In vitro sensitivity of leukemic cells to nucleoside derivatives in childhood acute leukemias: good activity in leukemic relapses

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Nucleoside analogues such as fludarabine and cladribine are used in therapy of indolent lymphomas and leukemias in adults, while cytarabine is used mainly in protocols for acute leukemias. Mechanisms of their activity is based on inhibition of enzymes involved in DNA, RNA and protein synthesis. The objective of the study was the analysis of *in vitro* cellular drug sensitivity in childhood acute lymphoblastic (ALL) and myeloid (AML) leukemia. Isolated leukemic cells obtained from 264 patients, including 152 initial ALL, 45 relapsed ALL, 54 initial AML and 13 relapsed AML were tested for cytotoxicity for fludarabine, cladribine, and cytarabine by the MTT assay. Drug concentration lethal to 50% of tested cells was regarded as a value of drug resistance. Three tested nucleoside analogues showed highest cytotoxicity against initial ALL samples. Samples of relapsed ALL and initial AML were more resistant than ALL de novo ones. Unexpectedly, no differences were observed between initial and relapsed AML samples for all tested drugs, what suggests that nucleoside analogues are active drugs in relapsed AML, which is commonly regarded as a resistant disease. All tested drugs presented significant cross-resistance in each of analyzed subgroups. In summary, tested nucleoside analogues presented relatively good activity against childhood leukemias at relapse stage.

Key words: nucleoside analogues, leukemia, resistance, sensitivity

Fludarabine, cladribine and cytarabine are nucleoside analogues used in therapy of leukemias and lymphomas (Fig. 1). Mechanisms of their activity is based on suppression of enzymes involved in DNA synthesis, as well as RNA and protein synthesis inhibition [1, 10]. F-AraA and 2-CdA are deoxyadenosine analogues regarded as active agents in lymphoproliferative disorders including chronic lymphocytic leukemia, low-aggressive non-Hodgkin lymphomas (NHL), Waldenstrom macroglobulinemia and hairy cell leukemia [10]. Both drugs are purine analogs, resistant to adenosine deaminase (ADA); both suppress activity of DNA polymerase, primase, ligase and ribonucleotide reductase. This finally leads to suppression of DNA, RNA

Abbreviations: F-AraA – fludarabine, AraC – cytarabine, 2-CdA – cladribine, ALL – acute lymphoblastic leukemia, iALL – initial ALL, rALL – relapsed ALL, AML – acute myelogenous leukemia, iAML – initial AML, rAML – relapsed AML, CLL – chronic lymphatic leukemia, NHL – non-Hodgkin lymphoma, RR – relative resistance, m – median, n – number of patients.

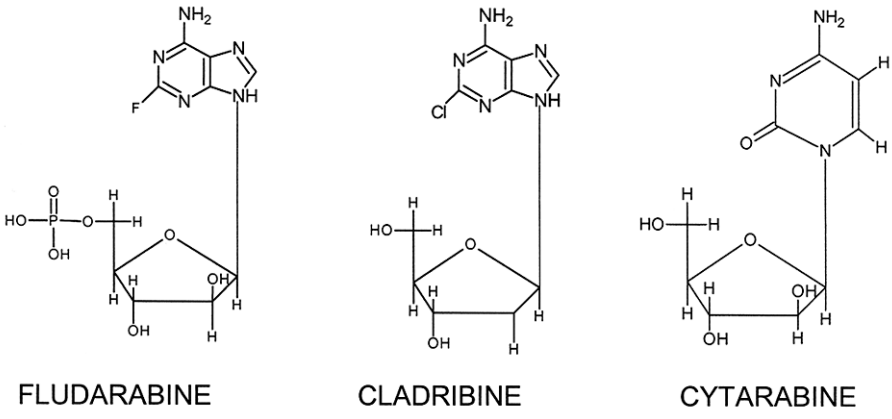

Figure 1. Chemical structure of nucleoside derivatives used in the study.

and protein synthesis. F-AraA and 2-CdA require intracellular phosphorylation by deoxycytidine kinase to be activated to cytotoxic form [3,6]. Active phosphorylated metabolites of 2-CdA (2-chloro-deoxyadenosine) are being accumulated in cells with high activity of deoxycytidine kinase, such as lymphocytes [10].

Pyrimidine analogue, cytarabine is a derivative of 2-deoxycytidine. It is metabolized by deoxycytidine kinase to pharmacologically active phosphorylated form, Ara-CTP, which inhibits DNA polymerase and reductase transforming CDP to dCDP, compound of DNA [1]. Inhibition of these enzymes suppresses DNA synthesis. Cytarabine is used in therapy of ALL, AML and NHL.

In contrast to AraC, there is little knowledge about possibilities of use F-AraA and 2-CdA in childhood acute leukemias. The objective of this study was the analysis of *in vitro* cellular drug sensitivity to these nucleoside derivatives in childhood acute lymphoblastic (ALL) and myeloid leukemia (AML) in a large cohort of patients.

Material and methods

Patients. Isolated leukemic cells from 264 children, including 111 males and 153 females aged 2 days–19 years (median 8 years). According to diagnosis, all patients were divided into four groups: initial ALL (iALL) – 152; relapsed ALL (rALL) – 45; initial AML (iAML) – 54; relapsed AML (rAML) – 13 patients.

Drugs. Cytotoxicity was tested by the cytotoxicity assay (MTT assay) for following drugs: Fludarabine (Fludara, Schering, concentration range: 0.019–20 μg/ml), Cytarabine (Alexan, Mack, range: 0.0097–10 μg/ml), Cladribine (Biodribine, Bioton, range: 0.0004–40 μg/ml). Drug concentration lethal to 50% of tested cells (LC50) was regarded as a value of drug resistance.

Separation of mononuclear cells from bone marrow (BM) and peripheral blood (PB). Samples were diluted 1:1 or more with RPMI-1640 (Sigma Chemical Co, St Louis, USA). In case of the presence of small clots, the sample of BM or PB was first filtered through a cell strainer (70 μm nylonfilter; Falcon, Franklin Lakes, New Jersey, USA) using RPMI-1640 to rinse off the strainer. Cells were separated on Ficoll gradient (Gradisol L density; 1.077 g/ml; Aqua-Medica, Lodz, Poland) at 540 g for 20 minutes at room temperature. After centrifugation, cells were washed twice with RPMI-1640.

Culture medium and culture suspension. The sample was finally resuspended in culture medium containing RPMI-1640, 20% FCS heat-inactivated, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.125 μg/ml amphotericine B, 200 μg/ml gentamycine, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (all: Sigma). The cell concentration for the MTT assay was finally adjusted to $2.5x10^6$ vital cells per ml.

In vitro drug resistance assay. The assay conditions were essentially the same as described previously [7]. Eighty μl of the cell suspension containing $2x10^6$ vital cells / ml was incubated with each drug concentration in 20 μl RPMI in duplicate wells of a 96-well round-bottomed microtiter plate (Profilab, Warszawa, Poland). Six wells containing only cells in a drug-free medium served as controls for cell survival, while other six wells containing only culture medium blanked the spectrophotometer. The outer wells of the microculture plate were filled with RPMI-1640 only. Plates were then wrapped in cling film and incubated for 4 days (96 hours) at 37 ˚C in humidified air containing 5% CO_2. After 4 days, 50 μg (10 μl of a solution of 5 mg/ml) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT, Sigma) was added to each well (final concentration 0.45 mg/ml); plates were shaken and incubated for another 6 hours at 37 ˚C. In such an exposure yellow MTT can be reduced into purple formazan by viable but not dead cells. The formazan crystals were dissolved with 80 μl of acidified (0.04 N HCl) 2-isopropanolol (Chemia, Bydgoszcz, Poland) and the quantity of reduced product was measured by an ELISA microplate spectrophotometer at 550 nm with reference wavelength of 720 nm (Assys, Eugendorf, Austria). The optical density (OD) at 550 nm is linearly related to the number of viable cells [4]. Cytospin slides from control wells, stained with MGG, were used to determine the percentage of blasts after 96-hours incubation. Samples with more than 70% leukemic cells in the control wells without drug after 4 days of culture and with an OD higher than 0.050 arbitrary units (adjusted for blank values) are suitable for evaluation [7]. The leuke-

mic cell survival was calculated by the equation: (OD drug well / mean OD control wells) x 100%. The OD of both control and tested wells were adjusted by OD of blank wells. The LC50, the concentration of drugs that was lethal to 50% of the cells (in μg/ml) was used as a measure for the in vitro drug cytotoxicity in each sample [4].

Criteria for a successful MTT assay. The MTT assay was performed with a cell suspension containing a minimum of 90% malignant cells in acute lymphoblastic leukemia samples and at least of 70% in acute myeloid leukemia samples. After a 4-day culture the cells in the control wells contained a minimum of 70% malignant cells and the optical density (OD) was above 0.050 [7].

Statistical analysis. Differences in resistance to specific drug between two analyzed groups was defined by relative resistance (RR), measured as median LC50 of one group divided by the median LC50 of the other group for tested compound. Comparison of cytotoxicity between groups was done by non-parametric U Mann-Whitney test for 2 groups and by Kruskal-Wallis H test for higher number of groups. Correlations between drug resistance was estimated by rho coefficient value and Spearman correlation test. P value less than 0.05 was regarded as significant.

The study was approved by Local Ethic Committee, Medical University of Bydgoszcz.

Results

A large variability between LC50 values was seen (Tab. 1). The best sensitivity to fludarabine was observed in iALL (median LC50=0.41 μg/ml), than in rALL (2.1-fold lower, p=0.07) and in iAML (4-fold lower, p<0.001). The best sensitivity to cytarabine was in iALL (median LC50=0.53 μg/ml), then in iAML (median LC50=0.61 μg/ml, ns) and in rALL (2-fold lower, p=0.009). The best sensitivity to cladribine was seen in iALL (median LC50=0.057 μg/ml), then in iAML (5-fold lower, p=0.042) and in rALL (6.1-fold, p=0.008 (Fig. 2). Activity of AraC in rAML was comparable to iAML, alike F-AraA and 2-CdA (ns). Kruskal-Wallis H test showed differences between LC50 values between 4 analyzed groups of patients for F-AraA (p=0.001), AraC (p=0.069) and 2-CdA (p=0.017).

As the number of rAML patients was low (and results were similar to iAML group), another comparison by Kruskal-Wallis H test was made also between 3 groups of patients, with exclusion of rAML children. In such condition, differences reached higher significance: for fludarabine (p<0.001), cytarabine (p=0.034), and cladribine (p=0.010). Differences between each subgroups compared by Mann-Whitney test are presented in Table 1. All tested pairs of drugs presented significant cross-resistance in each of subgroups analyzed (p<0.001 for each comparison by Spearman's correlation test) (Tab. 2, Fig. 3).

Table 1. Results of resistance to fludarabine, cytarabine and cladribine in childhood acute leukemias

	FLUDARABINE	CYTARABINE	CLADRIBINE
iALL	m=0.41 (0.21–2.12) n=152	m=0.53 (0.1–1.17) n=135	m=0.057 (0.02–1.86) n=144
rALL	m=0.89 (0.28–7.10) n=45 RR=2.15 p=0.07	m=1.06 (0.30–7.43) n=40 RR=2.0 p=0.009	m=0.35 (0.02–>40) n=40 RR=6.1 p=0.008
iAML	m=1.60 (0.36–>20) n=54 RR=3.9 p<0.001	m=0.61 (0.24–3.46) n=50 RR=1.15 p=0.225	m=0.29 (0.03–16.64) n=54 RR=5 p=0.042
rAML	m=1.46 (0.22–11.69) n=13 RR=3.6 p=0.001	m=0.62 (0.31–0.86) n=11 RR=1.17 p=0.315	m=0.16 (0.03–>40) n=13 RR=2.8 p=0.056

m – median (percentiles 25-75) LC50 [μg/ml], n – number of patients tested, RR – relative resistance in comparison to iALL, measures as median LC50 of analyzed group divided by the median LC50 for iALL for tested compound; RR>1 indicates that for the given drug, patients in analyzed group are more resistant than iALL, p-value by U Mann-Whitney test, counted in relation to iALL.

Table 2. Correlation matrix of cross-resistant cytotoxicity for each pair of tested nucleoside analogues

	All patients	iALL	rALL	iAML
FLUDARABINE – CYTARABINE	0.573 <0.001	0.547 <0.001	0.520 0.001	0.710 <0.001
FLUDARABINE – CLADRIBINE	0.642 <0.001	0.607 <0.001	0.544 <0.001	0.715 <0.001
CYTARABINE – CLADRIBINE	0.642 <0.001	0.607 <0.001	0.520 <0.001	0.715 <0.001

Upper value in each area is the value of Spearman's rho coefficient of correlation of LC50 values for a pair of drugs; lower value – p-value for this coefficient.

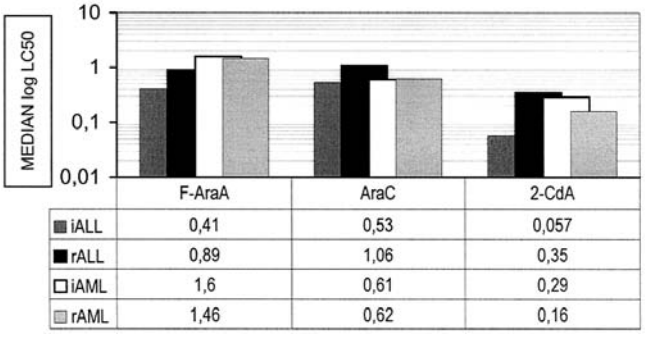

Figure 2. Comparison of in vitro resistance to nucleoside derivatives in childhood acute leukemias. Drug concentrations are given in μg/ml.

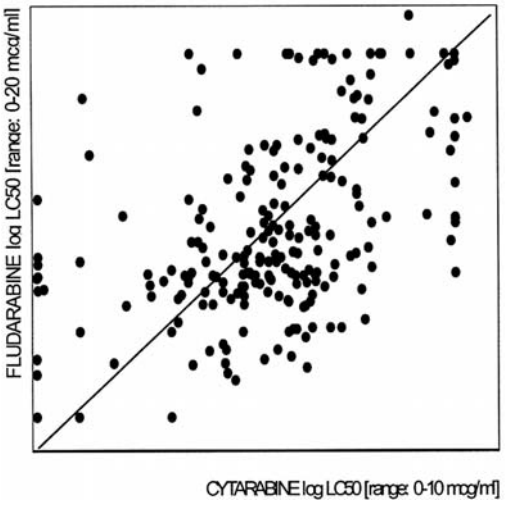

Figure 3. Correlation between LC50 values for fludarabine and cytarabine in iALL (rho=0.547, p<0.01, n=236 samples). A line shows values y=2x. Concentrations for both drugs are given in μg/ml.

Discussion

Leukemia is the most frequent group of neoplastic diseases in childhood. In spite of significant progress obtained over two last decades in therapy of childhood leukemias, still about 25% of patients with ALL and about 50% with AML relapse. As also high-dose therapy with hematopoietic stem cell transplantation is still a method with limited efficacy and unacceptable toxicity, there is a need for newer drugs and newer regimen.

We have previously shown, that glucocorticoids belong to groups of anticancer drugs with strong activity against childhood acute lymphoblastic leukemia, however cross-resistance was observed within this group [8]. Current study was focused on nucleoside analogues which belong to purine and pyrimidine antimetabolites, agents suppressing DNA elongation. There were at least 2 rationales for *in vitro* testing this group of drugs. First, because fludarabine and cladribine have distinctive antileukemic potential in patients with the chronic lymphoproliferative disorders such as chronic lymphocytic leukemia, hairy cell leukemia and non-Hodgkin lymphomas [3]. Second, because synergism between purine nucleoside analogues (such as fludarabine and cladribine) and cytosine nucleoside analogue, cytarabine, have been demonstrated [2].

In vitro activity of these 3 drugs in childhood acute leukemias were tested. All results were compared to the group of iALL patients, which is the best curable childhood leukemia subgroup. It has been shown that activity of cytarabine and fludarabine in rALL and iAML was satisfactory. Relatively good sensitivity of leukemic cells at relapse is quite unusual phenomenon [9].

Despite large interpatient variability of cytotoxicity assay, differences in median values of resistance between rALL and iALL were less than 4-fold for F-AraA and AraC, and 6-fold for 2-CdA. As usual doses of AraC in treatment protocols for childhood relapsed ALL are over 25-fold higher than used to be in protocols for initial ALL, differences obtained in current study were relatively not high. The AraC dosage in protocols for relapsed leukemia is aimed to overcome resistance of rALL cells to cytarabine.

It is assumed that relapsed acute leukemia is a disease more drug resistant than its primary form [11]. In this study, however, we did not find significant differences in drug resistance to nucleoside analogues between iAML and rAML samples. This might contribute to relatively good activity of fludarabine and cladribine in rAML, and might be a rationale for use of protocol FLAG in therapy of childhood rAML [5]. This can also be a rationale for use of cladribine in this disease. A pilot study of clinical use of cladribine in relapsed childhood AML is ongoing. On the other hand, fludarabine has also other pharmacological and clinical advantages. Combination of fludarabine with cytarabine gives synergistic effect, while combination of fludarabine with methotrexate or vincristine is antagonistic [2]. Another advantage of fludarabine is its low toxicity, with the most favorable profile out of 3 tested drugs. Toxicity of fludarabine is related mainly to myelosuppression, while nausea, vomiting, diarrhea and toxic hepatitis are rather mild [2].

In summary, when compared to lymphoblasts, all tested nucleotide analogues presented relatively good activity against childhood myeloblasts, both on initial diagnosis and at relapse. This might give a rationale for their use in clinical protocols for therapy of relapsed childhood acute myeloblastic leukemia.

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