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Different interaction of cisplatin and etoposide on *in vivo* and *in vitro* tumor systems

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The effect of cisplatin (CDDP) in combination with etoposide (VP16) was examined on four human cell lines (one colon adenocarcinoma, LoVo; one ovarian carcinoma, IGROV-1; two small cell lung cancers, NCI-H146 and NCI-N592; and two murine leukemias, P388 and L1210). Simultaneous exposure to CDDP and subtoxic concentrations of VP16 for 1 h produced a cell killing in all cell lines comparable to that achieved by CDDP alone. Sequential exposure of NCI-H146 and NCI-N592 to CDDP for 1 h followed by VP16 for 96 h again produced an additive effect. When two of these cell lines were treated in *in vivo* models (i.p. P388 leukemia, s.c. NCI-N592) with suboptimal doses of the two drugs, a potentiation of the antitumor effects of the two drugs in simultaneous combination was evidenced by the increase in survival time and in the number of 'cures' in P388 leukemia bearing mice and by the inhibition of tumor size in NCI-N592. This comparative study, using the same cell lines *in vitro* and *in vivo*, indicates that the CDDP–VP16 potentiation observed *in vivo* does not reflect a specific interaction at the cellular-biochemical level. The results support the therapeutic interest of this combination (presumably as a result from favorable pharmacological interactions) even despite the lack of potentiation at cellular level, under comparable conditions of treatment.

Key words: In vitro and *in vivo* interaction, cisplatin, etoposide.

Introduction

The combination of cisplatin (CDDP) and etoposide (VP16) has been shown to be synergistic *in vivo* against murine P388 leukemia and B16 melanoma.[1,2] Furthermore, this combination has proven effective as second line therapy for small cell lung cancer (SCLC) in patients unresponsive to primary therapy with cyclophosphamide, adriamycin and vincristine.[3,4] Whereas in *in vitro* systems synergism was reported in three-dimensional growing murine cells,[5] only additive cytotoxicity was observed in monolayer human cell cultures.[6,7]

In an attempt to better clarify the cellular basis of the interaction between the two drugs, the aim of this study was to examine: (a) the effects of the CDDP–VP16 combination on a variety of human and murine *in vitro* cell lines representative of different tumor histotypes, including two SCLCs in which the efficacy of the combination is known; and (b) the dependence of the cytotoxic effects on the schedule of cell exposure to the two drugs. Moreover the effects of the combination on selected cell lines growing *in vitro* were compared to those of the same cell lines growing *in vivo*.

Materials and methods

In vitro studies

Cell lines. P388 and L1210 murine leukemia cells (obtained from NCI, Bethesda, MD) were cultured in RPMI-1640 medium containing 15% heat-inactivated fetal calf serum (FCS) and 1% 2-mercaptoethanol. NCI-H146 and NCI-N592, two SCLCs, were obtained by Dr Minna (NCI, Bethesda, MD) and were maintained in RPMI-1640 supplemented with 10% heat-inactivated FCS. LoVo, an adenocarcinoma of the colon, obtained from American Tissue Culture Collection (Rockville, MD), was maintained in Ham's F12 medium supplemented with 15% FCS, vitamins and glutamine. IGROV-1 cells, kindly supplied by Dr Benard (Institut Gustave Roussy, Villejuif, France), were cultured in RPMI-1640 supplemented with 10% FCS. All cell lines were free from mycoplasma and were kept in 5% CO_2 solution in air at 37°C. Trypsin-EDTA was used to prepare single cell suspensions.

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Drugs. CDDP (Platinex) and VP16 (Vepesid) were obtained from Bristol Italiana (Sud) SpA. The drugs were diluted with tissue culture medium immediately before use.

Cytotoxic assay. NCI-H146 and NCI-N592 cell survival was assessed by tetrazolium dye (MTT) assay.[8] Cells were harvested from exponential-phase maintenance cultures, dispensed into tubes and treated for 1 h with the drugs. The cells were rinsed and dispensed into 96-well culture plates (Costar Plastics 3799) in 100 μl volumes using a repeating pipette (Multipette 4780, Eppendorf). In the experiments designed to examine the effect of continuous exposure to VP16 (i.e. 96 h), the drug was included in the medium after initial treatment for 1 h. Each plate had 16 control wells and 16 wells for each dose. After incubation of the microtiter plates for 96 h, 10 μl of MTT working solution was added to each culture well (resulting in 50 μl MTT/250 μl total medium volume) and cultures were incubated at 37°C for 4 h. The culture plates were then centrifuged, and the culture medium removed from the wells and replaced with 150 μg of DMSO, using a multichannel pipette. The absorbance of each well was measured using a microculture plate reader (SLT Labinstruments, Austria) at 540 nm interfaced with an Apple computer. Preliminary experiments were performed to determine the appropriate seeding number of each cell line, after confirming the linear relationship between the absorbance and number of cells in the growth curve of each cell line: 10^3 cells/well for both SCLC cell lines.

In all other cell lines cell survival was evaluated by growth inhibition test. Briefly, cells in the logarithmic phase of growth were placed in appropriate seeding number into 6-well plates (9.6 cm^2 Costar). P388 and L1210 cells were drug treated for 1 h before seeding into the well plates (six wells for each dose); IGROV-1 and LoVo monolayer cells were treated 24 h after seeding. The cells were harvested and counted by Coulter Counter 72 h following exposure to drugs, and cell numbers were expressed as a percentage of untreated control.

IC_{50} is the concentration of drug that produces 50% reduction of absorbance or cell number.

Analysis of drug interactions. According to the method of Kern *et al.*,[9] an expected value of cell survival (S_{exp}) defined as the product of the survival observed for CDDP alone and the survival observed for VP16 alone, and the actual survival observed (S_{obs}) for the combination of CDDP and VP16, were used to construct a synergistic ratio (R) as

$$R = S_{exp}/S_{obs}$$

Synergy was defined as any value of R greater than unity. An R value of 1.0 (additive effect) or less indicated an absence of synergy.

In vivo studies

Animals and tumors. The mice used in the study were purchased from Charles River Laboratories (Calco, Italy) and maintained in standard conditions. P388 murine leukemia cells were maintained i.p. in DBA/2 syngeneic mice. For chemotherapy studies BDF-1 hybrid mice were injected i.p. with 10^6 cells/mouse.

NCI-N592 human cells were injected s.c. in both flanks of 6–10-week old athymic nude Swiss mice, for line maintenance and for chemotherapy studies.

Chemotherapy. The drugs were dissolved in saline and administered in a volume of 10 ml/kg body weight. When drugs were combined, they were delivered simultaneously. Drugs were delivered i.p. to P388 bearing mice on days 5, 9 and 13 after tumor cells inoculum according to the schedule used by Schabel.[2] The antitumor effect was evaluated as the percentage increase of median survival time (MST) in treated *vs* control mice ($T/C\%$). Long-term survivors (LTS) were mice surviving 70 days after tumor cells inoculum.

For NCI-N592 bearing mice, drug treatments started when mean tumor weight was ≥ 250 mg. Treatments were delivered i.v. once a week for two weeks based on our previous experience in the treatment of this tumor model. Tumor weight (TW) was calculated biweekly by measurement of diameters with a vernier caliper, according to the formula $d^2 \times D/2$, where d and D are the shortest and the longest diameter, respectively.[10] One week after the last treatment, the percentage inhibition of mean TW ($TWI\%$) in treated (t) *vs* control (c) mice was calculated as $100 - (TW_t/TW_c \times 100)$.

Results

In vitro studies

The single-agent IC_{50} values of CDDP and VP16 for each cell line are reported in Table 1. The cytotoxicity caused by each compound varied greatly in the different cell lines, cell line NCI-N592 being the most resistant to both drugs.

In all the drug combination experiments, increasing doses of one drug were combined with a fixed dose of the other. The fixed dose used was chosen for being sub-toxic ($\leq IC_{20}$) in our assay system. For the purpose of comparison, the effects of single

Table 1. IC_{50} values (μg/ml) of CDDP and VP16 after 1 h exposure to single agent

Cell line	IC_{50} (μg/ml ± SD)[a]	
	CDDP	VP16
P388	4.5 ± 0.3	0.6 ± 0.06
L1210	2.8 ± 0.8	2.3 ± 0.5
IGROV-1	3 ± 0.4	6.6 ± 0.15
LoVo	11 ± 2	5.4 ± 0.6
NCI-H146	10 ± 3	6.6 ± 0.8
NCI-N592	34 ± 6	48 ± 5.5

[a] Mean values (±SD) from at least three experiments.

drug and combination were evaluated in separate experiments. Therefore, the results refer to representative experiments for each line.

Treatment with increasing concentrations of CDDP in combination with a fixed dose of VP16 caused a cell killing similar to that observed with CDDP alone in all six cell lines (Figure 1), indicating that the cytotoxicity of the combination was never supra-additive. The same pattern was observed treating the cells with increasing concentrations of VP16 and a fixed dose of CDDP (data not shown). The *R* values calculated according to Kern *et al.* did not exceed 1.12 and values as low as 0.86 were found (Table 2). When 1 h of incubation with CDDP was followed by continuous incubation (96 h) with VP16, again the survival curves (Figure 2) and the *R* values (Table 3) indicated an absence of synergy in both the cell lines tested.

In vivo studies

In advanced P388 leukemia bearing mice (Table 4), at the maximal tolerated dose (MTD) of 4 mg/kg, CDDP was inactive (T/C = 108%), whereas VP16 showed a dose-dependent activity (T/C = 200% and 250% at dose levels of 16 and 24 mg/kg, respectively). When the higher VP16 dose, i.e. 24 mg/kg, was combined with the MTD of CDDP or fractions of it, i.e. 2.6 or 1.3 mg/kg, an increase in mice survival time and in *LTS* was achieved. Even when lower doses of VP16 were combined with the MTD of CDDP, mice survived longer and some

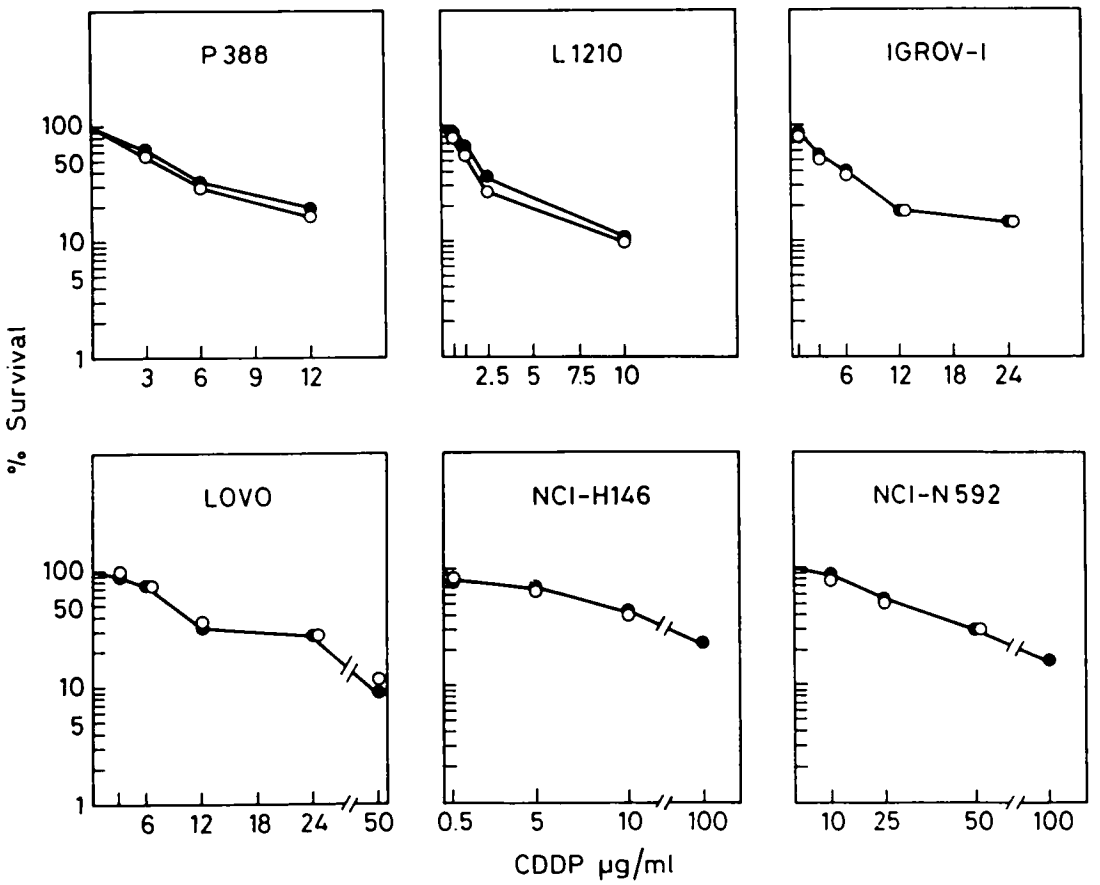

Figure 1. Dose–survival curves for 1 h exposure to CDDP alone or with simultaneous incubation with a fixed dose of VP16. CDDP alone (●—●); VP16 in combination with various concentrations of CDDP (○—○). SDs averaged 10%.

Table 2. Cytotoxicity of CDDP and VP16 as single agents and synergistic ratios of the agents in combination. Simultaneous treatment for 1 h

Cell line	Concentration (μ/ml)		% Survival[a]				Exp./obs. ratio
	CDDP	VP16	CDDP alone	VP16 alone	CDDP + VP16		
					expected	observed	
P388	12	0.15	20	89	18	17	1.05
	6	0.15	34	89	30	30	1
	3	0.15	63	89	56	57	0.98
L1210	10	0.75	11	89	9	10	0.9
	2.5	0.75	37	89	33	27	0.92
	1.25	0.75	69	89	61	54	1.12
	0.625	0.75	88	89	78	83	0.93
IGROV-1	12	0.3	18	95	17	18	0.94
	6	0.3	40	95	38	38	1
	3	0.3	54	95	51	53	0.96
	0.75	0.3	85	95	81	83	0.97
LoVo	24	0.6	28	95	27	29	0.93
	12	0.6	32	95	30	38	0.79
	6	0.6	74	95	70	72	0.97
	3	0.6	90	95	85	99	0.86
NCI-H146	10	0.25	47	90	42	42	1
	2.5	0.25	70	90	63	67	0.94
	0.5	0.25	78	90	70	80	0.9
NCI-N592	50	6	30	94	28	30	0.93
	25	6	56	94	53	51	1.03
	10	6	92	94	86	80	0.93

[a] SDs averaged 10%.

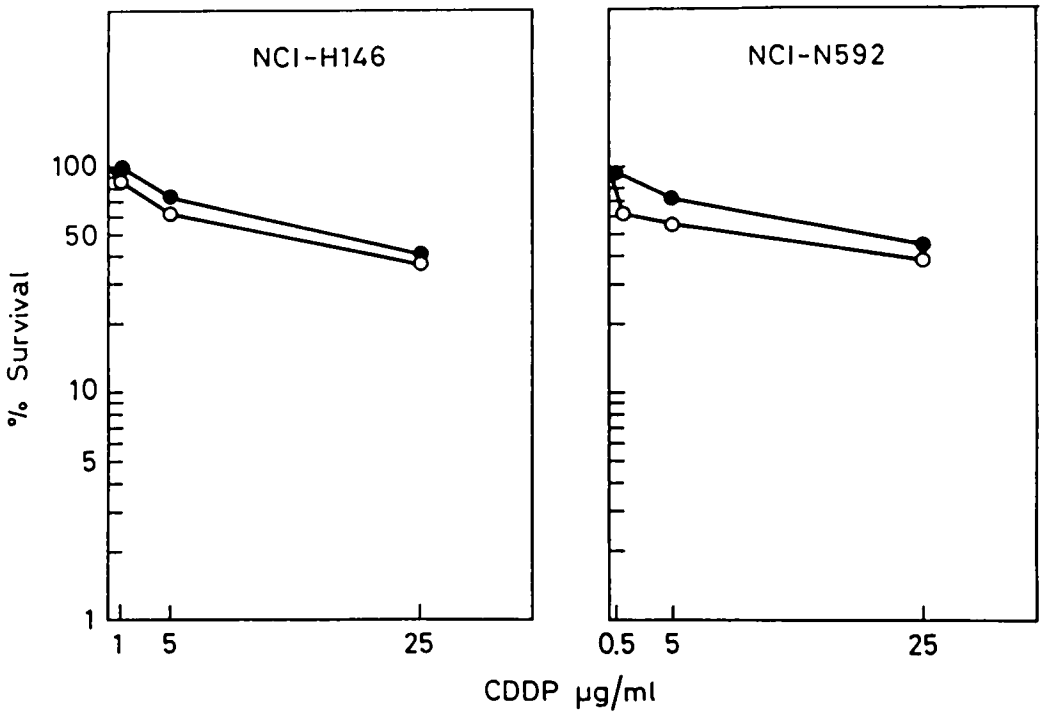

Figure 2. Dose–survival curves for 1 h exposure to CDDP alone or with subsequent incubation (96 h) with a fixed dose of VP16. CDDP alone (●—●); VP16 in combination with various concentrations of CDDP (○—○). SDs averaged 10%.

Table 3. Cytotoxicity of CDDP and VP16 as single agents and synergistic ratios of the agents in combination. CDDP treatment for 1 h and VP16 for 96 h

Cell line	Concentration (μ/ml)		% Survival[a]				Exp./obs. ratio
	CDDP	VP16	CDDP alone	VP16 alone	CDDP + VP16		
					expected	observed	
NCI-H146	25	0.006	41	92	38	38	1
	5	0.006	73	92	67	61	1.09
	1	0.006	101	92	92	87	1.05
NCI-N592	25	0.06	45	81	36	39	1.08
	5	0.06	71	81	57	55	1.03
	0.5	0.06	93	81	75	60	1.2

[a] SDs averaged 10%.

were 'cured'. Therefore, in the treatment of this murine tumor, the effect of the combination could be regarded as a 'therapeutic synergism'; i.e. the combination of CDDP and VP16 yielded an increase in survival time and a number of *LTS* (cures) greater than those observed with the single drug treatment at optimal doses. Moreover, even with the use of suboptimal doses (i.e. doses producing a marginal effect), a potentiation of VP16 (8 and 16 mg/kg) by CDDP (4 mg/kg) could be documented.

Unlike P388 leukemia, the NCI-N592 tumor was more sensitive to CDDP than to VP16 (Table 4). When two only slightly effective doses of VP16 (24 and 36 mg/kg) were combined with an inactive dose of CDDP (2.6 mg/kg), a significant tumor weight inhibition (>50% compared to control mice) was achieved, thus indicating a potentiation of CDDP effects by VP16. However, in the treatment of this human tumor, no therapeutic synergism (i.e. therapeutic advantage of the combination over single drug treatment) was observed, since the optimal dose of CDDP alone (6 mg/kg) was as effective as the combination (*TWI* = 74% *vs* 70% and 73%).

Table 4. Activity of CDDP and VP16, alone and in combination, in *in vivo* systems

Tumor	Treatment route and schedule	Drug	Dose (mg/kg)	*T/C*[a] (%)	*LTS*[b]	*TWI*[c] (%)
P388[d]	i.p. q4d × 3	CDDP	4	108	0/10	
		VP16	16	200	0/10	
			24	250	1/10	
		VP16 + CDDP	8 + 4	296	0/10	
			16 + 4	283	3/10	
			24 + 1.3	383	3/10	
			24 + 2.6	250	6/10	
			24 + 4	252[f]	3/10	
NCI-N592[e]	i.v. q7d × 2	CDDP	2.6			17
			4			53
			6			74
		VP16	24			44
			36			41
		VP16 + CDDP	24 + 2.6			70
			36 + 2.6			73

[a] *MST* of treated/*MST* of control mice × 100, calculated on dead mice only.
[b] Long-term survivors (mice surviving at 70 days).
[c] 100 (mean *TW* of treated/mean *TW* of control mice × 100), 7 days after the last drug treatment.
[d] 10^6 cells/mouse were delivered i.p. to BDF-1 mice. Drug treatments started on day 5. *MST* in control mice was 12 days.
[e] Tumor fragments were delivered s.c. by trocar in both flanks of Swiss athymic mice. Drug treatment started when *TW* was around 250–300 mg. Mean *TW* in control mice was 2.7 ± 1.3 (± SD) at the evaluation time.
[f] One mouse of this group died with reduced spleen and liver (i.e. for toxicity).

The efficacy of increased CDDP doses in the combination remains to be determined.

Discussion

The clinical efficacy of the combination CDDP–VP16 has been widely proven,[3,4] particularly in the treatment of SCLC, and a therapeutic synergism in *in vivo* experimental models has been reported already.[1,2] In our study, this combination was tested *in vitro* on cell lines of different origin, including human SCLC. In all cell lines concomitant exposure to the two drugs produced only an additive cell killing, thus suggesting that the nature of the CDDP–VP16 interaction is not tumor specific. Since in combined treatments the administration schedule could be a critical factor, and taking into account that VP16 is more effective when administered as repeated doses rather than as a single dose,[11] a CDDP treatment of 1 h plus continuous exposure to VP16 was also used to evaluate the sensitivity of SCLC cells. Even using this schedule the cytotoxicity of the drugs was not enhanced. The lack of *in vitro* synergistic interaction of this combination against human lung carcinoma cell lines has been reported by others.[6,7] Taken together, these observations do not support a cellular or biochemical basis for the therapeutic synergism achieved *in vivo* with the use of the CDDP–VP16 combination, at least with the schedules investigated.

In contrast to the cellular effects, a different interaction of the CDDP–VP16 combination was observed in the treatment of the same tumor lines growing *in vivo*. Indeed, combining suboptimal dose levels of each drug, a marked increase of survival and an appreciable number of 'cures' were achieved in P388 leukemia bearing mice compared to the effects of the single drug treatment. Moreover, a significant reduction of tumor size compared to control mice ($TWI > 50\%$) in NCI-N592 bearing mice was achieved combining low effective doses of the two drugs. Again, these results emphasize that the therapeutic advantage of the CDDP–VP16 combination does not reflect a specific interaction at the cellular-biochemical level, but may arise from favorable pharmacological interactions. Relevant to this point is the observed synergistic effect of this combination on the multicellular tumor spheroid system, an *in vitro* model in which tumor cells are arranged in three-dimensional structures and display a tumor-like organization.[5]

In conclusion, favorable pharmacological interactions of the two drugs at the tumor site must be considered in the clinical efficacy of the CDDP–VP16 combination, since an appreciable interaction could not be documented at the cellular level. Tumor heterogeneity might also play a role in the therapeutic interest of this combination, despite the lack of potentiation at cellular level, particularly in light of the reported collateral sensitivity to CDDP of VP16-resistant cells.[12,13]

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