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Verapamil potentiation of VP-16-213 in acute lymphatic leukemia and reversal of pleiotropic drug resistance*,**

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Summary. Verapamil, the calcium-influx-blocking agent, has previously been shown to have favorable interactions with antineoplastic drugs. Our study of human T cell acute lymphatic leukemia (ALL) GM3639 indicates that verapamil enhances the in vitro cytotoxicity of VP-16-213 against drug-sensitive ALL by reducing the concentration of VP-16-213, resulting in 50% cell viability from 104.5 ± 26.6 n*M* to 46.0 ± 2.7 n*M* ($P < 0.05$). The addition of verapamil to VP-16-213 treatment of BDF/1 mice bearing L1210 leukemia increases their mean survival from 21.2 ± 3.6 to 50.4 ± 4.3 days ($P < 0.01$) and the survival of $CD_2F/1$ mice bearing P388 leukemia from 27.8 ± 3.7 to 49.1 ± 5.0 days ($P < 0.01$). The 30-day survival is significantly increased in L1210 and P388 leukemia mice, and 60-day survival is significantly increased in P388 leukemic mice by verapamil.

We developed a vincristine (VCR)-resistant subline of GM3639 T cell ALL, L_{23}, by continuous exposure of drug-sensitive cells to VCR. This subline demonstrates pleiotropic cross resistance to VP-16-213 and daunorubicin. The addition of verapamil to VCR, to VP-16-213, and to daunorubicin completely restores responsiveness to these drugs, as indicated by the normalization of the VCR and VP-16-213 concentrations required for cytotoxicity and the concentration of daunorubicin required for inhibition of thymidine incorporation.

Introduction

VP-16-213 is an important new antitumor agent. It has clinical activity against a spectrum of human tumors, including small cell lung cancer, germinal neoplasia, Kaposi's sarcoma, neuroblastoma, and acute leukemia [6, 13]. Verapamil, the calcium-influx-blocking agent, potentiates the effect of vincristine and/or adriamycin in a variety of cultured human tumors and restores drug responsiveness to vincristine-resistant murine P388 leukemia and to daunorubicin-resistant Ehrlich ascites carcinoma in vivo [18, 19, 21, 22]. We now report verapamil enhancement of VP-16-213 cytotoxicity in cultured human T cell acute lymphatic leukemia. In addition, verapamil abrogates VP-16-213 and daunorubicin pleiotropic drug resistance in a vincristine resistant subline of this T cell ALL and enhances the therapeutic effect of VP-16-213 in murine L1210 and P388 lymphoid leukemias.

Methods

Tumor lines. Human acute lymphatic leukemia cell line GM3639 was obtained from the Human Genetic Mutant Cell Repository. This cell line was originally established by Huang et al. from a 16-year-old patient with acute lymphatic leukemia in relapse [9]. GM3639 cells express the T lymphocyte phenotype and are free of EB virus. We have maintained this tumor line in continuous suspension culture for 2 years in RPMI 1640 supplemented with 20% heat-inactivated FBS (Irvine Scientific), 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 100 μg/ml gentamicin, and 2 m*M* glutamine. Additional glutamine is routinely added to this glutamine-sensitive cell line. Cell doubling time is approximately 48 h, and cultures are refed every 2–3 days. The minimum seeding density for this line is 2×10^5 cells/ml, and cell growth reaches a plateau at $20–30 \times 10^5$ cells/ml. Cell viability was monitored by hemocytometer and trypan blue dye exclusion. For the development of drug resistance, GM3639 cells were suspended at 10×10^5 cells/ml in growth medium containing specified amounts of vincristine sulfate (Sigma Chemical Co.). Since 3 n*M* VCR was lethal to drug-sensitive cells (L_0) within 1 week of continuous culture, L_0 cells were exposed to 2 n*M* VCR (L_2) and continuously maintained at this sublethal drug dose. For 7 weeks, cell growth was significantly suppressed compared with that of cells cultured without drug or exposed to 1 n*M* VCR. At 12 weeks the L_2 cell line was exposed to 3 n*M* VCR (L_{23}), and the maximum growth rate was attained only 4 weeks later. After 21 weeks of continuous culture in VCR, L_{23} cell aliquots were reset at 10×10^5 cells/ml in 3 n*M* VCR or 5 n*M* VCR, or VCR was withdrawn. All three sublines attained maximum density within 1 week. The L_{23} subline has a doubling time of approximately 48 h and cell growth reaches a plateau at $20–30 \times 10^5$ cells/ml. P388 leukemia was obtained from Dr A. A. Ovejera, DCT

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Tumor Repository, NCI, Frederick Cancer Research Facility, Frederick, Md. Tumor cells grow IP and are passed weekly in $CD_2F/1$ mice (Simonsen, Gilroy, Calif). L1210 leukemia was used as previously described [17]. Groups of five or more female mice inoculated with 1×10^5 leukemia cells were used for survival time studies. Host mice were treated (a) 24 and 72 h after tumor inoculation with 10 mg/kg VP-16-213 with or without 25 mg/kg verapamil IP; (b) daily for 8 days starting 48 h after tumor inoculation with 0.25 mg/kg VCR with or without 25 mg/kg verapamil IP; or (c) every other day for three doses starting 24 h after tumor inoculation with 2.4 mg/kg daunorubicin with or without 25 mg/kg verapamil IP.

ED_{50} and IC_{50} assays. Cells are suspended in triplicate at 5×10^5 cells/ml culture medium with vincristine (0–250 n*M*) or VP-16-213 (0–300 n*M*) and verapamil (0–10 n*M*) in a 3-day cytotoxic assay, in which cell viability in increasing concentrations of drug is compared with cell viability in the absence of drug in the presence or absence of verapamil. After 3 days equivalent aliquots are taken from each triplicate culture and counted in duplicate with a hemocytometer, and cell viability is determined by means of trypan blue dye exclusion. In this system, the range between duplicate counts is less than 10%. Each data point reflects the average of these counts divided by counts from control cultures without drug, and is expressed as percentage viability. The median effective dose (ED_{50}) is defined as that concentration of drug resulting in 50% viable cells at 3 days compared with the originally seeded value. L_0 and L_{23} cell counts at 3 days show no significant difference in the presence or absence of 10 μ*M* verapamil. We have maintained aliuqots of GM3639 cells in 10 μ*M* verapamil for 12 weeks without impairment of cell growth. The daunorubicin concentration required to inhibit 50% of DNA synthesis (IC_{50}) was determined by measuring tritiated thymidine incorporation in tumor cells as previously described [18].

The significance of differences in ED_{50}, IC_{50}, and mean survival times was calculated by Student's *t*-test. The frequency of survival of leukemic mice at 30 and 60 days was compared between VP-16-213 and VP-16-213 plus verapamil treatment groups by Chi square analyis.

Results

Table 1 presents the ED_{50} values for VP-16-213 in the presence or absence of verapamil for drug-sensitive L_0 ALL cells. Verapamil shifts the VP-16-213 ED_{50} from 104.5 ± 26.6 to 46.0 ± 2.7 n*M*, ($P < 0.05$). Table 1 also presents the statistical analysis of the change in the ED_{50} of VCR with verapamil for vincristine-resistant L_{23} cells. In L_{23} cells the ED_{50} of VCR is 132.8 ± 19 n*M*, as against 2.4 ± 0.9 n*M* VCR in drug-sensitive L_0 cells ($P < 0.01$). This ED_{50} of VCR for L_{23} cells is reduced to 2.5 ± 0.7 n*M* in the presence of 10 μ*M* verapamil ($P < 0.01$). Figure 1 presents representative cytotoxic ED_{50} curves for L_0 cells, L_{23} cells, and L_{23} cells in the presence of verapamil. The ED_{50} of 111 n*M* for VCR-resistant L_{23} is shifted by the addition of 2 and 10 μ*M* verapamil to 11 and 1.6 n*M* VCR, respectively, as against an ED_{50} of 3.3 n*M* VCR for drug-sensitive L_0 cells without verapamil. The L_{23} subline shows persistent VCR resistance after 2 weeks of growth in the absence of VCR, ED_{50} 130 n*M* VCR. The L_{23} subline of ALL shows slight but statistically significant cross resistance to VP-

Table 1. Mean $ED_{50} \pm SD$ for VCR and VP-16-213 and $IC_{50} \pm SD$ for daunorubicin in the presence (+) or absence (−) of verapamil (10 μ*M*)

	Cell line	Verapamil	VP-16-213 (n*M*)	*P*
(A)	L_0	−	104.5 ± 26.6	
(B)	L_0	+	46.0 ± 2.7	<0.05 vs (A)
(C)	L_{23}	−	188.7 ± 15.8	<0.05 vs (A, D)
(D)	L_{23}	+	75.3 ± 13.4	>0.1 vs (A)
			VCR (n*M*)	
(A)	L_0	−	2.4 ± 0.9	
(B)	L_0	+	1.7 ± 1.3	>0.1 vs (A)
(C)	L_{23}	−	132.8 ± 19	<0.01 vs (A, D)
(D)	L_{23}	+	2.5 ± 0.7	>0.1 vs (A)
			Daunorubicin (μg/ml)	
(A)	L_0	−	1.4 ± 0.3	
(B)	L_0	+	0.6 ± 0.2	<0.05 vs (A)
(C)	L_{23}	−	2.9 ± 0.3	<0.001 vs (A), <0.01 vs (D)
(D)	L_{23}	+	1.6 ± 0.1	>0.1 vs (A)

L_0 and L_{23} denote drug-sensitive and drug-resistant ALL, respectively. The drug-resistant subline was developed by continuous exposure of drug-sensitive cells to sublethal concentrations of vincristine for 21 weeks. ED_{50} assays were performed on 5×10^5 cells/ml by comparing the viability of cells in increasing concentrations of drug, determined by hemocytometer count by trypan blue dye exclusion to the viability of cells in the absence of drug, in the presence or absence of 10 μ*M* verapamil at 72 h. Daunorubicin IC_{50} concentrations were determined by measuring the ability of daunorubicin to inhibit tritiated thymidine incorporation. ED_{50} and IC_{50} differences were calculated by Student's *t*-test and reflect averages of three or more independent experiments

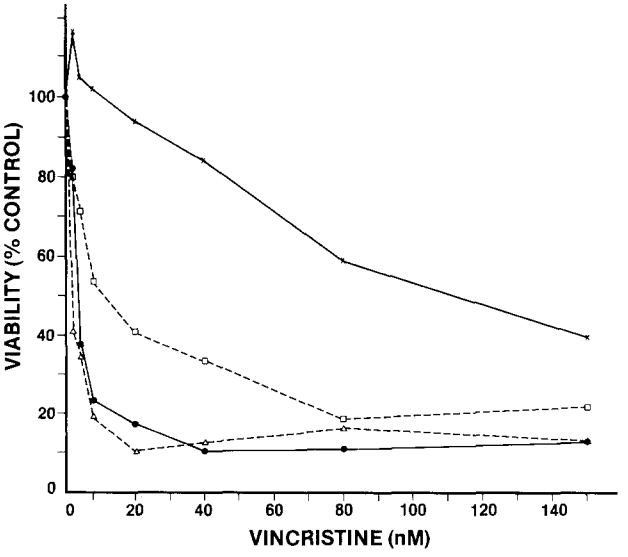

Fig. 1. Effect of verapamil on VCR cytotoxicity in VCR-resistant ALL cells. Viability of VCR-sensitive L_0 cells (●), VCR-resistant L_{23} cells (x) and VCR-resistant L_{23} cells in the presence of 2 μ*M* (□) and 10 μ*M* (△) verapamil at the indicated concentrations of VCR in a 72-h assay

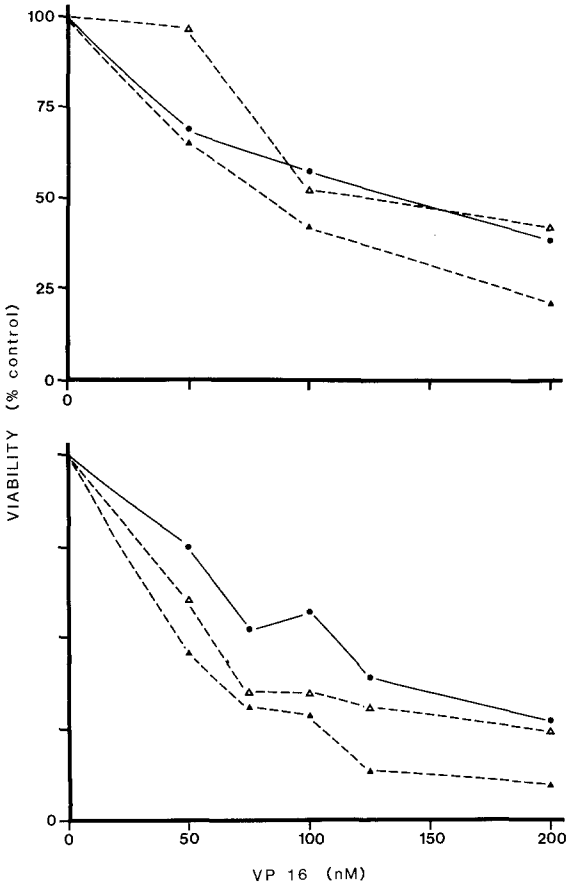

Fig. 2. Effect of verapamil on VP-16-213 cytotoxicity in L1210 and P388 cells. Viability of L1210 *(upper)* and P388 *(lower)* leukemia cells (●) in the presence of 1 μ*M* (△) and 10 μ*M* (▲) verapamil at the indicated concentrations of VP-16-213 in a 72-h assay

16-213. For L_{23} cells the ED_{50} of VP-16-213 is 188.7 ± 15.8 n*M* as against 104.5 ± 26.6 n*M* in drug-sensitive L_0 cells ($P < 0.05$). The addition of 10 μ*M* verapamil to L_{23} cells shifts their ED_{50} of 188.7 ± 15.8 n*M* VP-16-213 to 75.3 ± 13.4 n*M* ($P < 0.05$), a value characteristic of VP-16-213 sensitivity.

The concentration of daunorubicin required to inhibit 50% of thymidine incorporation by L_0 and L_{23} cells is shown in Table 1. The L_{23} VCR-resistant subline also shows a low but statistically significant degree of daunorubicin cross resistance. It has an IC_{50} of 2.9 ± 0.3 μg/ml daunorubicin, as against 1.4 ± 0.3 μg/ml daunorubicin for drug-sensitive L_0 cells ($P < 0.01$). The addition of verapamil to daunorubicin reduces the IC_{50} value of the resistant subline to that characteristic of drug-sensitive tumor, 1.6 ± 0.1 μg/ml daunorubicin.

Figure 2 demonstrates the in vitro effects of 1 and 10 μ*M* verapamil on the ED_{50} of VP-16-213 in drug-sensitive L1210 and P388 leukemias. The ED_{50} for L1210 and P388 cells is shifted from 132 and 111 n*M* VP-16-213 in the absence of verapamil to 113 and 61 n*M* by 1 u*M* verapamil, and to 79 and 46 n*M* by 10 μ*M* verapamil in these cell lines, respectively. The enhancement of VP-16-213 cytotoxicity by 10 μ*M* verapamil is similar to that of drug-sensitive human ALL cells. As can be seen in Table 2, this enhancement correlates with the increase in mean survival of

Table 2. Verapamil enhancement of VP-16-213 in vivo

Leukemia	Treatment	MST ± SEM (days)	*P*
L1210	(A) Untreated	9.1 ± 0.1	
	(B) Verapamil	8.6 ± 0.5	>0.1 vs (A)
	(C) VP-16-213	21.2 ± 3.6	<0.05 vs (A), (B)
	(D) VP-16-213 and verapamil	50.4 ± 4.3	<0.01 vs (A), (B), (C)
P388	(A) Untreated	9.8 ± 0.3	
	(B) Verapamil	10.4 ± 0.2	>0.1 vs (A)
	(C) VP-16-213	27.8 ± 3.7	<0.01 vs (A), (B)
	(D) VP-16-213 and verapamil	49.1 ± 5.0	<0.01 vs (A), (B), (C)

BDF/1 mice with L1210 and $CD_2F/1$ mice with P388 leukemia were treated with 10 mg/kg VP-16-213 on days 1 and 3 after inoculation with 100 000 tumor cells with or without 25 mg/kg verapamil. Control mice were untreated or treated with verapamil alone. Animals surviving over 60 days were calculated as having a 60-day survival

Chi-square values for VP-16-213 vs VP-16-213/verapamil-treated mice with L1210 leukemia surviving 30 and 60 days were 6.67 ($P < 0.01$) and 2.5 ($P > 0.1$) and for mice with P388 leukemia 4.46 ($P < 0.05$) and 8.13 ($P < 0.01$) in favor of the combined treatment, respectively

host mice bearing L1210 and P388 leukemia treated with combined VP-16-213 and verapamil compared with mice treated with verapamil alone ($P < 0.01$). This combined drug regimen also significantly increases the 30- and 60-day survival rates for leukemic mice ($P < 0.05$). The addition of verapamil fails to enhance the therapeutic responses of BDF/1 mice bearing L1210 leukemia to daunorubicin (14.2 ± 0.8 versus 14.6 ± 0.5 days mean survival) and of $CD_2F/1$ mice bearing P388 leukemia to VCR (15.2 ± 0.5 versus 16.1 ± 1.1 days mean survival).

Discussion

Our study demonstrates that the addition of verapamil, the calcium-influx-blocking drug, to VP-16-213 significantly enhances VP-16-213 cytotoxicity in drug sensitive cultured human ALL GM3639 and murine L1210 and P388 lymphoid leukemias. This in vitro enhancement of VP-16-213 correlates with, but is not as impressive as, the therapeutic use of verapamil and VP-16-213 in murine leukemia. In vivo, we observed a greater increase in mean survival in BDF/1 mice bearing L1210 leukemia and in $CD_2F/1$ mice bearing P388 leukemia treated with combined VP-16-213 and verapamil than in mice treated with VP-16-213 alone. L1210 leukemic mice treated with the combined drug regimen survived 130% longer and P388 leukemic mice 77% longer than mice treated with VP-16-213 alone. The frequency of prolonged survival was also significantly higher in the combined-treatment groups. Recently Yalowich et al. have described potentiation of VP-16-213-induced DNA damage and cytotoxicity in L1210 and K562 leukemias by verapamil in vitro. This potentiation was more selective for tumor cells than for normal human marrow CFU GM colony growth, suggesting that verapamil enhances the therapeutic index of VP-16-213 [23, 24]. Although verapamil has previously been shown to enhance in vitro responses of a variety of cultured tumors to VCR

and to adriamycin, our results are the first to demonstrate that verapamil significantly potentiates the chemotherapy of a drug-sensitive tumor in vivo.

For our drug-resistant L_{23} GM3639 ALL subline the ED_{50} of VCR is over 50 times greater than that for the parental leukemia. Complete VCR responsiveness is restored to the L_{23} subline by the addition of verapamil to VCR, as indicated by normalization of its ED_{50}. The L_{23} subline has a low but significant degree of cross resistance to VP-16-213 and to daunorubicin. Although the in vitro daunorubicin IC_{50} differences between drug-sensitive and drug-resistant ALL are small, we found that such changes correspond to large differences in drug response in Ehrlich ascites carcinoma in vivo [18].

Multiple or pleiotropic drug resistance, i.e., cross resistance of cells to natural compounds of varied molecular structure and different mechanisms of action, has previously been described in vincristine-resistant cultured leukemic lymphoblasts [1]. In the cell line described, as demonstrated in our L_{23} subline, the degree of resistance to secondary drugs is less than the degree of primary drug resistance [5]. Verapamil eliminates the pleiotropic drug resistance of our ALL L_{23} subline, in addition to eliminating its primary resistance to VCR. In the presence of verapamil, the concentration of VP-16-213 required for cytotoxicity and that of daunorubicin required for thymidine inhibition revert to values characteristic of drug-sensitive ALL.

Observations regarding reversal of resistance of vinca alkaloids and anthracycline antibiotics by calcium-influx-blocking agents were originally recorded in murine neoplasia. In 1981, Tsuruo et al. reported complete restoration of VCR cytotoxicity to VCR resistant P388 leukemia by verapamil in vitro and in vivo restoration of VCR responsiveness by verapamil [19]. The same group subsequently showed verapamil enhancement of adriamycin cytotoxicity in an adriamycin-resistant P388 leukemia variant in vitro [20]. We observed complete in vitro and in vivo restoration of daunorubicin sensitivity to daunorubicin resistant Ehrlich ascites carcinoma by verapamil [18].

Verapamil has been shown to completely reverse primary vincristine resistance in K562 leukemia, and partially reverse velban and adriamycin resistance in other leukemic cell lines [3, 4, 22]. Although K562 cells are VCR-sensitive in vitro they are not representative of lymphoid malignancy; they are capable of fetal and embryonic hemoglobin synthesis, possess the transferrin receptor, and lack lymphoid markers [2, 10, 11, 16]. Recently Rogan et al. described complete reversal of adriamycin resistance in human ovarian carcinoma cells that are three- to six-fold resistant to adriamycin, and partial reversal in 150-fold adriamycin-resistant cells [15]. The effects of verapamil on pleiotropic VP-16-213 resistance in these cell lines have not been studied.

Our observations and preliminary reports suggest a therapeutic role for calcium-influx-blocking drugs in the treatment of patients with malignancies that have acquired drug resistance. Fine et al. noted that verapamil does not potentiate suppression of human bone marrow myeloid macrophage colony formation by adriamycin, vinblastine, or vincristine [7]. Ozols et al. reported that no enhanced gastrointestinal or bone marrow toxicity followed the addition of verapamil to adriamycin in patients with advanced ovarian carcinoma [12]. Recently, Honda et al. described two patients with non-Hodgkin's lymphoma who experienced partial responses to combined vincristine-nicardipine therapy after having become refractory to vinca alkaloids[8], and Presant et al. described an adriamycin-resistant patient with carcinoma of the pancreas who responded to combined adriamycin-verapamil [14]. In view of the marked in vivo enhancement of VP-16-213 by verapamil in drug-sensitive ALL, the addition of verapamil to VP-16-213 therapy of acute lymphoproliferative disorders should also be considered.

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Note added in proof. Since acceptance of this manuscript, L_{23} cells have been grown in progressively increased concentrations of VCR. This ALL subline (L_{100}) is now resistant to 100 nM VCR. L_{100} cells have a 485.0 ± 48.1 nM VCR ED_{50} and 1018.6 ± 201.4 nM VP-16-213 ED_{50}. These values are reduced to 6.9 ± 0.8 and 577.0 ± 163.9 nM respectively by 10 μM verapamil, $P < 0.001$ and < 0.05. The daunorubicin IC_{50} of L_{100} cells is 4.7 ± 0.3 μg/ml, which is reduced to 1.8 ± 0.4 μg/ml, $P < 0.02$, by 10 μM verapamil.