Enhancement of Daunorubicin Accumulation, Retention, and Cytotoxicity by Verapamil or Cyclosporin A in Blast Cells From Patients With Previously Untreated Acute Myeloid Leukemia

By Douglas D. Ross, Patricia J. Wooten, Raji Sridhara, José V. Ordóñez, Edward J. Lee, and Charles A. Schiffer

This work was designed to discern the frequency of expression of classical multidrug resistance (MDR) in acute myeloid leukemia (AML) at the time of diagnosis, using Western blotting for P-glycoprotein (Pgp) and functional assays for an MDR phenotype (enhancement of daunorubicin [DNR] accumulation/retention and cytotoxicity by the known MDR modulators verapamil, cyclosporin A, and progesterone). Blast cells were studied from 49 newly diagnosed AML patients who were subsequently treated with the "3 and 7" combination of cytosine arabinoside (ara-C) and DNR. DNR accumulation (1 μg/mL; 3 hours) and retention (16 hours) were determined by flow cytometry. Cyclosporin A (CsA, 5 μmol/L) or verapamil (6.6 μmol/L) each caused significant enhancement of DNR accumulation and retention in these blast cell samples (P < .001, Wilcoxon's test). Verapamil or CsA caused greater than 20% enhancement of DNR accumulation or retention in over 25% or 50% of these patients, respectively; however, there was no correlation with the presence or degree of enhancement and response to treatment. Progesterone (10 μmol/L) caused no significant enhancement of DNR accumulation or retention. The effects of the MDR modulators on the cytotoxicity of DNR was also determined in blast cells from 40 of the patients, using a flow cytometric assay. CsA alone was cytotoxic (caused an approximate 20% decrease in cell survival compared with control, P < .001); CsA or verapamil caused enhancement of 1 μmol/L DNR cytotoxicity (P < .001). Greater than 40% enhancement of cell kill by CsA or verapamil was observed in over 75% of patients studied. There was no difference in the degree of enhancement of cytotoxicity between patients clinically sensitive or resistant to treatment. Progesterone caused no enhancement in DNR cytotoxicity. In contrast to the functional assays, highly sensitive immunoblots using the C219 antibody to Pgp showed evidence of low level expression of Pgp in blast cells from only 3 of these patients: 1 was chemotherapy resistant, 2 were sensitive. Thus, although the functional assays suggest a high frequency of expression of a classic MDR phenotype in AML patients at the time of diagnosis, with enhancement by CsA obtained at a clinically relevant concentration (5 μmol/L), the frequency of Pgp expression detectable by C219 Western blots was low in these patients. This could be interpreted either that the method used was not sufficiently sensitive to detect Pgp in all of the blast cell specimens that actually overexpressed mdrl, or that the accumulation-efflux-based MDR phenotype observed is not always mediated by Pgp in these previously untreated patients.

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DESPITE PROGRESS in our ability to produce complete remission, resistance of leukemic blast cells to chemotherapeutic agents remains the major reason for failure to cure the majority of patients with acute myeloid leukemia (AML). The mainstays of treatment of AML include the antimetabolite cytosine arabinoside (ara-C) and a variety of natural product drugs, particularly the anthracyclines (eg, daunorubicin, DNR), but also the anthraquinones (mitoxantrone), and the epipodophyllotoxins (etoposide, or VP-16). During the past 10 years, a large body of data has emerged regarding an intriguing form of cellular resistance to natural products, called multidrug resistance or, MDR. The MDR phenotype, demonstrable in experimental animal and in vitro systems, is one wherein selection with one drug results in resistance not only to the selecting agent, but also to other, often unrelated, drugs. "Classical" MDR is characterized by an impaired ability to accumulate intracellular drug in resistant cells compared with drug-sensitive cells as a result of active outward transport. "Classical" MDR is accompanied by overexpression of a gene (mdrl) coding for a 150- to 180-Kd membrane protein, P-glycoprotein (Pgp). Pgp is thought to function as an active outward transport mechanism for a variety of molecules, including certain chemotherapeutic drugs (eg, anthracyclines, vinca alkaloids, and epipodophyllotoxins).

Screening of an assortment of human tumors, including some studies of acute leukemia, suggest that the levels of Pgp, as can be assessed by immunohistochemical studies of individual cells, Western blots of cellular membranes, and indirectly by mRNA levels, are increased in patients refractory to chemotherapy. The exciting implications of this finding relate to the ability, at least in vitro, to reverse the effect of Pgp by exposure of cells to a variety of disparate compounds, including calcium channel blockers (eg, verapamil), progesterone, and cyclosporin A (CsA). This phenomenon is usually mediated by competitive inhibition of Pgp by these agents. Verapamil has been used in conjunction with chemotherapy in patients with refractory multiple myeloma and lymphoma with suggestive evidence of benefit for some patients, although cardiac toxicity is likely to limit the use of this agent. CsA, in contrast, can reverse the MDR phenotype in vitro at drug levels that are likely to...
be clinically acceptable in regard to toxicity. Clinical trials with CsA as an MDR reversing agent are currently in progress. 

Recent evidence has also suggested that mechanisms other than Pgp overexpression may account for an MDR phenotype in human leukemias and solid tumors. Particularly relevant are studies by two independent investigators, showing, in sublines of the cultured human AML cell HL-60 (HL-60/Adr, HL-60/AR), that a "classical" efflux-mediated MDR phenotype can develop in response to selection with an anthracycline without overexpression of mdrl. Although the precise cellular product(s) that mediate such non-Pgp resistance is/are currently unknown, Marquardt et al showed that HL-60/AR and HL-60/Adr cells contain a 190-Kd adenosine triphosphate (ATP) binding protein in their endoplasmic reticulum. The ability to detect such non-Pgp-mediated MDR may be of great clinical importance, because in our studies presented here we find that Pgp inhibitors such as verapamil and CsA are also able to enhance DNR accumulation/efflux and cytotoxicity in both HL-60/AR and HL-60/Adr cells. Thus, if one concentrates solely on mdrl expression, one may fail to recognize an efflux-mediated MDR phenotype in AML blast cell specimens that may be amenable to modulation by agents such as verapamil or CsA. Interestingly, an HL-60 subline with a "classical" MDR phenotype, produced by selection with vincristine (HL-60/Vcr), does overexpress Pgp, but has no detectable P190. Following the description of non-Pgp-associated efflux-mediated MDR in HL-60 cells, considerably more data are emerging in support of MDR mechanisms of this type. 

As a prelude to possible clinical trials in patients with AML, we systematically studied a consecutive series of previously untreated patients with AML using a variety of physiologic or "functional" assays to detect the MDR phenotype (effects of the MDR modulators verapamil, CsA, or progesterone on DNR accumulation/retention and cytotoxicity), coupled with a sensitive Western blot for detection of Pgp, using the C219 monoclonal antibody (MoAb). The MDR antagonists CsA and progesterone were chosen for study because they represent agents that have or would be expected to have acceptable toxicity when used in a clinical setting that achieve concentrations in plasma that are known to reverse MDR in vitro. Verapamil was included in this study as a point of reference for CsA and progesterone, because it represents a known and long-established antagonist of MDR.

MATERIALS AND METHODS

Patient Characteristics

We present data from 49 consecutive, previously untreated AML patients. Their median age was 64 years (range 24 to 87); 29 were male, 20 were female; The French-American-British (FAB) classifications are as follows: M0, 2 patients; M1, 10 patients; M2, 13 patients; M3, 5 patients; M4, 13 patients; M4e, 1 patient; M5, 3 patients; M6, 1 patient; unable to be classified, 1 patient.

Treatment and Response Categories

The "3 and 7" induction therapy (DNR, 45 mg/m²/d × 3d; ara-C, 200 mg/m²/d × 7d, continuous infusion) was used for all patients. Patients achieving complete remission received ara-C-based postremission consolidation therapy. Complete remission of leukemia (CR) was defined according to standard criteria. Thirty-two of the 49 patients (62%) obtained CR. 22 patients had CR duration of greater than 6 months; CR was of less than 6 months duration in 10 patients. The 22 patients having a CR of duration greater than 6 months were classified as chemotherapy sensitive. Twenty patients were classified as chemotherapy resistant, either by failing to obtain CR (10 patients), or by remaining in CR for less than 6 months (10 patients). Seven patients were not evaluable for assessment of drug sensitivity because of early death.

Materials

Verapamil HCl, from Abbott Labs, N Chicago, IL, was obtained as a stock solution of 2.5 mg/mL in 0.85% saline, and was added to achieve a final concentration in culture of 6.6 μmol/L. CsA was obtained as Sandimmune Injectable from the Sandoz Corp, East Hanover, NJ, as a stock solution of 50 mg/mL dissolved in a mixture of 32.9% ethanol in Cremophor EL. The final concentration of Cyclosporin A used in culture was 5 μmol/L. This corresponds to a final concentration of vehicle (Cremophor/alcohol mixture) of 0.012%. Progesterone was obtained from Eli Lilly, Indianapolis, IN, as a stock solution of 50 mg/mL in peanut oil, and was added to achieve a final concentration in culture of 10 μmol/L. This corresponds to a final concentration of vehicle (peanut oil) of 0.006%. DNR was obtained from Wyeth Laboratories, Philadelphia, PA. The C219 MoAb to Pgp, and the peptide epitope to C219 (NH2-R.V.Q.E.A.L.D.K.A.R.E.G.T.C.-COOH) were obtained from the Centocor Corporation (Malvern, PA).

Cell Culture Techniques

Cultured cells. All HL-60 human leukemic cells were cultured in RPMI 1640 medium, supplemented with 1% sodium pyruvate (Sigma Chemical Co, St Louis, MO), 1% nonessential amino acids (Sigma), and 10% (vol/vol) fetal calf serum (FCS), as described previously. Cells in logarithmic growth phase were used in all studies. HL-60/W and HL-60/AR cells were obtained from Drs Steven Grant, Alex Hindenberg, and Kapil Bhalla now of the Medical College of Virginia, Winthrop University Hospital, and the Medical University of South Carolina, respectively. HL-60/Vinc and HL-60/Adr cells were obtained from Dr Melvin Center, of Kansas State University. HL-60/W cells were maintained in culture between the original passages 30 to 80. HL-60/AR, HL-60/Adr and HL-60/Vinc were maintained between passages 1 to 60 from the time of receipt in our laboratory. Multidrug resistant Chinese hamster cells DC3F/ADX and CHF/C4 were obtained from Dr Peter Melera of the University of Maryland, and Dr Victor Ling, of the University of Toronto, respectively, along with the corresponding drug-sensitive parental cell lines, DC3F/W and AUXB, DC3F/W, DC3F/ADX, CHF/C4, and AUXB, cells were maintained in Dulbecco's F12/s minimal essential medium (Sigma), containing 10% (vol/vol) FCS. The cultured cells used in these studies were all tested to assure absence of contamination by Mycoplasma (Gen Probe, San Diego, CA). 

AML blast cells. Marrow aspirates (4 mL, collected in preservative-free heparin, 1:1,000) were diluted 1:1 with phosphate-buffered saline (PBS), then marrow mononuclear cells were collected by means of a Ficoll-Hypaque discontinuous gradient. The mononuclear cells (taken at the Ficoll-Hypaque/plasma interface) were then washed in PBS. If necessary, contaminating red blood cells (RBC) were lysed (RBC lysis buffer, Sigma). The cells were then suspended in RPMI 1640 medium, supplemented with 1% sodium pyruvate, 1% nonessential amino acids, and 10% FCS, for DNR accumulation/retention or cytotoxicity studies, or washed and resuspended in PBS for Western blotting (see below).
DNR Accumulation and Retention Methods

Forty-nine patient blast cell samples were studied. Blast cells were placed in RPMI 1640 culture medium as described above (median concentration was 750,000/mL, range 500,000 to 2,000,000/mL) with and without combinations of DNR, verapamil, CsA, or progestrone, as indicated in Results. After 3 hours in culture at 37°C, an aliquot was removed and intracellular DNR content was measured (3-hour DNR accumulation). The remainder of the cells were washed free of drugs, then placed back in culture at 37°C in the continued presence of the same MDR modulator that was used during accumulation. After 16 hours, intracellular DNR content was determined (16-hour DNR retention). Intracellular DNR content was quantified by flow cytometry (FACStar Plus flow cytometer, Becton Dickinson, San Jose, CA), using laser excitation of 488 nm, and reading fluorescence emission with the use of a 575-25 nm filter. Logarithmic amplification of red fluorescence signals was used throughout. Fluorescent beads (Propidium Iodide Alignment Microbead Standards, Flow Cytometry Standards Corporation, Research Triangle, NC) were used to insure precise day-to-day reproducibility of fluorescence measurements. Relative intracellular DNR content for a particular sample was obtained by dividing the channel number that represented mean red fluorescence for that sample by 256 (the number of channels per log decade), then obtaining the antilog of this value. Cell sorting and subsequent microscopic visualization of Wright-stained cytospin preparations of the sorted cells was performed in all cases to determine the scatter gate (forward v right angle) that contained leukemic blast cells. This scatter gate was then used for all subsequent determinations of intracellular DNR content (cellular accumulation or retention) for that sample.

Cytotoxicity Studies

Forty patient blast-cell samples were studied. Blast cells were placed in RPMI 1640 culture medium as described above for the accumulation_retention studies, with or without DNR and/or the MDR modulators. Initial cell concentration was 0.5 to 1.0 × 10⁶/mL. The cultures were incubated for 48 hours (9 samples), 72 hours (21 samples), 92 hours (8 samples), or 120 hours (2 samples) in the continuous presence of drugs, after which the number of viable cells per mL of culture was determined by the use of fluorescein diacetate (FDA) and propidium iodide (PI), as described below. This difference in incubation/exposure times occurred because of the imposition of holidays or weekends. For the 40 patients studied, no statistically significant differences were observed in cytotoxicity with respect to time of incubation. During these short-term cultures of AML blast cells, viable cell number deceased to a mean of 45% (median 38%) of the original cell inoculum in control cultures (no drugs added). There were no statistically significant differences in cell survival in drug-free control cultures among the groups incubated for different lengths of times.

A sensitive flow cytometric method that we had developed for determining the number of cells surviving in suspension culture was used. At the time of determination of the number of surviving viable cells in culture, FDA and PI in isotonic solution were added to the cells in culture, to achieve final concentrations of 0.5 and 50 μg/mL, respectively. Viable cells were identified as those that displayed a bright green fluorescence, induced by the intracellular metabolism of FDA to fluorescein, and a low red fluorescence, indicating cellular exclusion of PI. In this assay, only intact viable cells are counted; cell debris and dead cells are ignored. The number of viable cells per mL of culture medium sample is determined by a timed count and knowledge of the flow cytometer sample flow rate, as previously described. The analyses were performed with a FACStar Plus flow cytometer (Becton Dickinson).
where SurvDNRx_mod = cells surviving DNR dose X in the presence of a given MDR modulator, and SurvDNRx = 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 percent change data for cell survival were analyzed using the Kruskal-Wallis test for differences among the response categories. 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

Model System

To ascertain the effectiveness of our assays to detect an MDR phenotype, Western blots and the effects of the MDR modulators on the accumulation/retention and cytotoxicity of DNR were modeled in known MDR cell lines and their corresponding drug-sensitive parental cells. For the functional assays of a classical MDR phenotype, we studied the human AML cell line HL-60 and the MDR sublines HL-60/AR and HL-60/Vinc. Both sublines display a classical MDR phenotype, although HL-60/Vinc overexpresses Pgp, whereas HL-60/AR has no detectable Pgp. Figure 1 shows the 3-hour accumulation and 16-hour retention of DNR (1 μg/mL) in these three cell lines, and the effects of the MDR modulators verapamil, CsA, and progesterone. Note in Fig 1 that the intracellular accumulation and retention of DNR is fourfold to sixfold higher in drug sensitive HL-60/W cells, compared with HL-60/AR or HL-60/Vinc. None of the MDR modulators caused significant alteration in DNR accumulation or retention in the drug-sensitive HL-60/W cells. In contrast, verapamil and CsA caused significant enhancement of DNR accumulation and retention in both HL-60/AR and HL-60/Vinc cells, with levels of accumulation and retention approaching those seen in the drug-sensitive cells. Progesterone, which has been reported to antagonize Pgp in cultured cells, had no effects on DNR accumulation/retention in HL-60/W, HL-60/AR, or HL-60/Vinc cells.

The effects of the various MDR modulators on the cytotoxicity of DNR to these cells were consistent with the accumulation and retention results (Fig 2). Note that HL-60/AR and HL-60/Vinc cells are 80-fold to 90-fold more resistant to DNR than drug-sensitive HL-60/W cells. The concentrations of DNR causing 50% lethality (LC50) to HL-60/W, HL-60/AR, and HL-60/Vinc cells are 0.005, 0.4, and 0.45 μmol/L, respectively. None of the MDR modulators had an effect on the cytotoxicity of DNR in HL-60/W cells. In contrast, verapamil and CsA each caused some enhancement in the cytotoxicity of DNR in HL-60/AR cells, while progesterone caused no enhancement of cytotoxicity. In HL-60/Vinc cells, the effects of CsA or verapamil were more pronounced than seen in HL-60/AR cells. Verapamil caused substantial enhancement in DNR cytotoxicity, although the degree of enhancement was not sufficient to achieve the sensitivity of HL-60/W cells. The most dramatic enhancement seen was with CsA, where the cells became almost as sensitive as HL-60/W cells. Progesterone had no effect on the cytotoxicity of DNR in HL-60/Vinc cells.

For Western blot studies, we selected the MDR cell line DC3F/ADX, and its drug-sensitive, parental counterpart, DC3F/W, as the model system. These cells were used because DC3F/ADX displays marked overexpression of Pgp, and because we were able to detect Pgp expression in DC3F/W cells by Western blot. Hence, the detection of Pgp in DC3F/ADX was used as a positive control, and detection of Pgp in DC3F/W was used to assure sufficient sensitivity of the assay. Controls for the specificity of the reaction also included prereacting the C219 MoAb with a 10-fold molar excess of its peptide epitope. The results of a typical Western blot are shown in Fig 3. For DC3F/ADX cells, a very intense region of immunoreactivity is seen corresponding to a protein of 180 Kd, which is consistent with the molecular mass of Pgp. Using densitometric measurements, this dark band contains greater than 99% of the immunoreactivity observed. However, in addition to Pgp, two or three minor bands were observed in the 90- to 93- and 70-Kd range, which may represent degraded immunoreactive forms of Pgp. A 180-Kd species corresponding to Pgp is also clearly seen in the DC3F/W control. The immunoreactive bands observed in DC3F/W and DC3F/ADX reflect a specific reaction with C219, because preincubation of C219 with its
resistant parental cell line of the MDR cell line CHO/CH\textsuperscript{R}C\textsubscript{5} (data not shown).

Studies of AML Blast Cells

Accumulation and retention studies. The results of a DNR accumulation and retention study of blast cells from an AML patient are illustrated in Fig 4. Cell sorting was used to verify that the forward and side-scatter gates used for analysis of DNR accumulation and retention contained the viable AML blast cells. This example is typical of those samples that responded to verapamil or CsA. The accumulation distribution is typically relatively homogeneous, whereas in this case, the retention histogram displays two subpopulations, one with lower DNR retention than the other. The addition of verapamil or CsA not only enhanced DNR accumulation, but caused the low DNR retaining subpopulation to merge with the one with higher DNR content. With CsA, the overall retention was increased to a level higher than that of the subpopulation of the control that had the higher DNR retention. Progesterone had no effect on DNR accumulation or retention in this patient.

The median percent change and 95\% confidence interval in DNR accumulation or retention caused by the addition of the modulating agents are shown in Fig 5. Both verapamil and CsA caused a statistically significant enhancement in DNR accumulation and retention ($P < .001$), whereas progesterone did not enhance DNR accumulation or retention.

We also analyzed the relationship of DNR accumulation and retention in control cultures (no added MDR modulator) with clinical response group (Table 1). For these studies, DNR accumulation was measured in all 49 patients; retention studies were performed in 39 patients. The large standard deviations shown in Table 1 reflect true interpatient variability, because the coefficient of variation for experimental determinations within individual patients was routinely less than 10\%. There were no statistically significant differences between chemotherapy-sensitive and resistant patients (Table 1). This may be caused in part by interpatient variability and/or to the use of ara-C in the clinical induction regimen.

The degree and frequency of modulator enhancement of DNR accumulation and retention is displayed in Table 2. As one becomes more rigorous in the percent change required as evidence for an MDR phenotype, the effects of progesterone become minimal, whereas those of CsA or verapamil remain apparent in a relatively large proportion of patients. For example, if one selects greater than 20\% change in DNR accumulation in response to the modulator as evidence of a classical MDR phenotype, then 27\% and 33\% of patient samples showed this degree of change in response to verapamil or CsA, respectively, compared with only 5\% of patients with such a response to progesterone (Table 2). Similarly, for DNR retention, 58\% and 51\% of patient blast cell samples showed greater than 20\% enhancement with verapamil or CsA, whereas only 6\% showed a comparable percent change in response in progesterone. There was good correlation between the enhancement in DNR accumulation ($r = .84$) or retention ($r = .992$) caused by verapamil with that caused by CsA (Fig 6). These data suggest that in blast-cell samples from individual patients,
these two disparate agents (CsA and verapamil) are affecting the intracellular target to the same extent, and also serve to validate the reproducibility of this assay as a measure of Pgp or other efflux pumps.

*Cytotoxicity studies.* Of the 49 patient blast cell samples studied for DNR accumulation/retention, 40 were studied for cytotoxicity. To optimize the assessment of the effects of the MDR modulator on DNR toxicity, it is desirable to use a concentration of DNR that causes approximately 50% to 90% cytotoxicity to the cells when used alone. Thus, three

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**Western Blot – C219 Ab**

<table>
<thead>
<tr>
<th>Controls</th>
<th>Patients</th>
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<tr>
<td>DC3F</td>
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</tr>
<tr>
<td>W</td>
<td>#2</td>
</tr>
<tr>
<td>ADX</td>
<td>#3</td>
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**Western Blot – C219 + Epitope**

<table>
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<th>Controls</th>
<th>Patients</th>
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<tbody>
<tr>
<td>DC3F</td>
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<td>W</td>
<td>#2</td>
</tr>
<tr>
<td>ADX</td>
<td>#3</td>
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**Fig. 3.** Typical C219 Western blot for Pgp. The numbers below the figure describe the amount of membrane protein loaded in that particular well. The panel on the right shows the results of a Western blot study where the nitrocellulose blot was incubated with C219 and a 10-fold molar excess of the peptide epitope for C219, as described in Materials and Methods.

**Fig. 4.** Example of a patient whose blast cells displayed enhancement of DNR accumulation/retention by verapamil and cyclosporin A. Increasing intracellular DNR fluorescence is shown in log scale on the X axis. The top panels show the histogram distribution of intracellular DNR after 3 hours of accumulation, or 16 hours of retention, in control cells.
concentrations of DNR (0.1, 0.3, and 1.0 μmol/L) were used to ensure obtaining at least one informative data point per patient blast sample tested. Overall, survival was (mean ± SD) 56% ± 36%, 40% ± 31%, and 21% ± 31% for cells exposed to 0.1, 0.3, or 1.0 μmol/L DNR, respectively. This gives a concentration of DNR causing 50% lethality (LC50) of 0.13 μmol/L for all patients studied. The large SDs obtained for cell survival reflect interpatient variability, and not experimental variability, because the coefficients of variation for experimental determinations within individual patient samples were routinely less than 10%. There were no significant differences in cell survival to DNR among the various clinical response groups.

The percent changes in cell survival obtained in response to the MDR modulators at each concentration of DNR used are presented in Fig 7. In this case, an enhancement of DNR cytotoxicity caused by a given MDR modulator is reflected by a decrement in surviving cells. Hence, a negative percent change indicates the enhancement of cytotoxicity of a given concentration of DNR as a result of the modulator, or in the case of the controls that contained only MDR modulator, the decrement in cell survival caused by the modulator itself. In the absence of DNR, CsA caused an approximate 22% mean decrement in survival as compared to drug-free controls, P < .001. Neither verapamil nor progesterone had a significant effect on cell survival in the absence of DNR. When combined with DNR, both verapamil and CsA caused enhanced DNR cytotoxicity at all concentrations of DNR tested (P at least <.03 for all comparisons), compared with controls (modulator alone, no DNR). In contrast, progesterone caused no significant alteration in DNR cytotoxicity at any concentration of DNR tested. When the % change in DNR toxicity caused by the MDR modulators was analyzed with respect to the differing times of incubation used, there were no statistically significant differences among these groups. Similar to the DNR accumulation or retention results, there was a correlation with the degree of enhancement of DNR cytotoxicity caused by CsA with that caused by verapamil. This correlation was best at DNR concentrations of 0.3 and 1.0 μmol/L (r = .689 and .642, respectively).

An analysis of the magnitude and the frequency of modulator enhancement of 1 μmol/L DNR cytotoxicity among patients is shown in Table 3. Note that 62% and 78% of patient samples studied showed more than a 40% decrement in cell survival (percent change < −40) in response to verapamil or CsA, respectively, compared with only 18% of blast cell samples showing such a percent change in response to progesterone.

**Western blot studies.** We had sufficient cells from 38 of the 49 patients to perform Western blot studies (Table 4). Despite our Western blot assay being sensitive enough to detect Pgp in drug-sensitive DC3F/W cells, Table 4 illustrates that all but three of our patient samples showed no reactivity with the C219 MoAb, as illustrated in the patient lanes shown in Fig 3. In addition, the three reactive patient samples were at a level considerably less than observed for DC3F/W, when the densities of the immunoreactive bands were compared. Two of the patients with detectable Pgp were in the drug sensitive group, one was in the drug resistant group (short CR). All three of these patient blast cell samples displayed enhanced DNR accumulation and retention with CsA or verapamil.

**DISCUSSION**

In the current study, we present data showing that blast cells from a high percentage of patients with previously untreated AML have enhancement of DNR accumulation/retention and/or cytotoxicity following coincubation in vitro with verapamil or CsA. As such, these constitute a physiologic or "functional" assay for an accumulation/efflux-mediated MDR phenotype operative in these AML blast cell populations. No effect was seen using progesterone, another putative MDR modulator. This, in effect, served as a confirmatory negative control. Although there was no statistically significant difference in the enhancement of DNR accumulation/retention or cytotoxicity by verapamil or CsA amongst patients categorized according to their sensitivity to chemotherapy, this may be a consequence of the moder-

**Table 1. DNR Accumulation and Retention by Response Group**

<table>
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<tr>
<th>Group</th>
<th>Accumulation</th>
<th>Retention</th>
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<tr>
<td>Sensitive CR &gt;6 mo</td>
<td>22 ± 949</td>
<td>375 ± 280</td>
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<tr>
<td>Failed</td>
<td>10 ± 986</td>
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<tr>
<td>Short CR</td>
<td>10 ± 904</td>
<td>352 ± 482</td>
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<tr>
<td>Other NE</td>
<td>7 ± 1,067</td>
<td>457 ± 495</td>
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P = NS.

Abbreviations: NE, not evaluable; NS, not significant.
MDR PHENOTYPE IN AML

could be that other non-Pgp-based mechanisms of DNR mediated MDR phenotype present. Another explanation described by Cole or the P-95 protein described by Chen et al. MDR associated with expression of these proteins may not be ame-
nable to reversal by CsA or verapamil and hence may not be detected by our “functional” assays. This contrasts with our C219 antibody Western blot investigations, wherein only 3 of 9 patients expressed measurable Pgp. In distinction to the studies above reporting a high frequency of de novo AML samples expressing mdrl by slot-blot hybridization for mdrl message or immunohistochemi- cal/flow cytometric detection of Pgp with the MRK16 or C219 MoAbs, report a low frequency of mdrl expression in newly diagnosed AML patients. Other studies, using similar methods, found a high frequency (>30%) of expression of MDR in AML at time of presentation. These studies agree with our present assessment of MDR expression in untreated AML by “functional assays,” where we found enhancement of DNR accumulation/retention or cytotoxicity in more than half of de novo AML patients. One of the studies reporting a high frequency of expression of Pgp in de novo AML used Western blots and the C219 antibody to detect Pgp expression. This contrasts with our C219 antibody Western blot investigations, wherein only 3 of 8 patients expressed measurable Pgp. In distinction to the studies above reporting a high frequency of de novo AML samples expressing mdrl by slot blots, in one series of 51 patients, no detectable Pgp was observed by filter hybridization, whereas 27 of the 51 had a low level (1 to 2+) expression of Pgp detected by reverse transcriptase-polymerase chain reaction (RT-PCR).

In terms of correlation of mdrl expression with response to treatment, two large studies of 61 and 63 patients, respectively, found a significant association between low re-
mission rate/short remission duration with expression of mdrl as measured by slot-blot hybridization. In a follow-up of one of these studies, mdrl mRNA expression was found to be an independent prognostic factor. A study by Campos et al used flow cytometry and indirect immunofluorescence staining with the MRK16 MoAb to Pgp to examine marrow samples from 150 newly diagnosed AML patients who were subsequently treated with ara-C and DNR. Of these, 71 (47%) were “Pgp positive” defined as having greater than 20% of cells staining positive with MRK16. Pgp expression was found to correlate significantly with expression of the early hematopoietic stem-cell marker CD34, with lower CR rate/CR duration, and with decreased sur-
vival. In contrast to this, an earlier study by the Cancer and Acute Leukemia Group B of 205 newly diagnosed AML patients, using virtually the same analytic system and treatment with ara-C and DNR, found no adverse correlation of CD34 or Pgp expression with attainment of CR, CR duration, or survival. Lack of correlation of CD34 or mdrl expression with relapse-free or overall survival was observed in a study of 155 de novo AML cases by the Southwest

<table>
<thead>
<tr>
<th>Modulator</th>
<th>No.</th>
<th>&gt;0%*</th>
<th>&gt;5%</th>
<th>&gt;10%</th>
<th>&gt;20%</th>
<th>&gt;30%</th>
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<tr>
<td>Verapamil</td>
<td>49</td>
<td>40 (82%)</td>
<td>33 (67%)</td>
<td>27 (55%)</td>
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<td>Cyclosporin A</td>
<td>48</td>
<td>44 (92%)</td>
<td>37 (77%)</td>
<td>26 (54%)</td>
<td>16 (33%)</td>
<td>7 (15%)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>44</td>
<td>28 (64%)</td>
<td>8 (18%)</td>
<td>5 (11%)</td>
<td>2 (5%)</td>
<td>2 (5%)</td>
</tr>
<tr>
<td>Retention</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil</td>
<td>36</td>
<td>29 (81%)</td>
<td>26 (72%)</td>
<td>25 (69%)</td>
<td>21 (58%)</td>
<td>19 (53%)</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>37</td>
<td>26 (70%)</td>
<td>25 (68%)</td>
<td>21 (57%)</td>
<td>19 (51%)</td>
<td>18 (49%)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>35</td>
<td>18 (51%)</td>
<td>12 (34%)</td>
<td>6 (17%)</td>
<td>2 (6%)</td>
<td>2 (6%)</td>
</tr>
</tbody>
</table>

* % Change = % enhancement compared with control without MDR modulator.

ate number of patients studied and the relatively short follow-up to date. In addition, all patients treated with DNR also received concurrent therapy with ara-C, making it quite possible that the potent anti-leukemic effect of ara-C "masked" the potential clinical effect of any efflux-me-
diated MDR phenotype present. Another explanation could be that other non-Pgp-based mechanisms of DNR resistance were operative in some of these patients such as the overexpression of membrane transporters like MRP (multidrug resistance-associated protein) described by Cole et al29 or the P-95 protein described by Chen et al. MDR associated with expression of these proteins may not be ame-
nable to reversal by CsA or verapamil and hence may not be not be deleted by our “functional” assays. In this context, recent studies of ours (not presented here) have detected expression of P-95 protein in blast cells from approximately 30% of AML patients. P-95 expression was associated signifi-
cantly with reduced accumulation or retention of DNR, but not with enhancement of accumulation/retention by CsA or verapamil.

While the observed verapamil- or CsA-induced percent change in DNR accumulation/retention or cytotoxicity was relatively small in an absolute sense (20% to 40%), this still may be quite clinically significant. Because of dose-limiting toxicity, the degree of overall resistance in clinical tumors may be lower in absolute terms than that expressed in cell lines exposed to high selective pressures in vitro. Furthermore, it is possible, particularly at the time of diagnosis, that a drug-resistant phenotype might be present in only a small population of cells. Therefore, an overall mean enhance-
ment of 20% to 40% by an MDR modulator may reflect a small but clinically significant level of accumulation/efflux-
mediated drug resistance in the majority of cells, or might represent an extreme level of resistance in a subpopulation of the AML blasts.

In contrast to the “functional assay” findings, we were unable to show evidence of frequent expression of Pgp by C219 antibody Western blotting. Although these findings are in contrast to some reports in the literature, it must be emphasized that there is considerable discrepancy in published works as to the frequency of expression of MDR in AML, the relationship of MDR expression with response to treatment, and its significance as a prognostic indicator. Results obtained by different investigators using the same or similar methods vary. For example, some studies, using slot blot hybridization for mdrl message or immunohistochemi-
cal/flow cytometric detection of Pgp with the MRK16 or C219 MoAbs, report a low frequency of mdrl expression in newly diagnosed AML patients. Other studies, using similar methods, found a high frequency (>30%) of expression of MDR in AML at time of presentation. These studies agree with our present assessment of MDR expression in untreated AML by “functional assays,” where we found enhancement of DNR accumulation/retention or cytotoxicity in more than half of de novo AML patients. One of the studies reporting a high frequency of expression of Pgp in de novo AML used Western blots and the C219 antibody to detect Pgp expression. This contrasts with our C219 antibody Western blot investigations, wherein only 3 of 8 patients expressed measurable Pgp. In distinction to the studies above reporting a high frequency of de novo AML samples expressing mdrl by slot blots, in one series of 51 patients, no detectable Pgp was observed by filter hybridization, whereas 27 of the 51 had a low level (1 to 2+) expression of Pgp detected by reverse transcriptase-polymerase chain reaction (RT-PCR). In terms of correlation of mdrl expression with response to treatment, two large studies of 61 and 63 patients, respectively, found a significant association between low re-
mission rate/short remission duration with expression of mdrl as measured by slot-blot hybridization. In a follow-up of one of these studies, mdrl mRNA expression was found to be an independent prognostic factor. A study by Campos et al used flow cytometry and indirect immunofluorescence staining with the MRK16 MoAb to Pgp to examine marrow samples from 150 newly diagnosed AML patients who were subsequently treated with ara-C and DNR. Of these, 71 (47%) were “Pgp positive” defined as having greater than 20% of cells staining positive with MRK16. Pgp expression was found to correlate significantly with expression of the early hematopoietic stem-cell marker CD34, with lower CR rate/CR duration, and with decreased sur-
vival. In contrast to this, an earlier study by the Cancer and Acute Leukemia Group B of 205 newly diagnosed AML patients, using virtually the same analytic system and treatment with ara-C and DNR, found no adverse correlation of CD34 or Pgp expression with attainment of CR, CR duration, or survival. Lack of correlation of CD34 or mdrl expression with relapse-free or overall survival was observed in a study of 155 de novo AML cases by the Southwest
A. 3-hr dnr Accumulation

![Graph showing dnr Accumulation with CsA and Verapamil effects.]

B. 16-hr dnr Retention

![Graph showing dnr Retention with CsA and Verapamil effects.]

Fig 6. DNR accumulation and retention: Each point in the figure relates the percent change in DNR accumulation (A) or retention (B) caused by verapamil with that caused by CsA in a single AML patient. Of the 49 patients where DNR accumulation was studied, 47 were studied for both CsA and verapamil effects on DNR accumulation. Of the 39 patients where DNR retention was studied, 37 were studied for both CsA and verapamil effects on DNR retention. There are 36 x-y pairs shown in (B). Not shown is an x-y pair that would represent one patient where the percent change for verapamil was 1.270%, and for CsA was 2.072%.

Oncology Group. Furthermore, mdr1 expression was not significantly associated with attaining a CR, nor was it found to be an independent prognostic factor. In these studies, mdr1 expression was detected both by flow cytometry/MRK16 antibody, and by a semiquantitative RT-PCR assay that was specific for mdr1 transcripts. Confirmation of MRK16 immunoreactivity by RT-PCR for actual mdr1 transcripts may be important, because these same investigators found that MRK16 positivity is frequently associated with expression of the mdr2(mdr3) gene (not associated with drug resistance).47 Expression of mdr1 was closely correlated to the expression of CD34 in this study,44 which itself was found to predict CR attainment. These findings with CD34 agree with our own studies of AML patients treated at the University of Maryland Cancer Center. In

"functional" studies, Maruyama et al.52 used enhancement of DNR intracellular accumulation and cytotoxicity by verapamil as evidence of an MDR phenotype in blast cells from 30 AML patients. Like our studies, they found functional evidence of verapamil enhancement of DNR accumulation or cytotoxicity in a significant proportion (17% or 41%, respectively) of de novo AML patients. Unlike our studies, they were able to correlate the functional evidence of MDR with clinical response to treatment with thioguanine, ara-C, and DNR.

Further confounding interpretation of the above studies is the variability of the in vitro methodology, particularly the difficulty in defining "positivity" and the selection of appropriate controls. For example, the use of the "standard" 20% "cutoff" in flow cytometric assays may "miss" smaller populations of cells overexpressing Pgp that may be clinically significant. The use of a lower "cutoff" may result in "false positive" assessments because of the background fluorescence noted with many specimens with this technology.

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

<table>
<thead>
<tr>
<th>Modulator</th>
<th>No.</th>
<th>0%</th>
<th>&lt;-20%</th>
<th>&lt;-40%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>39</td>
<td>31 (80%)</td>
<td>29 (74%)</td>
<td>24 (62%)</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>40</td>
<td>37 (93%)</td>
<td>36 (90%)</td>
<td>31 (78%)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>38</td>
<td>22 (56%)</td>
<td>15 (38%)</td>
<td>7 (18%)</td>
</tr>
</tbody>
</table>
Table 4. Western Blot Data: 38 AML Patients
(C219 MoAb Against Pgp)

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Patients With Detectable Pgp</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>R-Fail</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>R-SCR</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>NE</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

One of the most salient observations of our studies is that CsA is effective in enhancing the accumulation/efflux and cytotoxicity of DNR in these blast cell specimens, and that its effects in this regard are equal to those of verapamil (Fig 6). Although the concentration of CsA used (5 μmol/L) is slightly higher than the plasma concentration range associated with acceptable toxicity in short-term exposure in a recent phase I clinical trial (2.5 to 4 μmol/L), in studies of HL-60/Vinc and HL-60/AR cells, we find that 3 μmol/L CsA is equally as effective as 5 μmol/L in enhancing DNR accumulation in both cell lines (data not shown). In addition, our studies show that CsA alone causes a small but statistically significant in vitro cytoreduction of blasts from our AML patients (Fig 7). Taken together, the cytoreductive effects of CsA alone, its antagonism of accumulation/efflux-mediated MDR, and its acceptable clinical toxicity, may make it an ideal candidate for future clinical trials in AML in combination with chemotherapeutic drugs susceptible to a classical MDR mechanism. However, given the effect of CsA on antineoplastic drug pharmacokinetics, it is necessary that phase I trials with pharmacokinetic monitoring be performed as an initial step. Despite the variability and sometimes contradictory results noted in the published studies, the "thrust" overall seems to be that overexpression of mdr1, detected by a variety of techniques, occurs frequently in patients with AML, and may influence treatment outcome. This, plus the demonstration, by our "functional" assays, that CsA significantly enhances DNR accumulation/retention and cytotoxicity in a high proportion of de novo AML patients at the time of presentation, warrants further clinical studies of CsA in combination with regimens that could be used as "front line" initial induction therapy for AML.

ACKNOWLEDGMENT

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REFERENCES


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47. Willman CL, Kopecky KJ, Weick J, Grover M, Applebaum F: Multiparameter analysis of the expression of the multidrug resistance genes mdr1 and mdr2(mdr3) in de novo AML by polymerase chain reaction (PCR) and multicolor flow cytometry: Identification of a biological subset of CD34+, mdr1+ AML cases. Blood 78:172a, 1991 (abstr, suppl 1)


Enhancement of daunorubicin accumulation, retention, and cytotoxicity by verapamil or cyclosporin A in blast cells from patients with previously untreated acute myeloid leukemia

DD Ross, PJ Wooten, R Sridhara, JV Ordonez, EJ Lee and CA Schiffer