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Cyclosporin A potentiation of VP-16: production of long-term survival in murine acute lymphatic leukemia*

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Summary. Our prior in vitro studies on the correction of multidrug resistance by cyclosporin A (CsA) prompted us to investigate the effect of CsA and VP-16 in vivo. CsA given simultaneously at 2 or 10 mg/kg with VP-16 to BDF/1 mice bearing parental drug-sensitive P388 or L1210 lymphatic leukemia produced a 100% increase in survival as compared with VP-16 treatment alone. CsA-containing regimens also promoted 60-day survival in a significant number of P388 or L1210 leukemia-bearing mice as compared with animals receiving VP-16 in the absence of CsA ($P < 0.02$ and $P < 0.001$, respectively). CsA enhancement of the survival of mice bearing these lymphatic leukemias is restricted to VP-16, since the addition of CsA to therapeutic agents such as vincristine, daunorubicin, methotrexate, or cisplatin had no effect on survival.

Introduction

We have previously reported that cyclosporin A (CsA) corrects multidrug resistance (MDR) in human acute lymphatic leukemia in vitro and that it improves responses to daunorubicin in resistant Ehrlich ascites carcinoma in vivo [9, 10]. Although there has been extensive investigation of chemotherapy modulators against MDR tumor sublines, few studies have addressed the efficacy of these agents against parental tumors. We report the results of our study of CsA as a chemotherapy modulator in drug-sensitive murine acute lymphatic leukemia and describe the unique activity it exhibits when used in combination with VP-16.

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Materials and methods

P388 and L1210 leukemia were maintained in vivo by sequential passage in host mice as previously described [8, 11]. Groups of ten or more female BDF/1 mice (Simonsen, Gilroy, Calif.) were used in all survival studies. For i.p. tumor inoculation, 1×10^5 L1210 cells or 1×10^6 P388 cells were used.

Mice were treated with vincristine (VCR) at 0.3 mg on day 3 in the P388 system or at 1 mg/kg every other day times three in the L1210 system or with 2.4 mg/kg daunorubicin (DNR) every other day times three, 1.5 mg/kg methotrexate (MTX) daily times four, 3 mg/kg cisplatin (CDDP) on day 2, or 5 mg/kg VP-16 on days 1 and 3. All treatments were carried out in the presence or absence of 10 mg/kg CsA given i.p. simultaneously with each chemotherapeutic drug. An additional treatment regimen consisting of 2 mg/kg CsA together with VP-16 was chosen because this dose is lower than the conventional i.v. dose used in man [1]. Animals were cared for in accordance with institutional guidelines.

Differences in the mean survival of mice were determined using Student's two-tailed *t*-test. For calculations of mean survival, animals that survived for more than 60 days were assigned a survival value of 60 days; 60-day survival values were determined by chi-square analysis.

Absolute granulocyte counts (ACG) were determined in peripheral blood samples collected by retro-orbital puncture from groups of ten nonleukemic BDF/1 mice into heparinized micro hematocrit tubes (Fisher Scientific, Irvine, Calif.) prior to i.v. treatment with 5 mg/kg VP-16 given alone or in combination with two doses of 10 mg/kg CsA separated by a 48-h interval. AGC were determined weekly thereafter for 6 weeks. Individual total WBC were determined in each sample using a Coulter S Plus automatic cell counter, and AGC were calculated from differential counts performed on Wright-stained smears by visual inspection. Results were pooled for each group, and differences between the means were determined using Student's two-tailed *t*-test.

In vitro cytotoxicity was measured by the following modification of the original MTT assay initially described by Mosman [6]. The assay is based on the principal that living cells can convert a soluble tetrazolium salt [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide, MTT] to an insoluble formazon precipitate. The purple-colored formazon crystals are then dissolved in dimethylsulfoxide (DMSO), and the optical density of the solution is measured using a multiwell spectrophotometer (Elisa plate reader). Peritoneal leukemia cells are harvested, washed, and counted by trypan blue dye exclusion. Suspensions of leukemia cells are prepared at $1.0-6.0 \times 10^3$ cells/ml, and triplicate 150 µl aliquots are placed in individual wells of 96-well microliter plates (Falcon Plastic). After a 2-h period of incubation at 37° C, 10 µl of the drug to be tested is added and the plates are incubated for 4 days. At the end of the exposure period, MTT (20 µl) is added to each well. After an

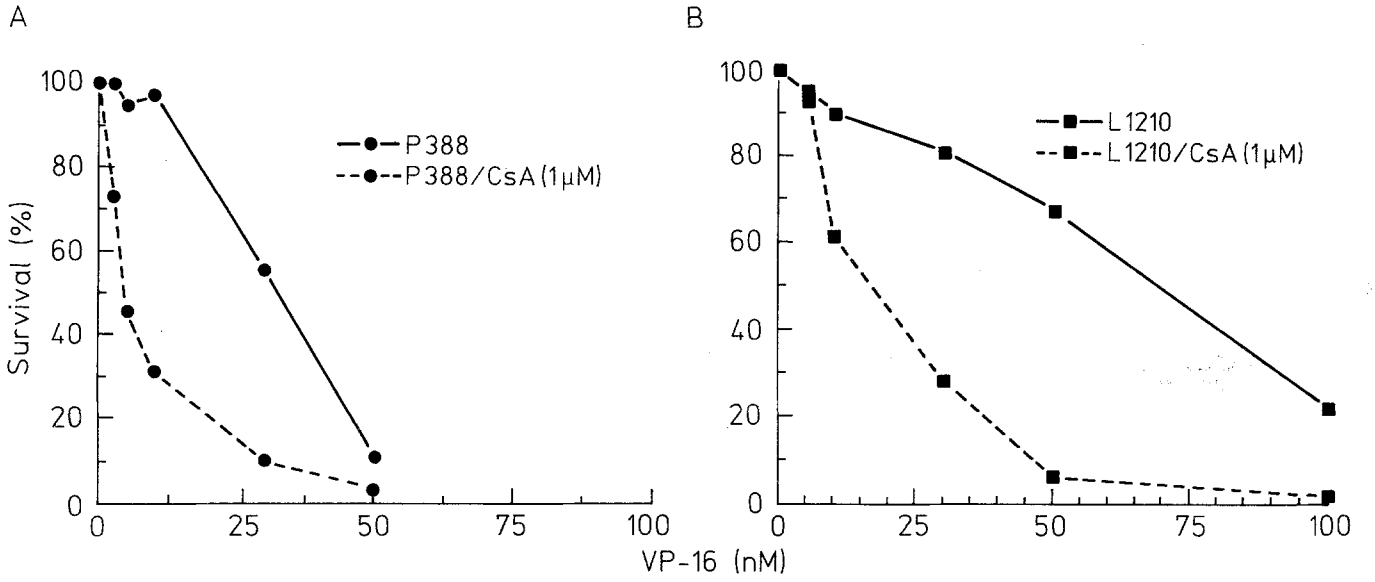

Fig. 1. In vitro enhancement of VP-16 cytotoxicity by CsA

incubation period of 4 h, the medium is removed, DMSO is added to solubilize the crystals, and the optical density is measured at 540 nm. The surviving fraction is then calculated by the following formula:

Mean $D_{\text{test sample}}$/mean D_{control}.

Results

Table 1 shows the mean survival of mice bearing sensitive parental P388 or L1210 leukemia that either were left untreated or were treated with CsA alone, VP-16 alone, or a combination of VP-16 and CsA. The use of 2 and 10 mg/kg CsA in combination with VP-16 had similar enhancing effects on VP-16 and increased the survival of mice with P388 leukemia to 46.7±14.6 and 43.1±15.6 days, respectively, as compared with the value of 21.4±2.2 days obtained using VP-16 alone. Similar results were obtained in mice bearing L1210 leukemia in that their survival was improved to 33.2±23.0 and 31.7±20.0 days, respectively, as compared with the value of 15.9±3.1 days obtained using VP-16 alone. The wide variation in the mean survival produced by combined VP-16/CsA treatment in mice bearing P388 and L1210 leukemia was attributable to the achievement in 30%–50% of the animals of survival values exceeding 60 days.

The chi-square values obtained for 60-day survival following the administration of VP-16 alone and after treatment with VP-16 in combination with 2 mg/kg CsA in mice bearing P388 and L1210 leukemia were 6.7 (P <0.01) and 5.0 (P <0.05), respectively. Similar analysis for pooled 60-day survival of leukemic mice treated with VP-16 alone as compared with the VP-16/CsA regimen yielded chi-square values of 5.45 (P <0.02) and 12 (P <0.001) for P388 and L1210 leukemia, respectively.

In vitro, the addition of CsA (1 μM) to VP-16 enhanced the cytotoxic efficacy of VP-16 against P388 and L1210 leukemia (Fig. 1). Table 2 shows the lack of effect of CsA in vivo following its use in combination with VCR, DNR, MTX, or CDDP in the therapy of mice bearing P388 or L1210 leukemia.

Table 3 compares the AGC values obtained in non-leukemic BDF/1 mice treated with VP-16 alone or VP-16

Table 1. Mean survival ± SD of BDF/1 mice treated with the indicated regimens

	Leukemia			
	P388		L1210	
Regimen	MS±SD (days)	P	MS±SD (days)	P
(a) –	11.0± 1.3	–	11.3± 2.1	–
(b) CsA (10 mg/kg)	11.1± 1.3	–	10.6± 1.7	–
(c) VP-16	21.4± 2.2	<0.001 vs (a)	15.9± 3.1	<0.001 vs (a)
(d) VP-16/CsA (2 mg/kg)	46.7±14.6	<0.001 vs (a), (c)	33.2±23.0	<0.02 vs (a), <0.05 vs (c)
(e) VP-16/CsA (10 mg/kg)	43.1±15.6	<0.001 vs (a),(c)	31.7±20.0	<0.01 vs (a) <0.05 vs (c)

Table 2. Mean survival of female BDF/1 host mice inoculated with P388 or L1210 cells and treated with VCR at 0.03 mg on day 3 in the P388 system or at 1 mg/kg q2d×3 in the L1210 system or with 2.4 mg/kg DNR q2d×3, 1.5 mg/kg MTX×4, or 3 mg/kg CDDP on day 2 in the presence or absence of 10 mg/kg CsA given i.p. simultaneously with each chemotherapeutic drug

	Regimen	Mean survival ± SD (days)	
		P388	L1210
(a)	–	9.8 ± 1.1	9.2 ± 0.8
(b)	VCR	19.1 ± 2.3	12.8 ± 1.8
	VCR/CsA	23.6 ± 1.7	4.9 ± 0.3
(c)	DNR	18.9 ± 2.7	14.7 ± 3.6
	DNR/CsA	15.3 ± 5.6	12.4 ± 1.4
(d)	MTX	15.6 ± 1.5	–
	MTX/CsA	15.8 ± 1.1	–
(e)	CDDP	–	14.3 ± 2.1
	CDDP/CsA	–	13.5 ± 1.5

Table 3. AGC values ± SD obtained in nonleukemic mice treated with VP-16 alone or in combination with CsA

Week	VP-16	VP-16/CsA	*P*
0	881 ± 231	814 ± 343	>0.1
1	836 ± 406	678 ± 225	>0.1
2	800 ± 239	820 ± 347	>0.1
3	834 ± 296*	572 ± 164*	<0.05
4	519 ± 306*	471 ± 220**	>0.1
5	582 ± 120**	576 ± 178*	>0.1
6	1284 ± 451	955 ± 320	>0.1

* $P > 0.05$; ** $P < 0.05$ vs the respective untreated control

combined with CsA. Although a significant difference was found between groups in the 3-week counts, no significant difference was observed either between the initial AGC obtained prior to treatment and that measured during week 3 in the combined-therapy group or in the 4-week nadir counts between the groups.

Discussion

VP-16 is an important antineoplastic agent that shows activity against lymphoproliferative disorders, germ-cell tumors, and lung cancer. Its clinical efficacy was predicted by its activity in early in vivo studies in murine tumors, including P388 and L1210 acute lymphatic leukemias [4]. In 1986 Osieka et al. [7] reported that CsA enhanced the cytotoxic effect of VP-16 and Adriamycin against L1210 leukemia as determined by clonogenic assay in vitro. The present in vivo study demonstrates that the addition of CsA to VP-16 promoted the survival of mice bearing sensitive acute lymphatic leukemia and produced long-term survival in a significant percentage of the animals. The addition of CsA to VP-16 resulted in a greater than 100% increase in the life span of host mice bearing P388 and L1210 leukemia as compared with VP-16 treatment alone. It is noteworthy that the combined regimen produced 30%–50% long-term survival as compared with the lack of 60-day survivors among the animals treated with VP-16 alone. The enhancing effects of CsA in P388 and L1210 leukemia seem to be uniquely restricted to VP-16, since we failed to observe alterations in the efficacy of VCR, DNR, MTX, or CDDP against these tumors on the addition of CsA.

The mechanism underlying this enhancement is uncertain but is probably not related to inhibition of the exaggerated active efflux of drug that is characteristic of multidrug-resistant cells, since these parental leukemias do not overexpress P-glycoprotein. However, CsA does have minor enhancing effects on drug uptake by parental drug-sensitive tumor cells. We have previously noted that CsA slightly enhances daunorubicin uptake by Ehrlich ascites carcinoma and murine hepatoma 129 cells under conditions of intact and impaired drug transport [5]. Osieka et al. [7] have obtained increases of up to 50% in VP-16 uptake by L1210 cells using CsA. Since VP-16 is a potent topoisomerase II inhibitor, CsA enhancement of drug-target interaction is an important possible alternative explanation for the effects described.

Over the last decade, the National Cancer Institute's program for the development of new antineoplastic agents has required an increase in life span of over 20% in initial drug screening against P388 leukemia. An increase in life span of 50% or greater in second-stage testing against L1210 leukemia, B16 melanoma, or Lewis lung carcinoma has been used to indicate sufficient antitumor drug activity for progression to clinical trial [3, 12]. The increase in life span produced by the addition of CsA to VP-16 in P388 and L1210 leukemias clearly meets these criteria if they are extended to chemotherapy-modulating agents.

The clinical toxicity of CsA as a single agent is well known. However, the toxicity of CsA and VP-16 used in combination have not been defined. Osieka et al. [7] reported that the lethality to 50% and 90% of the population (LD_{50} and LD_{90}, respectively) of a single oral dose of VP-16 at 30 days was increased by a nonspecified concentration of CsA in NMRI mice; however, the LD_{10} value was not affected by the addition of CsA. These VP-16 doses are far in excess of those used in the clinical setting and those used in the present study. In fact, we found no difference in the nadir AGC values obtained using VP-16 or the CsA/VP-16 regimen in our treatment groups. Although we did not perform other specific toxicity assays on these experimental groups, the animals failed to show evidence of debilitating toxicities such as dehydration, ruffling, or wasting. Mice treated with CsA alone showed no apparent side effects. Yahana et al. [13] recently reported the results of a phase I clinical trial in which CsA was added to VP-16 therapy in patients with solid tumors who had failed initial treatment with VP-16 given i.v. at 150–200 mg/m^2 daily for 3 days at 3- to 4-week intervals. The tolerable dose of CsA was quite high, and these authors recommended the use of a 5-mg/kg CsA loading dose followed by the continuous infusion of 15 mg/kg daily for 3 days for phase II clinical trials [13].

Eventual clinical trials should be directed at initial treatment rather than at relapsed patients or individuals with

progressive disease. Attempts at chemotherapy modulation would not be expected to promote responses beyond those seen during initial chemotherapy in the former group, and there is no evidence that modulation can overcome intrinsic drug resistance in the latter. Since maximal chemotherapeutic efficacy is obtained in patients undergoing initial treatment, we have begun a phase I trial in which CsA is added to VP-16 and cisplatin for initial therapy of patients with lung cancer [2]. This study is ongoing, but preliminary observations indicate that the maximum tolerated dose of CsA in this setting would be over 5 mg/kg daily.

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