Combination of prednisolone and low dosed dexamethasone exhibits greater *in vitro* antileukemic activity than equiactive dose of prednisolone and overcomes prednisolone drug resistance in acute childhood lymphoblastic leukemia

Michaela Spenerova[a,b,#], Petr Dzubak[a,b,#], Josef Srovnal[a,b], Lenka Radova[a], Renata Burianova[a], Petr Konecny[a], Sona Salkova[a], Zbynek Novak[b], Dagmar Pospisilova[b], Jan Stary[c], Bohumir Blazek[d], Jiri Hak[e], Tomas Votava[f], Pavel Timr[g], Emilia Kaiserova[h], Eva Bubanska[i], Vladimir Mihal[a,b], Marian Hajduch[a]

Introduction. Glucocorticoids, particularly prednisone/ prednisolone and dexamethasone, play a prominent role in the treatment of pediatric patients with acute lymphoblastic leukemia due to their ability to induce apoptosis in susceptible cells. Current therapeutic protocols use prednisone for both the prophase and the induction phase of the therapy because the greater antileukemic activity of dexamethasone is compromised by its high frequency of serious adverse reactions.

Aim. To compare, for the first time, the in vitro antileukemic activity of prednisolone alone to that of a combination of prednisolone and dexamethasone using dexamethasone at a very low and presumably safe dosage (1/50 w/w).

Methods. Lymphoblasts were isolated from bone marrow and/or blood samples from children with newly diagnosed acute lymphoblastic leukemia. The cytotoxic activity of prednisolone, dexamethasone and the prednisolone/dexamethasone combination against isolated leukemia cells was analyzed using the MTT cytotoxicity assay.

Results. We observed differences in the in vitro antileukemic activity of prednisolone and dexamethasone in 21% of the tested patients. 3% of the children were prednisolone sensitive but dexamethasone resistant, while 18% were prednisolone resistant and dexamethasone sensitive. 32% were sensitive to both glucocorticoids and 18% were resistant to both. Cells from patients with good *in vivo* responses to prednisone monotherapy were more responsive to prednisolone in vitro than were cells from patients with poor prednisone responses ($P<0.07$). Importantly, we demonstrated that the use of even a minimal dose (1/50 w/w) of dexamethasone with prednisolone dramatically increases the in vitro anti-leukemic activity of prednisolone ($P<0.0006$).

Conclusion. The high inter-individual variability of acute lymphoblastic leukemia responses to glucocorticoids suggest that either patients should be selected for prednisone or dexamethasone treatment on the basis of predictive biomarkers or that prednisone should be used directly in combination with a very low and safe dose of dexamethasone to potentiate its antileukemic activity. The latter option is likely to be cheaper and more efficient, and therefore warrants further clinical investigation to assess its efficacy and safety in treating childhood acute lymphoblastic leukemia.

[a]*Laboratory of Experimental Medicine, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University Olomouc and University Hospital Olomouc, Czech Republic*
[b]*Department of Pediatrics, Faculty of Medicine and Dentistry, Palacky University Olomouc and University Hospital Olomouc*
[c]*Department of Pediatric Haematology and Oncology, University Hospital Motol, Prague*
[d]*Department of Pediatrics, University Hospital in Ostrava*
[e]*Department of Pediatrics, University Hospital in Hradec Kralove*
[f]*Department of Pediatrics, Faculty Hospital in Pilsen*
[g]*Department of Pediatrics, Hospital in Ceske Budejovice*
[h]*Department of Pediatric Oncology, University Hospital in Bratislava, Slovak Republic*
[i]*Department of Pediatric Oncology and Hematology, Children's Medical University Department, Banska Bystrica, Slovak Republic*
[#]*These authors equally contributed to the work*
Corresponding author: Marian Hajduch, email: marian.hajduch@fnol.cz

INTRODUCTION

Glucocorticoids (GCs), and especially prednisone/ prednisolone (PRED) and dexamethasone (DEX), play an important role in the treatment of pediatric patients with acute lymphoblastic leukemia (ALL) because of their ability to induce apoptosis in susceptible cells[1]. GCs enter the cell by passive diffusion and bind to the glucocorticoid receptor (GR), which is localized in the cytoplasm and forms complexes with chaperone molecules such as heat-

shock proteins 70 and 90 (hsp 70 and hsp 90). The binding of GCs to the GR causes the chaperone proteins to dissociate. GR homodimers are then translocated into the nucleus where they interact with glucocorticoid response elements to induce gene transcription (transactivation) or interact with transcription factors (notably, activating protein-1 or AP-1) and nuclear factor κB (NFκB). The homodimers also interact with the c-*myc* proto-oncogene, which is involved in cell cycle regulation and proliferation and plays a causal role in cell survival. The expression of c-*myc* inhibits apoptosis and induces cell cycle arrest. These mechanisms lead to inhibition of cytokine production, alteration of oncogene expression, cell cycle arrest and programmed cell death[2].

The clinical significance of the GC response in ALL was first reported by Riehm in 1983, who introduced routine clinical evaluations of the "*prednisone response*" during the first week of PRED monotherapy as an independent prognostic factor in children with ALL. In approximately 90% of patients treated with prednisone, the number of blast cells in the peripheral blood decreases rapidly to below 1×10^9 / L by day 8 of the treatment program. Patients who respond in this way are said to exhibit a prednisone good response (PGR). Such patients have more favorable prognosis than those with a poor prednisone response (PPR) (ref.[3]).

A large number of publications have reported a correlation between the *in vitro* corticoid responses of leukemic lymphoblasts and *in vivo* responses to PRED monotherapy[4]. This includes our report published in 1999 which focused on differences in the antileukemic activities of PRED and DEX *in vitro* as assessed by the MTT cytotoxicity assay[5]. We found significant correlations between the cytotoxic activities of PRED and DEX for 69 patients undergoing treatment for ALL. However, the leukemic cells isolated from 30% of the ALL children exhibited different *in vitro* responses to PRED and DEX ($P<0.01$); 14% of the tested patients were PRED-sensitive but DEX-resistant, while 16% were PRED-resistant but DEX-sensitive[6].

Unfortunately, the clinical and laboratory studies published to date have only examined the anti-leukemic activities of PRED and DEX administered separately or consecutively, even though their pharmacological properties make it possible to apply them together as a combination therapy. In theory, the combination of the two should be effective in the 30% of patients who show resistance to one of the PRED/DEX pair but not the other. To test this hypothesis, we examined the *in vitro* cytotoxic responses of leukemia cells isolated from ALL children to PRED, DEX and a combination therapy (PRED&DEX) consisting of the two compounds in a 50:1 mass ratio, administered in parallel. The antileukemic activity of DEX in the PRED&DEX combination was calculated in PRED equivalents ($PRED_{EQ}$) with 1 mg of DEX being equivalent to 6.67 mg of PRED, as is done in most comparative clinical studies[7].

MATERIALS AND METHODS

Fresh peripheral blood/bone marrow samples were obtained from patients with ALL at primary diagnosis. Patients were characterized according to gender, age, and early response to prednisone, as well as the immunological, cytogenetic and molecular characteristics of their lymphoblasts. The bone marrow samples were obtained from the patients at the time of diagnosis before the PRED induction therapy (i.e. in day 0). Samples were provided by cooperative pediatric oncohematological centres in the Czech Republic (Prague, Ostrava, Hradec Králové, Plzeň, České Budějovice and Olomouc) and the Slovak Republic (Bratislava, Banská Bystrica) with the patients'/ parent's informed consent. The study was approved by the Ethics Committee of Palacky University and University Hospital in Olomouc.

To analyze the *in vitro* responses of leukemia cells to PRED, DEX and the PRED&DEX combination, we used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously[5,6]. Briefly, viable leukemia cells were isolated from bone marrow samples by gradient centrifugation and isolated lymphoblasts were incubated with PRED (prednisolone dinatrium-phosphate, Netherland), DEX (dexamethasonii natrii phosphas, Medochem Ltd.) or the PRED&DEX combination at 37 °C under a humidified atmosphere containing 5% CO_2 for 72 h in 96-well plates. The maximum tested drug concentrations were 242.4 µg/mL for PRED, 6 µg/mL for DEX, and 125&2.5 µg/mL for the PRED&DEX combination, respectively. The ratio of PRED:DEX in the combined treatment (50:1) was chosen because it corresponds approximately to the ratio of the median *in vitro* cytotoxic concentrations for the two drugs *in vitro*[5,6]. After 72 h incubation *in vitro*, MTT was added to the cell cultures; viable cells reduce the soluble MTT to insoluble blue formazan crystals. The crystals were dissolved in a solution of 10% SDS (sodium dodecylsulphate) in water (vol/vol) and a microplate reader (Labsystems iEMS Reader MF Chemorezist) was used to determine the solution's absorption at 540 nm, the magnitude of which is proportional to the number of surviving cells. The concentration of each drug or combination required to inhibit the survival of 50% of the leukemia cells (LCS_{50}; µg/mL) was calculated using the Chemorezist software package[8].

The activities of PRED and DEX in individual patients were expressed in terms of their LCS_{50} values. The activity of the PRED&DEX combination was expressed in terms of PRED equivalents ($PRED_{EQ}$), where 1 mg of DEX is equivalent to 6.67 mg of PRED. This is the approach taken in most comparative clinical studies[9,10]. Patients were classified as being drug sensitive or resistant based on the criteria used in our previous publications: LCS_{50} values <12/0.15 µg/mL indicated PRED or DEX sensitivity, while LC_{50} values equal to or below this threshold indicated resistance[5].

Comparisons between groups were made using the Mann-Whitney U test and Wilcoxon paired tests. The Spearman correlation coefficient was used to determine the correlations. Probability values <0.05 were considered

statistically significant. All statistical analyses were performed using the Statistica 8 software package (StatSoft, Inc.).

RESULTS

We examined 62 children with acute lymphoblastic leukemia as their primary diagnosis. Of these, 57 suffered from pre-B cell ALL and 5 from T-cell ALL. The age at diagnosis ranged from 6 months to 18 years; the median was 6 years. The patient population was predominantly male (64.6% boys; 35.4% girls). Molecular (cyto)genetics revealed that 9 of the leukemias expressed TEL/AML1, 3 were BCR/ABL positive, and 1 patient tested positive for the MLL/AF4 fusion gene. Hyperdiploidy, defined as the presence of >50 chromosomes per cell, was found in 16 patients. A prednisolone good response was observed in 56 patients; the remaining 6 did not respond well to prednisolone monotherapy and were classified as poor responders. Bone marrow/peripheral blood samples were obtained at diagnosis via our collaboration with the Pediatric Onco-Hematology Centers in the Czech and Slovak Republics over the period of June 2009 - November 2011. Table 1. summarizes the characteristics of the patient cohort.

We examined the *in vitro* responses of leukemia cells isolated from 62 bone marrow and/or blood samples from children with ALL. Successful tests of the *in vitro* response to glucocorticoids were performed using samples from all 62 patients for PRED. In addition, 57 samples were tested against DEX and 52 against the combination of PRED&DEX.

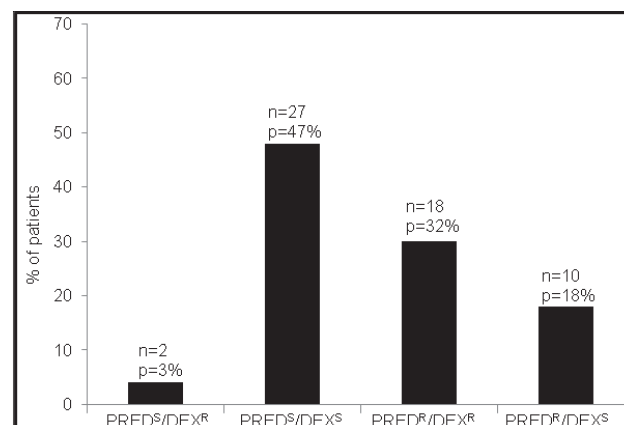In keeping with our previously published results[5], we observed different levels of antileukemic activity for the two drugs in 21% (12/57) of the tested patients: 3% (2/57) of the children were PRED sensitive but DEX resistant, while 18% (10/57) were PRED resistant and DEX sensitive. 47% (27/57) of the children were sensitive to both glucocorticoids and 32% (18/57) were resistant to both (Fig. 1.).

Cells from patients with good *in vivo* responses to PRED monotherapy exhibited greater sensitivity to PRED *in vitro* (median LCS_{50}=2.37 μg/mL) than those from patients with poor PRED responses (median LCS_{50}= 242.4 μg/mL). This difference was on the borderline of statistical significance for the number of patients examined ($P<0.07$, Fig. 2.).

We also compared the in vitro antileukemic activities of PRED, DEX and the PRED&DEX combination (50:1 ratio). The antileukemic activity of DEX in the PRED&DEX combination was calculated in PRED equivalents ($PRED_{eq}$), where 1 mg of DEX is considered equivalent to 6.67 mg of PRED (ref.[11]). The results are reported as pair-wise comparisons between the LCS_{50} values for PRED alone and the $PRED_{eq}$ values for the combination of PRED&DEX (Fig. 3.). The median LCS_{50} values for PRED, DEX and PRED&DEX were 19.6, 0.11 and 5.66 μg/mL, respectively, demonstrating that the PRED&DEX combination generates a substantially and significantly more pronounced *in vitro* response than does PRED alone. It was found that when administered in tandem with PRED, even a minimal dose (1/50 w/w) of DEX yields significantly increased antileukemic activity ($P<0.0006$).

DISCUSSION

Cure rates for child patients with ALL have improved dramatically over the last few decades. However, there are still approximately 10-15% of patients who do not respond to or do not tolerate complex chemotherapy and die due to disease progression and/or serious adverse reactions to intensive treatment. One of the most important risk factors in childhood ALL is the patient's response to PRED

Table 1. Clinical and laboratory characteristics of children with ALL enrolled in the study.

Patients	62
Age at diagnosis; range/median	0.5-18 (6)
Sex Female/Male ratio	22/40
Early response to PRED	
PGR	56
PPR	6
Immunophenotype	
PBC-ALL	57
T-ALL	5
Molecular genetics/cytogenetics	
TEL/AML1+	9
BCR/ABL+	3
MLL/AF4+	1
hyperdiploidy>50	16

Fig. 1. Antileukemic activity of PRED versus DEX under *in vitro* conditions in 57 children with ALL determined using the MTT assay. The figure shows the percentage of samples that are sensitive ([s]) or resistant ([R]) to individual glucocorticoids *in vitro*.
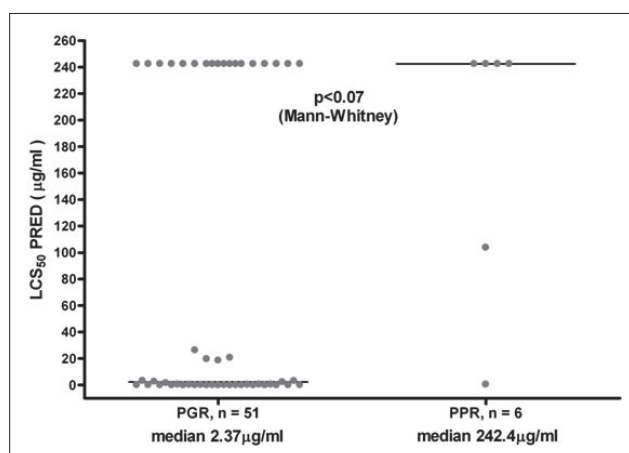
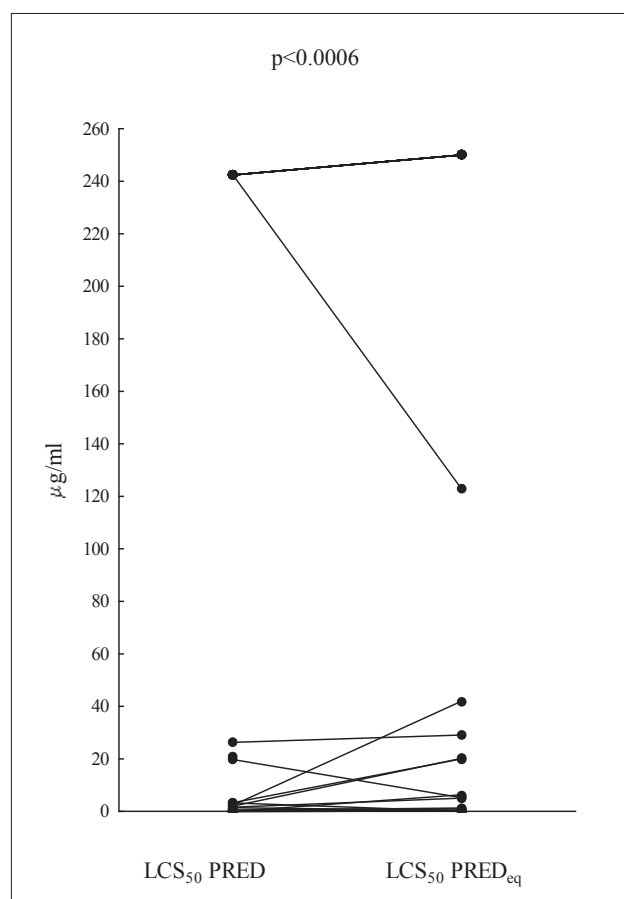|	n	Median	25% quantile	75% quantile
PGR	51	2.37	0.048	242.4
PPR	6	242.4	52.1	242.4

Fig. 2. Leukemia cells isolated from patients with poor *in vivo* responses to PRED (PPR) are also less responsive to the drug *in vitro* than are cells from individuals with good responses to PRED (PGR).

monotherapy during the first week of treatment. It has been shown that PRED poor responders benefit from more intensive chemotherapy[12], which usually involves replacing PRED with DEX (ref.[13]).

Two of the most important glucocorticoids used for treating childhood ALL are PRED and DEX. Although DEX consistently shows higher antileukemic activity both in vitro and *in vivo*[4,15-17], PRED is used in most chemotherapy protocols due to its better toxicity profile. DEX administration is associated with higher risk of myopathy, adverse neuro-psychiatric events, osteonecrosis, sepsis, fungal infections, diabetes and pancreatitis[7].

Several studies, including ours[4-6,16], have shown that leukemia cells from different patients can exhibit different responses to PRED and DEX. However, the development of personalized glucocorticoid therapies based on the in vitro response of leukemia cells to individual glucocorticoids is clinically challenging because of time constrains (MTT assay would delay onset of therapy for minimum 4 days). Additional problems arise from the difficulties associated with inter-laboratory standardization and quality control of cytotoxic assays since it is often necessary to transport samples between labs and/or employ cryo-preservation[5].

Following these initial in vitro reports, Schrappe et al.[18] initiated a clinical trial involving 3655 children, with the aim of comparing sequential administrations of PRED and DEX to treatment with PRED alone for the treatment of ALL. All children were pretreated with PRED for 7 days during the prophase of the trial and then randomized to either the PRED arm of the trial (in which they received a dosage of 60 mg/m²) or the DEX arm (10 mg/m²) during the induction phase. For a median follow-up

	n	median	25% quantile	75% quantile
LCS_{50} PRED (µg/mL)	63	18.6	0.059	242.4
LCS_{50} DEX (µg/mL)	58	0.11	0.007	0.35
LCS_{50} $PRED_{eq}$ (µg/mL) (PRED & DEX in combination)	52	5.77	0.08	250.0

Fig. 3. Antileukemic activity of PRED alone versus PRED&DEX in combination, expressed in terms of LCS50 for PRED or PREDeq for combination with DEX as described in the Materials and Methods. Treatment with low dosages of DEX in conjunction with PRED (1:50) dramatically increased in vitro potency relative to PRED alone.

time of 4.4 years, the 6-year event-free survival rate (6y-EFS) for individuals from the DEX group was 84.1% while that for the PRED group was 79.1% (*P*=0.0083). The 6-year cumulative incidence (CI) of relapse was 11% for the DEX group and 18% for the PRED group (*P*<0.001). More specifically, differences between the two groups were found in terms of isolated bone marrow relapses (8% versus 12%), CNS-relapses (2% versus 4%) and other relapses (2% versus 3%). Patients treated with DEX also experienced more adverse events due to toxicity: the CI for death during induction was 2.0% for DEX but only 0.9%

for PRED (*P*=0.003). Patients from the DEX group also experienced a greater number of severe but non-fatal toxicities, mostly due to infection. More detailed analyses revealed that the CI of relapse for patients treated with DEX was significantly lower for individuals who had T-ALL or TEL/AML1-positive or –negative precursor B-ALL. The reduction was most pronounced in T-ALL patients with good prednisone response after the prophase: the CI of relapse in DEX-treated patients from this group (n=135) was only 6%, compared to 20% for PRED-treated patients (n=138; *P*=0.003). In TEL/AML1-positive patients with good prednisone response, the CI for relapse was 4% in the DEX group and 13% for the PRED treated patients (*P*<0.001). In conclusion, although treatment with DEX at the same dosage as applied in delayed intensification (10 mg/m^2/d for 3 weeks) presented a greater risk of severe toxicity, it also significantly reduced the risk of relapse, yielding significant benefit in terms of event-free survival. This was most evident in patients with *in vivo* sensitivity to the prednisone prophase; the efficacy of DEX in patients who responded poorly was not convincing[18].

Although Schrappe et al.[18] and other authors (systematically reviewed in Teuffel et al.[19]) have clearly demonstrated that sequential replacement of DEX with PRED in the induction phase of therapy is highly beneficial in terms of decreasing the risk of disease recurrence, it is still not clear whether sequential or concomitant administration of both glucocorticoids would better eliminate leukemic cells resistant to DEX but sensitive to PRED and vice versa. We therefore conducted the study reported here to compare the in vitro antileukemic activity of PRED to that of PRED&DEX administered in tandem, using DEX at a very low and thus presumably clinically safe dosage. Our previous study and the data presented in this work demonstrate the high inter-individual variability of ALL responses to glucocorticoids, and suggest that either patients should be selected for PRED or DEX treatment on the basis of predictive biomarkers or that PRED should be administered in tandem with a very low and safe dosage of DEX. The latter option is likely to be a lot more clinically convenient and probably more efficient as well. The combination of PRED with low-dose DEX exhibited much greater *in vitro* potency than PRED alone (3.16 times, *P*<0.0006, Fig. 3.), suggesting that the combination of PRED and DEX merits further clinical investigation to assess its efficacy and safety in the treatment of childhood ALL.

ACKNOWLEDGMENTS

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