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Enhancement of vincristine- and adriamycin-induced cytotoxicity by verapamil in P388 leukemia and its sublines resistant to vincristine and adriamycin

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Recent findings from this laboratory describe an enhancement of vincristine (VCR) cytotoxicity in P388 luekemia and its VCR-resistant subline by the calcium antagonist verapamil [1]. Verapamil greatly enhances the cellular level of VCR in P388 leukemia cells, especially in the VCR-resistant P388 subline (P388/VCR), through an inhibition of the VCR-efflux function of the cells [1]. VCR- or adriamycin (ADM)-resistant tumor cells have been reported to possess an enhanced outward transport mechanism for these drugs [2–5]. Inhibition of the VCR-efflux function of the cells by verapamil is directly related to a high accumulation of VCR, especially in P388/VCR cells, leading to the overcoming of VCR-resistance in P388/VCR cells *in vivo* and *in vitro* [1].

In a variety of experimental tumor cells, vinca alkaloid resistant cells are also resistant to anthracyclines [2, 4, 6–9]. In this report we have examined the effect of verapamil on the cytotoxicity and the cellular accumulation of VCR and ADM in P388, P388/VCR and P388/ADM (adriamycin-resistant subline) cells.

Methods

P388, P388/VCR and P388/ADM cells, harvested from tumor-inoculated mice, were maintained in plastic dishes (Corning Glass Works, Corning, NY) containing Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY), 20 μM 2-mercaptoethanol, and kanamycin (100 μg/ml) (growth medium) [1]. The cultures were incubated at 37° in a humidified atmosphere of 5% CO_2.

For the drug treatment experiment, culture medium (2 ml) containing exponentially growing tumor cells (2×10^4) was transferred to Falcon No. 2054 culture tubes (Falcon Plastics, Oxnard, CA). Two tubes were used for each drug concentration. The tubes were incubated at 37° in a humidified atmosphere of 5% CO_2. Five hours later, verapamil and VCR or ADM dissolved in phosphate-buffered saline (PBS) were added successively to the culture, and cells were cultivated for another 72 hr. Cells were then counted with a Coulter counter, and the cytotoxic activity of VCR or ADM in the presence or absence of verapamil was measured by determining the IC_{50} (drug concentrations required for 50% inhibition of cell growth) [1].

For the drug uptake experiment, tumor cells (2×10^5) in a tube containing 1 ml of growth medium with 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer, pH 7.4, were incubated at 37° in the presence of [^{3}H]VCR (33 nM, Amersham Japan, Tokyo, sp. act. 6.3 Ci/mmole) for P388/VCR cells or [^{3}H]ADM (200 nM, contributed by Kyowa Hakko Kogyo, Co., Ltd. Tokyo, sp. act. 73 mCi/mmole) for P388/ADM cells with verapamil at a nontoxic concentration of 6.6 μM. At various time intervals, the amount of VCR or ADM incorporated into the cells was determined as described previously [1].

Results and Discussion

P388, P388/VCR and P388/ADM cells showed almost the same sensitivity to verapamil. No growth inhibition was observed up to 20 μM. The IC_{50} of verapamil for these cells was 48–50 μM.

The sensitivities of P388, P388/VCR and P388/ADM cells to VCR and ADM and the effects of verapamil on these sensitivities are shown in Fig. 1. The IC_{50} values of VCR and ADM toward P388, P388/VCR and P388/ADM cells in the absence and presence of verapamil are summarized in Table 1. P388/ADM cells were highly resistant to ADM (index of resistance = 27) and also highly resistant to VCR (index of resistance = 46.9). P388/VCR cells were highly resistant to VCR (index of resistance = 25.6). The cells were also resistant to ADM; however, the index of resistance (3.4) was relatively small, indicating a possible slight difference between VCR and ADM resistance of P388 cells.

Verapamil at nontoxic doses of 2.2 and 6.6 μM greatly enhanced the cytotoxicity of P388 cells to VCR, especially the P388/ADM cells, as was observed for P388/VCR cells [1]. At a final concentration of 6.6 μM verapamil, the IC_{50} of VCR shifted from 75 to 1.1 nM toward P388/ADM cells and also shifted from 41 to 1.0 nM toward P388/VCR cells. The resistance to VCR was completely overcome in these

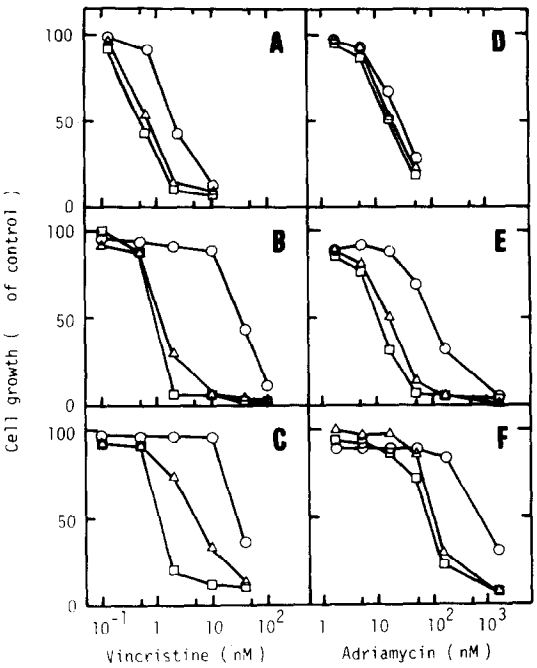

Fig. 1. Effects of verapamil upon growth-inhibitory action of VCR and ADM on P388, P388/VCR and P388/ADM cells. The cells were seeded in 2 ml of RPMI 1640 medium containing 10% fetal bovine serum, 20 μM 2-mercaptoethanol, and kanamycin (100 μg/ml) at 1×10^4 cells/ml of medium. Five hours later, P388 (A), P388/VCR (B) and P388/ADM (C) cells were treated with VCR at the following concentrations of verapamil: 0 (○), 2.2 (△) and 6.6 (□) μM. P388 (D), P388/VCR (E) and P388/ADM (F) cells were also treated with ADM and verapamil at the concentrations shown above. Cells were counted 72 hr after the drug treatment.

Table 1. Enhancement of vincristine- and adriamycin-induced cytotoxicity by verapamil in P388, P388/VCR, and P388/ADM leukemia cells*

Verapamil (μM)	IC_{50} (nM) of VCR			IC_{50} (nM) of ADM		
	P388	P388/VCR	P388/ADM	P388	P388/VCR	P388/ADM
0	1.6	41	75	31	105	850
2.2	0.55	1.3	5.4	20	20	110
6.6	0.42	1.0	1.1	18	11	90

* The IC_{50} values of VCR and ADM in P388, P388/VCR and P388/ADM cells at indicated verapamil concentrations were obtained from Fig. 1.

cells as the IC_{50} of VCR toward P388 cells was 1.6 nM. Verapamil also enhanced the cytotoxicity of ADM in P388/VCR and P388/ADM cells. At 6.6 μM verapamil, the IC_{50} value of ADM decreased to approximately 1/10 the values of those obtained in P388/VCR and P388/ADM cells in the absence of verapamil. However, in P388 cells, verapamil had only a slight effect on the enhancement of ADM cytotoxicity.

Uptake of [^{3}H]VCR by P388 cells increased time dependently (Fig. 2). However, uptake of VCR by P388/ADM cells was remarkably low, as was observed for P388/VCR cells [1]. The amounts of VCR found in 10^6 P388/VCR and P388/ADM cells were approximately 1/5 and 1/10 of that found in P388 cells at 3 hr. Verapamil greatly enhanced the cellular uptake of VCR by resistant cells. Approximately a 10- to 20-fold increase was observed in P388/VCR and P388/ADM cells, while only a 3-fold increase occurred in P388 cells with verapamil. Uptake of [^{3}H]ADM into P388 cells also increased time dependently. However, the uptake of [^{3}H]ADM into P388/VCR and P388/ADM cells was approximately 1/4 to 1/3 of that found in P388 cells at 2 hr. Verapamil also enhanced the cellular uptake of [^{3}H]ADM in resistant cells. Approximately a 2- to 2.5-fold increase occured in P388/VCR and P388/ADM cells. Verapamil, however, did not enhance the cellular uptake of [^{3}H]ADM in P388 cells.

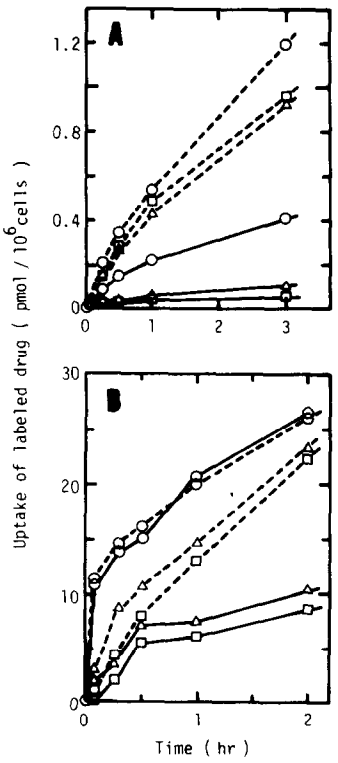

Fig. 2. Effects of verapamil on the uptake of [^{3}H]VCR and [^{3}H]ADM by P388, P388/VCR and P388/ADM cells, Each 2×10^5 of P388 (○), P388/VCR (△) and P388/ADM (□) cells was incubated in 1 ml of the medium containing 20 mM Hepes buffer (pH 7.4) at 37° with 33 nM [^{3}H]VCR or 200 nM [^{3}H]ADM in the absence (—) or presence (– –) of 6.6 μM verapamil. At time intervals, the cellular uptake of VCR (in A) and ADM (in B) was determined as described in Methods.

In P388/ADM cells, and also in P388/VCR cells, verapamil enhanced ADM cytotoxicity and accumulation, while in P388 cells the cellular uptake and cytotoxicity of ADM were not enhanced significantly by verapamil, although an evidently enhanced effect of VCR was obtained in P388 cells with verapamil [1]. These results suggest that the enhanced ADM-efflux mechanism in P388/ADM and P388/VCR cells may be somewhat different from the ADM-efflux mechanism in P388 cells in respect to sensitivity to verapamil. Further, it can be speculated that the ADM-efflux mechanism in sensitive P388 cells is somewhat different from the VCR-efflux mechanism of the cells.

In summary, a calcium antagonist, verapamil, enhanced the cellular uptake and cytotoxicity of vincristine (VCR) in adriamycin-resistant P388 leukemia (P388/ADM) cells and also enhanced the cellular uptake and cytotoxicity of adriamycin (ADM) in vincristine-resistant P388 leukemia (P388/VCR) and P388/ADM cells. The enhancement of cytotoxicity and cellular uptake of VCR in P388 and P388/VCR cells has been reported previously. [1]. VCR and ADM resistance was circumvented by verapamil. A common transport mechanism for VCR and ADM, which is responsive to verapamil, seems to exist in VCR- and ADM-resistant cells. However, the enhancement of ADM cytotoxicity and cellular uptake by verapamil was not evident in P388 cells.

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Metabolic tolerance as related to initial rates of ethanol metabolism

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It is well known that the rate of ethanol metabolism varies markedly in individuals of the same species [1, 2]. However, the within-group interindividual variance becomes less marked when one studies the rate of ethanol metabolism in inbred lines of experimental animals and in humans of the same ethnic group. American Indians and Orientals appear to have higher rates of ethanol metabolism than Caucasians [3]. It is also known that chronic ethanol consumption leads to metabolic tolerance in humans and experimental animals [4, 5], and the question arises whether tolerance develops to the same extent in individuals with different initial rates of ethanol metabolism.

We have examined this aspect in two rat lines inbred for their different central nervous system sensitivities to the motor-impairing effects of ethanol [6]. The MA (most affected) line is more sensitive than the LA (least affected) line. Apart from their different CNS sensitivities to ethanol, we have also found a sex/line difference in their initial ethanol metabolic rates (EMR), viz. the MA males had a 40–45% lower EMR compared to the other three groups of animals. Therefore, these animals offered a model to investigate the problem of extent of metabolic tolerance.

MA and LA rats used for this study were 60-day-old (at the start of experiment) offspring of the twenty-second generation. The origin stock (generation 19) was kindly provided by Dr. D. Lester of the Center of Alcohol Studies, Rutgers University, and then maintained by inbreeding in our own animal quarters. Animals for this experiment were housed individually and allowed continuous *ad lib.* access to standard rat chow and tap water. Food and water were withheld on days of measurement of EMR.

The ethanol metabolic rates for all four groups were determined before and after chronic ethanol treatment, following the i.p. injection of a test dose of ethanol [2.5 g/kg as a 12.5% (w/v) solution]. Samples of capillary blood (0.05 ml) from the cut tip of the tail of each animal were taken every hour for 7–8 hr after ethanol injection. Each sample was deproteinized, and ethanol was measured by the enzymatic method as described previously [7]. The disappearance rates of blood ethanol were calculated from the slope of the linear descending portion of each curve, and the rates of ethanol metabolism in mg per kg per hr were calculated as described previously [8]. Chronic ethanol treatment consisted of single daily ethanol (5 g/kg) intubations for up to 6 weeks for the males and 4 weeks for the females.

The initial body weights for the males were MA = 257 ± 10 and LA = 268 ± 9 g; for the females MA = 170 ± 4 and LA = 176 ± 4 g. Although body weights of males and females were different, no line difference was observed. While initial EMR values for the MA and LA females as well as LA males were similar, the MA males had a significantly lower EMR (40–45%). Following chronic ethanol treatment, all four groups showed significant increases in EMR, but the sex/line difference had disappeared (Table 1). As shown in Fig. 1, the increase in ethanol metabolism in the male LA animals occurred progressively over the first 3 weeks. On the other hand, a sharp increase in EMR occurred between 1 and 2 weeks of ethanol treatment in the male MA animals. While the same maximum value of ethanol metabolism was reached in both lines, after 6 weeks of chronic ethanol treatment, the temporal differences in the activation of ethanol metabolism in the two strains may represent two different mechanisms of metabolic tolerance.

The results indicate that the extent of metabolic tolerance is markedly dependent on the initial rates of metabolism which, in these rates lines, appear to be sex/line dependent. It is of interest to note that, despite differences in the initial (naive) rates of ethanol metabolism, the rates of metabolism after chronic ethanol treatment were virtually identical in all groups.

In another line of rats, a similar situation is seen when comparing males and females. Adult males of the spontaneously hypertensive line, which metabolize ethanol at

Table 1. Rates of ethanol metabolism in MA and LA rats before and after chronic ethanol treatment*

	Initial EMR ($mg \cdot kg^{-1} \cdot hr^{-1}$)	Final EMR ($mg \cdot kg^{-1} \cdot hr^{-1}$)	Increase (%)
Females			
LA	399 ± 21	473 ± 7	20
MA	388 ± 21	465 ± 7	20
Males			
LA	361 ± 15	455 ± 13	26
MA	244 ± 16	462 ± 5	89

* Values are means ± S.E.M.; N = six to ten animals per group.