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Correlation of the in vitro cytotoxicity of ethyldeshydroxysparsomycin and cisplatin with the in vivo antitumour activity in murine L1210 leukaemia and two resistant L1210 sublcones

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Summary. The cultured murine leukaemia L1210 cell populations used in the present study were derived from L1210 cells that had been grown in vivo. Subclones resistant to sparsomycin (L1210/Sm) or cisplatin (L1210/CDDP) were also developed in vivo. The doubling times of the cultured cell populations were identical. Fractions surviving after drug treatment in vitro were determined by colony formation in soft agar. The results, based on the differential sensitivity of the cell populations to ethyldeshydroxysparsomycin (EdSm) and CDDP, indicated that after a short exposure, cultured L1210/CDDP cells were cross-resistant to EdSm. L1210/Sm cells, however, were not cross-resistant to CDDP. The results obtained in cultured cell populations were confirmed in vivo. $CD2f_1$ mice bearing i.p. implants of 1×10^5 tumour cells were given EdSm or CDDP and a combination of the two agents. Drugs were given once daily every 4 days for 3 doses starting at 24 h after tumour implantation. Treatment of mice bearing L1210/wt leukaemia with combined EdSm and CDDP caused strongly synergistic amtitumour activity. In animals bearing the two resistant subclones, however, combined drug treatment did not improve the antitumour activity. The corresponding median survival of mice receiving combined drug treatment was 60 days in each group containing 6 mice bearing L1210/wt, with 4–6 cures being noted; 19 days in animals harbouring L1210/Sm, with 2 cures being recorded among 6 mice; and 11 days in mice bearing L1210/CDDP, with no cure being obtained. The results of this study indicate that the synergism resulting from combined treatment with CDDP and EdSm is a function of the cellular properties of the target tumour-cell populations and is independent of host factors.

Introduction

Cisplatin (CDDP) is a metallic antineoplastic agent that is used in the treatment of cancers such as testicular, ovarian and head and neck tumours. It is thought to act via inter- and intrastrand cross-linking of duplex DNA [19]. Sparsomycin (Sm) is a known inhibitor of ribosomal protein synthesis [7, 14]. Pretreatment of L1210 leukemia cells in vitro [27] with Sm strongly enhances the cytotoxic effects of CDDP. In vivo, Sm potentiates CDDP's antitumour activity when it is given 3–6 h prior to CDDP and results in prolongation of the median survival of mice (>60 days) and in a 66% cure rate [26]. Sm analogues that are more active in vitro than the parent drug have been tested for their antitumour activity in eight in vivo murine tumour models [28]. The most active compounds appeared to be deshydroxy-Sm (dSm), ethyldeshydroxy-Sm (EdSm) and *n*-pentyl-Sm (pSm).

In vivo potentiation of CDDP's antitumour activity has been studied for Sm and the three above-mentioned active analogues in s.c. implanted L1210 leukaemia [29]. In this tumour model, CDDP treatment (5 mg/kg) resulted in a 156% treated/control (T/C) value. Treatment with sparsomycin and its analogues alone resulted in an increase in survival for EdSm only (139%). Although Sm itself was incapable of potentiating the antitumour activity of CDDP in this model, two analogues, dSm and EdSm, were active. At a dose of 10 mg/kg, EdSm potentiated CDDP's antitumour activity by 2.8 times. PSm, the third analogue, showed no potentiation of the antitumour activity of CDDP in this tumour model.

On the basis of these results, EdSm was chosen for further preclinical studies on the synergism between sparsomycins and CDDP. To investigate the relationship between the results of combined drug treatment in vivo and the drug sensitivity of tumour cells in vitro, we derived two resistant sublines from L1210 murine leukaemia. Our further goal was to examine the correlation between in vitro sensitivity testing and in vivo antitumour activity. The outcome of this investigation might facilitate further

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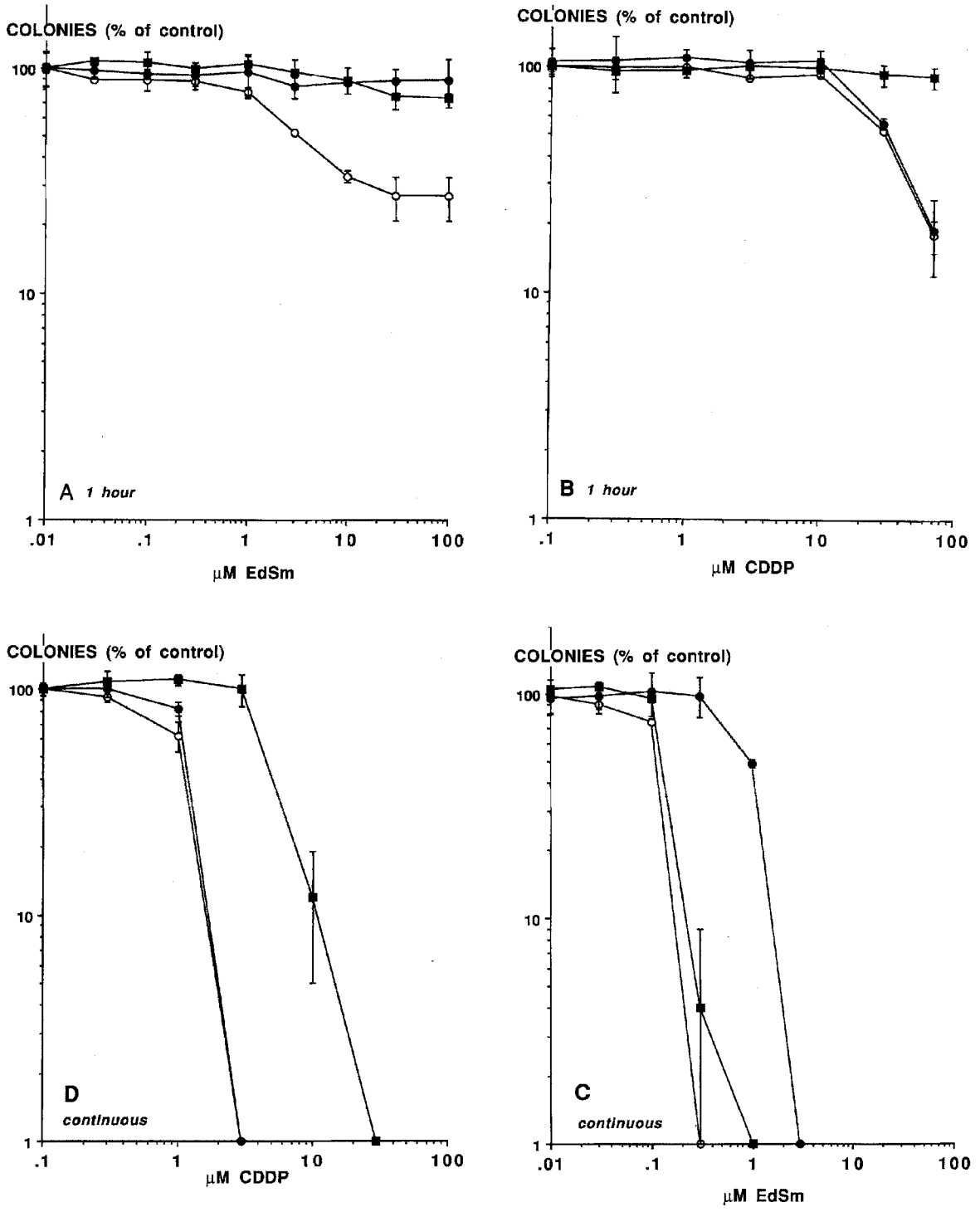

Fig. 1 A–D. In vitro chemosensitivity of L1210 leukaemia (○) and two drug-resistant subclones, L1210/Sm (●) and L1210/CDDP (■), exposed to EdSm and CDDP. **A, B** Effect of 1 h exposure. **C, D** Effect of continuous exposure. **A, C** Cytotoxicity of EdSm. **B, D** Cytotoxicity of CDDP. Data represent mean values for experiments performed in triplicate

studies on the synergistic antitumour effects of EdSm on other cytostatic agents.

Materials and methods

Cell culture. Murine L1210 leukaemia cells (L1210/wt) were kindly supplied by Dr. G. Atassi (Institute Jules Bordet, Laboratory for Experimental Chemotherapy, Brussels). Cells were maintained in logarithmic growth as suspension cultures in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. Cells were grown in a humidified atmosphere containing 5% CO_2 at 37° C.

Drug-resistant sublines. The murine L1210 cisplatin-resistant subclone (L1210/CDDP) was kindly supplied by Dr. G. Atassi and was developed in vivo. We established another subclone of mouse tumour cell line L1210 with acquired resistance to sparsomycin by repeated in vivo

Table 1. Resistance factors of EdSm and CDDP in the two resistant L1210 subclones in vitro

Subclone	RF[a]	
	EdSm	CDDP
L1210/Sm:		
1 h exposure	>25	1.2
Continuous exposure	26.0	1.4
L1210/CDDP:		
1 h exposure	>25	>3
Continuous exposure	2.6	10.2

[a] Calculated as the IC_{50} value for the subclone/IC_{50} value for the wild type [IC_{50} = drug concentration (μM) that gives 50% growth inhibition]

treatment with increasing doses of deshydroxysparsomycin (L1210/Sm). In vitro, these subclones are stably resistant for up to 6 months in drug-free media. The doubling times of the cultured cell populations (mean ± SD; $n = 40$) were 13.9 ± 1.2 h for L1210/Sm, 13.2 ± 1.4 h for L1210/CDDP and 13.6 ± 1.2 h for L1210/wt. Both subclones were evaluated for their response to these drugs using a clonogenic assay to measure cell survival.

Clonogenic assay. L1210 cells were exposed to different drug doses. After drug exposure, the drug-containing medium was removed and the cells were washed with balanced salt solution and counted (Coulter counter). The clonogenic ability of treated cells was evaluated by a soft-agar colony assay. Leukaemic colonies were grown by plating the cells in 0.3% agar in six-well plates (Costar) in drug-free medium. After 8 days of incubation, colonies (≥50 cells) were scored using an inverted microscope. The surviving fraction was calculated as the absolute survival of the treated sample divided by the absolute survival of the control sample at the same time. Each experimental point was determined in duplicate and all experiments were repeated twice.

Drugs. Ethyldeshydroxysparsomycin was synthesised as previously described [15, 25] and was acquired in a freeze-dried form. The drug was dissolved in phosphate-buffered saline (PBS) and kept in dark flasks at 4° C. Cisplatin was kindly provided by Pharmachemie B. V. (Haarlem, The Netherlands). These drugs were prepared just before administration and were diluted with isotonic NaCl until the dose per gram of mouse body weight was contained in 0.01 ml.

Animals. $CD2f_1$ mice weighing between 18 and 22 g were used in this study. In each experiment, PBS-treated tumour-bearing animals served as controls.

Antitumour activity. Mice were inoculated i.p. with 10^5 cells suspended in 0.2 ml PBS (pH 7.4). In each experiment, tumour-bearing mice were randomised into treatment and control groups; each group consisted of six animals. Drugs were injected i.p. into mice using 0.01 ml/g body weight at various doses. Drug treatment was started on day 1 after tumour implantation. In all experiments, drugs were given i.p. every 4 days until day 9 (q4dx3). As previously shown [26], Sm potentiates the activity of CDDP when the former is given 3–6 h prior to CDDP. The 3-h pretreatment schedule was used in the present experiments. Animal survival was recorded daily and the median survival of each group was calculated. The final results obtained in these tumours are presented as the median survival times (MST) expressed in days after tumour implantation. The MST value was computed on the basis of the entire population, and there was no deletion of early deaths or survivors.

Statistical analysis. For statistical analysis of the data, we used Wilcoxon's rank-sum test for independent samples followed by Student's *t*-test, and *P* values of ≤0.05 were considered to be significant.

Table 2. Median survival and number of 60-day survivors for combined drug treatments of $CD2f_1$ mice inoculated i.p. with 10^5 L1210 leukaemia cells

Treatment		Median survival[a] (days ± SEM)	60-day survivors[b]	
EdSm (mg/kg)	CDDP (mg/kg)		*n*	%
0	0	10.0 ± 0.34	0/60	0
5	0	15.8 ± 0.84	0/30	0
10	0	15.0 ± 1.6	1/12	8
0	1	12	0/ 6	0
0	2	17.5 ± 1.5	2/12	17
0	3	30.5 ± 7.5	8/24	33
0	4	39.7 ± 11.0	6/18	33
0	5	48.6 ± 7.0	14/30	47
5	2	47 ± 1.0*	5/12	42
5	3	51 ± 6.0*	18/30	60
5	4	60 ± 0.0*	15/18	83
5	5	60 ± 0.0[NS]	18/24	75
10	3	60	4/ 6	67
10	4	60	4/ 6	67
10	5	19	0/ 6 toxic	

Treated mice received drugs via i.p. administration, with EdSm being given 3 h before CDDP. Control animals received injections of saline only. NS, Not significant as compared with single-agent CDDP treatment

[a] Average of individual experiments using 6 mice each

[b] Tumour-free at the time of evaluation (number of survivors/total number of mice)

* Significant ($P < 0.05$) as compared with single-agent CDDP treatment

Results

Cytotoxicity

The in vitro results, based on the sensitivity of the cell populations to EdSm and CDDP, are shown in Fig. 1. The resistance factors (RF) for EdSm and CDDP in two resistant L1210 subclones are summarised in Table 1. Cultured L1210/CDDP cells were cross-resistant to EdSm following 1 h exposure (RF, >25). L1210/Sm cells, however, were not cross-resistant to CDDP (RF = 1.2). On continuous drug exposure, the magnitude of cross-resistance was decreased from >25 to 2.6.

Antitumour activity

The combination of EdSm and CDDP was studied in L1210 tumour-bearing mice. On the basis of the loss of body weight after single CDDP treatment (data not shown), we used 5 mg/kg CDDP as the maximal tolerable dose (MTD) for this treatment schedule. The MTD used for EdSm single treatment on this schedule was 10 mg/kg per injection. For combined drug treatment, the MTDs were 5 mg/kg CDDP and 5 mg/kg EdSm. Increasing the dose of one of the drugs in the combination resulted in early deaths as shown in Table 1 for EdSm given at 10 mg/kg per injection. All results obtained following single or combined treatment of mice bearing L1210/wt with EdSm and CDDP are presented in Table 2. Death of untreated tumour-bearing animals invariably began on day 9, and all mice had died by day 12. The MST values

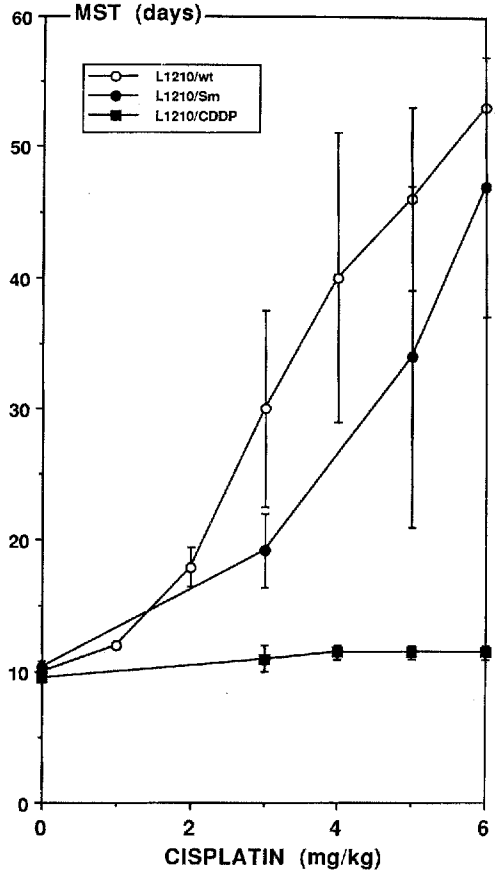

Fig. 2. Dose-response determination for CDDP in i.p. inoculated L1210 leukaemia cells. ○, L1210/wt; ●, L1210/Sm; ■, L1210/CDDP. Beginning at 1 day following the implantation of 10^5 tumour cells, mice were treated i.p. once daily every 4 days for 3 doses

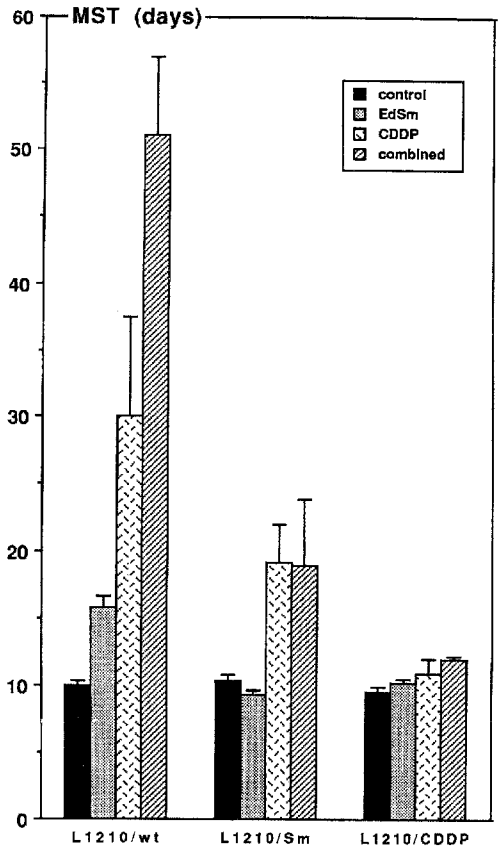

Fig. 3. In vivo antitumour activity of EdSm (5 mg/kg) and CDDP (3 mg/kg) after single-drug injection and combined drug treatment in L1210 leukaemia and two drug-resistant subclones. ○, L1210/wt; ●, L1210/Sm; ■, L1210/CDDP. Beginning at 1 day following tumour implantation, mice were treated i.p. once daily every 4 days for 3 doses. Results are expressed as median survival time (in days) as mean values for 2–4 experiments. Vertical bars represent the SEM

determined for untreated mice were 10 ± 1.1 days for L1210/wt, 10.4 ± 0.75 days for L1210/Sm and 9.5 ± 0.71 days for L1210/CDDP. The dose-response effects of CDDP in all three tumour populations are illustrated in Fig. 2. From a comparison of the CDDP data obtained in the L1210 clonogenic assay (Fig. 1) with those acquired in L1210 cells growing in vivo (Fig. 2), it can be concluded that a direct correlation of tumour cell sensitivity exists for CDDP.

The combination of EdSm and CDDP appeared to be highly effective against tumour cells sensitive to both drugs, resulting in a strong synergism between EdSm and CDDP in the L1210/wt cell population. Combination treatment of tumour cells resistant to EdSm (L1210/Sm) showed no improvement as compared with single-agent CDDP treatment (Fig. 3). Tumour cells resistant to CDDP (L1210/CDDP) were also resistant to combined drug treatment (Fig. 3); moreover, the L1210/CDDP cells were cross-resistant to EdSm treatment. Figure 4 shows the duration of host survival after treatment of mice with a combination of EdSm (5 mg/kg) and CDDP (3 mg/kg). The corresponding MST values were 60 days in mice bearing L1210/wt, with 4–6 cures being noted in each group consisting of 6 mice; 19 days in mice harbouring L1210/Sm, with 2 cures being recorded among 6 animals; and 11 days in animals bearing L1210/CDDP, with no cure being obtained. These observations suggest that synergistic improvement of the antitumour activity of CDDP by combined treatment with EdSm is successful only when the tumour cells are sensitive to both of the drugs used in combination.

Discussion

The combination of EdSm and CDDP produces potentiation of antitumour activity in L1210 leukaemia cells that are sensitive to both drugs but fails to do so in L1210 leukaemia cells that are resistant to one or both of these drugs. Furthermore, the present study indicates that the in vitro sensitivity of L1210 leukaemia cells after a short drug exposure correlates well with the antitumour activity of EdSm and CDDP obtained in vivo after i.p. bolus injection, whereas their sensitivity to continuous exposure in vitro does not. However, the factors that determine on a molecular level this observed increase in chemosensitivity are not yet known. Similarly, the mechanisms that are active in the CDDP resistance of L1210/CDDP or the EdSm resistance of L1210/Sm are unknown. CDDP inter-

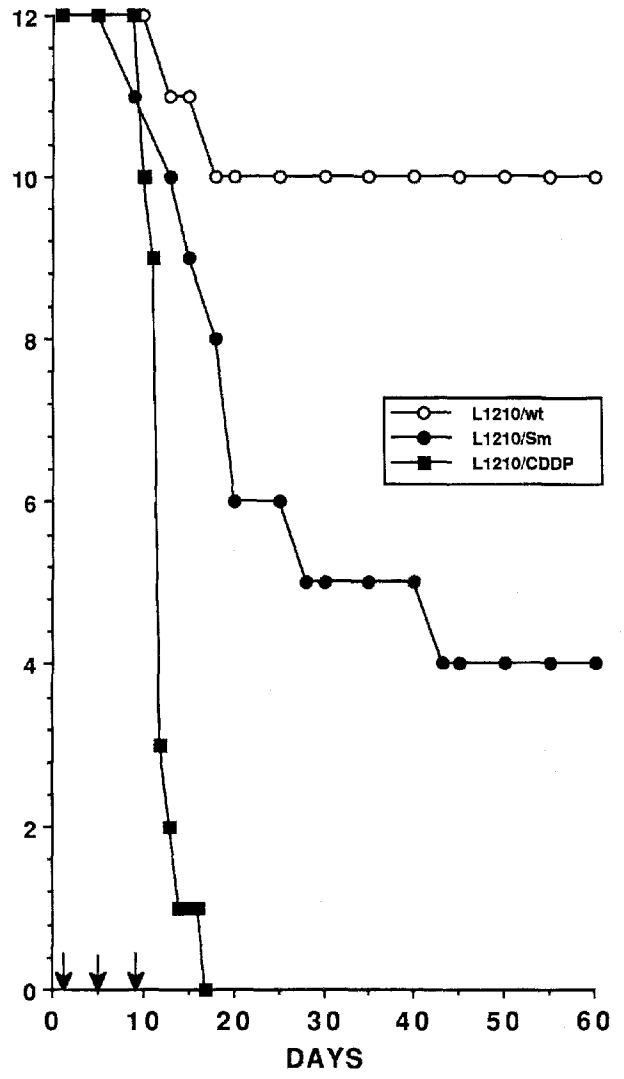

Fig. 4. Survival of mice inoculated i.p. with 10^5 L1210 cells as determined after combined drug treatment. Groups of 6 mice received i.p. injections of EdSm (5 mg/kg) and CDDP (3 mg/kg). Beginning at 1 day following tumour implantation, mice were treated once daily every 4 days for 3 doses. ○, L1210/wt; ●, L1210/Sm; ■, L1210/CDDP. Data represent the combined results of 2 experiments. *Arrows* indicate the days of treatment. Death was due to the toxicity of the tumours

feres at various levels with cell physiology, and on each of these levels, different mechanisms of resistance could be operative. A number of reports have indicated that tumour cells resistant to CDDP can show an elevated level of glutathione [9, 13, 16, 20], a decreased CDDP uptake [11, 18, 24] or, sometimes, an increased level of DNA repair [23], resulting in a decreased amount of DNA interstrand cross-links [6, 8]. Moreover, the activity of glutathione S-transferase (GST) in CDDP-resistant tumour cells is often increased in comparison with that in CDDP-sensitive tumour cells [17, 21].

Glutathione depletion can increase chemosensitivity to CDDP [2, 4, 9, 13] or partially reverse CDDP resistance [3]. On the other hand, it has recently been shown that in vivo glutathione depletion by D,L-buthionine sulfoximine (BSO) does not enhance the antitumour activity of CDDP [12], whereas another study has demonstrated increased sensitivity to CDDP [4]. In addition to reduced glutathione (GSH), metallothioneins (MT) may act as another nucleophile towards electrophilic agents. The involvement of MT in acquired resistance to CDDP is rather controversial. MT can be induced by heavy metals such as cadmium and zinc and has led to cross-resistance with CDDP in human ovarian carcinoma cells [1]. However, in vitro selection with CDDP does not trigger this mechanism [1]. Cells with acquired resistance to CDDP frequently show an increase in MT levels and overexpress MT mRNA [10, 11], but one study has failed to correlate overexpression of MT mRNA with CDDP resistance [22].

In the murine leukaemia cell line L1210, the degree of resistance has been associated with an elevated MT content [11]; however, in another study the basis for the resistance of L1210/CDDP cells to CDDP was neither an increased level of MT nor an enhanced ability to increase the synthesis of MT after CDDP exposure [5]. Thus, MT may be associated with the induction of CDDP resistance, but its causal role remains to be established.

EdSm is a potent inhibitor of ribosomal protein synthesis [25]. Due to this property, one would expect it to have a strong effect on enzymes such as GST and enzymes related to DNA repair. Decreased cellular enzyme activities might restore the sensitivity of CDDP-resistant tumour cells. L1210/CDDP cells are cross-resistant to EdSm after a short period of exposure, but on continuous exposure their sensitivity to EdSm is comparable with that of L1210/wt cells. These results indicate that changing the administration schedule of EdSm in vivo might increase its antitumour activity in L1210/CDDP leukaemia cells and might even change the sensitivity of these tumour cells to CDDP; in consequence, CDDP-resistant tumour cells might become sensitive to CDDP in the presence of EdSm.

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