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Comparison of the Antileukemic Activity In Vitro of Dexamethasone and Prednisolone in Childhood Acute Lymphoblastic Leukemia

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It is generally assumed that prednisolone (PRD) and dexamethasone (DXM) have equal glucocorticoid activity if PRD is given at sevenfold higher doses. Results of clinical studies of childhood acute lymphoblastic leukemia (ALL) suggested that DXM is more potent relative to PRD than assumed. The purpose of this study was to determine the relative antileukemic activity of PRD phosphate and DXM phosphate in 133 untreated childhood ALL samples in vitro, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. There was a marked variation in antileukemic activity of both agents among the patient samples. The median LC50 (drug concentration lethal to 50% of the ALL cells) for PRD phosphate was 3.50 μM , for DXM phosphate 0.20 μM . The individually calculated ratios of the LC50 values for PRD

and DXM phosphate showed a large range from 0.7 to >500, with a median of 16.2. This 16-fold difference could not be explained by differences between these glucocorticoids in stability, hydrolysis into unesterified drug, adhesion to the wall of the microculture plates, or protein binding. ALL cells were cross-resistant to PRD and DXM phosphate (correlation coefficient = 0.85, $P < 0.000001$).

We conclude that the in vitro antileukemic activity of DXM phosphate is median 16-fold higher than that of PRD phosphate, which contrasts to the generally assumed factor of 7. Based on the higher potency of DXM, and its more favorable pharmacokinetics as reported in the literature, DXM may be preferred to PRD as the glucocorticoid in the treatment of ALL. © 1996 Wiley-Liss, Inc.

Key words: leukemia, childhood, antileukemic activity, glucocorticoids, prednisolone, dexamethasone

INTRODUCTION

Glucocorticoids are effective drugs in the treatment of acute lymphoblastic leukemia (ALL). Response rates to single-agent glucocorticoid treatment range from 75–90% in newly diagnosed childhood ALL [1]. Prednisolone (PRD) is the most frequently used glucocorticoid, but few studies addressed the question whether other glucocorticoids, such as dexamethasone (DXM), might be of more value. It is assumed that DXM is seven times more potent than PRD, or in other words, PRD and DXM would have equal antileukemic activity if PRD is given at sevenfold higher doses. However, this assumption is not based on a comparison of the antileukemic activity of both agents, but rather on their antiinflammatory and thymolytic activities [2]. Results of two clinical studies showed that treatment results with DXM as part of a combination chemotherapy for leukemias were better than those with PRD, although “equivalent” doses were used [3,4]. The pharmacokinetics of DXM seem to be more favorable than those of PRD [5–7]. It may also be that DXM is more potent than is assumed.

The purpose of the present study was to compare the

in vitro antileukemic activity of PRD and DXM phosphate in childhood ALL, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. We also studied several aspects of their in vitro behavior—stability, hydrolysis, adhesion to plastic, and protein binding—to minimize the possibility of differences in the relative antileukemic activities of these glucocorticoids caused by artefacts. The MTT assay is an objective and reliable cell culture drug resistance assay suited for large-scale testing of leukemia and lymphoma samples [8,9]. Using this assay, we previously reported significant corre-

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lations between the in vitro antileukemic activity of PRD with the clinical response to a prednisone monotherapy [10], and with the clinical outcome after combination chemotherapy in childhood ALL [11].

MATERIALS AND METHODS

Samples and Patients

Bone marrow (BM) and peripheral blood (PB) samples as well as smears from children with newly diagnosed non-B ALL were sent by local institutions to the laboratory of the Dutch Childhood Leukemia Study group (DCLSG) for confirmation of diagnosis, classification according to the FAB criteria [12], and immunophenotyping [13]. Mononuclear cells were isolated by Ficoll density gradient centrifugation (Ficol Paque; density 1.077 g/ml; Pharmacia, Sweden). Until October 1, 1991, immunofluorescence was used for terminal deoxynucleotidyl-transferase (TdT) and surface immunoglobulin heavy chain, while an indirect immunoperoxidase staining technique on cytocentrifuge preparations was used for all other antibodies. From October 1, 1991 onwards, immunoperoxidase staining was replaced by flow cytometry after calibrating these techniques.

Samples from 159 patients were sent by the DCLSG laboratory to the research laboratory for pediatric hemato-onco-immunology of the Free University Hospital in Amsterdam for drug resistance testing, with informed consent. Samples from 133 (84%) of the 159 children with non-B ALL were successfully tested. A minority ($n = 60$) of these samples was included in a preliminary meeting report [14]. Twenty-six assays could not be evaluated because of infection ($n = 1$), laboratory error ($n = 1$), insufficient cells in the sample submitted ($n = 3$), percentage of leukemic cells below 70% after 4 days of culture ($n = 9$), and failure of ALL cells to reduce MTT into formazan in the drug-free control wells ($n = 12$).

The median age of the 74 male and 59 female patients was 5 years (range 0–15 years). The white blood cell count at diagnosis ranged from 2.5 to $900 \times 10^9/l$ (median $25.8 \times 10^9/l$). Three cases could not be classified morphologically, while 103 cases were diagnosed as FAB type L1 and 27 as FAB L2. Of 132 immunophenotyped ALL cases, 6 were pro-B ALL (positive for TdT, CD19, and HLA-DR), 97 were common- or pre-B ALL (positive for TdT, CD19, and HLA-DR, and positive for CD10 or cytoplasmic μ), and 29 T-ALL (positive for TdT, CD3, and CD7).

Reagents and Drugs

PRD and DXM sodium phosphate and acidified isopropanol were obtained from the hospital pharmacy, which purchased the glucocorticoids from Hyocint (Oss, The Netherlands). PRD phosphate was dissolved in saline,

TABLE I. Antileukemic Activity of PRD and DXM Phosphate In Vitro in Untreated ALL Samples.

	LC50 values (μM) and individual ratios		
	Median	Range	n^a
PRD phosphate	3.50	<0.13 to >4167	130
DXM phosphate	0.20	<0.0005 to >15.3	114
Individual ratios of LC50 PRD/DXM	16.2	0.7 to >500	74

^aBoth drugs were tested successfully in 111 samples, but individual ratios of the LC50 for PRD phosphate divided by the LC50 for DXM phosphate could not be calculated in 37 cases, because one ($n = 21$) or both ($n = 16$) LC50 values were outside the concentration ranges tested (if both, always at the same end of the ranges).

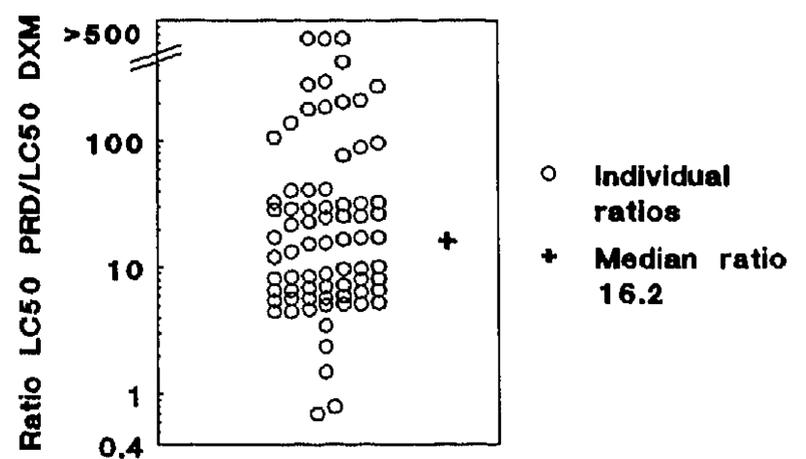


Fig. 1. Ratio of LC50 values for PRD and DXM phosphate in 74 untreated childhood ALL samples.

whereas DXM phosphate was obtained in solution in ampules as used in patients. Solvents for DXM phosphate were sodium pyrosulfate (1 mg/ml), sodium edetate (0.5 mg/ml), glycerol 85% (0.15 ml/ml), and sodium hydroxide 2N (2 $\mu\text{l/ml}$). These solvents were not cytotoxic nor did they influence the background of the culture medium in these concentrations.

Cells were cultured in RPMI 1640 (Dutch modification, Gibco, Uxbridge, UK) containing 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 0.125 $\mu\text{g/ml}$ fungizone, 200 $\mu\text{g/ml}$ gentamycin, all obtained from Flow Laboratories (Irvine, UK), and 5 $\mu\text{g/ml}$ insulin, 5 $\mu\text{g/ml}$ transferrin, and 5 ng/ml sodium selenite, obtained from Sigma. MTT was also obtained from Sigma.

MTT Assay

This cell culture drug resistance assay was started within 36 hours after collection of the sample. BM and PB samples were evaluated together, because they do not differ in drug resistance [15]. All samples tested with the MTT assay contained $\geq 80\%$ leukemic cells (as percentage of all viable cells) at the start of the assay, and results were considered evaluable in case of $\geq 70\%$ leukemic cells after culture. In addition, the minimum mean optical density (OD) of the six control

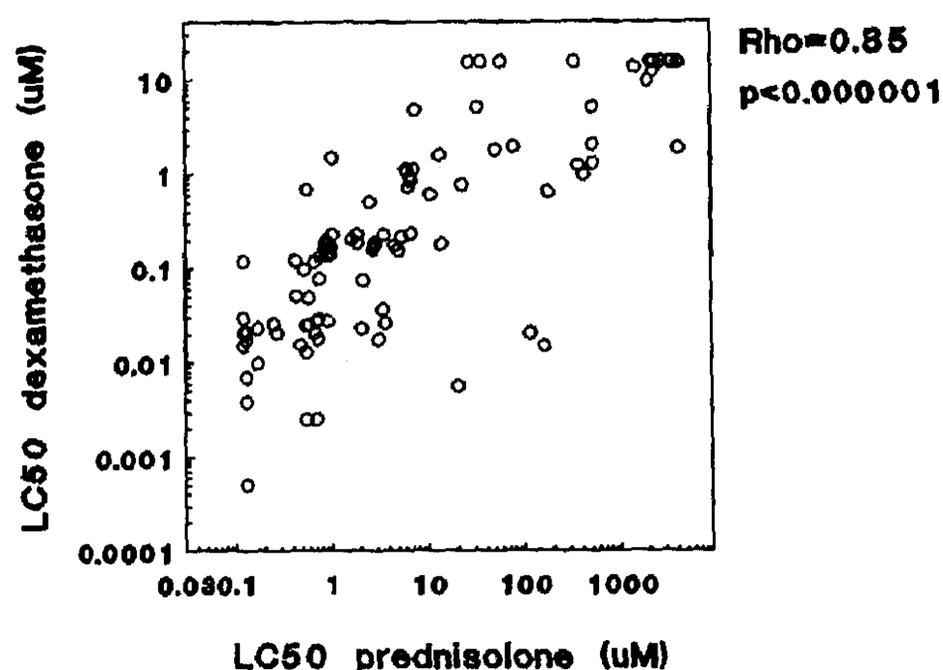


Fig. 2. Correlation between LC50 values for PRD and DXM phosphate in untreated childhood ALL samples.

TABLE II. Some Aspects of the In Vitro Behavior of PRD and DXM in Our Assay System

	PRD	DXM
Phosphates		
Functionally stable after storage of at least 6 months	Yes	Yes
Hydrolysis into unesterified glucocorticoid	10–15%	10–15%
Unesterified		
Adhesion to polystyrene	No	No
Protein binding	22.2%	21.6%

wells (see later) was 0.050. These criteria ascertain reliable assay results [8,16].

The MTT assay was performed at the research laboratory for pediatric hemato-onco-immunology of the Free University Hospital. An 80 μ l cell suspension (2×10^6 cells/ml) was added to 20 μ l of the drug solutions in 96-well (12 columns, 8 rows) microculture plates. These plates had been prepared before use and were stored at -20°C for not longer than 6 months. Each drug was tested in duplicate in six concentrations, which ranged from 0.13 μM to 4.2 mM for PRD (eightfold dilutions) and from 0.5 nM to 15.3 μM for DXM (also eightfold dilutions). These concentrations include clinically achievable peak and steady state plasma levels. Because evaporation had been observed from the outer wells, these were filled with RPMI only. Thus, columns 1 and 12 and rows 1 and 8 were not used. Columns 2 and 3, 6 and 7, 8 and 9, and 10 and 11 contained four different drugs, with duplicates of all concentrations on the same row, and with the higher concentrations in the upper rows, and the lower concentrations in the lower rows. Columns 4 and 5 contained in the upper six wells culture medium only to assess the background color of the medium, and in the lower six wells cells in medium without drugs to determine the control cell survival. The plates were incubated

in humidified air containing 5% CO_2 for 4 days at 37°C . Then 10 μ l MTT solution (5 mg/ml) was added and after shaking the plates until the cell pellet was resuspended, they were incubated for 6 hours. The formazan crystals formed were dissolved with 100 μ l acidified isopropanol. The OD of the wells, which is linearly related to the cell number [17], was measured with an EL-312 microplate spectrophotometer (Bio-tek Instruments Inc., Winooski, USA) at 562 nm. After correction for the background color of the medium, leukemic cell survival (LCS) was calculated by the equation: $\text{LCS} = (\text{OD treated well}/\text{mean OD control wells}) \times 100\%$, followed by averaging the two measurements at each concentration. The LC50, the drug concentration lethal to 50% of the cells, was used as the measure of resistance, and was calculated using the two concentrations from the dose-response curve which gave an LCS just above and below 50%.

In Vitro Behavior of PRD and DXM Phosphate

Functional stability of PRD and DXM phosphate was studied by repeated testing of cryopreserved cells from the same patients, using stock solutions which had been stored at -20°C between 2 months and 3 years, and in separate experiments using plates with these drugs in the wells stored at -20°C for 1–6 months.

Hydrolysis of PRD and DXM phosphate into unesterified glucocorticoid in culture medium as described above was studied at the laboratory of the Department of Endocrinology of the Free University Hospital in Amsterdam. The concentrations of PRD and DXM phosphate, and unesterified PRD and DXM, were determined without prior extraction by high-performance liquid chromatography (HPLC) and ultraviolet (UV) detection (254 nm), both before and after the 4 days of culture. The four compounds were separated completely, using an injection volume of 100 μ l; column C18 reversed phase microsphere 3 μm , 50×4.6 mm (Chrompack, The Netherlands); mobile phase methanol:water:acetic acid (40:58:2), 0.01 M tetrabutylammoniumsulphate, pH = 4.

Protein binding and adhesion to polystyrene—the material from which the microculture plates are made—were studied at the laboratory of the Department of Experimental and Chemical Endocrinology of the St. Radboud University Hospital in Nijmegen, using an equilibrium dialysis method as previously described [18]. For these experiments, tritiated unesterified PRD (Amersham TRK-691, 2.44 Ci/mmol) and DXM (Amersham TRK-417, 1.70 TBq/mmol) were used, and measurements were done in duplicate. Protein binding was measured after an incubation of 30 minutes, and adhesion to polystyrene after an incubation of up to 6 hours (times at which a plateau had been reached). These experiments were performed in culture medium as described above.

TABLE III. A Summary of Studies About the Relative Activities of PRD and DXM

Reference	Model	Effect	Activity of DXM/PRD
22	Fibroblasts	Growth inhibition	4
23	Human	Antirheumatic	8
24	Rat	Antiinflammatory	48
		Thymolysis	48
25	Thymus cells	Induction of pyknosis in vitro	11
26	Lymphocytes	Inhibition of PHA-induced stimulation	10
27	Rat	Depletion of glucocorticoid receptor	10
28	Lymphocytes	Immunosuppression in vitro	4
29	Rat	Antiinflammatory	39
		Thymolysis	12
		Glycogenesis	18
30	Human	Pituitary-adrenal function suppression	16
31	Mice	Inhibition of tumor induction and promotion	67
32	Rat	Glycogenesis	36
		Antiinflammatory	68
33	Human	Pituitary-adrenal function suppression	40
34	Rat	Antiinflammatory	240
		Thymolysis	69
		Adrenal suppression	218
		Glycogenesis	90
35	Rheumatoid synovial tissue	Prostaglandin production	3
36	Rat	Glycogenesis	6
		Antiinflammatory	60
37	Mouse thymocytes	DNA fragmentation	100
38	Mice	Antiinflammatory	5
	Mice	Thymolysis	26
	Mice	Eosinopenia	3
	Dogs	Eosinopenia	5
	Rat	Diuretic	10
39	Rat	Antiinflammatory	12
	Rat	Thymolysis	20
40	Rat	Dermal atrophy	4
	Rat	Antiinflammatory	10

Statistics

The Mann-Whitney U test for paired and unpaired data and the Spearman's rank correlation test (parameter, Rho) were used for two-tailed testing at a level of significance of $P = 0.05$.

RESULTS

MTT Assay

For 133 successful assays, the percentage leukemic cells in the control wells was median 94% at the start of the culture, and 89% at the end of the 4-day incubation period (percentages of the total number of viable cells). The coefficient of variation of the six control wells after 4 days of culture was median 5.2% (range 0.9–15.3%). The intra-assay (duplicates) and interassay (repeated testing of same frozen sample) variation in LC50 values was less than a factor of 5 for both PRD and DXM phosphate, which is well within one dilution factor of 8.

Relative Antileukemic Activity of PRD and DXM Phosphate

For both drugs, the LCS was generally dose related, but a wide concentration range (4-log) was necessary to obtain dose-response curves. The dose-response curves tended to be more steep with DXM, and plateaus in the antileukemic activity were more often seen with PRD. However, because of a very strong cross-resistance ($Rho > 0.95$) between LC50 values and area under the dose-response curves for both drugs, the LC50 was used as the well-known parameter. Table I shows the median and ranges of LC50 values, which were known for both drugs in 111 cases. For each drug, the LC50 values differed more than 1,000-fold between patients. Based on the group median LC50 values, DXM phosphate had a 17-fold higher antileukemic activity than PRD phosphate. Table I also shows the median and range of the individual ratios of the LC50 values for these glucocorticoids, which could be calculated for 74 individual samples (shown in Fig. 1). DXM phosphate was 16.2-fold more potent than

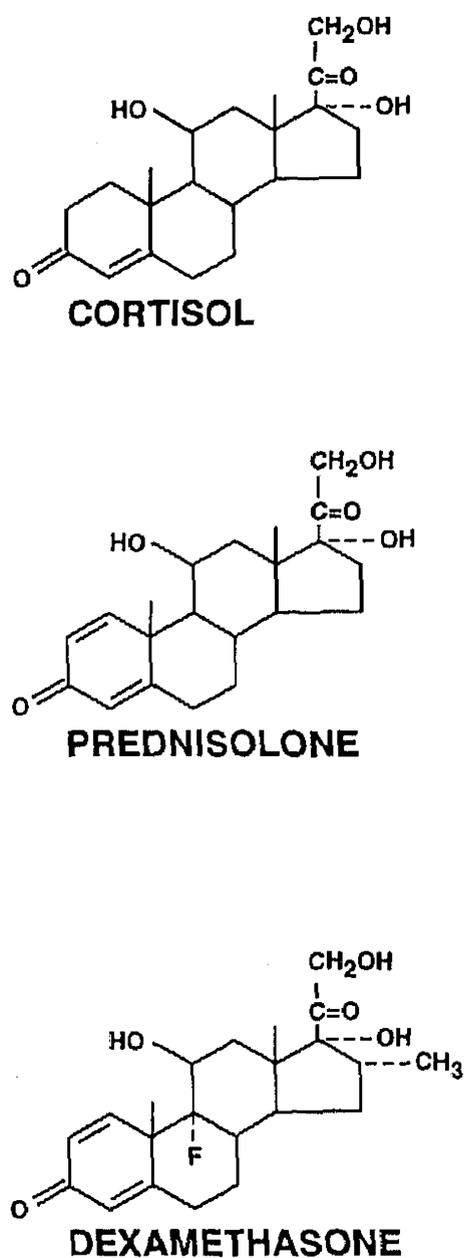


Fig. 3. Chemical structures of cortisol and of the synthetic glucocorticoids PRD and DXM.

PRD phosphate based on these individual numbers. In 16 (22%) of 74 patients this ratio was even more than 50. An absolute ratio could not be calculated in 16 cases because both LC50 values were outside the concentration ranges used (always at the same end of these ranges), and not in 21 cases because one LC50 value was outside the concentration range used. Including the latter group in the analysis (a ratio of >190 was included as 190) did not influence the results: median ratio 15.8, range 0.7–>500. Clinical and cell biological features were not significantly related to the ratio of LC50 values for PRD and DXM (not shown).

ALL cells were highly significantly cross-resistant to PRD and DXM phosphate (Fig. 2). The correlation coefficient for the LC50 values for both agents was 0.85 ($P < 0.000001$). This pattern was also found within several subgroups distinguished by FAB- or immunophenotype (not shown).

In Vitro Behavior of PRD and DXM Phosphate

PRD and DXM phosphate were functionally stable over the time period of 3 years studied. Thus, the antileukemic activity of both drugs did not decrease in time when stored in stock solutions or in microculture plates.

After 4 days of culture at 37°C, the glucocorticoid phosphates had been hydrolyzed into their corresponding unesterified forms to an extent of 15% at most. No difference in this percentage was found between PRD and DXM phosphate or between incubation with and without cells.

There was no significant adhesion of unesterified PRD or DXM to polystyrene after incubation up to 6 hours, as was demonstrated by the unchanged radioactivity of the tritiated glucocorticoids detected in the culture medium. Protein binding of unesterified PRD and DXM, at concentrations in the nanomolar or lower range, in culture medium as described above was mean 22.3% for PRD (duplicates 22.0 and 22.5%) and 21.6% for DXM (duplicates 21.2 and 22.0%). In the same medium, but without fetal calf serum, this binding was only 8.0% for PRD and 10.1% for DXM. Table II summarizes the results with respect to the in vitro behavior of PRD and DXM.

DISCUSSION AND CONCLUSIONS

Single-agent glucocorticoid treatment in newly diagnosed ALL results in a response rate of approximately 80%. At present, PRD is the most frequently used glucocorticoid in the first-line treatment of ALL, but few studies have compared the antileukemic activity of PRD with that of other glucocorticoids, such as DXM. Recently, results of two studies which addressed this question have been reported. PRD and DXM were used at what is generally considered equivalent doses, 40 mg/m² and 6 mg/m² daily, respectively. In a randomized study, Jones et al. [3] reported a significant reduction in central nervous system (CNS) relapses, but not in BM relapses, when DXM was substituted for PRD in the treatment of childhood ALL. Veerman et al. [4] reported the results of the Dutch ALL-VI protocol in which DXM was used, but which was preceded by a pilot study in which the only difference was the use of PRD instead of DXM. With DXM, the complete remission rate was higher and less relapses were observed both in the BM and in the CNS. The toxicity of DXM seemed to be more pronounced than that of PRD.

One explanation for the better CNS results with DXM has been provided by Balis et al. [5], who reported that in monkeys DXM had more favorable cerebrospinal fluid (CSF) pharmacokinetics than PRD. The half-life of DXM in the CSF was 1.5-fold longer, and the CSF:plasma ratio for DXM was 2-fold higher than for PRD. In addition, the plasma half-life of DXM is longer than that of PRD [19–22]. In summary, the pharmacokinetics of DXM are more favorable than those of PRD [5–7].

An additional explanation for the better results with DXM, investigated in the present study, may be that DXM has a higher cellular antileukemic activity compared to that of PRD than is generally assumed. Indeed, we found

that the antileukemic activity of DXM phosphate in vitro was 16-fold higher than that of PRD phosphate, which contrasts to the generally assumed factor of 7. We studied several aspects of the in vitro behavior of glucocorticoids, in order to minimize the possibility of the contribution of in vitro artefacts to this ratio of 16 (Table II). No differences between the two glucocorticoids were observed.

An extensive literature study did not reveal the source of the assumption that equivalent doses of PRD are seven times higher than those of DXM in the treatment of ALL. Apparently, the factor of 7 has been extrapolated from studies in which PRD and DXM were compared regarding other effects, such as their antiinflammatory or thymolytic potential. However, even these studies, summarized in Table III, do not support this factor of 7 [23–41]. Ratios from 3 up to 240 were found in various systems, mostly studying animals rather than human subjects. DXM was often more than sevenfold more potent than PRD.

It is unknown why DXM is more potent than PRD. These glucocorticoids are synthetic analogs of cortisol, and their molecules differ in only a few, although important, aspects (Fig. 3). Several authors reported that the glucocorticoid receptor of leukemic cells has a higher affinity for DXM than for PRD [42–45], but this affinity has also been reported to be similar [46] or even higher for PRD [47]. Ichii et al. [48] reported that the DXM-receptor complex was more stable than the PRD-receptor complex. The difference in potency between DXM and PRD might also be partly explained by a direct relation between the specific steroid nested in the steroid-receptor complex and events occurring at the postreceptor level [49].

We found that untreated ALL cells were significantly cross-resistant to PRD and DXM. This pattern was also found in our studies in childhood relapsed ALL and in childhood acute nonlymphoblastic leukemia [50,51]. Therefore, DXM should probably not be considered as a glucocorticoid which might circumvent PRD resistance. A more promising drug in this respect is cortivazol, a glucocorticoid which binds the glucocorticoid receptor at two sites, while PRD and DXM bind at only one site. A human leukemic cell line has been described which was resistant to DXM, but not to cortivazol [52].

In conclusion, the in vitro antileukemic activity of DXM phosphate was 16-fold higher than that of PRD phosphate in childhood ALL, with marked interindividual differences. Of course, our in vitro study does not consider several clinically important pharmacokinetic aspects of these drugs and therefore cannot give the final answer to the question whether DXM is more potent than assumed. However, two clinical studies in childhood ALL showed that DXM, at so-called equivalent doses, gave better treatment results than PRD. We suggest that DXM is to be preferred to PRD as a glucocorticoid in the treatment of

ALL, because of its higher potency, and because of its more favorable pharmacokinetics. However, a greater antileukemic activity of DXM could be associated with increased toxicity and increased drug interactions. Clinical studies are warranted to address the important question: Which glucocorticoid should be preferred in the treatment of ALL?

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