The effect of ethyldeshydroxy-sparsomycin and cisplatin on the intracellular glutathione level and glutathione S-transferase activity

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Ethyldeshydroxy-sparsomycin (EdSm) is a ribosomal protein synthesis inhibitor which synergistically enhances the antitumor activity of cisplatin against L1210 leukemia *in vivo*. Because cellular glutathione (GSH) and glutathione S-transferases (GST) are reported to interfere with the antitumor activity of cisplatin, we analyzed the effect of EdSm and cisplatin on GSH and GST activity in selected tumor cells. For this purpose we used three murine leukemia tumors with different sensitivities towards EdSm and cisplatin: L1210-WT, sensitive to both drugs, L1210-Sm, resistant to EdSm, and L1210-CDDP, resistant to cisplatin. No significant differences were detectable between these three cell lines regarding the population doubling time, the cell size, and the cellular level of protein and glutathione. Neither of the resistant L1210 subclones showed P-glyco-protein expression. Drug exposure, however, changed the intracellular dynamics. Exposure to EdSm strongly decreased the amount of cellular protein, decreased the overall GST activity and led to GSH depletion, whereas exposure to cisplatin induced a rise in the amount of protein, in GSH, and in the total GST activity. These effects are dose-dependent and correlate well with the sensitivity of the tumor cells for EdSm or cisplatin. In addition, exposure to EdSm lowered the V_{max} of GST in L1210-WT and L1210-Sm; however, in L1210-CDDP both the V_{max} and the K_m were increased. That this was not a direct effect of EdSm on GST was shown in a cell-free system, where EdSm did not influence the GST activity nor could it act as a substrate for GST. Our results suggest that the synergistic combination of EdSm and cisplatin might be explained by EdSm switching off the cellular detoxification mechanism for cisplatin, i.e. by inhibition of *de novo* synthesis and subsequent depletion of GSH and GST.

Key words: Cisplatin, drug-resistance, glutathione S-transferase, glutathione, sparsomycin.

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Introduction

Ethyldeshydroxy-sparsomycin (EdSm) is a potent inhibitor of ribosomal protein synthesis[1] and is one of the sparsomycin analogs which show enhanced antitumor activity in comparison to sparsomycin.[2] In addition, EdSm synergistically enhances the antitumor activity of cisplatin *in vivo* in mice bearing L1210 leukemia tumor cells sensitive to both drugs[3] but is unable to do so when these tumor cells are resistant to one of these drugs.[4] The *in vitro* sensitivity of L1210 leukemia cells correlates well with the antitumor activity of EdSm and cisplatin *in vivo*. The cellular factors of these leukemic tumors, which are responsible for the enhanced chemosensitivity to combined drug treatment, are unknown. Likewise, it is not known for these leukemic cell lines which mechanism is involved in cisplatin resistance or EdSm resistance.

Glutathione, a tripeptide containing a sulfhydryl group, protects cells from oxidative damage, participates in the transport of amino acids, and is a cofactor for ribonucleotide reductase, which is required for deoxyribonucleotide synthesis. Glutathione conjugation, established by a family of glutathione-S-transferases (GSTs), provides a defense mechanism because it removes potentially toxic low-soluble and/or electrophilic compounds by converting them into better water-soluble and non-electrophilic conjugates. A logical but undesirable effect of glutathione therefore is its potential involvement in cellular resistance after long or repeated exposure to anticancer agents. Tumor cells may adapt to chemical or nutritional depletion of GSH by producing a rebound elevation of GSH levels and the induction of elevated levels of specific GST iso-forms. Cellular concentrations are maintained by *de novo* synthesis of GSH from the constituent amino acids, using the

enzymes τ-glutamyl cysteine synthetase and glutathione synthetases. A number of reports describe an elevated level of glutathione in tumor cells, which are resistant to cisplatin,[5-8] but a decreased cisplatin uptake[9,10] and increased DNA repair have been reported as well, resulting in less DNA interstrand cross-links.[11-13] Moreover, the activity of GST in cisplatin-resistant tumor cells is often increased compared to cisplatin-sensitive tumor cells.[14,15] GSH depletion can increase the chemosensitivity to cisplatin[5,6,16-18] or partially reverse cisplatin resistance.[19] On the other hand, conflicting results were obtained upon *in vivo* glutathione depletion using DL-buthionine sulfoximine (BSO). One report failed to observe an increase of the antitumor activity of cisplatin,[20] yet another report showed an increase in sensitivity to cisplatin.[18] EdSm, being a potent inhibitor of ribosomal protein synthesis, might inhibit *de novo* synthesis of enzymes like GST and of enzymes needed for glutathione synthesis, thereby potentiating the antitumor activity of cisplatin.

In this study we addressed these questions using three murine leukemia tumors which show significant differences in cytotoxic IC$_{50}$ levels for EdSm or cisplatin, and great differences in antitumor responses.[4] The mechanism responsible for these sensitivity differences is unknown. Therefore, information about cellular changes in tumor cells after EdSm exposure could be very valuable in order to predict the chemomodulatorial antitumor activity of EdSm in drug combinations and possibly for other protein synthesis inhibitors as well. In an attempt to explain the patterns of synergistical drug interactions in these leukemic murine tumors we investigated the dynamics of a panel of cellular parameters: protein content, GSH and GSSG content, and GST activity.

Materials and methods

Cell culture

The culture conditions for murine L1210 leukemia cells have been described earlier.[1] The murine L1210 leukemia wild-type (L1210-WT) and the cisplatin-resistant subclone (L1210-CDDP) were kindly supplied by Dr G Atassi (Institute Jules Bordet, Laboratory for Experimental Chemotherapy, Brussels, Belgium). We have established another subclone of the mouse tumor cell line L1210 with acquired resistance to sparsomycin by repeated *in vivo* treatment with increasing doses of deshydroxysparsomycin (L1210-Sm). *In vitro* these subclones are stably resistant in drug-free media for up to 6 months.

Drugs

EdSm was synthesized at the Department of Organic Chemistry (University of Nijmegen, The Netherlands)[1] and was acquired in a lyophilized form. The drug was dissolved in phosphate buffered saline (PBS, pH 7.4) and kept in dark flasks at 4°C. Cisplatin was kindly provided by Pharmachemie (Haarlem, The Netherlands). Solutions with the required drug concentration were prepared just before administration by dilution with isotonic NaCl.

Drug treatment

The L1210 leukemia cells were seeded and allowed to grow in a fresh, drug-free medium for 24 h. Thereafter, EdSm or cisplatin were applied in increasing concentrations from 0.01 to 30 μM for a period of 20 h. To investigate the effect of drug exposure on enzyme kinetics of GST the tumor cells were exposed to 30 μM EdSm during 4 h. After drug exposure, the tumor cells were washed twice with PBS and used for different assays. The cell viability was checked by dye exclusion using Trypan blue and the cell numbers were determined using a hemocytometer. All experiments were performed in triplicate.

Cytotoxicity assays

The clonogenic ability of drug-treated cells was evaluated by a soft-agar colony assay. Leukemic colonies were grown by plating the cells in 0.3% agar in six-well plates (Costar) in a drug-free medium. After 8 days of incubation, colonies (greater than 50 cells) were scored using an inverted microscope. The surviving fraction was calculated by dividing the absolute survival (number of colonies) of the treated sample by the absolute survival of a parallel control sample. Each experimental point was determined in duplicate and all experiments were repeated twice.

Flow cytometry

Cell cycle distributions. Single-cell preparations were obtained from control cultures and fixed in

70% ethanol. The cells were removed from the fixative by centrifugation and washed with PBS. The cells were stained in 400 μl PBS containing 20 μg/ml propidium iodide and were measured with a flow cytometer 50H (Ortho Instruments, Weatwood, CA). Red fluorescence, for DNA content measurement, was detected through a 630 nm long pass filter and photomultiplier pulses were amplified linearly.

P-glycoprotein (Pgp). For flow-cytometric analysis of Pgp expression, tumor cells were labeled with the Pgp-specific monoclonal antibody MRK16 (a gift from Professor T Tsuruo) according to the method of Hamada.[21] Thereafter, cells were stained with fluoresceinated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark), FITC–RAM–F(ab')$_2$ fragments 1:50 diluted in PBS. Cells stained with FITC were analyzed by flow cytometry using a 515/530 band-pass filter. The area of the green fluorescence signal and both area and peak value of the red fluorescence signal were recorded in list mode and analyzed with a PDP11/34 computer (Digital Equipment, Galway, Ireland).

Cell size. Flow cytometry of the samples was done with a cytofluorograph equipped with on-line computer analysis. The exact relationship between scattered light and cell size is difficult to quantify. It depends on the light collection, angle, aperture and index of refraction of the suspending fluid. Usually, however, the scattered light increases monotonically with the cell volume. In this study, latex microspheres of 10 and 20 μm were used to calibrate any differences in cell size between these three cell lines. All samples were light-microscopically checked for differences in size.

Protein

For an estimation of the amount of protein we used the fluorometric assay of Udenfriend,[22] as modified by Lai.[23] Bovine serum albumin was used for calibration.

Glutathione

Glutathione consists of two forms, reduced (GSH) and oxidized (GSSG), and can be determined by established enzymatic techniques.[24] Cellular glutathione content was determined from the deprotei-

nated cell homogenates with the fluorometric method of Hissin and Hilf using *o*-phthalaldehyde.[25]

GST

GST catalyses the transfer of the -SH group of glutathione to specific substrates. The activities of GSH transferases were measured at 25°C spectrophotometrically with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the procedure of Habig.[26] The conjugate, CDNB–glutathione, has an absorbance band at 340 nm and the activity of the enzyme can therefore be estimated by measuring the kinetics of the change in optical density at this wavelength. Assays were performed in 100 mM potassium phosphate, pH 7.4, 1 mM EDTA, 5 mM GSH, 1 mM CDNB and 0.1 mg/ml cytosolic protein. Michaelis–Menten kinetics of cytosolic GSH transferases of sonicated cells were measured using CDNB as a substrate. GSH concentrations in the assay system were fixed at 1 mM. The K_m and V_{max} were calculated using the Lineweaver–Burk plot.

Statistics

A comparison of the different sample distribution is usually performed on the log-scale in order to reduce skewness, be more robust against outliers or make the SDs more comparable. Even if this transformation to the log-scale seems unnecessary—as in this study—there is hardly any harm in doing so. Differences between the means of different groups were compared using the analysis of variance (ANOVA) in combination with the Ryan multiple F-test. All computations were done by SAS, proc. ANOVA. The results have been expressed as the mean $\pm$ SE and $p < 0.05$ was considered to be significant.

Results

Biological characteristics of the L1210 leukemia cell lines

IC$_{50}$ levels of the various cell lines for EdSm and cisplatin are compiled in Table 1. The cisplatin-resistant L1210 line (L1210-CDDP) acquired some resistance to EdSm as well, resulting in a resistance factor of 4 compared to 18 in L1210-Sm. Yet, the L1210-Sm has normal sensitivity for cisplatin. No detectable, significant difference was found between

Table 1. Characteristics of three different murine leukemia cell lines, with regard to population doubling times, expression of plasma membrane Pgp, and sensitivity for EdSm and cisplatin during exponential cell growth [cell lines are wild-type (L1210-WT), cisplatin resistant (L1210-CDDP) and deshydroxy-sparsomycin resistant (L1210-Sm)]

Parameter	L1210-WT	L1210-CDDP	L1210-Sm	Unit
T doubling time[a]	13.6 ± 1.2[b]	13.2 ± 1.4	13.9 ± 1.2	h
Relative cell diameter	1.0 ± 0.17	1.0 ± 0.17	1.2 ± 0.17	
Pgp	< 1	< 1	2.2	% positive cells
IC_{50} EdSm[c]	0.22 ± 0.08	0.88 ± 0.16	4.0 ± 1.4	μM
RF EdSm[d]	1	4	18	
IC_{50} CDDP	2.0 ± 1.25	3.8 ± 0.3	1.5 ± 0.2	μM
RF CDDP	1	1.9	0.75	

[a]Based on 40 analyses for each cell line.
[b]Mean $\pm$ SE.
[c]IC_{50} is the drug concentration needed during an exposure time of 20 h to give 50% growth inhibition in a colony-forming assay.
[d]RF is the resistance factor, representing the fold increase in IC_{50} for a specific drug in a resistant cell line relative to the wild-type.

the three cell lines regarding population doubling times, cell diameter and Pgp expression. Only the cisplatin-resistant cell population harbors a few positive cells (2.2%). However, the intensity of their fluorescence was low as a consequence of which we ignored this information. Whilst the population doubling time is the same for all three cell lines, the analysis of the cell phase distribution during exponential growth (Table 2) revealed significant differences ($p = 0.03$) with respect to the number of cells in the S phase. To be more specific, the percentage of L1210-CDDP tumor cells in the S

phase cells is significantly higher than that of L1210-Sm cells, values which are 62.3 and 49.4%, respectively. Moreover, the percentage of L1210-CDDP cells (6.8%) in the G_2M phase is low compared to these values of L1210-WT (11.6%) and L1210-Sm (12.7%) cells. The percentage of G_1 phase cells is not significantly different within the three cell lines. Furthermore, no difference was found in the average protein content of these cell lines (Table 3). The average glutathione levels in the resistant cell lines also did not change significantly: $p = 0.35$ for GSH oxidized, $p = 0.48$ for GSSG and $p = 0.32$ for total

Table 2. Cell phase distribution of the three L1210 cell lines, during exponential cell growth in tissue culture, in the absence of any drug treatment (the p value is calculated by ANOVA in combination with the Ryan multiple F-test)

Cell phase	L1210-WT (10)	L1210-CDDP (4)	L1210-Sm (12)	p value
G_1	35.3 ± 6.6	31.0 ± 2.8	37.9 ± 1.8	0.19
S	53.1 ± 2.6	62.3 ± 3.0	49.4 ± 2.1	0.03
G_2M	11.6 ± 5.3	6.8 ± 1.4	12.7 ± 0.9	0.07

Table 3. Standard values in three different murine leukemia cell lines for the protein content, the specific activity of GST using 1 mM CDNB as a substrate and the glutathione levels during exponential cell growth (the p value is calculated by ANOVA in combination with the Ryan multiple F-test)

Parameter	L1210-WT	L1210-CDDP	L1210-Sm	Unit	p value
Protein	43.8 ± 4.8 (8)[a]	47.5 ± 2.3 (6)	39.5 ± 3.2 (6)	$\mu g/10^6$ cells	0.30
GST	2.09 ± 0.15 (9)	1.84 ± 0.13 (7)	2.36 ± 0.15 (7)	nmol/min/10^6 cells	0.09
GST	49.4 ± 5.0 (9)	38.2 ± 1.7 (7)	58.0 ± 5.9 (7)	nmol/min/mg protein	0.04
GSH	331 ± 55 (8)	371 ± 46 (7)	268 ± 46 (7)	ng/10^6 cells	0.35
GSSG	165 ± 26 (8)	188 ± 23 (7)	145 ± 19 (7)	ng/10^6 cells	0.48
Ratio GSH/GSSG	2.0 ± 0.2	2.0 ± 0.2	1.9 ± 0.2		
Total-GS	496 ± 74 (8)	560 ± 56 (7)	413 ± 58 (7)	ng/10^6 cells	0.32

[a]Mean $\pm$ SE, (n) is number of experiments.

glutathione (Table 3). Moreover, a 2:1 molar ratio between GSH and GSSG was found in all cell lines. The specific GST activities are different (Table 3). In particular, if we compare the L1210-Sm with the L1210-CDDP cell line, we notice an elevated level of 58 versus 38.2 nmol/min/mg protein, respectively ($p = 0.04$), or 2.36 and 1.84 nmol/min/10^6 cells, respectively ($p = 0.09$).

Effects of EdSm and cisplatin on the intracellular protein and glutathione levels and on the GST activity

The dose-effect curves for cisplatin and EdSm diverge in all three cell lines. Inhibition of the protein synthesis by EdSm has a strong impact on the cellular protein content (Figure 1). EdSm causes a dose-dependent decrease in protein level, which is most extensive and most rapid in L1210-WT, and already starts at submicromolar EdSm concentrations. This decrease is more gradual in L1210-CDDP. L1210-Sm is clearly less sensitive and requires at least 10 μM to see the same effect. Cisplatin causes the opposite effect, i.e. a dose-dependent increase in protein level in the L1210-CDDP and L1210-WT cell lines with little effect on L1210-Sm. The curves of

drug treatment with cisplatin or EdSm and the 100% control level were statistically analyzed. The curves of L1210-WT and L1210-CDDP are significantly different, $p = 0.04$ and 0.05, respectively. Worth mentioning is the situation in L1210-Sm, in which EdSm exposure only caused a moderate effect, while cisplatin had no effect at all ($p = 0.45$). Comparable effects were observed for GST activity. Because we are interested in the total effect per tumor cell, we decided to express the values for the glutathione level and GST activity in units per 10^6 cells instead of mg protein. The effect of both drugs on the overall cellular GST activity is represented in Figure 2. These results parallel the effects observed for the intracellular protein level, but significant differences between EdSm and cisplatin were found only in the L1210-WT cell line ($p = 0.01$), while the effect in the L1210-CDDP cell line comes close to significance ($p = 0.06$). Thus, the decrease in GST activity (when treated with EdSm) parallels the decrease in total protein. Similarly, the increase in GST activity (when treated with cisplatin) follows the increase in protein content. This implies that if the specific activity of GST was expressed in nmol/min/mg protein, no significant differences are found in these cell lines— neither between EdSm and cisplatin treatment, nor between drug-treated and control cells.
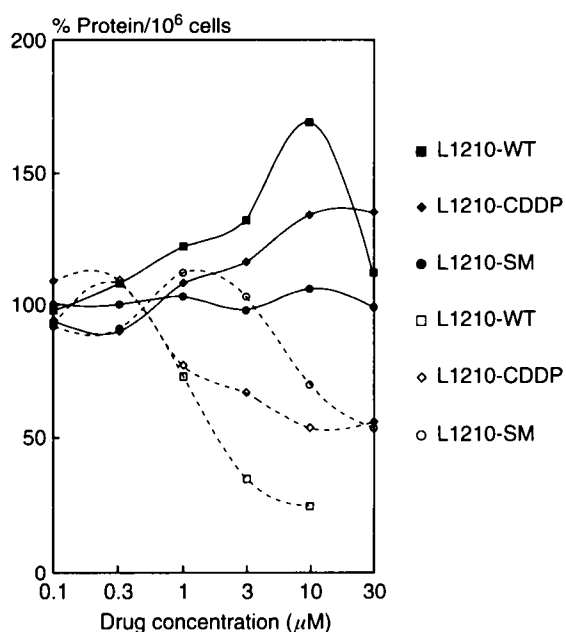

Figure 1. Relative protein content (ng/10^6 cells) of three different murine leukemic tumors after 20 h exposure to varying doses of EdSm (open symbols) or cisplatin (solid symbols).
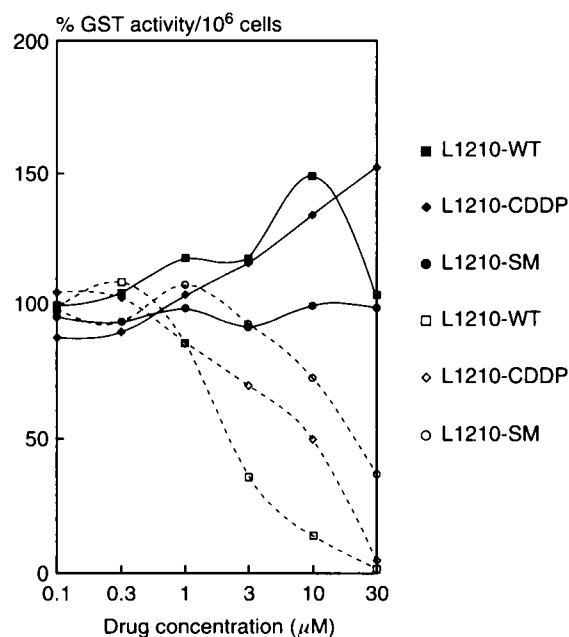

Figure 2. Relative cellular activity (nmol/min/10^6 cells) of GST in three different murine leukemic tumors after 20 h exposure to varying doses of EdSm (open symbols) or cisplatin (solid symbols).

Effects of EdSm and cisplatin on the intracellular glutathione dynamics

Figure 3 shows the results of drug treatment on the intracellular glutathione level in these leukemic tumors. Again, EdSm and cisplatin showed opposite effects in these three cell lines, and again these effects are dose- and cell line-dependent. Significant differences have only been observed in the L1210-WT cell line ($p = 0.05$). Only about 3 μM was required to induce strong GSH depletion in L1210-WT. In the sparsomycin-resistant tumor 3 μM EdSm evoked a 50% increase in GSH level and a significant decrease was only observed at 30 μM. In the cisplatin-resistant tumor the GSH depletion dropped with EdSm by 3 μM or greater, albeit slowly compared to the wild-type. The effect of cisplatin treatment on the GSH levels followed the same pattern as seen with GST and protein.

Enzyme kinetics

First we investigated whether EdSm and cisplatin could directly affect the activity of GST or the assay. The results in a cell-free system (Table 4) indicate that only a very high dose of cisplatin provides a
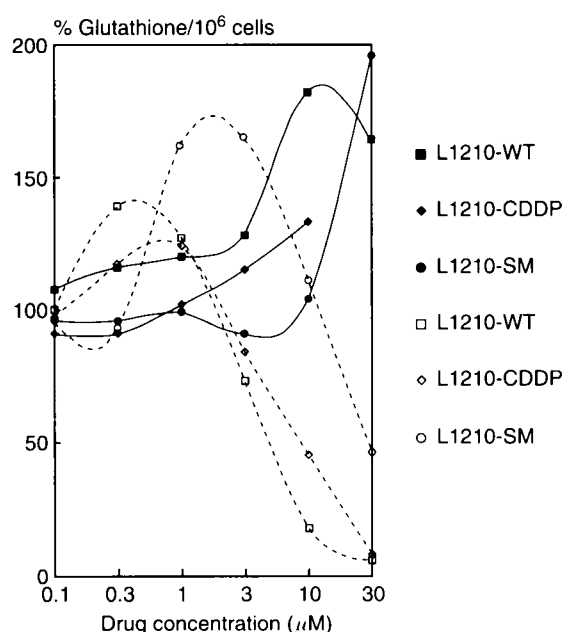

% Glutathione/10^6 cells

■ L1210-WT

♦ L1210-CDDP

● L1210-SM

□ L1210-WT

◇ L1210-CDDP

○ L1210-SM

Figure 3. Relative cellular glutathione level (ng/10^6 cells) in three different murine leukemic tumors after 20 h exposure to varying doses of EdSm (open symbols) or cisplatin (solid symbols).

Table 4. Influence of EdSm and cisplatin on the GST activity in a cell-free system relative to untreated control (+CDNB) in the presence (+CDNB) or absence (−CDNB) of substrate (1 mM CDNB)

Drug dose (μM)	EdSm		Cisplatin	
	−CDNB	+CDNB	−CDNB	+CDNB
30	0.0[a]	99.2%	0.0	102%
10	0.0	97.9%	0.0	69%

[a]The enzyme activity of GST (nmol/min/mg protein) is expressed as percentage of the treated sample over the +CDNB control (%T/C).

lower value, but we do not know whether this is due to inhibition of the enzyme, a low substrate potential or interference with the assay (e.g. reaction with GSH, CDNB or the product). We did not pursue this any further since, under our standard conditions (30 μM or lower), no effect was observed and the intracellular-free concentrations of CDNB will certainly not reach the 10 mM level. The next question we addressed was whether EdSm could interfere with intracellular GSH transferases. We therefore used the Michaelis–Menten kinetics to estimate the V_{max} and K_m of cellular GST in the presence of 30 μM EdSm for a period of 4 h. These results are summarized in Table 5. In the control situation we saw a significant difference in V_{max} between L1210-CDDP (150 nmol/min/mg protein) and the other cell lines, L1210-WT (10^- nmol/min/mg protein) and L1210-Sm (95 nmol/min/mg protein). This is even more pronounced for the K_m values. The K_m value for CDNB in the L1210-CDDP cell line is almost 3-fold higher than that of L1210-WT and L1210-Sm. Exposure to EdSm reduced the maximal activity on a protein base in L1210-WT and L1210-Sm, although not significantly, and increased the V_{max} in L1210-CDDP cell lines. These EdSm effects, however, were not significant, which is in part due to the small sample number.

Discussion

At the onset of our study no information was available about the mechanisms underlying resistance against cisplatin in L1210-CDDP and against EdSm in L1210-Sm. Our results show (Table 1) that these three leukemic tumor cell lines are comparable as to the population doubling time and cell size. Also, the protein content and total glutathione level are similar in these cell lines (Table 3). The GST

Table 5. Effect of 4 h incubation with 30 μM EdSm on Michaelis–Menten parameters of GST in the various L1210 cell lines (see Materials and methods)

		L1210-WT	L1210-CDDP	L1210-Sm	p value[a]
V_{max}[b]	control	107 ± 23 (3)	150 ± 10 (3)	95 ± 25 (2)	0.28
	+ EdSm[c]	77 ± 19 (3)	131 ± 41 (3)	72 ± 15 (2)	0.39
	p value[d]	0.34	0.63	0.35	
K_m[e]	control	0.62 ± 0.12 (3)	1.52 ± 0.34 (3)	0.58 ± 0.13 (2)	0.06
	+ EdSm[c]	0.66 ± 0.08 (3)	2.61 ± 1.05 (3)	0.52 ± 0.01 (2)	0.24
	p value[d]	0.65	0.78	0.18	

[a] p value given for one-way ANOVA between cell lines.
[b] nmol/min/mg protein.
[c] %T/C (treated over control).
[d] p value given for the contrast between the control group and the treated group.
[e] mM.

activities, however, are statistically significantly different. The GST in L1210-CDDP also shows a relatively high K_m. To explain these results, we should bear in mind that the GSTs are a multigene family of isoenzymes which catalyze the reaction between numerous electrophilic compounds and glutathione.[27,28] The cytosolic GST is divided into four gene families: the α, μ, π and θ class. The π-class is preferentially expressed in tumor cells. They function as dimers, with heterodimers occurring in the same class.[29] The various classes have different specific activities and different substrate specificity. We have measured the overall cellular GST pool and the differences we observed between the cell lines most likely indicate a substantial variation at the isoenzyme level. Another difference between these cell lines was detected in the cell phase distribution. L1210-CDDP cells contain more S phase cells and less G_2M cells than L1210-WT and L1210-Sm, which indicates a prolonged S phase, probably due to enhanced DNA repair. Another parameter often involved in resistance to chemotherapy is the elevated expression of a Pgp of 170 kDa, which acts as a broad spectrum membrane pump and removes chemotherapeutic drugs as well. Although Pgp expression has never been implied in cisplatin resistance, other antitumor agents have been shown to be able to induce Pgp expression in murine leukemic cells.[30] Even alkylating agents, like mitomycin C, can induce resistance in L1210 leukemia cells through changes in the membrane Pgp population,[31] but these tumor cells remain sensitive to cisplatin. A possible Pgp induction by Sm derivatives has not yet been investigated. Our results show what we expected, i.e. that the cisplatin-resistant L1210 subclone shows no Pgp induction. The sparsomycin-resistant L1210 cell line also does not show a

significant elevation of the Pgp level. Hence, we conclude that resistance towards EdSm does not involve Pgp.

As for glutathione, our results show that EdSm is not a likely target for GSH conjugation, since EdSm did not directly influence the GST activity (Table 4). Exposure of intact tumor cells to EdSm yielded a dose- and cell line-dependent decline in cellular protein content, glutathione level, as well as in the total GST activity. This dose-dependency of the decline in protein content in the individual cell lines upon EdSm treatment correlates directly with the sensitivity of each cell line for EdSm! Under normal conditions the cellular level of a protein is determined by the balance between the rates of its synthesis and degradation. The average turnover times for individual proteins range from several minutes to months or even years.[32] Many intracellular proteins that have half-lives of 10 min or less are proteins with key regulatory roles whose cellular level is rapidly regulated by modulating their rate of synthesis.[33] Inhibition of protein synthesis by EdSm will directly disturb short-lived protein balance, which could cause effects such as depletion of GSH and GST. The cellular glutathione level is maintained by *de novo* synthesis of GSH from the constituent amino acids, using the enzymes τ-glutamyl cysteine synthetase and glutathione synthetase. Furthermore, glutathione cycles between a reduced thiol form (GSH) and an oxidized form (GSSG) in which two GSH tripeptides are linked by a disulfide bond, and GSSG can be reduced to GSH by glutathione reductase.[34] Because GSH is non-ribosomally synthesized, depletion of GSH must be caused by inhibition of the synthesis of these enzymes. Because glutathione depletion parallels the declining protein content, EdSm apparently has a strong effect on

these enzymes which also implicates that these enzymes have relatively short turnover times and require a high level of *de novo* synthesis.

In combined chemotherapy we must be cautious not to interpret many of the effects as the result of simple direct interaction between cytostatic agents. As this study has also shown, administration of cisplatin can cause the induction of endogenous GSH synthesis, as well as enhanced levels of enzymes such as GST which can catalyze the detoxification of agents such as cisplatin.[35] GSH depletion by BSO suggests two roles for GSH in cisplatin resistance, i.e. cytosolic elimination, resulting in less DNA platination, and a nuclear effect on the formation and repair of DNA platinum adducts.[35] Cisplatin can also conjugate non-enzymatic with sulfhydryl compounds like glutathione.[35–37] This reaction involves nucleophilic displacement of chloride ligands, either directly or subsequent to a reaction with water. This type of reaction could explain the apparent reduction in GST activity that we observe at very high cisplatin concentrations. In addition to GSH, other thiols, like the metallothioneins (MT), can act as a nucleophile towards electrophilic agents like cisplatin, but the involvement of MT in acquired resistance to cisplatin is rather controversial.[9,38–40] In the murine leukemia cell line L1210, the degree of resistance was reported to be associated with the level of MT;[9] however, in another study the resistance of L1210-CDDP cell towards cisplatin could neither be based on an increased level of MT nor on an enhanced ability to increase the synthesis of MT after cisplatin exposure.[11] Thus, MT may be associated with the induction of cisplatin resistance, but its causal role remains to be established. It should be noted that resistance to cisplatin is a relative term, which is at least partially attributable to its narrow therapeutic index. Because of fatal toxicity, it is often not possible to successfully treat tumors, which exhibit even a small inherent resistance to cisplatin, by increasing the dose level of cisplatin. As a consequence, combination therapy with an agent like EdSm, which enhances cisplatin's antitumor effects with little or no enhancement of cisplatin's toxicity,[3,4] may be of value in the treatment of tumors which resist treatment with cisplatin alone.

To summarize, our results suggest that the synergistic combination of EdSm and cisplatin can, at least partly, be explained by the EdSm-induced block of protein synthesis, with subsequent depletion of the cellular detoxification mechanism for cisplatin, and will very likely involve a reduction in the intracellular level of GSH.

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