulation)? It may be that the accumulation defect in resistant lines is not due to downregulation of CTR1, but that it is downregulated or mislocalized in resistant lines as part of a pleiotropic resistance mechanism present in selected resistant cells (119).

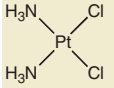
ORGANIC CATION TRANSPORTERS (THE SLC22 FAMILY)

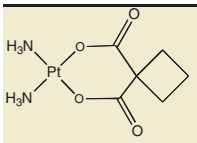
The solute carrier (SLC) gene series encodes a large family of passive transporters, ion-coupled transporters, and exchangers. To date, 45 gene families (SLC1–SLC45) with approximately 350 transporter genes have been identified (**http://www.gene.ucl.ac.uk/nomenclature**, 120). As discussed above, *SLC31A1*, which encodes the copper transporter CTR1, is an SLC family member. The SLC22 transmembrane transporters have also been shown to play a role in the uptake of Pt compounds. The human SLC22 family consists of 18 genes (*SLC22A1–18*), which include the organic cation transporters (OCTs), organic cation/carnitine transporters (OCTNs), and organic anion transporters (OATs). The transporters are predicted to contain 12 transmembrane helices with a large extracellular loop between helices 1 and 2 (121). These transporters are generally involved in the absorption and/or excretion of various endogenous and exogenous compounds in organs, including the kidney, liver, intestine, brain, lung, heart, and bone marrow (121).

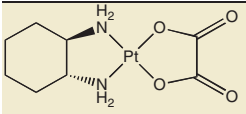
The SLC22 family members OCT1, 2, and 3, encoded by *SLC22A1*, *2*, and *3*, respectively, were first investigated as candidate uptake transporters of Pt compounds owing to their expression in tissues associated with cisplatin toxicities, and early observations that cisplatin inhibited the active uptake of tetraethylammonium (TEA), a prototypical substrate for OCTs, in rat kidney slices (122). OCT1 is expressed primarily in the liver (and also the intestine), OCT2 is found in the kidney, and OCT3 is detected in a range of tissues, such as the placenta and heart (121). Because OCT2 is expressed mainly in the proximal tubules of the kidney, which is the major site of cisplatin-induced renal injury, it has been assumed that OCT2 is one of the main transporters of cisplatin.

Given that there is some divergence in the observations on Pt drugs as OCT substrates, the results reported to date are detailed in the **Supplemental Section** on Organic Cation Transporters (the SLC22 Family) (123–131) and summarized in **Table 1**. Differences in the expression levels of OCTs in transfected cells (often not demonstrated in the reports), the specificity of inhibitors, media used for assays, and the types of drug sensitivity assays employed may be reasons for the discordant data. A further complication may arise from the cell lines employed for the above studies; kidney cell lines, by nature of their function, have a high background level of influx and efflux transporter expression (such as the ABC and MATE transporters

Table 1 Observations on cisplatin, carboplatin, and oxaliplatin substrate specificity for OCT1, OCT2, and OCT3 influx transporters (rat and human)[a]

 **Cisplatin**	OCT1	OCT2	OCT3
Pan et al. (128)	—	Yes	—
Yonezawa et al. (129)	No	Yes	—
Briz et al. (123)	No	No	No
Ciarimboli et al. (124)	No	Yes	—
Yonezawa et al. (130)	Yes (weak)	Yes	No
Zhang et al. (131)	Yes (weak)	No	No

 **Carboplatin**	OCT1	OCT2	OCT3
Ciarimboli et al. (124)	—	No	—
Yonezawa et al. (130)	No	No	No
Zhang et al. (131)	Yes (weak)	No	No

 **Oxaliplatin**	OCT1	OCT2	OCT3
Ciarimboli et al. (124)	—	No	—
Yonezawa et al. (130)	No	Yes	Yes (weak)
Zhang et al. (131)	Yes	Yes	No

[a]Positive or negative observations based on increases in drug accumulation (rather than sensitization) observed in transfected cell lines.

mentioned elsewhere), which may interfere with drug accumulation and account for discrepancies in the observations summarized in **Table 1**.

Cisplatin's clinical dose-limiting toxicity is predominantly renal toxicity, which correlates with the high renal expression of OCT2, whereas carboplatin and oxaliplatin (and other less reactive Pt drugs) are not generally OCT2 substrates, and their toxicities do not correlate with the OCT transporter tissue expression (carboplatin dose-limiting toxicity: thrombocytopeniea/myelosuppression; oxaliplatin: sensory neuropathy) (132). One of the common characteristics of SLC22 family members (OCTs, OCTNs, and OATs) is that certain members transport some of the same

compounds. Presumably, several SLC22 family members participate in the uptake of Pt compounds, some being more effective than others. To shed more light on the uptake of Pt compounds, a clear demonstration of drug uptake in a dose-dependent and saturable manner needs to be provided for each candidate uptake transporter of Pt compounds.

ARGUMENTS FOR ACTIVE EFFLUX

The reduced accumulation of cisplatin in resistant cell lines and the early evidence pointing toward passive diffusion into cells meant the search for a molecular cause of accumulation defects was not undertaken. The identification of P-glycoprotein (P-gp), the multidrug resistance transporter encoded by *MDR1*, probably slanted initial investigations toward the search for increased expression of an efflux transporter in resistant cells [it has been demonstrated that cisplatin is not a substrate of P-gp (133)].

A number of reports of increased efflux in cisplatin-resistant lines exist in the literature (summarized in the **Supplemental Section** on Arguments for Active Efflux), although the nature of the effluxed species (intact drug versus metabolites) has rarely been examined. The main complicating factor in the interpretation of these efflux studies (134–137) is the possibility that observed drug efflux is unreal passive efflux that only is observed in cells exposed to high doses (all three reports of efflux described in the **Supplemental Section** on Arguments for Active Efflux used ~500 mM cisplatin). To examine efflux, cells are generally rapidly washed to remove extracellularly associated drug, and then incubated in drug-free media for proscribed periods of time until the cells are harvested and their Pt content examined. In this situation, the drug concentration gradient favors movement out of the cell into the media, and this may account for an initial rapid efflux that would not occur when the cell is bathed in drug media. To examine this, cells initially treated with radiolabeled "hot" drug could then be treated with media containing cold drug, and efflux of hot drug in the presence of unchanged extracellular drug concentration could provide insight into whether efflux actually occurs against a concentration gradient.

There are also several reports of no difference in the exodus of cisplatin from sensitive and resistant cell pairs: L1210/0 and L1210/DDP (SRI) mouse lymphocytic leukemia (59), 2008 and 2008/DPP human ovarian carcinoma (treated at lower doses, 10 mM) (52), BEL7404 and 74049CP20 human hepatoma (9), H4-II-E and H4-II-E/CDDP rat hepatoma (87), A431 and A430/Pt human squamous carcinoma (138), A2780 and A2780cis ovarian cancer (112), and HeLa and HeLaCK cervical carcinoma (112) cell lines. This has also been shown for carboplatin in the BEL7404 and 7404 CP20 pair and is not temperature-dependent, further suggesting passive efflux (11).

ATP7A/7B COPPER EFFLUX TRANSPORTERS

In tandem with the copper influx transporter CTR1 described above, the copper efflux transporters ATP7A and ATP7B have also been examined—again reviews exist

in the literature, and only the key observations are discussed here (107–139). ATP7A and 7B are functionally conserved P-type ATPases involved in the sequestering and extrusion of excess Cu ions. They are homologous in structure, sharing eight transmembrane domains and ~65% amino acid sequence. However, their tissue expression differentiates them (ATP7A, intestinal epithelium of copper absorption; ATP7B, liver and kidney), and mutations in 7A and 7B are responsible for the Cu-related diseases Menkes and Wilson's disease, respectively (104). Under normal (Cu replete) conditions, ATP7A/B resides in the trans-Golgi network, where it receives Cu from the chaperone Atox1 and translocates it to the luminal side for incorporation into enzymes. When excess Cu exists in the cell, ATP7A/B is trafficked to the cell surface to directly efflux Cu from the cell (104). In both instances, the Cu is transferred directly to ATP7A/B by a metallochaperone; O'Halloran and coworkers have proposed that there are no free Cu atoms in the cell (140). ATP7A/B possesses six metal-binding sequences of ~100 amino acids each that include a GMTCXXCIE motif on the cytoplasmic side required for metal ion translocation, with a methionine and two cysteines capable of coordinating Pt (139).

Akiyama and coworkers transfected KB-3-1 cells with ATP7B cDNA and demonstrated resistance to both cisplatin and Cu, which coincided with diminished accumulation of cisplatin that could be recovered by ATP depletion (141). PC-5 prostate carcinoma cells were also shown to express increased levels of ATP7B relative to the sensitive PC-3 line and the revertant PC-5R. Nakayama subsequently examined ATP7B mRNA expression and cisplatin cytotoxicity in nine parental ovarian cancer cell lines and showed that cells with increased ATP7B expression were less sensitive to cisplatin (142).

Katano et al. showed that three selected resistant ovarian lines (2008/C13*5.25, IGROV-1/CP, and A2780/CP) expressed greater levels of either APT7A or 7B protein relative to their respective parental lines (143). Whereas increased Pt and Cu efflux was demonstrated in the 2008/C13*5.25, expression of other Pt efflux pumps, such as the GS-X family (described below), was not examined to deconvolute other potential contributors to efflux. There is not complete agreement on ATP7A/B expression changes in resistant lines; Zisowsky et al. observed that cisplatin-selected A2780cis cells show modest increases in expression of both ATP7A and 7B (by RT-PCR), whereas resistant HeLa cervical carcinoma cells expressed less of each gene in selected cells, rather than more (112), the authors concluding that ATP7B did not contribute to resistance in the lines examined.

Cells transfected with ATP7B accumulate less cisplatin and carboplatin than cells transfected with empty vector (144), whereas cells with ATP7A transfected into them are resistant to cisplatin, carboplatin, and oxaliplatin. However, the ATP7A transfectants had increased Pt accumulation, which coincided with the cell vesicular fraction, suggesting Pt is extruded into vesicles and effectively deactivated by ATP7A. This seems to suggest that simple overexpression of ATP7A alone may be sufficient to compartmentalize Pt, but not be enough to lower accumulation. ATP7A is not relocalized to the plasma membrane for Pt efflux as it is for Cu efflux, suggesting the Cu sensing domain associated with trafficking cannot sense Pt. This was confirmed by confocal microscopy using fluorescent Pt compounds (see below) that colocalized

with ATP7A signal in vesicles (145). The role of ATP7A/B is also Pt drug-type dependent; cells selected with the satraplatin analog JM118 did not show expression of either ATP7 gene (93). It was also recently shown that ATP7A transfection into Chinese hamster ovary cells conferred cross-resistance to a large range of organic drugs (etoposide, vincrisine, paclitaxel, doxorubixin, SN-38, CPT-11), reduced drug accumulation, and compartmentalized doxorubicin into Golgi vesicles only in ATP7A expressing cells, suggesting that ATP7A confers multidrug resistance (146). It may be that ATP7 gene expression alters underlying cellular processes (or vice versa), particularly given that cellular copper status regulates proteins, such as XIAP (X-linked inhibitor of apoptosis), that inhibit proapoptotic caspase-3 and -7 in the presence of Cu (147, 148).

The vesicular accumulation of Pt drugs by ATP7A/B presents a challenge to the traditional decreased influx and increased efflux model of drug transporter-dependent resistance. Increased vesicular sequestration of drug does not necessarily affect global cell Pt levels, although this should be measurable as decreased DNA platination as the cytosolic drug pool is depleted. The mechanism of Pt drug trapping with these vesicles is also a matter for debate and may rely on acidic pH trapping of charged drug as described elsewhere. Elimination of this vesicular Pt pool is achieved (although perhaps not necessary to confer resistance) by trafficking along the secretory pathway. The secretory pathway inhibitors wortmannin (inhibitor of endosomal maturation) and H89 (blocks Golgi vesicular secretion) both increase cisplatin accumulation dramatically in 2008 cells (145). In keeping with a lysosomal trapping and efflux of Pt, 2008/C13*5.25-resistant cells have significantly fewer lysosomes than parental cells and greater exosomal Pt levels than in wild-type cells (149). Significantly, steady-state drug levels in sensitive and resistant cells demonstrated little difference. However, once placed in drug-free media, resistant cells eliminated drug at a more rapid rate (149), suggesting that vesicular sequestration is responsible for lowered drug activity.

Unlike other putative transporters described already, there is a significant amount of clinical evidence for ATP7B expression relating to outcome prognosis in a range of human solid carcinomas (150), and some data is available for ATP7A (146), although prognostic significance has not been demonstrated (clinical observations are summarized in the **Supplemental Section** on ATP7A/B as Markers for Chemoresistance). For example, cisplatin is regularly used in ovarian cancer chemotherapy, and patients with APT7B-positive tumors demonstrated an inferior response to chemotherapy compared with ATP7B-negative patients (median survival of 33 months for ATP7B-positive versus 66 months for ATP7B-negative) (151).

Given the in vitro observation that ATP7A and ATP7B expression can result in lowered accumulation and/or efficacy of cisplatin, and the evidence that ATP7B expression in solid carcinomas is generally an indicator of poor response to chemotherapy, it is possible that disruption of ATP7A/B function may sensitize cells to Pt drugs. Although no known inhibitors exist (other than the obvious indirect ATP-depletion or vanadate inhibition of ATPase activity), Cu could act as a competitive substrate that would increase Pt drug accumulation. Farrell and coworkers have reported that coincubating subtoxic Cu concentrations with cisplatin increase cisplatin accumulation in A2780 and HCT116 cells, suggesting that Cu may be effluxed

preferentially over cisplatin. However, A2780 cells expressed elevated ATP7B with little effect on cisplatin uptake and cytotoxicity (152). Also interesting is that cells transfected with ATP7A/B do not result in the same Pt accumulation and efflux properties as selected cell lines, leading one to wonder whether ATP7A/B expression and Cu resistance to parallel cisplatin resistance in Pt-resistant cells is symptomatic of a broader upregulated detoxification response (153), and that ATP7A/B is more of a marker of resistance than the cause of it.

GLUTATHIONE-CONJUGATED (GS-X) EFFLUX TRANSPORTERS

In considering Pt drug efflux, one must consider the active efflux of glutathione-conjugated Pt, part of a larger generic detoxification pathway for *d*-block and the so-called heavy metals, along with metallothionein. The chelation of the Pt drugs by glutathione is generally accepted to be a deactivation pathway, and one which drugs can be designed to circumvent, as is the case with the sterically bulky ZD0473 that has reduced reactivity with glutathione.

Both Pt(II) and the glutathione thiol sulfur are relatively soft [according to Pearson's Hard-Soft-Acid-Base theorem (154–156)], and form highly stable 2:1 GS-Pt complexes (157). Many Pt-resistant cell lines express increased levels of glutathione (owing to increased expression of γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase synthetic enzymes) (158), and once the Pt drug is chelated (nonenzymatic conjugation) by glutathione, the glutathione-Pt complex is effluxed from the cell in an ATP-dependent fashion by a transporter family termed the GS-X pumps (157). The GS-X efflux pumps are responsible for the elimination of a range of glutathione-conjugated drugs, including metal-glutathione chelates.

Ling and coworkers first reported the overexpression of a 200-kDa plasma membrane glycoprotein in a murine thymic lymphoma cell line selected for resistance to cisplatin (termed CP^R-200), and showed that its expression correlated with the degree of resistance (159). Although CP^R-200 has not been further characterized, it is probably the same 200-kDa membrane protein that is overexpressed in cisplatin-resistant HL-60 human leukemia cells and is associated with the GS-X pump (160). GS-X pumps are ATP-dependent organic anion transporters, belonging mainly to the ABCC (or MRP) family [see **Supplemental Table 1** for list of the ABCC (MRP) transporters].

The first member of the family identified was the multidrug resistance-associated protein (MRP, MRP1, *ABCC1*), a 200-kDa transmembrane glycoprotein capable of effluxing a range of glutathione-conjugated molecules (hence the term GS-X), and a member of the ABC family of drug efflux transporters (161, 162). It is highly expressed in lung, adrenal, heart, and skeletal muscle tissue, and weakly expressed in liver and brain tissue (162), and its overexpression in multidrug-resistant tumors and cell lines was shown to confer resistance to natural product drugs (163).

Following the observation that Pt-conjugated glutathione is effluxed from cisplatin-treated cells in an ATP-dependent manner (157), Ishikawa et al. examined cisplatin-resistant human promyelitic leukemia HL-60/R-CP cells that expressed

high levels of glutathione, showing overexpression of a 200-kDa protein now known as MRP1 (160). The cells did not show altered P-glycoprotein expression, and doxorubicin was equipotent against both the parental and resistant cells. Membrane vesicles generated from the R-CP cells showed a marked increase in tritiated glutathione-conjugated Pt, and confocal microscopy revealed vesicular localization of the MRP1 substrate monochlorobimane [*syn*-($ClCH_2$,CH_3)-1,5-diazabicyclo-[3.3.0]-octa-3,6-dione-2,8-dione], suggesting that MRP1 may sequester substrates into vesicles that then fuse with the plasma membrane, ejecting their contents (160).

Cole et al. showed that HeLa cells transfected with MRP1 were not cross-resistant to cisplatin (164), suggesting that the efflux transporter alone is not sufficient to confer resistance to Pt drugs. Hamaguchi et al. examined a series of seven cell lines increasingly resistant to cisplatin established from A2780 cells by step-wise exposure to cisplatin (9–400-fold cytotoxicity resistance) and found no relationship between MRP1 gene expression and resistance to cisplatin, but they did observe that intracellular levels of glutathione correlated with resistance (accumulation was not assessed) (165) and depletion of cellular glutathione with buthionine sulfoximine (BSO) increases sensitivity to cisplatin (166). Ikuta et al. also found no relationship in NSCLC cell lines between MRP1 expression and cisplatin cytotoxicity or accumulation (167), and a number of investigations found no clinical correlation between MRP1 expression and Pt therapy outcome (7, 168, 169).

Seven more MRP1 homologs have subsequently been identified (MRP2–MRP8). MRP2, initially termed canalicular multispecific organic anion transporter (cMOAT), shares 49% sequence homology with MRP1, resides in the hepatocyte canalicular membrane, and exports glucuronide-conjugated bile acid and glutathione conjugates such as leukotriene C_4 into the bile (170, 171). Taniguchi initially reported cMOAT as a putative cisplatin efflux pump after isolating MRP2 cDNA from three cisplatin-resistant cell lines (172). A vector containing MRP2 antisense cDNA transfected into KB epidermoid carcinoma cells increased both sensitivity to, and accumulation of, cisplatin, along with a marked increase in cellular glutathione levels (173), and cisplatin has been shown to induce MRP2 gene expression (174). A range of cisplatin-resistant cell lines have been shown to express increased levels of MRP2 (171), and some reports of MRP2 levels correlating with DNA platination exist in melanoma and ovarian cancer lines (175, 176), although no definitive relationship between MRP2 expression and resistance or accumulation defects has been reported (177).

Analysis of clinical data gives mixed indications of MRP2 as a prognostic marker. Immunohistochemical and RT-PCR analyses of ovarian (178–180) and lung (181, 182) cancer samples have shown no correlation with response to chemotherapy or progression-free survival, although a study of Stage III ovarian carcinoma samples demonstrated that an absence of MRP2 transcript was generally associated with improved progression-free survival (183). One analysis of resected colon cancer found that MRP2 expression was associated with resistance to cisplatin therapy, again demonstrating the tissue-specific nature of transporter-mediated resistance (184). It was recently reported that ABCC2 expression in the nuclear membrane, rather than the plasma membrane, was associated with response to first-line chemotherapy

in ovarian carcinoma samples, and ABCC2 showed higher expression in cases with relapse (185).

Of the remaining MRP transporters, Kool et al. initially reported that MRP3 (expressed mainly in the liver), MRP4 (low level expression), and MRP5 (widely expressed in tissues including liver, kidney, heart, brain, and skeletal muscle) were not correlated with drug resistance, although a number of cell lines expressed high levels of MRP3 and MRP5 mRNA (186). Suzuki has reported increased MRP5 mRNA levels in Pt-treated lung tumor samples, and that MRP5 expression correlated with glutathione synthesis in said samples (7); little is known about the relationship between MRP6 and MRP7 expression and Pt drug resistance.

Based on these observations, it seems that overexpression of MRP1 alone is not sufficient to confer resistance to Pt drugs—a concomitant increase in glutathione expression to complex Pt is required for efflux of the detoxified drug (187), and the cross-resistance to metal ions observed in cisplatin-resistant cell lines is probably conferred by the expression of GS-X pumps (described in the **Supplemental Section** on Cross-Resistance to Metal Ions). Glutathione-conjugated Pt is already deactivated, so efflux by the MRP (GS-X) transporters is not necessarily a part of the accumulation-resistance phenotype; however, there is evidence in specific cases that their expression is associated with clinical resistance to cisplatin, and more work needs to be done.

INTRACELLULAR TRAFFICKING

Examination of cellular accumulation of cisplatin is performed readily thanks to the availability of elemental spectroscopies, such as atomic absorption spectroscopy (AAS) and inductively-coupled plasma–optical emission spectroscopy (ICP-OES). Some groups have also used radioactive ^{14}C-labeled diamine groups (such as ^{14}C ethane-1,2-diamine) or leaving groups (^{14}C-cyclobutyldicarboxylic acid in carboplatin). Unfortunately, these spectroscopic handles do not readily lend themselves to imaging of cisplatin. Few reports of electron microscopy (188) and synchrotron elemental imaging (189–191; H.L. Daly, M. Zhang, R.A. Alderden, D.M. Pursche, "Monitoring the biological reduction of cis and trans Pt(IV) complexes using X-ray absorption near edge spectroscopy (XANES)," manuscript submitted) (SRIXE, XRF) that can directly monitor Pt exist; however, live samples cannot be imaged, and in the case of SRIXE, resolution is limiting (192). Chen et al. used SRIXE to show that malignant melanoma cells sequester Pt into large subcellular compartments (probably melanosomes) that can then be extruded from the cell (193).

A number of Pt complexes have been synthesized with a fluorophore conjugated to allow cellular trafficking studies. Although isolated subcellular components have been assessed for Pt levels, little other information is available. The first of these, reported by Reedijk and coworkers, incorporated carboxyfluoresceindeacetate, termed CFDA-Pt (**Supplemental Figure 4**) (194), to monitor cellular distribution over time in U2-OS and U2-OS/Pt cisplatin-resistant osteosarcoma cell lines; importantly, the U2-OS/Pt line demonstrates an accumulation defect (195). CFDA-Pt demonstrated rapid uptake into cells, and there was no obvious difference in uptake or localization between CFDA-Pt, or the nonplatinated fluorophore CFDA-Boc, for

6–8 h when CFDA-Pt levels are lower and punctate staining in the cytoplasm appears that colocalizes with a Golgi-specific stain (194). The U2-OS/Pt-resistant line did not demonstrate differences in uptake, distribution, secretion, or localization of drug compared with the parental cell line. Aside from the rapid uptake (indicated by strong fluorescence intensity) (194), it is unlikely that CFDA-Pt behaves analogously to cisplatin. The cytotoxicity of CFDA-Pt was not reported, the metabolism of the fluorophore (breakdown or dissociation from the Pt center) is not known, and the lack of differences in CFDA-Pt between resistant and sensitive cells may be due to the large lipophilic fluorophore altering the pharmacokinetic properties of the complex and allowing more rapid uptake.

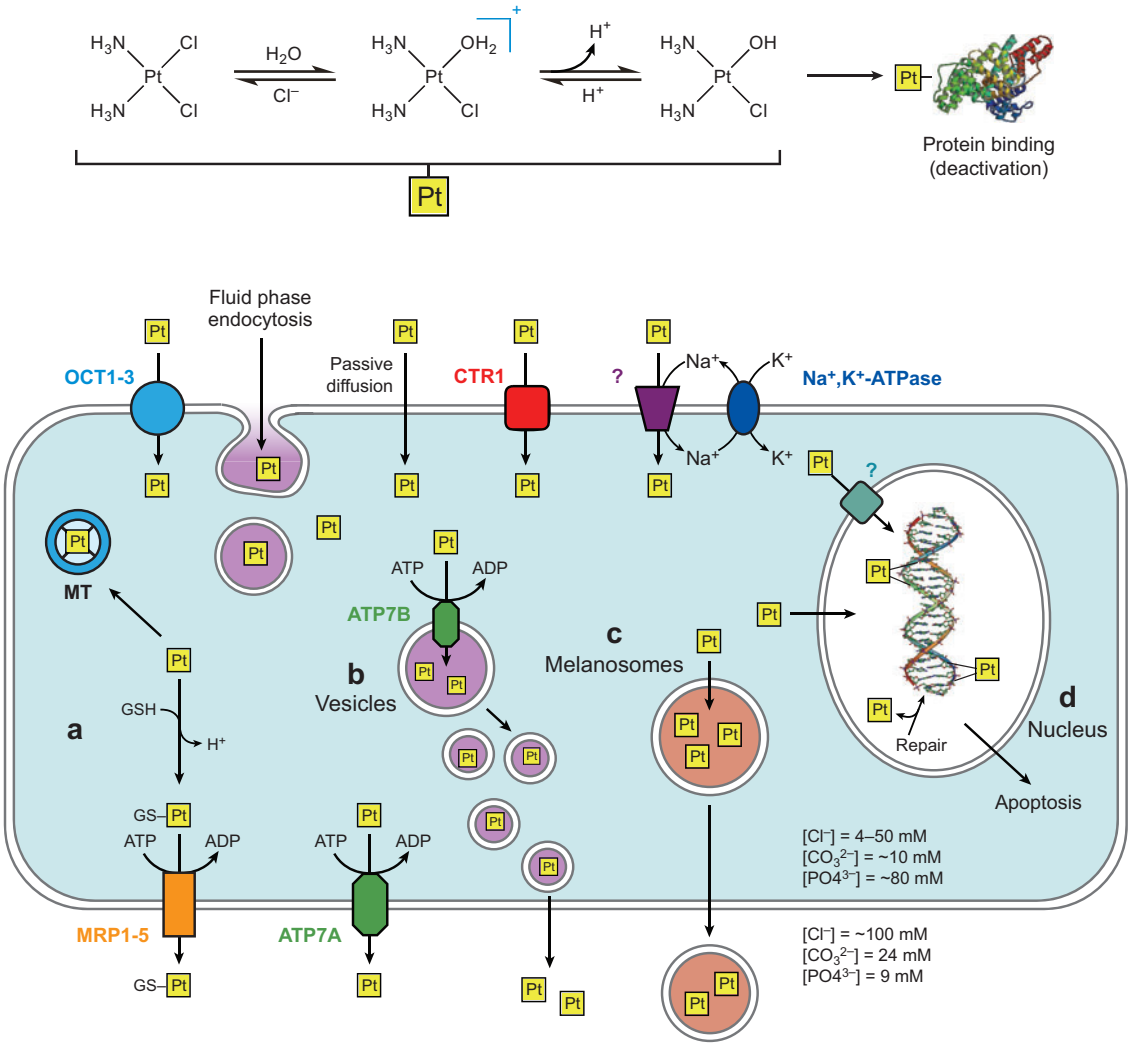
Howell and coworkers addressed some of these issues, using another fluorescein-conjugated Pt complex, FDDP (196). FDDP demonstrated cross-resistance with cisplatin to 2008/C13*5.25 ovarian cancer cells compared with parental lines (~fivefold resistance) in terms of both cytotoxicity and accumulation but lower absolute activity, and the free, nonplatinated fluorophore demonstrated altered localization (196). FDDP was again noted to be concentrated in small vesicular structures in the cytoplasm, and FDDP colocalized in part with the copper efflux transporter ATP7B (described above). FDDP was subsequently used to identify the vesicles as those belonging to the Golgi, the secretory export pathway, and lysosomes (145).

Aside from the fluorescein-conjugated complexes above, Liang et al. utilized the ULYSIS nucleic acid label (Molecular Probes) Alexa Fluor 546 (**Supplemental Figure 4**), developed by Houthoff and coworkers (197), which contains a Cy3 dye coordinated to the Pt center in lieu of a chloro ligand. Monofunctional compounds, typified by $[PtCl(dien)]^+$, have been shown to be inherently inactive (20), and whether cellular processing of inactive complexes is physiologically relevant is difficult to assess, although the decreased uptake in resistant cell lines argues for the relevance of labeled Pt compounds. Liang observed lowered accumulation of Alexa Fluor 546 in KB-CP.5 and KB-CP20 cells, the vesicular localization observed by others, which merged with the signal from the fluid-phase endocytosis marker Fluorescein Dextran-10, and partial localization on the Golgi (as shown for CFDA-Pt) (198). Liang et al. believe the vesicular localization is endocytic (as less was observed in resistant cells); as such, the vesicles may represent a reservoir of drug available to the cell over time. Other researchers observe decreasing vesicular staining over time, consistent with a drug extrusion process.

A number of studies of novel Pt complexes tethered to bioactive intercalators such as anthraquinones have utilized their native fluorescence to track uptake and distribution in cells; however, these are beyond the scope of this review (199–202). Yet the punctate staining of fluorescent drug observed in the above studies is analogous to that observed by Hambley and coworkers in A2780 cells with Pt-1C3 [*cis*-$[PtCl_2(NH_3)$(1-{[3-Aminopropyl]amino}-anthracene-9,10-dione}], which colocalizes with LysoTracker Green, a lysosome-specific stain (J. Zhang and T.W. Hambley, in preparation). Lysosomes are acidic vesicles with an intravesicular pH around 4.8 that accumulate molecules (lysomotropic agents), especially weak bases, via passive diffusion (predominately), autophagocytosis, active transport, and endocytosis, and then trap them as charged species (owing to the acidic environment)

(204, 205). Lysosomes are then secreted to the plasma membrane and their contents exocytosed. If the protonated species within the lysosome is protonated at low pH, as aquated species of Pt drugs, and amines on fluorophores would be, the compounds are effectively membrane impermeable and trapped by a process termed pH partitioning (204).

There is certainly scope for greater use of fluorescent analogs of Pt drugs, especially in comparing trafficking in sensitive and resistant cell lines to gain insight into altered drug accumulation. Caution must be exercised to ensure that the effects observed are related to the Pt center and not due to the fluorophore (i.e., that the tail is not wagging the dog).



PLEIOTROPIC RESISTANCE TO PLATINUM DRUGS

Cells selected in our laboratory for cisplatin resistance, including single-step mutants, express a complex pleiotropic phenotype consisting of cross-resistance to antimetabolites and heavy metals, and reduced energy-dependent accumulation of cisplatin and other compounds, including nutrients such as sugars and amino acids (11, 206, 207). In examining this phenotype, it became clear that fluid-phase endocytosis was reduced in these cells (208); proteins normally localized to the cell surface were mislocalized to intracellular vesicular compartments because they fail to recycle back to the cell surface (**Supplemental Figure 5**) (119); biophysical measurements of plasma membrane function were altered (64); some genes, such as the folate binding protein gene, were hypermethylated (209); actin and filamin networks were disorganized (210); and endocytic recycling was abnormal (211). Fluorescent cisplatin complexes showed delayed uptake and altered distribution in the resistant cells, consistent with a role for defective uptake and endocytosis in causing the reduced cisplatin accumulation (198). Such a phenotype suggesting alterations in recycling of membranes and membrane proteins would explain many of the observations reported in the literature, including energy-dependence of uptake, a defect in several different putative cisplatin uptake transporters, and reduced passive diffusion (i.e., decreased endocytosis) in cisplatin-resistant cells.

In searching for a single abnormality that could account for such a pleiotropic phenotype, we examined the expression of several small GTPases thought to be involved in membrane recycling and found decreases in several (209). Microarray experiments suggested that K^+ channels were consistently altered in cisplatin-resistant cells, but

Figure 5

A schematic of the mechanisms affecting and controlling the cellular accumulation of platinum chemotherapeutics (exemplified here by cisplatin). In the extracellular environment, cisplatin can be aquated (*shown*) or react with biomolecules such as carbonate, resulting in a complex speciation profile. These species may enter the cell or cross-react with extracellular proteins such as serum albumin (*shown*), reducing the bioavailable pool of drug. Given the limited knowledge of the ability of these species to be actively or passively translocated into cells, or the intracellular speciation of exported drug, they are collectively represented here by yellow squares (Pt). Neutral platinum drugs can enter the cell by passive diffusion across the lipid bilayer, and a number of carrier-mediated import proteins have been identified, the main players being organic cation transporters (OCT1–3, SLC22A1–3), CTR1, and an as-yet unidentified sodium-dependent process, as well as a number of putative transporters such as hMATE1 and the aquaporins. Inside the cell, (*a*) platinum drugs can be deactivated by binding to the thiol-rich metallothionein (MT) or chelated by glutathione (GSH) and effluxed from the cell via the GS-X pumps (MRP1–5). Platinum drugs can also be ensnared in subcellular organelles such as (*b*) vesicles via ATP7B influx and/or acidic trapping followed by exocytosis to expel the platinum from the cell, or (*c*) melanosomes in melanoma cells that are subsequently exported from the cells. Drug that evades these detoxification and efflux processes can enter the nucleus by an as-yet undetermined mechanism and bind to DNA, eliciting apoptosis if the DNA lesion is not repaired. The global pool of platinum drug present in the cell (and therefore the cytotoxic potential) is dependent on both the rate of influx and efflux, and a shift in the balance of these in resistant cells (decreased influx, increased extrusion processes) collectively leads to a reduction in cellular accumulation of cisplatin.

modulation of the function of these channels with antibodies had no effect on cisplatin resistance (198). The amount of γ-catenin protein was strikingly reduced in the resistant cells and relocalized away from the plasma membrane but re-expression of gamma-catenin did not have a strong effect on cisplatin resistance (212). The complex phenotype associated with cisplatin resistance has the hallmarks of a programmed cellular response to environmental adversity, including reduced glucose uptake because of a reduction in Glut1 transporters on the cell surface, increased SirT1 levels in mitochondria, and reduced oxygen utilization by mitochondria (X.J. Liang and M.M. Gottesman, unpublished data), but the proximal trigger and/or regulator of this response remains unknown.

CONCLUDING REMARKS

Cisplatin probably enters the cell via two pathways: (*a*) passive diffusion and (*b*) facilitated and active uptake by a number of transport proteins. The relative contributions of the two uptake pathways to total cellular accumulation of drug are probably dependent on the lipophilicity and speciation of the drug. Energy-dependent uptake is supported by evidence that lowered accumulation of cisplatin is observed in energy-depleted parental cells; if the accumulation defect were due to increased efflux, energy depletion would result in increased drug accumulation. As a number of transporters are probably capable of accommodating cisplatin uptake, molecular, genomic, and proteomic studies of sensitive and resistant cell lines have not been able to identify a single transporter whose decreased presence on the plasma membrane significantly contributes to a reduction in accumulation. This is probably compounded by the fact that, along with diminished expression, transporters are probably not recycled correctly in resistant cells, but mislocalized to intracellular compartments, making ready detection of resistance candidates more difficult. Following this process of resistance development, a drug can only enter resistant cells at a baseline rate owing to passive diffusion and the modicum of transporters that persist on the membrane. Even this mode of entry will be compromised by defects in fluid-phase endocytosis observed in resistant cell lines.

Resolving cisplatin resistance with molecular biological or gene therapeutic approaches will rely on an understanding of drug design and the specific carriers present at the cell surface to both control drug efficacy in cancer cells and ameliorate deactivation and drug clearance. The enthusiasm for creative Pt drug design and development has cooled over the past 10 years, as a large number of analogs have been tested in vitro that failed to offer any improvement over cisplatin, carboplatin, and their congeners. That the platinums will remain in the clinic for a long time to come appears to be a certainty, particularly with the recent finding of carboplatin as an effective therapy for colon cancer (213). If cellular resistance to cisplatin and its congeners can be considered to be a generic response to Pt drugs, then features of resistance not associated with other drugs may point to potential non-Pt therapies that can circumvent resistance. Given that accumulation of drug in resistant cells probably relies in large part on passive diffusion, effective lipophilic drugs that do not compromise cytotoxic potency should be considered, although increasing lipophilicity always raises the risk

of improving recognition by ATP-dependent efflux pumps. A greater understanding of the homeostatic regulation of transporters at play in cisplatin accumulation could also allow manipulation of their expression by coadministration of nontoxic substrates that alter expression to favor drug accumulation.

Pt drugs probably enter cells by a number of influx transporters along with passive diffusion, and they can be extruded after chelation by glutathione and in an unknown form via the Cu efflux system (**Figure 5**). It is clear from a review of the literature that disagreement exists about the relative importance of each of these transport pathways to cisplatin accumulation, which is probably tissue-dependent. Additionally, our understanding of the transportome and endocytic regulation is incomplete, and almost certainly new candidates for Pt drug passage will appear. Although resistant cells selected in vitro provide a good platform for probing accumulation resistance defects, clinical resistance develops in tandem owing to combinations of Pt and natural product drugs, and a comprehensive understanding of genome and proteome factors that correlate with response to chemotherapy is required. Perhaps the most important challenge for the future is to demonstrate in primary tumor samples that accumulation defects owing to specific mechanisms correlate with clinical outcome.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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