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Short communication

Synergistic interaction of cyclosporin A and verapamil on vincristine and daunorubicin resistance in multidrug-resistant human leukemia cells in vitro*

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Summary. We studied the effects of cyclosporin A and verapamil on the modulation of vincristine and daunorubicin resistance in a multidrug-resistant subline of human T-cell acute lymphatic leukemia GM3639. Our results show that cyclosporin A is more effective than verapamil as a modulator of the high degree of primary vincristine resistance and the low degree of daunorubicin cross-resistance expressed by this cell line. Isobologram analysis revealed that the combined modulators act synergistically in correcting both vincristine and daunorubicin resistance.

Introduction

Although cyclosporin A (CsA) and verapamil (Vp) are well-known modulators of multidrug resistance (MDR) [10, 14], recent controversy has arisen over the 1990 report by Hu et al. that these agents act synergistically as vincristine resistance modulators [2, 6]. We present our observations on the synergistic effect of these modulators on vincristine (VCR) as well as daunorubicin (DNR) resistance in an MDR subline of a human T-cell acute lymphatic leukemia (ALL).

Materials and methods

Drug-sensitive BM3639 acute lymphatic leukemia cells (L_0) were originally obtained from the Human Genetic Mutant Cell Repository (Camden, N.J.). A resistant subline (L_{100}) was developed and maintained as described elsewhere [9]. DNR sensitivity is measured by the ability of DNR to inhibit thymidine incorporation into DNA as previously described [8]. Since the tritiated thymidine ([^{3}H]-Tdr)-inhibition short-term assay fails to detect VCR's cytotoxic effect, VCR cytotoxicity is measured in a 3-day viability assay. In this assay, cell viability determined in increasing concentrations of drug is compared with that found in the absence of drug and in the simultaneous presence or absence of chemotherapy modulator [9]. Vp hydrochloride (Knoll Pharmaceutical Co., Whippany, N. J.) was dissolved in normal saline. CsA (Sandimmune; Sandoz Inc., East Hanover, N. J.) was further diluted with normal saline from its solubilized cremaphor EL preparation.

For comparison of the extent of CsA- and/or Vp-induced reversal of resistance to specific concentrations of VCR or DNR, both a VCR cytotoxicity-reversal index and a DNR DNA-inhibition-reversal index were defined. These indices compare the effect of a specific concentration of chemotherapeutic drug in the presence vs the absence of resistance-modulating agent. The VCR cytotoxicity-reversal index is defined as:

$$\frac{\text{Cell count, VCR+modulator/Cell count, modulator}}{\text{Cell count, VCR/Cell count, control}} \times 1000$$

The DNA-inhibition-reversal index is similarly defined, except that the count per minute of [^{3}H]-Tdr is substituted for cell count. Since our prior studies had obtained an ED_{50} (effective dose in 50% of the cell population) viability value of 2.4 ± 0.9 nM for VCR in drug-sensitive parental ALL L_0 cells as compared with 485.0 ± 48.1 nM in MDR, L_{100} cells [9], VCR cytotoxicity-reversal assays used an incubation dose of 300 nM VCR. The DNR ED_{50} value for DNA inhibition in L_0 cells is 1.4 ± 0.3 μg/ml vs 4.7 ± 0.3 μg/ml in L_{100} cells [9]. DNR DNA-inhibition-reversal assays were therefore performed at a DNR concentration of 4 μg/ml. Resistance-reversal curves were individually developed for CsA, Vp, and various mixtures the two drugs as plots of the total molar concentration of either CsA or Vp alone or the combination of CsA and Vp vs the percentage of resistance defined as 100 minus the reversal index. Using the resistance-reversal curves, we determined the observed concentration of reversing agent(s) required to obtain equivalent degrees of reversal of resistance (e.g., 25%, 50%, 75%) to 300 mM VCR or 4 ng/ml DNR. The expected values for various ratios of CsA/Vp required to achieve 25%, 50%, or 75% resistance reversal were calculated as the sum of the multiples of the observed concentrations of CsA and Vp alone and their respective percentage of contribution to each ratio.

To assess the possibility of interaction between CsA and Vp, we used the isobologram technique. This method has the advantage of being independent of the shape of the dose-response curves. The experimental resistance-reversal curves were used to construct an isobologram for the combined doses of CsA and Vp required to produce 50% reversal of drug resistance. Using previously described methods [3, 12, 13], we constructed two isoeffect lines for the combined effects of the two drugs. Under the assumption that the two drugs act independently and their

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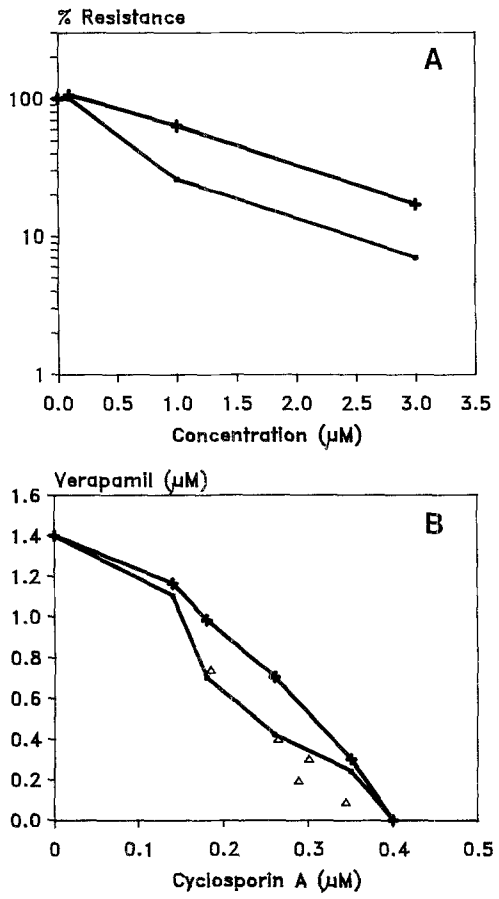

Fig. 1. A Dose-response curve for the effect of CsA (—●—) and Vp (—+—) on the reversal of VCR resistance in L_{100} cells. **B** 50% isobologram for L_{100} cells treated with 300 nM VCR, —●—, Model I; —+—, model II; △, experimental data

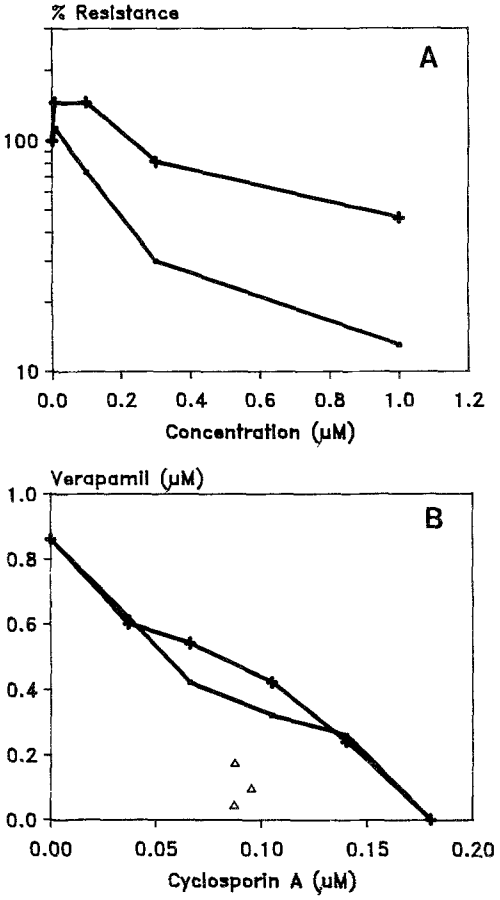

Fig. 2. A Dose-response curve for the effect of CsA (—●—) and Vp (—+—) on the reversal of DNR resistance in L_{100} cells. **B** 50% isobologram for L_{100} cells treated with DNR at 4 μg/ml. —●—, Model I; —+—, model II; △, experimental data

effects are therefore additive, the doses of CsA and Vp that together produce 50% resistance were plotted as Model I. Model II assumes that the addition of the first modulator (CsA) has reduced the increment of the second modulator (Vp) that is needed to produce the desired total effect by shifting over onto the second drug's dose-response curve. The area between the two curves thus represents an envelope of additivity. Experimental isoeffect points falling within this area are consistent with an additive effect for the two modulators. Experimental data points falling to the left of the curves indicate that the two modulators have a supra-additive or synergistic effect on the reversal of drug resistance.

Results

Figure 1 A illustrates the dose-response curve constructed for the effect of CsA and Vp on the reversal of resistance to a concentration of 300 nM VCR. The 50% isobologram for the combined effect of CsA and Vp on the reversal of resistance to VCR is shown in Fig. 1 B. The envelope of additivity is bounded on the left by the Model I isoeffect line and on the right by the Model II isoeffect line. The experimental isoeffect points fall along the boundaries of the additive area at lower doses of CsA. At higher concentrations of CsA, the isoeffect points fall to the left of the envelope of additivity, indicating a supra-additive or synergistic effect for the two modulators. The dose-response curves constructed for the effects of varying concentrations of CsA and Vp on the reversal of resistance to a 4-μg/ml concentration of DNR are presented in Fig. 2 A. The effect of combined doses of CsA and Vp on the reversal of DNR resistance are shown in the 50% isobologram illustrated in Fig. 2 B. The experimental isoeffect points fall far to the left of the area of additivity, clearly supporting a supra-additive effect for CsA and Vp in the reversal of resistance to DNR.

Discussion

We used the isobologram technique, which is independent of the mechanism of action and of the dose-response curve shape [3, 12, 13], to analyze the combined effects of CsA and Vp on the resistance of MDR L_{100} ALL cells to VCR and to DNR. L_{100} cells show a high degree (200-fold) of primary VCR resistance, are 3- to 4-fold cross-resistant to DNR, and overexpress P-glycoprotein [5, 9]. Our results demonstrate that the effect of the combined modulators on both the high-degree VCR resistance and the low-degree DNR cross-resistance of L_{100} cells is supra-additive.

Hu et al. [2] have proposed a synergistic interaction for CsA and Vp in the correction of doxorubicin resistance in MDR sublines of human T-cell CEM/CCRF cells using the fractional product method of Webb. Samuels and Ratain

[6] subsequently pointed out that the application of this method should be restricted to the analysis of simple exponential dose-response data and that isobologram analysis was the more appropriate technique for the analysis of the complex exponential dose-response findings evaluated by Hu et al. [2]. When applied to the latter data, isobologram analysis indicated additive or subadditive rather than synergistic interactions [6]. Ishida et al. [3] studied the action of CsA and Vp on the resistance of four P-glycoprotein-positive MDR human tumor cell lines to VCR and found that CsA reversed this resistance more efficiently than did Vp in each case. The effect of combined CsA and Vp was evaluated in one of these cell lines (16-fold VCR-resistant HEL erythroleukemia cells) and was found to be supra-additive by isobologram analysis [3]. However, the effects of a combination of modulators on resistance to other drugs included in the MDR phenotype were not studied.

Our current understanding of the mechanisms underlying chemotherapy modulation by CsA and Vp is incomplete, but recent observations strongly suggest the involvement of more than one mechanism of action [1, 7]. Both agents have been noted by Larrson and Nygren [4] to potentiate the effects of VCR, DNR, and etoposide in a human carcinoma cell line that fails to overexpress P-glycoprotein in vitro. We have found that CsA and Vp potentiate the effect of etoposide in parental-drug-sensitive murine acute leukemias in vivo [9, 11]. Chemotherapeutic enhancement beyond the correction of P-glycoprotein-mediated drug resistance by these agents therefore does occur. It has recently been reported that cremophor EL, the agent used to solubilize CsA in Sandimmune, shows activity in reversing MDR [15]. We do not believe that cremophor EL is responsible for the reversal of drug resistance observed for CsA, as our original experiments were performed using powdered CsA [10] and since we have more recently noted that the effect of Sandimmune in reversing the VCR resistance of L_{100} cells is 12-fold that found for equivalent concentrations of cremophor EL alone.

As clinical studies of chemotherapy modulation are developed, the combination of different modulators at subtoxic doses will be attractive. Since both CsA and Vp are effective resistance modulators in murine tumor systems in vivo, their synergistic activity in reversing MDR may provide a rationale for the use of combinations of these agents in clinical trials [11, 14].

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