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# Therapeutic efficacy of combination of antitumor agent with AHC-52 against multidrug-resistant cells in the intravenously inoculated P388 leukemia model

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**Summary.** To predict the clinical effect on leukemic disease of a combination regimen developed to circumvent multidrug resistance (MDR), we tested various antitumor agents in the presence and absence of AHC-52, a sensitizing agent for multidrug-resistant cells, in the i.v.-i.v. model of murine leukemia. In this model system, sensitive and resistant P388 murine leukemia cells are inoculated i.v. into mice, and each antitumor agent is injected via the i.v. route. Vincristine (VCR) had no effect on the survival of mice bearing VCR-resistant P388, a relatively poorly resistant subline, when given either as a single agent or in combination with AHC-52. In contrast, adriamycin (ADR) alone had no effect on these mice, but its combination with AHC-52 resulted in significant survival, the maximal value achieved being 196% (treated mice/control animals, T/C). Etoposide (VP-16) strongly enhanced survival, even when used alone, and this effect was markedly potentiated by AHC-52. Combination of any antitumor drug with AHC-52 was ineffective in mice bearing ADR-resistant P388, a highly resistant subline. On the other hand, AHC-52 strongly augmented the therapeutic efficacy of these antitumor agents in mice bearing the sensitive parent P388 leukemia, producing some curative effects. On the basis of these results, the feasibility of this type of combination therapy is discussed.

## Introduction

It is widely accepted that drug resistance is a major obstacle to cancer chemotherapy. Recurrence of drug-resistant cancers often occurs, severely limiting the curative effects of the chemotherapy, and relapses are frequently encountered, even following remarkable responses of drug-sensitive cancers such as leukemia. Therefore, the development of effective therapeutic regimens for these resistant cancers is a very urgent subject in cancer chemotherapy.

The mechanism underlying multidrug resistance (MDR) has been elucidated at the molecular level, and a number of compounds that can sensitize resistant cells have been reported (for recent reviews, see [2, 3, 12, 20, 23]). We have recently reported that AHC-52, a partial analogue of nifedipine that exhibits much lower calcium-antagonizing activity and produces less host toxicity, inhibits the active efflux of vincristine (VCR) from VCR-resistant P388 leukemia cells and potentiates the therapeutic efficacy of VCR in mice bearing VCR-resistant P388 leukemia as well as in parent P388 leukemia-bearing mice in the i.p.-i.p. model, in which leukemic cells are inoculated i.p. and drugs are also injected i.p. [19].

However, in general, this is said to be a quite artificial therapeutic model in which relatively high therapeutic effects can be observed. Therefore, to assess the therapeutic effects of this type of combination therapy under more severe conditions, we used the i.v.-i.v. model of P388 leukemia in the present study because this model seems to reproduce in mice the pharmacological as well as pathological features of clinical leukemia. Furthermore, we investigated whether AHC-52 could potentiate the therapeutic effects of Adriamycin (ADR), etoposide (VP-16), and VCR in this model.

In this study, we used VCR- and ADR-resistant sublines of P388, that is, P388/VCR and P388/ADR. Characteristic phenotypes for MDR, including broad cross-resistance to a number of MDR-related antitumor agents [9, 24], active efflux of these agents [5, 6], expression of P-glycoprotein [16, 17], and reversal of resistance by various agents such as verapamil [21, 22] were observed in both cell lines. These observations clearly indicate that these cell lines are MDR leukemia cells. The only apparent difference between them involves the degree of their resistance; P388/ADR is much more resistant to various MDR-related agents and expresses more P-glycoprotein than P388/VCR [17]. It should be noted that slight but nonnegligible expression of P-glycoprotein has been detected even in parent P388 cells by Western-blot analysis [16].

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**Table 1.** Effect of AHC-52 on the in vitro sensitivity to VCR, ADR, and VP-16 of P388, P388/VCR, and P388/ADR cells

| Antitumor agent | AHC-52 | $IC_{50}$ (nM) | | |
|---|---|---|---|---|
| | | P388 | P388/VCR | P388/ADR |
| VCR | – | 4.2 (1.0) | 110 (26) | 270 (64) |
| | + | 0.63 (0.15) | 4.6 (1.1) | 9.5 (2.3) |
| ADR | – | 5.7 (1.0) | 33 (5.8) | 460 (81) |
| | + | 4.6 (0.8) | 4.1 (0.7) | 57 (10) |
| VP-16 | – | 54 (1.0) | 370 (6.9) | 6,300 (117) |
| | + | 31 (0.6) | 15 (0.3) | 6,600 (122) |

Cells were cultured for 72 h with various concentrations of antitumor agent in the presence or absence of AHC-52 (1 μg/ml), and $IC_{50}$ values were then determined by the tetrazolium-based assay. Each value represents the mean of 3 determinations. Numbers in parentheses represent relative values as compared with the $IC_{50}$ value for P388 cells incubated with antitumor agent in the absence of AHC-52

## Materials and methods

*Drugs.* VCR, ADR, and VP-16 for clinical use were purchased from Shionogi and Co. (Osaka), Kyowa Hakko Kogyo Co. (Tokyo), and Nihon Kayaku Co. (Tokyo), respectively. AHC-52 [methyl-2-(*N*-benzylamino)ethyl-2,6-dimethyl-4-(2-isopropylpyrazolo[1,5a]-pyridine-3-yl)-1,4-dihydropyridine-3,5-dicarboxylate] was synthesized according to a previously reported method [7]. It was dissolved in a small volume of 1 *N* HCl solution, diluted with 0.85% NaCl solution containing 0.2% Emulphor (GAF Co., New York, N.Y.), pH-adjusted to 4.0 with 1 *N* NaOH, and sterilized through a Millipore filter (pore size, 0.45 μm).

*Animal and tumor cells.* Female BALB/c × DBA/2 (hereafter referred to as $CD2F_1$) mice weighing 20–23 g were purchased from Charles River Japan, Inc. (Atsugi, Japan). P388, P388/VCR, and P388/ADR cell lines were supplied by the National Cancer Institute (NIH, Bethesda, Md., USA). The parent line and these resistant sublines were passaged weekly through $CD2F_1$ mice.

*In vitro MTT assay.* Cytotoxicity was evaluated by the tetrazolium-based assay [13]. First, cells were plated into 96-well microtiter plates at 6,000 cells/well in 60 μl culture medium. Next, 60 μl antitumor-agent solution diluted with the same medium in the presence or absence of AHC-52 was added, and the mixture was incubated at 37° C. RPMI 1640 supplemented with 10% fetal calf serum was used as the culture medium. At 72 h after plating, 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 2.5 mg/ml) was added to each well, and the plate was incubated at 37° C for 3 h. The dark-colored crystals that formed were solubilized by vigorous pipetting following the addition of 2-isopropanol containing 0.04 *N* HCl (180 μl/plate). After the solution had been allowed to stand for a few minutes at room temperature, the plates were read on a microplate reader (Titertek Multiskan MCC/340 MKII; Dainippon Pharmaceutical Co., Ltd., Osaka) using a sample wavelength of 540 nm. The percentage of cytotoxicity was determined according to optical density (*D*) using the following equation:

$$\text{Cytotoxicity (\%)} = \frac{D_{\text{sample}} - D_{\text{blank}}}{D_{\text{control}} - D_{\text{blank}}} \times 100.$$

*Colony-forming assay.* In the colony-forming assay, 0.25 ml 0.3% agarose (Sea Plaque; FMC Corporation, Rockland, Me., USA) prepared by mixing 1 vol. melted 3% agarose with 9 vol. RPMI 1640 supplemented as described above was added to each well of a 24-well plate (Corning Laboratory Sciences Co., New York, N.Y., USA) as an underlayer, and plates were refrigerated for 1 h at 4° C. Onto this layer, 0.25 ml of a mixture consisting of cell suspension (2,000 cells), a concentration of drug solution 10-fold that of the final concentration, and 3% agarose (8:1:1, by vol.) were added, and the plates were again refrigerated for 1 h at 4° C. The plates were then transferred to a $CO_2$ incubator and the cells were cultured for 4 days at 37° C, after which 40 μl MTT solution (5 mg/ml distilled water) was added to each well. After further incubation for 4 h at 37° C, the stained colonies were counted with a Model CA-7 Colony Analyzer (Oriental Instruments, Ltd., Tokyo).

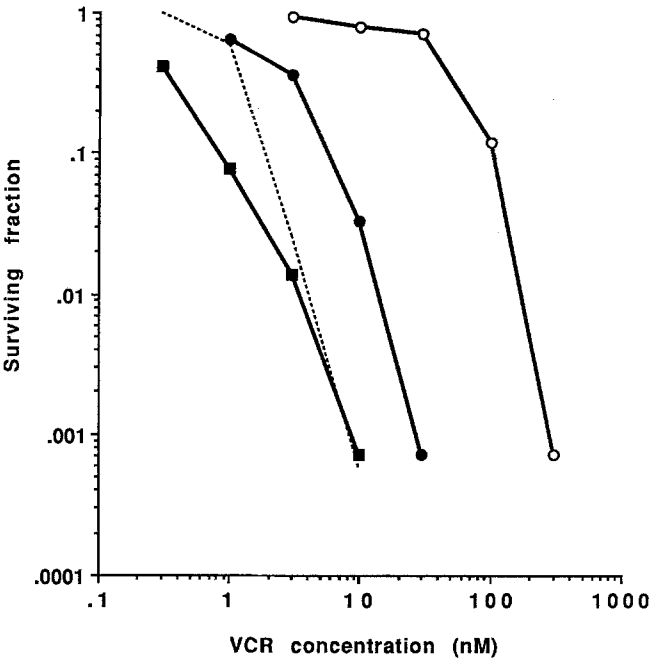


**Fig. 1.** Effects of AHC-52 on resistance to VCR of P388/VCR cells in a colony-forming assay. P388/VCR cells were cultured on a double layer of soft agarose containing various concentrations of VCR together with AHC-52 concentrations of 0 (○), 1 (●), or 2 μg/ml (■). After 4 days of culture, the colonies formed were counted. Parent P388 cells (*broken line*) were cultured in the absence of AHC-52. Each point represents the mean value for 3 determinations whose coefficient of variation was <10%

*In vivo experiments.* Leukemia cells ($2 \times 10^5$ cells) were inoculated i.v. into $CD2F_1$ mice. Antitumor agent was injected daily i.v. on days 1–4. In the case of ADR, a single i.v. injection given on day 1 only was also attempted. AHC-52 was injected i.p. twice a day for 4 days; the first injection was given just prior to the administration of the antitumor agent, and the second was given approximately 8 h later.

## Results

### *Degrees of resistance and resensitization of cells by AHC-52 in vitro*

The sensitivity of P388, P388/VCR, and P388/ADR cells to VCR, ADR, and VP-16 was measured by the tetrazolium-based assay. The degree of resistance was determined as a ratio of the 50% growth-inhibitory concentration ($IC_{50}$) in resistant cells to that in sensitive cells. As shown in Table 1, 26-, 5.8-, and 6.9-fold degrees of resistance or cross-resistance to VCR, ADR, and VP-16, respectively, were found for P388/VCR cells, and the respective values determined for P388/ADR cells were 64-, 81-, and 117-fold degrees of resistance. AHC-52 (1 μg/ml) was

**Table 2.** Comparison of single-agent and combination therapy with VCR and VCR plus AHC-52, respectively, in P388/VCR-bearing mice

| Single treatment | | | Combined treatment | | | |
|---|---|---|---|---|---|---|
| VCR (mg/kg) | Median survival in days (range) | T/C (%) | VCR (mg/kg) | AHC-52 (mg/kg) | Median survival in days (range) | T/C (%) |
| 0 | 10.1 ( 9–11) | 100 | 0 | 75×2 | 10.9 (10–11) | 108 |
| 0.1 | 9.8 ( 9–11) | 97 | 0.1 | 75×2 | 11.9 (10–12) | 118 |
| 0.2 | 10.5 (10–12) | 104 | 0.2 | 75×2 | 12.0 (10–12) | 119 |
| 0.3 | 10.0 (10) | 99 | 0.3 | 75×2 | 12.5 (12–13)* | 124 |
| 0.5 | 11.5 (10–12) | 114 | 0.5 | 75×2 | 7.0 (7) | 69 |

Six mice in a group transplanted i.v. with $2 \times 10^5$ P388/VCR cells were treated with VCR (i.v.) and AHC-52 (i.p., twice daily at intervals of 8 h) for 4 consecutive days

* Significantly different from the result obtained using the same dose of VCR alone ($P < 0.01$; Wilcoxon's rank-sum test)

**Table 3.** Comparison of single-agent and combination therapy with ADR and ADR plus AHC-52, respectively, in P388/VCR-bearing mice

| Experiment | Single treatment | | | Combination treatment | | | |
|---|---|---|---|---|---|---|---|
| | ADR (mg/kg) | Median survival in days (range) | T/C (%) | ADR (mg/kg) | AHC-52 (mg/kg) | Median survival in days (range) | T/C (%) |
| 1 | 0 | 9.7 ( 9–10) | 100 | 0 | 75×2 | 10.9 (10–11) | 100 |
| | 1 | 10.0 ( 9–10) | 103 | 1 | 75×2 | 11.4 (11–13)* | 117 |
| | 2 | 10.4 (10–11) | 107 | 2 | 75×2 | 15.0 (12–17)* | 155 |
| | 3 | 11.1 (10–12) | 114 | 3 | 75×2 | 19.0 (13–23)* | 196 |
| 2 | 0 | 10.4 (10–11) | 100 | | | | |
| | 2.5 | 10.7 ( 9–11) | 103 | 2.5 | 100×2 | 12.3 (11–13) | 118 |
| | 5.0 | 11.0 (10–12) | 106 | 5.0 | 100×2 | 12.9 (11–13) | 124 |
| | 7.5 | 11.0 (10–12) | 106 | 7.5 | 100×2 | 14.0 (13–16)* | 135 |
| | 10.0 | 11.0 (11) | 106 | 10.0 | 100×2 | 16.0 (15–19)* | 154 |

Six mice in a group transplanted i.v. with $2 \times 10^5$ P388/VCR cells were treated with ADR (i.v.) daily (experiment 1) or once on day 1 (experiment 2) and AHC-52 (i.p., twice daily at intervals of 8 h) for 4 consecutive days

* Significantly different from the result obtained using the same dose of ADR alone ($P < 0.01$; Wilcoxon's rank-sum test)

capable of completely restoring the sensitivity of P388/VCR cells to all of these drugs. However, in P388/ADR cells, the high degree of resistance to VCR and ADR was not completely reversed by AHC-52, and that to VP-16 remained entirely unchanged. In the colony-forming assay, sensitizing activity was also observed for AHC-52 as shown in Fig. 1. At 2 μg/ml, AHC-52 restored the sensitivity to VCR of P388/VCR cells to levels almost the same as those shown by the sensitive parent P388 cells.

*Therapeutic effects of antitumor agent and AHC-52 on resistant cells*

In the present study, leukemic cells were inoculated i.v. and antitumor agents were injected via the i.v. route, but AHC-52 was injected i.p. because bolus i.v. injection significantly reduced the maximum tolerated dose. Although we recognized that a slow i.v. infusion enables safe administration of the same dose, in this study we injected AHC-52 only by the i.p. route for technical reasons. Since some detergents are known to show MDR-reversal activity, in a control experiment we treated P388- or P388/VCR-bearing mice with VCR and the vehicle solution containing Emulphor (for AHC-52). As a result, potentiation of therapeutic effects by the vehicle solution was not observed.

In contrast to the results we previously obtained in the i.p.-i.p. model [19], the combination of VCR and AHC-52 did not appreciably affect the survival of P388/VCR-bearing mice (Table 2). Table 3 shows the results obtained using ADR and AHC-52. Following either daily or single administration, ADR alone did not have any effect on the survival of P388/VCR-bearing mice; however, when given in combination with AHC-52, it significantly improved the survival (maximally, 196% and 154% for the respective schedules) of these mice. VP-16 alone significantly enhanced the survival of mice, and its effectiveness was greatly increased when it was given in combination with AHC-52 (Table 4). When given in combination with AHC-52, neither VCR, ADR, nor VP-16 affected the survival of mice bearing the highly resistant P388/ADR line (data not shown).

*Therapeutic effects of antitumor agent and AHC-52 on parent P388 cells*

In the i.v.-i.v. model, the therapeutic effects of the present combination treatments were also tested on mice bearing the parental P388 cells. In this model, VCR did not considerably affect the survival of sensitive P388-bearing mice. These results differ greatly from those previously

**Table 4.** Comparison of single-agent and combination therapy with VP-16 and VP-16 plus AHC-52, respectively, in P388/VCR-bearing mice

| Single treatment | | | Combination treatment | | | |
|---|---|---|---|---|---|---|
| VP-16 mg/kg | Median survival in days (range) | T/C (%) | VP-16 (mg/kg) | AHC-52 (mg/kg) | Median survival in days (range) | T/C (%) |
| 0 | 9.0 ( 9) | 100 | 0 | 75×2 | 9.7 ( 9–10) | 108 |
| 5 | 12.0 (12) | 133 | 5 | 75×2 | 15.1 (14–16)* | 168 |
| 10 | 13.3 (13–14) | 149 | 10 | 75×2 | 20.0 (19–23)* | 222 |
| 20 | 16.0 (16) | 178 | 20 | 75×2 | 24.3 ( 6–28) | 270 |

Six mice in a group transplanted i.v. with $2\times10^5$ P388/VCR cells were treated with VP-16 (i.v.) and AHC-52 (i.p., twice daily at intervals of 8 h) for 4 consecutive days

* Significantly different from the result obtained using the same dose of VP-16 alone ($P$ <0.01; Wilcoxon's rank-sum test)

**Table 5.** Comparison of single-agent and combination therapy with VCR and VCR plus AHC-52, respectively, in P388-bearing mice

| Single treatment | | | Combined treatment | | | | |
|---|---|---|---|---|---|---|---|
| VCR (mg/kg) | Median survival in days (range) | T/C (%) | VCR (mg/kg) | AHC-52 (mg/kg) | Median survival in days (range) | T/C (%) | Survivors |
| 0 | 7.8 (7–9) | 100 | 0 | 75×2 | 9.0 ( 8–19) | 115 | 0/6 |
| 0.1 | 8.0 (7–8) | 103 | 0.1 | 75×2 | 13.8 (13–16)* | 177 | 0/6 |
| 0.2 | 8.1 (7–9) | 104 | 0.2 | 75×2 | 19.0 (16–21)* | 244 | 0/6 |
| 0.3 | 8.9 (8–9) | 114 | 0.3 | 75×2 | 21.0 (20–23)* | 269 | 0/6 |
| 0.4 | 9.7 (9–10) | 124 | 0.4 | 75×2 | >60.0 (16)* | >769 | 4/6 |

Six mice in a group transplanted i.v. with $2\times10^5$ P388 cells were treated with VCR (i.v.) and AHC-52 (i.p., twice daily at intervals of 8 h) for 4 consecutive days

* Significantly different from the result obtained using the same dose of VCR alone ($P$ <0.01; Wilcoxon's rank-sum test)

**Table 6.** Comparison of single-agent and combination therapy with ADR and ADR plus AHC-52, respectively, in P388-bearing mice

| Experiment | Single treatment | | | Combined treatment | | | | |
|---|---|---|---|---|---|---|---|---|
| | ADR (mg/kg) | Median survival in days (range) | T/C (%) | ADR (mg/kg) | AHC-52 (mg/kg) | Median survival in days (range) | T/C (%) | Survivors |
| 1 | 0 | 7.4 ( 7–8) | 100 | | | | | |
| | 1 | 8.4 ( 8–9) | 114 | 1 | 75×2 | 13.9 (11–14) | 188 | 0/6 |
| | 2 | 8.4 ( 8–9) | 114 | 2 | 75×2 | 18.3 (16–20) | 247 | 0/6 |
| | 3 | 11.3 (10–13) | 153 | 3 | 75×2 | 22.0 (19–28) | 297 | 0/6 |
| 2 | 0 | 7.4 ( 7–8) | 100 | | | | | |
| | 2.5 | 8.9 ( 8–9) | 120 | 2.5 | 100×2 | 11.8 ( 9–13)* | 159 | 0/6 |
| | 5.0 | 10.3 ( 9–12) | 139 | 5.0 | 100×2 | 17.0 (15)* | 230 | 1/6 |
| | 7.5 | 14.8 (13–17) | 200 | 7.5 | 100×2 | 22.3 (19–27)* | 310 | 0/6 |
| | 10.0 | 15.8 (15–17) | 214 | 10.0 | 100×2 | >60 (22)* | >811 | 3/6 |

Six mice in a group transplanted i.v. with $2\times10^5$ P388/VCR cells were treated with ADR (i.v.) daily (experiment 1) or once on day 1 (experiment 2) and AHC-52 (i.p., twice daily at intervals of 8 h) for 4 consecutive days

* Significantly different from the result obtained using the same dose of ADR alone ($P$ <0.01; Wilcoxon's rank-sum test)

obtained in the i.p.-i.p. model. At the highest dose tested (0.4 mg/kg), VCR alone had only a marginal effect on survival (124%); however, when combined with AHC-52, it produced survival values as high as 177%, even at a dose of 0.1 mg/kg. It should especially be noted that this combination therapy had a curative effect on four of six mice at 0.4 mg/kg (Table 5).

P388-bearing mice were treated with ADR on two different schedules (Table 6). Following daily treatments, ADR had no significant effect on survival at doses of 1 and 2 mg/kg, but a significant increase in survival (153%) was noted at 3 mg/kg. However, the combination administration of ADR and AHC-52 produced a remarkable survival value of 188% at an ADR dose as low as 1 mg/kg. At higher ADR doses, this combination resulted in much greater survival benefits as compared with those provided by ADR alone. On the other hand, a single injection of ADR alone at 7.5 and 10 mg/kg produced a maximal survival value of about 200%. Coadministration of AHC-52 potentiated the effect of ADR, providing significantly greater effects at all ADR doses tested, and a curative effect was seen at 10 mg/kg.

In the case of VP-16, the effect of combined treatment was clearly observed in P388-bearing mice. VP-16 alone produced a survival value of 176% at 5 mg/kg, but the same dose given in combination with AHC-52 significant-

**Table 7.** Comparison of single-agent and combination therapy with VP-16 and VP-16 plus AHC-52, respectively, in P388-bearing mice

| Single treatment | | | | Combined treatment | | | | |
|---|---|---|---|---|---|---|---|---|
| VP-16 (mg/kg) | Median survival in days (range) | T/C (%) | Survivors | VP-16 (mg/kg) | AHC-52 (mg/kg) | Median survival in days (range) | T/C (%) | Survivors |
| 0 | 8.8 ( 8–9) | 100 | 0/6 | 0 | 75×2 | 9.2 ( 9–10) | 102 | 0/6 |
| 5 | 15.7 (15–17) | 176 | 0/6 | 5 | 75×2 | 21.0 (18–27)* | 233 | 0/6 |
| 10 | 27.6 (24) | 333 | 1/6 | 10 | 75×2 | >59.9 (23) | >666 | 4/6 |

Six mice in a group transplanted i. v. with $2 \times 10^5$ P388 cells were treated with VP-16 (i. v.) and AHC-52 (i. p., twice daily at intervals of 8 h) for 4 consecutive days

* Significantly different from the result obtained using the same dose of VP-16 alone ($P < 0.01$; Wilcoxon's rank-sum test)

ly enhanced this effect (233%). At 10 mg/kg, VP-16 alone had a curative effect on one of six mice, but when the same dose was used in combination with AHC-52, four of six mice survived (Table 7).

## Discussion

Acute leukemia is one of the most sensitive cancers to chemotherapeutic treatments. Extremely high response rates are generally obtained during standard chemotherapy using anthracyclines, vinca alkaloids, and antimetabolites as the major drugs, resulting in significant life-prolonging effects. However, the cure rates remain unsatisfactory in spite of the high chemosensitivity of this disease. This observation strongly suggests that acquisition of drug resistance is involved in the restricted clinical effects of such chemotherapy on leukemia. In fact, the MDR phenotype has been reported to be expressed often in human acute myeloid and lymphatic leukemias and to correlate with their chemotherapeutic responsiveness [1, 4, 8, 10, 11, 14, 15, 18]. Therefore, it seems that leukemia should be the first target for the current combination regimen developed to circumvent MDR.

From such a standpoint, we tested the therapeutic efficacy of the combination of VCR, ADR, or VP-16 with AHC-52 using the i. v. inoculated P388 leukemia model. Unexpectedly, VCR alone did not have any significant effect on the survival of sensitive P388-bearing mice in the present i. v.-i. v. model. However, at the highest dose tested, this drug promoted marked survival, when given in combination with AHC-52, with four of six mice surviving. This remarkable effect was probably caused by further sensitization of the parent P388 cells by AHC-52 as suggested by the results obtained in vitro. In addition, the killing of a small number of resistant cells preexisting in the parent P388 cell population by the combination of both drugs may also have contributed to the therapeutic effect on P388-bearing mice. On the other hand, P388/VCR cells were also highly sensitized in vitro by AHC-52, but VCR had little or no therapeutic effect on P388/VCR-bearing mice, even when it was combined with AHC-52. These data suggest that in vivo sensitization by AHC-52 was not sufficient to produce the therapeutic combined effect.

In contrast to VCR, ADR produced prominent survival values of maximally 153% and 214% in mice bearing the parent P388 leukemia when given on daily and single-treatment schedules, respectively. Irrespective of the slight sensitization of P388 cells to ADR by AHC-52 in vitro, the therapeutic effect of ADR on P388-bearing mice was markedly enhanced by AHC-52. ADR given at 10 mg/kg as a one-time injection had a curative effect on three of six mice, again suggesting circumvention of the heterogeneous drug sensitivity of the parent P388 cell population. It should particularly be noted that ADR alone had no effect on the survival of P388/VCR-bearing mice when given on either schedule, whereas the combination of ADR and AHC-52 was quite effective. The remarkable effect produced by this combination suggests that P388/VCR cells were sensitized in vivo by AHC-52 to a level close to that shown by the parent P388 cells.

VP-16 alone promoted significant survival of P388-bearing as well as P388/VCR-bearing mice, although evident cross-resistance to VP-16 was observed in P388/VCR cells in vitro. It is likely that the potent therapeutic activity of VP-16 exceeded the cellular level of cross-resistance. Such therapeutic effects on both P388- and P388/VCR-bearing mice were further potentiated by AHC-52.

In contrast to the results obtained in mice bearing P388 and P388/VCR, none of the antitumor agents had a significant therapeutic effect on mice bearing the highly resistant subline P388/ADR, even when they were given in combination with AHC-52.

These results may be summarized as follows. First, the combination of antitumor agent with AHC-52 obviously produced better therapeutic effects on mice bearing sensitive parent P388 leukemia than did the antitumor agent alone. Practically, the concurrent use of AHC-52 further potentiated the therapeutic activity of ADR and VP-16, and whereas VCR alone had no effect on survival, its combination with AHC-52 endowed it with potent therapeutic activity. These findings indicate that AHC-52 was capable of effectively sensitizing not only resistant but also sensitive cells in the i. v. inoculated leukemia model.

Second, although the present combination therapy was effective in vivo against resistant P388 leukemia in some cases, it was ineffective in other cases. Sensitization of cells to a given antitumor drug requires that they be exposed to an effective concentration of the sensitizing agent together with the antitumor drug for a certain period; the more resistant the cells, the more severe the minimal requirement for effective sensitization. Furthermore, the satisfaction of this requirement seems to be more difficult (a) in vivo than in vitro and (b) in the i. v. model than in the

i.p. model. In vitro studies (Table 1) demonstrated that P388/VCR was more resistant to VCR than to ADR and that P388/ADR was highly resistant to all three of the drugs tested. Considering these points, the slight response, if any of P388/VCR to VCR plus AHC-52 as well as that of P388/ADR to the combination of any antitumor agent with AHC-52 are easily understandable.

In conclusion, the present results raise the possibility that this type of combination regimen might be effective in the clinical treatment of leukemia, since AHC-52 was capable of sensitizing resistant cells even under therapeutic conditions nearly as difficult as those encountered in the clinical setting. Our results also suggest that this combination therapy should be performed not only during the late stage for recurrent resistant diseases but also during the early stage for previously untreated sensitive leukemia. For the achievement of curative effects, the latter approach seems more desirable. Furthermore, as a typical drug-sensitive cancer, leukemia is perhaps the most suitable disease in which the clinical efficacy of this novel combination therapy developed to circumvent multidrug resistance should be initially tested. We are currently investigating the pharmacokinetic basis underlying the present therapeutic results and the effectiveness of this combination regimen against experimental solid tumors.

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