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Synergistic antitumor activity of vincristine and VP-16-213

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Summary. *The potential interaction of the antitumor agents vincristine and VP-16-213 was investigated in vivo. DBA/2 mice were inoculated with 10^6 P388 murine leukemia cells, after which single IP injections of saline only, vincristine only, VP-16-213 only, or a combination of vincristine and VP-16-213 were administered. Long-term survival (> 60 days) was observed in 0/45, 1/45 (2%), 9/135 (7%), and 44/135 (33%) mice, respectively ($P < 0.001$). Treatment was most effective when VP-16-213 was administered 0–72 h after vincristine. A similar trend was observed in mice bearing P1534 murine leukemia. These data demonstrate synergistic antitumor activity between vincristine and VP-16-213 in a murine model.*

Introduction

Vincristine (VCR) is a vinca alkaloid that has been shown to have a wide spectrum of antitumor activity over the past two decades. It is also used in many combination chemotherapy programs, because of the lack of myelosuppression when it is given in conventional dosage. VCR binds avidly to microtubules and its major mechanism of action appears to be arrest of cells in metaphase [7].

VP-16-213 is a semsynthetic derivative of podophyllotoxin, which is derived from podophyllin, an extract of the roots and rhizomes of the mandrake (or May apple) plant. Unlike podophyllotoxin or the vinca alkaloids, however, it does not cause arrest of cells in metaphase. The major mechanism of action appears to be arrest of cells in the G_2 phase of the cell cycle [7]. VP-16-213 has been in clinical trial during the past decade and has shown antitumor activity in small cell cancer, testicular carcinoma, acute nonlymphoblastic leukemia, and the lymphomas [9]. The dose-limiting toxicity of this agent is myelosuppression.

The combination of VCR and VP-16-213 in cancer chemotherapy is appealing, given the differences in their mechanisms of action and toxicity and their wide spectrum of clinical activity. In a preliminary report, Chiuten et al. suggested a positive interaction of these two agents in P388 murine leukemia in vivo [3]. This potential interaction has been further explored in the current study.

Materials and methods

Experimental animals. Male DBA/2 mice approximately 6–9 weeks of age (Cumberland View Farms, Clinton, Tenn and Jackson Laboratories, Bar Harbor, Me) and weighing approximately 20 g were caged in groups of five with water and Purina mouse chow ad libitum. Alternating light-dark cycles were maintained automatically.

Drugs. VCR was the generous gift of Eli Lilly Company, Indianapolis, Ind. Stock solutions of VCR were prepared by its addition to phosphate-buffered saline (PBS) and filtration through a 0.20 μm membrane filter (Nalge Sybron Corp., Rochester, NY). VP-16-213 was kindly provided by Bristol Myers Company, Syracuse, NY. Procedures for its preparation for injection were modified from those of Hacker et al. [5]. In a typical experiment, 25.6 mg VP-16-213 was mixed with 2.0 ml dimethyl sulfoxide, after which 2.0 ml Tween 80 was slowly added during vortexing. Finally, 16.0 ml sterile saline was added during continuous vortexing and the resulting soluting was filtered through a 20 μm membrane filter. Stock solution of VP-16-213 was prepared daily.

Tumor cells. Murine leukemia cell lines (P-388 and P-1534) obtained from the E.G.G. Mason Research Institute, Worcestor, Mass were inoculated IP into DBA/2 mice. With a sterile technique, tumor cells were harvested weekly from the ascites of two donor mice. The cell line was continued by inoculation of 10^6 cells IP into donor mice weekly.

Treatment design. The experimental format used was that of Chiuten et al. [3] with modifications. Experimental groups consisted of tumor-bearing animals which each received one of four treatments: VCR only, VP-16-213 only, the combination of VCR and VP-16-213, and saline (PBS) only. Following appropriate dilution with sterile PBS, 10^6 murine leukemia cells were inoculated IP 24 h before the administration of VCR. In the drug combination group, a single injection of VP-16-213 was given at time 0–96 h after VCR. A sham injection with PBS was given 24 h after tumor inoculation in the VP-16-213 only group, after which a single injection of VP-16-213 was administered at times 0–96 h after PBS. The saline control group consisted of animals receiving PBS only following tumor cell inoculation.

The dose of VCR used was 1.0 mg/kg, which is $< LD_{10}$ (2.3 ± 0.7 mg/kg) in our laboratory for DBA/2 male mice with body weight 20 g receiving a single IP injection. The dose of VP-16-213 used in these experiments was 32.0 mg/kg, which was previously found to be $< LD_{10}$ (47.0 mg/kg) in mice with a single IP injection [4]. Drug doses $< LD_{10}$ were selected in an attempt to avoid toxic deaths among the experimental

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Table 1. Overall survival results in mice bearing P388 leukemia[a]

Experiment/Time of administration of VP-16	Survival after								
	VCR[b]			VP-16[b]			VCR + VP-16		
	Median (days)	ILS	No. > 60 days	Median (days)	ILS	No. > 60 days	Median (days)	ILS	No. > 60 days
No. 1	18	64%	0	–	–	–	–	–	–
4 h				21	91%	0	13	18%	1
24 h				12	9%	0	31	182%	2
48 h				19	73%	0	10	–	0
72 h				18	39%	0	14	27%	0
96 h				12	9%	0	18	64%	0
No. 2	16	33%	0	–	–	–	–	–	–
4 h				11	8%	0	29	142%	2
24 h				21	75%	0	19	58%	0
48 h				16	33%	0	25	108%	0
72 h				15	25%	0	25	108%	2
96 h				16	33%	0	14	17%	0
No. 3	18	64%	0	–	–	–	–	–	–
4 h				26	136%	1	60+	>445%	3
24 h				21	91%	0	60+	>445%	3
40 h				21	91%	1	18	64%	2
72 h				18	64%	0	39	256%	1
90 h				19	73%	0	21	91%	0
No. 4	16	52%	1	–	–	–	–	–	–
0 h				23	119%	2	27	157%	3
4 h				30	186%	4	47+	>448%	5
24 h				23	119%	1	27	157%	3
48 h				23	119%	0	60+	>471%	7
72 h				16	52%	0	60+	>471%	6
96 h				16	52%	0	24	129%	3

[a] Mice were inoculated with 10^6 murine leukemic cells IP 24 h before drug administration. Median survival times of control animals receiving an injection of saline only 24 h after tumor cell inoculation were 11, 12, 11, and 10.5 days for expts. 1–4, respectively, and there were no long-term survivors. There were 5 mice in each treatment group in expts. 1–3 and 10 mice in each treatment group in expt 4. The increase in life-span (ILS) was determined by comparing the median survival time (MST) of treatment mice with the MST of control (saline only) mice (MST): ILS = 100% $(MST\text{-}MST_c)/MST_c$

[b] VCR (vincristine) 1.0 mg/kg was given IP 24 h after tumor cell inoculation and single injections of VP-16 32.0 mg/kg were given at various times (0–96 h) after the 24 h period following tumor cell inoculation

subjects. The volume of all treatment solutions for injection was 0.5 ml; all injections were given via a 25-gauge needle.

In all, five experiments involving 420 mice were performed. In expts 1–3, VP-16-213 was given at 4, 24, 48, 72, and 96 h after administration of VCR; each treatment group consisted of five mice. In expt 4, VP-16-213 was given at 0, 4, 24, 48, 72, and 96 h after administration of VCR; each treatment group consisted of ten mice. The tumor cell line employed in expts 1–4 was P388 murine leukemia. In expt 5 P 1534 murine leukemic cells were used.

Treatment groups were observed daily until the death of all subjects or until 60 days from the start of the experiment. Survival of the animals was calculated form the day of inoculation with tumor cells. Long-term survivors ('probable cures') were considered to be those animals surviving 60 + days. The percentage increase in life-span (ILS) was determined by comparison of the median survival of treatment animals with the median survival of animals that had received saline (PBS) only; ILS was determined separately for the specific treatment group as a whole (including survivors) and for nonsurvivors only.

Statistical analysis. The data for each of the first four experiments were examined separately for consistency of results and were subsequently combined for overall summary statistics and statistical tests of hypotheses. The two sample Wilcoxon rank sum one-sided test adjusted for ties [6] was used to compare median survival times. Differences in proportions of long-term survivors were evaluated using the one-sided Fisher's exact test for 2×2 tables [2].

Results

Overall treatment results in mice bearing P388 leukemia are shown in Table 1 and cumulative results are given in Table 2. Administration of VCR 24 h after tumor cell inoculation resulted in a 45% increase in life-span compared with control animals receiving saline only. VP-16-213 alone also resulted in an appreciable ILS for each time point when it was administered as a single injection beginning 24 h after tumor cell inoculation. An ILS greater than that recorded after either VCR alone or VP-16-213 alone was generally observed with the combination of VCR and VP-16-213 for each time point of VP-16-213 administration after VCR.

The cumulative median survival of mice (Table 2) bearing 10^6 P-388 leukemia cells without treatment (saline only) was 11 days; administration of VCR only and VP-16-213 only resulted in modest increases of survival to 16 and 19 days, respectively.

Table 2. Cumulative survival results in mice bearing P388 leukemia[a]

Time of administration of VP-16	Survival after								
	VCR			VP-16			VCR + VP-16		
	Median (days)	ILS	No. > 60 days	Median (days)	ILS	No. > 60 days	Median (days)	ILS	No. > 60 days
–	16	45%	1/45 (2%)		–			–	
0 h		–		23	109%	2/10 (20%)	27	141%	3/10 (30%)
4 h		–		24	118%	5/25 (20%)	32	191%	11/25 (44%)
24 h		–		21	91%	1/25 (4%)	27	145%	8/25 (32%)
48 h		–		19	73%	1/25 (4%)	34	209%	9/25 (36%)
72 h		–		17	55%	0/25 (0%)	38	245%	9/25 (36%)
96 h		–		16	45%	0/25 (0%)	21	91%	3/25 (12%)

[a] The cumulative results from four experiments involving 360 animals are shown. In the first three experiments there were five mice in each treatment group and there were 10 mice per treatment group in the fourth experiment. The median survival of control animals receiving an injection of saline only after tumor cell inoculation was 11 days. Further experimental details are given in the footnote to Table 1

Table 3. Treatment results in nonsurviving mice bearing P388 leukemia[a]

Experiment/ Time of administration of VP-16	Survival after					
	VCR		VP-16		VCR + VP-16	
	Median (days)	ILS	Median (days)	ILS	Median (days)	ILS
No. 1	18	64%				
4 h			21	91%	13	18%
24 h			12	9%	21	91%
48 h			19	73%	10	–
72 h			18	64%	14	27%
96 h			12	9%	18	64%
No. 2	16	33%				
4 h			11	–	27	125%
24 h			21	75%	19	58%
40 h			16	33%	25	108%
72 h			15	25%	22	83%
96 h			16	33%	14	17%
No. 3	18	64%				
4 h			26	136%	20	82%
24 h			21	91%	27	145%
48 h			20	82%	18	64%
72 h			18	64%	32	191%
96 h			19	73%	21	91%
No. 4	14	40%				
0 h			23	130%	24	140%
4 h			26	160%	27	170%
24 h			23	130%	26	160%
48 h			23	130%	18	70%
72 h			16	60%	28	180%
96 h			16	60%	21	110%

[a] Mice were inoculated with 10^6 P388 murine leukemic cells IP 24 h before drug administration. The median survival times of control animals receiving an injection of saline only 24 h after tumor cell inoculation were 11, 12, 11, and 10 days for experiments 1–4, respectively, and there were no long-term (> 60 days) survivors. The increase in life-span (ILS) for mice not surviving treatment was determined by comparing the median survival time (MST) of nonsurviving mice with the MST of control (saline only) mice (MST_c): ILS = 100% (MST-MST_c)/MST_c

Table 4. Cumulative survival results in nonsurviving mice bearing P388 leukemia[a]

Time of administration of VCR	Survival after					
	VCR		VP-16		VCR + VP-16	
	Median (days)	ILS	Median (days)	ILS	Median (days)	ILS
–	16	45%	–	–	–	–
0 h			23	109%	24	118%
4 h			21	91%	20	82%
24 h			21	91%	24	118%
48 h			19	73%	20	82%
72 h			17	55%	24	118%
96 h			16	45%	19	73%

[a] The cumulative results from four experiments are shown. The median survival time of control animals receiving an injection of saline only after tumor cell inoculation was 11 days. Further experimental details are given in the footnote to Table 3

However, the median survival following the combination of VCR and VP-16-213 was 26 days, which was significantly longer than that after placebo, VCR only, or VP-16-213 only ($P < 0.001$). Median survival times when VP-16-213 was given 0, 4, 24, 48, 72, or 96 h after VCR were 27, 32, 27, 34, 38, and 21 days, respectively. That the observed increase in median survival with the drug combination was not attained at the expense of greater toxicity beyond that seen with either drug alone is supported by two findings: (1) there were no obviously toxic deaths, as all animals died with tumor ascites; (2) median survival times of non-long-term survivors in the combination group were not significantly different from those of the individual drug groups (Tables 3 and 4). The median survival time when VP-16-213 was given 96 h after VCR was significantly shorter than in groups given VP-16-213 at 0, 4, 24, 48, or 72 h after VCR ($P < 0.02$).

Probable cures (> 60d survival) were observed infrequently (2%–4%) after VCR only or after VP-16-213 only when these were given as single injections 24 or 48 h after the 24-h period following tumor cell inoculation (Tables 1 and 2 and Fig. 1). There were no long-term survivors in the 72-h and 96-h VP-16-213 only groups. However, 42 of 135 (31%) animals receiving the combination of VCR and VP-16-213 experienced

probable cure, a significantly greater proportion of long-term survivors than observed in the placebo, VCR only, and VP-16-213 only groups ($P < 0.001$). Figure 1 graphically demonstrates the synergistic activity of the combination in this murine system by comparison of the observed percentage of long-term survivors with the proportion of long-term survivors predicted by combining the results with the separate drugs. A modest percentage (12%–20%) of long-term survivors was seen in the 96-h VCR + VP-16-213 group and in the 0-h and 4-h VP-16-213 only groups. When VP-16-213 was given 0–72 h after VCR a high percentage (32%–44%) of long-term survivors was observed. The proportion of long-term survivors when VP-16-213 was given 96 h after VCR was significantly lower than in any of the each groups given VP-16-213 at 0, 4, 24, 48, and 72 h after VCR ($P < 0.05$).

Similar trends were observed in a single experiment with mice bearing P1534 murine leukemia (Table 5). The ILS was quite modest with VCR alone or VP-16-213 alone; no improved survivorship was observed when VP-16-213 was given alone 96 h after VCR. Whereas no probable cures (> 60d survival) occurred following either VCR alone or VP-16-213 alone, long-term survival was observed at each time point of VP-16 administration following VCR with the exception of the 4-h time point.

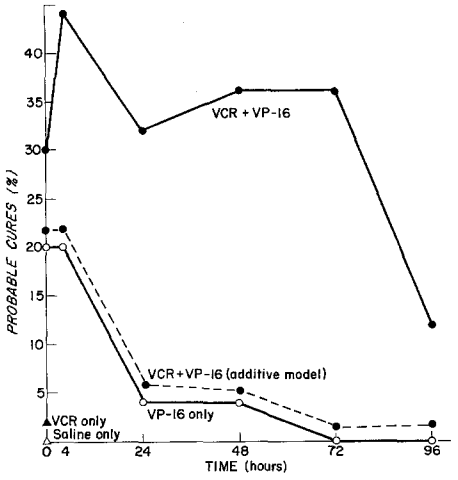

Fig. 1. Long-term survivorship in mice bearing P388 leukemia. The cumulative results of four experiments involving 360 animals are shown (see footnote to Table 1 for treatment design). Mice were inoculated with 10^6 tumor cells IP prior to all treatments. The percentage of long-term survivors (> 60 days) receiving VCR + VP-16 (●–––●), VP-16 only (○–––○), VCR only (▲), and saline (PBS) only (△) are shown, as is the proportion of long-term survivors expected (●–––●) on the basis of an additive model [8] for VCR and VP-16: (1-expected proportion of survivors VCR + VP-16) = (1-proportion survivors receiving only VCR) (1-proportion of survivors receiving VP-16)

Discussion

The current study demonstrates synergistic antitumor activity with the combination of VCR and VP-16-213 in this murine model. Each treatment interval between the administration of VCR and VP-16-213 explored was associated with some long-term survivors among mice bearing P388 leukemia; the greatest proportion of probable cures was seen when VP-16-213 was given 0–72 h after VCR. In a limited trial with P1534 leukemia a similar trend was observed.

These data are somewhat at variance with those of Chiuten et al., who observed the greatest number of long-term survivors in mice bearing P388 murine leukemia when VP-16-213 was given 96 h after VCR [3]. Also, these investigators noted increased toxicity without improvement in survival when VP-16-213 was given 4 h after VCR. However, as in the current study, 'more than additive therapy' was observed with lower dose combinations when VP-16-213 was given 24 h after VCR. Perhaps the differences between these investigations are attributable in part to the variations in drug dosage. An attempt to avoid toxicity by using $< LD_{10}$ doses of both agents was made in the current study.

That the interaction of these two antitumor agents may vary with different types of tumors in the same animal is suggested by the finding of a lack of synergistic activity in L1210 murine leukemia by Dombernowsky and Nissen [4] in an investigation using $\leqq LD_{10}$ for both agents; these authors observed more than additive results with VP-16-213 in combination with either cyclophosphamide or BCNU; actinomycin D, cytosine arabinoside, daunorubicin, 5-fluorouracil, 6-mercaptopurine, methotrexate, and VCR were unimpressive. However, it should be noted that the antitumor activity of VCR in L1210 murine leukemia in vivo is minimal and much less marked than in the P388 or P1534 types [1].

The known wide spectrum of activity of VCR and VP-16-213 as single agents in cancer chemotherapy and the therapeutic advantage demonstrated in the current murine

Table 5. Survival results in mice bearing P388 leukemia[a]

Time of administration of VP-16	Survival after								
	VCR			VP-16			VCR + VP-16		
	Median (days)	ILS	No. > 60 days	Median (days)	ILS	No. > 60 days	Median (days)	ILS	No. > 60 days
–	16	78%	0/5 (0%)		–			–	
4 h		–		17	89%	0/5 (0%)	9	26%	0/5 (0%)
24 h		–		19	111%	0/5 (0%)	60+	566+%	3/5 (60%)
48 h		–		19	111%	0/5 (0%)	60+	566+%	3/5 (60%)
72 h		–		19	111%	0/5 (0%)	23	156%	1/5 (20%)
96 h		–		9	0%	0/5 (0%)	19	111%	1/5 (20%)

[a] The results of one experiment involving 60 animals are shown. Mice were inoculated with 10^6 P1534 murine leukemia cells IP prior to drug administration. The median survival time of control animals ($n = 5$) receiving an injection of saline only 24 h after tumor cell inoculation was 9 days and there were no long-term (> 60 days) survivors. See footnotes to Table 1 for other details of treatment design

study suggests the need for further investigation of this drug combination. Low doses of each of these agents were evaluated in the current study to avoid toxicity. The potential benefit of increasing the doses of VCR and VP-16-213 both singly and in combination remains to be evaluated in this murine model.

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