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Metallothionein-like proteins and cell resistance to *cis*-dichlorodiammineplatinum(II) in L1210 cells*

Paul Farnworth, Brian Hillcoat, and Ian Roos

Experimental Chemotherapy Unit, Peter MacCallum Cancer Institute, 481 Little Lonsdale Street, Melbourne, Victoria, 3000, Australia

Summary. Our studies on the mechanism of resistance of the murine leukemia L1210-PDD line to *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) have not shown why it is 10-fold more resistant to the drug than the L1210 line. For this reason we investigated metallothionein-like proteins ('MTs') in these cells. Soluble protein extracts from cultures treated for 24 h with *cis*-DDP, zinc sulphate or saline were anaerobically eluted from columns of chemically reduced Sephadex G-75, and the profiles of zinc, copper and platinum were determined along with those for incorporated radioactive cyst(e)ine and tyrosine. Both saline-treated cell lines contained similar levels of 'MTs', which were induced by exposure to a minimally toxic level of zinc (100 μM). Zinc induction of 'MTs' was nearly 4-fold greater in L1210 than in L1210-PDD cells. The levels of mRNA for metallothionein I (MTI) and II (MTII) in uninduced cells were measured by dot-blotting with a cDNA probe. The L1210-PDD cells contained 80% of the MTI and 41% of the MTII compared with L1210 cells, confirming the similar levels in uninduced cells. L1210-PDD cells were 2-fold more sensitive than L1210 cells to cadmium and equally sensitive to zinc. Thus, the resistance of L1210-PDD cells to *cis*-DDP was not associated with cross-resistance to group II_b metals, whereas their sensitivity to cadmium did reflect the relative inability of the cells to synthesize 'MTs'. The L1210 cells produced 'MTs' when treated with 0.5 and 5.0 μM *cis*-DDP, but the L1210-PDD cells did not when treated with 5.0–40 μM *cis*-DDP. Small amounts of platinum (<21% of the total eluted) were bound to 'MTs' in both cell lines, but platinum provided a minor portion of the 'MT'-bound metals, with zinc and copper contributing the bulk. The basis for the resistance of L1210-PDD cell to *cis*-DDP is neither an increased level of 'MTs' in the resistant cells nor an enhanced ability to increase the synthesis of 'MTs' after drug exposure.

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Offprint requests to: P. Farnworth: C/- Medical Research Centre, Prince Henry's Hospital, St. Kilda Road, Melbourne, Victoria. 3004, Australia

Introduction

Cells may develop resistance to cytotoxic drugs by numerous mechanisms, including alteration of the membrane transport of the drug [41, 46], reduction in the affinity of a drug for its binding site on a critical target protein through point mutation of the coding gene [8] and an increase in the pool of target protein through amplification of the same gene [8, 12, 43]. Several mechanisms are described for *cis*-DDP resistance, but we have found no significant difference in drug uptake, drug egress, glutathione content or repair of DNA interstrand cross-links in the L1210-DDP cells [47]. Although DNA is believed to be the critical target on which *cis*-DDP exerts its cytotoxic action(s) [42], the platinum nucleus of the drug also has a high affinity for sulphur-binding sites on protein, as do other "soft" or class "b" Lewis acids such as copper(I) and cadmium [5, 27].

A further mechanism of resistance to cytotoxic agents involves the amplification of genes encoding proteins that can sequester and thus detoxify the drugs. This mechanism is exemplified by clonal cell sublines resistant to cadmium that have been isolated after the continuous exposure of cadmium-sensitive parent cell lines to increasing concentrations of the metal in tissue culture [7, 13, 20, 23, 25]. The resistant clones can markedly increase their synthesis of metallothioneins (MTs) in response to group I_b or II_b metals relative to that observed with the parent cell line [7, 13, 20, 23]. MTs are a family of small, cytoplasmic proteins that are particularly rich in heavy metal-binding cysteine residues. Their ability to reduce the toxicity of cadmium has been amply documented [22, 29]. Cadmium-resistant cell sublines that have amplified MT genes and/or an enhanced ability to synthesize MTs show cross-resistance to various cytotoxic agents, including X-rays [4], alkylating agents [15, 38] and other metals that bind the MTs [13, 16, 20, 21]. In accordance with its known chemistry [5, 27], platinum derived from *cis*-DDP binds to and displaces zinc from MTs [33, 48]; hence, it is not surprising that cadmium-resistant, MT-overproducing cell lines show cross-resistance to *cis*-DDP in tissue culture [3, 38].

Several research groups have investigated the hypothesis that resistance to *cis*-DDP, like that to cadmium, involves MTs [3, 17, 33, 34, 37, 44, 48]. However, the interaction between *cis*-DDP and MTs and its role in resistance to the drug have mostly been studied under circumstances

that are indirect, involving cadmium as the primary inducer of MTs or of the resistance phenotype.

We have previously found that *cis*-DDP and/or its hydrolysis products not only bind to but also induce biosynthesis of MTs in mouse liver and kidneys [17]. In the present work we sought to determine whether cells primarily resistant to *cis*-DDP have an enhanced ability to synthesize MT-like proteins ('MTs') in response to known MT inducers and/or *cis*-DDP. In addition, the question as to whether cells that are primarily resistant to *cis*-DDP show cross-resistance to MT-inducing metal ions was investigated.

Materials and methods

Drugs and reagents. The complex *cis*-DDP was obtained as a gift from the Institute of Drug Technology, Parkville, Australia. Stock solutions of *cis*-DDP and the supplied chloride or sulphate salts of zinc and cadmium (B. D. H. Chemicals, Australia) were freshly prepared in 0.9% sodium chloride and then sterilized by filtration through a 0.22-μ*m* filter. Radioisotopes and stock drug solutions were appropriately diluted in 0.9% sodium chloride to achieve a standard 1 : 50 dilution to the required concentration in the medium.

Chelex-100 (100–200 mesh, Bio-Rad) was used for metal decontamination of chromatography reagents. Water with a specific resistance of >15 Mohm-cm, obtained from a four-cartridge NANOpure-A water purification system (Sybron/Barnstead), was used throughout the procedure. Water for buffers was degassed by boiling for at least 30 min, then cooled under a stream of nitrogen. Buffer A (10 m*M* TRIS HCl, pH 8.0) and buffer B (buffer A plus 250 m*M* sucrose and 5 m*M* mercaptoethanol) were prepared and, where possible, maintained under an atmosphere of nitrogen. All chemicals were analytical grade or purer. Acids used in atomic absorption procedures were "Suprapur" grade (Merck).

Cell lines and culture conditions. L1210 murine leukemia cells and a *cis*-DDP-resistant subline, L1210-PDD (obtained from Dr. J. Burchenal of the Memorial Sloan-Kettering Institute, New York) [9] were grown as suspension cultures in Eagle's modified minimum essential medium containing gentamicin (20 μg/ml) and supplemented with 15% fetal bovine serum and 2 m*M* L-glutamine (all obtained from Flow Laboratories). L1210-PDD cultures additionally contained *cis*-DDP (2 μg/ml). Stock cultures were kept in Costar tissue-culture flasks at 37° C in a humidified atmosphere of 10% CO_2, 5% O_2 in nitrogen and sub-cultured once in the absence of antibiotics (and *cis*-DDP in the case of L1210-PDD cells) before use. Both cell lines were confirmed to be free of *Mycoplasma* contamination at regular intervals.

Drug inhibition of cell growth. L1210 and L1210-PDD cells from log-phase cultures were pelleted by centrifugation and resuspended at about 5×10^4 cells/ml in fresh medium containing gentamicin (20 μg/ml), and 2.0-ml aliquots of cell suspension were dispensed by Cornwall syringe into 16-mm (diameter) wells of 24-well cluster dishes (Costar), each well containing 40 μl test drug in graded concentrations. After growth for four doubling times, the single-cell suspensions obtained from each well were counted on a Coulter model ZM counter. The L1210-PDD cells took 72 h for four doublings compared with 48 h for the L1210 cells due to an initial lag period, although their actual doubling time was similar (13–14 h). Mean cell numbers from duplicate drug-treated cultures for each drug concentration were compared with the mean cell population from four saline-treated control cultures grown in the same cluster dish. The drug concentration at which cell proliferation was 50% of that in control cultures (IC_{50}) was determined by interpolation from graphs of log (drug concentration) vs percentage of growth inhibition.

Preparation of metallothionein-like proteins from cell cultures. Cells obtained from log-phase cultures were resuspended at $2-5 \times 10^5$ cells/ml in 50–100 ml fresh, antibiotic-free medium containing the test compound and maintained under normal tissue-culture conditions for 24 h. During the final 2 h, cells were incubated with 0.5 μCi/ml [^{3}H]-cystine dihydrochloride (876 mCi/mmol) and 0.1 μCi/ml [^{14}C]-tyrosine (504 mCi/mmol), both isotopes having been obtained from Amersham. The final cell number in each culture was determined by haemocytometer counting, then cells were washed and harvested by centrifugation, resuspended in 3.0 ml ice-cold buffer B and sonicated at output 4, 40% duty cycle for 20 pulses using a model B-15 Branson Sonifier Cell Disruptor in the pulsed mode. After this treatment, no intact cells were evident by light microscopy. The resulting sonicate was centrifuged for 20 min at 11,600 *g* in a Micro-Centaur microcentrifuge (MSE) and the supernatant (extract) was removed and either chromatographed immediately or stored at −70° C for later assay. Aliquots of each sonicate and extract were taken for metal and radioactivity measurements.

Analytical gel-filtration chromatography and assay of metallothionein-like proteins. Cell extracts were chromatographed on a 1.6×68 cm column of Sephadex G-75 (superfine). The gel had previously been treated with alkaline potassium borohydride at 80° C for 2 h to reduce residual metal-binding carboxylate groups to hydroxyl groups [35]. A volume of cell cytosol corresponding to the soluble fraction of 2×10^7 cells was run onto the column of reduced Sephadex under a stream of nitrogen and was eluted with buffer A under an atmosphere of nitrogen. The column was routinely washed with 70–150 ml 0.05 *M* hydrochloric acid (Merck, Suprapur grade) between each sample elution and then regenerated with several column volumes of buffer A to eliminate metal carry-over between experiments. Rabbit cadmium, zinc-MTs (Sigma) eluted later from the chemically reduced Sephadex G-75 (Ve/Vo, ca. 2.7) than from the native gel (Ve/Vo, ca. 2.1). The buffer flow rate through the gravity-feed column was about 5 ml/h and 3.0-ml fractions were collected. The void volume was determined with blue dextran and the column was calibrated by standard methods using molecules of known molecular weight, including [^{3}H]-glutathione (1.0 Ci/mmol, New England Nuclear).

Chromatography fractions were monitored for UV light absorbance at 280 and 215 nm using a Cary model 219 spectrophotometer (Varian). Aliquots (0.50 ml) of each fraction were prepared in 3.0 ml PCS (Amersham) acidified with glacial acetic acid (1 ml/l), and radioactivity was measured in a 1215 Rack Beta II liquid scintilla-

tion counter (LKB/Wallac) using an external standard channels ratio for the correction of quenching.

Measurement of MTI and MTII mRNAs. DNA probes for murine MTI and MTII mRNAs were obtained from Dr. Julian Mercer, Murdoch Institute, Melbourne, and used with the permission of Dr. Richard Palmiter. A ribosomal RNA probe was used to standardize the amount of RNA applied to the filters. Probes were labelled by the random primer method [18, 19] using alpha-^{32}P-ATP. Cellular RNA was prepared from 4×10^7 cells of each line using guanidine isothiocyanate [11] and 10 µg RNA dissolved in 50 µl water, treated with formaldehyde, heated to 65° C and applied to a nitrocellulose membrane, which was baked for 45 min after washing. The probes were hybridized by a standard method [28] and autoradiographs were developed from the filters; the resulting X-rays were scanned by laser densitometer. The amounts of mRNA for MTI and MTII were compared by setting the lesser amount as being equal to 1, after correcting for the amount of RNA on the filter as shown by the probe for ribosomal RNA.

Metal analyses. Platinum and copper were measured by graphite-furnace atomic absorption spectrophotometry using a Perkin-Elmer 3030 spectrophotometer with an HGA-400 furnace and an AS 40 autosampler. Pyrolytically coated graphite tubes purged with nitrogen gas were used throughout the procedure. The absorption-peak area for each metal was measured, and a deuterium continum source was used to obtain simultaneous background correction. For platinum estimations, 20- or 50-µl samples were charred at 1,400° C and vaporized at 2,500° C, and atomic absorption was monitored at the 265.9-nm line with the gas flow stopped. In the case of copper, 10-µl samples were charred at 900° C and vaporized at 2,100° C with the gas flow stopped and the lower sensitivity, 327.4-nm line was used. Samples and standards were analysed in triplicate.

Zinc was measured by conventional flame atomic absorption at 213.7 nm using a Varian model AA-5 atomic absorption spectrophotometer modified with AA-6 components. The absorption signal was integrated over 3 s without background correction. Standards for atomic absorption assays were prepared from commercial 1,000 ppm stock solutions (B. D. H.) by dilution in 5% HCl.

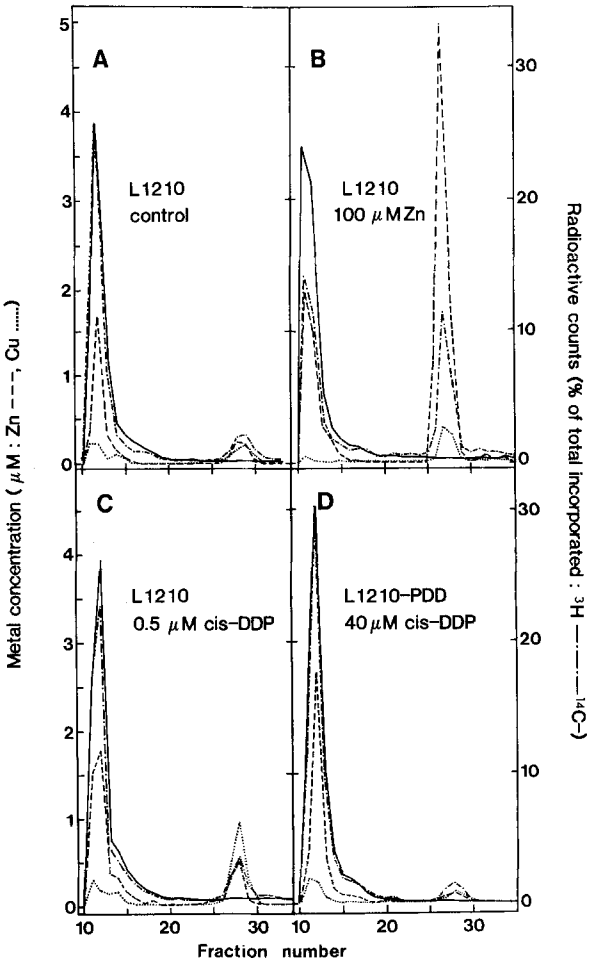

Fig. 1. Distribution of zinc and copper (µ*M*) and of [^{14}C]-tyrosine- and [^{3}H]-cystine-derived radioactivity (percentage of total incorporated) across the soluble protein profile of L1210 (*A*–C) and L1210-PDD (*D*) cells following growth for 24 h in the presence of saline (*A*), zinc sulphate (100 µ*M*, *B*) or *cis*-dichlorodiammine-platinum(II) (*cis*-DDP: 0.5 µ*M*, *C*; 40 µ*M*, *D*), Extracts prepared by sonication and ultracentrifugation of cells in the presence of mercaptoethanol were anaerobically fractionated on a 1.6 × 68 cm column of borohydride-reduced Sephadex G-75 (superfine) that was previously washed with dilute HCl and were then equilibrated and eluted with 10 m*M* TRIS-HCl (pH 8.0). Fractions of 3.0 ml were collected and analysed for zinc by flame, for copper and platinum by graphite-furnace atomic absorption spectrophotometry and for isotopes by liquid scintillation counting. Proteins in the second peak shared many characteristics with metallothioneins

Results

Status of MT-like proteins in cell extracts

Cell extracts from the L1210 and L1210-PDD cell lines were fractionated anaerobically on a column of chemically reduced Sephadex G-75 (superfine). The profiles of soluble material in these extracts included two main peaks. The second of these peaks from saline-treated L1210 cultures (Fig. 1 A) contained material that bound zinc and copper at a ratio of 1.4:1, incorporated [^{3}H]-cystine-derived radioactivity but no [^{14}C]-tyrosine, absorbed light at 215 nm but not at 280 nm and eluted in the same volume as rabbit cadmium, zinc-MTs. The material from the second peak of the profiles thus had the characteristics of MTs, which are distinct from other known proteins. However, since its amino acid composition has not been determined, it cannot be unequivocally identified as such [22] and will hereon be signified as 'MTs'. The levels of 'MTs' were compared on the basis of their content of zinc and copper. It is assumed that if any of these metals were lost from 'MTs' during chromatography, the loss would be small, given their tight binding to 'MTs', and similar in each preparation. Total recovery of zinc, copper and platinum from the columns was 87%, 94% and 87%, respectively.

The levels of 'MTs' in the two cell lines after various treatments are summarised in Fig. 2. A similar level of 'MTs' to that found in the control L1210 cell extracts also occurred in the extracts from saline-treated cultures of L1210-PDD cells. Zinc predominated over copper (1.5 : 1) in the latter case, but the relative rate of basal 'MT' syn-

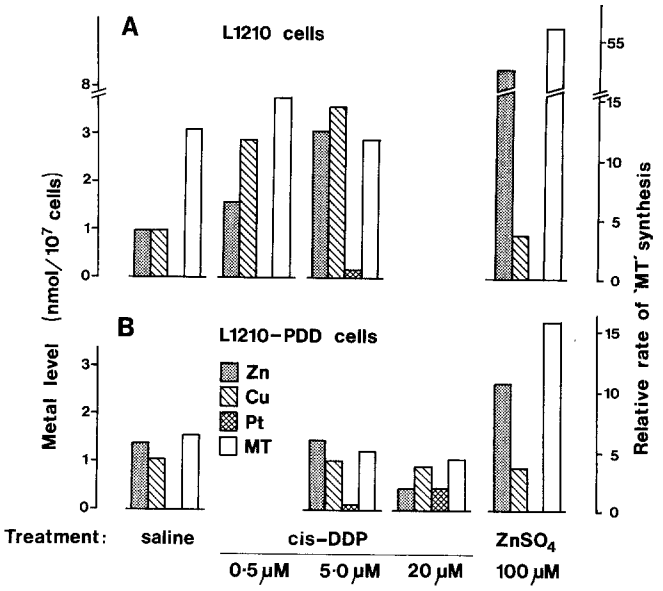

Fig. 2. Zinc, copper and platinum content and relative rate of synthesis of metallothionein-like protein(s) (*'MT(s)'*) isolated by Sephadex G-75 chromatography from L1210 (*A*) and L1210-PDD (*B*) cell cytosols after cultures were exposed for 24 h to saline, zinc sulphate (100 μ*M*) or *cis*-DDP (0.5–20 μ*M*). See legend to Fig. 1 for experimental details. The relative rate of 'MT' synthesis = 100× [^{3}H]-cystine-derived d.p.m. in the 'MT' peak/[^{3}H]-cystine-derived d.p.m in earlier fractions, as determined for MTs by Hildebrand et al. [25]. Triplicate assays of control L1210 and L1210-PDD cultures gave the following respective coefficients of variation for 'MT' parameters: zinc content, 2.2% and 5.1%; copper content, 33% and 24%; relative rate of synthesis, 17% and 18%. Triplicate assays of L1210-PDD cultures treated with 5.0 μ*M* *cis*-DDP yielded a coefficient of variation of 9% for 'MT' platinum content. Results shown for these groups represent the means of three determinations. Other 'MT' assay results are from single assays, each run against an independent control prepared on the same day

thesis in L1210-PDD cells was only about half that in L1210 cells. Two dot-blotting experiments with DNA probes showed that L1210-PDD cells contained 0.8 times the level of MTI mRNA and 0.4 times the level of MTII mRNA determined in L1210 cells, relative to equal amounts of ribosomal mRNA.

The ability of each cell line to synthesize 'MTs' in response to known inducers of true MTs was tested using zinc sulphate at 100 μ*M*, a concentration that caused minimal growth inhibition of either culture. 'MT' synthesis was stimulated by zinc challenge in both L1210 (Fig. 1 B) and L1210-PDD cells (see Fig. 2). The former cells contained 2.8 times more 'MT' than did the latter after treatment for 24 h with zinc. When compared with that in the appropriate control, the increase in 'MT' content was 3.5-fold greater in L1210 than in L1210-PDD cells. Furthermore, the relative rate of 'MT' synthesis at the end of the 24-h exposure to zinc was nearly 4-fold higher in the L1210 cell culture. The copper content of the 'MTs' from each zinc-treated cell line differed little from the respective control, such that zinc accounted for almost all of the additional 'MT'-bound metal (Fig. 2).

After a 24-h exposure of L1210 cells to *cis*-DDP (0.5–5 μ*M*), the amounts of zinc and copper that were associated with 'MTs' were greater than those in saline-treated control cultures (Fig. 2), whereas incorporation of [^{3}H]-cystine was similar and that of [^{14}C]-tyrosine, negligible (see Fig. 1 C). The increase of 'MTs' produced by *cis*-DDP in L1210 cell depended on the drug concentration, but the relative rate of 'MT' synthesis 24 h after the addition of *cis*-DDP differed little from that in controls (Fig. 2). The distribution of platinum across the soluble protein profile for the L1210 cells exposed to <5.0 μ*M* *cis*-DDP could not be determined reliably, because the levels of platinum in individual chromatography fractions were mostly at or below the limit of detection. In cells exposed to 5.0 μ*M* *cis*-DDP, around 60% of the total eluted platinum was associated with high-molecular-weight proteins, 13% was bound to 'MTs' and 23% occurred in low-molecular-weight forms; other minor peaks were also found, one each side of the 'MTs', with associated zinc and/or copper. The eluted 'MTs' bound zinc, copper and platinum at ratios of 0.9:1.0:0.04, respectively. The amounts of zinc, copper and platinum per 10^7 cells in the L1210 cell extract rose progressively in response to increasing concentrations of *cis*-DDP, whereas the ratio of Zn : Cu in the extract fell from 3.9 to 1.7.

The 'MT' content of L1210-PDD cells after a 24-h continuous exposure to 5.0 μ*M* *cis*-DDP barely exceeded that observed in saline-treated cultures (Fig. 2). This contrasts with the 4-fold increase in the level of 'MTs' found in L1210 cells given the same drug treatment. Also, there was no evidence for 'MT' induction in L1210-PDD cells treated with either 20 μ*M* (Fig. 2) or 40 μ*M* (Fig. 1 D) *cis*-DDP. The latter concentration of the drug caused a level of growth inhibition in the L1210-PDD culture that was similar to that produced by 5 μ*M* *cis*-DDP in L1210 cells. The relative rate of 'MT' synthesis in L1210-PDD cell cultures after a 24-h exposure to *cis*-DDP declined slightly as the drug concentration was increased (Fig. 2). Under 25% of the platinum in the treated L1210-PDD cell extracts was 'MT'-bound, whereas nearly 30% was found to be associated with high-molecular-weight proteins and the rest of the platinum with low-molecular-weight species. The 'MTs' in L1210-PDD cells treated with 5.0 μ*M* *cis*-DDP bound zinc, copper and platinum at ratios of 1.5 : 1 : 0.12, respectively, but apart from the presence of platinum, there was no consistent change in the metal content of the cell extract or in its Zn : Cu ratio.

Metal sensitivity studies

The results of cytotoxicity assays in which L1210 and L1210-PDD cells were continuously exposed for four doubling times to metal salts and complexes are given in Table 1. Under these conditions, L1210-PDD cells were 10-fold more resistant than L1210 cells to growth inhibition by *cis*-DDP. Curves for growth of the two cell lines in the presence of *cis*-DDP showed that at a concentration of 5.0 μ*M*, the drug completely inhibited growth of L1210 cultures but caused only 35% inhibition of L1210-PDD cell proliferation. Based on their respective IC_{50}s (Table 1), L1210-PDD cells were twice as sensitive as L1210 cells to cadmium, thus showing collateral sensitivity to this metal, but they still retained the same sensitivity to zinc as L1210 cells.

Discussion

The L1210-PDD cell line was generated from a parent L1210 murine ascites leukemia by repeated exposure to

Table 1. Inhibition of culture growth produced by the continuous exposure of *cis*-DDP-sensitive (L1210) and -resistant (L1210-PDD) murine leukemia cells to platinum complex or metal ion over four doubling times and the relative resistance shown by L1210-PDD cells

Agent	Tissue culture IC_{50} (μM):		Relative resistance
	L1210 (= I)	L1210-PDD (= II)	(II/I)
cis-DDP	1.0 ±0.05	9.8 ±0.3	9.8
Zn^{2+}	141 ±3	161 ±5	1.1
Cd^{2+}	97 ±2	45 ±1	0.5

IC_{50}, interpolated drug concentration that reduces the cell population to 50% of that in matched, saline-treated controls
Data represent the means ± SD from four experiments

cis-DDP [9] and is thus primarily resistant to that drug (or related hydrolysis products). A 10-fold difference in sensitivity was consistently found in growth inhibition assays carried out under the stated conditions in this laboratory, although differences of up to 60-fold have been reported [14]. Our results further show that L1210-PDD cells are not cross-resistant to zinc and show a collateral sensitivity to cadmium. Human ovarian carcinoma cell lines derived from the parent lines 2008 and COLO 316 and made primarily resistant to *cis*-DDP likewise show no cross-resistance to cadmium [38]. This pattern for *cis*-DDP-resistant cells differs from that found in cells made primarily resistant to cadmium and for which cross-resistance to *cis*-DDP has been reported [3, 38].

Primary resistance to the cytotoxic actions of cadmium and other group I_b and II_b metal ions commonly reflects an enhanced ability of the resistant cells to synthesize MTs that bind and detoxify the metals [7, 13, 20, 23, 25, 29]. It is well established in vivo and in tissue culture that the pre-existence of high levels of MTs within cells coincides with enhanced platinum binding by the MTs and resistance to the cytotoxicity of *cis*-DDP [3, 34, 39, 48]. However, published evidence in favour of MT involvement in resistance to the toxic actions of *cis*-DDP has generally been complicated by the prior exposure of the resistant cells to other metals, such that the detoxifying MTs have to be induced by a third agent. For example, the reported cross-resistance of cadmium-resistant, MT-rich cells to *cis*-DDP was demonstrated in vitro soon after the removal of cadmium from the growth medium [3]; the cells would thus have had elevated MTs induced by their exposure to the cadmium. In vivo, prior induction of MTs by the injection of cadmium [34, 48] leads to greater MT binding of platinum after a subsequent injection of *cis*-DDP and, as a corollary, pre-administration of the MT-inducing metals zinc and cadmium to mice results in protection against the toxicity of *cis*-DDP [39]. Despite these findings, there is no evidence to date that implicates 'MTs' in resistance to *cis*-DDP without the involvement of other agents in the prior induction of the proteins.

'MTs' have been identified in normal lymphocytes [1, 24, 32] and neoplastic lymphoid cells [45]. The mouse leukemia L1210 and L1210-PDD cells were shown in the present work to contain proteins that resemble MTs in many respects. These 'MTs' co-elute with true MTs from chemically reduced Sephadex G-75, have high zinc and copper content, incorporate much cyst(e)ine and negligible tyrosine, have low light absorbance at 280 nm and high absorbance at 215 nm and are induced by exposure to zinc. Although absolute confirmation of their status as MTs will depend on the determination of their amino acid composition, their identification with true MTs is very likely. It seemed plausible that the resistance of L1210-PDD cells to the cytotoxic actions of *cis*-DDP would reflect a more effective induction of the synthesis of the 'MTs'. Our results, therefore, were unexpected.

Firstly, the amounts of 'MTs' in the two cell lines were similar as judged from their metal contents, but the L1210 cells had a roughly 4-fold higher capacity than L1210-PDD cells to produce 'MTs' during a 24-h challenge with a minimally toxic level of zinc. This relative deficiency in L1210-PDD cells may contribute to their 2-fold greater sensitivity to cadmium as compared with L1210 cells. It is not clear why collateral sensitivity to zinc was not also found in the L1210-DDP cells. It is possible that zinc inhibited the proliferation of the two cell lines by different mechanisms since the zinc growth-inhibition curves were not parallel, unlike the cases of matched cadmium and *cis*-DDP curves for the two cell lines.

Secondly, we found that the 'MT' pool in L1210 cells expanded in response to toxic levels of *cis*-DDP but that the resistant L1210-PDD cells lacked this ability. As with *cis*-DDP induction of MTs in the mouse [17], substantial increases in 'MT'-bound intracellular zinc and copper accompanied the exposure of L1210 cells to *cis*-DDP. The failure to show an increased rate of cysteine incorporation into 'MTs' 24 h after the addition of *cis*-DDP to the cultures does not invalidate this conclusion, since the rate of induced 'MT' synthesis falls towards control levels within 24 h in cells continuously exposed to cadmium or zinc in vitro [25, 30]. This effect was probably increased in the L1210 cells at *cis*-DDP levels of 5 μM and above, which were cytotoxic to most of the cells (IC_{50} = 1.0 μM). In addition, MT induction by *cis*-DDP could be self-limiting since the drug not only forms stable and inert complexes with the induced proteins but should also do so with their nuclear metal-sensitive regulating elements, which are rich in guanosine [31], the kinetically and thermodynamically favoured site of *cis*-DDP binding to DNA [36].

Although L1210 cells are better able than L1210-PDD cells to synthesize 'MTs', they remain more sensitive to the effects of *cis*-DDP. This anomaly may reflect limitations to the effectiveness of 'MTs' in binding and detoxifying *cis*-DDP within cells [17]. Firstly, the drug reacts slowly with 'MTs' in vitro [48]; this is consistent with the requirement for the formation of an intermediate platinum species. As well, relative to zinc and copper, platinum occurs at a low concentration inside the treated cells; thus, the probability of 'MT' binding sites, initially becoming occupied by platinum would be lower. Lastly, platinum-protein complexes are relatively inert [10, 27], which limits the possibility of transferring the metal from non-'MT' protein onto newly synthesized 'MTs', such as occurs during detoxification of the much more rapidly exchanging cadmium ion by MTs [26, 40].

The 'MTs' induced by *cis*-DDP in L1210 cells, as with *cis*-DDP-induced MTs in the mouse liver [17], bound much more zinc and copper than platinum, making this process an inefficient way of detoxifying *cis*-DDP. It is possible that the transfer of cellular zinc and copper into an expanding

pool of 'MTs' actually disadvantages *cis*-DDP-treated cells by causing a functional depletion of readily available metal. Under these circumstances, cells in which the *cis*-DDP-sensitive 'MT' induction was impaired would acquire greater resistance to the drug. Our results suggest that the zinc-sensitive mechanism for inducing 'MTs' in L1210-PDD cells is either insensitive to or inactivated by *cis*-DDP.

Discrepancies have been reported in the literature concerning the correlation of an enhanced capacity for de novo MT synthesis and resistance to MT-binding metal ions and drugs [13, 16, 40]. Indeed, the protective effect of MT-inducing metals on the lethal toxicity of *cis*-DDP in mice correlates poorly with the levels of MTs in either the liver or the kidneys [39]. Other molecules that react with *cis*-DDP and may play a role in resistance to the drug include glutathione [2] and glutathione-S-transferase [6]; their levels do not differ significantly in L1210 and L1210-PDD cells (F. Stone and B. L. Hillcoat, unpublished data). Other, as yet unidentified, metal-induced factors may also play some role in this process of *cis*-DDP detoxification. Species of lower molecular weight than 'MT' bound nearly half of the soluble platinum in L1210-PDD cells in contrast to L1210 cells, where platinum binding by this fraction of the extract was minor. Whether this difference is related to the resistance of L1210-PDD cells to *cis*-DDP remains to be determined.

In summary, our results show that 'MTs' are present in L1210 cells and the *cis*-DDP resistant subline, L1210-PDD, and that their synthesis is induced by exposure of both cell types to zinc in vitro. In comparison with that in L1210 cells, zinc induction of 'MTs' is less effective in L1210-PDD cells, which show no cross-resistance to zinc and a heightened sensitivity to cadmium. L1210 cells also produce 'MTs' in response to *cis*-DDP, whereas L1210-PDD cells do not. This pattern suggests that enhanced synthesis of 'MTs' is not the mechanism by which L1210-PDD cells are resistant to *cis*-DDP cytotoxicity. It is possible that the synthesis of 'MTs' does not benefit and may, in fact, disadvantage cells treated with *cis*-DDP.

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Note added in proof: Overexpression of metallothioneins has recently been shown to be the likely basis for resistance to *cis*-DDP in some cell lines [SL Kelley et al. (1988) Science 241: 1813]