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Concentration and sequence dependent synergism of ethyldeshydroxy-sparsomycin in combination with antitumor agents

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The modulating effect of ethyldeshydroxy-sparsomycin (EDSM), an inhibitor of ribosomal protein synthesis, on cytostatic agents was studied on cultured B16 melanoma cells using the microculture tetrazolium test (MTT). The data were analyzed for true synergism using the combination index and the median effect principle. The extent of cytotoxic drug interaction was influenced by the duration of drug exposure, the dose ratio, as well as the treatment schedule. When drug ratios were used, synergism was observed upon pre- and post-treatment in combination with cisplatin, cytosine arabinoside (Ara-C), methotrexate (MTX) and 5-fluorouracil (5-FU). The combination of a fixed dose of EDSM was synergistic with cisplatin, Ara-C, vincristine (VCR) and MTX, in the order MTX > Ara-C > VCR > cisplatin, while the combinations with doxorubicin, 5-FU and etoposide (VP-16) were shown to be antagonistic. These results suggest that only certain drugs and treatment schedules might be worthwhile for combination studies with EDSM *in vivo* and indicate a role for EDSM as modifier of antitumor responses in cancer chemotherapy.

Key words: Antineoplastic agents, combination index, median effect, protein synthesis inhibitors, sparsomycin.

Introduction

Sparsomycin and several derivatives are well-established inhibitors of ribosomal protein synthesis.[1–4] Previous reports from our laboratory clearly demonstrated that sparsomycin and some analogs can potentiate the antitumor activity of cisplatin *in vivo*.[5–7] Furthermore, it was shown that combined treatment of cisplatin and ethyldeshydroxy-sparsomycin (EDSM) was order-independent.[7] Recently, it has been described for leukemia cells[8] that this interaction did not directly depend on pharmacological effects, but rather strongly on the cellular properties of the target tumor cell populations. A comparison of the cellular basis of drug sensitivity of human colon, pancreatic and renal carcinoma cell lines with that of leukemia cell lines[9] suggests that the refractoriness of solid tumors to chemotherapy compared with leukemia cells is, at least in part, due to drug resistance of the tumor cell itself. A second important factor might be, however, that the sensitivity of tumor cells as well as the cytotoxicity of drug combinations depends on the schedule of administration as well as the doses used.[10,11]

The purpose of this study was to investigate at the cellular level the optimal dose ratio and schedule dependency for maximal cytotoxic interaction of EDSM with antitumor agents with different intracellular targets. To analyze the experimental data of the drug combination studies and to quantify synergism or antagonism, we applied computer software based on the median effect principle developed by Chou.[12]

Materials and methods

Cell culture

Murine B16 melanoma cells were kindly supplied by Dr G Atassi (Institute Jules Bordet, Laboratory for Experimental Chemotherapy, Brussels, Belgium). These cells were grown routinely as monolayer culture in closed tissue culture flasks (Costar, Cambridge, MA) at 37°C in a humidified atmosphere (Heraeus incubator) of 5% CO_2 in air. As growth medium we used Eagle minimum essential medium with Earle's salts (Gibco, Grand Island, NY) supplemented with 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid)

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buffer, 10% heat-inactivated fetal calf serum (Gibco), 2 mM glutamine (Boehringer-Mannheim, Mannheim, Germany) and 50 μg gentamycin/ml (Boehringer-Mannheim). Cells were harvested by treatment with 0.025% trypsin (type III; Sigma, St Louis, MO) and 0.05% EDTA for 3 min at 37°C.

Drugs

EDSM was synthesized by the department of Organic Chemistry of the University of Nijmegen[13] and was acquired in a lyophilized form. The drug was dissolved in phosphate buffered saline (PBS) and kept in dark flasks at 4°C. The antitumor agents we used were formulated for clinical use, and were stored as indicated for each drug. Cisplatin (Platinol), 5-fluorouracil (5-FU) and methotrexate (MTX) were kindly provided by Pharmachemie BV (Haarlem, The Netherlands). The other antineoplastic agents were derived from Bristol-Myers [etoposide (VP-16)], Upjohn [cytosine arabinoside (Ara-C)], Eli Lilly [vincristine (VCR)] and Pharmacia [doxorubicin (DX)]. All drug solutions were prepared immediately before use to prevent loss of activity due to decay. The agents were first dissolved in the prescribed solvent and subsequently diluted in growth medium.

Before final conclusions can be drawn concerning the schedule dependency of an anticancer drug based on *in vitro* studies using the MTT, it is essential to document drug stability under *in vitro* assay conditions. The drugs used in these studies, with the exception of VP-16, cisplatin and DX, maintain their chemical and biological stability for up to 10 days.[14–16] Degradation of VP-16 is rapid under *in vitro* culture conditions, where it shows a half-life of 2 days.[17] Cisplatin has a half-life of 18.5 h under *in vitro* culture conditions.[19] Definitive statements about DX stability are confusing, but it probably slowly degrades in medium.[18] Thus, apart from DX, the drugs used in our experiments will exert their cytotoxic action during the entire treatment period of 18 h.

Microculture tetrazolium test (MTT)

We used the MTT assay as previously described by Mosmann.[18] This assay is based on the reduction of a yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, by mitochondrial dehydrogenase in viable cells to a blue formazan product that can be measured spectrophotometrically. This reaction directly indicates cell viability.[19] The relation between cell density and formazan crystal formation was determined using increasing cell densities (100–20 000 cells/well, plated in 96-well culture plates). Seven days after cell plating, 0.02 mg (20 μg of 1 mg/ml in growth medium) MTT was added to each well and incubated at 37°C for a further 4 h. The formazan was dissolved in dimethylsulfoxide (DMSO) and measured spectrophotometrically at 550 nm. Underestimation of the antitumor efficacy of certain compounds can occur in the MTT assay due to a possible effect of the drugs on cell size.[20] Therefore, all microwell plates were microscopically checked at the end of each experiment.

Drug treatment

For drug treatment, B16 melanoma cells were seeded at 1000 cells/well, allowed to grow for 24 h and then drugs were applied. In the single-agent experiments, cells were exposed to increasing concentrations of the individual drugs. EDSM treatment was 3 or 24 h. The duration of exposure of the other drugs was always 18 h. Following drug exposure, the cells were washed with PBS and fresh medium was added. Combination treatments were always performed using sequential exposure to the two agents. EDSM was given before (pretreatment) or after (post-treatment) the exposure of the second drug. Treatment schedules: A, exposure to EDSM for 3 h, followed by exposure to an antitumor agent for 18 h; B, exposure to an antitumor agent for 18 h, followed by exposure to EDSM for 3 h; C, exposure to EDSM for 24 h, followed by exposure to an antitumor agent for 18 h; D, exposure to an antitumor agent for 18 h, followed by exposure to EDSM for 24 h. Cells were washed with PBS after each exposure step and allowed to grow in fresh growth medium. All experiments were performed in triplicate.

Quantitation of synergism and antagonism of two drugs

Dose–effect relationships were analyzed by the median effect equation derived by Chou[12] and is given by: $D = D_m[f_a/(1 - f_a)]^{1/m}$, in which D is dose of the drug used, D_m is the median effect dose signifying the potency, f_a is the fraction affected (% decreased formazan production compared to

untreated cells) and m is a coefficient signifying the shape of the dose–effect curve, i.e. $m = 1$ for hyperbolic (first-order or Michaelis–Menten) systems; $m > 1$ for sigmoidal and $m < 1$ for negatively sigmoidal shapes. The median effect equation describes the behavior of many biological systems and has been effectively used to analyze multiple drug effects.

Synergism or antagonism for drug combinations is determined by the combination index (CI), on the basis of the multiple drug effect equation of Chou and Talalay.[21–23]

$$CI = \frac{(D_{comb})_1}{(D_{alone})_1} + \frac{(D_{comb})_2}{(D_{alone})_2} + \alpha \frac{(D_{comb})_1(D_{comb})_2}{(D_{alone})_1(D_{alone})_2}$$

$CI = 1$ indicates the additive effects, $CI < 1$ indicates synergism and $CI > 1$ indicates antagonism. CI can be presented as a function of the fractional effect, f_a. $(D_{alone})_1$ is the dose of drug 1 alone required for a given effect (f_a), $(D_{comb})_1$ is the dose of drug 1 in the combination required for a given effect (f_a) $(D_{alone})_2$ is the dose of drug 2 alone required for a given affect (f_a) and $(D_{comb})_2$ is the dose of drug 2 in the combination required for a given effect (f_a). $\alpha = 0$ if the effects of the two drugs are mutually exclusive (which means that the median effect plots for parent drugs and their mixtures are parallel, indicating the same mode of actions) and 1 if the effects of the two drugs are mutually non-exclusive (independent or different mode of actions).

The dose reduction index (DRI) defines the extent (folds) of dose reduction in a combination for a given degree of effect as compared with the dose of each drug alone

$$DRI_{f_a} = \frac{(D_{alone})_{f_a}}{(D_{comb})_{f_a}}.$$

Results

Single drug treatment

The chemotherapeutic agents have been chosen because their antiproliferative activity is based on different mechanisms of action and the cytotoxicity of each drug in B16 melanoma tumor cells has been evaluated (Table 1). These results show a broad difference in sensitivity of B16 cells for the drugs used. The individual D_ms varied from 0.12 μM for DX to 8.30 μM for 5-FU. Although the shape of the dose–response curves are all sigmoidal ($m > 1$), their specific m values vary and increased in steepness from 1.5 for VP-16 to 3.84 for DX.

Table 1. Cytotoxic effects of antitumour drugs on B16 melanoma cells treated for 18 h (EDSM treatment was 3 or 24 h)

Agent	D_m (μM)[a]	m[b]	R[c]
EDSM (3 h)	22 ± 4	3.4 ± 0.8	0.993 ± 0.004
EDSM (24 h)	1.8 ± 0.3	2.4 ± 0.9	0.984 ± 0.009
Cisplatin	6.1 ± 0.7	3.6 ± 0.6	0.970 ± 0.012
Ara-C	1.3 ± 0.2	2.2 ± 0.2	0.980 ± 0.006
VCR	0.13 ± 0.04	1.9 ± 0.5	0.976 ± 0.007
MTX	0.35 ± 0.04	3.0 ± 0.4	0.956 ± 0.011
5-FU	8.3 ± 0.6	2.7 ± 0.3	0.982 ± 0.003
VP-16	0.88 ± 0.06	1.5 ± 0.1	0.969 ± 0.016
DX	0.12 ± 0.03	3.8 ± 0.4	0.980 ± 0.012

[a]Median effect dose (ED_{50}).
[b]Coefficient signifying the shape of the dose–effect curve for the agent used ($m = 1$, >1 and <1 indicates a hyperbolic, sigmoidal and negatively sigmoidal curve, respectively).
[c]Linear correlation coefficient of the median effect plots.

Drug combinations at fixed dose ratios

B16 melanoma cells were treated with different drugs and drug doses for a period of 18 h. The modulating capacity of EDSM in combination with a specific agent was evaluated after pre-treatment and post-treatment of the 18 h period of cystostatic drug exposure. EDSM was given at a fixed dose ratio of the drug in that combination and the dose ratios were based on their individual D_m values. Computer-generated curves describe the combined effects at these fixed ratios. Computed regression coefficients for the linearized dose–effect curves of the drug combinations were >0.9, which means that the data fulfilled the criteria for computation of the CI according to Chou. Figure 1 shows an example of the results of such an analysis when tumor cells are exposed to drug combinations dependent of drug dose, exposure time and treatment schedule. B16 cells were treated with EDSM for either 3 or 24 h, before or after exposure to cisplatin or MTX. These results are plotted as a function of the fraction of treated cells affected (f_a) versus the CI (f_a–CI plot) under mutually non-exclusive assumption, which means that we assume that the mode of action of the drugs in the combinations are not comparable. Time-dependent cytotoxicity effects were found. The combination of EDSM with cisplatin showed no preferent treatment schedule when the EDSM treatment was only 3 h at a dose ratio of cisplatin to EDSM of 1:1 (Figure 1A) and resulted in synergistic interactions when the f_a was above 0.2. On the other hand, when the EDSM treatment was prolonged to

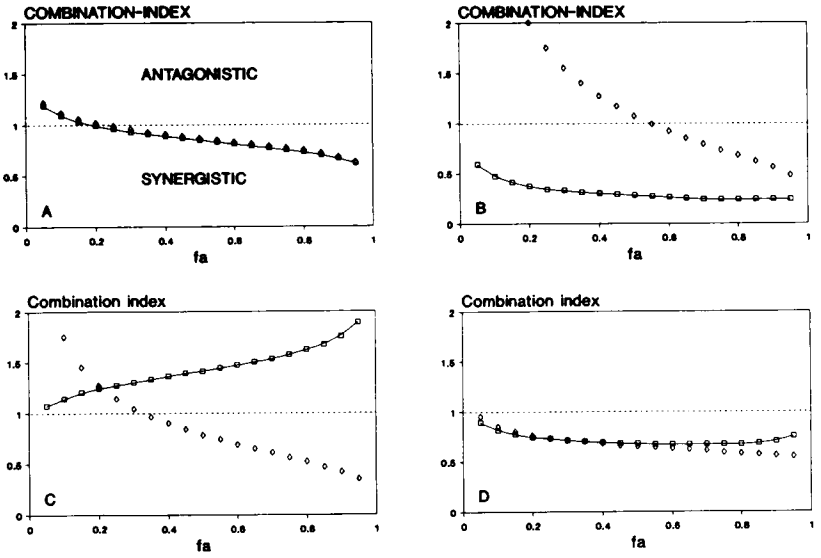

Figure 1. Antagonistic and synergistic effects of EDSM in combination with cisplatin and MTX based on median effect analysis. (A) and (B): combination of EDSM with cisplatin at a molar drug ratio for CDDP:EDSM of 1:1 in (A) and 3:1 in (B). (C) and (D): combination of EDSM with MTX. The molar drug ratio used for MTX:EDSM is 1:100 in (C) and 1:10 in (D). EDSM treatment was 3 h in (A) and (C), and 24 h in (B) and (D). CI > 1, antagonistic; CI < 1, synergistic; CI = 1, additive. □, EDSM pre-treatment; ◇, EDSM post-treatment.

24 h at a dose ratio of cisplatin to EDSM of 1:3 (Figure 1B), it becomes important whether the EDSM treatment is before or after the cisplatin treatment. EDSM pre-treatment gives strong synergistic interaction over the whole range of the affected fraction, while EDSM post-treatment results in strong antagonistic effects from $f_a = 0$ to 0.5, but changes to synergism when the affected fraction is greater than 0.5. The combination of EDSM with MTX shows the opposite result. The 24 h EDSM treatment gives schedule-independent synergism over the complete f_a area, while the EDSM 3 h pre-treatment gives increasing antagonism and increasing synergism after post-treatment.

The results of EDSM in combination with Ara-C and 5-FU are summarized and presented in Table 2, together with the cisplatin and MTX effects. Instead of presenting all CI results from $f_a = 0$ to 1, we show the results at the levels of 0.5 and 0.9 fraction affected, based on their computer-calculated combination index. The combination of EDSM with Ara-C results mainly in antagonistic

Table 2. Summary of computer derived data analysis[a]

Agent	Scheme[b]	Ratio[c]	DRI-ED_{50}		CI	DRI-ED_{90}		CI
			agent	EDSM		agent	EDSM	
Cisplatin	A	1:1	1.7	3.9	1.00	2.3	4.2	0.78
	B	1:1	1.6	3.8	1.05	2.3	4.2	0.78
	C	3:1	8.1	6.5	0.39	5.6	16.3	0.19
	D	3:1	2.2	1.6	1.34	2.4	7.3	0.61
Ara-C	A	1:3	0.8	6.4	1.60	1.4	4.9	1.06
	B	1:3	1.0	9.3	1.22	3.9	7.6	0.42
	C	1:10	11.0	2.0	0.64	23.0	2.3	0.50
	D	1:10	7.1	1.3	1.02	12.4	4.6	0.32
MTX	A	1:100	1.8	1.8	1.42	1.5	1.6	1.71
	B	1:100	4.2	2.3	0.78	12.1	3.2	0.42
	C	1:10	5.4	2.4	0.68	2.9	3.7	0.71
	D	1:10	7.6	2.2	0.65	4.9	1.8	0.87
5-FU	A	1:3	2.8	2.9	0.83	1.4	8.6	0.91
	B	1:3	4.4	3.6	0.57	3.5	2.5	0.80
	C	1:1	6.0	1.8	0.81	7.6	5.2	0.35
	D	1:1	1.6	1.3	1.88	4.3	4.0	0.54

[a]The results are expressed as the individual DRI and calculated CI at the 50% and 90% effect level (ED_{50} and ED_{90}). B16 melanoma cells were exposed to EDSM in combination with individual agents at a fixed dose ratio.
[b]See Materials and methods for detailed information.
[c]Molar ratio of the dose of the antitumor agent to the dose of EDSM.

effects at the 50% effect level, which reverses to strong synergism at the 90% effect level, except for short EDSM pre-treatment. In combination with 5-FU we see synergism in all four treatment schedules, but there seems to be preference for long EDSM treatment. At short EDSM exposure the CI increases from ED_{50} to ED_{90}, while at long EDSM exposure the opposite effect is obtained, resulting in strong synergism.

In addition to the CI values we get information about the individual DRI of the drugs in combinations. In Table 2, we see not only a modulating effect of EDSM on the drugs used in the combination, but often these drugs have a modulating capacity on EDSM. This phenomenon is even more obvious at the 90% effect level, where a maximum dose reduction of 16.3-fold is calculated for EDSM in combination with cisplatin. Synergistic results are obtained when the drugs in the combination have mutual dose reduction effects.

Drug combinations with fixed EDSM doses

In these combinations we used 1 μM EDSM in the 24 h and 3 or 10 μM in the 3 h treatment period. These exposure periods give less than 10% growth inhibition of B16 cells ($f_a < 0.1$). The CI is only calculated at the D_m. Broad differences in the cytotoxic modulations were demonstrated at the level of D_m and the results are summarized in Table 3. In contrast to dose ratios, we see that a fixed EDSM dose and exposure during 24 h always resulted in antagonistic drug interaction. Different results were obtained after short EDSM exposures. Pre-treatment with 3 μM EDSM for 3 h (data not shown) always resulted in antagonistic cytotoxicity which was more obvious in combination with 10 μM EDSM. Synergistic interactions were seen when EDSM was given as the second drug in combination with Ara-C, MTX, VCR and moderately with cisplatin. The combinations of EDSM with DX, 5-FU and VP-16 were shown to be antagonistic.

Discussion

Cell culture laboratories have adopted the MTT assay to predict the clinical effectiveness of various chemotherapeutic agents against human malignancies,[24–26] as well as to study synergistic interactions between cytotoxic agents.[27] Several reports have indicated that use of the appropriate drug sequence and drug doses can produce potentiation of cytotoxic action,[28–31] but completely different patterns of synergistic interaction were seen. Our results give an impression of the complexity of drug combination studies. The importance of this analysis is that the results give information about the character of drug interaction instead of absolute numbers. It will be obvious that when drugs are used in other dose ratios the curve of the f_a–CI plot will change. For EDSM it is clear (Figure 1 and Table 2) that its modulating capacity is present and increasing when the affected cell fraction is 50% or more, which indicates that synergism will occur when EDSM reaches an active concentration at the cellular level. This might be

Table 3. Summary of the calculated individual DRI at the 50% effect level (ED_{50}) of the combination, when EDSM was given as a fixed dose in combination with individual agents at various doses

Drugs	Treatment schedule[a]			
	EDSM pre-treatment		EDSM post-treatment	
	1 μM (24 h)	10 μM (3 h)	1 μM (24 h)	10 μM (3 h)
Cisplatin	1.08 ANT[b]	2.10 ANT	1.06 ANT	4.30 SYN
Ara-C	0.92 ANT	0.68 ANT	2.81 ANT	3.62 SYN
5-FU	1.80 ANT	1.40 ANT	4.60 ANT	1.50 ANT
DX	1.79 ANT	1.12 ANT	1.65 ANT	2.38 ANT
VP-16	1.08 ANT	0.52 ANT	0.95 ANT	1.25 ANT
VCR	1.29 ANT	0.95 ANT	1.47 ANT	3.70 SYN
MTX	1.17 ANT	1.32 ANT	1.76 ANT	15.7 SYN

[a] B16 melanoma cells were exposed to EDSM, 1 μM for 24 h or 10 μM for 3 h, before (pre-treatment) or after (post-treatment) treatment with antitumor agents for 18 h.

[b] For each combination we calculated the CI, but instead of the exact number we indicate the tendency of drug interaction. ANT, antagonistic, CI > 1; SYN, synergistic, CI < 1.

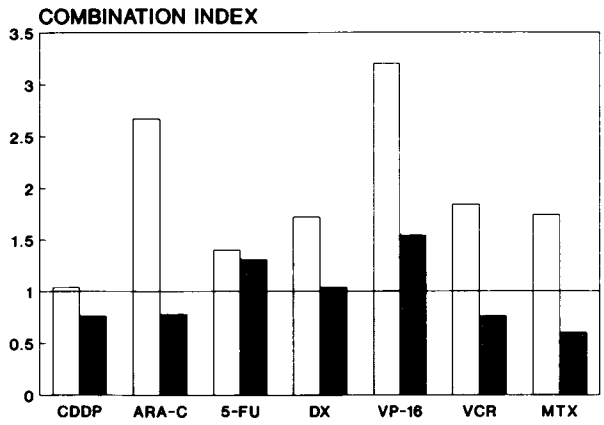

Figure 2. Interaction of 10 μM EDSM (3 h) with increasing doses of seven different cytostatic agents. The CI value for each pair of drugs is calculated at the D_m. CI > 1, antagonistic interaction; CI < 1, synergistic interaction. □, pre-; ■, post-.

the reason for the mainly antagonistic effects when a fixed EDSM dose is used which has less than 10% growth inhibition on its own (Table 3 and Figure 2).

There does not seem to be a general optimal treatment schedule for EDSM in combination with antitumor drugs. However, the CI results in Table 2 and Figure 2 show preference for EDSM post-treatment. An understanding of the mechanism underlying synergistic interaction may help us to anticipate the selectivity of a given combination of drugs against neoplastic cells relative to normal tissues (therapeutic ratio) prior to the initiation of clinical trials or to develop additional strategies designed to enhance synergy through the addition of other agents or the implementation of changes in the schedule of drug administration.

Regarding cisplatin, additive antitumor effects and no enhancement of toxicities have been found in combination with *N*-methylformamide,[32] synergism has been described for compounds that interfere with plasma membrane functions like inhibitors of protein kinase C[33] and inhibition of nucleoside membrane transport.[34] The synergism of combinations with EDSM and cisplatin might be explained on the basis of cytokinetic considerations. Inhibitors of protein synthesis as well as protein kinase C block the cell transition from the G_0–G_1 to the S Phase of the cell cycle.[35,36] It is known that G_1 phase cells are more sensitive to platinum bound to their DNA than mid-S phase or asynchronously treated cells.[37]

Synergistic drug interactions *in vitro* suggest greater therapeutic activity. However, drug–drug interactions of anticancer agents studied *in vitro* provides precise information on drug potency, but none on drug selectivity. The DRI is a more intuitive measure of drug dose reduction due to synergism and is particularly helpful when attempting to minimize toxic side-effects of one of the agents.

Although the MTT assay may be used to select a specific drug-dosing schedule for clinical use, the *in vitro*/*in vivo* correlation of drug exposure studies still has to be established. Factors other than the inherent chemosensitivity of tumor cells significantly influence the outcome of chemotherapy *in vivo*.[38] An improvement of clinical response might be secondary chemotherapy based on *in vitro* selected drug sensitivity of individual patients' tumor specimens.[39] Attention to the doses of the agents used in sequential combinations is important because the synergistic effects observed in cell culture may translate into formidable toxicity when combinations are used *in vivo*.[40] This makes pre-clinical animal studies and phase I trials necessary even when the maximal tolerated doses for the individual agents are well known.

The underlying mechanism of the interaction between antitumor agents and EDSM is unknown and might be different for each drug combination. Our results indicate that interactions between EDSM and antitumor drugs are phenomenona which must be dealt with separately for each combination. *In vivo* pharmacokinetic aspects also have to be considered in view of both enhancing the therapeutic ratio and of detecting any potential deleterious combinations. There are a large number of non-chemotherapeutic agents that have potentiated the efficacy of chemotherapy in pre-clinical studies — too few clinical trials have been done to conclusively prove or disprove the value of any of these approaches in humans.[41,42]

Clearly, the application of data from model systems is limited, but it is a step toward less empiric use of chemotherapy modifiers.

References

1. Contreras A, Carrasco L. Selective inhibition of protein synthesis in virus-infected mammalian cells. *J Virol* 1979; **29**: 114–22.
2. Bitonti AJ, Kelly SE, Flynn GA, *et al.* Inhibition of *Trypanosoma brucei* peptidyl transferase activity by sparsomycin analogueues and effects on trypanosome protein synthesis and proliferation. *Biochem Pharmacol* 1985; **34**: 3055–60.

3. Goldberg IH, Mitsugi K. Inhibition by sparsomycin and other antibiotics of the puromycin-induced release of polypeptide from ribosomes. *Biochemistry* 1967; **6**: 383–91.
4. Tada K, Trakatellis AC. Mechanism of action of sparsomycin on protein synthesis. *Antimicrob Agents Chemother* 1970; **3**: 227–30.
5. Zylicz Z, Wagener DJTh, Rennes H van, *et al. In vivo* potentiation of *cis*-diamminedichloroplatinum (II) antitumour activity by pretreatment with sparsomycin. *Cancer Lett* 1986; **32**: 53–9.
6. Zylicz Z, Hofs HP, Wagener DJTh. Potentiation of cisplatin antitumour activity on L1210 leukemia s.c. by Sparsomycin and three of its analogues. *Cancer Lett* 1989; **46**: 153–57.
7. Hofs HP, Wagener DJTh, Valk-Bakker V de, *et al.* Potentiation of cisplatin antitumour activity by Ethyldeshydroxy-sparsomycin in L1210 leukemia. *Anticancer Res* 1992; **12**: 167–70.
8. Hofs HP, Wagener DJTh, Valk-Bakker V de, *et al.* Correlation of the *in vitro* cytotoxicity of ethyldeshydroxy-sparsomycin and cisplatin with the *in vivo* antitumour activity in murine L1210 leukemia and two resistant L1210 subclones. *Cancer Chemother Pharmacol* 1993; **31**: 289–94.
9. Ozawa S, Yasuda T, Inaba M. Comparison of cellular basis of drug sensitivity of human colon, pancreatic, and renal carcinoma cell lines with that of leukemia cell lines. *Cancer Chemother Pharmacol* 1988; **22**: 41–6.
10. Daoud SS, Forde NH. Synergistic cytotoxic actions of cisplatin and liposomal valinomycin on human ovarian carcinoma cells. *Cancer Chemother Pharmacol* 1991; **28**: 370–6.
11. Jayaram HN, Murayama K, Pillwein K, *et al.* Schedule-dependent synergistic action of tiazofurin and dipyridamole on hepatoma 3924A cells. *Cancer Chemother Pharmacol* 1992; **31**: 93–6.
12. Chou TC. Derivation and properties of Michaelis–Menten type and Hill type equations for reference ligands. *J Theor Biol* 1976; **39**: 253–76.
13. Broek LAGM van den, Lazaro E, Zylicz Z, *et al.* Lipophilic analogues of sparsomycin as strong inhibitors of protein synthesis and tumour growth: a structure–activity relationship study. *J Med Chem* 1989; **32**: 2002–15.
14. Yang LY, Drewinko B. Cytotoxic efficacy of reconstituted and stored antitumour agents. *Cancer Res* 1985; **45**: 1511–5.
15. Bosanquet AG. Stability of solutions of antineoplastic agents during preparation and storage for *in vitro* assays. II. Assay methods, adriamycin and the other antitumour antibiotics. *Cancer Chemother Phamacol* 1986; **17**: 1–10.
16. Bosanquet AG. Stability of solutions of antineoplastic agents during preparation and storage for *in vitro* assays. III. Antimetabolites, tubulin-binding agents, platinum drugs, amsacrine, L-asparaginase, interferons, steroids and other miscellaneous antitumour agents. *Cancer Chemother Pharmacol* 1989; **23**: 197–207.
17. Mader RM, Steger GG, Moser K, *et al.* Instability of the anticancer agent etoposide under *in vitro* culture conditions. *Cancer Chemother Pharmacol* 1991; **27**: 354–60.
18. Mosmann T. Rapid calorimetric assay for cellular growth and survival: application of proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–63.
19. Carmichael J, DeGraff WG, Gazdar AF, *et al.* Evaluation of Tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987; **47**: 936–42.
20. Sobottka SB, Berger MR. Assessment of antineoplastic agents by MTT assay: partial underestimation of antiproliferative properties. *Cancer Chemother Pharmacol* 1992; **30**: 385–93.
21. Chou TC, Talalay P. Quantitative analysis of dose–effect relationships; the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Reg* 1984; **22**: 27–55.
22. Chou J. Quantitation of synergism and antagonism of two or more drugs by computerized analysis. In: Chou T-C, Rideout DC, eds. *Synergism and antagonism in chemotherapy.* Academic Press: New York 1991: 223–44.
23. Chou J, Chou T-C. *Dose–effect analysis with microcomputers: quantitation of ED50, LD50, synergism, antagonism, low-dose risk, receptor ligand binding and enzyme kinetics (manual and software).* Cambridge: Biosoft 1987.
24. Campling BG, Pym J, Baker HM, *et al.* Chemosensitivity testing of small cell lung cancer using the MTT assay. *Br J Cancer* 1991; **63**: 75–83.
25. Alley MC, Scudiero DC, Monks A, *et al.* Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. *Cancer Res* 1988; **48**: 589–601.
26. Wilson JK, Sargent MJ, Elgie AW, *et al.* A feasibility study of the MTT assay for chemosensitivity testing in ovarian malignancy. *Br J Cancer* 1990; **62**: 189–94.
27. Basu A, Lazo JS. Sensitization of human cervical carcinoma cells to *cis*-diamminedichloroplatinum(II) by bryostatin 1. *Cancer Res* 1992; **52**: 3119–24.
28. Fischel JL, Formento P, Berlion M, *et al.* Sequence-dependent cytotoxic effects of the combination of a new nitrosourea, foemustine, with 5-fluorouracil plus folinic acid. *Cancer Chemother Pharmacol* 1991; **28**: 448–54.
29. Barranco SC, Townsend Jr CM, Ho BY, *et al.* Schedule dependent potentiation of antitumour drug effects by α-difluoromethylornithine in human gastric carcinoma cells *in vitro. Invest New Drugs* 1990; **8**: S9–S18.
30. Ludwig R, Alberts DS, Miller TP, *et al.* Evaluation of anticancer drug schedule dependency using an *in vitro* human tumour clonogenic assay. *Cancer Chemother Pharmacol* 1984; **12**: 135–41.
31. Durand RE, Vanderbyl SL. Schedule dependence for cisplatin and etoposide multifraction treatments of spheroids. *JNCI* 1990; **82**: 1841–5.
32. Harpur ES, Langdon SP, Fathalla SAK, *et al.* The antitumour effect and toxicity of *cis*-platinum and *N*-methylformamide in combination. *Cancer Chemother Pharmacol* 1986; **16**: 139–47.
33. Hofmann J, Doppler W, Jakob A, *et al.* Enhancement of the antiproliferative effect of *cis*-diamminedichloroplatinum(II) and nitrogen mustard by inhibitors of protein kinase C. *Int J Cancer* 1988; **42**: 382–8.
34. Jekunen A, Vick J, Sanga R, *et al.* Synergism between dipyridamole and cisplatin in human ovarian carcinoma cells *in vitro. Cancer Res* 1992; **52**: 3566–71.
35. Scambia G, Ranelletti FO, Panici PB, *et al.* Synergistic antiproliferative activity of quercetin and cisplatin on ovarian cancer cell growth. *Anti-Cancer Drugs* 1990; **1**: 45–8.
36. Miyamoto KI, Nakamura S, Ikeda K, *et al.* Inhibition of the G_1/S transition in A65 cells by H-7, a protein kinase C inhibitor. *Biochem Pharmacol* 1993; **45**: 772–5.
37. Fraval HNA, Roberts JJ. G_1-phase chinese hamster V79-379A cells are inherently more sensitive to platinum bound to their DNA than mid S phase or asynchronously treated cells. *Biochem Pharmacol* 1979; **28**: 1575–80.
38. Philips RM, Bibby MC, Double JA. A critical appraisal of

the predictive value of *in vitro* chemosensitivity assays. *J Natl Cancer Inst* 1990; **82**: 1457–68.

39. Gazdar AF, Steinberg SM, Russell EK, *et al.* Correlation of *in vitro* drug-sensitivity testing results with response to chemotherapy and survival in extensive-stage small cell lung cancer: a prospective clinical trial. *J Natl Cancer Inst* 1990; **82**: 117–24.
40. Gerson SL, Zborowska E, Norton K, *et al.* Synergistic efficacy of O^6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in a human colon cancer xenograft completely resistant to BCNU alone. *Biochem Pharmacol* 1993; **45**: 483–91.
41. Stewart DJ, Evans WK. Non-chemotherapeutic agents that potentiate chemotherapy efficacy. *Cancer Treat Rep* 1989; **16**: 1–40.
42. Hill BT. Modulation of antitumour drug resistance: experimental laboratory data and results of clinical evaluation. *Cancer Treat Rev* 1990; **17**: 197–202.

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