Differential Gene Expression Profile Associated to Apoptosis Induced by Dexamethasone in CLL Cells According to IGHV/ZAP-70 Status

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Translational relevance

Some patients with chronic lymphocytic leukemia (CLL) are refractory to conventional treatments and in this setting one of the therapeutic options are the glucocorticoids. Herein we provide the first ‘gene/molecular fingerprint’ of dexamethasone in CLL cells. Our results corroborate the better response to glucocorticoids of CLL cells from patients from the poor outcome subgroup with unmutated IGHV genes/high ZAP-70 expression and describe some genes associated to this differential response.

The better understanding of the effect of dexamethasone in CLL cells can unveil new therapeutic targets for chemotherapy combinations and can facilitate the development of predictive markers of response to this drug.
ABSTRACT

Purpose
Glucocorticoids are part of the therapeutic armamentarium of chronic lymphocytic leukemia (CLL) where it has been suggested that cells with unmutated IGHV genes exhibit higher sensitivity. The mechanisms by which glucocorticoids are active in CLL are not well elucidated. We aimed to ascertain the activity of dexamethasone in CLL cells according to prognosis and to identify the molecular mechanisms that are influencing the response to this drug.

Experimental design
Sensitivity to dexamethasone was analyzed ex vivo in 50 CLL and compared according to IGHV mutational status and/or ZAP-70 expression. The response was further compared by gene expression profiling (GEP) of selected cases. Expression of genes of interest was validated by quantitative reverse transcriptase PCR.

Results
Response to dexamethasone was higher in cases with unmutated IGHV/high ZAP-70 expression, and the levels of induction the pro-apoptotic Bim protein correlated with the degree of cell death. GEP analysis showed few genes differentially expressed after dexamethasone treatment between mutated and unmutated cases. However, functional annotation analysis showed that unmutated cases had significant enrichment in terms related to apoptosis. Specific analysis of genes of interest performed in a large series disclosed that in unmutated IGHV cells FKBP5 expression was higher at baseline and after...
dexamethasone exposure, and that GILZ was more induced by dexamethasone treatment in these cases.

**Conclusions**

Unmutated IGHV/high ZAP-70 CLL cells exhibit better response to dexamethasone treatment, which is accompanied by a differential expression of genes involved in the glucocorticoid-receptor pathway and by an increased induction of genes related to apoptosis.
INTRODUCTION

Treatment of patients with chronic lymphocytic leukemia (CLL) has dramatically changed during the last decade with the introduction of monoclonal antibodies. Chemoimmunotherapy regimens like FCR (fludarabine, cyclophosphamide and rituximab)(1, 2), FCR plus mitoxantrone(3, 4), or FCR plus alemtuzumab(5) have proved to be highly effective in the treatment of this disease. Despite the excellent overall response and complete response rates obtained with these regimens, patients with 17p13.1 deletion and/or TP53 mutations usually exhibit a lower response rate, and shorter progression-free survival and overall survival (2, 6, 7). There is no standard salvage treatment for patients with refractory disease, particularly those with TP53 abnormalities, and therapeutic options are based on non-genotoxic drugs like alemtuzumab(8, 9), flavopiridol(10-12), lenalidomide(13, 14) or glucocorticoids(15-17), alone or in combination with monoclonal antibodies(5, 18-21). Patients that respond to these salvage treatments are recommended to undergo allogeneic stem-cell transplantation(22).

The mechanisms by which glucocorticoids induce CLL cell death are still not well understood. Glucocorticoids bind to a multiprotein complex receptor present in the cytoplasm constituted by the receptor itself and several cochaperones(23). After binding, the glucocorticoid-receptor dissociates from some of the cochaperone proteins and translocates into the nucleus, where it acts as a transcription factor modulating gene expression(24). Several studies have been conducted to identify genes that are regulated by glucocorticoids and have the ability to trigger lymphoid cell death, particularly in acute lymphoblastic
leukemia (25-30). In CLL, it has been found that dexamethasone up-regulates mRNA and protein expression of the pro-apoptotic BH3-only gene Bim (31). Of note, cell death induced by glucocorticoids is higher in CLL with unmutated IGHV genes (UCLL)/high ZAP-70 expression than in cases with mutated IGHV genes (MCLL)/low ZAP-70 (32-35), although the molecular mechanisms that could explain these differences have not been uncovered.

With this background, we aimed to widely analyze the modulation of gene expression induced by dexamethasone in CLL cells according to the different IGHV mutational status/ZAP-70 expression groups. For that, genome-wide gene expression profile (GEP) analysis was performed after dexamethasone treatment and genes of interest were retrieved for further study.

MATERIALS AND METHODS

Patient selection and sample collection

A group of 50 patients diagnosed with CLL was selected on the basis of the availability of frozen samples for biological studies. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, United Kingdom) density gradient and stored in liquid nitrogen until analysis. Informed consent from all patients was obtained according to the Declaration of Helsinki and the study was approved by the local clinical investigation ethical committee. The mean percentage of CLL cells (CD19+/CD5+ cells) in the series was 82.5% ± 9.7.
**Ex-vivo treatment with dexamethasone and evaluation of the response**

PBMCs from CLL patients were thawed at 37°C and resuspended in standard culture medium (RPMI-1640 medium (Gibco, Paisley, Scotland, UK) supplemented with 10% heat-inactivated FBS (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Lonza, Viviers, Belgium), 2 mM L-glutamine and 1 mM sodium pyruvate (Gibco)) and cultured at 37°C in a 5% CO₂ atmosphere at a density of 1 x 10⁶ cells/ml. PBMCs were allowed to recover from the thawing process for one hour before manipulation. Samples were split in two for control and incubation with the glucocorticoid dexamethasone (Merck KGaA, Darmstadt, Germany) at a concentration of 13.25 µM based on previous reports of CLL treatment ex-vivo (36). After 24 hours, cell viability was evaluated by surface annexin V binding and propidium iodide staining assessed by flow cytometry (rh Annexin V/FITC kit, Bender MedSystems, Vienna, Austria). Cell viability was measured as the percentage of double-negative cells for annexin V and propidium iodide. Dexamethasone response was calculated as the percentage of live cells after treatment with dexamethasone relative to the percentage of live cells in the untreated cells (left with standard media).

**ZAP-70 and IGHV mutational status analysis**

Mutational status of the IGHV genes and ZAP-70 expression by flow cytometry were determined as previously described(37). Patients with more than 98% germline identity for IGHV genes were considered to be unmutated. CLL cases were considered to have high ZAP-70 expression when ZAP-70 was ≥20%(37).
Quantitative reverse transcriptase PCR (QRT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen Life Technologies, Paisley, Scotland, UK) according to manufacturer instructions. For QRT-PCR analysis, complementary DNA was synthesized from 1µg RNA. Expression of Bim (BCL2-like 11 (apoptosis facilitator)), FKBP5 (FK506 binding protein 5) and GILZ (glucocorticoid-induced leucine zipper) was analyzed using pre-developed Taqman assays (Applied Biosystems, Foster City, CA): Hs00197982_m1, Hs01561001_m1 and Hs00608272_m1, respectively; and the ABI PRISM 7900 sequence detector instrument (Applied Biosystems). The comparative Ct method (ΔΔCt) for relative quantification of gene expression was used. β−Glucoronidase gene expression (GUSB, Applied Biosystems) was used as internal control, and mRNA-expression levels were given as arbitrary units (AU) refereed to a commercial standard mRNA (Control RNA (Human), Applied Biosystems). Fold change (gene induction) was determined as the ratio between expression in dexamethasone treated cells and expression in untreated cells.

Microarray analysis

Total RNA (2 µg) was converted into biotin-labeled cRNA and further fragmented and hybridized to oligonucleotide Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix Inc, Santa Clara, CA). Expression measures were normalized and summarized using the frozen robust multiarray analysis (fRMA) methodology(38). Clustering and heatmaps were performed with the TM4 Software Suite(39). Gene expression data with log values lower than 5 were discarded. Differential expression analysis was carried out by a linear model
using empirical Bayes method to moderate the standard errors of the estimated log-Ratio changes with the limma package(40). The online tool David (41) was used for the functional annotation analysis using the BP_FAT category of Gene Ontology (GO). The GEP data has been deposited at the National Centre for Biotechnology Information's Gene Expression Omnibus (GEO ID: GSE33135).

**RNA interference experiments**

Small interference RNA (Dharmacon, Lafayette CO) targeting FKBP5, GILZ, non-targeting or rhodamine-labelled as positive control for transfection (mean at 48h 35%) was transfected to 5·10^6 primary CLL cells in 500µl RPMI-10%FBS. Briefly, 500nM of siRNA was mixed with 4.5 µl of Hiperfect Transfection Reagent (Qiagen, Hilden, Germany) and added drop wise to the cells after 10 minutes incubation at room temperature. The cells were used 48 hours post-transfection only when viability was superior to 50% as analyzed by annexinV-PI staining by flow cytometry. Gene silencing efficiency was analyzed by QRT-PCR.

**Statistical analysis**

Comparisons between groups were done using the Mann-Whitney test. Correlations between measures were performed using a parametric linear regression model. For all comparisons, P-values were two-sided and the type I error was set at 5%. Statistical analyses were performed with the use of SPSS v18.0 software (IBM, Somer, NY) and GraphPad Prism v5.0 software (La Jolla, CA).
RESULTS

**CLL cases with unmutated IGHV genes and/or high ZAP-70 expression show a higher response to dexamethasone ex-vivo**

Samples from 50 patients diagnosed with CLL were treated ex-vivo with dexamethasone and the response was evaluated after 24 hours. The characteristics of the series are shown in Supplementary Table 1. Briefly, median age at diagnosis was 58 years (range, 30-82 years) and there was a male predominance (72%). ZAP-70 expression was considered high in 48% of the patients. IGHV mutational status was assessed in 47 cases, 23 of them (49%) being considered as UCLL. All the MCLL cases had low ZAP-70 expression, whereas only one UCLL case showed a low expression of ZAP-70. FISH analysis for the main CLL chromosomal abnormalities was performed in 48 out of 50 patients at the time the samples were obtained. According to the hierarchical model(6), 45.8% of the patients showed isolated 13q14.3 deletion, 10.4% 17p13.1 deletion, 10.4% trisomy 12, 4.2% 11q22.3 deletion and 29.2% presented no abnormality. After 24 hours of treatment with 13.25 µM dexamethasone, the percentage of live cells relative to untreated cells ranged from 42% to 100%. Notably, UCLL cases (n=23) had a significantly better response to dexamethasone than MCLL cases (n=24) (mean of cell viability ±SD: 68% ±14.0 vs 85% ±11.3; P<0.001; Figure 1A). In agreement, response to dexamethasone was also better in cases with high ZAP-70 expression (n=24) than in those with low ZAP-70 (n=26) (mean of cell viability ±SD: 68% ±13.9 vs 85% ±11.0; P<0.001; Figure 1B). Remarkably, cases with 17p13.1 and 11q22.3 deletion (n=7) had a better response to dexamethasone than cases without
these high-risk genetic abnormalities (n=41) (mean of cell viability ±SD: 64% ±16.2 vs 79% ±13.9; P=0.026). Of note, the only case with 17p13.1 deletion and low ZAP-70 expression disclosed a poor response to dexamethasone (Supplementary Table 1). Moreover, after excluding the cases with high-risk genetic abnormalities (17p13.1 and 11q22.3 deletions), ZAP-70 expression retained its predictive value for response to dexamethasone (mean of cell viability ±SD: high ZAP-70 (n=17) 71% ±13.1 vs low ZAP-70 (n=24) 85% ±11.4; P=0.001). In absolute terms, the mean percentage of live cells after thawing in this series was 72% ± 14, whereas after 24 hours of culture the mean viability was 56% ± 15 for control cells and 44% ± 16 for treated cells.

**Induction of Bim expression by dexamethasone correlates with the extent of apoptosis in CLL cells**

The expression of the pro-apoptotic BH3-only gene Bim has been reported to be induced by dexamethasone at both mRNA and protein level in different cellular models, including CLL cells (26, 31, 42-44). To study the kinetics of induction of Bim after treatment with dexamethasone, levels of Bim mRNA were analyzed by QRT-PCR at different time points in primary cells from 7 patients with CLL. As early as after 3 hours of treatment, an increase in Bim mRNA was already detected; in five of the cases, levels kept increasing up to 9 hours and then remained stable, whereas in the other two cases an additional increase in Bim levels was observed from 9 to 24 hours (Figure 2A). Furthermore, the relationship between the magnitude of the response to dexamethasone and the degree of Bim induction was evaluated in 43 CLL samples after 24 hours of
treatment. An inverse correlation between Bim induction and the percentage of live cells was observed ($P=0.001$; Figure 2B). Moreover, levels of Bim induction were higher in the subgroup of CLL cases with high ZAP-70 expression ($n=19$) than in the subgroup with low ZAP-70 (mean Bim fold change $\pm$SD: $3.75 \pm 1.89$ vs $2.61 \pm 0.78$; $P=0.042$; Figure 2C) which is in agreement with the better response to dexamethasone observed in this subgroup. Altogether, these results indicate that Bim may be part of the apoptotic pathway triggered by dexamethasone.

**Gene expression profiling of CLL samples treated with dexamethasone**

GEP analysis was performed in a series of CLL patients to identify genes potentially implicated in the differential response to dexamethasone. For this, we selected 7 CLL samples with high ZAP-70 expression and 5 with low ZAP-70 expression (Supplementary Table 1). Tumor cells were treated with dexamethasone or left with standard media for 6 hours; this time point was selected on the basis that it preceded the highest levels of Bim induction observed after dexamethasone treatment (Figure 2A).

The unsupervised analysis of the expression data performed using the 1,000 probe sets showing the highest variability defined two main branches of samples according to ZAP-70 expression (Figure 3A).

The effect of dexamethasone treatment was then independently analyzed in the high and low ZAP-70 groups by means of supervised analysis considering only those changes in gene expression with a false discovery rate (FDR) value lower than 0.05 and a logRatio>$|0.75|$. We found that dexamethasone treatment up-regulated the expression of 314 probe sets (153 genes) in the group with high
ZAP-70 expression, whereas in the low ZAP-70 group a total of 226 probe sets (118 genes) resulted up-regulated (Supplementary Table 2). Among up-regulated genes, 190 probe sets were shared by both ZAP-70 expression groups. We conducted functional annotation analysis of genes differentially expressed using gene ontology (GO) categories for ‘biological process’. This allows for the discovery of overrepresented categories of genes. Functional annotation analysis of up-regulated genes revealed that the most significant GO categories in the high and low ZAP-70 groups were related to apoptosis. Interestingly, the specific analysis of the common 190 probe sets showed that the most enriched category was regulation of lymphoid activation, which included genes such as IL7R and CTLA4. Of note, analysis of the 124 probe sets solely up-regulated in samples with high ZAP-70 expression showed a significant enrichment in genes involved in positive regulation of apoptosis, whereas analysis of the 36 probe sets only up-regulated in cases with low ZAP-70 disclosed that the most enriched GO category was related to ion homeostasis, a term that includes genes that participate in any process involved in the maintenance of an internal steady state of metal ions at the level of a cell, thus the relevance of apoptosis in this subgroup was less notorious. In conclusion, the enrichment in the apoptosis GO category observed in conjunctural analysis of high and low ZAP-70 groups was predominantly due to genes up-regulated only in cases with high ZAP-70 expression and better response to dexamethasone.

Dexamethasone treatment induced the down-regulation of 219 probe sets (153 genes) in CLL cases with high ZAP-70 expression and of 222 probe sets (155 genes) in cases with low ZAP-70 expression (Supplementary Table 2).
all down-regulated genes, a total of 132 probe sets were shared by both ZAP-70 groups. GO analysis of down-regulated probe sets showed that in both high and low ZAP-70 groups the most significant term was *immune response*. Of note, probe sets that were exclusively down-regulated in CLL cases with low ZAP-70 expression (n=90) were significantly enriched in genes related to *regulation of apoptosis*, being the majority of them involved in the positive regulation of this process.

The top 10 probe sets with the highest variation caused by the treatment with dexamethasone were subsequently selected (Table 1). Three genes were commonly up-regulated in high and low ZAP-70 groups, namely FKBP5, DDIT4 and TMEM2. In addition, 4 genes were commonly down-regulated by dexamethasone in both ZAP-70 expression groups: KMO, PALM2-AKAP2, IFIT2 and SAMD9L. Of note, FKBP5 was the most up-regulated gene in both ZAP-70 groups and was represented by three different probe sets. Interestingly, FKBP5 expression was also higher in the high ZAP-70 CLL group in both untreated (224840_at, logRatio=0.958, FDR=0.0129) and treated cells (probe sets: 224840_at, logRatio=0.734, FDR=0.0390 and 224856_at, logRatio=1.068, FDR=0.0416). The above mentioned results led us to hypothesize that the levels of FKBP5 could be involved in the differential response to dexamethasone observed in CLL cases.

Finally, we aimed to identify genes that had a significant differential regulation after treatment with dexamethasone in the two ZAP-70 expression groups. For this, the interaction term was calculated by assessing the difference between the genes induced/repressed by dexamethasone in the high ZAP-70 expression group and the genes induced/repressed by dexamethasone in the low ZAP-70
group. Considering P-values lower than 0.001, 45 probe sets (38 genes) were identified as differentially regulated (Figure 3B). GO analysis revealed a significant enrichment in genes related to regulation of apoptosis. Among these 38 differentially regulated genes we observed that TSC22D3 (alias GILZ) clustered with the pro-apoptotic gene BCL2L11 (alias Bim) (Figure 3B) indicating that the two genes were altered in a similar way by dexamethasone. In addition, only in the high ZAP-70 group, GILZ was one of the most up-regulated genes by dexamethasone (Table 1). These data suggested that GILZ may be implicated in the different response to dexamethasone observed in the ZAP-70 expression groups.

**Increased levels of FKBP5 at baseline and after dexamethasone treatment correlate with enhanced apoptosis and high ZAP-70 expression**

The GEP analysis revealed that FKBP5 was the most inducible gene by dexamethasone in CLL cells, its levels being higher in the high ZAP-70 subgroup. FKBP5 gene codifies for a cochaperone of the glucocorticoid-receptor complex which maintains the receptor complex in the cytoplasm. After glucocorticoid binding, FKBP5 is replaced by FKBP4 which allows for the nuclear translocation of the glucocorticoid-receptor complex(24). To further analyze the relationship between FKBP5 and the response to dexamethasone in CLL samples, we ascertained the expression of this gene by QRT-PCR in 46 CLL samples, 22 with high ZAP-70 expression, at baseline, at 6 hours after treatment with dexamethasone, and at 6 hours with media only. At baseline, levels of FKBP5 were higher in CLL cases with high ZAP-70 expression (n=16) than in those with low ZAP-70 (n=22) (mean levels of FKBP5 mRNA-expression...
FKBP5 baseline levels correlated with an increased apoptotic cell death at 24 hours of treatment with dexamethasone (n=38; P=0.027; Figure 4B). Interestingly, and in accordance with the GEP results, FKPB5 expression was induced 10 fold in mean after 6 hours of dexamethasone treatment (mean FKBP5 mRNA-expression ±SD: treated cells (n=43) 3.04AU ±2.12 vs untreated cells (n=41) 0.36AU ±0.22; P<0.001; Figure 4C). Of note, levels of FKBP5 reached after 6 hours of treatment were significantly higher in cases with high ZAP-70 expression (n=20) than in those with low ZAP-70 (n=23) (mean FKBP5 mRNA-expression ±SD: 4.31AU ±2.51 vs 1.95AU ±0.65; P<0.001; Figure 4D).

**GILZ expression highly correlates with the induction of apoptosis by dexamethasone in CLL**

GILZ is a transcription regulator directly targeted by the glucocorticoid-receptor (45) which negatively controls important mediators of cell proliferation (46). We found that GILZ was one of the top ten most inducible genes only in the high ZAP-70 group (Table 1). Moreover, GILZ was one of the few genes differently regulated by dexamethasone in the two ZAP-70 subgroups (Figure 3B).

To further assess the relationship between GILZ expression, ZAP-70 expression and the response to dexamethasone, levels of GILZ mRNA were determined by QRT-PCR in 40 CLL samples with or without treatment with dexamethasone for 6 hours. In untreated samples, levels of GILZ were higher in the low ZAP-70 group (n=20) than in the high ZAP-70 group (n=20) (mean GILZ mRNA-expression ±SD: 40.45AU ±21.46 vs 32.71AU ±22.05; P=0.040; Figure 4E). Conversely, and according to GEP results, induction of GILZ after
treatment with dexamethasone was significantly higher in samples with high ZAP-70 expression (n=20) than in those with low ZAP-70 (n=20) (mean GILZ fold change ±SD: 5.59 ±2.16 vs 3.92 ±0.83; P=0.002; Figure 4F). Moreover, we observed that this induction of GILZ correlated with cell viability (n=40; P<0.001; Figure 4G). Finally, and reinforcing that GILZ clustered with Bim in the GEP interaction term analysis, GILZ induction correlated with the increase of Bim expression (n=34; P=0.001; Figure 4H) determined after 24 hours of treatment with dexamethasone.

Inhibition of FKBP5 or GILZ expression by siRNA in primary CLL cells impairs response to dexamethasone treatment ex vivo.

In order to investigate if FKBP5 and GILZ are directly participating in the apoptotic response to dexamethasone observed in primary CLL cells, we analyzed the response to this treatment ex vivo after 48h of transfection with siRNA targeting FKBP5 or GILZ in four CLL cases. As can be observed in Figure 5, the percentage of live cells after 24 hours of treatment with 13.25 µM dexamethasone is higher in CLL cells transfected with siRNA targeting FKBP5 or GILZ as compared to cells transfected with non-targeting RNA. The mean downregulation of FKBP5 was 26% and of GILZ 32%, as assessed by QRT-PCR, which led to a discrete but consistent decrease in the response to dexamethasone in all the cases analyzed. These results indicate that both FKBP5 and GILZ are indeed involved in the apoptotic response of CLL cells to dexamethasone ex vivo.
DISCUSSION

Herein we report that the degree of apoptosis induced by dexamethasone in neoplastic B CLL lymphocytes ex-vivo is significantly higher in patients with UCLL/high ZAP-70 expression than in patients with MCLL/low ZAP-70 expression, which have a better prognosis. This is in agreement with what has been previously described using prednisone and methylprednisolone(32-35). Interestingly, we showed that IGHV unmutated genes/high ZAP-70 expression conferred higher susceptibility to dexamethasone independently of the presence of 17p13.1 or 11q22.3 deletion. These results corroborated the clinical experience on the use of glucocorticoids in patients with high-risk cytogenetics (17, 19).

Induction of expression of Bim protein has been shown to be implicated in apoptosis induced by dexamethasone in ALL(26, 42-44) and this protein appeared to be the unique pro-apoptotic protein involved in cell death induced by glucocorticoids in CLL(31). In our study, besides confirming the early up-regulation of Bim expression on treatment with dexamethasone, we showed that dexamethasone-induced cell death positively correlated with levels of Bim induction. Altogether, these findings indicate that Bim is probably a downstream effector of dexamethasone in CLL. Since Bim pro-apoptotic mechanism has been demonstrated to be independent of p53 (47), its up-regulation could explain in part the response to glucocorticoids observed in some CLL cases with TP53 abnormalities(17, 19).
GEP analysis revealed high similarities between ZAP-70 subgroups in terms of genes regulated after dexamethasone treatment, indicating that the different response to dexamethasone may not be due to an independent biological targeting of dexamethasone but to a differential capacity to induce cell death while inducing/repressing the same genes.

GEP results allowed us to select genes with significant levels of modulation along with biological relevance in the glucocorticoid pathway for further studies in larger series of patients. FKBP5, the cochaperone of the glucocorticoid-receptor(23), resulted to be the most inducible gene after dexamethasone treatment in both ZAP-70 subgroups. Moreover, we observed that baseline levels of expression of FKBP5 were higher in cases with high ZAP-70 expression by GEP and QRT-PCR experiments, the levels correlating with the extent of cell death. Interestingly, the downregulation of FKBP5 by siRNA decreased CLL cells sensitivity to dexamethasone. Our results are in line with previous studies performed in ALL, where the levels of glucocorticoid-receptor have been correlated with the degree of induced apoptosis(48). FKBP5 maintains the glucocorticoid-receptor in the cytoplasm in an active conformation(24), thus the higher levels of FKBP5 observed in cases of CLL with high ZAP-70 expression can be in part responsible for their better response to dexamethasone, however, in some cellular systems an overexpression of FKBP5 can actually reduce the transcriptional activity of the glucocorticoid-receptor, probably because of modification of the access of FKBP4 protein to the receptor, which allows nuclear translocation of the complex (24). Finally, GILZ, a previously know target of glucocorticoids (30, 49, 50) was identified in GEP analysis as differentially induced by dexamethasone, being higher in CLL
samples with high ZAP-70 expression. Moreover, induction of GILZ was correlated with the induction of the downstream apoptotic effector Bim. GILZ has been directly implicated in cell death after glucocorticoid treatment since its inhibition by siRNA impaired the apoptotic response in our and previous studies in multiple myeloma (49). Altogether these findings point toward a role of GILZ in apoptosis induced by glucocorticoids in CLL.

In summary, the induction of apoptosis by dexamethasone was higher in the cells from patients with UCLL/high ZAP-70 expression, being the induction of Bim positively correlated with the extent of apoptosis. The increased response to dexamethasone observed in cases with UCLL/high ZAP-70 expression is probably attributable to differences in baseline expression and induction of genes involved in the glucocorticoid and apoptosis pathways.
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**TABLES**

**Table 1.** The top 10 most up-regulated and down-regulated probe sets in CLL groups with high and low ZAP-70 expression after treatment with dexamethasone

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Genes are ranked according to their logRatio values calculated as the difference in log expression value using the untreated cells group as baseline. *common probe sets in high and low ZAP-70 expression groups.
FIGURE LEGENDS

Figure 1. CLL cases with poor prognostic factors show better response to treatment with dexamethasone. CLL cells from 50 cases were treated with 13.25 µM dexamethasone for 24 hours and the percentage of live cells was determined by annexinV/PI staining. (A) Response to dexamethasone in UCLL and MCLL. UCLL cases show significantly higher response to dexamethasone in terms of percentage of live cells than MCLL cases. (B) Response to dexamethasone in high and low ZAP-70 expression groups. CLL cases with high ZAP-70 expression have better response to dexamethasone than cases with low ZAP-70. Horizontal bars represent the mean values of live cells.

Figure 2. Bim is induced after treatment with dexamethasone and correlates with response and ZAP-70 expression. (A) Time-course of the induction of Bim after treatment with dexamethasone. CLL cells from 7 cases were treated with 13.25 µM dexamethasone and Bim levels were evaluated at 3, 6, 9 and 24 hours by QRT-PCR. Results are expressed as the Bim fold change. HZ-CLL stands for high ZAP-70 expression and LZ-CLL for low ZAP-70 expression. The induction of Bim is high in the initial hours of treatment with dexamethasone and stabilizes after 9 hours. (B) Correlation of induction of Bim with the response to dexamethasone. CLL cells were treated with 13.25 µM of dexamethasone for 24 hours and then both Bim fold change and response to treatment with dexamethasone were determined. The scatter-plot shows a linear correlation between induction of Bim and response to dexamethasone. (C) Induction of Bim in CLL cells with high or low ZAP-70 expression. CLL cells from 43 cases were
treated with 13.25 µM of dexamethasone for 24 hours and then both Bim fold change and response to dexamethasone treatment were determined. CLL cases with high ZAP-70 expression have significantly higher induction of Bim than cases with low ZAP-70. Horizontal bars represent the mean values of induction of Bim.

**Figure 3.** Gene expression profile analysis of CLL cells treated with dexamethasone according to ZAP-70 expression. (A) Dendogram representing the unsupervised analysis of the 1,000 probe sets with the most variable expression applying the hierarchical clustering algorithm. DXM stands for dexamethasone treated cells and UNT stands for untreated cells; (B) Unsupervised cluster analysis of the 45 probe sets retrieved in the analysis of the interaction term. Changes in expression due to dexamethasone treatment for each probe set are displayed as LogRatios. HZ stands for high ZAP-70 expression and LZ stands for low ZAP-70 expression; in both (A) and (B) the number after CLL is the sample number according to Supplementary Table 1.

**Figure 4. (A-D)** FKBP5 levels at baseline and after treatment with dexamethasone correlate with higher response to dexamethasone and with ZAP-70 expression. CLL cells were treated with 13.25 µM of dexamethasone for 24 hours. Dexamethasone responses were determined at 24 hours. The levels of FKBP5 expression were determined by QRT-PCR. (A) At baseline, cases with high expression of ZAP-70 show higher levels of FKBP5 than cases with low ZAP-70. (B) Scatter-plot showing a significant negative correlation between the percentage of live cells after 24 hours of treatment with...
dexamethasone and baseline FKBP5 levels. (C) After 6 hours, treated cells show higher levels of FKBP5 than untreated cells. (D) After 6 hours of treatment with dexamethasone, cases with high ZAP-70 expression show higher levels of FKBP5 than cases with low ZAP-70. In (A), (C) and (D) horizontal bars represent the mean value of the y-axis units.

(E-H) Induction of GILZ after 6 hours of treatment with dexamethasone correlates with the response to the treatment. The levels of expression of GILZ were determined by QRT-PCR after 6 hours of treatment. Fold change of Bim expression was determined by QRT-PCR at 24 hours. (E) The untreated cells from the cases with low expression of ZAP-70 show higher levels of GILZ mRNA than the cells from those with high ZAP-70. (F) After 6 hours of treatment with dexamethasone, cases with high ZAP-70 expression show higher induction of GILZ than cases with low ZAP-70. In (E) and (F) horizontal bars represent the mean value of the y-axis units. (G) Scatter-plot showing a significant negative correlation between GILZ induction and percentage of live cells after treatment with dexamethasone. (H) Scatter-plot showing a significant positive correlation between the induction of Bim and GILZ.

**Figure 5.** Inhibition of FKBP5 and GILZ by siRNA attenuates the response to dexamethasone. Primary CLL cells from 4 patients were transfected with siRNA targeting FKBP5, GILZ or with a non-targeting control. After 48 hours cells were treated with 13.25 µM dexamethasone and the response was evaluated 24 hours later.
Figure 1

(A) Percent of live cells in MCLL (n=24) and UCCL (n=23). P < 0.001

(B) Percent of live cells in ZAP-70 (<20%) (n=26) and ZAP-70 (≥20%) (n=24). P < 0.001
Figure 2
Figure 4
Clinical Cancer Research

Differential Gene Expression Profile Associated to Apoptosis Induced by Dexamethasone in CLL Cells According to IGHV/ZAP-70 Status

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