In vitro sensitivity and cross-resistance to deoxynucleoside analogs in childhood acute leukemia

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Background and Objectives. Cytarabine (ara-C) is a key drug in the treatment of acute leukemia. Resistance to ara-C might be circumvented by the use of other deoxynucleoside analogs.

Design and Methods. Using the MTT assay, we determined *in vitro* sensitivity and cross-resistance to deoxynucleoside analogs in 362 acute leukemia samples from untreated children and 32 normal bone marrow mononuclear cell samples.

Results. Normal bone marrow samples were significantly more resistant to ara-C, cladribine and fludarabine than were acute myeloid leukemia (AML) samples and significantly more resistant to ara-C and fludarabine than were acute lymphoblastic leukemia (ALL) samples. The only drug to which AML samples were more sensitive *in vitro* than ALL was cladribine. AML FAB M5 was significantly more sensitive *in vitro* to ara-C and cladribine than FAB M1/2 or FAB M4. T-ALL was significantly more resistant to cladribine than B-cell precursor ALL. A paired analysis of 60 AML and 99 ALL samples demonstrated significant cross-resistance between all four deoxynucleoside analogs. Cross-resistance was also observed between ara-C and etoposide (Rp=0.54, $p<0.0001$), and ara-C and daunorubicin (Rp=0.48, $p<0.0001$) in AML. In ALL blasts, cross-resistance was observed between ara-C and vincristine (Rp=0.50; $p<0.0001$), and between ara-C and daunorubicin and L-asparaginase (Rp=0.25; p=0.01; Rp=0.28; p=0.005).

Interpretation and Conclusions. Cladribine appears to be a useful drug in AML, particularly in FAB M5. We observed cross-resistance between ara-C and other deoxynucleoside analogs, as well as between ara-C and drugs with different modes of action in childhood acute leukemia.

Key words: childhood acute leukemia, drug sensitivity, cytarabine, deoxynucleoside analogs, cross-resistance.

Haematologica 2006; 91:17-23

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The introduction of refined schedules of combination chemotherapy has considerably improved the prognosis of childhood acute leukemia during the past decades. Long-term survival has reached 80% for acute lymphoblastic leukemia (ALL) and 60% for acute myeloid leukemia (AML).[1-3] The deoxynucleoside analog cytarabine (ara-C) forms the backbone of treatment for AML and is also frequently given in high-risk *de novo* and relapsed ALL. Ara-C enters the cell via the nucleoside transporter. Inside the cell, ara-C is phosphorylated by deoxycytidine kinase (dCK). Ara-C cytotoxicity results from DNA polymerase alpha inhibition and incorporation into DNA, leading to inhibition of chain elongation and a blockade in DNA synthesis.[4] Ara-CTP levels correlate with prognosis, and improved clinical outcome has been associated with increased ara-CTP accumulation and retention.[5,6] Resistance to ara-C may, therefore, contribute to treatment failure. In addition, we have previously reported *in vitro* ara-C resistance in some types of childhood leukemia, such as T-ALL and AML FAB types M1 and M2.[7,8] The use of deoxynucleoside analogs other than ara-C may circumvent resistance to ara-C, this was addressed in our *in vitro* study.

Cladribine, fludarabine and gemcitabine share several characteristics with ara-C (e.g. transport mediated by membrane transporters, intracellular activation by dCK and formation of active phosphate derivatives), but also differ with respect to their preferential transporters and interaction with intracellular targets (Figure 1).[9] Because of their unique properties these deoxynucleoside analogs may be able to circumvent resistance to ara-C in childhood leukemia. With the aim of identifying particular AML and/or ALL subtypes that might preferentially benefit from subtype-directed therapy with deoxynucleoside analogs, we investigated *in vitro* sensitivity patterns in different AML (FAB type) and ALL (immunophenotype) subtypes. In addition, we studied toxicity of these drugs in normal bone marrow mononuclear cells from healthy children in order to determine

therapeutic indices. Finally, we investigated whether or not ara-C shows cross-resistance *in vitro* to deoxynucleoside analogs (cladribine, fludarabine and gemcitabine) and other drugs used in the treatment of acute leukemia, in the leukemic blasts of children with newly diagnosed acute leukemia.

Design and Methods

Patients' samples

Bone marrow and peripheral blood samples were collected from children with acute leukemia, with informed consent. Several collaborative groups participated in the study: (i) the Dutch Oncology Group (DCOG), The Hague, The Netherlands; (ii) the MRC Childhood Leukaemia Working Party, UK; (iii) the AML Berlin-Frankfurt-Münster (BFM) Study group, Münster, Germany; and (iv) the COALL Study group, Hamburg, Germany. AML (DCOG/AML-BFM/MRC) samples were collected between December 1993 and September 2002. Patients were all treated with intensive cytarabine/anthracycline-based protocols: AML-BFM 93 and 98; DCOG AML 87, 94 and 97.[1,2,10-12] ALL (DCOG/COALL) samples were collected between January 1994 and October 2002. The diagnosis, data collection, FAB-classification and immunophenotype were centrally reviewed by the reference laboratories and data centers of these study groups. The FAB classification was assigned according to the criteria described by Bennett *et al.,* including the diagnostic modifications for FAB M0 and FAB M7.[13] The immunophenotype of ALL blast cells was determined by either flow cytometry or immunocytochemistry.

B-cell precursor ALL (BCP-ALL) was defined as surface immunoglobulin $(sIg)^-$/terminal deoxynucleotidyl transferase$^+$/human leukocyte antigen-DR$^+$/CD19$^+$, and included both CD10$^-$ (pro-B ALL), as well as CD10$^+$ and/or cytoplasmic μ-chain$^+$ (common/pre-B) ALL. T-cell ALL was defined as cytoplasmic CD3$^+$/CD7$^+$. The patients' characteristics (gender, age, white blood cell count at diagnosis) were recorded by the study centers. Normal bone marrow samples were collected from children undergoing elective eye surgery, with informed consent. Samples were collected at the VU University Medical Center between August 1999 and December 2002. This study was performed with the approval of the local Medical Ethical Committee.

Cells

Mononuclear cells were separated by density gradient centrifugation at 480 g for 15 minutes (Lymphoprep, 1.077 g/mL, Nycomed Pharma, Oslo, Norway). After washing, the cells were resuspended in culture medium consisting of RPMI 1640 (Dutch modification without L-glutamine; Gibco BRL, Breda, The Netherlands) containing 20% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 2 mM L-glutamine (ICN Biochemicals, Costa Mesa CA, USA), ITS media supplement (5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite) (Sigma, St Louis MO, USA), 80 IU/mL penicillin (Gibco BRL, Breda, The Netherlands) 80 μg/mL streptomycin (Gibco BRL), 0.1 μg/mL fungizone (ICN Biochemicals) and 0.2 mg/mL gentamycin (Flow laboratories, Irvine, Scotland). Contaminating lymphocytes were removed using immunomagnetic beads as described previously.[14] Isolated cells were suspended in culture medium. Only samples that contained more than 80% blasts, as determined by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) staining, were included.

MTT-assay

In vitro drug resistance was determined using the 4-day total cell kill MTT assay, as described previously.[15] Briefly, isolated cells were exposed to six concentrations of a selected drug. After 4 days of incubation in 5% CO_2 humidified air at 37°C, 3-(4,5-dimethylthiazol-2,5-diphenyl) tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) was added and cells were incubated for another 6 hours. Subsequently, the formazan crystals that had developed were dissolved in acidified isopropanol. The optical density (OD) is linearly related to the number of viable cells. Control wells, containing leukemic cells with culture medium but no drugs were used to determine the control cell survival. Wells with culture medium only were used as blanks. Results were considered evaluable only if the control wells contained ≥70% leukemic cells (determined by morphology after May-Grünwald Giemsa staining) after 4 days of culture and if the mean control OD, after correction for the background, at day 4 exceeded 0.05 arbitrary units. The LC_{50} value, the drug concentration needed to kill 50% of the leukemia cells, was used as a measure of resistance. Sample source (bone marrow or peripheral blood) and cryopreservation do not influence the results obtained by cellular drug resistance testing and the results were therefore analyzed together.[15] The following drugs were tested (minimal and maximal concentration) in duplicate: ara-C 0.04–41.1 μM (Cytosar®; Pharmacia&Upjohn, Woerden, The Netherlands), cladribine 0.001–40.0 μM (Leustatin®, Ortho Biotech, US), daunorubicin 0.004–3.5 μM (Cerubidine®, Rhone Poulenc Rorer, Amstelveen, The Netherlands), etoposide 0.08–85.0 μM (Vepesid®, Bristol-Myers Squib, Woerden, The Netherlands), fludarabine 0.04–43.8 μM (Fludara®, Schering AG, The Netherlands), gemcitabine 0.04–1335.0 μM (Gemzar®, Eli Lilly, Houten, The Netherlands), L-asparaginase 0.003–10 IU/mL (Christiaens, Breda, The Netherlands), vincristine 0.05–54.2 μM (Oncovin®, Eli Lilly, Houten, The Netherlands) and prednisolone disodium phosphate 0.0008–2.58 μM (Brocacef, The Netherlands).

Statistical analysis

Differences in the distribution of variables between groups were analyzed by the Mann-Whitney U test.

Cross-resistance patterns were determined in a group of AML samples in which all four deoxynucleoside analogs had been tested successfully with the MTT-assay. Similarly, cross-resistance between ara-C and 2-CdA was evaluated in a group of 144 ALL samples, in which all samples were tested successfully for these two drugs. The Pearson correlation coefficient was calculated, after log transformation of the data. The therapeutic index of the effect of ara-C, gemcitabine, fludarabine and cladribine on normal bone marrow and blast cells was calculated by the following equation: therapeutic index=LC_{50} normal bone marrow/median LC_{50} value leukemic blasts cells. All analyses were two-tailed, with a significance level of $p \leq 0.01$.

Results

Patients' characteristics

In vitro sensitivity to deoxynucleoside analogs was studied in 362 acute leukemia samples from untreated children and 32 normal pediatric bone marrow mononuclear cell samples. The patients' characteristics are listed in Table 1. In a subgroup of these patients we studied cross-resistance patterns between ara-C and deoxynucleoside analogs, and other drugs used in the treatment of acute leukemia. Sixty AML and 99 ALL samples were selected for which the *in vitro* drug sensitivity (MTT-assay) had been tested successfully for all drugs in each patient. In this group, the median white cell count was 58.1×10^9/L and 35.7×10^9/L in the AML and ALL patients, respectively. These values did not differ significantly from those of the whole group of patients described in Table 1 (AML, p=0.28 and ALL, p= 0.27). The median age of the AML patients (10.4 years, p=0.99) in the cross-resistance group was similar to that of the total group. The ALL patients (median age 6.2 years) in the cross-resistance group were, however, older than the patients in the total group (p=0.01).

Table 1. Patients' characteristics.

	AML	*ALL*	*Normal bone marrow*
Number	134	228	32
Sex (male:female), n	73:60 (1 unknown)	124:104	14:16 (2 unknown)
Median age, yrs	8.9	4.8	7.2
25^{th}-75^{th} percentile	(2.7-13.3)	(3.0-9.5)	(5.1-9.6)
Median WBC, $\times10^9$/L	51.8	28.3	
25^{th}-75^{th} percentile	(17.9-125.5)	(7.2-71.9)	
FAB-type, n			
M0	7		
M1	15		
M2	28		
M3	7		
M4	46		
M5	27		
RAEB-t	1		
Unknown	3		
Immunophenotype, n			
Pro-B		4	
C/preB		184	
T		39	
Unknown		1	

In vitro sensitivity to deoxynucleoside analogs in normal bone marrow mononuclear cells and leukemic blasts (AML and ALL)

We selected samples that had tested successfully for at least one drug. We have previously published data on potential selection bias of samples due to different assay failure rates in specific AML and ALL subtypes.[7,16] In AML, failure rates were higher in samples with a low white cell count, in FAB M6/7 subtypes (not included in the present study) and in morphologically unclassified samples.[7] In ALL, failure rates were higher

Table 2. Comparison of *in vitro* drug sensitivity to deoxynucleoside analogs between samples from children with newly diagnosed AML and ALL, and normal bone marrow (NBM) samples.

Drug	*LC_{50} value (μM)*	*AML*	*ALL*	*NBM*	*AML vs ALL*		*AML vs NBM*		*ALL vs NBM*	
					p-value	*RR*	*p-value*	*RR*	*p-value*	*RR*
Ara-C	Median	1.88	2.34	12.18	0.02	0.80	<0.0001*	0.15	<0.0001*	0.19
	*p*25-p75	0.97-3.07	1.11-6.24	9.28-56.56						
	n	134	228	32						
Cladribine	Median	0.07	0.10	0.19	0.0001*	0.7	<0.0001*	0.37	0.34	0.53
	*p*25-p75	0.03-0.10	0.07-0.74	0.08-0.28						
	n	129	149	21						
Fludarabine	Median	1.27	0.70	2.56	0.06	1.81	0.0001*	0.50	<0.0001*	0.27
	*p*25-p75	0.64-2.26	0.42-1.68	1.98-5.38						
	n	81	22	27						
Gemcitabine	Median	7.06	12.26	11.50	0.91	0.58	0.08	0.61	0.24	1.07
	*p*25-p75	2.06-20.86	1.51-24.70	2.55-69.54						
	n	83	20	27						

*Results are expressed as LC_{50} values (μM). Median indicates the median LC_{50} value; p25, the 25^{th} percentile; p75, the 75^{th} percentile; n, the number of patients. RR (resistance ratio) indicates the median LC_{50} for AML divided by the median LC_{50} value for ALL etc. RR>1.0 indicates that for the given drug AML is more resistant than ALL. *p-values determined with the Mann-Whitney U-test at a significance level of 0.01 (2-tailed).*

Table 3. Therapeutic index of deoxynucleoside analogs.

*Therapeutic index**	*AML*	*ALL*
Ara-C	6.5	5.2
Cladribine	2.7	1.9
Fludarabine	2.0	3.6
Gemcitabine	1.6	0.9

**Therapeutic index = median LC_{50} normal bone marrow/median LC_{50} value blast cells*

in samples with a low white cell count and in hyperdiploid samples.[16] Dose-response curves could be obtained for all four deoxynucleoside analogs and we observed marked differences in LC_{50} values between individual patients (Table 2). Normal bone marrow mononuclear cells were significantly more resistant to ara-C (6.5 fold; $p<0.0001$), cladribine (2.7 fold; $p<0.0001$) and fludarabine (2.0 fold; $p<0.0001$) than were the AML blasts, but they were not more resistant to gemcitabine (1.6 fold; $p=0.08$). Normal bone marrow mononuclear cells were also more resistant to ara-C (5.2 fold; $p<0.0001$) and fludarabine (3.7 fold; $p<0.0001$) than were ALL samples but they were not more resistant to cladribine (1.9 fold; $p=0.34$) or gemcitabine (0.9 fold; $p=0.24$). The therapeutic index was highest for ara-C in both AML and ALL, indicating a relative high sensitivity of leukemia cells to this drug compared to the sensitivity of normal bone marrow cells (Table 3).

AML blasts were more sensitive *in vitro* to cladribine (1.4 fold; $p<0.0001$) than were ALL blasts (Figure 2). Additionally, AML blasts tended to be more sensitive to ara-C than were ALL blasts (1.2 fold; $p=0.02$). AML and ALL blasts did not, however, differ in sensitivity to gemcitabine or fludarabine (Table 2).

In vitro sensitivity to deoxynucleoside analogs in different AML and ALL subtypes

AML is a heterogeneous disease and different AML FAB type subgroups display different *in vitro* sensitivity profiles.[7] We therefore also compared the drug sensitivity profiles of different AML FAB-type subgroups (Table 4). Patients were divided into three subgroups: FAB M1/2, FAB M4 and FAB M5. FAB M1 and FAB M2 were combined for analysis because they did not differ in drug sensitivity (*data not shown*). FAB M0 and FAB M3 were not included because of low sample numbers. *In vitro*, FAB M5 was significantly more sensitive to ara-C and cladribine than was FAB M1/2 (2.2 fold; $p<0.0001$ and 2.0 fold; $p=0.001$, respectively) or FAB M4 (1.8 fold; $p=0.002$ and 1.8 fold; $p=0.003$, respectively). Sensitivity to gemcitabine and fludarabine did not differ significantly between AML FAB types, although FAB M1/2 tended (3.2 fold; $p=0.03$) to be more resistant than FAB M4.

Previous studies have reported a relationship between both immunophenotype and *in vitro* drug resistance in ALL.[17] Therefore, ALL samples were divided into two subgroups based on immunophenotype: B-cell precursor (BCP) ALL or T-ALL (Table 5). T-ALL was more resistant to cladribine (7 fold; $p=0.001$), and tended to be more resistant to ara-C

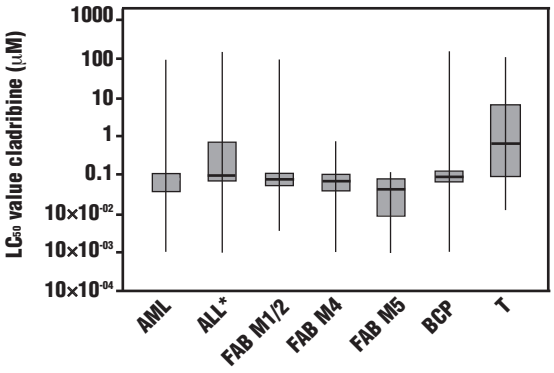

Figure 2. Sensitivity (expressed as the LC_{50} value) to cladribine in AML and ALL, as well as in different acute leukemia subgroups, determined with the MTT assay. *Indicates a significant difference (*p*-value determined with the Mann-Whitney U-test).

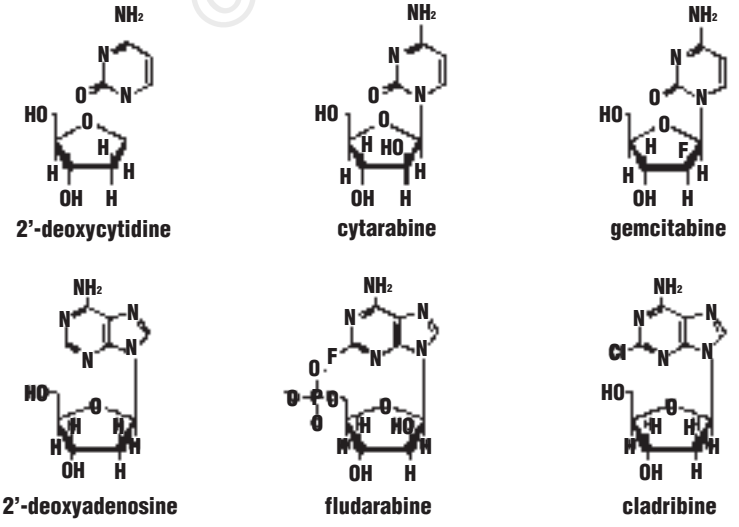

Figure 1. Structures of 2'-deoxycytidine, 2'-deoxyadenosine, cytarabine, gemcitabine, fludarabine and cladribine.

Table 4. Comparison of *in vitro* drug sensitivity to deoxynucleoside analogs between samples from different childhood AML (FAB) and ALL (immunophenotype) subgroups.

	LC_{50} value (μM)	Ara-C	Cladribine	Fludarabine	Gemcitabine
			AML		
FAB M1/2	Median	2.21	0.08	1.42	11.17
	p25-p75	1.39-5.61	0.05-0.10	0.66-2.29	2.51-50.00
	n	43	40	24	26
FAB M4	Median	1.80	0.07	1.22	3.51
	p25-p75	1.16-2.75	0.04-0.11	0.57-2.36	0.11-15.15
	n	46	43	29	29
FAB M5	Median	0.99	0.04	1.29	3.79
	p25-p75	0.45-2.01	0.01-0.07	0.65-2.03	2.15-13.08
	n	27	28	16	16
FAB M1/2 vs FAB M4	*p*-value	0.28	0.60	0.69	0.03
	RR	1.23	1.14	1.16	3.18
FAB M1/2 vs FAB 5	*p*-value	<0.0001*	0.001*	0.77	0.06
	RR	2.23	2.00	1.10	2.95
FAB M4 vs FAB M5	*p*-value	0.002*	0.003*	0.94	0.87
	RR	1.82	1.75	0.95	0.93
			ALL		
BCP	Median	2.13	0.09	0.55	8.53
	p25-p75	1.09-5.81	0.06-0.13	0.32-0.89	1.59-28.96
	n	188	111	16	14
T	Median	4.66	0.63	1.92	20.09
	p25-p75	1.55-8.10	0.09-6.10	0.64-31.74	0.46-66.63
	n	39	38	6	6
BCP vs T	*p*-value	0.04	0.001*	0.03	0.60
	RR	0.46	0.14	0.29	0.42

*Results are expressed as LC_{50} values (μM). Median indicates the median LC_{50} value; p25, the 25th percentile; p75, the 75th percentile; n, the number of patients. RR (resistance ratio) indicates the median LC_{50} for FAB M1/2 divided by the median LC_{50} value for FAB M4, etc. RR>1.0 indicates that for the given drug FAB M1/2 is more resistant than AML FAB M4. *p-value determined with the Mann-Whitney U-test at a significance level of 0.01 (2-tailed).*

Table 5. Cross-resistance between deoxynucleoside analogs in childhood AML.

Pearson's correlation coefficient	*Ara-C*	*Cladribine*	*Fludarabine*	*Gemcitabine*
Ara-C	–	0.53 *	0.44*	0.67*
Cladribine	0.53*	–	0.72*	0.59*
Fludarabine	0.44*	0.72*		0.49*
Gemcitabine	0.67*	0.59*	0.49*	–

**indicates a significant correlation (p≤0.01).*

(2.2 fold; p=0.04) and fludarabine (3.5 fold; p=0.03), than BCP ALL. BCP and T-ALL did not significantly differ in their sensitivity to gemcitabine.

Cross-resistance patterns

We studied cross-resistance patterns between deoxynucleoside analogs in AML (n=60) and ALL (n=99) samples. In each patient *in vitro* drug sensitivity for all deoxynucleoside analogs (ara-C, cladribine, fludarabine and gemcitabine) was successfully tested with the MTT assay. In AML, we observed cross-resistance between ara-C and cladribine (Rp=0.53; p<0.0001), fludarabine (Rp=0.44; p<0.0001) and gemcitabine (Rp=0.67; p<0.0001). Moreover, we observed considerable cross-resistance between all four deoxynucleoside analogs (Table 5), with the highest level of cross-resistance being between cladribine and fludarabine (Rp= 0.72; p<0.0001). In ALL, we also observed moderate cross-resistance between ara-C and cladribine (Rp=0.49; p<0.0001). Gemcitabine and fludarabine sensitivity was not tested in this group of ALL samples.

We also tested cross-resistance between ara-C and other drugs frequently used in the treatment of acute leukemia. In AML, cross-resistance was observed between ara-C and etoposide (Rp=0.54; p<0.0001), as well as with daunorubicin (Rp=0.48; p<0.0001). In ALL blasts we observed cross-resistance between ara-C and vincristine (Rp=0.50; p<0.0001). Weak cross-resistance was observed between ara-C and daunorubicin and L-asparaginase (Rp=0.25; p=0.01 and Rp=0.28; p=0.005, respectively). We did not observe cross-resistance between ara-C and prednisolone (Rp=0.12; p=0.24) in ALL.

Discussion

In the present study we compared *in vitro* sensitivity to deoxynucleoside analogs in childhood AML and ALL, and in subgroups of these leukemias. In addition, we studied whether cladribine, fludarabine or gemcitabine could circumvent *in vitro* resistance to ara-C in childhood AML and ALL. Previous studies have shown that for most drugs AML shows more resistance *in vitro* than ALL. However, AML and ALL are equally sensitive to a few drugs including ara-C.[7] While our results show that AML cells tend to be more sensitive than ALL cells to ara-C, cladribine is the only drug to which AML cells are significantly more sensitive. Like ara-C, cladribine is phosphorylated intracellularly and incorporated into the DNA of dividing cells, resulting in inhibition of DNA synthesis and cell death. However, cladribine is resistant to deamination and is able to induce apoptosis in non-dividing cells, by impairing DNA repair and causing strand breaks. Other groups have studied *in vitro* cladribine sensitivity in leukemic blasts. Nagourney *et al.,* found no difference in cladribine sensitivity between blasts from five AML and seven ALL patients, using a differen-

tial staining cytotoxicity (DISC) assay.[18] Larsson *et al*. tested 17 ALL and 34 AML samples using a fluorimetric microculture cytotoxicity assay, and found cladribine to be active against both diseases.[19]

Clinically, cladribine administered by continuous infusion resulted in a 59% response rate (27% complete remission) in pediatric casses of relapsed AML, but in pediatric ALL the response was poor (one out of seven cases responding).[20] Cladribine was able to induce complete or partial responses in approximately 60% of children with *de novo* AML.[21] In adults results with cladribine have been disappointing[22-24] and further studies have focused on the role of cladribine in combination chemotherapy. Gandhi *et al*. reported that ara-CTP accumulation increased in adult patients with AML when cladribine was given before ara-C.[25] In pediatric AML the combination of cladribine and ara-C also seemed effective.[26]

Another purine analog that has shown efficacy in AML in combination with ara-C is fludarabine. In our *in vitro* study, fludarabine was equally effective against AML and ALL. The FLAG regimen, which consists of fludarabine, ara-C and granulocyte colony-stimulating factor is one of the most active regimens in AML.[27,28]

Experience with gemcitabine in AML has been limited, although phase I studies have suggested activity in heavily pre-treated patients with acute leukemia.[29-31] Our *in vitro* results demonstrated that the therapeutic index for gemcitabine was low. ALL and AML samples were not significantly more sensitive to gemcitabine than were normal bone marrow cells. Moreover, we could not identify a subgroup of acute leukemia patients with increased sensitivity to gemcitabine *in vitro*. However, this drug may have a role as a modulator of ara-C activity. Gemcitabine has been reported to increase the activity of dCK, resulting in a three-fold increase in intracellular accumulation of ara-CTP and a subsequent increase in cytotoxicity in leukemic cell lines.[32,33]

We observed several subgroup-related differences in sensitivity to deoxynucleoside analogs in both AML and ALL. Acute monocytic leukemia, or FAB M5, is a distinct AML subtype with characteristic clinical features and a specific *in vitro* drug resistance profile.[7,34] Within AML, we found FAB M5 to be significantly more sensitive to both ara-C and cladribine. These *in vitro* data are in line with clinical studies showing that FAB M5 patients are sensitive to cladribine and have a high complete response rate after treatment with cladribine.[35] Within ALL, T-ALL blasts were significantly more resistant to cladribine than were BCP ALL blasts. This was not the case for ara-C, suggesting that cladribine might not be an effective alternative to ara-C in the treatment of T-ALL.

In vitro drug sensitivity testing has been shown to provide significant prognostic information in childhood acute leukemia and can also be used to determine cross-resistance patterns between drugs.[36,37] Our paired analysis in 60 AML samples suggests that gemcitabine, fludarabine and cladribine could not circumvent ara-C resistance. We observed significant cross-resistance between all deoxynucleoside analogs. A high degree of cross-resistance among deoxynucleoside anaologs has been reported previously in resistant variants of the human leukemic cell line K562.[38] Ara-C and gemcitabine displayed the highest degree of cross-resistance, which is consistent with their structural similarity. The purine analogs fludarabine and cladribine also displayed considerable cross-resistance.

Furthermore, we observed cross-resistance between ara-C and several structurally unrelated drugs in both AML and ALL samples. Cross-resistance between ara-C and other classes of anti-leukemic drugs has been described in leukemic cell lines.[39-41] Our results show that cross-resistance also occurs in primary cells. Although we observed cross-resistance between ara-C and cladribine, fludarabine and gemcitabine in this study, deoxynucleoside analogs can be used in combination with ara-C as resistance modulators. We have previously demonstrated synergistic interactions between ara-C and cladribine, and ara-C and gemcitabine in the HL60 leukemic cell line[42] and samples from ten AML patients (*Hubeek et al., Br J Haematol, in press*).

In conclusion, we observed several subgroup-related differences in sensitivity to deoxynucleoside analogs in childhood acute leukemia. *In vitro* resistance to ara-C does not appear to be circumvented by cladribine, fludarabine or gemcitabine. Our results suggest that cladribine is a useful drug in AML, and in FAB type M5 in particular. Cross-resistance between ara-C and other deoxynucleoside analogs, and also between ara-C and drugs with different modes of action, suggests that downstream factors might be involved. Therefore drug resistance in childhood acute leukemia may be explained by drug-type specific but also by more general downstream mechanisms (e.g. the apoptosis machinery or DNA repair pathways).

All persons designated as authors qualified for authorship by contributing to the design and development of the study as well as the interpretation of data. All of them revised the manuscript critically for important intellectual content and approved the final version of the manuscript.

The authors declare that they have no potential conflict of interest.

The authors thank all hospitals and clinicians participating in the MRC Childhood Leukaemia Working Party, the DCOG, the AML-BFM Study Group and the COALL Study Group who provided us with the patients' samples. We also thank the technicians of the Research Laboratory of Pediatric Oncology at the VU University Medical Center for their expertise in testing the patients' samples. Part of this study was performed within the setting of the International I-BFM Study Group (chaired by M Schrappe), especially the AML committee (chaired by GJL Kaspers).

Manuscript received July 25, 2005. Accepted October 19, 2005.

References

1. Hann IM, Webb DK, Gibson BE, Harrison CJ. MRC trials in childhood acute myeloid leukaemia. Ann Hematol 2004; 83 Suppl 1:S108-12.
2. Creutzig U, Zimmermann M, Ritter J, Henze G, Graf N, Loffler H, et al. Definition of a standard-risk group in children with AML. Br J Haematol 1999; 104:630-9.
3. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med 2004;350:1535-48.
4. Grant S. Ara-C: cellular and molecular pharmacology. Adv Cancer Res 1998; 72: 197-233.
5. Estey E, Plunkett W, Dixon D, Keating M, McCredie K, Freireich EJ. Variables predicting response to high dose cytosine arabinoside therapy in patients with refractory acute leukemia. Leukemia 1987;1:580-3.
6. Plunkett W, Liliemark JO, Estey E, Keating MJ. Saturation of ara-CTP accumulation during high-dose ara-C therapy: pharmacologic rationale for intermediate-dose ara-C. Semin Oncol 1987;14 Suppl 1:159-66.
7. Zwaan CM, Kaspers GJ, Pieters R, Ramakers-Van Woerden NL, Den Boer ML, Wunsche R, et al. Cellular drug resistance profiles in childhood acute myeloid leukemia: differences between FAB types and comparison with acute lymphoblastic leukemia. Blood 2000; 96: 2879-86.
8. Pieters R, Kaspers GJ, Van Wering ER, Huismans DR, Loonen AH, Hahlen K, et al. Cellular drug resistance profiles that might explain the prognostic value of immunophenotype and age in childhood acute lymphoblastic leukemia. Leukemia 1993;7:392-7.
9. Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues and nucleobases in cancer treatment. Lancet Oncol 2002;3: 415-24.
10. Creutzig U, Harbott J, Sperling C, Ritter J, Zimmermann M, Loffler H, et al. Clinical significance of surface antigen expression in children with acute myeloid leukemia: results of study AML-BFM-87. Blood 1995;86:3097-108.
11. Creutzig U, Ritter J, Zimmermann M, Hermann J, Gadner H, Sawatzki DB, et al. Idarubicin improves blast cell clearance during induction therapy in children with AML: results of study AML-BFM 93. AML-BFM Study Group. Leukemia 2001;15:348-54.
12. Slats AM, Egeler RM, Van der Does-Van den Berg, Korbijn C, Hahlen K, Kamps WA, et al. Causes of death-other than progressive leukemia-in childhood acute lymphoblastic (ALL) and myeloid leukemia (AML): the Dutch Childhood Oncology Group experience. Leukemia 2005;19:537-44.
13. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med 1985;103:620-5.
14. Kaspers GJ, Veerman AJ, Pieters R, Broekema GJ, Huismans DR, Kazemier KM, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. Br J Cancer 1994;70:1047-52.
15. Kaspers GJ, Pieters R, van Zantwijk CH, De Laat PA, de Waal FC, Van Wering ER, et al. In vitro drug sensitivity of normal peripheral blood lymphocytes and childhood leukaemic cells from bone marrow and peripheral blood. Br J Cancer 1991; 64:469-74.
16. Kaspers GJ, Veerman AJ, Pieters R, van Zantwijk CH, Smets LA, Van Wering ER, et al. In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood 1997;90:2723-9.
17. Pieters R, Den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, et al. Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia - implications for treatment of infants. Leukemia 1998;12:1344-8.
18. Nagourney RA, Evans SS, Messenger JC, Su YZ, Weisenthal LM. 2 chlorodeoxyadenosine activity and cross resistance patterns in primary cultures of human hematologic neoplasms. Br J Cancer 1993; 67:10-4.
19. Larsson R, Fridborg H, Liliemark J, Csoka K, Kristensen J, de la Torre M, et al. In vitro activity of 2-chlorodeoxyadenosine (CdA) in primary cultures of human haematological and solid tumours. Eur J Cancer 1994;30A:1022-6.
20. Santana VM, Mirro J Jr., Kearns C, Schell MJ, Crom W, Blakley RL. 2-Chlorodeoxyadenosine produces a high rate of complete hematologic remission in relapsed acute myeloid leukemia. J Clin Oncol 1992; 10:364-70.
21. Kumagai M, Manabe A, Coustan-Smith E, Blakley RL, Beck WT, Santana VM, et al. Use of stroma-supported cultures of leukemic cells to assess antileukemic drugs. II. Potent cytotoxicity of 2-chlorodeoxyadenosine in acute lymphoblastic leukemia. Leukemia 1994;8:1116-23.
22. Vahdat L, Wong ET, Wile MJ, Rosenblum M, Foley KM, Warrell RP Jr. Therapeutic and neurotoxic effects of 2-chlorodeoxyadenosine in adults with acute myeloid leukemia. Blood 1994;84:3429-34.
23. Kornblau SM, Gandhi V, Andreeff HM, Beran M, Kantarjian HM, Koller CA, et al. Clinical and laboratory studies of 2-chlorodeoxyadenosine +/- cytosine arabinoside for relapsed or refractory acute myelogenous leukemia in adults. Leukemia 1996; 10:1563-9.
24. Van Den NE, Martiat P, Mineur P, Delannoy A, Doyen C, Zenebergh A, et al. 2-Chlorodeoxyadenosine with or without daunorubicin in relapsed or refractory acute myeloid leukemia. Ann Hematol 1998;76:19-23.
25. Gandhi V, Estey E, Keating MJ, Chucrallah A, Plunkett W. Chlorodeoxyadenosine and arabinosylcytosine in patients with acute myelogenous leukemia: pharmacokinetic, pharmacodynamic, and molecular interactions. Blood 1996;87:256-64.
26. Crews KR, Gandhi V, Srivastava DK, Razzouk BI, Tong X, Behm FG, et al. Interim comparison of a continuous infusion versus a short daily infusion of cytarabine given in combination with cladribine for pediatric acute myeloid leukemia. J Clin Oncol 2002; 20:4217-24.
27. Montillo M, Mirto S, Petti MC, Latagliata R, Magrin S, Pinto A, et al. Fludarabine, cytarabine, and G-CSF (FLAG) for the treatment of poor risk acute myeloid leukemia. Am J Hematol 1998;58:105-9.
28. Ferrara F, Palmieri S, Pocali B, Pollio F, Viola A, Annunziata S, et al. De novo acute myeloid leukemia with multilineage dysplasia: treatment results and prognostic evaluation from a series of 44 patients treated with fludarabine, cytarabine and G-CSF (FLAG). Eur J Haematol 2002;68:203-9.
29. Steinherz PG, Seibel NL, Ames MM, Avramis VI, Krailo MD, Liu-Mares W, et al. Phase I study of gemcitabine (difluorodeoxycytidine) in children with relapsed or refractory leukemia (CCG-0955): a report from the Children's Cancer Group. Leuk Lymphoma 2002;43: 1945-50.
30. Gandhi V, Plunkett W, Du M, Ayres M, Estey EH. Prolonged infusion of gemcitabine: clinical and pharmacodynamic studies during a phase I trial in relapsed acute myelogenous leukemia. J Clin Oncol 2002;20:665-73.
31. Grunewald R, Kantarjian H, Du M, Faucher K, Tarassoff P, Plunkett W. Gemcitabine in leukemia: a phase I clinical, plasma, and cellular pharmacology study. J Clin Oncol 1992; 10:406-13.
32. Gandhi V, Plunkett W. Modulatory activity of 2',2'-difluorodeoxycytidine on the phosphorylation and cytotoxicity of arabinosyl nucleosides. Cancer Res 1990;50: 3675-80.
33. Santini V, D'Ippolito G, Bernabei PA, Zoccolante A, Ermini A, Rossi-Ferrini P. Effects of fludarabine and gemcitabine on human acute myeloid leukemia cell line HL 60: direct comparison of cytotoxicity and cellular Ara-C uptake enhancement. Leuk Res 1996;20:37-45.
34. Tallman MS, Kim HT, Paietta E, Bennett JM, Dewald G, Cassileth PA, et al. Acute monocytic leukemia (French-American-British classification M5) does not have a worse prognosis than other subtypes of acute myeloid leukemia: a report from the Eastern Cooperative Oncology Group. J Clin Oncol 2004;22:1276-86.
35. Krance RA, Hurwitz CA, Head DR, Raimondi SC, Behm FG, Crews KR, et al. Experience with 2-chlorodeoxyadenosine in previously untreated children with newly diagnosed acute myeloid leukemia and myelodysplastic diseases. J Clin Oncol 2001;19:2804-11.
36. Bresters D, Broekhuizen AJ, Kaaijk P, Faircloth GT, Jimeno J, Kaspers GJ. In vitro cytotoxicity of aplidin and crossresistance with other cytotoxic drugs in childhood leukemic and normal bone marrow and blood samples: a rational basis for clinical development. Leukemia 2003;17:1338-43.
37. Kaspers GJ, Veerman AJ. Clinical significance of cellular drug resistance in childhood leukemia. Recent Results Cancer Res 2003;161:196-220.
38. Dumontet C, Fabianowska-Majewska K, Mantincic D, Callet BE, Tigaud I, Gandhi V, et al. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol 1999;106:78-85.
39. Takemura H, Urasaki Y, Yoshida A, Fukushima T, Ueda T. Simultaneous treatment with 1-β-D-arabinofuranosylcytosine and daunorubicin induces cross-resistance to both drugs due to a combination-specific mechanism in HL60 cells. Cancer Res 2001; 61:172-7.
40. Martin-Aragon S, Mukherjee SK, Taylor BJ, Ivy SP, Fu CH, Ardi VC, et al. Cytosine arabinoside (ara-C) resistance confers cross-resistance or collateral sensitivity to other classes of anti-leukemic drugs. Anticancer Res 2000; 20:139-50.
41. Mansson E, Paul A, Lofgren C, Ullberg K, Paul C, Eriksson S, et al. Cross-resistance to cytosine arabinoside in a multidrug-resistant human promyelocytic cell line selected for resistance to doxorubicin: implications for combination chemotherapy. Br J Haematol 2001;114:557-65.
42. Hubeek I, Peters GJ, Broekhuizen AJ, Kaspers GJ. Modulation of cytarabine induced cytotoxicity using novel deoxynucleoside analogs in the HL60 cell line. Nucleosides Nucleotides Nucleic Acids 2004;23:1513-6.