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# Sequence-dependent Antitumor Effect of VP-16 and 1-β-D-Arabinofuranosylcytosine in L1210 Ascites Tumor

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**Abstract**—*The sequence-dependence of the antitumor effect of etoposide (VP-16) and 1-β-D-arabinofuranosylcytosine (ara-C) was investigated against the L1210 ascites tumor in BDF1 mice. Treatment with VP16 (7.5 or 15 mg/kg) and ara-C (25 or 500 mg/kg) was administered intraperitoneally on days 1, 4 and 7 after tumor inoculation. Six hour pretreatment with 15 mg/kg VP16 followed by 500 mg/kg ara-C yielded a 100% cure rate, but only a 20% cure rate was obtained with the reverse sequence. Simultaneous administration of 15 mg/kg of VP-16 and 500 mg/kg ara-C interacted synergistically, producing a 70% cure rate. In contrast with the results obtained with VP-16 and 500 mg/kg ara-C, simultaneous administration of 25 mg/kg ara-C neither antagonized nor potentiated the antitumor effect of VP-16. Twenty-five mg/kg ara-C was too low to produce any antitumor effect with VP-16 in simultaneous administration. At every dose investigated, pretreatment with VP-16 followed by ara-C was the most effective antitumor schedule in L1210 leukemia. This sequence of drug administration did not cause greater toxicity as measured by weight loss or toxic death.*

## INTRODUCTION

THE COMBINATION of ara-C with VP-16 has been shown to be promising in recent clinical studies [1–3], but conflicting reports certainly exist as to whether this combination of drugs is synergistic or antagonistic in experimental systems [4, 5]. VP-16 produces topoisomerase II-mediated protein-linked DNA strand breaks and the breakage of DNA is rejoined in a relatively short time by the removal of the drug [6–13]. Recent studies have shown that VP-16-induced DNA breakage and cytotoxicity is dependent on the proliferation of the cells. Markovits *et al.* [14] found that VP-16-induced DNA breaks were higher in nuclei from exponentially growing than in those from quiescent L1210 cells, and that VP-16 was most cytotoxic in proliferative cells and during DNA replication. VP-16 also inhibits nucleoside transport reversibly. Loike and Horwitz [15] found that 30 μM VP-16 inhibits nucleoside transport by 50% in HeLa cells. Ara-C is a drug with a cell cycle phase-specific action [16–18]. It inhibits DNA polymerase and also can be incorporated into DNA [17, 19–24]. Its cytotoxicity depends not only on the percentage of cells in S phase but also on the rate of DNA synthesis [25].

Although sequential administration is essential for some other combination therapies, such as MTX:5-fluorouracil [26–28] and MTX: ara-C [28–30] combination chemotherapy, little attention has been paid to the timing of administration of VP-16 and ara-C. Since ara-C is an analog of deoxycytidine and the effect of VP-16 on membrane transport is reversible, and furthermore ara-C is a strong inhibitor of DNA synthesis and VP-16-induced DNA damage is proliferation dependent, there is reason to believe that sequential administration is more potent than simultaneous administration. In the present study in the L1210 ascites tumor, the experiments were designed to see whether time sequence benefits exist between VP-16 and ara-C.

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Abbreviations used: ara-C, 1-β-D-arabinofuranosylcytosine; VP-16, VP16-213, etoposide; MTX, methotrexate.

## MATERIALS AND METHODS

*Mice and tumor*

Male BDF1 mice weighing from 20 to 22 g each

were kept at Mie University Animal Center under constant conditions (12 h light:dark regimen, Oriental Chow pellet food and water *ad libitum*). The strain of leukemia L1210 used was obtained from Shionogi Pharmacological Laboratory, Osaka and has been maintained in BDF1 mice by weekly intraperitoneal (i.p.) inoculation of $1 \times 10^6$ cells. Mice which were to be used in experiments received $1 \times 10^6$ ascites tumor cells i.p. in 0.2 ml 0.9% NaCl solution as described previously [30].

*Drug and drug administration*

VP-16 was supplied from Bristol Myers Co., Tokyo, Japan in vials containing 100 mg VP-16, and ara-C from Nippon Shin'yaku Co., Kyoto, Japan. The drug was prepared just before administration and diluted with isotonic NaCl solution until the dose per gram of mice was contained in 0.01 ml. After transplantation, mice were randomized into a control group of 20 mice and various treatment groups of 10 mice. On days 1, 4 and 7 after tumor inoculation mice received the drug treatment intraperitoneally. The doses of VP-16 (7.5, 15 mg/kg) and ara-C (25, 500 mg/kg) were decided based on the optimal dose for antitumor effect by single use of each drug. According to previous reports 18 and 1200 mg/kg are the doses that cause maximum antitumor effect respectively in the treatment schedule used in this study [4, 31]. When VP-16 and ara-C were given in combination, the drugs were injected either simultaneously (VP-16 + ara-C) or with a time interval in between (VP-16-6 h-ara-C or ara-C-6 h-VP-16). The time intervals between VP-16 and ara-C administration were 3 and 6 h. Mice in the control group were given 0.2 ml isotonic NaCl solution in the same schedule.

*Drug effectiveness and toxicity*

The antitumor effect of the drugs was evaluated by comparing cure rate and prolongation of the postinoculation life span, and percentage increase in lifespan (percentage ILS) was calculated. Deaths were attributed to tumor on the basis of autopsy findings, such as bloody ascites with leukemic cells, organomegaly and solid tumor masses. One hundred and twenty-day survivors from inoculation were defined as 'cures'. All mice were weighed on days 0 and 7 to evaluate acute toxicity. The data were analyzed by *t*-test for life prolongation of uncured mice without inclusion of cured mice, and by chi-square test for cure rate.

## RESULTS

The therapeutic results are summarized in Table 1.

*Effect of individual drugs*

As compared with 7.20 days in the control group, the life span of the mice treated with 7.5 and 15 mg/kg VP-16 was extended significantly to 17.50 days (143% ILS, $P < 0.01$ by *t*-test) and 19.80 days (175% ILS, $P < 0.01$) respectively. Treatment with 25 and 500 mg/kg ara-C also gave a dose-dependent increase in life span to 13.55 days (88% ILS, $P < 0.01$) and 20.05 (167% ILS, $P < 0.01$) respectively. Cures were not obtained by single drug treatment.

*Effect of* 15 mg/kg *VP-16 and* 500 mg/kg *ara-C*

Of 50 mice treated with 15 mg/kg VP-16 and 500 mg/kg ara-C, 20 died with tumor and 30 were cured. Although the mean life span of uncured mice did not differ significantly among the treatment groups, the cure rate was strikingly sequence dependent. Pretreatment with VP-16 given 6 h before ara-C produced the best therapeutic results with a 100% cure rate, but the reverse sequence produced only a 20% cure rate (different significantly by chi-square test, $P < 0.01$). Three hour pretreatment with VP-16 produced a 70% cure rate, which is better than that obtained with the reverse sequence (40%), but there was no statistical difference. Simultaneous administration produced a 70% cure rate, suggesting a synergistic interaction. The mean survival time of uncured mice ranged from 23.67 (229% ILS) to 25.63 days (256% ILS), and was improved significantly ($P < 0.01$) compared with that of mice given 15 mg/kg VP/16 and 500 mg/kg ara-C alone. Mice in all treatment groups gained weight slightly, and no significant difference was observed among the treatment groups.

*Effect of combination of* 15 mg/kg *VP-16 and* 25 mg/kg *ara-C*

Of 50 mice treated with 15 mg/kg VP-16 and 25 mg/kg ara-C, 26 died with tumor and 24 were cured. The mean life span of uncured mice ranged from 18.50 (157% ILS) to 41.50 (476% ILS) in five treatment groups. Although the mean life span of all combination groups was significantly longer ($P < 0.01$) than that of the group treated with 25 mg/kg ara-C (13.55 days), only when VP-16 preceded ara-C (41.50 and 27.67 days) and when ara-C preceded VP-16 by 6 h (24.33 days) did survival of the mice significantly exceed that of VP-16 alone (19.80 days, $P < 0.01$). The cure rate of mice treated with VP-16-6 hr-ara-C, VP-16-3 h-ara-C, VP-16 + ara-C, ara-C-3 h-VP-16, and ara-C-6 h-VP-16 was 80, 70, 0, 20, 70% respectively. In contrast with the results of treatment with VP-16 and 500 mg/kg ara-C, simultaneous administration of 25 mg/kg of ara-C showed the worst antitumor effect, and 6 h and 3 h pretreatment with VP-16 and 6 h pretreatment with ara-C showed a synergistic antitumor effect (statistically different from simultaneous treatment, $P < 0.01$, and 3 h pretreatment with ara-C, $P < 0.05$). The mean life

*Table* 1. *Effect of sequence of administration of VP-16 and ara-C on survival of mice with L1210 leukemia*

| Group | No. of mice | No. of deaths | Mean survival ± 1 S.D., days (% ILS over control) | | Cure rate | Weight gain (g) |
|---|---|---|---|---|---|---|
| 1 Control | 20 | 20 | 7.2 ± 0.26 | | 0% | +2.20 |
| 2 VP-16 15 mg/kg | 10 | 10 | 19.80 ± 3.50 | (175%) | 0% | +0.97 |
| 3 VP-16 7.5 mg/kg | 10 | 10 | 17.50 ± 3.26 | (143%) | 0% | +1.02 |
| 4 ara-C 500 mg/kg | 10 | 10 | 20.05 ± 1.57 | (186%) | 0% | +1.09 |
| 5 ara-C 25 mg/kg | 10 | 10 | 13.55 ± 0.44 | (88%) | 0% | +1.58 |
| VP-16 15 mg/kg; ara-C 500 mg/kg | | | | | | |
| 6 VP-16-6 h-ara-C* | 10 | 0 | | | 100% | +0.51 |
| 7 VP-16-3 h-ara-C | 10 | 3 | 23.67 ± 0.58 | (229%) | 70% | +0.60 |
| 8 VP-16 + ara-C | 10 | 3 | 25.33 ± 0.58 | (252%) | 70% | +0.48 |
| 9 ara-C-3 h-VP-16 | 10 | 6 | 25.00 ± 2.19 | (247%) | 40% | +0.52 |
| 10 ara-C-6 h-VP-16 | 10 | 8 | 25.63 ± 4.93 | (256%) | 20% | +0.62 |
| VP-16 15 mg/kg; ara-C 25 mg/kg | | | | | | |
| 11 VP-16-6 h-ara-C | 10 | 2 | 41.50 ± 9.19 | (476%) | 80% | +0.74 |
| 12 VP-16-3 h-ara-C | 10 | 3 | 27.67 ± 5.03 | (284%) | 70% | +0.63 |
| 13 VP-16 + ara-C | 10 | 10 | 18.50 ± 2.05 | (157%) | 0% | +0.65 |
| 14 ara-C-3 h-VP-16 | 10 | 8 | 20.44 ± 1.05 | (184%) | 20% | +0.49 |
| 15 ara-C-6 h-VP-16 | 10 | 3 | 24.33 ± 0.58 | (238%) | 70% | +0.57 |
| VP-16 7.5 mg/kg; ara-C 500 mg/kg | | | | | | |
| 16 VP-16-6 h-ara-C | 10 | 1 | 23.0 | (219%) | 90% | +0.44 |
| 17 VP-16-3 h-ara-C | 10 | 3 | 23.33 ± 1.25 | (224%) | 70% | +0.62 |
| 18 VP-16 + ara-C | 10 | 4 | 23.25 ± 1.25 | (223%) | 60% | +0.73 |
| 19 ara-C-3 h-VP-16 | 10 | 6 | 22.13 ± 1.14 | (207%) | 40% | +0.53 |
| 20 ara-C-6 h-VP-16 | 10 | 7 | 23.83 ± 1.21 | (231%) | 30% | +1.06 |
| VP 16 7.5 mg/kg; ara-C 25 mg/kg | | | | | | |
| 21 VP-16-6 h-ara-C | 10 | 5 | 25.00 ± 2.53 | (247%) | 50% | +1.04 |
| 22 VP-16-3 h-ara-C | 10 | 8 | 23.38 ± 3.20 | (225%) | 20% | +0.71 |
| 23 VP-16 + ara-C | 10 | 10 | 16.60 ± 2.46 | (131%) | 0% | +0.66 |
| 24 ara-C-3 h-VP-16 | 10 | 10 | 16.30 ± 1.73 | (126%) | 0% | +0.71 |
| 25 ara-C-6 h-VP-16 | 10 | 8 | 19.30 ± 2.86 | (168%) | 20% | +0.04 |

Groups of 10 mice were inoculated i.p. with $10^6$ L1210 cells 24 h before treatment. Treatment as indicated in the table was administered on days 1, 4 and 7. The control group of mice received saline injection.
*Ara-C was given 6 h after VP-16.

span of the mice treated with 3 h pretreatment with VP-16 at these doses was longer than that of the mice treated with the same schedule at a dose of 500 mg/kg ara-C (group 7 in Table 1), but there was no statistical difference. No significant difference was observed in the body weight gain of five treatment groups.

*Effect of* 7.5 mg/kg *VP-16 and* 500 mg/kg *ara-C*

Of 50 mice treated with 7.5 mg/kg VP-16 and 500 mg/kg ara-C, 21 died with tumor and 29 were cured. The interaction between 7.5 mg/kg VP-16 and 500 mg/kg ara-C was quite similar to that between 15 mg/kg VP-16 and 500 mg/kg ara-C. Treatment with VP-16 6 h before ara-C produced the best therapeutic results with a 90% cure rate and the reverse sequence produced only a 30% cure rate (different significantly, $P < 0.01$). A 3 h pretreatment with VP16 produced a higher cure rate as compared with the reverse sequence, but there was no statistical difference. Simultaneous treatment produced a 60% cure rate. The mean survival time of uncured mice of five combination groups ranged from 22.13 (207% ILS) to 23.83 (231% ILS), and was significantly expanded as compared with that of single drug treatment with 7.5 mg/kg VP-16 and 500 mg/kg ara-C.

*Effect of* 7.5 mg/kg *VP-16 and* 25 mg/kg *ara-C*

Of 50 mice treated with 7.5 mg/kg VP-16 and 25 mg/kg ara-C, 41 died with tumor and nine were cured. The relationship between the antitumor effect and the treatment schedule at these doses was similar to that between 15 mg/kg VP-16 and 25 mg/kg ara-C. A 6 h pretreatment with VP-16 showed the best antitumor effect (25.00 days mean lie span and a 50% cure rate, statistically different from simultaneous treatment and 3 h pretreatment with ara-C, $P < 0.05$). Simultaneous treatment and 3 h pretreatment with 25 mg/kg ara-C did not potentiate the antitumor effect of 7.5 mg/kg VP-16.

## DISCUSSION

Our studies demonstrated the sequence- and

dose-dependence of the antitumor effect of VP-16 and ara-C in L1210 ascites tumor. Pretreatment with VP-16 produced the best therapeutic results at every dose investigated without greater toxicity as measured by weight loss. The interaction between VP-16 and 500 mg/kg ara-C was different from that between VP-16 and 25 mg/kg ara-C. Simultaneous administration of 500 mg/kg ara-C produced an impressive increase in the cure rate as compared with single-agent treatment, i.e. with a combination of 15 mg/kg VP-16 and 500 mg/kg ara-C a 70% cure rate was found as compared with 0% for single-agent treatment with VP-16 or ara-C, respectively. But simultaneous administration of 25 mg/kg ara-C failed to potentiate the antitumor effect of VP-16, and sequential administration was essential. Although pretreatment with 500 mg/kg ara-C was the worst therapeutic schedule, 6 h pretreatment with ara-C showed better results than simultaneous treatment. The therapeutic results obtained with combinations of 15 mg/kg VP-16 and ara-C were qualitatively similar to those obtained with combinations of 7.5 mg/kg VP-16 and ara-C.

When treated with VP-16 and 25 mg/kg ara-C, the mean postinoculation life span of uncured mice was increased in parallel with the cure rate, but when treated with VP-16 and 500 mg/kg ara-C, mean life span did not suffer significantly among the treatment schedules. It may be due to the toxicity of high dose ara-C.

This sequence- and dose-dependence may result from a number of factors, including the metabolic modulation of the drugs and cytokinetic effects. Ara-C is one of the most potent anticancer agents for remission induction in acute nonlymphocytic leukemia [21, 32]. After penetration into the cell via a membrane nucleoside carrier, ara-C is phosphorylated to ara-CTP which inhibits DNA polymerase. Ara-C is also incorporated into DNA, and the amount of ara-C incorporation correlates closely with cytotoxicity in L1210 cells *in vitro* [20, 33, 34].

VP-16 inhibits nucleoside membrane transport. In an early investigation, Loike and Horwitz [15] found that 30 μM VP-16 inhibits thymidine transport by 50% in HeLa cells and that the inhibition is completely reversible. Yalowich and Goldman [35] demonstrated that VP-16 inhibits both transport and accumulation of thymidine at low concentrations in Ehrlich ascites cells. White *et al.* [36] reported the significance of transport effects of VP-16 on ara-C accumulation in freshly isolated human leukemic blasts. But at high ara-C concentrations, the transport capacity was in excess of the phosphorylation capacity, and VP-16 inhibition of ara-C transport did not affect ara-C accumulation. This reversible and dose-dependent effect of VP-16 in diminishing the membrane transport of ara-C may explain why simultaneous administration of the 25 mg/kg ara-C failed to potentiate the antitumor effect of VP-16, and simultaneous administration of 500 mg/kg ara-C did interact synergistically.

VP-16 produces topoisomerase II-mediated DNA strand breaks, and a correlation has been observed between the degree of drug-induced DNA breaks and cytotoxicity [7, 8, 11–13]. Recent studies have shown the proliferation-dependence of VP-16-induced DNA breakage and cytotoxicity. Duguet *et al.* [37] reported that topoisomerase II activity increased in regenerating rat liver, suggesting a possible correlation between the activity of topoisomerase and cell proliferation. Sullivan *et al.* [38] found that VP-16-induced DNA breaks was several-fold higher in the exponential phase versus plateau phase CHO cells. Markovits *et al.* [14] demonstrated that VP-16-induced DNA breaks increased concomitantly with the peak of DNA synthesis in L1210 cells, making the cells more vulnerable to VP-16 at this time. After intraperitoneal injection of 200 mg/kg ara-C, L1210 DNA synthesis decreases to nearly zero with 1 h, leading to a block of G1 cells at the G1–S border in the cell cycle, and it starts to increase again at about 8 h [39]. In this period cells may remain in a VP-16-insensitive stage. Inhibition of DNA synthesis by 25 mg/kg ara-C is weaker as compared with that caused by 500 mg/kg ara-C, and the cells recover from the inhibition more rapidly. This may be the reason why pretreatment with 500 mg/kg ara-C led to the worst therapeutic results and 6 h pretreatment with 25 mg/kg of ara-C produced better results.

From 3 to 6 h after intraperitoneal administration of 15 mg/kg VP-16, the sedimentation rate of L1210 DNA increased on alkaline sucrose gradients, suggesting the rejoining of strand breaks induced by VP-16 (data not shown). This period of repair of DNA damage might be the time when the cell is vulnerable to ara-C.

Further work will be necessary to explain the results obtained in this study completely.

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