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Synergistic Interactions between Cyclophosphamide or Melphalan and VP-16 in a Human Rhabdomyosarcoma Xenograft[1]

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ABSTRACT

Based on previous work demonstrating the activity of cyclophosphamide and melphalan in a series of human medulloblastoma and rhabdomyosarcoma cell lines and transplantable xenografts, investigations were conducted to define the effects of combining cyclophosphamide or melphalan with VP-16. These studies demonstrated a synergistic interaction between cyclophosphamide and VP-16 and melphalan and VP-16 in the treatment of the human rhabdomyosarcoma cell line TE-671 growing in athymic mice. The combination of cyclophosphamide or melphalan with VP-16 may warrant consideration as a therapeutic strategy for solid tumors sensitive to bifunctional alkylating agents.

INTRODUCTION

Bifunctional alkylating agents have shown considerable activity against a broad spectrum of human neoplasms both in the laboratory and in clinical trials (1–4). However, despite the pronounced antineoplastic activity of alkylating agents, it is unlikely that a single agent or class of agents will produce significant increases in disease-free survival, due to the presence or emergence of drug-resistant cell populations (5). Efforts to find new active agents as well as to increase the activity of current drugs are clearly warranted. One of the most effective approaches designed to increase the efficacy of antineoplastic drugs has been the use of combinations of two or more agents. Combination chemotherapy has been successfully utilized to prevent or overcome the development of clinically relevant drug resistance (with subsequent curative intervention) in many tumor types, particularly in the childhood malignancies. However, the choice of drug combinations in cancer chemotherapy has often been made empirically, with few clinical regimens based on laboratory analysis (6).

Previous studies have demonstrated cyclophosphamide and melphalan to be among the most active single agents in the treatment of a broad spectrum of solid tumors (1–4), indicating that interventions enhancing the activity of these bifunctional alkylating agents might provide a therapeutic advantage. We now report the therapeutic response of the human rhabdomyosarcoma cell line TE-671, grown s.c. in athymic mice, to cyclophosphamide or melphalan given alone or in combination with VP-16, demonstrating a synergistic interaction between each of these two alkylating agents and VP-16.

MATERIALS AND METHODS

Animals. Male or female athymic BALB/c mice (*nu/nu* genotype, 6 weeks or older) were used for all *in vivo* studies and were maintained as previously described (7).

Xenograft Transplantation. TE-671, a subline of the human rhabdomyosarcoma-derived continuous cell line RD, grown as s.c. xenografts, was used for all studies (8–10). Tumor-bearing animals were killed by cervical dislocation and tumors removed in a laminar flow hood. Tumor fragments were passed through a bilayered 20-mesh screen in a tissue press. The tissue was passed through consecutively smaller gauge needles (16-, 19-, and 20-gauge) and the tumor homogenate placed into a 500-μl Hamilton syringe (Hamilton Co., Reno, NV). Thirty μl of tumor homogenate were injected with a 19-gauge needle into the right flank of recipient mice.

Tumor Measurements. Tumors were measured every 3 to 4 days with Vernier calipers (Scientific Products, McGaw Park, IL) until the volume exceeded 2000 mm^3. Width and length in millimeters were measured, and volume was calculated by the formula:

$$\frac{\text{width}^2 \times \text{length}}{2}$$

Drug Toxicity and Tumor Therapy. The lethal toxicity of cyclophosphamide, melphalan, and VP-16 were assessed by probit analysis as previously described (11, 12). A minimum of four dose levels with 10 animals per dose was used to calculate the LD_{10}[3] of each drug. These values were as follows: cyclophosphamide, 1391.0 mg/m^2 (single dose); melphalan, 71.3 mg/m^2 (single dose); VP-16, 170.2 mg/m^2 (days 1, 4, 7). The regimen used in each experiment was a fraction of the calculated LD_{10} (as shown in Tables 1 and 2), administered as i.p. injections in a volume of 90 ml/m^2. Cyclophosphamide and melphalan were each given as a single dose facilitating comparison to a series of bifunctional alkylators tested against human rhabdomyosarcoma and medulloblastoma xenografts using an identical single dose regimen (13). VP-16 was given on a 3-day regimen (for three doses), which represents a modification of a schedule active in preclinical studies with L1210 leukemia. VP-16 and cyclophosphamide were administered in a 0.9% NaCl solution, and melphalan was delivered in 17% dimethylsulfoxide.

VP-16 and Cyclophosphamide. Groups of nine to 10 randomly assigned mice were treated when the median tumor volume exceeded 200 mm^3 with vehicle alone (Day 1), VP-16 alone (Days 1, 4, 7), cyclophosphamide alone (Day 4), or VP-16 and cyclophosphamide. For combination studies with cyclophosphamide plus VP-16, cyclophosphamide was given concomitant with the second dose of VP-16.

VP-16 and Melphalan. Groups of nine to 10 randomly assigned mice were treated with vehicle alone (Day 1), VP-16 alone (Days 1, 4, 7), melphalan alone (Day 1), or melphalan and VP-16. For combination studies with melphalan and VP-16, melphalan was administered with the first dose of VP-16 due to the longer time interval to peak interstrand cross-link formation following melphalan (12–24 h) as opposed to cyclophosphamide (2–4 h) administration (14–16). VP-16 was administered on a schedule designed to potentially increase alkylating agent activity by treatment prior to repair of these cross-links.

Assessment of Response. Response of xenografts was assessed by growth delay, the difference in days between the median of individual treated animals' and individual control animals' tumors to reach a volume of five times the treatment volume (T-C) and treated *versus* control tumor regressions. Tumor regression was defined by a smaller volume on two consecutive measurements to minimize the effect of measurement errors. T-C and regressions were further examined for

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[3] The abbreviations used are: LD_{10}, 10% lethal dose; T-C, difference in days between the median time for the tumors of treated (T) and control (C) animals to reach a volume five times greater than the volume at the time of treatment; BCNU, 1,3-bis(2-chloroethyl)nitrosourea.

Table 1 *Chemotherapeutic responses of s.c. TE-671 xenografts to varying doses of cyclophosphamide ± VP-16 (0.50 LD_{10})*

Treatment[a] (fraction LD_{10})			
Cyclophosphamide	VP-16	T-C[b]	Regressions[c]
0.25	0	6.1	0/10 (NS)[d]
0	0.50	3.3	0/10 (NS)
0.25	0.50	11.1	7/10[f]
0.25	0	6.9	0/10 (NS)
0	0.50	3.6	0/9 (NS)
0.25	0.50	14.3[e]	5/7[f]
0.375	0	9.9	3/10 (NS)
0	0.50	2.9	0/10 (NS)
0.375	0.50	14.8[e]	9/10[f]
0.50	0	9.0	7/9
0	0.50	2.6	0/10 (NS)
0.50	0.50	19.0[e]	8/8
0.50	0	10.4	7/9
0	0.50	3.0	1/9 (NS)
0.50	0.50	14.1[e]	8/8
0.50	0	8.4	2/10 (NS)
0	0.50	1.4	0/10 (NS)
0.50	0.50	11.6[e]	2/10 (NS)
0.75	0	12.4	3/8 (NS)
0	0.50	1.0	0/10 (NS)
0.75	0.50	13.5	7/9

[a] Experiment regimen: VP-16 days 1, 4, 7; cyclophosphamide day 4 (i.p.).
[b] T-C (days): The difference in days between the median of individual, treated animals and the controls to reach a volume of five times the treatment volume.
[c] Number of animals with regressing tumors/number of surviving treated animals. Regressing tumors have at least two consecutively decreasing volume measurements.
[d] P values for all experiments were <0.02 unless otherwise designated. NS, not significant; $P > 0.020$.
[e] Growth delay (T-C) for cyclophosphamide + VP-16 significantly longer than cyclophosphamide alone: $P \leq 0.020$.
[f] Regressions for cyclophosphamide + VP-16 significantly greater than cyclophosphamide alone: P value ≤ 0.010.

Table 2 *Chemotherapeutic responses of s.c. TE-671 xenografts to varying doses of melphalan ± VP-16 (0.50 LD_{10})*

Treatment[a] (fraction LD_{10})			
Melphalan	VP-16	T-C[b]	Regressions[c]
0.10	0	4.00	0/10 (NS)[d]
0	0.50	1.4	0/10 (NS)
0.10	0.50	8.3	5/10[f]
0.10	0	3.4	0/9 (NS)
0	0.50	2.7	0/9 (NS)
0.10	0.50	13.3[e]	5/9[f]
0.25	0	11.1	8/10
0	0.50	1.1	0/10 (NS)
0.25	0.50	21.0[e]	7/7[f]
0.25	0	9.3	4/9
0	0.50	1.9	0/9 (NS)
0.25	0.50	17.1[e]	7/7[f]
0.375	0	18.6	10/10
0	0.50	4.3	0/10 (NS)
0.375	0.50	24.9[e]	9/9
0.375	0	16.4	8/9
0	0.50	1.6	0/9 (NS)
0.375	0.50	23.1[e]	9/9
0.5	0	20.4	5/7
0	0.50	2.9	0/9 (NS)
0.5	0.50	28.4[e]	9/9
0.5	0	21.2	7/7
0	0.50	2.7	0/9 (NS)
0.5	0.50	36.3[e]	8/8
0.75	0	29.6	8/9
0	0.50	2.0	0/9 (NS)
0.75	0.50	29.3	8/8

[a] Experiment regimen: VP-16 days 1, 4, 7; melphalan day 1 (i.p.).
[b] T-C (days): The difference in days between the median of individual treated animals and the controls to reach a volume of five times the treatment volume.
[c] Number of animals with regressing tumors/number of surviving treated animals. Regressing tumors have at least two consecutively decreasing volume measurements.
[d] P values for all experiments were <0.02 unless otherwise designated. NS, not significant; $P > 0.020$.
[e] Growth delay (T-C) for cyclophosphamide + VP-16 significantly longer than cyclophosphamide alone: $P \leq 0.020$.
[f] Regressions for cyclophosphamide + VP-16 significantly greater than cyclophosphamide alone: P value ≤ 0.010.

each drug combination effect *versus* individual drug alone. Statistical significance for T-C was determined by the Wilcoxon rank sum test and for tumor regressions by the Fisher exact test.

Data Analysis. Isobolograms were generated by the method of Deen and Williams (17) and Steel and Peckham (18) for the special case in which the dose of one agent is held constant. Envelopes of additivity (supraadditive and additive) were generated and assessed as described by Eder *et al.* (19) and Teicher *et al.* (20).

RESULTS

Drug Toxicity. Twenty-one deaths in 446 tumor-bearing treated animals were attributed to drug toxicity. Animals bearing s.c. tumors do not die as a consequence of these tumors until the tumor size is at least 5000 mm^3. TE-671 does not metastasize and was not responsible for the deaths seen at the times noted in our experiments. In all experimental groups, death rate was 10% or less, except as follows (fraction LD_{10}): cyclophosphamide (0.50) 6 of 50; cyclophosphamide (0.75) 2 of 10; cyclophosphamide plus VP-16 (0.25 + 0.50) 3 of 20; cyclophosphamide plus VP-16 (0.50 + 0.50) 4 of 30; melphalan (0.50) 2 of 16. Mean nadir weight loss was less than 15% in all groups except for cyclophosphamide (0.375) 16.6%; and cyclophosphamide plus VP-16 (0.75 + 0.50) 16.9%; and melphalan plus VP-16 (0.75 + 0.50) 15.7%.

Tumor Therapy. The response to chemotherapy is summarized in Tables 1 and 2. The combination of cyclophosphamide or melphalan plus VP-16 is synergistic as determined for the case in which the dose of one agent is held constant (Figs. 1 and 2). Representative growth delay curves for cyclophosphamide, melphalan, and VP-16, alone and in combination, are shown in Figs. 3 and 4.

DISCUSSION

Recent laboratory and clinical trials have demonstrated the activity of bifunctional alkylating agents in the treatment of many human neoplasms, including ovarian carcinoma, neuroblastoma, rhabdomyosarcoma, and medulloblastoma (1–4). Since it is unlikely that a single agent or class of agents will prove curative for rhabdomyosarcoma (or any other malignancy), modulations effective in enhancing alkylator activity are warranted. The current studies were designed to determine whether the activity of the bifunctional alkylating agents cyclophosphamide and melphalan against TE-671 xenografts growing s.c. in athymic mice could be enhanced by administration of VP-16. Both alkylating agents demonstrated a synergistic interaction with VP-16.

VP-16, an epipodophyllotoxin with known clinical anticancer activity against a number of neoplasms including testicular cancers, leukemia, and lymphoma (21), has been shown to stimulate topoisomerase II-mediated DNA cleavage via a nonintercalative, ATP-independent reversible reaction. The cyto-

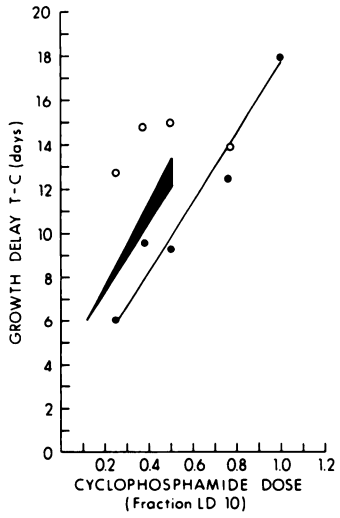

Fig. 1. Isobologram for the simultaneous treatment of athymic mice bearing s.c. TE-671 xenografts with VP-16 (0.5 LD_{10}) in combination with a range of cyclophosphamide doses (0.25–0.75 LD_{10}). Lower line with ●, tumor treatment with cyclophosphamide alone (mean values). *Shaded area*, envelope of additivity for treatment with VP-16 and cyclophosphamide. ○, mean values for combination treatment of VP-16 (0.5 LD_{10}) plus variable doses of cyclophosphamide (0.25–0.75 LD_{10}).

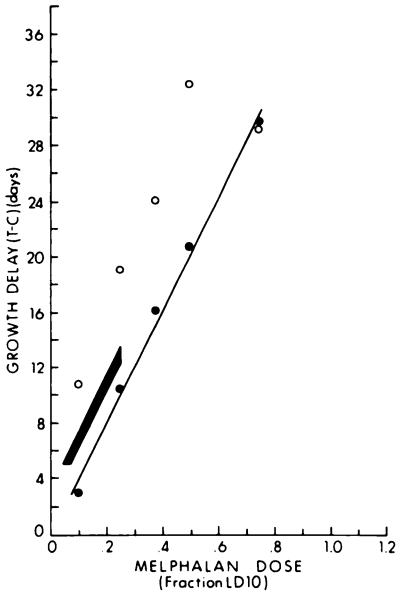

Fig. 2. Isobologram for the simultaneous treatment of athymic mice bearing s.c. TE-671 xenografts with VP-16 (0.5 LD_{10}) in combination with a range of melphalan doses (0.10–0.75 LD_{10}). Lower line with ●, tumor treatment with melphalan alone (mean values). *Shaded area*, envelope of additivity for treatment with VP-16 and melphalan. ○, mean values for combination treatment of VP-16 (0.5 LD_{10}) plus variable doses of melphalan (0.10–0.75 LD_{10}).

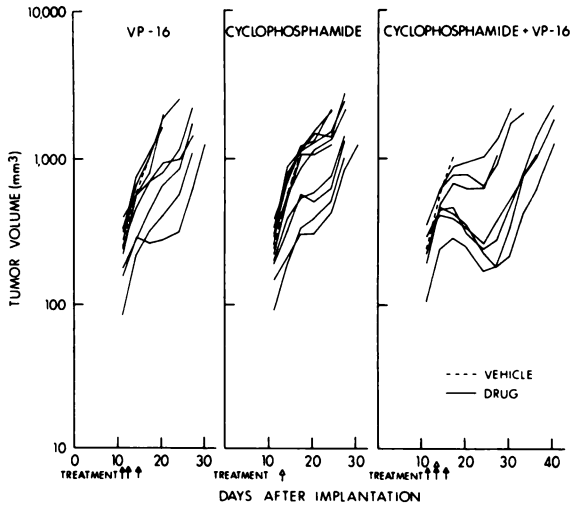

Fig. 3. Groups of 10 randomly assigned mice bearing s.c. TE-671 xenografts were treated when the median tumor volume exceeded 200 mm^3 with either VP-16 at 0.5 LD_{10} (day 1, 4, 7); cyclophosphamide at 0.25 LD_{10} (day 4); VP-16 plus cyclophosphamide; or drug vehicle (day 1).

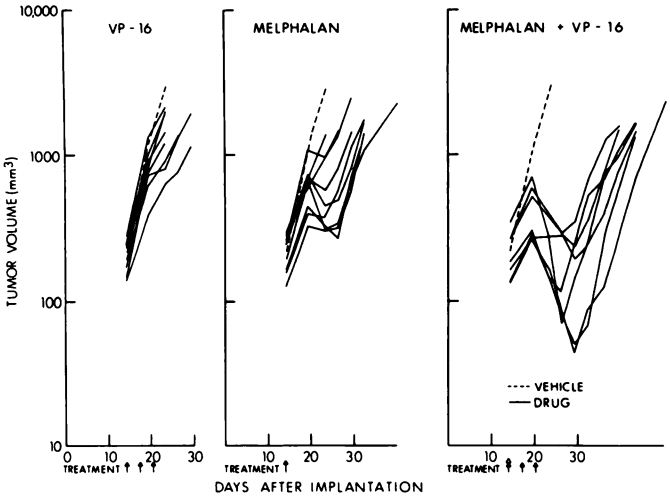

Fig. 4. Groups of 10 randomly assigned mice bearing s.c. TE-671 xenografts were treated when the median tumor volume exceeded 200 mm^3 with either VP-16 at 0.5 LD_{10} (day 1, 4, 7); melphalan at 0.25 LD_{10} (day 1); VP-16 plus melphalan; or drug vehicle (day 1).

toxicity of VP-16 is believed to result from a subsequent stabilization of the topoisomerase II-DNA "cleavable complex," thus preventing DNA repair. VP-16 also inhibits the normal strand-passing activity of topoisomerase II (22). Despite minimal activity of VP-16 at the 0.5 LD_{10} against TE-671 xenografts (with growth delays of 1.0–3.6 days), the combination of this agent with cyclophosphamide or melphalan produced significant (and synergistic) increases in tumor growth delay. Although the mechanism for this synergistic interaction remains speculative, VP-16-mediated inhibition of the repair of cyclophosphamide or melphalan-induced DNA cross-links is a probable explanation.

Previous laboratory investigations have explored the potential therapeutic benefits resulting from combination therapy with VP-16 and cyclophosphamide. Dombernowsky and Nissen (23) demonstrated that the combination of VP-16 and cyclophosphamide resulted in a more than additive effect against L1210 leukemia. Similarly, Chang *et al.* (24) demonstrated a synergistic effect between VP-16 and 4-hydroperoxycyclophosphamide against HL-60 leukemic cells. No reports of similar interaction between melphalan and VP-16, to our knowledge, have been published.

Clinical trials combining cyclophosphamide or melphalan with VP-16 have yielded equivocal results. Trials utilizing cyclophosphamide plus VP-16 in patients with lung cancer resulted in no definite conclusions regarding the interaction of these two agents (25–27). Current Phase II trials conducted by the Pediatric Oncology Group using the alkylating agent ifosfamide combined with VP-16 are too premature for meaningful analysis. Melphalan has been employed with VP-16 and BCNU prior to bone marrow transplanation in the successful therapy of patients with refractory Hodgkin's disease (28). Whether the success of this regimen is due in part to the synergistic interaction of melphalan with VP-16 is unclear due to the incorpo-

ration of a third drug. The benefit of combination therapy using cyclophosphamide or melphalan plus VP-16 thus remains inconclusive, with further studies needed to define the role of this approach.

Our studies suggest that combination therapy with cyclophosphamide or melphalan plus VP-16 may represent a synergistic chemotherapeutic intervention for solid tumors sensitive to alkylating agents. The potential role of VP-16 and cyclophosphamide or melphalan is less clear in alkylator-resistant tumors, particularly since the mechanisms responsible for resistance to these agents and for the synergy between them and VP-16 are unknown. Nevertheless, confirmation of the synergistic relationship between VP-16 and cyclophosphamide or melphalan in additional preclinical models of human tumors would justify translation into clinical trials.

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