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Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells*

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Summary. We have determined the ability of two human ovarian carcinoma cells to over-express metallothioneins (MTs) and the subsequent effect this elevation has on DDP cytotoxicity. Cells of 2008 and COLO 316 human ovarian carcinomas that were resistant to $CdCl_2$ were obtained by stepwise selection and chronic culture in $CdCl_2$ and $ZnCl_2$. The 2008/MT cells were 3.2-fold resistant to $CdCl_2$ and 4.1-fold resistant to DDP; they had 23-fold elevated MTs. The COLO/MT cells were 1.2-fold resistant to $CdCl_2$ and 3.3-fold resistant to DDP, and they had 9-fold elevated MTs. Glutathione (GSH) was also elevated in the Cd-resistant sublines. However, four times more intracellular thiols were contributed by the MTs than by the GSH. 2008 and 2008/MT cells were examined in more detail to elucidate the mechanism of DDP resistance. Depletion of GSH with D,L-buthionine-S,R-sulfoximine (BSO) had no effect on the sensitivity of these cells to either $CdCl_2$ or DDP. Uptake of [^{195m} Pt]DDP in 2008 and 2008/MT cells was identical. Fractionation of the cytosol from [^{195m}Pt]DDP-exposed cells on Sephadex G-75 revealed that 17% of the total cellular Pt in 2008/MT cells was associated with the MT fraction, as against 4% in the parent 2008 cells. This increase corresponded to a concomitant loss of Pt from the particulate fraction. Fractionation of 2008 cells selected with DDP (2008/DDP cells) indicated that elevated MTs did not contribute to the DDP resistance of these cells. Only 2% of the total cellular Pt was in the MT fraction in 2008/DDP cells. These results showed that elevation of MTs may be one mechanism of DDP resistance in ovarian carcinoma; however, in vitro selection with DDP does not trigger this mechanism.

Introduction

Ovarian carcinoma is the fourth leading cause of death in women [11]. Cisplatin (DDP) is an important drug for the treatment of this disease [24]; however, despite the good response rates of this tumor to DDP, complete remissions are limited by the frequent emergence of DDP- resistance [10, 17, 28]. DDP platinates nucleic acids, amino acids, and many proteins, and is particularly reactive towards methionine and cysteine residues [15]. Given the sulfur-reactive properties of DDP, alterations in intracellular thiol components may be potentially useful defense mechanisms for resistant cells to acquire. There is some evidence that elevated GSH levels may contribute to the DDP-resistant phenotype [13, 16, 35].

Metallothioneins (MTs) comprise a class of isoproteins with molecular weight 6000–7000, which are involved in Zn^{+2} homeostasis and the detoxification of heavy metals [19, 21]. They are composed of 30% cysteine and can account for large percentages of the intracellular thiol content. The major forms in humans, MT-I and MT-II, are acutely and differentially induced by Cd^{+2}, Zn^{+2}, and glucocorticoid hormones through increased transcription [21]. In addition to this acute response, MTs can be elevated as a result of gene amplification triggered by chronic exposure to heavy metals [4]. Owing to their high content of reactive thiol groups, MTs are prime potential targets for electrophilic agents such as DDP. Bakka et al. [3] have shown that human epithelial cells and mouse fibroblasts, made resistant to Cd^{+2} in vitro and containing large amounts of MTs, were indeed cross-resistant to DDP. Pt was found to be associated with the MT fraction in both types of cells, and the DDP-resistance of the mouse fibroblasts was maintained in vivo [3, 8]. Other studies have shown that in both rat liver and kidneys, 25% of the total cytosolic Pt was bound to MT-like proteins [25, 34, 37]. A recent report has shown that in Ehrlich cells, which have high endogenous levels of MTs, a significant fraction of the intracellular Pt was bound to MTs [23].

We have been interested in whether MTs can play a role in mediating acquired resistance to DDP in human ovarian carcinoma [27]. In this report, we present evidence that MTs were inducible with Cd^{+2} and Zn^{+2} in human ovarian carcinoma cells in vitro and that this elevation was associated with the appearance of DDP resistance. However, DDP-resistant cells generated by in vitro DDP selection did not have elevated MT levels.

Materials and methods

Drugs and chemicals. DDP was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI (Bethesda, Md). [^{195m}Pt]DDP was ob-

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tained from Oak Ridge National Laboratories (Oak Ridge, Tenn). $CdCl_2$, $ZnCl_2$, Sephadex G-10, and Sephadex G-75 were from Sigma Chemical Co. (St Louis, Mo). [^{203}Hg]Mercuric chloride was from New England Nuclear (Boston, Mass).

Cell lines. COLO 316 and 2008 cells established from patients with serous cystadenocarcinoma of the ovary were used in these studies [6, 36]. Cells were grown as monolayer cultures in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 m*M* freshly added glutamine, and 1% Fungi-bact (Irvine Scientific, Santa Ana, Calif). Cultures were equilibrated with humidified 5% CO_2 in air at 37 °C. Cells in exponential growth were used in all experiments. Clonogenic assays were performed as previously described [2]. Levels of resistance were determined by the ratio: IC_{50} subline/IC_{50} parent cells, where the IC_{50} is the concentration causing 50% inhibition of colony formation.

Cadmium chloride-resistant cells were developed by stepwise selection with increasing $CdCl_2$ and $ZnCl_2$ concentrations, administered to the cells at each passage for 2 months. When toxic levels of these metals were reached, as indicated by vacuolization, cells were chronically exposed to the metals for over 9 months, at which time the studies described in this report were done. 2008/MT cells were grown chronically in 25 μ*M* $CdCl_2$, 200 μ*M* $ZnCl_2$ and COLO/MT cells were grown in 35 μ*M* $CdCl_2$. Clonogenic assays and MT determinations were performed 2 days after passage in metal-containing media. 2008/DDP cells were generated as previously described [2] and were approximately 3-fold resistant to DDP.

Metallothionein determination. MT levels were determined as described by Patierno et al. [29]. Trypsinized cells from one 75-cm^2 flask were resuspended in 10 ml phosphate-buffered saline (PBS; Oxoid, Colombia, Md), and an aliquot removed for cell counting by hemacytometer. The cells were pelleted again, resuspended in 1.0 ml PBS, rapidly frozen in an ethanol/dry ice bath, and stored at −20 °C until analysis. Samples were thawed, sonicated, and 100 μl lysate incubated with 50 μl of 64 μ*M* $^{203}HgCl_2$ in an Eppendorf tube at room temperature. After 15 min, 30 μl 12% TCA was added and the solution vortexed. After another 5 min, the sample was sedimented in a microcentrifuge and 100 μl of the supernatant applied to Sephadex G-10 mini-columns [29]. MTs were quantified with a horse kidney MT standard (Sigma).

DDP binding to intracellular MTs was determined by gel filtration of cytosol from approximately 55×10^6 cells. 2008/MT and 2008/DDP cells treated with 10 μ*M* [^{195m}Pt]DDP or 2008 cells treated with 20 μ*M* [^{195m}Pt]DDP for 3 h were trypsinized, washed three times with cold PBS, resuspended in 2.0 ml 20 m*M* Tris-HCl, pH 8.0, and lysed by rapid freeze/thawing. The cell lysate was centrifuged at 105 000 *g* for 90 min. The supernatant was applied to a Sephadex G-75 column (1.5 × 40 cm) and eluted with 20 m*M* Tris-HCl, pH 8.0 at 4 °C. The flow rate was 10.5 ml/h and 15-min fractions were collected. The fractions were mixed with an equal volume of 3a70B scintillation fluid (Research Products International, Corp., Mount Prospect, Ill) and the radioactivity determined on an LS-1000 beta counter (Beckman, Fullerton, Calif) with the windows set on wide open.

Glutathione determination and modulation. Glutathione (GSH) was determined in cell extracts by the method of Reed et al. [30], modified as previously described (P. A. Andrews, M. P. Murphy, S. B. Howell 1986, to be published). Cells were passaged, and for each of the following 4 days one flask was harvested for GSH measurement. The values reported are the averages of these four flasks. GSH levels were depleted in 2008 and 2008/MT cells by treating monolayers with 0.5 m*M* BSO for 24 h prior to clonogenic assay [2]. Although our clonogenic assay procedure released the cells from the GSH-depleting effects of BSO at the time of plating and drug exposure, GSH levels did not rebound past 34% of controls for at least an additional 48 h in 2008 cells (P. A. Andrews, M. P. Murphy, S. B. Howell 1986, to be published). In GSH-depleted 2008/MT cells, the GSH rebounded to 59% of controls at 31 h after removal of BSO. GSH-transferase (EC 2.5.1.18) levels were determined by the method of Habig and Jakoby with 1-chloro-2,4-dinitrobenzene as substrate [12]. One unit was defined as conjugating 1 μmol substrate with GSH per min.

DDP uptake. Uptake of DDP was determined in parent and resistant cells as described by the method of Frei et al. [9]. Parental and resistant cells were seeded into 100-mm tissue culture dishes. After 3–4 days, when the plates had approached confluency, the medium was aspirated and replaced with 3.0 ml 37 °C RPMI 1640 medium containing 20 μ*M* [^{195m}Pt]DDP. The dishes were promptly returned to the incubator and then at 15, 30, 45, and 60 min they were removed, the medium aspirated, and the cells washed three times with 4 °C PBS (Oxoid, Colombia, Md). Then 3 ml 1 *N* NaOH was added and the cells allowed to digest overnight. An aliquot was then removed for the determination of protein content [5], and the remainder of the digest was mixed with 1.0 ml acetic acid and 8.0 ml 3a70B scintillation fluid. Radioactivity was determined by liquid scintillation counting.

Results

We generated Cd-resistant sublines of 2008 and COLO 316 cells by selecting cells with stepwise increases in $CdCl_2$ and $ZnCl_2$ concentrations. The 2008/MT cells became 3.2-fold resistant to $CdCl_2$ and were 4.3-fold cross-resistant to DDP as determined by clonogenic assay (Fig. 1). Despite the fact that COLO/MT cells grew normally in concentrations of $CdCl_2$ that kill the parent cells (35 μ*M* $CdCl_2$), the COLO/MT cells were only 1.2-fold resistant to $CdCl_2$ as determined by clonogenic assay (Fig. 2). These cells were, however, 3.3-fold cross-resistant to DDP (Fig. 2).

Since Cd-resistance is only an indirect indication that MTs may be elevated in these cells, we next examined the changes in the MT content of these cells directly. The MTs were elevated 23-fold in 2008/MT cells and 9.4-fold in COLO/MT cells versus their respective parent cells (Table 1). Both acute and chronic heavy metal exposure is known to affect GSH and GSH-transferase levels [22, 31, 32] so we also examined the GSH and GSH-transferase content in these cells. GSH transferase levels were 0.20 mU/mg protein in both COLO 316 cell types and 0.14 mU/mg protein in both 2008 cell types. GSH was elevated 3.4-fold in the 2008/MT cells and 2.8-fold in the

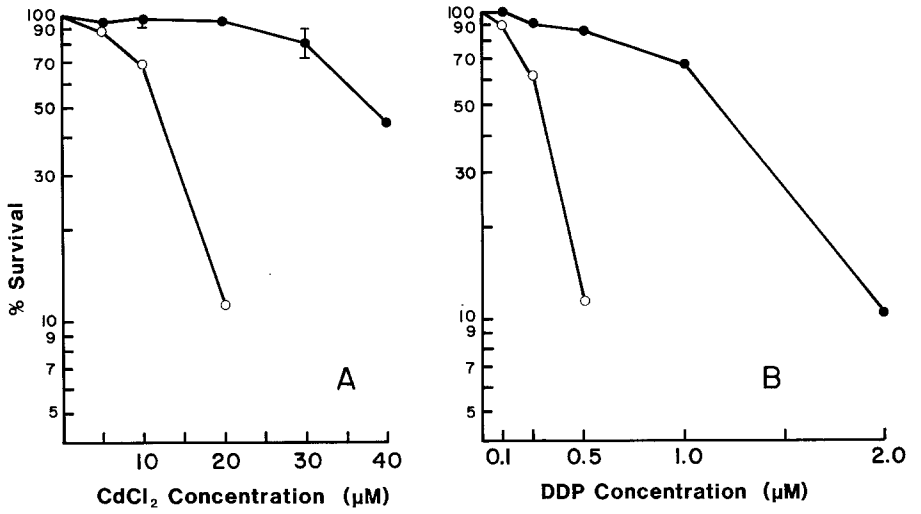

Fig. 1 A, B. Typical dose response of 2008 and 2008/MT cells to **A** $CdCl_2$ and **B** DDP. Drug cytotoxicity was determined by clonogenic assay on plastic. *Points* are means of triplicate plates. SE *(bar)* was less than 5% except where indicated. ○, 2008 cells; ●, 2008/MT cells

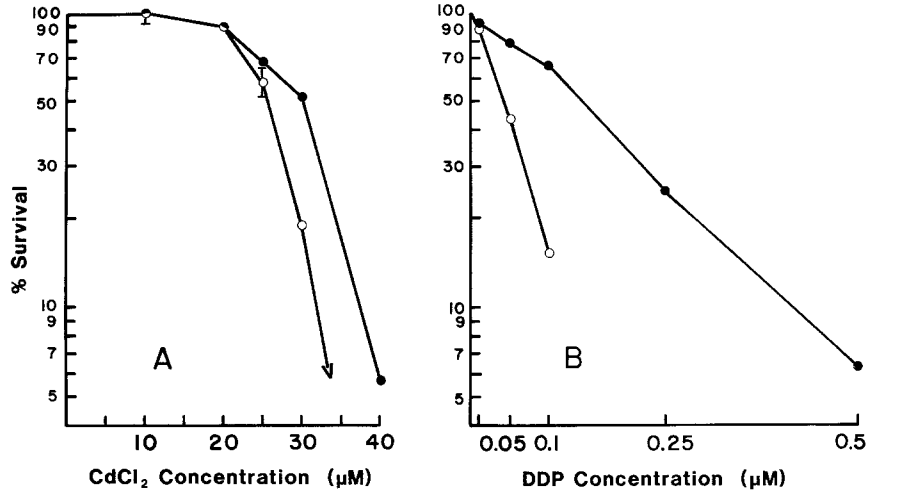

Fig. 2 A, B. Typical dose response of COLO 316 and COLO/MT cells to **A** $CdCl_2$ and **B** DDP. Drug cytotoxicity was determined by clonogenic assay on plastic. *Points* are means of triplicate plates. SE *(bar)* was less than 5% except where indicated. ○, COLO 316 cells; ●, COLO/MT cells

COLO/MT cells (Table 1). However, when the total thiols from the MTs were accounted for, the MT contribution to the total thiol content was 3 to 4 times the GSH thiol contribution (Table 1).

To determine what importance the elevated GSH had in mediation of the $CdCl_2$ and DDP resistance, we depleted GSH and 2008 in 2008/MT cells with the γ-glutamylcysteine synthetase inhibitor BSO. Treatments that depleted GSH to 12% of the control levels did not reverse either the $CdCl_2$ or DDP resistance of 2008/MT cells (Fig. 3).[1] With this BSO treatment the GSH levels did not rebound past 50% of control cells until 17 h after drug exposure. GSH depletion caused the already low levels of MTs in 2008 cells to drop approximately 50%, but had no effect on MT levels in 2008/MT cells (data not shown).

Decreased Cd^{+2} transport has been implicated as a factor in the development of Cd resistance [14]. To determine whether decreased uptake could account for the DDP-resistant phenotype, we next examined the uptake of DDP into 2008 and 2008/MT cells. Uptake of 20 μ*M*

Table 1. Correlation of MT[a] and GSH[b] levels with DDP resistance[c]

Cell line	Fold resistance[d]		Metallothionein level		Glutathione level
	$CdCl_2$	DDP	(nmol)[e]	(nmol SH)[e]	(nmol)[e]
2008	1.0	1.0	0.37	7.4	12.7
2008/MT	3.2	4.1	8.4 (23)[f]	168	43.2 (3.4)[f]
COLO 316	1.0	1.0	1.9	38	45.4
COLO/MT	1.2	3.3	17.9 (9.4)[f]	358	127.5 (2.8)[f]

[a] Determined by the method of Patierno et al. [29]
[b] Determined by the method of Reed et al. [30]
[c] Determined by clonogenic assay
[d] Fold restistance = IC_{50} subline/IC_{50} parent
[e] nmol are expressed per 10^6 cells
[f] Fold elevation versus parent

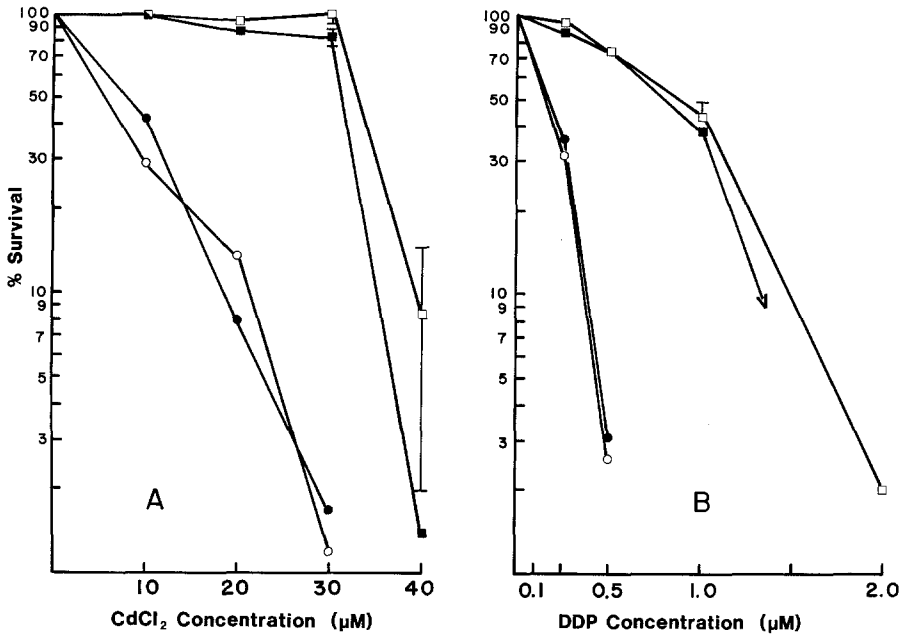

Fig. 3 A, B. Typical dose response of 2008 and 2008/MT cells to **A** $CdCl_2$, and **B** DDP following depletion of GSH with 0.5 m*M* BSO for 24 h. *Points* are means of triplicate plates. SE *(bars)* were less than 5% except where indicated. ○, ●, 2008 cells; □, ■, 2008/MT cells. *Closed symbols,* control cells; *open symbols,* GSH-depleted cells. Figures 1A and 3A do not match precisely: day-to-day sensitivity to $CdCl_2$ frequently varied

[^{195m}Pt]DDP was identical in both these cell types at 2.1 pmol/mg protein per min for up to 60 min (data not shown). Alterations in DDP uptake were therefore not a factor in the DDP resistance of these cells.

If elevated MTs mediate resistance to DDP directly, then these proteins should bind and inactivate significant amounts of the intracellular drug. To confirm that MTs were acting as an intracellular sink for DDP we treated 2008 cells and 2008/MT cells with [^{195m}Pt]DDP for 3 h. Fractionation of the cytosol by gel filtration revealed that a significant amount of the intracellular Pt in 2008/MT cells was associated with the MT fraction at $V_e/V_o = 1.8$ to 2.2 (Fig. 4). The 2008 cells had very little Pt associated with the MT fraction, even when cells were exposed to 20 μ*M* DDP, twice the concentration of DDP used with the 2008/MT cells (Fig. 4). Likewise, 2008/DDP cells had very little PT associated with the MT fraction. As shown in Table 2, the Pt content of the 2008 and 2008/DDP cells was almost equally divided between the particulate and cytoplasmic fractions. Only 4% and 2% of the total Pt was associated with MTs in the 2008 and 2008/DDP cells, respectively. In the 2008/MT cells, however, the Pt content of the particulate fraction dropped approximately 10% and there was a corresponding increase to 17% in the Pt content of the MT fraction. MT-associated Pt accounted for 28% of the cytosolic Pt in 2008/MT cells, as against 7% in 2008 cells and 3% in 2008/DDP cells. Interestingly, the 2008/DDP cells exhibited a shift in the cytoplasmic Pt (approximately 10%) away from the high-molecular-weight fraction into the low molecular weight fraction.

Discussion

MTs are inducible, thiol-rich proteins that present attractive targets to electrophilic compounds. Other workers have shown that normal human epithelial cells, mouse fibroblasts, and Ehrlich ascites cells that possess high levels of MTs bind a significant portion of the intracellular Pt in the MT fraction [3, 23]. In normal tissues, Pt has been found associated with the MT fraction in rat liver and kidney following dosing with DDP [25, 34, 37]. This evidence suggests that MTs may be an important determinant of DDP cytotoxicity. The treatment of ovarian carcinoma with DDP frequently fails due to the acquisition of DDP resistance. However, the biochemical mechanisms of DDP resistance are poorly understood. In this report we have

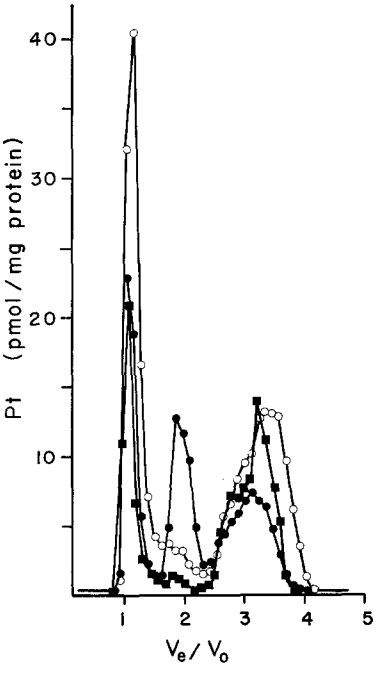

Fig. 4. Sephadex G-75 elution profile of cell lysates following exposure of cells to [^{195m}Pt]DDP for 3 h as described in "Materials and methods". ○, 2008 cells; ●, 2008/MT cells; ■, 2008/DDP cells

[1] Figure 3A does not exactly match Fig. 1A. For unknown reasons, the day-to-day sensitivity to $CdCl_2$ by clonogenic assay frequently varied. The absolute differences between cell lines, as reflected by the IC_{50} values, were however consistent.

Table 2. Distribution of platinum in 2008, 2008/DDP, and 2008/MT cells

	Percent of total Pt			Percent of cytosolic Pt		
	2008	2008/DDP	2008/MT	2008	2008/DDP	2008/MT
Particulate	48	48	40	–	–	–
Cytosolic	52	52	60	100	100	100
High molecular weight	22	18	20	43	35	33
MT fraction	4	2	17	7	3	28
Low molecular weight	26	32	23	50	62	39
Total Pt (pmol/10^6 cells)	77[a]	N.D.	35			

Cells were fractionated as described in "Materials and methods". Pt was determined following 3 h exposure to [^{195m} Pt] DDP

[a] 2008 cells were treated with twice the concentration of [^{195m} Pt] DDP as the 2008/DDP and 2008/MT cells

addressed the issue of whether human ovarian carcinoma cells can over-express MTs and whether this can be a potential mechanism of resistance of these cells to DDP.

Elevated MT levels were found in 2008 and COLO 316 ovarian carcinoma cells selected with $CdCl_2$ and $ZnCl_2$. These cells were resistant to $CdCl_2$ and cross-resistant to DDP as determined by clonogenic assay. Fractionation of 2008 and 2008/MT cells by gel filtration revealed that the Pt in the MT-containing cells was distributed into a new molecular weight region which had very low levels of Pt in the parent 2008 cells. The elution volume of these fractions ($V_e/V_0 = 2.0$) was typical for that reported for authentic MTs [18]. The covalent binding of DDP to purified rat liver MT-I or MT-II in cell-free incubations has been previously documented [23, 37]. DDP will displace Zn from MTs but not Cd [23, 33, 37]. Zelazowski et al. [37] have shown that Pt bound to MTs migrates with the MTs on gel electrophoresis, generates a UV band typical of Pt-S bonds, and cross-reacts with MT antibodies in a competitive RIA. The evidence that DDP binds to MTs both in vitro and in vivo is thus compelling and strengthens the argument that authentic MTs are covalently binding DDP in our cells. The increase in the proportion of the intracellular Pt that was bound to MTs in 2008/MT cells was at the expense of the Pt in the particulate fraction. With the crude cell lysis procedure used, the particulate fraction would be expected to contain any intact nuclei and the majority of the chromatin. This redistribution of Pt in the 2008/MT cells thus suggests that less DDP was reaching what is believed to be the critical target for DDP-induced cytotoxicity, DNA.

Approximately four times as much total cellular Pt was found in the MT fraction from 2008/MT cells as from 2008 cells. The 2008/MT cells were also approximately 4-fold resistant to DDP. However, we believe that it is inappropriate to attempt to examine this data for a direct correlation between MT levels and the degree of resistance. DDP or its aquation products react with a myriad of intracellular components; these reactions will depend on the DDP concentration in different regions of the cell and the inherent rate constant for each target. The intracellular binding and localization of DDP is therefore a complex process. A priori, it will be impossible to predict whether a large or small increase in a particular nucleophilic sink such as GSH or MTs will be needed to have a singificant impact on the platination of critical targets such as DNA.

Chronic exposure of CHO cells to $CdCl_2$, besides increasing MT levels, in some instances has been shown to elevate GSH levels [31]. Acute exposure to $ZnCl_2$ has also been shown to elevate GSH and GSH-transferase levels and antagonize melphalan cytotoxicity [32]. We thus measured the GSH levels in our cells and found that our selection procedure was elevating GSH in addition to MTs. The effect of GSH depletion on the DDP and Cd resistance of 2008/MT cells was negligible even though the GSH was depleted for a period that would allow these agents ample opportunity to produce a considerable amount of damage. This indicated that GSH was not playing an important role in defending these cells against DDP and $CdCl_2$, and that the primary defense was therefore elevated MTs. No change was found in the GSH-transferase levels. While a role for GSH in mediating the resistance of these cells cannot be ruled out, it is possible that the GSH is elevated due to the heavy demand for cysteine required for MT synthesis [22, 31]. GSH is known to serve as a ready source of cysteine through the operation of the γ-glutamyl cycle [26].

While it is clear that DDP will bind to preformed MTs, several studies have shown that DDP itself does not induce MTs [7, 25, 37]. Our observations indicate that 2008 cells selected in vitro for resistance to DDP do not possess elevated MTs. Even if the mechanisms of resistance expressed in vitro to DDP do not involve MTs, the in vivo processes that select for DDP resistance are certainly much more complex. MTs are induced by a wide variety of stresses besides heavy metals and glucocorticoid hormones [20]. Any one of these stress-related factors, if present during DDP therapy, could serve to elevate MTs in tumor cells. DDP is often given in combination with other agents that delay the traverse of cells through S phase; S phase delay is known to promote the amplification of many genes. Cells with amplified MT genes may then be selected with the DDP given in combination; proliferation of these cells could then lead to clinically overt DDP resistance. In addition, patients frequently receive dexamethasone, a known MT inducer, to control emesis. Aapro et al. [1] have shown that dexamethasone does not affect sensitivity of P388, human endometrial, or human colon cancers to DDP in mice, but this issue has not been addressed for ovarian carcinoma either in vitro or in vivo. Studies to define the role of MTs in DDP resistance in vivo more closely and to define the role of dexamethasone in modulating DDP response are being pursued.

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