Synergistic Reversal of Multidrug-Resistance Phenotype in Acute Myeloid Leukemia Cells by Cyclosporin A and Cremophor EL

By Douglas D. Ross, Patricia J. Wooten, Yi Tong, Brian Cornblatt, Chanan Levy, Rajeshwari Sridhara, Edward J. Lee, and Charles A. Schiffer

Cremophor (Crem) EL, the vehicle for intravenous delivery of cyclosporin A (CsA), has been reported to counteract multidrug resistance (MDR) in P-glycoprotein (Pgp)-overexpressing cell lines. Because of this, we sought to determine whether Crem functions independently as a modulator of MDR in blast cells obtained from acute myelogenous leukemia (AML) patients, and the nature of its interaction in combination with CsA in reversing an MDR phenotype. In the phenotypically classical MDR AML cell lines HL-60/Vinc (overexpresses Pgp) or HL-60/AR (does not overexpress Pgp), the dose causing half-maximum enhancement (D50) of daunorubicin (DNR, 1 µg/mL, 3 hours) accumulation was achieved by the combination of CsA and Crem (CsA/Crem) at 1.2 µmol/L CsA. In contrast, the D50 for Crem alone was approached at an amount that would be needed to suspend 6.2 µmol/L CsA for HL-60/Vinc, and 81 µmol/L CsA for HL-60/AR. The D50 concentrations for CsA alone (dissolved in ethanol, which does not alter DNR accumulation) were also higher than those for CsA/Crem, being 6.5 µmol/L for HL-60/Vinc, and 3.1 µmol/L for HL-60/AR. The maximum absolute level of enhancement of DNR accumulation (Emax) in each cell line was approximately equivalent for CsA/Crem or CsA alone, and was equal to the 3 hr intracellular DNR accumulation observed in parental, drug sensitive HL-60/W cells. For Crem alone, HL-60/AR and HL-60/Vinc cells showed markedly different responses: HL-60/Vince cells attained intracellular DNR content comparable to HL-60/W, whereas HL-60/AR cells achieved only approximately 35% of this level. Multiple-drug effects were analyzed by calculation of the Combination Index (Chou and Talalay, Adv Enzyme Regul 22:27, 1984), which indicated that CsA and Crem are synergistic in causing enhancement of DNR accumulation in these MDR HL-60 cell lines. In blasts from AML patients, 5 µmol/L CsA/Crem or an equivalent amount of Crem alone each caused significant (P < .001) enhancement of DNR accumulation (60 AML-patient marrow samples) or DNR retention (51 AML-patient marrows). Similarly, CsA/Crem or Crem alone caused significant (P < .01) enhancement of the cytotoxicity of DNR in 36 AML blast cell specimens. The degree of enhancement of accumulation retardation or cytotoxicity by CsA/Crem was approximately equivalent to that obtained with Crem alone. These studies indicate that Crem can reverse an MDR phenotype in patient AML blast cells. The equivalent degree of enhancement observed for Crem alone versus CsA/Crem also suggests that the Emax for DNR accumulation or retention was reached in AML cells by Crem alone (relative to 5 µmol/L CsA). Hence, synergism of CsA and Crem, indicated by the HL-60 studies, may permit the plasma concentrations obtained in recent phase I/II clinical trials (1 to 4 µmol/L CsA in Crem), to achieve maximal reversal of MDR in AML blast cells.

I N THE CONTINUING effort to develop better chemotherapeutic strategies against cancer, much interest has recently focused on cell membrane-associated xenobiotic transporters as potential mediators of multidrug resistance (MDR) to anticancer drugs.1-7 The most extensively characterized of these carriers is P-glycoprotein (Pgp), the product of the multidrug-resistance gene MDR1,1,4 although very recently, other membrane transporters such as multidrug-resistance-associated protein (MRP)1 and P-955,6 have been described that may also act as transporters responsible for MDR. Anticancer drugs transported out of the cell by Pgp include daunorubicin (DNR), mitoxantrone, vinca alkaloids, etoposide, and taxol. Data are currently emerging that may implicate Pgp expression as a factor contributing to resistance to chemotherapy in acute myeloid leukemia (AML).8-16 Recently, we found evidence for transport-mediated MDR in a large proportion of previously untreated AML patients, using functional assays for the MDR phenotype.17 Particularly exciting to the clinical arena is the discovery of a variety of inhibitors of Pgp-mediated anticancer drug transport that may prove useful in rendering Pgp-expressing resistant cells sensitive to chemotherapy.18-21 In our investigation of blast cells from previously untreated AML patients cited above,17 we found that one such inhibitor of Pgp, the immunosuppressive agent cyclosporin A (CsA), caused enhancement of the cytotoxicity, intracellular accumulation, and retention of DNR in approximately half of the patients studied, suggesting that a CsA-responsive MDR phenotype is present in a substantial proportion of AML patients at the time of disease presentation. Indeed, phase I/II clinical trials of CsA in combination with etoposide22 or with DNR23 have been published recently, with maximum sustained CsA plasma levels reported to be 2.5 to 4 µmol/L by the first study22 and 2.2 µmol/L by the other.23 One of these studies23 involved the combination of CsA with the “3 and 7” regimen of cytosine arabinoside (ara-C) and DNR in poor-risk AML patients, and obtained complete remission (CR) rates and remission durations (62% and 13 months, respectively)

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that may be better than expected with the "3 and 7" regimen alone in this group of patients.

Like most substances transported by Pgp, CsA is highly lipid soluble, and only sparingly soluble in water. In order to be given parenterally, CsA is suspended in a vehicle that is miscible with aqueous solutions. This vehicle consists of a mixture of 32.9% ethanol in Cremophor (Crem) EL. Cremophor EL is a polyethoxylated castor oil, produced by treating castor oil with ethylene oxide. Crem itself has been shown recently to be a modulator of Pgp-mediated MDR.24-27 Because the phase I/II clinical trials with CsA22,23 and our own in vitro studies of CsA modulation of the MDR phenotype in patient-derived AML blast cells17 used CsA suspended in Crem, the present studies were conceived to determine the degree to which (if any) Crem plays a role in reversing the MDR phenotype in AML blast cells treated with CsA/Crem, and the nature of the effects of CsA alone, Crem alone, or their combination on the expression of the MDR phenotype in MDR human leukemia cell lines, one which overexpresses Pgp, but not MRP (HL-60/Vinc)28 and one that overexpresses MRP, but not Pgp (HL-60/AR).29

MATERIALS AND METHODS

Patient Characteristics

These data are from 60 marrow specimens obtained from 51 previously untreated AML patients, of whom 9 were studied a second time (at time of relapse or progressive disease). The median age was 63 years (range 19 to 81); 25 were men, 26 were women; The French-American-British classifications24 are as follows: M1, 18 patients; M2, 12 patients; M3, 5 patients; M4, 8 patients; M5, 4 patients; M7, 1 patient; unable to be classified, 3 patients. Six of the patients reported here were also included in the cohort of 49 previously untreated AML patients whose marrows were studied for the effects of verapamil, CsA, and progesterone in culture corresponding to the following concentrations (in mL/dL) of the ethanol and Crem alone, or their combination on the expression of the MDR phenotype in patient-derived AML blast cells" used CsA from Sandoz) per mL of absolute ethanol was prepared. The survival of the AML blast cells in culture was then determined by the method that we had developed,30 exactly as described previously.17 The survival of the AML blast cells in culture was then determined by the method that we had developed,30 exactly as described previously.17

Cytotoxicity Studies

Thirty-six patient blast cell samples were studied. Blast cells or HL-60 cells were placed in RPMI 1640 culture medium as described above (median concentration was 750,000/mL) with and without combinations of DNR, CsA/Crem, CsA, or Crem. The 3-hour accumulation and 16-hour retention of DNR were determined exactly as described previously, using flow cytometry to determine intracellular DNR content.17

Statistical Methods

The relationships between the dose of modulator (CsA/Crem, Crem alone, or CsA alone) and enhancement of DNR accumulation (defined as percent change, described below) were modeled to a modification of the Hill equation, using the Adapt software package (Biomedical Simulations Resource, University of Southern California).35 as follows:

\[ \text{Percent Change} = \left( \frac{E_{\text{max}} \times D_D}{D_D + D_o} \right) \]

where \(E_{\text{max}}\) is the maximum effect (maximum enhancement), D is the dose of modulator, \(D_o\) is the dose of modulator that causes...
half-maximum effect, and H is Hill's constant, which describes the sigmoidicity of the curve.

For assessing drug interactions, we used the Combination Index (CI), which may describe synergistic, additive, or antagonistic interactions of CsA and Crem with respect to enhancement of DNR accumulation. For the interaction of CsA and Crem were calculated with the use of the “Dose-Effect Analysis with Microcomputers” program (Elsevier-BIOSOFT, Cambridge, UK) as follows:

\[ CI = \frac{\left(\frac{D_1}{D_1^*}\right) + \left(\frac{D_2}{D_2^*}\right) + \alpha\left(\frac{D_1}{D_1^*}\right)\left(\frac{D_2}{D_2^*}\right)}{\left(\frac{D_1}{D_1^*}\right)\left(\frac{D_2}{D_2^*}\right)} \]

where \(D_1\) and \(D_2\) are doses of drug 1 or drug 2 in a mixture that affects the system x percent, and \((D_1^*)\) and \((D_2^*)\) are doses of drug given individually that affect the system x percent. For mutually exclusive drugs, \(\alpha = 0\); for mutually nonexclusive drugs, \(\alpha = 1\). Hence, for any given effect or fraction affected \((Fa)\), the CI represents the ratio of the doses of drugs in combination to the doses of drugs alone that achieve that particular effect. When the CI = 1, the effects of the two drugs are additive, for CI less than 1, synergism is suggested, and for CI greater than 1, the effects of the drugs are antagonistic.

As a criterion for measuring the effect of an MDR modulator based on 3 hour accumulation and/or 16 hour retention of DNR, we defined the percent change in accumulation or retention as:

\[ \text{Percent change} = \frac{\text{AccDNR}_{\text{mod}} - \text{AccDNR}_{\text{nom}}}{\text{AccDNR}_{\text{nom}}} \times 100 \]

where \(\text{AccDNR}_{\text{mod}}\) and \(\text{AccDNR}_{\text{nom}}\) = 3-hour intracellular accumulation or 16-hour intracellular retention of DNR, respectively, in the presence of the MDR modulator, and \(\text{AccDNR}_{\text{mod}}\) or \(\text{AccDNR}_{\text{nom}}\) = accumulation or retention of DNR, respectively. A positive percent change reflects the percent enhancement of DNR accumulation or retention caused by a particular MDR modulator. The data were analyzed using the Wilcoxon signed rank test for the effect of modulators as measured by percent change, over the 60 marrow specimens.

For analysis of the cytotoxicity studies, the criterion for measuring the effect of an MDR modulator was similarly defined as:

\[ \text{Percent Change in Cell Survival} = \frac{\text{SurvDNR}_{X_{\text{mod}}} - \text{SurvDNR}_{X_{\text{nom}}}}{\text{SurvDNR}_{X_{\text{nom}}}} \times 100 \]

where \(\text{SurvDNR}_{X_{\text{mod}}}\) = cells surviving DNR dose \(X\) in the presence of the MDR modulator, and \(\text{SurvDNR}_{X_{\text{nom}}}\) = cells surviving DNR dose \(X\). In this case, an enhancement in DNR cell kill by the MDR modulator is reflected by a negative percent change. The Wilcoxon signed rank test was used to analyze the percent change data for any statistically significant effects of the various MDR modulators at any specified dose of DNR.

**RESULTS**

**CsA and/or Crem Effects on MDR HL-60 Human Leukemia Cells**

The sublines HL-60/Vinc and HL-60/AR were selected for these studies because both display a classical drug-transport–mediated MDR phenotype, yet the molecular mechanism responsible for this phenotype differs in the two cell lines. HL-60/Vinc cells overexpress the product of the MDRI gene, Pgp. In contrast, HL-60/AR cells have no detectable Pgp expression but do overexpress a 190-kD membrane protein that has been recognized recently to be the product of a transporter gene called MRP.

Intracellular accumulation (3 hour) of DNR (1 \(\mu\)g/mL) is threefold to sevenfold higher in the parental, drug-sensitive HL-60/W cells compared with HL-60/AR or HL-60/Vinc (Fig 1, 0 \(\mu\)mol/L CsA). The addition of the mixture CsA/Crem caused a concentration-dependent enhancement of intracellular DNR accumulation in HL-60/AR and HL-60/Vinc cells, but had no effect on the DNR accumulation in HL-60/W cells (Fig 1A). Concentrations shown in Fig 1 are relative to the final concentration of CsA in the cultures. Concentrations in excess of 3 \(\mu\)mol/L CsA/Crem brought the intracellular DNR content of HL-60/AR and HL-60/Vinc cells to levels achieved in HL-60/W. Crem alone (Fig 1B), added in amounts that would be obtained at the indicated concentrations of CsA (see Materials and Methods), enhanced DNR accumulation in HL-60/Vinc cells to levels approaching those obtained in HL-60/W, but higher concentrations were required than when combined with CsA (compare with Fig 1A). Crem alone had only a slight enhancement effect on DNR accumulation in HL-60/AR cells (Fig 1B).

To determine the effects of CsA alone, it was dissolved in ethanol (Fig 1, C and D), because ethanol alone in amounts required to achieve the indicated concentrations of CsA in culture (when the mixture is used) had no effect on DNR content in any of the three cell lines (Fig 1D). Hence, data displayed in Fig 1C should reflect changes caused by CsA alone. CsA in the absence of Crem enhanced DNR accumulation in both HL-60/Vinc and HL-60/AR cells to levels comparable with HL-60/W cells (Fig 1C), although higher concentrations were required than when combined with Crem.

To evaluate the degree of enhancement in DNR accumulation caused by CsA and/or Crem, data in Fig 1 were expressed as percent change, as described in Materials and Methods (Fig 2). A positive percent change represents the percent enhancement of DNR accumulation caused by the modulator. Note that enhancement of DNR accumulation is seen only in the MDR cell lines in response to CsA/Crem (Fig 2A), Crem alone (Fig 2B), or CsA alone (Fig 2C). Note also that the percent-change curves for CsA/Crem, Crem alone, or CsA alone possess a sigmoidal shape, with a plateau in percent change occurring at higher doses suggesting that a maximal enhancement \((E_{\text{max}})\) in DNR accumulation occurs as a result of saturation of the membrane transporter of DNR by the modulator. Using Adapt, we fitted the data for CsA/Crem, Crem alone, or CsA alone in Fig 2 to a sigmoid \(E_{\text{max}}\) model as described by a modified Hill equation (Table 1). Initially, we modeled the data for the combination CsA/Crem. For both HL-60/AR and HL-60/Vinc, the model fit these data very well, with coefficients of determination (CD) of 0.993 (Table 1). The model calculated the maximum effect (which we define here as maximum enhancement, or \(E_{\text{max}}\)), the dose causing half-maximum effect \((D_{0.5})\), and Hill’s constant (H), which represents the degree of sigmoidicity of the curve. Hence, for the combination of CsA/Crem, the model estimate of \(E_{\text{max}}\) was 737.9% change.
Effects of CsA dissolved in Cremophor EL (A), Cremophor EL alone (B), CsA dissolved in ethanol (C), or ethanol alone (D) on DNR accumulation (1 μg/mL, 3 hours) in HL-60 cells. Vertical bars represent SEM. Intracellular DNR content was determined by flow cytometry (excitation 488 nm, emission 575-595, logarithmic amplification) and is expressed as a relative value obtained by dividing the channel number that represented mean red fluorescence for a given sample by 256 (the number of channels per log decade), then obtaining an antilog of this value. When used alone, the concentrations of Crem EL or ethanol used are given relative to the amount that would be required to achieve a particular concentration of CsA (see Materials and Methods). Points shown in (A) and (B) in the figure represent the means of values obtained in four identical experiments, done on different days. Points shown in (C) and (D) in the figure represent the means of values obtained in two identical experiments, done on different days.

The ability of CsA/Crem or Crem to enhance the cytotoxicity of DNR was also studied in these HL-60 cell lines (Fig 4). Both HL-60/AR and HL-60/Vinc display approximately 80-fold resistance to DNR, compared with HLd0/W cells, with concentrations causing 50% lethality (LC50) being 0.005 μmol/L for HL-60/W, 0.4 μmol/L for HL-60/AR, and 0.45 μmol/L for HL-60/Vinc, as we reported previously. In general, the effects of CsA/Crem or Crem alone on DNR cytotoxicity paralleled their effects on DNR accumulation. Neither CsA/Crem nor Crem had an effect on DNR cytotoxicity in HL-60/W cells, consistent with the lack of enhancement in DNR accumulation observed with these agents in these cells (Figs 1 and 2). In HL-60/AR cells, only the combination of CsA and Crem was able to enhance DNR cytotoxicity significantly. Similar to the modest DNR accumulation enhancing effect of Crem in these cells, Crem caused very minimal enhancement of DNR cytotoxicity. Both CsA/Crem and Crem alone enhanced DNR cytotoxicity in HL-60/Vinc cells, with the greatest effect caused by the combination, where a sensitization comparable with that of HL-60/W was observed (Fig 4).

Studies of AML Blast Cells

Accumulation and retention studies. The effects of CsA/Crem or Crem alone on 3-hour DNR accumulation was
studied in 60 AML marrow specimens. Of the 60 specimens, 51 were also studied for the effects of these MDR modulators on 16-hour DNR retention. Figure 5 displays the median percent change in intracellular DNR accumulation or retention and the 95% confidence interval for all patient marrows studied. Both CsA/Crem and Crem alone caused significant enhancement of DNR accumulation and retention (Wilcoxon signed rank test, \( P < .001 \)). The magnitude of these effects for CsA/Crem in Figure 5 are consistent with our previous report of the effects of CsA (in Crem) on DNR accumulation and retention in blast cells from previously untreated AML patients.\(^{17}\) Note that over all marrows studied, the median enhancement in DNR accumulation or retention caused by Crem alone was equal to that caused by the combination of CsA/Crem (Fig 5). To determine the equivalency of response to CsA/Crem or Crem alone in an individual marrow specimen, the percent change caused by CsA/Crem in each specimen was plotted against the percent change caused by Crem alone for DNR accumulation (Fig 6A) or DNR retention (Figure 6B). Good correlation was obtained for both DNR accumulation \((r = .9, P < .01)\) and DNR retention \((r = .6, P < .01)\).

The frequency and degree of enhancement of DNR accumulation or retention caused by CsA/Crem or Crem alone are provided in Table 2. Note that approximately half of the marrows studied exhibited greater than 10% change in response to CsA/Crem or Crem alone. At all cut-off levels, the number of specimens exhibiting that degree of percent change with CsA/Crem was approximately equal to those exhibiting the same amount of percent change in response to Crem alone (Table 2). The data shown in Table 2 for CsA/Crem are in agreement with our observations for CsA (in Crem) in our recent study of newly diagnosed AML patients.\(^{17}\)

Cytotoxicity studies. Of the 60 marrow specimens studied for DNR accumulation, 36 were also investigated for the effects of CsA/Crem or Crem alone on DNR cytotoxicity.

Table 1. Hill Equation Modeling of Percent Change Curves Displayed in Fig 2 for HL-60/Vinc or HL-60/AR Cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>CsA</th>
<th>Crem</th>
<th>CsA/Crem</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60/Vinc</td>
<td>( E_{max}^* )</td>
<td>—</td>
<td>—</td>
<td>737.9 (34%)†</td>
</tr>
<tr>
<td>( D_0 ) (umol/L)</td>
<td>6.5 (6.6%)†</td>
<td>6.2 (6.6%)†</td>
<td>1.2 (10.2%)</td>
<td></td>
</tr>
<tr>
<td>( H^* )</td>
<td>2.7 (16.4%)</td>
<td>0.9 (6.9%)</td>
<td>1.5 (13.2%)</td>
<td></td>
</tr>
<tr>
<td>( CD^* )</td>
<td>0.991</td>
<td>0.993</td>
<td>0.993</td>
<td></td>
</tr>
<tr>
<td>HL-60/AR</td>
<td>—</td>
<td>—</td>
<td>232.3 (4.1%)</td>
<td></td>
</tr>
<tr>
<td>( D_0 ) (umol/L)</td>
<td>3.1 (16.8%)</td>
<td>81 (44%)</td>
<td>1.2 (12.8%)</td>
<td></td>
</tr>
<tr>
<td>( H )</td>
<td>1.4 (24.7%)</td>
<td>0.55 (19.1%)</td>
<td>1.2 (12.7%)</td>
<td></td>
</tr>
<tr>
<td>( CD )</td>
<td>0.964</td>
<td>0.911</td>
<td>0.993</td>
<td></td>
</tr>
</tbody>
</table>

The percent change data for HL-60/Vinc or HL-60/AR cells from Fig 2 were modeled by the Hill equation, using the Adapt software package.

*\( E_{max} \) values were obtained by modeling the observations of percent change for CsA/Crem for HL-60/Vinc or HL-60/AR in the Hill equation. The \( E_{max} \) values so obtained were then used to model the curves for CsA alone or Crem alone. \( D_0 \) is the concentration of drug (CsA alone, Crem alone, or CsA/Crem) causing half-maximal enhancement, calculated by the model; \( H \), the Hill constant; \( CD \), coefficient of determination, defined as the correlation coefficient for goodness of fit for model estimates with the observed data.

† Numbers in parentheses represent coefficients of variation.
Three concentrations of DNR (0.1, 0.3, and 1.0 μmol/L) were used in combination with the MDR modulators. The overall LC₅₀ of DNR observed for all marrows studied was 0.044 μmol/L, which is comparable with the LC₅₀ observed for our previous cohort of newly diagnosed AML patients. The medians and 95% confidence intervals for percent change caused by CsA/Crem or Crem alone are given in Fig 7. For evaluation of the effects of the modulators on DNR cytotoxicity, a negative percent change indicates enhancement in the cytotoxicity (ie, percent decrease in cell survival) of a given concentration of DNR in the presence of the modulator, or in the case of the controls that contained only CsA/Crem or Crem alone, the percent decrement in cell survival caused by the modulator itself (see formula given in Materials and Methods). In the absence of DNR, CsA/Crem or Crem were themselves cytotoxic to the blast cells, with median survival decreasing by approximately 50% for CsA/Crem, and 20% for Crem (P < .01). The cytotoxicity produced by the combination CsA/Crem was significantly greater than that of Crem alone (Mann-Whitney test, P < .01). When the modulators were combined with DNR, at all combinations of DNR plus modulator, absolute cell survival was significantly lower for DNR combined with CsA/Crem than when combined with Crem alone (Mann-Whitney test, P < .01). This effect may be caused by the greater cytotoxicity of CsA/Crem compared with Crem alone in the absence of DNR. To assess whether CsA/Crem or Crem were effective in enhancing DNR cytotoxicity (ie, antagonizing an MDR phenotype), one must compare the percent change for modulator in combination with DNR with that of modulator alone (control). For both CsA/Crem or Crem, there were significant increases in cytotoxicity among the various DNR levels with respect to control (P < .01, Kruskal-Wallis test). Had the modulators been ineffective in altering DNR cytotoxicity, there would have been no further increase in cell killing compared with the modulator alone, because the values depicted in Fig 7 include the “baseline” cytotoxicity at each DNR concentration in the calculation of percent change (see formula in Materials and Methods). Inspection of Fig 7 shows that the degree of enhancement of DNR cytotoxicity by either CsA/Crem or Crem alone is approximately equal (over all DNR levels, approximately a 20% to 40% decrement in cell survival relative to the controls containing modulator but no DNR). There was no significant difference in the degree of enhancement of DNR cytotoxicity by CsA/Crem compared with Crem alone (Mann-Whitney test).

Table 3 displays the analysis of the magnitude and frequency of CsA/Crem or Crem enhancement of 1 μmol/L DNR cytotoxicity among patients. At all levels of percent change, the number of specimens exhibiting a given percent change in response to CsA/Crem was approximately equal to the number of specimens exhibiting the same degree of percent change resulting from Crem alone.

DISCUSSION

Insight into the activity of surfactants and solvents such as Crem EL in reversing MDR was gained in 1972 with observations by Rhien and Biedler in MDR Chinese hamster cells (subsequently shown to overexpress Pgp) that tewen 80 enhanced the sensitivity of these cells to DNR and actinomycin D. In 1990, reports appeared from three different laboratories indicating that Crem EL could reverse the MDR phenotype of Pgp-overexpressing cells. In MDR CCRF-CEM cells or K562 cells that overexpress Pgp, Woodcock et al found that Crem dilutions of 1:3,000 to 1:
Fig 4. Effects of 5 pmol/L CsA/Crem versus an equivalent amount of Crem alone (0.012%) on the cytotoxicity of DNR to HL-60 cells. Cells were exposed to DNR and/or CsA/Crem or Crem alone for 72 hours, after which the number of surviving cells was determined by flow cytometry. The coefficient of variation for each experimental point shown in the figure is less than 10% of the mean value.

1,000 (0.03% to 0.1%) were necessary to effect reversal of the MDR phenotype. Of the published studies describing Crem as an MDR modulator, only two investigated the interaction of CsA and Crem, and neither were in AML-derived cell lines. Schuurhuis et al. found Crem concentrations of 33 and 132 μg/mL (0.005% or 0.02%, respectively) to be effective in reversing the MDR phenotype in Pgp overexpressing human nonsmall lung cancer cells (SW-1573/2R160), or human myeloma cells (8226Dox4 or 8226Dox40), and found the MDR-reversing effects of Crem and CsA to be additive when given as Sandimmune. Friche et al. found that concentrations of 0.001% to 0.01% Crem were effective in reversing the MDR phenotype in Pgp overexpressing Ehrlich ascites tumor cells, that 0.003% Crem inhibited the binding of radiolabeled azidopine to a 170-kD membrane protein in a photoaffinity assay (indicating that Crem EL may be a substrate for Pgp), and that the combination of CsA and Crem was synergistic in sensitizing the MDR cells to DNR. Our studies reported here indicate that the D₀ for maximum enhancement of DNR accumulation in Pgp-overexpressing HL-60/Vinc cells is 0.016% (6.2 μmol/L relative to CsA in Sandimmune, Table 1), which is comparable with the concentrations reported above for other Pgp-overexpressing MDR cell lines, and that the combination of Crem and CsA is synergistic in achieving this effect when given as Sandimmune at all drug-effect levels (Fig 3). In contrast to the MDR1-overexpressing HL-60/Vinc cells, the HL-60/AR cell line, which overexpresses MRP but has no detectable Pgp or MDR1 transcripts, exhibited only a small degree of enhancement of DNR accumulation in response to Crem alone (Eₘₐₓ was not reached with the concentrations of Crem used [Fig 2, Table 1]). However, like HL-60/Vinc cells, the combination of CsA/Crem displayed synergism in enhancing DNR accumulation in these MRP-overexpressing cells (Fig 3).

Recently, we reported that CsA (5 μmol/L, Sandimmune Injectable) was equally as effective as verapamil (6.6 μmol/L) in enhancing DNR accumulation, retention, and cytotoxicity in BM blast cells obtained from a cohort of 49 previously untreated AML patients, with a good correlation of the enhancement caused by verapamil with that caused by...
A. 3-hr dnr Accumulation

B. 16-hr dnr Retention

Table 2. Frequency of 5 μmol/L CsA/Crem or Crem Enhancement of DNR (1 μg/mL) Accumulation and Retention Among Patients

<table>
<thead>
<tr>
<th>Modulator</th>
<th>n</th>
<th>&gt;0%</th>
<th>&gt;5%</th>
<th>&gt;10%</th>
<th>&gt;20%</th>
<th>&gt;30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accumulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA/Crem</td>
<td>36</td>
<td>52 (88%)</td>
<td>44 (75%)</td>
<td>31 (53%)</td>
<td>21 (36%)</td>
<td>13 (22%)</td>
</tr>
<tr>
<td>Crem</td>
<td>36</td>
<td>53 (88%)</td>
<td>40 (67%)</td>
<td>29 (48%)</td>
<td>19 (30%)</td>
<td>11 (18%)</td>
</tr>
<tr>
<td>Retention</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA/Crem</td>
<td>36</td>
<td>51 (87%)</td>
<td>32 (55%)</td>
<td>24 (47%)</td>
<td>19 (37%)</td>
<td>18 (35%)</td>
</tr>
<tr>
<td>Crem</td>
<td>36</td>
<td>51 (87%)</td>
<td>30 (69%)</td>
<td>23 (45%)</td>
<td>19 (37%)</td>
<td>13 (25%)</td>
</tr>
</tbody>
</table>

CsA in a single BM specimen. We now describe, in another cohort of patients, that 0.012% Crem alone (the equivalent concentration that would be obtained in 5 μmol/L CsA using Sandimmune Injectable) produces enhancement of DNR accumulation, retention, and cytotoxicity in AML blast cells to an extent comparable with that caused by the combination CsA/Crem. Hence, our studies show for the first time that Crem functions independently as a modulator of the MDR phenotype displayed in blast cells obtained from AML patients, and that by virtue of the synergism of Crem and CsA observed in HL-60 cell lines, Crem may be an important or crucial contributor to the activity of the MDR-modulating effects of Sandimmune Injectable. The approximate equivalency of the enhancement response in a given marrow sample caused by verapamil or CsA/Crem or by CsA/Crem or Crem alone (Fig 6) indicates that E_max or saturation of the MDR membrane transporter by the modulator as illustrated by the HL-60 model, may also occur in blast cells from AML patients at the concentrations of modulator used in these studies (5 μmol/L CsA, 0.012% Crem). In contrast, in the MDR HL-60 cells, E_max was achieved by 5 μmol/L CsA/Crem, but not by the equivalent amount of Crem alone (Fig 2, Table 1). Thus, it could be that AML blast cells are more sensitive to the effects of Crem alone in terms of enhancement of DNR accumulation, because E_max appears to have been achieved in response to 0.012% Crem (the concentration equivalent that in 5 μmol/L CsA as Sandimmune Injectable). The last point leads to a number of possibilities. First, the synergism of CsA and Crem, as shown in the MDR HL-60 cells, may permit the

Table 3. Frequency of Modulator Enhancement of 1 μmol/L DNR Cytotoxicity Among Patients

<table>
<thead>
<tr>
<th>Modulator</th>
<th>n</th>
<th>&lt;0%</th>
<th>&lt;−20%</th>
<th>&lt;−40%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA/Crem</td>
<td>36</td>
<td>34 (94%)</td>
<td>29 (81%)</td>
<td>28 (78%)</td>
</tr>
<tr>
<td>Crem</td>
<td>36</td>
<td>36 (100%)</td>
<td>29 (81%)</td>
<td>27 (75%)</td>
</tr>
</tbody>
</table>

* % Change is the alteration in the number of cells surviving exposure to DNR in the presence of the modulator compared with those surviving in the absence of the modulator. Hence, a negative percent change reflects enhancement of DNR cytotoxicity by the MDR modulator (see Materials and Methods).
plasma concentrations obtained in recent phase I/II clinical trials (1 to 4 μmol/L CsA/Crem)\textsuperscript{22,23} to achieve maximal reversal of MDR in AML blast cells. Second, should the side effects attributed to high-dose CsA prove to be undesirable, a trial of Crem alone as an MDR modulator in AML may be contemplated. However, in clinical trials using CsA as an antagonist of MDR, it is reasonable to consider CsA combined with Crem.

Our studies in the MDR HL-60 cell lines showed that the enhancement of DNR accumulation by CsA/Crem, Crem alone (with the exception of HL-60/AR) or CsA alone was a saturable process: Using the Hill equation to calculate the maximum concentration for the combination CsA/Crem in each MDR cell line, we found that the % change for Crem in HL-60/Vinc cells or CsA alone in HL-60/Vinc and HL-60/AR cells displayed an excellent fit to a Hill equation model in which the \( E_{\text{max}} \), values calculated for CsA/Crem were imposed (Table 1).

The MDR HL-60 cells used in these studies were chosen because they display a drug-transport-mediated MDR phenotype, yet the transporters supposedly responsible for this phenotype differ in the two cell lines.\textsuperscript{28,39} In preliminary studies, we have observed that Pgp and MRP (or MRK16, UIC2, and C219), we confirmed that the HL-60/Vinc cells used in these studies overexpress \( MDR1 \) mRNA and Pgp, whereas the HL-60/AR cells used have no detectable \( MDR1 \) transcripts or Pgp. Using primer sequences specific for MRP suggested by Dr Susan P.C. Cole and Dr Caroline E. Grant of Queen’s University (Kingston, Ontario, Canada), we have been able to show by an RT-PCR assay, distinct overexpression of MRP transcripts in HL-60/AR cells, but not in HL-60/Vinc cells. The studies of HL-60/AR cells presented here and in our previous paper\textsuperscript{17} clearly show that these MRP-overexpressing cells display an alteration in overall DNR accumulation and retention that is reversible by MDR modulators such as verapamil and CsA. In contrast, the MDR human small cell lung cancer cells H69AR, from which MRP was first isolated and cloned,\textsuperscript{3} display a difference in the intracellular distribution of the drug compared with the parental drug-sensitive H69 cells (the drug is localized in cytoplasmic vesicles in H69AR cells that are associated with nuclear localization in H69), but no difference in total cellular accumulation of the drug, and no alteration in the MDR phenotype in response to MDR modulators.\textsuperscript{43} Like H69AR cells, HL-60/AR cells also have an alteration in intracellular distribution of the drug (eg, DNR) compared with HL-60/W,\textsuperscript{43} but in addition, have an overall decrease in intracellular drug accumulation and retention as illustrated here. Perhaps this illustrates the spectrum of expression of the phenotype associated with MRP. Future studies aimed at transfecting and expressing the MRP gene in HL-60 cells and other cell lines may serve to elucidate this possibility. If, in HL-60/AR cells, CsA/Crem was not able to effect complete restoration of the wild-type intracellular distribution of DNR (and hence of the ability of the drug to reach targets in the nucleus), this may explain the apparent restoration of overall intracellular DNR to levels found in HL-60/W cells (Fig 1), this combination caused only partial sensitization of HL-60/AR cells to DNR compared with HL-60/W (Fig 4). Failure of the drug to reach its intracellular target because of localization in the cytoplasm may also explain why HL-60/AR cells display a higher intracellular steady-state level of DNR accumulation than HL-60/Vinc (Fig 1), but still display the same degree of resistance to DNR (Fig 4).

The two HL-60 MDR cell lines differed greatly in their response to enhancement of DNR accumulation to CsA (see above), with CsA/Crem and CsA alone having a much greater effect in HL-60/AR cells than Crem alone, where the \( D_{50} \) (81 μmol/L) for Crem was not reached with the concentrations of Crem used in these studies. The studies of AML blast cells, however, suggest that the responses to CsA/Crem or Crem alone were approximately equivalent. This implies that the pattern of response of the MDR phenotype displayed in the AML blast cells is more similar to that displayed in the Pgp-expressing HL-60/Vinc cells than to the MRP-expressing HL-60/AR cells. Although our recent studies of a cohort of blast cells from 49 previously untreated AML patients showed a low degree of expression of Pgp as detected by Western blots using the C219 antibody,\textsuperscript{17} other investigators reported a higher frequency of expression of \( MDR1 \) in blast cells from de novo AML patients,\textsuperscript{8,10} and we are currently investigating \( MDR1 \) expression in AML blast cells using RT-PCR, which may be even more sensitive than the Western blot. In addition, we are also evaluating AML blast cell specimens prospectively for expression of MRP, using RT-PCR. These studies may allow correlation of MDR phenotypic characteristics in AML blast cells detected by functional assays with the expression of these MDR transporters. In this respect, we have recently detected, by means of Western blotting, the expression of P-95 protein in 16 of 46 (35%) AML blast cell specimens,\textsuperscript{7} and found that P-95 expression correlated with diminished accumulation of DNR in these blast cells. P-95 expression did not correlate with enhancement of DNR accumulation by CsA/Crem or verapamil in the blast cell samples. In agreement with this, in the human breast carcinoma cell lines used as a model for P-95 expression, CsA/Crem had only minimal enhancing effects on DNR accumulation on the P-95 expressing MCF-7/AdrVp, despite these cells having a marked reduction in DNR accumulation and retention compared with the drug-sensitive parental cells MCF-7/W.\textsuperscript{7}

The recognition of Crem EL as a modulator of MDR and its synergism with CsA has relevance to other anticancer agents in addition to DNR, and to other MDR modulators as well. For example, the cytotoxic activity of taxol against MDR neuroblastoma cells is enhanced by Crem EL.\textsuperscript{44} This is fortuitous, because taxol is formulated for intravenous delivery by suspension in Crem EL. In effect, therefore, one may already be administering significant ‘doses’ of an MDR modulator with standard taxol intravenous regimens. Finally, should Cremophor EL prove to be synergistic with...
other MDR modulators, the combination may allow reduction in the dose necessary to achieve maximal reversal of the MDR phenotype, and hence may alleviate toxicities that may be associated with higher doses of the given modulator.

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Synergistic reversal of multidrug-resistance phenotype in acute myeloid leukemia cells by cyclosporin A and cremophor EL

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