Hydrocortisone does not influence glucocorticoid sensitivity of acute lymphoblastic leukemia cells

Dexamethasone is the preferred glucocorticoid in the treatment of pediatric acute lymphoblastic leukemia (ALL), as its anti-leukemic activity *in vitro* is 7- to 16-fold higher than that of prednisolone[1] and it is associated with a higher event-free survival than prednisone.[2] However, serious neuropsychological side-effects have been described in 5%-75% of children with ALL during treatment with dexamethasone, as reflected by obsession with food, sleeping disorders, and by mood, cognition and behavioral problems, sometimes even resulting in psychosis and depression.[3,4] This may have a tremendous effect on patient quality of life during the two years of ALL treatment.[3]

These neuropsychological side-effects may be caused by binding of dexamethasone to the glucocorticoid receptor (GR) expressed in brain tissues.[4] However, recent studies imply that the mineralocorticoid receptor (MR) is important in the regulation of mood, behavior and sleep.[5,6] Cortisol can bind to both the MR and the GR, although cortisol has a 10-fold higher affinity for the MR.[5] In contrast, dexamethasone does not bind to the MR and its potency to activate the GR is 30- to 40-fold higher than that of cortisol.[7] Similarly, prednisolone has a higher binding-affinity (aprox. 5-fold) for GR compared to MR.[7] Recent data suggest that the MR in dexamethasone-treated patients is not fully saturated by endogenous cortisol.[7] This is caused by a reduced production of cortisol due to the fact that dexamethasone triggers a negative feedback-loop affecting the hypothalamus-pituitary-adrenal axis.[7] Studies in animals and small case series of patients with depression suggest that a reduced level of cortisol has serious effects on mood, behavior and sleep.[5,6] Based on these findings, we hypothesize that dexamethasone-induced depletion of cortisol in the brain may cause or exacerbate the neuropsychological side-effects in children suffering from ALL. It is, therefore, feasible that administration of hydrocortisone (i.e. the naturally occurring cortisol now used as medicine) may reduce the neuropsychological side-effects associated with dexamethasone treatment by circumventing this negative feedback-loop through direct activation of MR-mediated signaling as shown in *Online Supplementary Figure S1*. To enable the clinical application of such an intervention strategy, however, an absolute prerequisite is that MR activation does not interfere with the anti-leukemic efficacy of glucocorticoids (dexamethasone and prednisolone). To this aim, we examined the MR and GR levels and the effect of hydrocortisone on the cytotoxicity induced by dexamethasone and prednisolone in leukemic cell lines and freshly obtained ALL cells of children with newly diagnosed ALL.

The mRNA levels of MR and GR expressed in leukemic patients' cells were estimated by probeset 205259_at for MR and 232431_at for GR from a previously published set of gene expression data generated using Affymetrix U133 plus 2.0 gene arrays.[8,9] These levels were confirmed by RTqPCR (*Online Supplementary Appendix*). The genetic subtype of each patient was determined by means of FISH, RT-PCR and by utilizing a 110-probeset gene expression signature which enables the classification of ALL in (cytogenetic) subtypes.[8] A methyl-thiazol-tetrazolium salt drug cytotoxicity assay (MTT-assay) was used to select cases being either *in vitro* highly sensitive, intermediate resistant or highly resistant to prednisolone, using exactly the same cut-off levels as previously reported.[9] These lethal concentrations for 50% of the cells (LC50) have been shown to be predictive for clinical outcome of children with newly diagnosed ALL.[9-11]

There was no difference in mRNA levels of MR and GR between glucocorticoid sensitive, intermediate and resistant patients' ALL cells. Interestingly, the *ETV6-RUNX1*$^{+}$ subtype expressed higher MR mRNA levels than the other ALL subtypes ($P \leq 0.001$), although expression levels were still relatively low (Figure 1A). RT-qPCR analysis of MR and

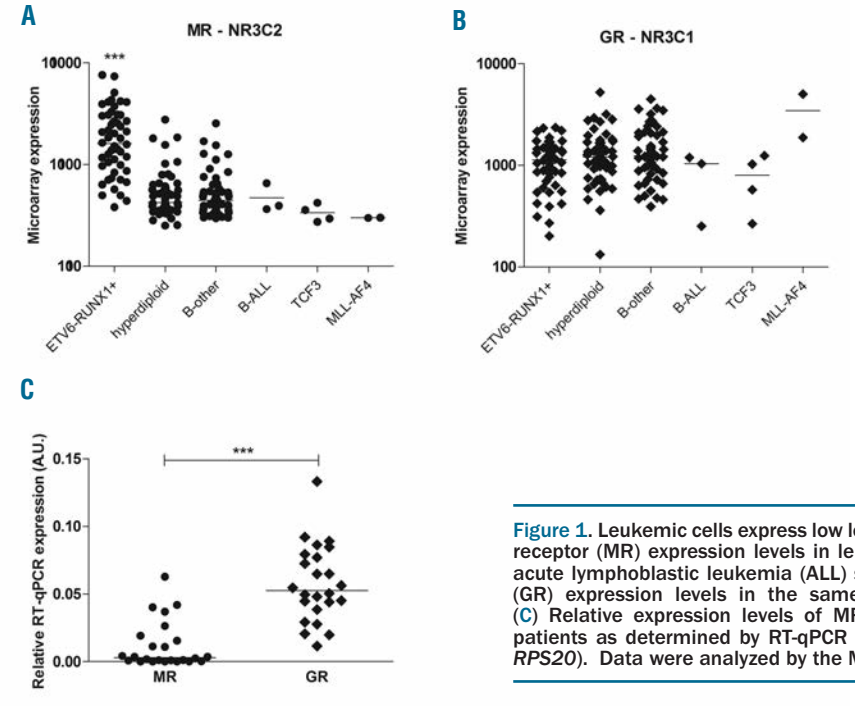

Figure 1. Leukemic cells express low levels of the MR. (A) Mineralocorticoid receptor (MR) expression levels in leukemic cells of different cytogenetic acute lymphoblastic leukemia (ALL) subtypes. (B) Glucocorticoid receptor (GR) expression levels in the same leukemic cell samples as in (A). (C) Relative expression levels of MR and GR in leukemic cells of ALL patients as determined by RT-qPCR (compared to the input-control gene *RPS20*). Data were analyzed by the Mann-Whitney U test (***$P \leq 0.001$).

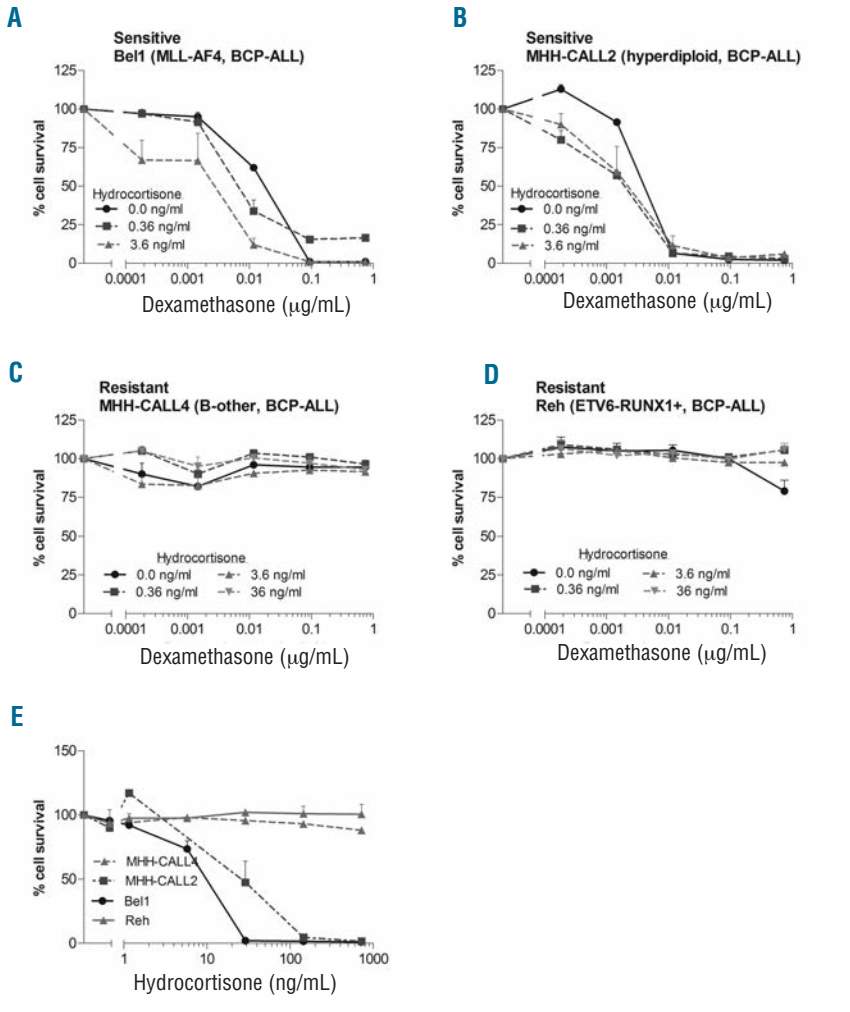

Figure 2. Hydrocortisone does not induce resistance to glucocorticoids in leukemic cell lines. (A) Cytotoxicity of hydrocortisone in combination with dexamethasone in a glucocorticoid-sensitive Bel1 cell line. (B) Cytotoxicity of hydrocortisone in combination with dexamethasone in a glucocorticoid-sensitive MHH-CALL2 cell line. (C) Cytotoxicity of hydrocortisone in combination with dexamethasone in a glucocorticoid-resistant MHH-CALL4 cell line. (D) Cytotoxicity of hydrocortisone in combination with dexamethasone in a glucocorticoid-resistant Reh cell line. (E) Hydrocortisone dose-response curve of glucocorticoid-sensitive (Bel1, MHH-CALL2) and 2 glucocorticoid-resistant (MHH-CALL4, Reh) cell lines. Responsiveness of leukemic cell lines was determined by a 4-day total cell kill MTT-assay. The experiment was performed twice for each of the 4 depicted cell lines. Sensitivity to dexamethasone was corrected for cell death induced by hydrocortisone as single agent to determine the synergistic or antagonistic effect of the drug combination. Data are presented as mean+SEM.

GR levels indicated that the mRNA levels obtained by both methods were strongly correlated for MR (R=0.88, $P<0.0001$) (*Online Supplementary Figure S2A*) and moderately for GR (R=0.48, $P=0.02$) (*Online Supplementary Figure S2B*). RT-qPCR also confirmed that *ETV6-RUNX1*$^+$ cells have higher MR mRNA levels than other subtypes of ALL ($P\leq0.001$) (*Online Supplementary Figure S2A*). GR mRNA levels were higher compared to MR mRNA levels, in both microarray and RT-qPCR analyses (Figure 1C).

In order to study whether hydrocortisone can be safely combined with dexamethasone in the treatment of ALL, we first tested hydrocortisone and corticosteroids in four leukemic cell lines purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany): Bel1 (*MLL-AF4*$^+$, BCP-ALL), MHH-CALL2 (hyperdiploid, BCP-ALL), MHH-CALL4 (B-other, BCP-ALL), Reh (*ETV6-RUNX1*$^+$, BCP-ALL). An MTT-assay was used using previously reported conditions.[9-11] MTT assays are suitable to test the sensitivity of leukemic cells for prednisolone and dexamethasone and these results can indicate the prognosis of children with newly diagnosed ALL.[1,9,10] MTT assays measure total cell kill, but do not reveal the underlying mechanism of cell death. We previously showed that prednisolone induces apoptosis in leukemic cells of patients as illustrated by a decrease in mitochondrial membrane potential, increased caspase 3 cleavage and Annexin V positivity/propidium iodide negativity.[12] Hydrocortisone (Bufa Pharmaceutical Products) induced cell death in the glucocorticoid sensitive cell lines Bel1 and MHH-CALL2, whereas it did not affect the viability of glucocorticoid resistant cell lines MHH-CALL4 and Reh (Figure 2E). In combination, hydrocortisone did not affect or even sensitize glucocorticoid sensitive cell lines to dexamethasone (Sigma) or prednisolone (Bufa Pharmaceutical Products) (Figure 2A and B and *Online Supplementary Figure S3C*), whereas the level of resistance remained unaffected in resistant cell lines upon increasing concentrations of hydrocortisone (Figure 2C and D and *Online Supplementary Figure S3A and B*).

Subsequently, we aimed to confirm these results in primary patients' cells of different ALL subtypes (*ETV6-RUNX1*$^+$, B-other, hyperdiploid and T-ALL). Hydrocortisone sensitized glucocorticoid sensitive patients' cells to dexamethasone or prednisolone (Figure 3), including *ETV6-RUNX1*$^+$ cells (*Online Supplementary Figure S4 and S5*). Hydrocortisone did not affect the level of resistance to glucocorticoids of resistant patients' leukemic cells (Figure 3).

Our *in vitro* studies show that addition of hydrocortisone does not interfere or even sensitize ALL cells to glucocorticoids. The (sensitizing) effect of hydrocortisone is found for both types of glucocorticoids tested, i.e. dexamethasone and prednisolone. The expression level of the receptor for hydrocortisone, i.e. MR, is very low compared to the

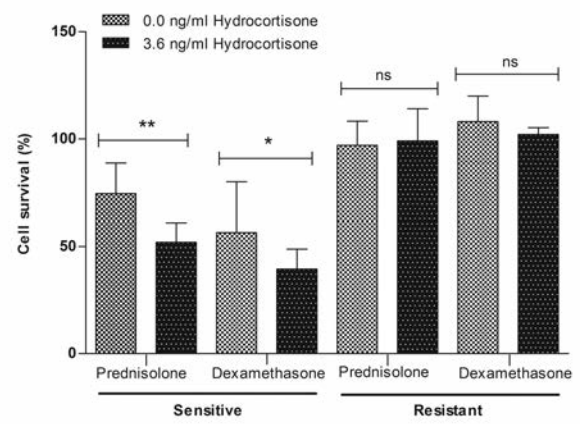

Figure 3. Hydrocortisone does not induce resistance to glucocorticoids in patients' leukemic cells taken at initial diagnosis. An MTT-assay was used to determine the cytotoxic effect of hydrocortisone (3.6 ng/mL) in combination with dexamethasone (0.012 μg/mL) or prednisolone (0.061 μg/mL for sensitive patients and 0.49 μg/mL for resistant patients) in patients' leukemic cells. Patients' cells of different acute lymphoblastic leukemia (ALL) subtypes were included (*ETV6-RUNX1*⁺ B-ALL, B-other, hyperdiploid B-ALL, T-ALL). Statistical analysis was performed with a Mann-Whitney U test (=*P*≤0.01, *=*P*≤0.05, ns=not significant). Data are presented as median and interquartile range. Sensitivity was corrected for cell death induced by hydrocortisone as single agent to determine the synergistic or antagonistic effect of the drug combinations. Further details on the drug sensitivity of different ALL subtypes are described in the *Online Supplementary Appendix*.**

expression levels found for GR in leukemic patients' cells. The MR levels were remarkable high in *ETV6-RUNX1*⁺ leukemic cells compared to other subtypes of ALL, for which there is still no explanation. The higher MR levels in *ETV6-RUNX1*⁺ patients' leukemic cells did not affect the response to hydrocortisone and glucocorticoids.

In conclusion, addition of hydrocortisone does not interfere with the response of leukemic cells to dexamethasone and prednisolone. Our next aim is to determine whether dexamethasone-induced neuropsychological side-effects can be prevented by co-administration of hydrocortisone. To this aim, we have initiated a double-blinded randomized control trial in which we test the effect of physiological dosages of hydrocortisone during dexamethasone treatment on neuropsychological symptoms in children with newly diagnosed ALL (*clinicaltrials.gov identifier 3280*).

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