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SHORT COMMUNICATIONS

The role of metallothionein, glutathione, glutathione *S*-transferases and DNA repair in resistance to platinum drugs in a series of L1210 cell lines made resistant to anticancer platinum agents

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Abstract—The glutathione contents, glutathione *S*-transferase activities and metallothionein contents have been measured in a series of L1210 cell lines which show decreased sensitivities to platinum drugs. Resistance to cisplatinum cisDDP, *cis*-diamminedichloroplatinum (II)] and chip [ioproplatin, *cis*-dichloro-bis-isopropylamine-*trans* dihydroxy platinum IV] was found to correlate with glutathione levels but not metallothionein. Conversely, resistance to tetraplatin was found to be correlated with metallothionein but not glutathione levels. However, depletion of glutathione by buthionine 1-sulphoximine sensitizes all cell lines to the effects of cisDDP, chip and tetraplatin [*d*, 1-*trans*-tetrachloro-1,2-diamino-cyclohexanplatinum (IV)]. Inhibition of DNA repair by aphidicholin or caffeine also partially restored sensitivity to these platinum drugs. These results indicate the complexity of the changes occurring upon the development of drug resistance.

The platinum drugs cisplatinum [cisDDP,* *cis*-diamminedichloroplatinum (II)] and its derivative carboplatin [*cis*-diammine (1,1-cyclobutanedicarboxylato)platinum (II)] are widely used in the treatment of a number of human malignancies, especially ovarian and testicular tumours. The clinical effectiveness of these agents is reduced by their toxicities and by the development of drug resistance. The mechanisms by which tumours can overcome the effects of anticancer drugs have been the subject of much research. It is becoming apparent that tumours can adopt a number of methods to circumvent the effects of these drugs. These include alterations in glutathione [1], metallothionein [2], cellular drug uptake [3], DNA repair [4] and oncogene expression [5]. This work investigates the glutathione content, metallothionein levels and glutathione *S*-transferase (GST) activities in a series of L1210 cell lines which show decreased sensitivity to platinum drugs. The effect of glutathione depletion and the inhibition of DNA repair by caffeine or aphidicolin on platinum cytotoxicity are also described.

Materials and Methods

Cell culture. Cell lines showing decreased sensitivity to platinum drugs have been developed from the parental L1210 line by incremental challenge with drug *in vitro* [6]. The lines are maintained in RPMI 1640 medium supplemented with 10% horse serum (Gibco, Uxbridge, U.K.). The characteristics of these lines, together with the selecting drugs, are shown in Table 1. The resistances are stable and do not require routine challenge with the selecting drug. The cell lines used were derived from the parental (L1210SE) line and made resistant to cisDDP (L1210RC), chip (iproplatin, *cis*-dichloro-bis-isopropylamine-*trans* dihydroxy platinum IV, L1210RH), platuran (*trans*-1,2,-diaminocyclo-hexane-platinum II glucarate, L1210RU), platinex (*trans*-1,2,-diaminocyclohexane-platinum II citrate, L1210RX), methotrexate (L1210RM), cisDDP then chip (L1210RCH), chip then cisDDP (L1210RHC), or cisDDP then methotrexate (L1210RCM).

Drugs. cisDDP was used as the commercial preparation (1 mg/mL in saline, David Bull Laboratories). Tetraplatin [(*d*, l-*trans*-tetrachloro-1,2-diamino-cyclohexanplatinum (IV)] and iproplatin (chip) were gifts from Dr L. Kelland, ICR, Sutton, U.K., and were stored as stock solutions (2.5 mM). Tetraplatin was kept frozen (−22°) whilst cisDDP and chip were at room temperature. Caffeine (100 mM) and buthioneine 1-sulphoximine (BSO, 25 or 2.5 mM) were stored frozen in saline (−22°). Aphidicolin, in dimethyl sulphoxide, was also frozen (1 or 10 mg/mL, −22°).

Cytotoxicity. The sensitivities of the cell lines were determined by the MTT assay [7]. Briefly cells were plated in 96 well dishes, drug or drugs added and the cell numbers measured colourimetrically following five days exposure by addition of the metabolically activated dye MTT [(3,4,5-dimethylthiozole-2-yl)-2,5-diphenyl tetrazolium bromide, 150 μg/well)]. A dual wavelength plate reader (Titertek Multiscan) was used and absorbances at 540 and 690 nm recorded. Cell numbers were calculated from appropriate standard curves. All experiments were performed in triplicate. The variations in these assays were routinely <5%.

Glutathione content. Total glutathione (reduced + oxidized) was determined enzymatically according to the method of Tietze [8]. Cells in exponential growth were collected by centrifugation, washed (×2) in ice-cold phosphate-buffered saline, and sonicated in 10 mM HCl. Particulate matter was removed by centrifugation (12,000 *g*, 5 min) and the supernatant deproteinized by the addition of sulphosalicylic acid (10%). Following recentrifugation the glutathione content of the supernatant (20 μL) was assayed using 5,5′-dithiobis-(2-nitrobenzoic) acid (0.6 mM) in a glutathione-reducing buffer (4.4 mM Na_4 EDTA, 250 μM NADPH, glutathione reductase 3 U/mL, in sodium phosphate buffer pH 7.5). Glutathione concentration was determined spectrophotometrically at 412 nm. Standard glutathione solutions were used on each occasion to calibrate the assay. All results were standardized to either cell number or per unit protein.

GST activity. This was determined spectrophotometrically ($\lambda = 340$ nm) using 1-chloro,-2,4-dinitrobenzene (1 mM) and glutathione (1 mM) as substrates [9]. Enzyme activity was standardized to protein content.

Metallothionein. Metallothionein was measured indirectly by ^{203}Hg binding [10] following trichloroacetic acid treatment. Metallothionein-bound ^{203}Hg was separated by

* Abbreviations: cisDDP, cisplatinum; BSO, buthioneine 1-sulphoximine; GST, glutathione-*S*-transferase.

Table 1. Drug sensitivity patterns in L1210 cell lines

Cell line	Resistance selected by	IC_{50} (cisDDP)	RI (cisDDP)	IC_{50} (chip)	RI (chip)	IC_{50} (tetraplatin)	RI (tetraplatin)	RI [selecting drug(s)]
L1210	—	1.3	1.0	5.0	1.0	0.72	1.00	—
L1210 RC	cisDDP	18.2	14.0	10.5	2.1	0.68	0.94	14.1
L1210 RX	Platinex	4.3	3.3	17.0	3.4	12.1	16.8	49.0
L1210 RU	Platuran	1.4	1.1	6.0	1.2	5.9	8.2	8.2
L1210 RH	Chip	1.7	1.3	15.5	3.1	1.5	2.1	3.1
L1210 RHC	Chip, then cisDDP	8.5	6.5	60.0	12.0	7.9	11.0	12/6.5
L1210 RCH	cisDDP, then chip	25.4	19.5	70.0	14.0	9.4	13.0	19.5/14
L1210 RM	Methotrexate	1.0	0.8	ND	ND	ND	ND	81.0
L1210 RCM	cisDDP, then methotrexate	22.1	17.0	ND	ND	ND	ND	17/72

RI (resistance index) = IC_{50}/IC_{50} L1210 SE.
IC_{50}, micromolar.
ND, not determined.

spinning column chromatography using Sephadex G10 minicolumns. All experiments were performed until errors were less than 10% and the results expressed as moles Hg bound per unit protein concentration.

Statistical analysis. This was carried out on a Microvax minicomputer using Minitab statistical analysis.

Results

The cytotoxicity profiles are shown in Table 1. The cell lines show a range of sensitivities towards cisDDP, chip and tetraplatin spanning nearly 20-fold compared to the parental L1210SE line. The glutathione contents, metallothionein levels, GST activities and cell volumes of the cell lines are shown in Table 2. An analysis of these data together with drug sensitivity (Table 3) showed a correlation between the glutathione content of the cell lines and their sensitivities towards cisDDP and chip ($P < 0.001$, regression analysis). Resistance to tetraplatin, however, was shown to be correlated with metallothionein content ($P < 0.001$) but not glutathione level ($P = 0.94$). Cell volume and GST activity were not related to drug sensitivity towards cisDDP, chip or tetraplatin. A more detailed analysis of the drug sensitivity data showed that resistance to cisDDP was not associated with resistance to tetraplatin ($P = 0.34$, regression analysis). However, resistances to chip and tetraplatin were associated ($P = 0.003$). Sensitivities to cisDDP and chip were related ($P < 0.001$) but this was mainly due to the L1210 RCH and L1210RHC cell lines which had been selected for resistance to both drugs.

The effects of glutathione depletion by BSO and DNA repair inhibition by caffeine and aphidicolin on the cell lines are shown in Table 4. The concentrations of these agents were chosen to approach the maximal level which gave minimal toxicity. These concentrations varied for each cell line and were [in the order caffeine (mM)/aphicicolin (nM)/BSO (μM)]: for L1210SE 0.2/90/40, L1210RC 0.4/150/80, L1210RX 0.4/120/30, L1210RU 0.4/90/30 and L1210RH 0.3/120/30. It can be seen that the resistant cell lines are in general less sensitive to the effects of BSO, aphicicolin and caffeine.

It can be seen (Table 4) that all three agents are capable of increasing platinum cytotoxicity. The level of caffeine potentiation is lower than that observed for aphidicolin or BSO. Indeed, the effect only becomes apparent in the L1210 SE, RC and RX cells at caffeine concentrations which are themselves toxic. The platuran- and chip-resistant cell lines, however, show significant enhancement of toxicity by caffeine at non-toxic levels.

Aphidicolin causes a potentiation of platinum toxicity at levels which are not themselves cytotoxic, except in the case of the L1210RC cell line treated with tetraplatin. Aphidicolin itself, however, shows cytotoxicity in this line. The cytotoxicity of cisDDP is, however, increased by aphidicolin in this line.

BSO potentiates the cytotoxicity of cisDDP, chip and tetraplatin in all the cell lines tested. The glutathione content of the cell lines was reduced to less than 5% of the normal value following treatment with BSO under the conditions described in the text (data not shown).

Discussion

The failure of cancer chemotherapy to dramatically increase survival with many common tumours can, at least in part, be explained by the development of drug resistance. The mechanisms by which tumours can overcome the cytotoxic effects of drugs have been the subject of much research interest. Two broad classes of resistance have been proposed. The first of these concerns tumours which undergo no regression, even on initial chemotherapy, and are said to show intrinsic resistance. Many tumours, however, show a good initial regression, but upon relapse the resulting tumour is found to be much less responsive

Table 2. Glutathione, metallothionein and GST

Cell lines	Glutathione	Metallothionein	GST	Relative cell volume (L1210 SE = 100)
L1210 SE	4.1 ± 1.2	2.43 ± 0.32	53.4 ± 9.0	100
L1210 RC	28.0 ± 5.6	4.33 ± 1.48	61.0 ± 4.9	86
L1210 RX	6.6 ± 2.5	8.82 ± 1.09	86.2 ± 14.1	121
L1210 RH	17.7 ± 2.0	1.75 ± 0.23	59.9 ± 4.3	154
L1210 RHC	21.5 ± 2.1	4.05 ± 1.18	47.6 ± 3.8	149
L1210 RCH	28.7 ± 4.2	2.13 ± 0.60	48.1 ± 5.0	129
L1210 RM	4.6 ± 1.4	2.00 ± 0.31	65.8 ± 7.7	147
L1210 RCM	16.3 ± 1.1	6.58 ± 1.35	45.2 ± 5.9	128
L1210 RU	12.7 ± 1.5	6.00 ± 2.27	57.2 ± 8.8	—

All results, means ± SD.
Glutathione, nmol/mg protein (N = 4).
Metallothionein, nmol Hg/mg protein (N = 6).
GST, pmol conjugate formed/mg protein/min (N = 9).

Table 3. Correlation between drug sensitivity and glutathione content, GST activity, metallothionein levels and cell volume in L1210 SE, L1210 RC, L1210 RX, L1210 RU, L1210 RH, L1210 RHC, L1210 RCH, L1210 M and L1210 RCM cell lines

Drug	Glutathione level (P value)	GST activity (P value)	Metallothionein level (P value)	Cell volume (P value)
cisDDP	<0.001*	0.27	0.62	0.72
Chip	<0.001*	0.29	0.11	0.58
Tetraplatin	0.94	0.40	<0.001*	0.47

* Statistically significant correlation.

Table 4. Effect of caffeine, aphidicolin and BSO sensitivity to platinum drugs

		% decrease in IC_{50} (cell kill drug 2 alone)			IC_{50}/IC_{50}L1210SE			
Drug	Cell line	Caffeine	Aphidicolin	BSO	Platinum drug only	+Caffeine	+Aphidicolin	+BSO
cisDDP	L1210 SE	17.8 (4.4)	39.5 (0)	60.5 (0)	1.0	0.82	0.60	0.40
	L1210 RC	22.5 (9.1)	63.7 (26.1)	39.0 (0)	14.1	10.9	5.1	8.6
	L1210 RX	23.3 (13.3)	50 (0)	58.1 (6.1)	3.3	2.6	1.7	1.4
Tetraplatin	L1210 SE	18.1 (4.4)	52.8 (0)	43.1 (0)	1.0	0.8	0.5	0.6
	L1210 RC	13.2 (9.1)	22.1 (26.1)	20.6 (0)	0.9	0.9	0.7	0.8
	L1210 RX	32.6 (23.2)	29.8 (0)	52.9 (6.1)	16.2	11.3	11.8	7.9
	L1210 RU	22.0 (0)	25.4 (0)	55.1 (13.0)	8.2	6.4	6.1	3.7
Chip	L1210 RH	32.3 (0)	38.1 (0)	39.4 (0)	3.1	2.1	1.9	1.9

to subsequent chemotherapy. These are said to demonstrate acquired resistance. The mechanisms by which the resistance arises are complex and as yet not fully understood.

The platinum drugs are amongst the most active agents available, showing particular activity in testicular and ovarian malignancies. These agents are believed to act by platination of DNA, resulting in the formation of DNA–Pt–DNA cross-links (both inter- and intrastrand), with resultant disruption of vital cellular processes. Several mechanisms of resistance to platinum drugs have been proposed including decreased drug accumulation [11], increased drug deactivation by elevated intracellular glutathione [11] or metallothionein [2], and increased DNA repair [4, 12]. Drug resistance is a complex process involving many alterations in tumour biochemistry. This paper examines the relationships between drug sensitivity and metallothionein, glutathione, GST activity and DNA repair in a series of L1210 cell lines.

The cell lines show a range of glutathione (7-fold) and metallothionein (5-fold) contents. The GST activities are more homogeneous (<2 fold variation).

Glutathione content was found to be a strong predictor of the sensitivity of cells towards both cisDDP ($P < 0.001$) and chip ($P < 0.001$) but not tetraplatin ($P = 0.94$). Conversely, the metallothionein content of the cells was a predictor of response to tetraplatin ($P < 0.001$) but not cisDDP or chip. Cell volume and GST activity were not predictors of response. However, the sensitivity of the cell lines was increased for both tetraplatin and cisDDP when cellular glutathione was depleted by BSO (Table 4). Therefore, glutathione is capable of protecting against the toxic effects of both cisDDP and tetraplatin. However, the

induction of resistance by platinex induced only a low elevation of glutathione in the L1210RX cell line (Table 2). This line, however, showed high levels of metallothionein, an increase also seen in L1210RX, a cell line which shows a high level of cross-resistance to tetraplatin.

Inhibition of DNA repair by aphicholin [13] caused a potentiation of cytotoxicity towards cisDDP, and to a lesser degree tetraplatin. Caffeine [14] was less potent than aphidicolin in these cell lines. Also, it is important to note that the increase in cytotoxicity seen in the parental L1210SE cell line, although similar in percentage terms to that seen in the resistant cell lines, represents a much smaller change in molar drug concentration than that seen in the drug-resistant cells. The role of drug accumulation in these cell lines has not been fully investigated although the parental cell line accumulates more (2-fold) cisDDP than its resistant (L1210RC) counterpart (data not shown). However, these studies have not been extended to the other drugs or cell lines.

The diversity of mechanisms of resistance which are available to tumour cells are reflected in this study. The cell lines have been derived from the parental (L1210SE) line by treatment with a variety of platinum drugs. These lines show that glutathione, metallothionein and DNA repair can be involved in resistance to platinum drugs. It is also interesting to note that where resistance is seen, glutathione depletion by BSO and DNA repair inhibition by caffeine or aphidicolin do not individually restore the sensitivity of the cell lines to that of the parental L1210SE line. BSO and aphidicolin increased the sensitivity towards cisDDP by an average of approximately 50% in the L1210SE, RC and RX cell lines (Table 4). However, the cisDDP-resistant L1210 RC cells were still 8-fold and 5-fold more resistant to the effects of the drug following BSO or aphicicolin treatment when compared to the L1210SE cell line.

In conclusion, cells have been shown to be capable of adopting several ploys to circumvent the toxic effects of platinum drugs. The diversity and complexity of these changes may have repercussions on the clinical circumvention of resistance to anticancer drugs.

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CRC Dept of Experimental Chemotherapy
Paterson Institute for Cancer Research
Christie Hospital NHS Trust
Manchester M20 9BX, U.K.

M. Hrubisko*
A. T. McGown†
B. W. Fox

* Present address: Department of Haematology, Postgraduate School of Medicine, Partizanska 4, 811 03 Bratislava, Czechoslovakia.

† Corresponding author. FAX (061) 446 3109.

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