

Mitoxantrone, Etoposide, and Cyclosporine Therapy in Pediatric Patients With Recurrent or Refractory Acute Myeloid Leukemia

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Purpose: To determine the remission rate and toxicity of mitoxantrone, etoposide, and cyclosporine (MEC) therapy, multidrug resistance-1 (*MDR1*) status, and steady-state cyclosporine (CSA) levels in children with relapsed and/or refractory acute myeloid leukemia.

Patients and Methods: MEC therapy consisted of mitoxantrone 6 mg/m²/d for 5 days, etoposide 60 mg/m²/d for 5 days, and CSA 10 mg/kg for 2 hours followed by 30 mg/kg/d as a continuous infusion for 98 hours. Because of pharmacokinetic interactions, drug doses were decreased to 60% of those found to be effective without coadministration of CSA. *MDR1* expression was evaluated by reverse transcriptase polymerase chain reaction, flow cytometry, and the ability of CSA at 2.5 μmol/L to increase intracellular accumulation of ³H-daunomycin in blasts from bone marrow specimens.

Results: The remission rate was 35% (n = 23 of 66). Overall, 35% of patients (n = 23) achieved complete remission (CR), 12% of patients (n = 8) achieved partial

remission, and 9% of patients (n = 6) died of infection. Exposure to CSA levels of greater than 2,400 ng/mL was achieved in 95% of patients (n = 56 of 59). Toxicities included infection, cardiotoxicity, myelosuppression, stomatitis, and reversible increases in serum creatinine and bilirubin. In most who had relapsed while receiving therapy or whose induction therapy had failed, response was not significantly different for *MDR1*-positive and *MDR1*-negative patients.

Conclusion: Serum levels of CSA capable of reversing multidrug resistance are achievable in children with acceptable toxicity. The CR rate of 35% achieved in this study is comparable to previously reported results using standard doses of mitoxantrone and etoposide. The use of CSA may have improved the response rate for the *MDR1*-positive patients so that it was not different from that for the *MDR1*-negative patients.

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WITH CURRENT chemotherapy regimens, approximately 75% to 85% of newly diagnosed children with acute myeloid leukemia (AML) will achieve complete remission (CR).¹ Further consolidation/maintenance chemotherapy results in 5-year event-free survival (EFS) of 35% to 60%.²⁻⁶ The major reason for treatment failure is leukemic relapse, which is most likely to occur within the first 2 years after diagnosis. Only 30% to 50% of patients who relapse will achieve a second remission with chemotherapy, and these remissions are not durable. The likelihood of achieving a second remission is directly proportional to the length of the first remission.⁷ The lack of response to chemotherapy suggests the development of acquired drug resistance.

Drug resistance may be present at the time of diagnosis or it may arise by somatic mutations during tumor growth. Numerous reports implicate the increased expression of the multidrug resistance-1 (*MDR1*)-encoded P-glycoprotein (P-gp) multidrug transporter as one cause of both intrinsic and acquired drug resistance.⁸⁻¹⁰ P-gp is a transmembrane ATP-dependent efflux pump that has broad substrate specificity. Increased amounts of P-gp confer multidrug resistance (MDR) in cells by reducing intracellular accumulation of a variety of cytotoxic drugs. Several key drugs used in the therapy of AML are transported by P-gp. These include anthracyclines and anthracenediones (daunorubicin and mi-

toxantrone, respectively), the vinca alkaloids (vincristine and vinblastine), and the epipodophyllotoxins (etoposide and teniposide).

Several noncytotoxic drugs, such as verapamil and phenothiazines, have been shown to modulate MDR, at least in part by competitive inhibition of P-gp-mediated drug efflux.¹¹ Verapamil and phenothiazines modulate MDR and P-gp at drug concentrations that produce unacceptable clinical toxicities (heart block and depression of the CNS). Cyclosporine (CSA) has also been shown to be a potent inhibitor of P-gp drug efflux in vitro. CSA concentrations of

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1,000 to 2,000 ng/mL, which reverse P-gp in vitro, are readily achievable with short-term intravenous administration in adults patients with acceptable toxicities.¹²

Several studies in patients with AML have shown that P-gp overexpression is relatively frequent, especially in refractory and relapsed leukemia.⁸⁻¹⁰ In several adult AML studies, CR rates were significantly lower in AML patients with overexpression of *MDR1*/P-gp, but the overall prognostic significance of P-gp expression on outcome remains controversial. Interpretation of the clinical data has been hampered by lack of well-defined criteria for detecting a level of P-gp expression considered positive and inadequate standardization of drug efflux assays.¹³ Two recent reports of phase II trials using cyclosporines as MDR modulators in adult AML patients have shown improved remission rates in high-risk patients.^{14,15}

We present the results of the largest pediatric study of an MDR reversal agent, and the first in childhood AML. The goals of this phase II study were (a) to determine the remission rate and toxicity of mitoxantrone, etoposide, and CSA (MEC) in children with refractory or relapsed AML, (b) to document steady-state CSA levels of greater than 2,400 ng/mL, (c) to determine *MDR1* mRNA and P-gp expression in leukemic blasts, and (d) to perform functional P-gp assays with and without CSA.

A phase III AML study—Pediatric Oncology Group (POG) 9421—with etoposide and mitoxantrone with/without CSA during the consolidation phase has just been completed.

PATIENTS AND METHODS

Between April 1992 and June 1994, a total of 68 pediatric patients with AML were entered onto the MEC protocol, a POG phase II multicenter trial (POG 9222). These children had received unsuccessful induction therapy with initial therapy or had relapsed during therapy or after one or more CRs. Forty-nine of the 66 assessable patients were initially treated on or as per POG 8821.¹⁶ In this study, patients received induction therapy with daunorubicin, cytarabine (Ara-C), and thioguanine, followed by a second induction with high-dose Ara-C (total anthracyclines, 135 mg/m²). Patients who entered remission were eligible for randomization. After randomization, an additional course of etoposide and azacytidine with intrathecal Ara-C was given, followed by either intensive chemotherapy (cumulative anthracyclines, 360 mg/m²), autologous transplantation, or allogeneic transplantation. Eight patients were treated according to POG pilot study 9194 (three cycles with daunorubicin, high-dose Ara-C, and thioguanine; etoposide and high-dose Ara-C; and high-dose Ara-C and daunorubicin [cumulative anthracyclines, 225 mg/m²]). The remaining patients were treated on various anthracycline-containing induction regimens. Relapse or induction failure was documented by bone marrow aspirate and biopsy in all patients. The characteristics of this patient population are listed in Table 1. In 68% of the assessable patients, either induction therapy failed or the patient relapsed while on therapy; the remainder had first remission duration of less than 1 year.

Table 1. Patient Characteristics

Characteristics	No. of Patients	CR	
		No. of Patients	%
Registered	68		
Assessable	66	23	35
Age			
0-5 years	29	11	38
5-10 years	8	4	50
> 10 years	29	8	28
Male	34	11	32
Female	32	12	38
Morphology			
M0	3	0	
M1-M2	21	9	43
M3	1	0	
M4-M5	24	10	42
M7	7	3	43
tAML	2	0	0
Other	8	1	13
Disease status			
Induction failure	18 (27%)	6	33
On-therapy relapse	27 (41%)	4	15
Off-therapy relapse	21 (32%)	13	62

The diagnosis of AML was made on bone marrow smears routinely stained and evaluated according to the revised French-American-British (FAB) criteria.¹⁷ All patients had normal cardiac, renal, liver, and pulmonary function, and all but two had received less than 500 mg/m² of prior daunorubicin therapy. The median prior daunorubicin dose was 265 mg/m² (range, 72 to 500 mg/m²), and four patients had received additional cardiotoxic therapy (amsacrine in three and idarubicin in one). This study was approved by the institutional review boards of each participating POG institution, and the patient and/or parent or guardian was required to sign an approved consent form.

Criteria for Response

The National Cancer Institute criteria for CR and partial remission (PR) were used. A CR was defined as the presence of a morphologically normal bone marrow, a granulocyte count of at least 1,500/ μ L, and a platelet count of 100,000/ μ L. A PR was defined as 5.1% to 25.0% of bone marrow blast cells with recovering counts and no circulating blasts. Relapse was defined as more than 25% blasts.¹⁷

Cardiac Monitoring

Echocardiography or planar radionuclide ventriculography (multi-gated angiogram scan) was required of all patients before the start of the study and before each course of therapy. If the shortening fraction was greater than 27% or ejection fraction was greater than 50%, therapy could be initiated. If clinical heart failure developed or the shortening fraction dropped below 25%, the second cycle of therapy was held until cardiac function improved.

Induction Therapy

MEC therapy consisted of mitoxantrone 6 mg/m²/d for 5 days and etoposide 60 mg/m²/d for 5 days in combination with CSA 10 mg/kg for 2 hours followed by 30 mg/kg/d continuous infusion for 98 hours

(total of 100 hours). The CSA dose was adjusted at hours 14, 26, 38, 50, and 74 in an attempt to maintain a steady-state serum CSA level above 2.5 $\mu\text{mol/L}$ (range, 3,000 to 5,000 ng/mL).

A bone marrow aspirate and biopsy samples were obtained 10 to 14 days from the start of the 5-day MEC course. If the aspirate had more than 25% blasts, patients were removed from study regardless of the cellularity. If patients had fewer than 25% blasts and cellularity of less than 25%, they were immediately given a second 5-day MEC course. If fewer than 5% blasts were noted and the biopsy was hypocellular (5% to 20%), a second course was delayed until the counts recovered. Patients who recovered with greater than 25% blasts before the second course were removed from the study. If there was residual leukemia after two courses, the patient was taken off the study. Patients who achieved a CR with one or two courses could receive two or one subsequent course(s), respectively, for consolidation. No more than three courses of MEC were given to any patient.

MDR1 Expression

Flow cytometric analysis of MDR1 expression. MDR1 expression was determined by both flow cytometric analysis and reverse transcriptase polymerase chain reaction (RT-PCR). Analysis of MDR1 P-gp cell surface expression was performed on a FACSCAN flow cytometer using Lysis II software (Becton Dickinson, Thousand Oaks, CA) and the 4E3 monoclonal antibody (MoAb) directed to an external epitope of P-gp.^{18,19} Similar results were obtained using MRK16, another external epitope anti-P-gp MoAb. Although there were subtle differences, the results reported here are only for the 4E3 antibody because expression of surface P-gp was observed to be similar and did not change the number of patients who were positive.

Fresh bone marrow samples were layered over Ficoll-Hypaque cushions and centrifuged at $600 \times g$ for 15 minutes to separate mononuclear cells, which were then washed in cold phosphate-buffered saline containing 2% fetal bovine serum. These cells were then used for staining with 4E3, other hematopoietic markers (including CD34, CD13, CD15, CD33, CD14, CD2, CD19, and CD10), or isotype controls. 4E3 or MRK16 was detected using secondary phycoerythrin-conjugated goat-antimouse immunoglobulin (Fab)2 fragment (Tago, Burlingame, CA) at a 1/30 dilution. All other MoAbs were directly conjugated to fluorescein (Becton Dickinson or AMAC, Inc, Miami, FL). 4E3 staining of gated leukemic blasts was compared with isotype control as well as control cells expressing different levels of MDR1 P-gp (CEM, CEM/VBL-100, and CEM/VBL-300).¹⁸

Quantitation was accomplished initially by comparing mean fluorescence intensities or using the Kolmogorov-Smirnov statistic, referred to as D, which measures the difference between the means of two distributions.^{13,20-22} The ratio of the mean fluorescence intensity for 4E3 expression compared with the value using an isotype control antibody was then calculated. This ratio never exceeded 1.2 for the drug-sensitive (P-gp-negative) cell line, CEM. AML blasts were therefore considered positive if they had a shift of ≥ 1.3 times a mean fluorescent intensity for P-gp compared with the isotype control. In addition, $\geq 10\%$ of marrow mononuclear cells in the leukemic blast population had to be positive for a sample to be considered positive for P-gp expression.²³ The observed ratios of the mean fluorescence intensity for 4E3 staining compared with the isotype control for all patient samples considered to be positive by these criteria ranged from 1.3 to 5, with a mean of 2.2 and a median value of 1.65. The values for percentage of positive cells in the leukemic blast populations from different patients ranged from 10% to 100%, with a mean of 70% and a median of 100% (values included 10%, 11%, 13%, 20%, 53%, and

75%; eight samples showed 100% positive blasts). Similar results were obtained when D values were calculated, with most samples falling in the low expression range (D values > 0.1 and < 0.2).²⁰ Because the number of positive leukemia samples was relatively low (a total of 14 patient samples), samples were not broken down into subgroups of differential P-gp positivity.

RT-PCR analysis of MDR1 expression. The level of MDR1 RNA was determined in bone marrow samples by RT-PCR using MDR1 and beta-2-microglobulin specific oligonucleotide primers as described by Noonan et al.²⁴ Total RNA (100 ng) was used to make cDNA followed by amplification in a 4800 Thermocycler (Perkin Elmer, Norwalk, CT) set for a 30-second denaturation at 94°C, 1 minute of primer annealing at 55°C, and 2 minutes of extension/synthesis at 72°C. Primers used for amplification of MDR1 RNA were from nucleotides 2596 to 2615 for the sense direction and nucleotides 2733 to 2752 for the antisense direction, yielding a 167-nucleotide product. Primers used for amplification of β 2-microglobulin were from nucleotides 1544 to 1563 for the sense and from nucleotides 2253 to 2263 for the antisense direction. This combination yielded a 120-nucleotide product. PCR products were electrophoretically separated in a 3% agarose gel and the specific PCR amplicons were excised after ethidium bromide staining. The amount of radioactivity incorporated into each amplicon was then determined by liquid scintillation counting. Analyses were done after 25 and 35 cycles of amplification. A sample was considered positive for MDR expression if a band was visibly present after ethidium bromide staining and/or the MDR1 to β 2-microglobulin ratio of incorporated radioactive counts was greater than 0.7 (based on comparison with the drug-sensitive [P-gp-negative] CEM cell line, which had a ratio of less than 0.7 under the RT-PCR conditions used). The range of values varied from 0.8 to 1.8, with an average of 1.2 and a median of 1.1.

Cytotoxic Drug Accumulation Studies

Although the functional analysis of MDR1/P-gp has evolved over the past several years with the discovery of a variety of dyes effluxed by the MDR transporter, when this clinical study was begun these methods had not been established.^{19,25-27} The determination of intracellular drug accumulation was used to measure the effect of verapamil and/or CSA on reversing the level of cytotoxic drug accumulation, even though, as many studies have now shown, the correlation with P-gp and the reversal of drug uptake or accumulation with these inhibitors expression is not perfect, in part due to the non-MDR1 transport systems.^{19,27,28}

The intracellular accumulation of cytotoxic agents was determined in the absence and presence of P-gp inhibitors verapamil and CSA, as previously described.^{18,27-30} ³H-Daunomycin (specific activity, ~ 1 to 5 Ci/mmol; Dupont, Wilmington, DE) and ³H-vinblastine (specific activity, ~ 5 to 25 Ci/mmol; Amersham International, Les Uris, France) were used in these studies. Isolated bone marrow mononuclear cells were resuspended at 1×10^6 cells/mL of RPMI medium containing 10% fetal bovine serum. Aliquots of 0.25 mL were then dispensed into 75-mm round-bottom plastic tubes and MDR reversal agents were added to a final concentration of 2.5 $\mu\text{mol/L}$. After a 30-minute incubation with gentle agitation at 37°C, tritiated cytotoxic drug was added to a final concentration of 8 nmol/L in the presence of the same but unlabeled drug at a final concentration of 10 $\mu\text{mol/L}$. After a 2-hour incubation, the entire contents of each tube were overlaid on a 200- μL cushion of Dow 550 silicone oil (Dow Corning, Midland, MI) and mineral oil (4:1) in a 1.5-mL Eppendorf tube and centrifuged at $10,000 \times g$ at 4°C for 1 minute to separate the cells from drug-containing medium. The medium and oil mixture was removed by aspiration, and the cellular pellets were solubilized in 1 mL of 1 M

NaOH at 60°C overnight before the amount of radioactivity accumulated in the cells was determined by liquid scintillation counting.

The "fold increase" of radiolabeled drug accumulation was calculated as follows: The ratio of the drug accumulation as described earlier without a reversal agent compared to with a reversal agent was calculated. Along with each patient sample, drug accumulation for the drug-sensitive cell line, CEM, and the drug-resistant derivative, CEM/VBL 300, was simultaneously determined. The values for each patient's drug accumulation ratio and for CEM cells were normalized by dividing by the ratio for CEM/VBL 300 cells. Last, a fold increase for the patient sample compared with CEM cells was determined. Values reported are the average of at least three determinations. A fold increase value of greater than 1.2 was considered positive, as this was the upper level observed using the drug-sensitive cell line, CEM.^{18,30} The range of values for patient samples greater than 1.2 varied from 1.23 to 6.59, with a median of 1.56.

Statistical Analysis

The significance of observed differences in proportions was tested using the χ^2 statistic and, when appropriate for small sample size, Fisher's exact test. The Mantel-Haenszel test³¹ was used to compare the CR rate in *MDR1*-negative patients with that in *MDR1*-positive patients with adjustment for imbalance of prior relapse status. The test is based on the difference between the observed number of CR in *MDR1*-negative patients and the expected number of CRs in the same group under the hypothesis that the remission rate in *MDR1*-negative patients is the same as that in *MDR1*-positive patients.

Kendall's tau-b³² was used to evaluate the correlation between the various measurements associated with the *MDR1* status. The estimator of Kendall's tau-b is based on the fraction of concordant versus discordant pairs of observations on two variables. A value of the estimator close to -1 , $+1$, or 0 indicates strongly negative, strongly positive, or no correlation, respectively, between the variables.

RESULTS

Patient Characteristics

Sixty-eight patients (age range, 1.4 months to 20 years) were entered onto the study and 66 were assessable for response and toxicity (Table 1). No patient was considered ineligible for entry onto the study, but two patients were administered nonprotocol cytotoxic therapy and therefore were considered nonassessable. The distribution of FAB subtypes was consistent with previously published experience.³³ There were two cases of treatment-related secondary AML (tAML). The "other" category includes one patient with refractory anemia with excess blasts developing after a 3-month remission of AML and seven AML cases not given a FAB designation.

Response

Overall, 23 patients (35%) achieved CR, eight patients (12%) achieved PR, and six patients (9%) died of infection during marrow hypoplasia. Among the complete responders, median time to CR was 39 days (range, 14 to 62 days). Eighteen (27%) achieved CR after a single course of

therapy; five achieved CR after the second course. CR rates by patient characteristics are listed in Table 1. The remission rates were 62%, 15%, and 33 for patients with off-therapy relapse, on-therapy relapse, and primary refractory AML or secondary AML%, respectively. The overall remission rate was 35% (23 of 66 patients), with a 95% confidence interval of 23% to 46%. The median EFS and median survival times were 2.5 months and 4.5 months, respectively. Fifty-seven (86%) of 66 patients died during the first year. When patients were stratified based on either the status of *MDR1* or status of prior relapse, the number within each stratum was too small to provide a useful survival estimate.

Marrow samples were received for determination of *MDR1* status in 59 (90%) of 66 patients. Table 2 lists the disease status and response of patients with AML according to their *MDR1* status as defined in the Materials and Methods. Fifty-five patients were tested by flow cytometry for the presence of 4E3 staining; 14 (25%) were positive and 41 (75%) were negative. Of these patients, CR was achieved by four (29%) of the 4E3-positive and by 14 (34%) of 4E3-negative patients. Fifty patients were tested for *MDR1* status by RT-PCR. Ten (20%) were positive and 40 (80%) were negative. Of these patients, CR was achieved in four (40%) of the PCR-positive patients and 13 (33%) of the PCR-negative patients. Fifty-two patients were tested for *MDR1* reversal by CSA in vitro. Twenty (38%) exhibited reversal and 32 (62%) did not. Of these patients, CR was achieved in nine (45%) with positive reversal by CSA and in nine (28%) with negative reversal by CSA in blast cells. In samples from 48 patients, results of all three *MDR1*/P-gp detection methods outlined earlier were available. When *MDR1*/P-gp positivity was defined as either 4E3+, PCR+, +CSA reversal, or any combination thereof, there were 27 (56%) positive and 21 (44%) negative samples. Of these patients, CR was achieved in 10 (37%) of *MDR1*/P-gp-positive patients and in seven (33%) of *MDR1*/P-gp-negative patients.

Only five patients were positive for *MDR1* expression by all three methods, and 21 were negative for *MDR1* by all three methods. The response (CR + PR) rates were 60% (three of five patients) and 38% (eight of 21 patients) for these two groups of patients. The difference was insignificant (two-sided $P = .62$ by Fisher's exact test).

The Mantel-Haenszel test shows that after adjustment for prior relapse status the CR rate in *MDR1*/P-gp-negative patients was not higher than that in *MDR1*/P-gp-positive patients when *MDR1*/P-gp positivity is defined as +4E3, +RT-PCR, +CSA reversal, or the presence of at least one of the above (one-sided P values were .30, .55, .86, and .39, respectively). In addition, the variables of age, sex, and FAB group were not significantly correlated with CR in

Table 2. CR Outcome Versus MDR1 Status

MDR1 Status	Induction Failure		On-Therapy Relapse		Off-Therapy Relapse	
	No. of Patients	CR	No. of Patients	CR	No. of Patients	CR
4E3 MoAb staining						
Positive*	3	2	6	0	5	3
Negative	10	3	18	3	13	8
RT-PCR						
Positive†	0		5	0	5	4
Negative	11	4	18	2	11	7
Cytotoxic drug accumulation						
Reversed by CSA‡	2	1	9	0	9	8
Not reversed by CSA	10	3	13	3	9	3
MDR1						
Positive§	4	1	11	0	12	9
Negative	6	3	10	2	5	2

*Defined as > 10% positive cells.

†Defined as a PCR value > 0.7 relative to 1.0 for positive control.

‡Defined as a CSA value > 1.2×, which is above that of P-gp-negative cells.

§A combination of antibody and PCR-positive samples, or reversible to CSA.

univariate analysis. The two-sample binomial test shows that the remission rate (13 of 21 patients, 62%) in patients with off-therapy relapse was higher than the CR rate (10 of 45 patients, 22%) in patients with on-therapy relapse or induction failure (one-sided $P = .0006$).

Correlation Among 4E3 Staining, RT-PCR, and Cytotoxic Drug Accumulation With or Without CSA

The Kendall's tau-b correlation coefficients among the 4E3 staining, RT-PCR, and CSA analyses were not significantly different from zero, except for the correlation coefficient ($r = .49$) between 4E3 staining and RT-PCR ($P = .0005$) and the correlation coefficient ($r = .34$) between 4E3 staining and CSA modulation ($P = .0023$).

CSA Blood Levels

Whole blood or serum CSA levels were performed by clinical laboratories at each participating institution. No standard measurement method was suggested. For the 59 patients with available data, the median serum CSA level after a 2-hour loading dose was 3,449 ng/mL and the mean was 2,820 ng/mL (range, 1,050 to 16,599 ng/mL). For the 5-day continuous infusion, the median level was 3,187 ng/mL and the mean was 3,446 ng/mL (range, 1,397 to 6,534 ng/mL). The median peak CSA level during the 5-day infusion was 4,911 ng/mL (range, 2,160 to 22,296 ng/mL). Although steady-state concentrations revealed significant interpatient variability, prolonged exposure to levels of greater than 2,400 ng/mL was achieved in 56 of 59 patients.

Toxicity

Table 3 lists toxicities related to the induction regimen. Nausea and vomiting associated with headache and tingling

of the hands and lips was frequently reported and resolved with completion of the CSA infusion. All patients experienced profound myelosuppression, with an absolute neutrophil count of zero and platelet counts of less than 20,000/mm³. In the responding patients, the median time to granulocyte and platelet recovery was 35 days (range, 21 to 55 days) and 36 days (range, 22 to 69 days), respectively. The major toxicity was infection, with 28 episodes; among these, 25 patients developed documented bacterial or fungal infections. As determined using National Cancer Institute toxicity criteria, grade 4 mucositis developed in eight children. Significant increases in serum creatinine occurred in three patients; one was grade 4 in a 19-month-old boy and was associated with severe mucositis, candidiasis, cardiac failure, and early death. The other two were related to poor state of hydration before the start of the CSA infusion, and both resolved with adequate parenteral fluid administration.

Cardiotoxicity, manifested by decreased ejection fraction or shortening fraction, was noted in 15 (23%) of the patients after induction therapy. In 12 (80%) of these patients, cardiotoxic events were transient, were due to multifactorial clinical problems, and resolved without specific therapy. Three children developed congestive heart failure that required therapy with digitalis.

Table 3. Grades 3 to 4 Toxicity Among Assessable Patients

Category	No. of Episodes	%
Infection	28	42
Stomatitis	29	44
Hepatic (bilirubin > 2.1 mg/100 mL)	45	68
Cardiac	3	4.5

For the 55 patients with available data, the median total bilirubin level during CSA infusion was 2.2 mg/100 mL (range, 0.6 to 9.7 mg/100 mL). The mostly direct hyperbilirubinemia was rapidly reversible once the CSA infusion was discontinued and was not accompanied by any other evidence of additional hepatic dysfunction. There was no relationship between the level of bilirubin, response rate, CSA level achieved, or *MDR1*/P-gp status.

DISCUSSION

The major objective of this protocol was to determine whether the *MDR1*/P-gp reversal agent CSA could be safely tolerated and produce significant response rates in pediatric patients when given in high doses with an active reinduction regimen of mitoxantrone and etoposide. In this study, high-dose CSA was administered continuously in a group-wide setting and found to be generally safe with acceptable toxicity. Grades 3 to 4 cardiac toxicity, likely related to chemotherapy, occurred in three patients (4.5%), who developed congestive heart failure and required treatment with digitalis. Although hyperbilirubinemia was common and directly related to CSA administration, it quickly normalized when the infusion was discontinued.

In addition, the combination of CSA, mitoxantrone, and etoposide was determined to be an active induction regimen for children with relapsed and refractory AML, even though the doses of mitoxantrone and etoposide given during induction were decreased to 60% of those found to be effective without coadministration of CSA. Dose modifications were necessary because high-dose CSA produces significant increases in etoposide and mitoxantrone systemic exposure and resultant leukopenia and mucositis. Our group has performed pharmacokinetic analysis of patients receiving etoposide and mitoxantrone with and without CSA in an ongoing phase III trial.³⁴ CSA produced significant increases in the area under the curve for etoposide and mitoxantrone, thus confirming the need for the dose adjustments used in this study and in our ongoing phase III trial. The CR rate of 35% achieved in this study is comparable to other results reported with mitoxantrone/etoposide.³⁵ As in other studies, the likelihood of achieving a CR was higher in patients who relapsed off therapy, compared with those who relapsed on therapy or did not go into remission (62% v 15% and 33%, respectively). Approximately 50% of patients achieved a CR or PR with this MEC regimen, which allowed most to go on to bone marrow transplant.

A second aim of this study was to determine the P-gp status of blasts in refractory childhood AML by several methods, including *MDR1* mRNA, P-gp expression, and drug efflux studies. The methodology used to assess P-gp expression has evolved over the last several years. Early studies reported the

level of *MDR1* transcripts by RT-PCR or RNase protection assays. Later, protein detection by immunoblotting and immunohistochemistry was introduced. More recently, functional assays have been developed. Despite these advances, it is difficult to assess the role that P-gp plays in clinical drug resistance due to tumor cell heterogeneity, sensitivity, and specificity of methods of detection. The best approach, as recommended by the international workshop on *MDR1* detection methods, is to use tissue-specific controls, antibody controls, standardized *MDR1* cell lines to calibrate detection methods, and two or more vendor-standardized anti-P-gp antibodies, and to report immunostaining data as staining intensity and the percentage of positive cells.¹¹ Although this study was completed before these consensus recommendations, many of these guidelines were followed. The RT-PCR data were complemented with immunostaining and drug accumulation studies with and without modulators. Standardized cell lines with low expression of *MDR1*/P-gp were used as a reference for clinical samples.

Flow cytometric assessment of P-gp had an adequate correlation with both RT-PCR and CSA enhancement of drug accumulation. Although adequate, the level of correlation highlights the need for protein and functional assays as necessary tools in evaluating the *MDR1* status in clinical samples and in validating the analysis of *MDR1* modulation trials.

Limited data are available on the expression of *MDR1*/P-gp in marrow or blood samples from pediatric patients with AML. This series includes the largest sample of relapsed or refractory pediatric AML samples analyzed for *MDR1*/P-gp expression and function. There is one published report of *MDR1*/P-gp expression in pediatric patients with de novo AML.³⁶ In this study, samples from 130 patients were evaluated, and 30% of infants and 8% of children older than 1 year expressed *MDR1*/P-gp at diagnosis. This study was limited to the use of the MRK-16 antibody with a fluorescein isothiocyanate-conjugated second-step reagent. *MDR1*/P-gp positivity did not have an effect on induction failure, incidence of relapse, EFS, or overall survival in this group of patients. The method of analysis was substandard because it relied on a single technique to study *MDR1*/P-gp status. If the data from this Children's Cancer Group study are corroborated, then our group of relapsed and/or refractory pediatric AML patients shows an increased expression of *MDR1*/P-gp, similar to what is seen when comparing de novo and relapsed AML in adult patients.

Although the response rate for *MDR1*/P-gp-negative patients is not significantly higher than that for *MDR1*/P-gp-positive patients, it is interesting to note that the patients relapsing off therapy before entry onto this regimen were

more likely to achieve remission and more likely to be *MDR1/P-gp* positive. *MDR1/P-gp* status as a predictor of response to induction may be important for patients for whom induction therapy fails or for patients who relapse on therapy, but it may have little prognostic importance in those with late relapses. It is interesting to note that the prognostic importance of *MDR1/P-gp* expression and function was not seen in this relatively large group of patients treated with an *MDR1/P-gp* modulator during induction. It may be that the modulator improved the response rate for the *MDR1/P-gp*-positive patients so that it was not different from that of the *MDR1/P-gp*-negative patients. However, the contribution of dose-intensity to this outcome needs to be considered.

It is impossible to determine the prognostic importance of *MDR1/P-gp* status and response to this therapy because of variations in treatment history and drug exposure before admission to this protocol. The induction failure and on- and off-therapy relapse groups were not balanced for *MDR1/P-gp* expression. The second largest subgroup of patients consisted of those who relapsed off therapy. These patients were more likely to be *MDR1/P-gp*-positive and historically have a higher chance of achieving remission. To determine the prognostic importance of *MDR1/P-gp* status and reinduction rate, a randomized trial, in which the target plasma concentration of the chemotherapeutic agents is matched in the group given the reversal agent and the control, is needed.

In summary, the results of this study indicate that levels of CSA capable of reversing *MDR1/P-gp* in vitro are achievable in children with acceptable toxicity. Mucositis

and hyperbilirubinemia were found to be major treatment-related toxicities in this population. Remissions were seen in heavily pretreated patients and in children who had failed to achieve an initial remission. The remission rate in *MDR1/P-gp*-negative patients was not significantly greater than that in *MDR1/P-gp*-positive patients.

The correlation among 4E3 staining, RT-PCR, and CSA inhibition of drug efflux was adequate. Our methods of analysis using several diagnostic tools to determine the *MDR1* status illustrate the pitfall of relying on a single method to diagnose the *MDR1/P-gp* status. Future studies will require the use of new flow cytometric analysis reagents and strict guidelines regarding clinical sample collection, preservation, and amount of sample to perform state-of-the-art analyses.

In the laboratory setting it has been shown that the use of *MDR1/P-gp* modulators during cytotoxic therapy can abolish the selection of *MDR1*-mediated drug resistance in cancer cell lines.³⁷ Thus a possible benefit of introducing *MDR1/P-gp* modulators in the therapy of patients with de novo AML may be a decrease in acquired drug resistance based on *MDR1/P-gp* overexpression. To follow up this study and to study further the incidence of P-gp positivity and the effect of P-gp reversal with CSA on outcome in de novo childhood AML, a randomized phase III study (POG 9421) was recently completed at POG institutions. When they become available, results from this study may help to determine the prognostic significance of *MDR1/P-gp* expression and the effect of modulation by CSA in children with AML.

APPENDIX

Institution	Grant No.	Institution	Grant No.
All Children's Hospital		POG Operations Office	CA-30969
Baylor	CA-03161	Swiss Pediatric Oncology Group/Lausanne	
Cancer Center of Hawaii		State University of New York Syracuse	
Children's Hospital Michigan	CA-29691	San Antonio Military Pediatric Center & Blood Disorders Center	
Children's Memorial Hospital (Chicago)	CA-07431	Stanford University	CA-33603
Children's Hospital (San Diego)	CA-28439	POG Statistical Office	CA-29139
Dana-Farber Cancer Institute	CA-41573	University of Alabama	CA-25408
Duke University	CA-15525	University of Arkansas	
Fairfax Hospital	CA-28476	University of Kansas	
Hackensack Medical Center		University of Miami	
Kaiser/San Diego	CA-28439	University of Mississippi Medical Center	CA-15989
Massachusetts General Hospital		University of South Alabama	
Medical University South Carolina	CA-69177	University of South Florida	
McGill University	CA-33587	University of Vermont	CA-29293
Miami Children's Hospital		University of California/San Diego	CA-28439
Midwest Children's Cancer Center	CA-32053	University of Texas/San Antonio	
Mount Sinai Medical School	CA-69428		
Nemours/Jacksonville			

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