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Etoposide prior to *cis*-Diamminedichloroplatinum in Combination Chemotherapy: *in Vitro* and *in Vivo* Studies*

GABRIELLA ZUPI,†‡ CLAUDIA GRECO,† ADA SACCHI§ and FEDERICO CALABRESI‖

†*Laboratorio Servizi Tecnici Complementari and* §*Laboratorio Biofisica, Istituto Regina Elena per lo Studio e la Cura dei Tumori, Roma, Italy and* ‖*Centro Oncologico "G. Porfiri", Ospedale di Latina, Latina, Italy*

Abstract—*Antitumor effect and toxicity of etoposide (VP 16) in combination with* cis-*diamminedichloroplatinum (DDP) were studied on the* in vitro *C108 line and its* in vivo *counterpart, the M1087 line, both deriving from Lewis lung carcinoma. A colony-forming assay was used to assess the* in vitro *cytoxicity of each drug and, on the basis of survival curve shape indications, different drug sequences were analyzed in order to find the optimal combination. Tumor growth inhibition, growth delay, metastasis reduction and percentage increase in lifespan were considered as parameters of therapeutic effect. From the present work it can be derived that the VP 16–DDP combination produces a poor antitumour effect against the Lewis lung carcinoma lines, otherwise associated with a highly acute toxicity. The sequence in which VP 16 is given before DDP induces a significant antimetastatic effect together with acceptable toxicity.*

INTRODUCTION

DURING the last few years, increasing interest has been devoted to the combination of etoposide (VP 16) and *cis*-platinum (DDP) for lung cancer therapy [1–4]. However, the antitumor activity elicited by this combination is often limited by heavy toxic effects [1, 5, 6].

That is why we have investigated the antitumor effectiveness of the VP 16–DDP combination on an experimental model system, using different regimens of treatment, in order to determine the scheduling influence on the therapeutic index.

The tumor line used throughout this study has been selected in our laboratory from lung metastases of Lewis lung carcinoma (3LL). This line, which shows a higher metastatic potential than the parent line, can grow both as an *in vitro* culture (C108 line) and as a solid tumor (M1087 line), thus allowing the drug effect to be assessed from different points of view. In addition, the relative chemoresistance elicited by the *in vivo* line makes it suitable as a model for mimicking human lung tumors [7, 8].

In the present report the effectiveness of various VP16–DDP combinations has been assessed on the same *in vitro–in vivo* model system either in terms of cytotoxicity or in terms of therapeutic activity.

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‡To whom correspondence and requests for reprints should be addressed.

MATERIALS AND METHODS

Tumor lines

Monolayer culture of C108 line are grown in Waymouth's medium supplemented with 15% calf serum. Cells are harvested by trypsinization (0.25% at 37°C for 5 min). The plating efficiency ranges between 45 and 60%. The C108 line when injected into syngeneic animals gives rise to an *in vivo* line (M1087) characterized by both a high artificial and a spontaneous ability to colonize the lungs [9]. This line is maintained in C57BL/6 mice by biweekly transplantation of 2.5×10^5 viable cells in the muscle of the hind leg. The isolation and selection procedure, as well as the main biological properties of these lines, have been published elsewhere [10, 11].

Drugs

Etoposide (VP 16–213, Vepesid, Bristol-Myers, U.S.A.) and *cis*-diamminedichloroplatinum (DDP, Platinex, Bristol-Myers, U.S.A.) ampoules supplied for clinical use were used.

The VP 16 solution (etoposide 100 mg in 5 ml of absolute ethanol, benzyl alcohol, polyethylene glycol 300, citric acid and Tween 80) and the DDP solution (DDP powder dissolved in saline) were further diluted with 5% dextrose in water and with Earle's saline to the final concentrations for *in vivo* or *in vitro* studies, respectively. Each drug was prepared just before an experiment took place. VP

16 solvent, at its final concentration, did not affect the cell viability in the *in vitro* experiments, and no toxicity was observed in the animals.

Cell survival studies

All *in vitro* experiments were performed on exponentially growing cells. Freshly prepared drug solutions were added to the dishes; in combination experiments, drugs were diluted conjointly with growth medium to reach the right concentration. In all experiments drug treatments were carried out for 1 hr at 37°C. After incubation the medium was discarded, cells were washed, harvested as a single-cell suspension and counted. Known aliquots of the cell suspension were dispersed into 60-mm Petri dishes so that colonies would appear after 8–9 days of incubation. In each experiment the plating efficiency of at least six control cultures was assessed simultaneously; the survival fractions of the various drug concentrations were normalized with respect to the individual control for each experiment.

All experiments were repeated at least three times with triplicate samples for each drug concentration.

Statistical analysis

The statistical analysis was performed according to Drewinko *et al.* [12]. For each drug and each selected concentration value, data relating to the surviving fraction as observed under single and combined drug administrations were recorded and compared to each other.

A performance index (P.I.) was then introduced, as the best parameter to evaluate deviation from additivity. This was compared to each time to unity by means of the Student test. The significance of the observed deviations was checked within a 5% confidence interval, thus assuming that one or two kinds of error occurred at a sufficiently low probability level. As a result of this procedure, for each set of data, a condition of additivity (P.I. not significantly different from 1), synergism (P.I. > 1) or antagonism (P.I. < 1) was statistically assumed.

In vivo studies

Acute toxicity of VP 16 and DDP was assessed in tumor-free C57BL/6 mice. For each dose, ten animals were injected and scored for at least 60 days. The LD_{50} was calculated according to the Spearman and Karber method [13]. The LD_{10}, amounting to 40 mg/kg for VP 16 and 8 mg/kg for DDP, was used for both drugs. Drugs were administered i.p. at a late stage of tumor growth, when the neoplastic mass had reached a weight of 800 ± 20 mg and pulmonary metastases had already occurred [9].

The antitumor activity was assessed according to the following end points: (i) tumor weight inhibition (TWI = T/C − 1 × 100). The average tumor weight of both treated and control groups were obtained by caliper measurements using the formula $w = ab^2/2$; (ii) tumor growth delay (T − C). The time taken to reach 1.000 mg was used; (iii) metastasis reduction, by counting the lung nodules fixed in a Bouin's solution with the aid of a dissecting microscope; and (iv) median survival time.

Early deaths (i.e. deaths occurring before the first death of control group) were considered as a parameter of acute toxicity and were disregarded in the median survival time calculation [14].

RESULTS

In vitro studies

The dose–response curves in which cell survival of C108 line is drawn as a function of the increasing concentrations of VP 16 or DDP are shown in Fig. 1. The survival curve trend of C108 after 1 hr of exposure to DDP is of the sigmoid type, with a D_0 value of 1.72 μg/ml and an initial shoulder region characterized by an extrapolation number (n) of 4.39. On the contrary, the survival curve trend of C108 line to VP 16 is of the biphasic exponential type, characterized by an initial concavity with a D_{01} value of 0.954 μg/ml and an exponential region with a D_{02} value of 1.93 μg/ml. In both slopes the extrapolation number is negative ($n_1 = 0.9$; $n_2 = 0.25$).

Figure 2 shows the effect of simultaneous exposure of cells to both drugs at concentrations ranging from 0.2 to 1.5 μg/ml for VP 16 and from 1.0 to 5.0 μg/ml for DDP. No increase in lethal activity is gained by the two-drug combination with respect to the single-drug administration; on the contrary, when C108 line is exposed to 1 μg/ml DDP plus 0.2 μg/ml VP 16 an increased percentage of cell survival fraction is observed, as compared to the cell survival of cells treated with 0.2 μg/ml VP 16 alone. The statistical analysis performed to compare the expected with the observed response demonstrates an antagonistic effect as regards the concentrations used ($P < 0.05$). On the contrary, a potentiation effect was obtained when the two drugs were administered in sequence.

Table 1 summarizes the values of cell survival fraction where DDP is given before or after VP 16 within a 0, 30 or 60-min interval. In the VP 16→DDP sequence the observed cell survival fraction significantly differs from the expected one, thus indicating a strong synergistic effect. This effort is particularly evident when DDP is administered immediately after VP 16 exposure (time interval = 0). No significant difference is otherwise

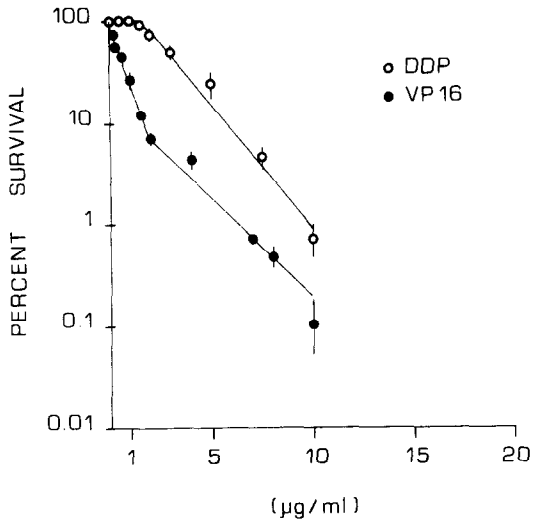

Fig. 1. Survival curves of the C108 line with increasing doses of DDP or VP 16.

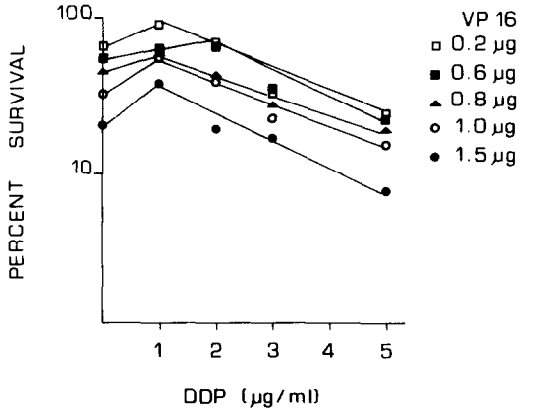

Fig. 2. Survival curves of the C108 line with increasing doses of DDP used in simultaneous combination with different doses of VP 16.

observed in the DDP→VP 16 sequence, demonstrating an additive effect.

In vivo studies

Antitumor and toxic effects of VP 16 and DDP given alone or in combination have also been assessed on the *in vivo* M1087 line, originating from the C108 line. When individually administered, both drugs did not show a relevant antitumor effect. In particular, DDP administered in a fractionated regimen is less toxic than the single dose, while repeated low doses of VP 16 (10 mg/kg dose, 4 hr × 4 on day 11) determines a highly toxic effect.

The only schedules giving rise to acceptable toxicity (lower than 10%) were: 40 mg/kg VP 16 in a single dose on day 10 after tumor implantation and 8 mg/kg DDP in repeated low doses on day 11 after tumor implantation (Table 2, schedules a and

Table 1. Lethal effect of different treatment schedules with VP 16 and DDP

First drug	Dose (μg/ml)	Time interval between administrations (min)	Second drug	Dose (μg/ml)	Observed survival* (% mean ± S.E.)	Expected survival	Significant deviation† from predicted response	Performance index
DDP	3.0	0	VP 16	1.0	16.2 ± 1.3	18.0		
DDP	3.0	0	VP 16	1.5	7.9 ± 1.3	11.1	N.S.	—
DDP	5.0	0	VP 16	1.0	9.5 ± 0.9	11.7		
DDP	5.0	0	VP 16	1.5	5.7 ± 2.2	7.2		
VP 16	1.0	0	DDP	3.0	4.0 ± 0.1	18.0		
VP 16	1.0	0	DDP	5.0	0.6 ± 0.1	11.7	S; $P < 0.05$	9.5
VP 16	1.5	0	DDP	3.0	1.7 ± 0.1	11.1		
DDP	3.0	30	VP 16	1.0	19.0 ± 1.1	18.0		
DDP	3.0	30	VP 16	1.5	20.5 ± 0.4	11.1		
DDP	5.0	30	VP 16	1.0	18.5 ± 2.5	11.7	N.S.	—
DDP	5.0	30	VP 16	1.5	8.1 ± 0.5	7.2		
VP 16	1.0	30	DDP	3.0	10.9 ± 1.0	18.0		
VP 16	1.0	30	DDP	5.0	3.3 ± 0.1	11.7	S; $P < 0.05$	2.7
VP 16	1.5	30	DDP	3.0	4.3 ± 0.2	11.1		
DDP	3.0	60	VP 16	1.0	17.8 ± 0.7	18.0		
DDP	3.0	60	VP 16	1.5	7.9 ± 0.3	11.1		
DDP	5.0	60	VP 16	1.0	15.1 ± 1.5	11.7	N.S.	—
DDP	5.0	60	VP 16	1.5	6.6 ± 0.3	7.2		
VP 16	1.0	60	DDP	3.0	10.7 ± 0.5	18.0		
VP 16	1.0	60	DDP	5.0	2.7 ± 0.2	11.7	S; $P < 0.05$	2.6
VP 16	1.5	60	DDP	3.0	3.1 ± 0.2	11.1		

*Observed survival for single dose was: DDP 3.0 μg/ml = 56.0 ± 2.0; DDP 5.0 μg/ml = 36.5 ± 4.5; VP 16 1.0 μg/ml = 32.1 ± 3.2; VP 16 1.5 μg/ml = 19.9 ± 1.9.

†N.S. = not significant; S = synergistic.

b, respectively). These regimens of VP 16 and DDP were therefore chosen for combination therapy studies.

As shown in Table 2, the sequence in which DDP is given before VP 16 proves to be totally ineffective because of the tremendous toxicity induced by this schedule. All treated animals, in effect, died significantly earlier than controls. Similarly, no therapeutic benefit followed the simultaneous administration of the drugs (schedule e) in that, in spite of a 62% tumor inhibition, a high percentage of early death occurred. On the contrary, the VP 16→DDP sequence (schedule d), although eliciting only a modest local tumor control (tumor inhibition less than 50%), determined a low toxicity together with a remarkable reduction of pulmonary metastases (94%).

DISCUSSION

The results of the present paper confirm the usefulness of an *in vitro–in vivo* model system for combination therapy studies. In effect, by using the colony-forming method to quantify the cell-killing, antagonistic or additive effects can be assessed. In addition, as described by other authors, the dose–response curve may provide helpful information about the optimal scheduling determining biologic potentiation [12]. On the other hand, toxic effects of drug combinations can easily be assessed on *in vivo* models.

The different survival curve shapes of the C108 line exposed to DDP or VP 16 makes it reasonable to combine these drugs. In fact, they represent a more complex situation than, for instance, that of two drugs characterized by a simply exponential killing trend, where only additivity could be expected [15].

Three different kinds of effect are elicited by the VP 16–DDP combination at the same doses, related to the various sequences used. The drug antagonism observed after the simultaneous exposure to VP 16 and DDP (Fig. 2) can be explained in two ways at least: it may well be due to some competition between the two drugs for the same cellular target(s) or to induction of a non-cumulative sublethal damage so that only a small fraction of viable cells could have been additionally affected. The different killing effect caused by the two sequences — VP 16 as first agent or DDP as first agent — agrees with the response analyzed in terms of the shape of the survival curves. In effect, the most effective sequence is VP 16 used as first agent (Table 1), and once again it appears to interfere with the repair mechanism.

In other words, the effectiveness of the sequence is related to the shape of the curve of the first agent: synergism if the curve is exponential, simple additivity if the curve is sigmoid. The other hypothesis is related to the possibility of a proliferation delay or block of cells in the premitotic phase by the first agent of the sequence (VP 16). However, such a hypothesis is not mutually exclusive with that related to the repair mechanism. Furthermore, it is unlikely that this hypothesis plays a major role because of the short interval time between exposure of the two drugs and because the maximum effect is observed in an interval of zero time. *In vitro* experiments, although not necessarily predictive of *in vivo* results, can be used to select treatment sequences according to cell survival kinetics.

Table 2. Antitumor activity and toxicity of the VP 16-DDP combination in the M1087 line

Treatment schedule	TWI at nadir*	TC (days)†	Median survival time (range)‡	Median No. of metastases (range)§	%MR‖	Early deaths¶
(a) VP 16 40 mg/kg on day 10	57	3.5	22.0 (14–28)	70.5 (25–104)††	50	1/12
(b) DDP 2mg/kg/dose, q 4 hr × 4 on day 11	11	1.0	28.0 (25–28)	113.0 (57–151)‡‡	19	1/15
(c) DDP 2 mg/kg/dose, q 4 hr × 4 on day 10→ VP 16 40 mg/kg on day 11	55	N.E.**	16.0 (14–16)	N.E.	N.E.	16/16
(d) VP 16 40 mg/kg on day 10→DDP 2 mg/kg/ dose, q 4 hr × 4 on day 11	34	4.0	31.0 (16–35)	8.0 (6–19)††	94	1/15
(e) DDP 2 mg/kg/dose, q 4 hr = 4 + VP 16 40 mg/kg on day 10	62	N.E.	15.0 (12–28)	N.E.	N.E.	12/13

* TWI = tumor weight inhibition.
† T – C = differences in days between untreated control mice (C) and drug-treated mice (T) to grow tumors to a predetermined size.
‡ Median survival time of controls = 27.0 (19–38).
§ Median No. of lung metastases in the control group = 140.0 (58–194).
‖ MR = metastases reduction.
¶ Deaths occurring before the first death of the controls.
** N.E. = not evaluable.
†† $P = 0.01$ vs control and ‡‡ not significantly different from the control (Mann–Whitney U test).

Data obtained on the *in vivo* M1087 line confirm interpretations made on the basis of *in vitro* results. In fact, fractionated treatments with DDP alone are less toxic than single doses, while fractionated treatments with VP 16 alone are more toxic than single doses. This finding could be explained by the short interval time between the VP 16 doses, as observed by other authors [16].

The VP 16–DDP combination has been described as synergistic in some animal tumor models [17], but as far as the antitumor effect of this combination on the *in vivo* line is concerned, the two drugs elicit a slight activity against the chemoresistant M1087 lung tumor, associated with severe toxicity. The only sequence which can be reasonably used is the VP 16 before DDP, according to the schedule d. As a matter of fact, this schedule determines an improvement in the therapeutic ratio by decreasing toxicity to acceptable levels and increasing the antimetastatic effect.

Finally, from the present work we can conclude that: (a) drug sequence is a critical factor to the optimal combination of VP 16 and DDP, as demonstrated for other antineoplastic drugs [18]. This notwithstanding, the schedule in which VP 16 is given as first agent needs further investigation on experimental models in order to achieve a better local control; and (b) the *in vitro–in vivo* tumor lines from Lewis lung carcinoma confirm their validity as predictive models for drug-schedule-dependent toxicity [9, 19].

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