Jpn. J. Cancer Res. 81, 834–841, August 1990

Synergistic Effect of Cyclosporin A and Verapamil in Overcoming Vincristine Resistance of Multidrug-resistant Cultured Human Leukemia Cells

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Reversal of vincristine (VCR) resistance by cyclosporin A (CyA) or the combination of CyA and verapamil (VER) was investigated by using four P-glycoprotein (P-gp)-associated human multidrug-resistant (MDR) cell lines (K562/ADM, KYO-1, HEL and CMK). Drug sensitivity was expressed as 50% inhibitory concentration (IC_{50}). The degree of reversal of resistance was expressed as *x*-fold decrease by dividing the IC_{50} value without modifier(s) by that with modifier(s). CyA overcame P-gp-associated MDR significantly in all four MDR cell lines. Reversal of VCR resistance by CyA appeared to be dose-dependent. In the case of low-grade MDR cell lines (KYO-1, HEL and CMK), CyA at the low concentration of 0.5 μg/ml was still effective. The degree of reversal of VCR resistance in this condition was greater (6.3- to 16-fold decrease) in the low-grade MDR cell lines than in a high-grade MDR cell line (K562/ADM) (2.9-fold decrease). At a high concentration (5 μg/ml) of CyA, however, it was greater (240-fold decrease) in the high-grade MDR cell lines than in the low-grade MDR cell line (20- to 100-fold decrease). This indicates that concentration of CyA required for overcoming drug resistance in MDR cells was dependent on the degree of drug resistance. CyA overcame VCR resistance more efficiently than VER. The combination of CyA and VER enhanced reversal of VCR resistance in a supra-additive or at least an additive manner and overcame VCR resistance at low concentrations of both modifiers that are clinically achievable with safety.

Key words: Multidrug resistance — Cyclosporin A — Verapamil — Leukemia — Lymphoma

The complete remission rate achieved in adult leukemias and lymphomas by the application of recently developed combination chemotherapeutic methods has steadily increased, but many patients still relapse and eventually die. The most common and important cause of treatment failure is drug resistance. Various mechanisms of drug resistance have been reported to date.[1,2] Among them, multidrug resistance (MDR) has been studied extensively.[3–5] MDR presents a serious problem in cancer chemotherapy, because MDR cells are resistant to a group of anticancer agents such as *vinca* alkaloids, anthracyclines, mitoxantrone, etoposide (VP16) and actinomycin-D (ACT-D), which are most commonly used clinically. Classical MDR is caused by decreased intracellular drug concentration as a result of increased drug efflux, which is closely associated with a 170 kilodalton glycoprotein termed P-glycoprotein (P-gp).[3–5] Identification of P-gp has provided a target for current efforts aimed at overcoming MDR in cancer. It has been reported that various agents overcame P-gp-associated drug resistance. They include calcium antagonists,[6,7] calmodulin inhibitors,[7] quinidine,[8] reserpine,[9] cyclosporin A (CyA),[10,11] some isoprenoids,[12] cepharanthine[13] and so on. Among them, we are interested in CyA, firstly because CyA has a stronger binding capacity with P-gp than verapamil (VER),[14] secondly because CyA has been widely used as a immunosuppressant for bone marrow transplantation and various organ transplantations,[15,16] and thirdly because CyA can be maintained at a relatively high blood concentration.

In the present study we found that CyA had much greater activity for reversal of vincristine (VCR) resistance of MDR cells than VER, and that the combination of CyA and VER overcame VCR resistance in a synergistic or at least an additive manner.

MATERIALS AND METHODS

Drugs VCR and vinblastine (VLB) were obtained from Shionogi & Co., Ltd. (Osaka), doxorubicin (ADM) from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo), daunorubicin (DRN) from Meiji Seika Kaisha, Ltd. (Tokyo), ACT-D from Banyu Pharmaceutical Co., Ltd. (Tokyo), VP16 and bleomycin (BLM) from Nippon Kayaku Co., Ltd. (Tokyo), methotrexate (MTX) from Lederle (Japan), Ltd. (Tokyo), and nimustine hydrochloride (ACNU) from Sankyo Co., Ltd. (Tokyo). CyA and VER were purchased from Sandoz Pharmaceutical Co., Ltd. (Tokyo) and Eisai Pharmaceutical Co., Ltd. (Tokyo), respectively. Each drug was dissolved in physiological saline and diluted in RPMI-1640 growth medium (GIBCO, Grand Island, N.Y.) to an appropriate concentration immediately before each experiment.

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Table I. Characterization of Cell Lines

Cell line[a]	Doubling time (h)	Relative resistance index to VCR[b]	P-glycoprotein[c]	
			MRK16-F $(ab')_2$ (positive percent)	mRNA
K562/ADM	31	500	100	overexpressed
KYO-1	40	23	74	overexpressed
HEL	27	16	62	overexpressed
CMK	36	6.5	41	overexpressed
K562	24	1.0	0	negative
P3HR-1	22	0.3	0	negative

a) Original papers on each cell line were listed in a previous report.[17]
b) IC_{50} of each cell line was divided by IC_{50} of K562 to get the degree of relative resistance as reported previously.[17]
c) Data were cited from our previous report.[17]

Cell lines In this study we used six human cultured cell lines derived from various leukemias and lymphoma. The characteristics of these cell lines are summarized in Table I. As reported previously,[17] K562/ADM is an ADM-resistant subline of K562 derived from chronic myelogenous leukemia in blastic crisis (CMLbc), and is characterized as a high-grade MDR cell line, having a relative resistance index of as high as 500. KYO-1 derived from CMLbc, HEL derived from erythroleukemia and CMK derived from acute megakaryoblastic leukemia are P-gp-associated MDR cell lines.[17] The three cell lines were characterized as low-grade MDR cell lines with relative resistance index values of about 10 to 20. P3HR-1 derived from Burkitt's lymphoma and K562 were used as control cells which had no overexpression of P-gp.[17] The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (GIBCO, Lot No. 27N8433), 100 μg/ml aminobenzyl penicillin and 20 μg/ml gentamicin, in a floating state at 37°C under a humidified atmosphere in a 5% CO_2 incubator (Forma Scientific Ltd., Marietta, Ohio). K562/ADM has been maintained in the presence of 300 ng/ml of ADM. None of the cell lines except K562/ADM had ever been exposed to any anticancer agent *in vitro*. These cells were used for experiments in the exponentially proliferating phase.

Drug sensitivity The cells were suspended in a fresh growth medium in a 24-well dish (Corning Glass Works, N.Y.) at an appropriate concentration of cells ($1.0–2.0 \times 10^5$/ml) according to the growth rate and the cell density required for exponential growth. The cells were cultured in the presence of graded concentrations of drug for 72 h and counted with a Coulter Counter (Multisizer, Coulter Electronics Ltd., Luton, England) as described previously.[17,18] Drug sensitivity was expressed in terms of the concentration of drug required for 50% inhibition of cell growth (IC_{50}) as reported before.[17,18] The relative resistance index of each cell line to drugs was calculated by dividing the IC_{50} value of the test cell line by that of K562 cell line as reported previously.[17] All experiments were performed in triplicate.

Reversal of resistance by CyA In order to observe the degree of reversal of VCR-resistance, each cell line was treated with graded concentrations of CyA in the presence of a given concentration of VCR. In the case of other anticancer agents, two representative MDR cell lines, K562/ADM and HEL, were treated with graded concentrations of anticancer agents in the presence or absence of a given concentration of CyA. The degree of reversal of resistance by CyA was expressed as an x-fold decrease by dividing the IC_{50} value in the absence of CyA by that in the presence of CyA. The cells treated with CyA alone served as one control to determine if CyA alone had a growth-inhibitory effect on the cultured cells.

Reversal of VCR resistance by the combination of CyA and VER A P-gp-overexpressing cell line, HEL,[17] was used to investigate reversal of VCR-resistance by the combination of CyA and VER. It was treated with VCR at 2 ng/ml (2.17 n*M*), which corresponded to about 1/14 IC_{50} value of VCR for HEL. The growth-inhibitory activity of VCR at 2 ng/ml was determined in the presence of graded concentrations of VER at a given concentration of CyA.

Isobologram analysis Isobologram analysis of the interaction of two drugs was performed by constructing "an envelope of additivity"[19,20] on the isobologram as described elsewhere.[19–21] Based on available dose-response curves, we analyzed the combined effect of two drugs (CyA and VER) on reversal of VCR resistance at the point of IC_{50}. As described elsewhere,[21] three isoeffect curves (Fig. 1) can be drawn as follows:

(1) Model I line: When the dose of drug A is chosen, there remains an increment in effect to be produced by drug B. If the two drugs act independently, the addition

is performed by taking the increment in doses, starting from zero, that gives a surviving fraction that adds up to IC_{50}.

(2) Model II(A) line: When the dose of drug A is chosen, an isoeffect curve can also be calculated by taking the dose increment of drug B that gives the required contribution to the total effect up to the limit, in this case, IC_{50}.

(3) Model II(B) line: Similarly, when the dose of drug B is chosen, there remains an increment in effect to be produced by drug A that gives the required contribution to IC_{50}.

When drug A has a linear dose-response curve, the Model II(B) line will be identical to the Model I line, and *vice versa*. When both drugs have linear dose-response curves, all three lines will be converted to a straight line. The total area enclosed by these three lines conceivably represents the additive response or the "envelope of additivity" (Fig. 1).

With the combination of graded doses of drug A and a chosen dose of drug B, a single dose-response curve can be drawn. When the experimental IC_{50} concentration in this combination falls to the left of the envelope (Fig. 1, point Pa), the two drugs have supra-additive interaction. When the experimental data point is within the envelope, this combination is considered to be non-interactive (additive) (Fig. 1, point Pb). When this point is in the area to the right of the envelope, but within the square produced by 1.0 on the ordinate and abscissa, the drugs have subadditive interaction (Fig. 1, point Pc). When the point is outside the square, both drugs are considered to be mutually protective (Fig. 1, point Pd).

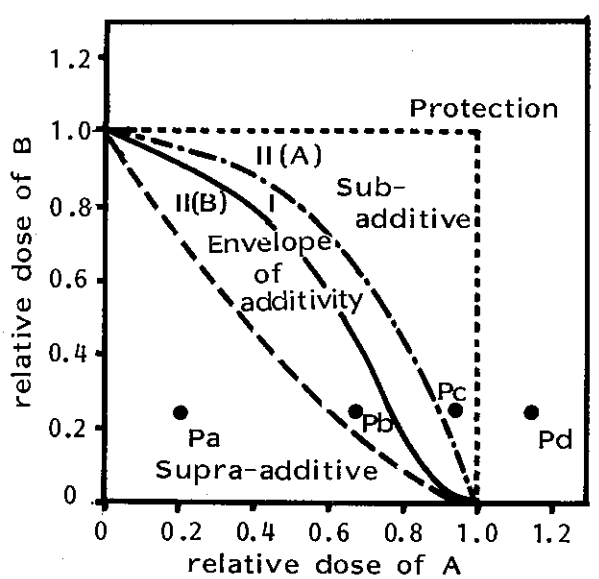


Fig. 1. Isobologram analysis. An envelope of additivity is constructed from the dose-response curve of two drugs (A and B). If the experimental data points (Pa, Pb, Pc, and Pd) fall in the areas specified in the diagram, the interaction of the two drugs can be classified as supra-additive, additive, subadditive, and mutually protective, respectively.

RESULTS

Dose-response effect of CyA on reversal of VCR-resistance Figure 2 shows the dose-response effect of CyA on the growth-inhibitory activity of VCR in two representative MDR cell lines (K562/ADM and HEL) and in one control sensitive cell line (P3HR-1). CyA enhanced the growth-inhibitory activity of VCR in a

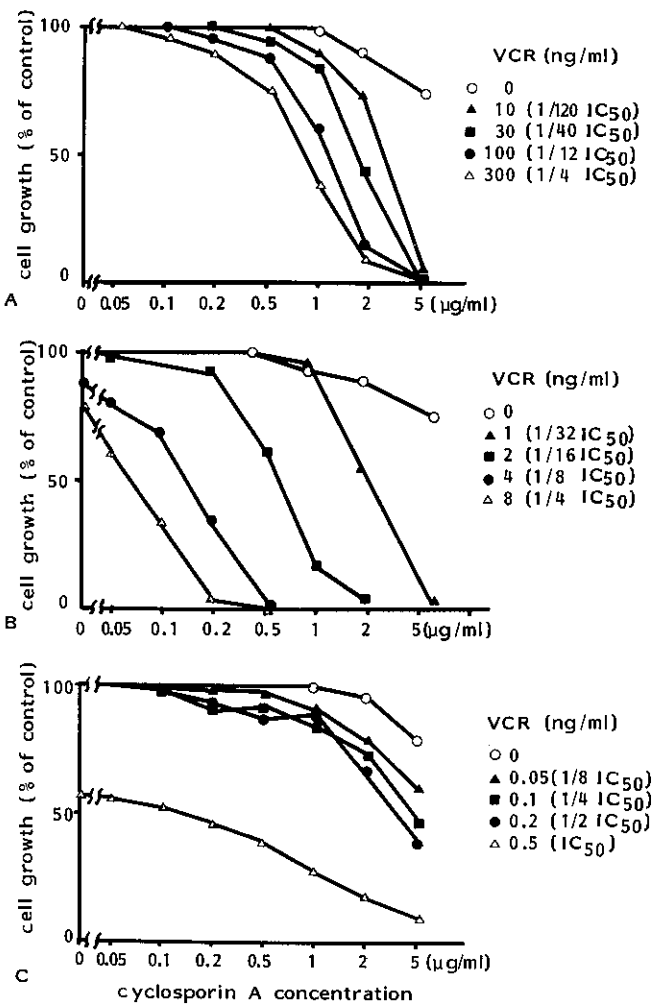


Fig. 2. Dose-response effect of CyA on reversal of VCR resistance. A. High-grade MDR K562/ADM cells. B. Low-grade MDR HEL cells. C. Control (non-MDR) P3HR-1 cells.

Table II. Dose-Response Effect of CyA on the Growth-inhibitory Activity of VCR

Cell line	IC_{50} (nM) of VCR (x-fold decrease)[a]			
	CyA 0	CyA 0.05	CyA 0.5	CyA 5.0 μg/ml
K562/ADM	1190	920 (1.3)	410 (2.9)	5.0 (240)
KYO-1	42	24 (1.8)	2.7 (16)	0.41 (100)
HEL	31	22 (1.4)	3.0 (10)	0.60 (52)
CMK	8.8	8.1 (1.1)	1.4 (6.3)	0.44 (20)
K562	1.7	1.9 (0.9)	1.2 (1.4)	0.50 (3.4)
P3HR-1	0.4	0.4 (1.0)	0.3 (1.3)	0.18 (2.2)

a) x-Fold decrease was obtained by dividing the IC_{50} value in the absence of CyA by that in the presence of CyA. It was used as an indicator of the degree of reversal of resistance by a modifier.
Significant potentiation ($P<0.01$) of VCR sensitivity was observed in all four P-gp-associated MDR cell lines (K562/ADM, KYO-1, HEL and CMK) by CyA at 0.5 and 5.0 μg/ml as compared to those in the control (non-MDR) cell lines (K562 and P3HR-1).

dose-dependent manner. In the two MDR cell lines, as the concentration of CyA increased, the growth-inhibitory activity VCR was greatly enhanced. However, a high concentration of CyA was necessary to overcome VCR resistance in K562/ADM, while a low concentration of CyA of 0.5 μg/ml was effective in HEL. In the control cell line (P3HR-1), on the other hand, no significant potentiation of the growth-inhibitory activity of VCR by CyA was observed.

The IC_{50} value of VCR against all cell lines in the presence or absence of a given concentration of CyA is shown in Table II. The IC_{50} value of VCR against four MDR cell lines, K562/ADM, KYO-1, HEL and CMK, decreased dramatically in the presence of CyA. The degree of reversal of VCR resistance by CyA at a low concentration (0.5 μg/ml) was greater in HEL (a low-grade MDR cell) than in K562/ADM (a high-grade MDR cell), but at a high concentration (5 μg/ml) of CyA it was greater in K562/ADM than in HEL. The

Table III. Significant Reversal of MDR by CyA at 5 μg/ml

	IC_{50} (nM) without CyA/IC_{50} (nM) with CyA (x-fold decrease)			
	K562/ADM	HEL	K562	P3HR-1
VCR	**1190/5.0 (240)**	**31/0.6 (52)**	1.91/0.35 (5.6)	0.67/0.22 (3.0)
VLB	**143/0.67 (240)**	**13/<1.1 (>11)**	3.20/1.16 (2.8)	1.98/1.20 (1.7)
ADM	**760/10.2 (74)**	**55/11 (5.0)**	12.4/6.97 (1.8)	2.28/2.21 (1.0)
DNR	**540/9.9 (54)**	**41/8.3 (4.9)**	12.8/9.75 (1.3)	3.83/4.56 (0.84)
ACT-D	**240/1.43 (170)**	**6.5/1.4 (4.6)**	3.51/4.95 (0.71)	0.73/1.17 (0.62)
VP-16	**1510/146 (10)**	**390/130 (3.0)**	59.8/32.3 (1.9)	8.66/13.9 (0.62)
BLM	1140/510 (2.2)	2950/3240 (0.91)	870/690 (1.3)	94/57 (1.6)
MTX	7.03/8.08 (0.87)	2.82/3.74 (0.75)	28.2/35.6 (0.79)	22.7/70.5 (0.32)
ACNU	6530/5210 (1.3)	20400/11000 (1.9)	2750/2780 (0.99)	1650/1910 (0.86)

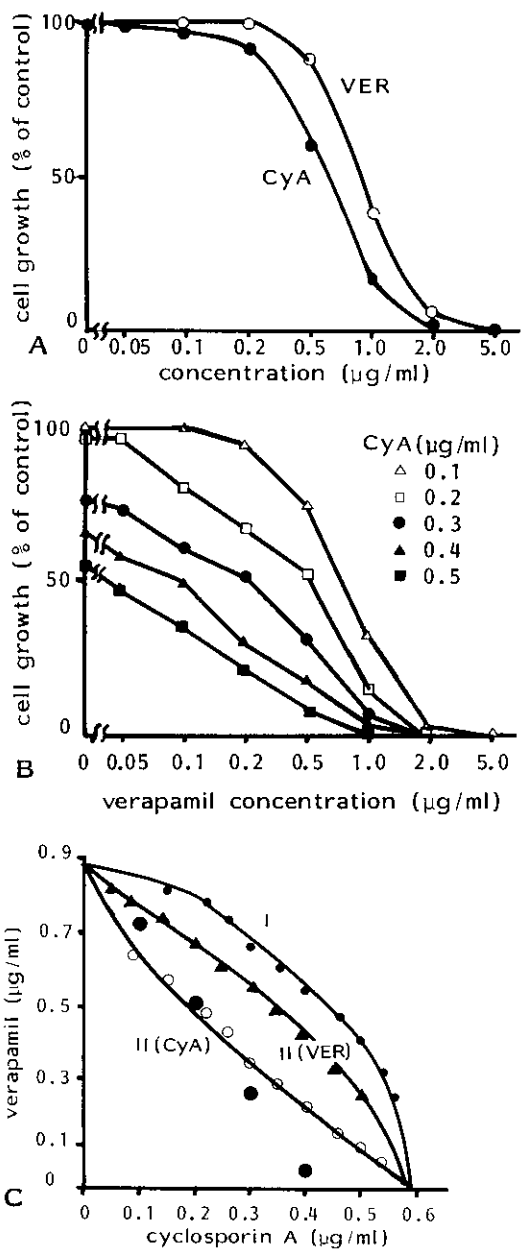
Date in bold face indicate significant reversal of resistance by CyA ($P<0.01$) in K562/ADM and HEL as compared to that in K562 and P3HR-1.

Table IV. Effect of 5.0 μg/ml of VER and 5.0 μg/ml of CyA on the Reversal of VCR Resistance

Cell line	IC_{50} (nM) of VCR		x-Fold decrease[a]	IC_{50} (nM) of VCR		x-Fold decrease[a]
	VER(−)	VER(+)		CyA(−)	CyA(+)	
K562/ADM	1100	23	48	1190	5.0	240
KYO-1	35	1.1	32	42	0.41	100
HEL	29	1.6	18	31	0.60	52
CMK	8.0	0.42	19	8.8	0.44	20
K562	1.7	0.62	2.7	1.7	0.50	3.4
P3HR-1	0.4	0.2	2.0	0.4	0.18	2.2

a) Significant potentiation ($P<0.01$) of drug sensitivity to VCR by both modifiers was observed in all four P-gp-associated MDR cell lines (K562/ADM, KYO-1, HEL and CMK) in comparison with those in the control (non-MDR) cell lines (K562 and P3HR-1).

IC_{50} value of VCR for KYO-1, HEL and CMK decreased as the concentration of CyA was increased. It was still high, ranging from 1.4 to 3.0 n*M*, when CyA was present at 0.5 μg/ml. When the CyA concentration was 5 μg/ml, the IC_{50} value of VCR for the three MDR cell lines decreased to 0.4 to 0.6 n*M*, which was almost equivalent to the IC_{50} value of sensitive P3HR-1 without CyA. On the other hand, the IC_{50} value of VCR for K562/ADM remained high (5 n*M*) even in the presence of CyA at 5 μg/ml.



Reversal of MDR by CyA The effect of CyA in overcoming MDR in the two representative MDR cell lines (K562/ADM and HEL) and two non-MDR cell lines (K562 and P3HR-1) was examined. The IC_{50} values of nine anticancer agents including VCR, VLB, ADM, DNR, ACT-D, VP-16, BLM, MTX and ACNU were measured for each cell line in the presence or absence of CyA at 5 μg/ml. As shown in Table III, resistance to VCR, VLB, ADM, DRN, ACT-D and VP16 was significantly reversed by CyA in the two MDR cell lines in terms of a high *x*-fold decrease, as compared to those of the two non-MDR cell lines.

In the cases of BLM, MTX, and ACNU, there was no significant change between the IC_{50} values that were measured in the presence and absence of CyA for each cell line. The results indicate that CyA could reverse P-gp-associated MDR specifically.

VCR resistance-reversal ability of CyA and VER The effect of two modifiers (VER and CyA) on the growth-inhibitory activity of VCR is shown in Table IV. Both VER and CyA enhanced the growth-inhibitory activity of VCR more markedly in the four MDR cell lines than in the two control (non-MDR) cell lines. In all four MDR cell lines, the degree of reversal of VCR resistance by CyA at 5 μg/ml was greater than that by VER at 5.0 μg/ml, indicating that CyA overcame VCR resistance more efficiently than VER. The greater the degree of VCR resistance, the greater the degree of reversal expressed as the *x*-fold decrease. However, with increase in the degree of resistance, greater concentrations of the modifier were required to overcome it.

Effect of combination with CyA and VER on reversal of VCR resistance Figure 3A shows the effect of CyA or

Fig. 3. Synergistic or at least additive effect of CyA and VER on reversal of VCR resistance. A. An MDR HEL cell line was treated with VCR at 2 ng/ml, which corresponded to 1/14 IC_{50} value. The growth-inhibitory activity of VCR at 2 ng/ml was determined in the presence of graded concentrations of VER or CyA. The concentration required for the IC_{50} value for HEL in the presence of VCR at 2 ng/ml was 0.9 μg/ml for VER and 0.6 μg/ml for CyA. B. The growth-inhibitory activity of VCR at 2 ng/ml was determined in the presence of graded concentrations of VER at each given concentration of CyA. CyA enhanced the effect of VER on the growth-inhibitory activity of VCR. C. Isobologram analysis of the interaction of two drugs (CyA and VER) was performed by constructing "an envelope of additivity" on the isobologram consisting of three lines, I (●), II (CyA) (○), and II(VER) (▲), which were obtained from the data shown in Fig. 3A by the method described the legend to Fig. 1 and in "Materials and Methods." The isoeffect points (IC_{50} in this case) (●) in combination with CyA and VER were obtained from the experiment shown in Fig. 3B. The points fell to the left of or within "the envelope of additivity," indicating a synergistic or at least additive effect of the two modifiers in overcoming VCR resistance.

VER on the growth-inhibitory activity of VCR at 2.0 ng/ml (2.17 n*M*) against HEL cell line. The IC_{50} value of VCR for HEL was approximately 30 n*M* in the absence of the modifiers (Tables II, III and IV), hence the concentration of 2.0 ng/ml was about 1/14 of the IC_{50} value. The modifier concentration required for the IC_{50} value for HEL in the presence of VCR at 2 ng/ml was 0.9 μg/ml for VER and 0.6 μg/ml for CyA. Figure 3B shows the combination effect of CyA and VER on the growth-inhibitory activity of VCR at 2.0 ng/ml against HEL cell line. The VER concentration required to obtain the isoeffect (50% growth inhibition) was reduced when CyA was used in combination. CyA enhanced the effect of VER on the growth-inhibitory activity of VCR, and *vice versa.*

According to isobologram analysis as shown in Fig. 3C, the experimental isoeffect points (50% growth inhibition) in the combination of CyA and VER fell to the left of "the envelope of additivity" when the CyA concentration was 0.3 μg/ml or more, or within "the envelope of additivity" when the CyA concentration was 0.2 μg/ml or less. This means that the two modifiers worked synergistically or at least additively to overcome VCR resistance.

DISCUSSION

MDR tumor cells are a major obstacle to curative cancer chemotherapy. Recent studies have shown that drug resistance in MDR cells can be circumvented by many agents.[6-13] Most of them inhibit the P-gp-associated active efflux of anticancer drugs. Some of them reverse MDR by competing with anticancer drugs for a P-gp binding site.[22, 23]

Among them, VER has been studied most extensively.[6, 7, 24-28] Most clinical studies, however, have failed to achieve an objective response with combinations of anticancer agents and VER.[27, 28] The reason for these unsuccessful results has been supposed to be that the concentration of VER is not enough to overcome the resistance; further, tumor cells of the patients have not been proved to be P-gp-associated MDR cells. The concentration of VER required for circumvention of MDR *in vitro* was at least about 1 to 5 μg/ml from our experiments described here (Fig. 3) and elsewhere.[17] This concentration of VER is too high to achieve with safety in the clinical setting, because the clinically achievable peak concentration of standard dose therapy of VER was reported to be 100 to 250 ng/ml by intravenous administration and 60 to 80 ng/ml by mouth. Dose-limiting toxicities of VER are hypotension, and cardiac toxicities such as arrhythmia, congestive heart failure, etc. These toxicities are so life-threatening that it is very difficult to perform higher-dose trials of VER with safety and to achieve an effective concentration of VER for circumvention of MDR. Therefore combined use of VER and anticancer agents may not be available clinically for circumvention of MDR.

In this study we investigated the reversal ability of CyA and of combined use of CyA and VER in MDR, especially VCR resistance, using four human P-gp-associated MDR cell lines with different degrees of resistance. The results indicate that CyA can overcome P-gp-associated MDR more effectively than VER, and that a low concentration of CyA (0.5 μg/ml) was effective to overcome VCR resistance in a low-grade MDR cell line, HEL.

CyA has been widely used in bone marrow transplantation and various organ transplantations.[15, 16] CyA can be administered orally, and higher concentrations than 0.5 μg/ml can be achieved by standard dose therapy. In this therapy, however, nephrotoxicity, hypertension and neurotoxicity are reported to be major adverse effects of CyA. In contrast to VER, a pharmacokinetic study on CyA has revealed that a peak concentration of 1–2 μg/ml and whole blood trough levels of 0.2–0.5 μg/ml can be achieved without serious adverse effects. In this study, 0.5 μg/ml of CyA enhanced the VCR sensitivity of the low-grade MDR cell lines (KYO-1, HEL and CMK) by 6.3–16 times. As reported previously,[17] these three cell lines had never been exposed to anticancer agents in culture. Clinically acquired MDR seems to be low grade, resembling that of the three cell lines. CyA was also reported to overcome etoposide resistance.[29] Therefore, a low concentration (0.5 μg/ml) of CyA might overcome P-gp-associated drug resistance in the clinical setting.

Our results also indicated that the combination of CyA and VER increased the growth-inhibitory activity of VCR synergistically or at least additively. The experiment revealed that the synergistic effect of combined use of VER and CyA was observed at the concentration of CyA of 0.25 to 0.5 μg/ml and that of VER of 0.05 to 0.3 μg/ml. These concentrations of both drugs are clinically achievable with safety. Therefore, the combined use of VER and CyA to circumvent MDR may be clinically safer and more efficient than single use of VER or CyA.

In recent studies *in vivo,* calcium channel blockers (nicardipine, verapamil and diltiazem) resulted in an increase in blood level of CyA without increasing nephrotoxicity.[30-32] A calcium blocker, diltiazem, has been found to have protective effects against post-transplant tubular necrosis by CyA in cadaveric kidney transplantation and resulted in a significant increase in whole blood through level of CyA.[32] These reports and the present results suggest that the combination of CyA and VER enhances the sensitivity of MDR cells to VCR without increasing their adverse effects. Although CyA and VER have various drug interactions, the mechanism

of the synergistic effect of CyA and VER on circumvention of MDR is unknown at present. In addition to the competitive inhibition between VER or CyA and anticancer agents,[22,23] many possibilities can be raised, such as correction of altered plasma membrane potentials,[33] allosteric effect on P-gp, suppression of P-gp production and antigenic modulation of P-gp that might be induced by combined use of VER and CyA. As most of these possible mechanisms are not supported by evidence, further investigations are necessary to clarify the exact mechanisms of the synergistic effect.

In summary, CyA was superior to VER in terms of reversal of P-gp-associated MDR. CyA overcame VCR resistance in a dose-dependent manner. Low-grade MDR could be overcome by CyA at 0.5 μg/ml. Combination of CyA and VER at a low concentration that is clinically achievable with safety is a promising way to overcome P-gp-associated MDR without increasing the adverse effects.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research (62S-1, 2S-1, 1-1 and 1-11) and for the Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare.

(Received March 8, 1990/Accepted April 27, 1990)

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