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Studies on the resistance of a murine leukemia L1210 cell line to *cis*-diamminedichloroplatinium(II)

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Metal coordination complexes, particularly those of platinum, have been shown to be effective antineoplastic agents in both experimental animals and man. The most extensively investigated complex is *cis*-diamminedichloroplatinum(II) (*cis*-DDP)* whose mechanism of action has been reviewed recently [1]. *cis*-DDP reacts bifunctionally with DNA to form both DNA-interstrand and DNA-intrastrand cross-links as well as DNA–protein cross-links. In most previous investigations of the nature of the critical lesion(s), *cis*-DDP has been compared to its isomer, *trans*-DDP, which is ineffective as an anticancer agent. An alternative approach is made possible by the development of resistant cell lines in which the mechanism of resistance may reflect the mechanism of action of a drug. In this regard, a murine leukemia L1210 cell line (L1210/DDP) has been developed which is resistant to concentrations of *cis*-DDP 30-fold higher than its parent cell line (L1210/0) [2]. Previous authors have reported that sublines derived from the Walker carcinoma [3] and the murine L1210 cell lines [4], both with acquired resistance to melphalan (L-phenylalanine mustard), another drug which causes DNA-interstrand cross-links, were also cross-resistant to *cis*-DDP. Cross-resistance is not, however, as once thought to be [5], universal for all alkylating agents but instead many cell lines exhibit variable patterns of cross-resistance [4, 6]. This communication therefore analyzes the possible cross-resistance of both *cis*-DDP-sensitive and -resistant L1210 cells to a variety of DNA damaging agents.

The drugs were obtained from the following sources: *cis*-DDP (NSC 119875), Bristol Laboratories, Syracuse, NY; *trans*-DDP and *cis*-dichloroethylenediamineplatinum(II) (*cis*-DEP), Alfa Ventron, Danvers, MA; melphalan (NSC 8806), Burroughs Wellcome Co., Research Triangle Park, NC; neocarzinostatin (NSC 69856) and aziridinyl benzoquinone (NSC 182986), Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD; methyl methanesulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), Aldrich Chemical Co., Milwaukee, WI. All culture media and supplies were obtained from Gibco, Grand Island, NY.

A murine leukemia L1210/0 and a *cis*-DDP-resistant subline (L1210/DDP) were obtained from Dr. J. Burchenal of the Sloan Kettering Institute, New York, and have been maintained in the Vermont Regional Cancer Center, University of Vermont, for 2 years. Cells were grown in plastic culture tubes in 6 ml McCoys 5a (modified) medium supplemented with penicillin, streptomycin, fungizone and 15% calf serum. Twice weekly, stock cultures of L1210/0 were diluted 1:11 (v/v) with fresh medium. The L1210/DDP cells were diluted 1:5 with fresh medium containing 2 μg/ml *cis*-DDP.

Growth inhibition studies were performed by a modification of the method of Burchenal *et al.* [2]. L1210/DDP cells were always grown for 3 days in the absence of *cis*-DDP to permit recovery from previous drug treatment. Cells from stock cultures were pelleted at 900 *g* for 5 min and resuspended in fresh medium; aliquots of 2.5 ml were added to 2.5 ml of drug-containing medium to yield approximately 50,000 cells/ml. A range of drug concentrations was obtained by serial 2-fold dilutions with four tubes used at each dose. Tubes were tightly capped and incubated at 37° for 3 days. Cell numbers were then obtained on a Coulter counter. The cells were in logarithmic growth throughout, with a mean doubling time in untreated cells of about 18 hr. The growth rate slowed in untreated cells after 3 days due to high density and could thereby change the apparent ID_{50} values. The results are expressed as ID_{50}, defined as the drug concentration that caused 50 per cent inhibition of cell growth relative to untreated control cells as measured after 3 days.

An alternative method was also employed that facilitated various post-incubation experiments. Cells from stock cultures were centrifuged, resuspended in medium containing drug, and incubated at 37° for 1 hr. The cells were then recentrifuged, washed once in medium, resuspended at approximately 50,000 cells/ml, and incubated at 37° for 3 days.

Ultraviolet irradiation of cells was performed in plastic petri dishes with a germicidal lamp emitting predominantly 254 nm light at a dose rate of 1 J/m^2 per sec. The cells were then transferred to plastic culture tubes for the 3-day incubation period.

As with many chemotherapeutic agents, cellular resistance to platinum coordination complexes develops upon continued use of the drug. In this experimentally derived resistant cell line, a 30-fold resistance to *cis*-DDP and *cis*-DEP was observed, whereas only a 2-fold change in sensitivity to *trans*-DDP was apparent (Table 1). Resistance was therefore specific for the active chemotherapeutic drug rather than for the ineffective isomer, emphasizing the value of using this resistant cell line to investigate the specific mechanism of action of *cis*-DDP. This resistance was also phenotypically stable as L1210/DDP cells have been grown for eighty doublings in the absence of *cis*-DDP without change in sensitivity.

Unlike previous reports of cross-resistance between *cis*-DDP and melphalan [3, 4], the resistant cell lines used in the present study exhibited the same sensitivity to melphalan as the L1210/0 cells (Table 1). More recently, the resistance to melphalan in the previously reported L1210 cell line was shown to depend on reduced uptake by the cell [7]. Leucine and glutamine competed with melphalan for the transport mechanism and reduced melphalan toxicity simultaneously. We therefore repeated the toxicity experiments in culture medium containing up to 5 mM L-glutamine but observed no change in *cis*-DDP toxicity in either the sensitive or resistant cell lines.

The other drugs tested included aziridinyl benzoquinone, a drug at present undergoing Phase 1 clinical trials. This drug affected both the sensitive and resistant cells similarly. The family of compounds from which this drug derives has been shown by renaturation studies to cause DNA-interstrand cross-links [8]. Aziridinyl benzquinone has also been shown by alkaline elution analysis of DNA to cause interstrand cross-links (M. Strandberg and A. Eastman, unpublished observations). Neocarzinostatin, a protein antibiotic that causes breakage of DNA in cultured cells and degradation of extracted DNA [9], was equally toxic to the two

* Abbreviations used: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *cis*-DEP, *cis*-dichloroethylenediamineplatinum(II); and MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Table 1. Effects of various agents on the growth of *cis*-DDP-sensitive and -resistant L1210 cells*

Drug treatment	ID_{50} (μg/ml)		Fold resistance
	L1210/0	L1210/DDP	
cis-DDP (3 days)	0.04	1.25	31
cis-DDP (1 hr)	0.45	12	27
trans-DDP (3 days)	7	17	2.4
cis-DEP (3 days)	0.29	7.9	27
Melphalan (3 days)	0.17	0.33	1.9
Melphalan (1 hr)	1.3	1.8	1.4
Aziridinyl benzoquinone (3 days)	0.16	0.17	0
Neocarzinostatin (1 hr)	0.3	0.28	0
MNNG (3 days)	0.5	0.85	1.7
Methyl methanesulfonate (3 days)	7.5	9.25	1.2
Caffeine (3 days)	220	190	0
Ultraviolet irradiation (J/m^2)	13	19.5	1.5

* Each value is the mean of at least two experiments with four determinations made at each dose in each experiment.

cell lines. Two direct acting methylating agents, methyl methanesulfonate and MNNG, were also tested, but neither exhibited cross-resistance. Ultraviolet irradiation was also equitoxic to the two cell lines.

The results presented here are of interest as they show a cell line with resistance specific for *cis*-oriented platinum coordination complexes. This appears to be the first report of a *cis*-DDP resistant cell line that is not cross-resistant with melphalan. This, together with previous reports [3, 4, 6], emphasizes that different patterns of drug resistance can be present in similar cell lines. There must therefore be multiple mechanisms by which resistance can develop to a particular drug. Resistance to melphalan in one line that is cross-resistant to *cis*-DDP has been associated with changes in a transport mechanism [7]. The lack of cross-resistance in our cells requires that an alternative mechanism of resistance be proposed. It seems improbable that a transport mechanism would be specific for the *cis*-isomers of platinum and discriminate against *trans*-DDP. Previous workers have shown that *cis*- and *trans*-DDP react equally with DNA and that the difference between them is in a specific type of lesion that results [1]. The mechanism of resistance to *cis*-DDP may therefore reflect a differential modification of DNA or a differential removal of DNA-bound platinum in the two cell lines. In this regard, several reports relating to repair of platinated DNA are of interest. Xeroderma pigmentosum is a DNA repair deficiency disease in which inadequate repair of ultraviolet irradiation damage results in neoplasia. These cells were more sensitive than normal cells to DNA bound platinum [10]. It therefore appears that *cis*-DDP lesions are repaired, at least in part, by a pathway similar to that for pyrimidine dimers. In the present study, however, both cell lines exhibited approximately equal sensitivities to ultraviolet light (Table 1) suggesting that some difference would have to exist in the DNA repair pathway, if this were a mechanism of resistance. A difference in the repair of the various types of lesions was suggested previously by the inability of an endonuclease from *Micrococcus luteus* to recognize *cis*-DDP damage while still recognizing ultraviolet damage in DNA [10]. To test for the involvement of either a transport mechanism or a repair process will require direct measurement of uptake and DNA binding of *cis*-DDP.

The enhancement by caffeine of *cis*-DDP toxicity in Chinese hamster [11], but not in HeLa cells [12], suggested a possible mechanism of resistance involving post-replication repair. HeLa cells are apparently more sensitive to *cis*-DDP because they lack a caffeine-sensitive pathway. We therefore looked for a caffeine-sensitive pathway in L1210/DDP cells. After a 1-hr incubation in *cis*-DDP, cells were reincubated in medium containing 50 μg caffeine/ml. This is the maximum dose that caused a minimal effect on cell growth. Growth was monitored for 4 days; the growth curves are shown in Fig. 1. No caffeine-sensitive process was observed in either L1210/0 or L1210/DDP. This experiment was repeated with either *cis*-DDP or MNNG in the medium concurrently with caffeine throughout the 4-day

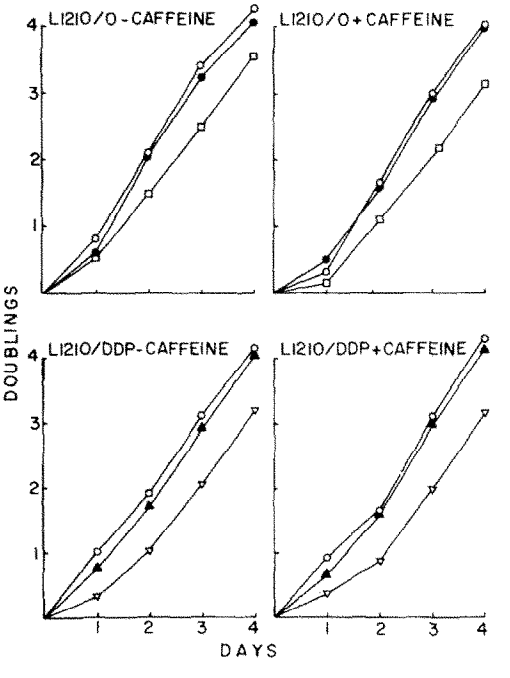

Fig. 1. Effect of caffeine (50 μg/ml) on the growth of L1210/0 and L1210/DDP cells after a 1-hr incubation in the following concentrations of *cis*-DDP: 0 μg/ml (○), 0.1 μg/ml (●), 0.3 μg/ml (□), 3 μg/ml (▲) and 10 μg/ml (▽).

incubation. In neither case was enhancement of toxicity observed. This inability to detect enhancement of cytotoxicity by caffeine is contrary to the previous report using neocarzinostatin in L1210 cells [13]. We therefore applied neocarzinostatin to our L1210 cells for 1 hr followed by post-incubation in caffeine-containing medium. Again, no enhancement of toxicity was observed. The only reason offered for this discrepancy is that significant differences exist between our cell lines and that previously reported. We must conclude that resistance is not related to a caffeine-sensitive pathway in our cells.

The present study characterizes a system in which an L1210 subline is specifically resistant to certain *cis*-platinum coordination complexes. Several *cis*-platinum complexes have been shown previously to be equitoxic to these two cell lines [2, 14]. These equitoxic compounds have a bulky group attached to the amines. One such example, 1,2-diaminocyclohexanedichloroplatinum(II), is now being considered for clinical trials in the hope that it will overcome resistance. This communication shows, however, that various mechanisms of resistance may exist and raises the possibility that a patient who had been treated previously with *cis*-DDP may have developed or may develop tumor cells that are resistant to the diaminocyclohexane derivative.

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Sulfate depletion after acetaminophen administration and replenishment by infusion of sodium sulfate or *N*-acetylcysteine in rats

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Humans and animals have a limited capacity to convert phenolic drugs to their sulfate conjugates [1–6]. The formation of sulfate conjugates of such drugs can be increased by administration of inorganic sulfate or of a sulfate donor substance, suggesting that the capacity-limiting factor is the availability of free sulfate in the body rather than the activation of sulfate or the transfer of the activated sulfate to the drug [1, 3, 6, 7]. This interpretation was questioned recently because the serum concentration of inorganic ("free") sulfate in rats decreased only slightly (from 0.92 to 0.71 mM) after intravenous injection of phenol (266 μmoles/kg) [8]. A study was initiated therefore to determine the effect of various doses of acetaminophen on the concentration of free sulfate in the serum of rats.

Acetaminophen is eliminated from the body primarily by conversion of the drug to glucuronide and sulfate conjugates [4]. In parallel with these processes, there also occurs formation of a quantitatively minor but highly reactive metabolite of acetaminophen that can cause serious and sometimes fatal hepatotoxicity following overdoses of the drug [9]. Because the processes are parallel and therefore competing, impaired formation of acetaminophen con-