Cytotoxicity of Cortivazol in Childhood Acute Lymphoblastic Leukemia

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Abstract. Background: Glucocorticoids are the most important group of drugs used in the treatment of childhood acute lymphoblastic leukemia (ALL), however, resistance to this group remains the main obstacle in curing the disease. One of the possibilities to circumvent glucocorticoid resistance is the use of new compounds, such as cortivazol (CVZ), which has two binding sites for the glucocorticoid receptor. Aim: Analysis of ex vivo sensitivity to cortivazol and other glucocorticoids in childhood acute lymphoblastic leukemia, as well as the relationship to anticancer therapy outcome. Patients and Methods: Leukemic samples from 60 children with ALL were tested by the MTT assay for glucocorticoid resistance. Cell cycle before and after ex vivo glucocorticoid treatment was analyzed by flow cytometry. Results: Although all tested glucocorticoids presented significant cross-resistance, CVZ showed high antileukemic activity. The equivalent activity of CVZ was 165-fold higher than prednisolone, 7.5-fold higher than dexamethasone and 2.8-fold higher than betamethasone. CVZ showed relatively better cytotoxicity than other glucocorticoids in prednisolone-poor-responders. CVZ, like other glucocorticoids, caused cell cycle arrest in the G1-phase, and increased the percentage of apoptotic cells to a greater extent than other glucocorticoids. The results of antileukemic therapy were strongly related to the ex vivo resistance to all tested glucocorticoids. Conclusion: Cortivazol has potent antileukemic activity in childhood ALL. Its activity is related to cell cycle arrest and induction of apoptosis.

Glucocorticoids are the most important agents used in the therapy of acute lymphoblastic leukemia (ALL) in children (1, 2). Resistance to this group of drugs is regarded as a one of the strongest prognostic factors, both in in vivo (3, 4) and in vitro conditions (5, 6).

The mechanisms of antileukemic glucocorticoid activity are related, in general, to activation of the glucocorticoid receptor (2, 7), heat shock proteins (2, 8), transcription factors (NF-ÎB, AP1) (9), transactivation or transrepression of genes (10) and induction of apoptosis (10). Most investigations on the antileukemic activity of glucocorticoids have been carried out on prednisolone and dexamethasone. A number of clinical studies in childhood ALL were also based on methylprednisolone (11) and high dose of dexamethasone (12).

Cortivazol (CVZ, RU 3625, Figure 1) is a pyrazolosteroid with two binding sites for the glucocorticoid receptor (13, 14), which induces the nuclear translocation and transactivation function of the glucocorticoid receptor, but not of the mineralocorticoid receptor. CVZ interacts with the distinct portion of the ligand binding domain (LBD) and differentially modulates the ligand-dependent interaction between transcription intermediary factor 2 and the LBD when compared with cortisol, dexamethasone and aldosterone (15). CVZ showed significant activity in a glucocorticoid-receptor-deficient cell line (16) and resistance to extrusion by P-glycoprotein (17). It has been shown that CVZ was more active in vitro than dexamethasone (13) and betamethasone (18) in cell lines, thus suggesting its promise against childhood ALL (14). Juneja et al. (19) have shown that CVZ was successfully used in ex vivo purging before autologous stem cell transplantation.

Since most previous studies were performed on cell lines, the in vitro activity of CVZ in childhood de novo and relapsed ALL samples was analyzed here. The impact of the tested glucocorticoids on the cell cycle was also analyzed. We found that CVZ has potent antileukemic activity and might have good cytotoxic profile in prognostically unfavorable patients.

Patients and Methods

Patient samples. Fresh bone marrow samples from the day of first diagnosis of ALL or relapse were taken from 60 children aged 0.1-17.5 years. The patient characteristics are shown in Table 1.
Leukemic cells were isolated on Ficoll gradient. Only samples which contained at least 90% lymphoblasts on initial diagnosis and at least 80% at relapse were included in the study. Morphology was based on the French-American-British criteria. According to immunophenotype, 51 children were classified as pre-B-lineage, and 9 as T-lineage. Cytogenetics was done by G-banding analysis. The DNA index was calculated by Multicycle software. Children at first diagnosis were treated by the New York II protocol (n=13), or by the ALL-BFM-90 protocol (n=33) (20, 21). For patients treated by BFM-based protocols, prednisolone in vivo response after 7 days of monotherapy (with one dose of intrathecal methotrexate) was determined: those who had less than 1000 blasts per µl in peripheral blood were diagnosed as prednisolone good responders (PGR); otherwise, as prednisolone poor responders (PPR). Relapsed patients were treated according to the ALL-BFM-REZ-96 protocol (n=14).

**Chemicals.** The following glucocorticoids and concentrations were tested: 0.0212–694 µM prednisolone (Fenicort, Jelfa, Jelenia Gora, Poland), 0.5 nM – 15.3 µM dexamethasone (Dexaven, Jelfa, Jelenia Gora, Poland), 0.5 nM – 15.3 µM betamethasone (Bedifos, Jelfa, Jelenia Gora, Poland), 0.094 nM – 9.43 µM cortivazol (Altim, Hoechst Marion Roussel/Aventis, Swindon, UK).

The MTT viability assay. Cytotoxicity was measured by a viability and cell proliferation assay by measuring the ability of the cells to cleave the soluble compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT; Serva, Heidelberg, Germany) into an insoluble salt. The ability of cells to cleave MTT is indicative of the degree of mitochondrial/cellular respiration within those cells. The conditions of the assay were similar to those described previously (22). Briefly, cells were incubated for 3 days with various concentrations of glucocorticoids in 96-well plates. Ten µl of MTT (5 µg/ml) were added to each well. The reaction was stopped after 4-h incubation by adding 100 µl of 0.04 N HCl in isopropanol, and the optical density (OD) was measured at 550 nm (reference wavelength 720 nm) with a Multiscan Bichromatic plate reader (Asys Hitech GmbH, Eugendorf, Austria) and DigiWin software (Asys Hitech). All experiments were performed in triplicate, and the data were confirmed to be reproducible. The cytotoxicity was expressed as LC50, i.e., lethal concentration for 50% of cells.

**Cell cycle analysis.** For DNA content analysis, cells were stained with hypotonic propidium iodine solution (20 µg/ml, DNA-Prep Kit, Lot number PN 6607055, Coulter, Miami, FL, USA) and 20000 events were analyzed with an Epics XL flow cytometer (Coulter) after 24-h incubation. This flow cytometer is equipped with an argon laser with an excitation wavelength of 488 nm. The cell cycle was calculated by Multicycle software (Phoenix Flow Systems, San Diego, CA, USA). The percentage of cells in the G1-, G2- and S-phases were expressed as mean ± s.d.

**Statistics.** Differences in drug sensitivity were compared by Mann-Whitney and Kruskal-Wallis tests. Correlations in resistance between groups were determined by Spearman’s rho coefficient. The Wilcoxon matched-pairs signed ranks test was used to compare changes in cell cycle phases in treated and untreated cells. The probability of disease-free survival (pDFS) was calculated by the Kaplan-Meier method, and differences between curves were compared by log-rank test. Multivariate analysis was performed by the Cox regression model in a backward stepwise manner using the likelihood ratio test at a 0.05 level until all factors in the model were significant. All tests were two-sided with p<0.05 regarded as significant.

**Results**

**Ex vivo sensitivity of lymphoblasts to glucocorticoids in childhood ALL samples.** Lymphoblasts at relapse showed higher resistance to all glucocorticoids, with relative resistance...
ranging at least 11 to 23-fold for each compound (Table II). Equivalent doses of glucocorticoids in de novo ALL were as follows: CVZ:BET:DX:PRN = 1 : 2.8 : 7.5 : 165.

Cells of prednisolone-poor-responders (PPR) were more resistant than cells of prednisolone-good-responders (PGR): median 5-fold to prednisolone, 3-fold to dexamethasone and betamethasone, and 1.6-fold to cortivazol. PPR had a higher WBC count (155 G/L vs 34.4 G/L, \( p = 0.035 \)) than PGR.

Common-ALL samples have shown a trend towards better sensitivity to: prednisolone in comparison to pro-B samples (median 4.1-fold) and T-ALL (median 1.3-fold); to dexamethasone (1.5-fold and 1.3-fold, respectively, ns) and to betamethasone (3-fold and 1.8-fold, respectively, ns). No differences in ex vivo sensitivity to cortivazol between immunophenotype subgroups were observed.

A highly significant cross-resistance was observed between the tested glucocorticoids in patients with ALL. As the results obtained on cell lines, for each pair of drugs Spearman’s rho value was over 0.8 and \( p \) value was less than 0.001 (Table III).

**Analysis of glucocorticoid cell cycle arrest in patient samples.** Both on day “0”, and after 24 hours, cell cycle phases did not differ between untreated ALL cells. After 24 hours, the percentage of cells undergoing spontaneous apoptosis, presented as sub-G1-phase, increased in all patients (\( p < 0.001 \), Wilcoxon matched-pair test) (Figure 2). The apoptotic phase, expressed as sub-G1-phase, was significantly higher for CVZ than for all other tested glucocorticoids (\( p < 0.001 \) in each case). Each of the tested glucocorticoids caused cell cycle arrest in G0/G1-phase in

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**Table II. Sensitivity to glucocorticoids in ALL initial and relapsed samples.**

<table>
<thead>
<tr>
<th></th>
<th>ALL de novo</th>
<th>Relapsed ALL</th>
<th>RR</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRN</td>
<td>29.7 (0.5-&gt;694)</td>
<td>390.5 (25-610.5)</td>
<td>13.1</td>
<td>0.015</td>
</tr>
<tr>
<td>DX</td>
<td>1.35 (0.0005-&gt;15.3)</td>
<td>&gt;15.3 (0.07-&gt;15.3)</td>
<td>&gt;11.3</td>
<td>0.003</td>
</tr>
<tr>
<td>BET</td>
<td>0.51 (0.0007-&gt;15.3)</td>
<td>11.7 (0.087-&gt;15.3)</td>
<td>22.9</td>
<td>0.001</td>
</tr>
<tr>
<td>CVZ</td>
<td>0.18 (0.0002-&gt;9.4)</td>
<td>3.11 (0.004-&gt;9.4)</td>
<td>17.3</td>
<td>0.003</td>
</tr>
</tbody>
</table>

The value of sensitivity is expressed as median and range of LC50 values, given in \( \mu M \); RR - relative resistance, calculated as median LC50 value for relapsed samples divided by median LC50 value for de novo samples; \( p \)-value - calculated by Mann Whitney U-test.

**Table III. Correlation matrix of LC50 values for tested glucocorticoids.**

<table>
<thead>
<tr>
<th></th>
<th>PRN</th>
<th>DX</th>
<th>BET</th>
<th>CVZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRN</td>
<td>0.893</td>
<td>&lt;0.001</td>
<td>0.882</td>
<td>0.867</td>
</tr>
<tr>
<td>DX</td>
<td>0.893</td>
<td>0.915</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BET</td>
<td>0.882</td>
<td>0.915</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CVZ</td>
<td>0.867</td>
<td>0.893</td>
<td>0.932</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Upper value - Spearman’s rho; lower value - level of significance (\( p \)).

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**Figure 2. Cell cycle analysis in childhood ALL samples.** (A) Histograms of DNA content and cell cycle arrest after 72 hours of therapy ex vivo. (B) Percentage of cells in G1-phase. (C) Percentage of cells in sub-G1-phase. Each graph shows (from the left), effect in untreated cells (K) and treated with P-prednisolone, D-dexamethasone, B-betamethasone and C-cortivazol.
ALL (p≤0.001, for each compound). Cytotoxicity correlated with the percentage of cells in sub-G1-phase (Figure 3).

Relationship between cellular drug resistance and antileukemic therapy. With cut-off values of 275 μM for prednisolone, 2 μM for cortivazol, 12 μM for dexamethasone and 5 μM for betamethasone, we divided all the tested samples as sensitive or resistant to each respective agent. The impact of \textit{ex vivo} glucocorticoid cellular resistance on pDFS was significant for each of the four agents tested (Table IV). The prognostic significance of cellular resistance to each of the four glucocorticoids was further analyzed in the Cox model. By univariate analysis, sensitivity to each of the glucocorticoids had a significant value (except borderline value for dexamethasone), with \textit{ex vivo} sensitivity to cortivazol being the strongest factor (Table IV). No factor reached significance by multivariate analysis.

Discussion

Glucocorticoids are the most important group of drugs used in the therapy of ALL in children, however, cellular resistance of lymphoblasts to these drugs remains one of the main obstacles in successful therapy. Cortivazol is a glucocorticoid with two binding sites for the glucocorticoid receptor, and is regarded as a potent drug with anti-inflammatory properties. In this study, we compared the antileukemic activity of cortivazol, prednisolone, dexamethasone and betamethasone, as well as their impact on apoptosis induction and cell cycle arrest. We have shown that cortivazol has potent antileukemic properties, and is approximately 165-fold more active than prednisolone and 7.5-fold than dexamethasone in childhood ALL. We have also shown that betamethasone was 2.6-fold more potent in antileukemic activity than dexamethasone. In a few cases, betamethasone was also active against cells resistant either to prednisolone or dexamethasone. All these comparisons were made for childhood initial ALL samples, which are relatively more sensitive to glucocorticoids, even if marked interindividual differences were observed.

Cross-resistance between the tested glucocorticoids was also observed in cell cycle analysis. The apoptotic phase, expressed by sub-G1-phase, was highest after CVZ \textit{ex vivo} therapy in comparison to other glucocorticoids, while cell cycle arrest caused by CVZ was comparable to other compounds.

It is believed that resistance to glucocorticoids plays a role in the etiology and course of various diseases such as autoimmunologic disorders, AIDS, Nelson syndrome, sclerosis multiplex and leukemias (23). It has been shown that primary (hereditary) abnormalities in the glucocorticoid receptor gene make 6.6% of the normal population relatively 'hypersensitive' to glucocorticoids, while 2.3% are relatively 'resistant' (24). These abnormalities might explain the well-known phenomenon that some individuals develop severe adverse effects during therapy with a low dose of glucocorticosteroids, while others do not develop side-effects even during long-term therapy.

Table IV. Estimated 2-year pDFS and univariate analysis in ALL patients with respect to sensitivity vs resistance to each of four glucocorticoids.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimated 2-year pDFS(*)</th>
<th>HR (95%CI)(#)</th>
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<tbody>
<tr>
<td>\textit{Ex vivo} prednisolone sensitivity</td>
<td>0.77±0.09 vs 0.50±0.10, p=0.0075</td>
<td>1.86 (1.14-3.04), p=0.0132</td>
</tr>
<tr>
<td>\textit{Ex vivo} cortivazol sensitivity</td>
<td>0.75±0.08 vs 0.45±0.12, p=0.0034</td>
<td>1.90 (1.19-3.03), p=0.0069</td>
</tr>
<tr>
<td>\textit{Ex vivo} dexamethasone sensitivity</td>
<td>0.71±0.08 vs 0.53±0.12, p=0.0486</td>
<td>1.57 (0.98-2.50), p=0.0594</td>
</tr>
<tr>
<td>\textit{Ex vivo} betamethasone sensitivity</td>
<td>0.76±0.10 vs 0.52±0.10, p=0.0226</td>
<td>1.71 (1.04-2.80), p=0.0317</td>
</tr>
</tbody>
</table>

(*) Analyzed by Kaplan-Meier method and log-rank test.
(#) HR-hazard risk, given with 95% confidence interval in univariate analysis.
therapy with a much higher dose (24). In childhood initial ALL, about 10% of children are regarded as prednisolone-poor-responders to initial therapy, and this rate is much higher at relapse (3, 25). Kaspers et al. have shown that glucocorticoid in vitro resistance in childhood leukemia, as measured by the MTT assay, is related to both clinical and cell biological features, and to the clinical outcome after multi-drug chemotherapy (26).

In vivo sensitivity to glucocorticoids is thought to be one of the most important prognostic factors in various therapeutic protocols for children with ALL. Several reports have shown that prednisolone resistance, expressed as absolute number of blasts in peripheral blood over 1 g/L in eight days of prednisolone monotherapy, is the strongest single adverse prognostic factor (3, 4, 27). It has been shown previously that therapy with high doses of prednisolone (1 g/day) did not improve the remission rate, but was related to serious adverse effects (28). In a study by Schwartz et al., it was shown that response of ALL to glucocorticoid therapy increased with dose (12). Higher-dose corticosteroid treatment abrogated the effect of relative drug insensitivity and of low glucocorticoid receptor number on peripheral blasts. This was presented with doses of dexamethasone of 18 or 150 mg/m²/day. In two recent series of ex vivo studies, it was shown that a combined resistance profile to prednisolone, vincristine and L-asparaginase was the strongest independent prognostic factor (29, 30).

Our study, based on clinical material, determined an in vitro cross-resistance pattern between prednisolone, dexamethasone, betamethasone, and cortivazol in samples of childhood acute leukemia, using the MTT assay. In summary, we have shown that cortivazol cannot circumvent leukemia, using the MTT assay. In summary, we have shown that cortivazol cannot circumvent glucocorticoid resistance in samples of childhood acute leukemia. Adv Exp Med Biol 277: 615-619, 1999.

Acknowledgements

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References


