

Schedule-dependent enhancement of antitumor activity of ethyldeshydroxy-sparsomycin in combination with classical antineoplastic agents

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The efficacy of the protein synthesis inhibitor ethyldeshydroxy-sparsomycin (EDSM) as a biochemical response modifier of several antitumor agents against L1210 leukemia and B16 melanoma is described. Seven drugs with different intracellular targets were selected for this combination study. Tumor implantation and drug treatment were both i.p., and the time interval between the administration of EDSM and the cytostatic agent was varied. Our results show that in the B16 tumor model EDSM is not able to potentiate any of these drugs, whereas antagonism is seen in combination with doxo-rubicin (DX). In the L1210 tumor model, however, no loss of activity is seen for this specific combination. The effect of the combination of cytosar (Ara-C), 5-fluorouracil (5-FU) or vincristine (VCR) with EDSM in the L1210 model is strongly time interval dependent. Loss of 5-FU antitumor activity is seen when EDSM is given 3 or 24 h after 5-FU; however, no effect is observed when EDSM is given 6 h after 5-FU. Enhancement of the 5-FU activity is not noticed. The VCR activity is potentiated when EDSM is given at least 6 h after VCR administration, which increases the antitumor response from 32 to >60 days and the percentage survivors from 33 to 83% ($p = 0.04$). In combination with Ara-C, potentiation of antitumor activity is seen only when EDSM is given 24 h after Ara-C, which increases the antitumor response from 32 to >55 days and the percentage survivors from 11 to 50% ($p = 0.008$). No modulatory effects are found when EDSM is combined with carmustine or DX. Our results suggest that EDSM changes the antitumor efficacy of selected antitumor agents (Ara-C and VCR) in a schedule-dependent way and that potentiation is largely restricted to cell-cycle phase-specific cytostatic agents.

Key words: Antineoplastic agents, combined chemotherapy, murine tumors, protein synthesis inhibitor, sparsomycin, synergism.

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Introduction

Incomplete response of malignancies to antineoplastic agents remains a major clinical problem. Sometimes their efficacy can be improved by combining cytostatic agents with different modes of action, but non-chemotherapeutic agents are also capable of improving the efficacy of cytostatics, e.g. by reversing the resistance to chemotherapy.

Sparsomycin and its analogs are inhibitors of ribosomal protein synthesis,^{1–4} and previous reports from our laboratory clearly demonstrated that these compounds potentiate the antitumor activity of cisplatin *in vivo*.^{5–7} Furthermore, it was shown that the effect of combined treatment of cisplatin and ethyldeshydroxy-sparsomycin (EDSM) was independent of administration order.⁷ This potentiation was not a result of pharmacological factors, but was strongly dependent on the cellular properties of the target tumor-cell populations.⁸ Comparison of the cellular basis of drug sensitivity of solid tumors with that of leukemia cells suggests that a large part of the resistance of solid tumors to treatment is due to low drug sensitivity of the tumor cell itself.⁹ Another parameter might be that the sensitivity of tumor cells as well as the cytotoxicity of drug combinations may depend on the schedule of administration as well as the doses used.^{10,11}

To investigate whether the synergistic effect of EDSM on cisplatin's efficacy is a general phenomenon of EDSM that is applicable to other anticancer drugs, we studied the effect of EDSM on the antitumor activity of agents with different intracellular targets. Moreover, we searched for the optimal dose ratio and schedule dependency for maximal antitumor enhancement by EDSM.

Material and methods

Tumor cells

Murine L1210 leukemia cells and B16 melanoma were kindly supplied by Dr G Atassi (Institute Jules Bordet, Laboratory for Experimental Chemotherapy, Brussels, Belgium). L1210 leukemia cells were maintained in logarithmic growth as suspension culture and B16 melanoma cells were grown routinely as a monolayer culture at 37°C in a humidified atmosphere of 5% CO₂ in air as described previously.⁸

Animals

CD2F₁ mice weighing 18–22 g were used as hosts for the L1210 leukemia and C57Bl/6 mice were used for the B16 melanoma tumor. In each experiment, phosphate-buffered saline (PBS)-treated tumor-bearing animals served as controls. All mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany).

Antitumor activity

Mice were inoculated i.p. with 10⁵ L1210 cells or 10⁶ B16 melanoma cells suspended in 0.2 ml PBS, pH 7.4. Acceptable control median survival times (MSTs) were 8–11 days for the L1210 i.p. tumor model and 14–22 days for the B16 i.p. tumor model.¹² Tumor-bearing mice were randomized in the treatment and control group in each experiment. Each group consisted of six animals. Drugs were administered at various doses i.p. into mice using 0.01 ml/g body weight. Different treatment schedules were used, starting on day 1, which was 24 h after tumor implantation. Animal survival was recorded daily during 60 days and the MST of each group was calculated. Final antitumor results were computed as MST in days after tumor implantation. A T/C value of $\geq 135\%$ is generally taken as a positive indication for antitumor activity according to the NCI criteria.¹² T/C is the MST of the test group divided by that of the control group. L1210 and B16 experiments were terminated after day 60. Mice alive at the end of an experiment were autopsied and judged to be cured if no signs of tumor were visible.

Drugs

EDSM was synthesized at the department of Organic Chemistry, University of Nijmegen, The Netherlands,¹³ and was acquired in a lyophilized form. The drug was dissolved in PBS and kept in dark flasks at 4°C. Seven drugs with different intracellular targets were selected for our combination studies: two S-phase specific antimetabolites, i.e. cytosar (Ara-C) and 5-fluorouracil (5-FU), one mitose-phase specific agent, i.e. vincristine (VCR), two topoisomerase II inhibitors, i.e. etoposide (VP-16) and doxorubicin (DX), and two DNA crosslinking compounds, i.e. cisplatin and carmustine (BCNU). Cisplatin and 5-FU were kindly provided by Pharmachemie BV (Haarlem, The Netherlands). BCNU and VP-16 were obtained from Bristol-Myers (Syracuse, NY), Ara-C from Upjohn (Kalamazoo, MI), VCR from Eli Lilly (Indianapolis, IN) and DX from Farmitalia (Milan, Italy). All drug solutions were prepared freshly before each use. The agents were dissolved in the prescribed solvent. Subsequently, solutions with the required drug concentration were prepared from these stocks by dilution with isotonic NaCl just before administration.

Statistical analysis

Our experimental data have been analyzed by Cox's proportional hazards regression model (PH model).¹⁴ Each presented data point was calculated on the basis of repeated experiments. As usual, dummy variables were used in this PH model to indicate the given doses of EDSM and drugs. Product terms of these dummy variables were included in the model to account for interaction effects of both drugs on survival. A tumor-specific hazard has been obtained by censoring death within 6 days for L1210 and within 14 days for B16 as due to drug toxicity. This occurred only in some B16 experiments. For each treatment schedule the MSTs were calculated within this (complete) PH model. The percentages of long-term survivors (LTSs) were also calculated using the PH survival function and were in very good agreement with the observed number of LTSs after 60 days. The risk ratios of individual as well as of combined drug treatments, compared with the non-treated animals, are directly given by PH analysis. All *p* values were calculated according to Wald's χ^2 test. *p* < 0.05 was considered to be significant.

Results

Combined chemotherapy in B16 melanoma

The tumor-take of i.p. inoculated B16 melanoma was 100% and resulted in a MST of 18 ± 0.13 days for 66 placebo-treated control animals. B16 melanoma is relatively resistant to single-drug treatment, except for DX. To determine whether EDSM could improve the antitumor activity of these drugs against B16 melanoma in mice, we varied drug doses as well as the treatment schedules. The effect of EDSM was studied using doses of 2.5 or 5 mg EDSM/kg, given on day 1, 4 and 7 (D1,4,7) or on day 1, 5 and 9 (D1,5,9). The results of these experiments are summarized in Table 1. Drug doses were increased until toxicity was observed. The classical antitumor agents gave acceptable antitumor effects ($\geq 135\%$ T/C, i.e. MST > 24 days, NCI protocol), except for Ara-C. The combination studies with EDSM showed that in comparison with single-drug treatment, none of the combinations gave improved results. Whereas our results showed that in this B16 tumor model EDSM is not capable of potentiating

any of the drugs studied, a pronounced effect was seen when EDSM was combined with DX, producing loss of the single-dose (1 mg/kg/injection) DX activity from 60 to 22 days, and reducing the number of LTSs from 4 to 0 (out of six animals). This antagonistic effect of EDSM was less obvious in combination with a DX dose of 2 mg/kg/injection.

Combined chemotherapy in L1210 leukemia

The treatment schedules of EDSM in combination with cytostatic agents were varied in several experiments performed with i.p. implanted L1210. The MST of 66 placebo-treated control animals was 10 ± 0.35 days and the tumor-take was 100%. EDSM at 5 mg/kg/injection showed a moderate antitumor response by increasing the MST to 12 days using the D1,5,9 treatment schedule whereas no response was observed at 2.5 mg/kg/injection. The results of combined drug treatment in L1210 are summarized in Table 2. Although we have described earlier¹⁴ that the modulatory capacity of EDSM in combination with cisplatin was independent of drug

Table 1. Dose- and schedule-dependent antitumor activity of several cytostatic agents in combination with EDSM against B16 melanoma (i.p.)

Drugs	Treatment ^a	Dose ^b	MST (days)		
			Single	2.5 + EDSM ^{b,c}	5
Control ^d			18	20	18
VCR	D1,4,7	0.5	24	—	5 TOX
		1.0	25	—	6 TOX
	D1,5,9	0.25	20	22	6 TOX
		0.50	20	22	—
		0.75	22	—	—
VP-16	D1,4,7	10	32	—	6 TOX
	D1,5,9	2.5	29	29	6 TOX
		5.0	29	28	5 TOX
5-FU	D1,4,7	50	23	—	7 TOX
		100	TOX	—	8 TOX
	D1,5,9	50	19	19	—
	D1,8,15	100	22	19	—
ARA-C	D1,4,7	50	19	—	19
		100	19	—	18
Cisplatin	D1,5,9	3	23	—	22
		5	26	—	13
					TOX
DX	D1,5,9	0.5	32 1/6 ^e	—	22 0/6
		1	60 4/6	—	22 0/6
		2	60 4/6	—	54 2/6

^a In the treatment schedules D1,4,7 and D1,5,9 these drugs were given i.p. on day 1, 4 and 7 or on day 1, 5 and 9 after tumor implantation.

^b mg/kg/injection.

^c EDSM was given i.p. 3 h after injection of the first drug.

^d The MST of i.p.-implanted B16 melanoma was 18 ± 0.13 days for control animals.

Table 2. Antitumor activity of several cytostatic agents in L1210 leukemia (i.p.), in combination with EDSM post-treatment after 3, 6 or 24 h (treatment schedule was D1,5,9; the number of animals treated is given in parentheses)

Drugs	Dose (mg/kg/ injection)	Single drug MST ^a	+EDSM 2.5 mg/kg/injection			p value
			+3 h	+6 h	+24 h	
Control	0	10 (66)				
EDSM	2.5	10 (12)				
VCR	0.5	32 (18)	24 (12)	>60 (12)	>60 (12)	0.04
	1.0	46 (6)	18 (6)	—	—	0.23
5-FU	50	>60 (18)	26 (12)	>60 (12)	30 (12)	0.16
Ara-C	100	14 (6)	14 (6)	—	—	0.88
	500	32 (18)	24 (12)	24 (12)	>55 (12)	0.008
BCNU	10	>60 (12)	>60 (6)	>60 (6)	>44 (6)	0.63
DX	3	>60 (24)	>60 (18)	>60 (6)	>60 (6)	0.20

^a The MST of 66 placebo-treated control animals was 10 ± 0.35 days.

sequence administration as well as of time interval between drug administration, we now see the importance of timing. In this L1210 model, EDSM modified the antitumor activity of Ara-C, 5-FU and VCR, and the time interval between drug administration is crucial for potentiation of antitumor activity. Even an antagonistic effect was seen when EDSM was given 3 or 24 h after 5-FU administration, although this antagonistic effect was not observed when EDSM was given 6 h after 5-FU. The MST of single 5-FU treatment was >60 days; therefore potential enhancement by EDSM could not be studied at this 5-FU dose. The antitumor activity of VCR, on the other hand, could be potentiated when EDSM was given at least 6 h after VCR (Figure 1), thereby increasing the MST from 32 to >60 days and the percentage survivors from 33 to 83% ($p = 0.04$). Potentiation of Ara-C's antitumor activity was only noticed when the EDSM administration was postponed until 24 h after the administration of Ara-C (Figure 2), increasing the MST from 32 to >55 days and the percentage survivors from 11 to 50%

($p = 0.008$). Potentiation of the antitumor activity could not be measured due to maximal activity of BCNU as a single drug. On the other hand, our results show that EDSM has no antagonistic effect on the BCNU activity.

As in both tumor models DX is a very strong antitumor agent on its own, we investigated the modulatory effect of EDSM in more detail using lower DX doses. Table 3 shows the results of this study. EDSM (5 mg/kg) given before or after certain DX doses did not significantly improve the activity of the latter drug. The overall p value for EDSM pre-treatment was 0.97 and for EDSM post-treatment was 0.20. Finally, a reduction in DX antitumor activity, as seen in the B16 model, was not noticed in the L1210 model.

Discussion

The results of the present study show that the modulating capacity of EDSM on antineoplastic agents is

Table 3. Effect of EDSM (5 mg/kg) on the antitumor activity of DX in L1210 (i.p.)

Dose DX ^a	EDSM = 0	EDSM = 5, -3 h ^b	EDSM = 5, +3 h
0	10 ^c (66) ^d	12 (48)	
0.5	20 (12)	21 (12)	14 (6)
1	19 (12)	19 (12)	17 (6)
2	19 (12)	19 (12)	44 (6)
3	>60 (24)	>60 (18)	>60 (18)
4	23 (12)	24 (6)	18 (6)
p value	0.0001 ^e	0.97 ^f	0.20 ^f

^a EDSM was given i.p. 3 h before or 3 h after DX injection.

^b Treatment schedule was i.p. on day 1, 5 and 9 after tumor implantation (D1,5,9).

^c MST in days.

^d Total number of animals used for analysis.

^e Overall p value for DX compared with the control level.

^f Overall p value of the combination compared with single DX treatment.

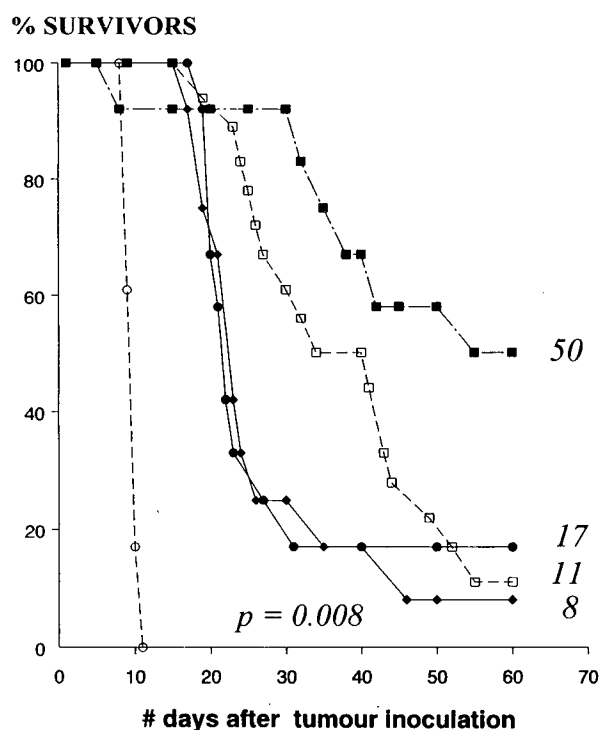


Figure 1. Survival of mice inoculated i.p. with 10^5 L1210 cells. Mice were treated i.p. with Ara-C (500 mg/kg/injection) as a single drug (□) and in combination with EDSM (2.5 mg/kg/injection), given 3 (●), 6 (◆) or 24 h (■) after Ara-C administration. Drug treatment started 24 h after tumor implantation, once daily every 4 days for three doses. (○) Untreated control group.

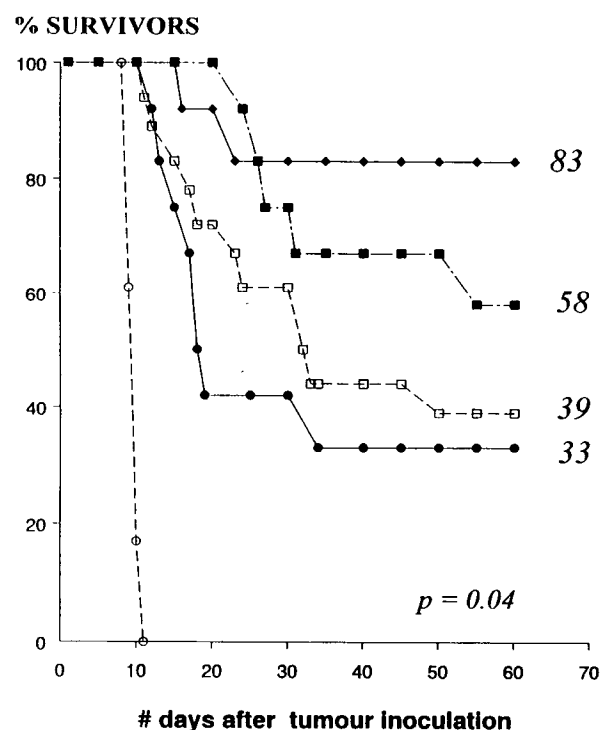


Figure 2. Survival of mice inoculated i.p. with 10^5 L1210 cells. Mice were treated i.p. with VCR (0.5 mg/kg/injection) as a single drug (□) and in combination with EDSM (2.5 mg/kg/injection), given 3 (●), 6 (◆) or 24 h (■) after VCR administration. Drug treatment started 24 h after tumor implantation, once daily every 4 days for three doses. (○) Untreated control group.

schedule dependent. The treatment schedule used in our experiments was based upon the results of the combination study of EDSM with cisplatin;¹⁵ these results were independent of time and order of drug administration. In combination with cisplatin EDSM could be given from 24 h before up to 24 h after cisplatin injections, without loss of synergy.⁷ However, recent results from our laboratory revealed that when EDSM was combined *in vitro* with drugs other than cisplatin the outcome was dose and schedule dependent,¹⁶ and synergism was most pronounced when EDSM was given as the second drug in the combination.

In this report we discuss the efficacy of these drug combinations *in vivo* in two murine tumor models. In contrast to what we have seen with cisplatin, the time interval between the administration of the two drugs is very important and determines whether the antitumor activity of the classical cytostatic agents is potentiated or inhibited. These effects were most obvious in the combinations with 5-FU, Ara-C, VCR

or DX. No significant effects were found in combination with BCNU or VP16. In order to explain the occurrence of the chemomodulatory effects caused by EDSM we must consider the intercellular response to treatment with the various drugs. Metabolism of 5-FU produces two critical intermediates: fluorouridine-5'-triphosphate (FUTP), which is incorporated into RNA and interferes with its function, and fluorodeoxyuridylate (FdUMP), which prevents normal DNA replication.¹⁷ 5-FU is phase-specific for the S-phase of the cell cycle, which may explain why the effects of 5-FU are schedule dependent.¹⁸ The cytotoxic activity of Ara-C is due to its triphosphorylated metabolite Ara-CTP, which blocks DNA synthesis through competitive inhibition of DNA polymerase. Ara-C nucleotides may also become incorporated into DNA.¹⁹ Intracellular deamination by the enzyme cytidine deaminase converts Ara-C to an inactive metabolite, uracil arabinoside (Ara-U). Hence Ara-C is also S phase-specific and again its efficacy was shown to be schedule-dependent.²⁰

Vincristine produces metaphase arrest by binding to tubulin, which results in a marked increase in the population of cells in mitosis, which is maximal 6–12 h after drug administration.²¹ Inhibitors of protein synthesis are known to cause a reversible cell-cycle arrest in G₁,^{22,23} and as long as tumor cells remain in G₁, no antitumor activity of S phase-specific agents like 5-FU and Ara-C can be expected. This might be the rationale for the reduction in antitumor activity that we observed when EDSM was given too soon (3 h) after the first drug, blocking the cell transfer from G₁ to S phase. In addition, protein synthesis inhibition may also decrease the cellular deaminase activity, which leaves more Ara-C nucleotides to be incorporated into DNA, increasing the cytotoxicity of Ara-C, assuming that the phosphorylation of Ara-C by nucleoside mono- and diphosphate kinase is not inhibited. On the other hand, if EDSM was given before Ara-C treatment the DNA-replicative synthesis would have been reversibly turned down by EDSM as well as the phosphate kinase activity, and temporarily the intracellular Ara-C molecules cannot be incorporated into DNA, which thus means loss of Ara-C antitumor activity. These biochemical implications indicate the importance of timing when EDSM is given in combination with 5-FU, Ara-C or VCR. In this context the synergism of EDSM and cisplatin could, for instance, result from glutathione depletion by EDSM, inhibition of *de novo* synthesis of glutathione-S-transferases and/or inhibition of the repair of cisplatin-induced DNA damage. The apparent induction by EDSM of the cytotoxic effects of DX and BCNU is less obvious. The mechanism of cytotoxicity of doxorubicin is very complex, including inhibition of DNA synthesis,²⁴ DNA intercalation,^{25–27} DNA single-strand breaks,²⁸ the production of superoxide free radicals binding to the cell membrane and altering its function,³⁰ and interaction with topoisomerase II.^{29,31,32} EDSM might contribute to this cytotoxicity by inhibition of *de novo* topo II synthesis and DNA repair proteins, but the extent of these effects can only be small to effect the miscellaneous DX targets and the strong activity of DX as a single drug in L1210 as well as B16 melanoma. BCNU, on the other hand, undergoes spontaneous chemical degradation to a carbonium ion that alkylates DNA and an isocyanate intermediate that carbamoylates proteins, such as DNA repair enzymes.^{33,34} DNA alkylation by BCNU subsequently results in DNA crosslinking, which is critical for cancer cell lethality. The cytotoxic DNA damage induced by BCNU is normally repaired by the DNA repair enzyme O⁶-alkylguanine-DNA al-

kyltransferase. The use of a direct alkyltransferase inhibitor, such as O⁶-methylguanine, inactivates the alkyltransferase and sensitizes cells to BCNU.³⁵ One of the effects of EDSM might be based on an inhibition of *de novo* synthesis of this repair enzyme and thereby indirectly increasing the antitumor activity of BCNU. This effect is dependent on the turnover time of this alkyltransferase. In the L1210 tumor model BCNU by itself gave complete antitumor responses at a dose of 10 mg/kg. Thus, improvement by EDSM cannot be expected under these conditions. On the other hand, our results did not show any antagonistic interaction between EDSM and BCNU, as was clearly observed in the combination with 5-FU.

The two tumor models used in this report are hardly comparable. L1210 leukemia is a rapidly growing tumor with a high percentage of cells synthesizing DNA. Because all cells are actively progressing through the cell cycle, its life cycle is consistent and predictable.³⁶ The relationship between cell number and survival in L1210 leukemia is linear. The time to death of animals bearing L1210 leukemia is the interval required to achieve a population size of about 10⁹ cells. With a growth fraction of 100%, 10⁹ cells will accumulate by 10 days after the injection of 10⁵ cells. The i.p. inoculated B16 melanoma cells will grow as a solid tumor, have a longer cell cycle and therefore grow less rapidly than L1210 cells. Injection of 10⁶ cells requires 18 days to reach lethality. This tumor model has a low percentage of cells synthesizing DNA and will be less sensitive to S phase-specific drugs like 5-FU and Ara-C. Moreover, in the L1210 model drug treatment starts on day 1 and continues until the number of tumor cells in control animals has reached the maximum cell number. The B16 tumor model is more advanced (10⁶ cells) at the start and the last drug treatment is given 9 days before MST of the control animals. EDSM by itself has no measurable antitumor activity against B16 tumors, probably because, contrary to the L1210 cell cycle time, the reversible inhibition of protein synthesis does not last long enough to cause measurable problems for the viability of B16 tumor cells.

Summarizing, we can conclude that modulation of antitumor activity by EDSM is tumor-type-dependent and is limited to cell-cycle phase-specific drugs like Ara-C and VCR, with the exception that it also potentiates the activity of cisplatin. Moreover, this report shows that the antitumor activity of drug combinations depends on the timing of the drug administration.

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