Resistance to glucocorticoids in childhood acute lymphoblastic leukemia: impact of relationship between *ex vivo* sensitivity and *in vivo* concentration on risk factor analysis*

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Resistance to glucocorticoids remains one of the main obstacles in therapy of childhood acute lymphoblastic leukemia (ALL). The aim of the study was the analysis of relationship between *ex vivo* drug resistance of prednisolone and dexamethasone and exposure to these drugs in childhood ALL, with respect to risk factor analysis. *Ex vivo* resistance to both glucocorticoids was compared to maximum drug concentration achievable in body fluids, calculated in mathematical model. Drug resistance to vincristine and L-asparaginase, expression of multidrug resistance and apoptosis proteins was also determined. Concentration of both glucocorticoids in extracellular fluid was higher than drug resistance in the following groups of patients: in initial ALL patients, in patients staying in remission during follow-up, and in prednisolone good responders. Factors significant by multivariate analysis were early bone marrow response by day 15 and concentration of prednisolone higher than *ex vivo* prednisolone resistance. For initial ALL patients with determined response to initial prednisolone monotherapy, factors significant by univariate analysis were early bone marrow response, and exposure to glucocorticoids higher than *ex vivo* resistance to these drugs. No factor was significant by multivariate analysis in this group. Risk factor analysis showed that concentration of prednisolone and dexamethasone higher than respective *ex vivo* drug resistance, is a strongest prognostic factor in childhood ALL.

Key words: acute lymphoblastic leukemia, prednisolone, dexamethasone, glucocorticoids, drug resistance, risk factors

Childhood acute lymphoblastic leukemia (ALL) is a disease stratified according to prognostic factors. Over the decades, some factors lost their value, while new ones were found. Currently, following factors are regarded as the most important: response to one-week prednisolone monotherapy [1, 2], *in vitro* cellular drug resistance profile [3, 4], minimal residual disease [5–7], gene expression profile results [8–10], and presence of bcr-abl rearrangement [11]. High cost of obtaining the data is, however, a disadvantage for standard use of some factors [12]. Some other factors have still no well-established role. There are contradictory results concerning the role of multidrug resistance or apoptosis proteins in childhood ALL [13–15].

Glucocorticoids are the group of drugs with specific activity against childhood ALL, however resistance to these agents remains one of the main obstacles to reach the success [16–18]. Several mechanisms of action related to glucocorticoids contribute to *ex vivo* and *in vivo* resistance to prednisolone [16, 19, 20], including changes in glucocorticoid receptor expression [21], alternative splicing of glucocorticoid receptor [18], changes in expression of heat shock protein HSP-90 [22], upregulation of cell cycle regulators such as retinoblastoma protein [23], however prognostic value of each separate factor is doubtful [22]. Several clinical trials aimed to circumvent glucocorticoid resistance in childhood ALL by administration of high-dose dexamethasone [24] or high-dose methylprednisolone [25] were undertaken with promising results.

The aim of the study was assessing the *ex vivo* drug resistance to prednisolone and dexamethasone and comparison with maximum achievable concentration of these drugs in body fluids with respect to risk factor analysis.

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Material and methods

Patient samples. A total number of 60 children with ALL, including 46 initial and 14 relapsed patients were included in the study. Their baseline characteristics is shown in Table 1. Twelve children with normal bone marrow (NBM) were included in the study as control groups.

Drugs. Following drugs were used: prednisolone (Jelfa, Jelenia Gora, Poland, in concentration range 0.0076–250 μg/ml), dexamethasone (Jelfa, 0.00018–6 μg/ml), L-asparaginase (Medac, Hamburg, Germany, concentration range 0.0032–10 IU/ml), and vincristine (Gedeon Richter, Budapest, Hungary, concentration range 0.019–20 μg/ml).

Viability assay. Fresh lymphoblasts obtained from the bone marrow were isolated by Ficoll gradient. Only samples with at least of 90% of lymphoblasts were included in the study. *Ex vivo* drug resistance profile for prednisolone, dexamethasone, vincristine and L-asparaginase was done by the MTT assay, as described previously [26]. *Ex vivo* resistance was expressed by LC50 value, which is the concentration of the drug lethal to 50% of tested cells after 72 hours of incubation. Combined drug resistance profile for prednisolone, vincristine and L-asparaginase (PVA score) was calculated for each patient, as reported previously [3, 4]. PVA was scored from 3 to 9; the higher the score, the higher the resistance to these 3 drugs. For the purpose of this study, lymphoblasts were regarded as sensitive, with cut-off value of PVA score ≤6; while resistant, when PVA scored 7–9.

Exposure to prednisolone and dexamethasone. Exposure to prednisolone and dexamethasone was expressed as maximum achievable concentration of prednisolone in body fluids and calculated in mathematical model. It was expressed as a ratio of a standard dose of prednisolone or dexamethasone and volume of the body fluids: plasma, extracellular fluid (ECF) and total body fluid (TBF) determined according to standard physiology formulas [27]. Briefly, TBF forms about 60% of total body weight in children, while ECF and plasma about 20% and 5% of total body weight, respectively. Doses of prednisolone and dexamethasone delivered to the body were assumed 60 mg/m^2/24 hours and 10 mg/m^2/24 hours, respectively, as these dosages are most often used in pediatric protocols. Maximum achievable concentrations of prednisolone and dexamethasone in respective body fluid were compared with the results of *ex vivo* drug resistance assay.

Multidrug resistance and apoptosis proteins. Expression of intracellular epitopes of three multidrug resistance proteins: P-glycoprotein PGP (clone JSB-1), Multidrug-resistance Related Protein MRP1 (clone MRPr1), Lung Resistance Protein LRP (clone LRP-56) (all: Alexis Biochemicals, Lausanne, Switzerland), and three proteins regulating apoptosis: Bcl-2 (DakoCytomation, Glostrup, Denmark), pRb (PharMingen, Becton Dickinson Biosciences, San Diego, CA, USA) and p53 (Dako) was analyzed by flow cytometry on diagnosis and after 72 hours of incubation with prednisolone at concentration of 250 μg/ml. Value of protein expression was presented as mean fluorescence intensity, corrected by expression of respective isotype controls. Negative control for multidrug resistance proteins was CCRF-CEM cell line, while positive control was adnocarcinoma LoVo-Dx cell line. Negative control for apoptosis proteins was Jurkat cell line.

Statistical analysis. Baseline characteristics of all patients were summarized using descriptive statistics. Comparisons of paired samples were done by Wilcoxon matched pair test. Associations between categorical variables were analyzed using chi-square analysis or Fisher exact test. Confidence intervals around a single proportion were calculated using exact binomial formulas. Survival curves were calculated by Kaplan-Meier method and compared by log-rank test. Cox proportional hazards regression model was used to correlate each potential prognostic factor with a survival in univariate analysis. The factors that appeared to be important were then fitted together, and dropped one at a time in a backward stepwise manner using the likelihood ratio test at a 0.05 level until all factors in the model were significant. A final check was made to ensure that no excluded factors would improve the fit. All tests were 2-sided with p-value of 0.05.

Table 1. Characteristics of patients with ALL

Characteristics	Number of patients (n=60)
Sex (male : female)	30 : 30
Age (median, range) in years	7.8 (0.1 – 17.2)
Initial : relapse	46 : 14
FAB subtype	L1 – 36, L2 – 24
Phenotype	precursor-B-lineage – 51, T-lineage-ALL – 9
BCR-ABL rearrangement	present – 5, absent – 55
Cytogenetics*	good risk – 5, poor risk – 8, standard – 47
PVA score	sensitive – 39, resistant – 21
In vivo prednisolone response (n=33)	good – 27, poor – 6, not done – 27
Bone marrow early response by day 15	M1 – 37, M2 – 11, M3 – 11, not done – 1
Bone marrow response by day 33	M1 – 53, M2 – 4, M3 – 1, not done – 2

Cytogenetics*: good risk was defined as hyperdiploidy over 50 chromosomes, DNA index ≥1.16 and translocation t(12;21). Poor risk included: translocation t(9:22), bcr-abl rearrangement, translocation t(4:11), hypodiploidy below 45 chromosomes, DNA index ≤0.95. Standard risk was all others.

Results

Comparison of exposure to prednisolone and dexamethasone and ex vivo drug resistance. Concentration of prednisolone and dexamethasone was higher than LC50 value, respectively in 32/60 and 57/60 patients in plasma; in 26/60 and 24/60 patients in ECF; while in 25/60 and 20/60 patients in TBF. All patients from control group showed extremely high resistance to both glucocorticoids.

Both for prednisolone and dexamethasone, ECF concentration was higher than LC50 only in 1/14 relapsed patients, and only in 2/14 patients who relapsed during follow-up (Tab. 2) (Fig. 1).

Expression of multidrug resistance and apoptosis proteins. There was no correlation between expression of PGP, MRP1, LRP and risk factors as well as therapy outcome and drug resistance both on diagnosis and after 72 hours of incubation with prednisolone. During *ex vivo* therapy, upregulation of PGP, MRP1 and LRP was observed in five, seven and two patients, respectively. There changes were, however, not correlated with *ex vivo* drug resistance. After prednisolone therapy, expression of anti-apoptotic protein Bcl-2 decreased in 51/60 patients, including 11 with at least >2-fold downregulation. Expression of pro-apoptotic proteins pRb and p53 was upregulated in 50 and 55 patients ($p<0.01$), respectively, during therapy with prednisolone. Values of expression of tested proteins are shown in Table 3.

Disease free survival. Mean follow-up was 17.9 months (95% CI=15.6–20.3). pDFS for all patients was 0.65±0.07; for *de novo* ALL patients pDFS=0.82±0.06 (mean survival 20.8 months, 95% CI=18.6–23.0); in relapsed patients pDFS=0.17±0.11 (mean survival 8.5 months, 95% CI=5.0–12.0). Clinical response to 7-day prednisolone monotherapy (with one dose of intrathecal methotrexate) was assessed in the group of 33 children with initial ALL. For *in vivo* prednisolone good responders, pDFS was 0.963±0.036, while for prednisolone-poor-responders 0.25±0.20 ($p<0.0001$) (Tab. 4) (Fig. 2).

Uni- and multivariate analysis. Factors prognostic by univariate analysis are shown in Table 4. All others analyzed factors, such as gender, initial leukocytosis, Langermann risk factor, expression of PGP, MRP1, LRP, p53 and pRb both on day "0", and day "3" had no predictive value on pDFS. By multivariate analysis, two factors reached statistical significance: early bone marrow response by day 15 (HR=0.54, 95% CI=0.33–0.89, p=0.0122) and ECF concentration of prednisolone higher than LC50 value (HR=0.42, 95% CI=0.20–0.88, p=0.005).

Separate analysis was done for 33 children with initial ALL, for whom *in vivo* response to one-week prednisolone therapy was assessed. Three factors reached value $p<0.1$: resistance to prednisolone monotherapy (HR=4.96, 95% CI=1.63–15.08, p=0.0047), ECF concentration of prednisolone higher than LC50 value (HR=0.45, 95% CI=0.22–0.96, p=0.0196), ECF concentration of dexamethasone higher than LC50 value (HR=0.54, 95% CI=0.32–0.90, p=0.0379). No factor reached significance by multivariate analysis.

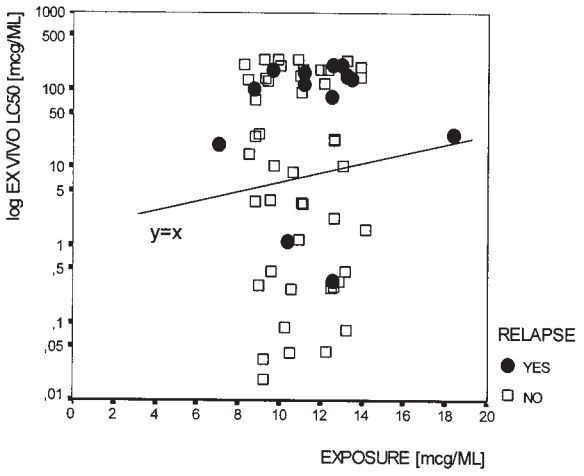

Figure 1. Distribution of prednisolone exposure (calculated as drug dose divided by volume of extracellular fluid) and *ex vivo* resistance of lymphoblasts to prednisolone (expressed by LC50 value obtained by the MTT assay) with respect to relapse of leukemia during follow-up.

Discussion

A number of risk factors analyses in childhood ALL have shown high importance of results of *in vitro* drug resistance

Table 2. Relationship between *ex vivo* drug resistance and *in vitro* prednisolone and dexamethasone concentration in extracellular fluid

Characteristics of patients	Prednisolone	Dexamethasone
Number of patients in whom: concentration > LC50	26/60	24/60
De novo ALL (n=46) vs relapsed (n=14)	25/46 vs 1/14 OR=15.48 p=0.001	23/46 vs 1/14 OR=14.64 p=0.002
Remission (n=46) vs relapse during follow-up (n=14)	24/46 vs 2/14 OR=6.55 p=0.012	22/46 vs 2/14 OR=5.50 p=0.024
Ex vivo sensitivity (n=39) vs resistance of lymphoblasts (n=21), determined by PVA score	22/39 vs 4/21 OR=5.50 p=0.005	20/39 vs 4/21 OR=4.47 p=0.015
In vivo prednisolone good (n=27) vs poor responder (n=6)	19/27 vs 1/6 OR=11.84 p=0.024	16/27 vs 2/6 OR=2.91 ns

OR – odds ratio, p – calculated by Fisher exact test, PVA – combined *ex vivo* drug resistance profile to prednisolone, vincristine and L – asparaginase.

Table 3. Expression of multidrug resistance and apoptosis proteins before and after 72 hours of *ex vivo* therapy with prednisolone

	MFI (untreated cells)	MFI (after prednisolone treatment)
PGP	0.74 (0.17 – 5.45)	0.69 (0.25 – 4.21)
MRP1	0.66 (0.22 – 5.70)	0.72 (0.31 – 2.87)
LRP	0.80 (0.21 – 10.65)#	0.59 (0.21 – 2.38)#
Bcl-2	2.22 (0.30 – 4.48)#	1.47 (0.47 – 3.00)#
p53	1.15 (0.70 – 12.95)#	2.00 (1.00 – 11.65)#
pRb	1.00 (0.40 – 4.76)#	1.36 (0.93 – 6.63)#

Median values and range of mean fluorescence intensity (MFI), corrected by isotype control are shown. Value MFI=1 indicates expression of negative control. (#) – $p<0.01$ calculated by Wilcoxon matched pair test.

Table 4. Risk factors positive for disease-free-survival by univariate analysis in the group of 60 children with ALL

Factor	Characteristics	Estimated 2-years pDFS	p	HR (95%CI)
Age	Age 2 – 10 years vs	0.82±0.07 vs	0.0229	
	< 2 years vs	0.0±0.0 (<2 years)		2.52 (1.19–5.33)
	> 10 years	0.57±0.12 (>10 years)		2.38 (1.18–4.55)
bcr-abl rearangement	Presence of bcr-abl fusion	0.70±0.07 vs 0.0±0.0	0.0922	1.61 (0.86–3.02)
Early bone marrow response (day 15)	Bone marrow M1 by day 15	0.85±0.06 vs 0.50±0.12	0.0085	0.51(0.32–0.85)
Remission by day 33	Bone marrow M1 by day 33	0.70±0.07 vs 0.20±0.18	0.0173	0.51 (0.29–0.89)
Combined ex vivo drug sensitivity profile to prednisolone, vincristine and L-asparaginase	PVA score ≤6	0.795±0.07 vs 0.43±0.12	0.0096	0.53 (0.33–0.85)
In vitro resistance to prednisolone (PRN)	LC50 PRN >100μg/ml	0.77±0.09 vs 0.50±0.10	0.0132	1.86 (1.14–3.04)
In vitro resistance to dexamethasone (DX)	LC50 DX >5μg/ml	0,75±0,08 vs 0,45±0,12	0.0340	1.54 (1.04–2.15)
Relation between ex vivo sensitivity and concentration of prednisolone in extracellular fluid (ECF)	Maximum prednisolone concentration achievable in ECF higher than LC50 value	0.84±0.12 vs 0.60±0.09	0.0150	0.40 (0.19–0.84)
Relation between ex vivo sensitivity and concentration of dexamethasone in ECF	Maximum dexamethasone concentration achievable in ECF higher than LC50 value	0.85±0.10 vs 0.58±0.09	0.0272	0.43 (0.20–0.91)
Decrease of Bcl-2 expression	At least 50% decrease of Bcl-2 expression after 3 days of ex vivo therapy with prednisolone	0.74±0.06 vs 0.33±0.17	0.0835	0.65 (0.40–1.05)

HR – hazard ratio

[4, 28–29]. In the present study, relationship between *ex vivo* sensitivity to prednisolone and dexamethasone, and maximum achievable concentration of these drugs in body fluids of children with acute lymphoblastic leukemia was analyzed. In presented mathematical model, results of *ex vivo* drug resistance were combined with clinical possibilities of delivering the drug dose, which is able to exert antileukemic effect. We have shown that patients in whom calculated exposure to glucocorticoids was higher than the respective value of *ex vivo* drug resistance, had better therapy outcome. Results obtained both for prednisolone and dexamethasone were similar, however statistical significance was higher for factors related to prednisolone. In control group of patients with normal bone marrow cells, *ex vivo* resistance to glucocorticoids was extremely high, while calculated achievable maximal concentrations of tested drugs in body fluids were far lower.

Mathematical model, constructed for this study, was based on the observation of PETERSEN et al that predicted and observed plasma concentration of prednisolone after oral administration in childhood ALL had presented very strong

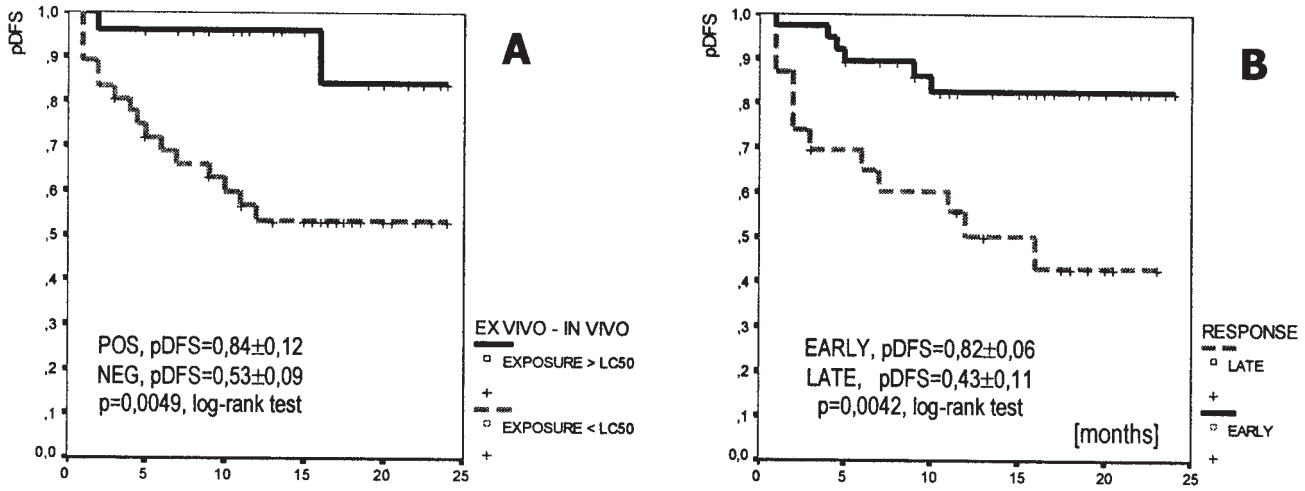

Figure 2. Probability of disease-free-survival (pDFS) in ALL patients related to (A) relationship between *ex vivo* drug resistance and *in vitro* prednisolone concentration in extracellular fluid, (B) early bone marrow response by day 15.

correlation due to complete absorption and bioavailability of this drug [30]. Thus, prednisolone concentration in extracellular fluid is related to the dose of drug delivered to the body. Systemic clearance of prednisolone, normalized for body surface area, is not related to sex, age, initial white blood cell count, risk group and body weight in population study of pharmacokinetics of this agent in children with acute lymphoblastic leukemia [30]. PETERSEN et al also showed that plasma protein binding was independent of the albumin concentration. They concluded that due to the small inter-patient and intra-individual variations in the pharmacokinetic parameters, body surface area-based dosing is sufficient to obtain similar systemic exposure among patients [30], what is used in clinical practice in commonly adopted treatment protocols.

DEN BOER et al [4] reported that combined *in vitro* drug resistance profile to prednisolone, vincristine and L-asparaginase (PVA score) can be used for stratification of children with ALL, and PVA score can predict early and very early relapses. *In vitro* drug resistance seems to be a factor with predictive value for identifying patients at higher risk of leukemia-related events within 2.5 years after diagnosis [4]. Persistence of minimal residual disease is probably a mechanism responsible for development of late relapses. Though we have shown that relationship between *in vitro* drug resistance to glucocorticoids and maximal achievable concentration in body fluids has prognostic value, we were not able to compare this factor with the impact of gene-expression profile and minimal residual disease, which are being currently of great interest [8, 31, 32]. Nonetheless, the *in vitro* drug resistance to prednisolone, vincristine, L-asparaginase and daunorubicin is strongly correlated with gene-expression profile in childhood ALL and with therapy outcome, as it has been presented in recently published reports by groups from Rotterdam and Memphis [28, 33].

In conclusion, we have shown that *in vitro* resistance to glucocorticoids is a strong adverse prognostic factor in childhood acute lymphoblastic leukemia, however the value of this variable depends on drug concentration achievable in body fluids. Factor showing that calculated drug concentration is higher than *ex vivo* drug resistance, easily determinates a group of patients with unfavorable prognosis. This group of patients should be identified as being at high risk of treatment failure and who, therefore, may benefit from more intensive treatment already during the initial phase of therapy.

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