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

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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 of 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 conducted 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. Clin Cancer Res; 18(21); 1–10. ©2012 AACR.

Introduction

Treatment of patients with chronic lymphocytic leukemia (CLL) has dramatically changed during the last decade with the introduction of monoclonal antibodies. Chemoimmuno-therapy regimens such as FCR (fludarabine, cyclophosphamide, and rituximab; refs. 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 such as alemtuzumab (8, 9), flavopiridol (10–12), lenalidomide (13, 14), or glucocorticoids (15–17), alone or in combination with monoclonal antibodies (5, 18–21). Patients who 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
Some patients with chronic lymphocytic leukemia (CLL) are refractory to conventional treatments, and in this setting, one of the therapeutic options is 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.

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 biologic studies. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque Plus (Amersham Biosciences) 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 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.

Ex vivo treatment with dexamethasone and evaluation of the response

PBMCs from patients with CLL were thawed at 37°C and resuspended in standard culture medium [RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated FBS (Gibco), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Lonza), 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate (Gibco)] and cultured at 37°C in a 5% CO₂ atmosphere at a density of 1 × 10⁶ cells/mL. PBMCs were allowed to recover from the thawing process for 1 hour before manipulation. Samples were split in 2 for control and incubation with the glucocorticoid dexamethasone (Merck KGaA) at a concentration of 13.25 μmol/L 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 (PI) staining assessed by flow cytometry (rh Annexin V/FTITC kit, Bender MedSystems). 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

Total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies) according to manufacturer instructions. For quantitative reverse transcriptase PCR (QRT-PCR) analysis, cDNA was synthesized from 1 μg RNA. Expression of Bim [BCL2-like 11 (apoptosis facilitator)], FKBP5 (FK506 binding protein 5), and GILZ (glucocorticoid-induced leucine zipper) was analyzed using predeveloped TaqMan assays (Applied Biosystems): Hs00197982_m1, Hs01561001_m1, and Hs00608272_m1, respectively; and the ABI PRISM 7900 Sequence Detector Instrument (Applied Biosystems). The comparative Ct method (∆∆Ct) for the 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) referred to a commercial standard mRNA [control RNA (Human), Applied Biosystems]. Fold change (gene induccion) 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.). Expression measures were normalized and summarized using the frozen robust multiarray analysis (RMA) methodology (38). Clustering and heatmaps were conducted with the TM4 Software Suite (39). Gene expression data with log values lower than 5 were discarded. Differential expression analysis was conducted 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 have been deposited at the National Centre for Biotechnology Information’s Gene Expression Omnibus (GEO ID: GSE33133).

RNA interference experiments

The siRNA (Dharmacon) targeting FKBP5, GILZ, non-targeting or rhodamine-labeled as positive control for transfection (mean at 48 hours, 35%) was transfected to 5 × 10⁶ primary CLL cells in 500 μL RPMI-10%-FBS. Briefly, 500 nmol/L of siRNA was mixed with 4.5 μL of HiPerfect Transfection Reagent (Qiagen) and added drop wise to the cells after 10-minute incubation at room temperature. The cells were used 48 hours after transfection only when viability was superior to 50% as analyzed by Annexin V-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 conducted using a parametric linear regression model. For all comparisons, P values were 2-sided and the type I error was set at 5%. Statistical analyses were conducted with the use of SPSS v18.0 software (IBM) and GraphPad Prism v5.0 software.

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 S1. Briefly, median age at diagnosis was 58 years (range, 38–82 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 conducted in 48 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 μmol/L 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; Fig. 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; Fig. 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 S1). 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.

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 μmol/L dexamethasone for 24 hours, and the percentage of live cells was determined by Annexin V/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.
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 5 of the cases, levels kept increasing up to 9 hours and then remained stable, whereas in the other 2 cases, an additional increase in Bim levels was observed from 9 to 24 hours (Fig. 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 ($P = 0.001$; Fig. 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 ± SD: 3.75 ± 1.89 vs. 2.61 ± 0.78; $P = 0.042$; Fig. 2C) which is in agreement with the better response to dexamethasone observed after dexamethasone treatment (Fig. 2A).

The unsupervised analysis of the expression data conducted using the 1,000 probe sets showing the highest variability defined 2 main branches of samples according to ZAP-70 expression (Fig. 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 log ratio $> |0.75|$. We found that dexamethasone treatment upregulated the expression of 314 probe sets (153 genes) in the group with high ZAP-70 expression.
expression, whereas in the low ZAP-70 group, a total of 226 probe sets (118 genes) resulted in upregulation (Supplementary Table S2). Among upregulated genes, 190 probe sets were shared by both ZAP-70 expression groups. We conducted functional annotation analysis of genes differentially expressed using gene ontology categories for "biologic process." This allows for the discovery of overrepresented categories of genes. Functional annotation analysis of upregulated 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 upregulated in samples with high ZAP-70 disclosed that the most enriched GO category was related to positive regulation of apoptosis, whereas analysis of the 36 probe sets only upregulated 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 conjunctional analysis of high and low ZAP-70 groups was predominantly due to genes upregulated only in cases with high ZAP-70 expression and better response to dexamethasone.

Dexamethasone treatment induced the downregulation 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 S2). Among all downregulated genes, a total of 132 probe sets were shared by both ZAP-70 groups. GO analysis of downregulated 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 downregulated in CLL cases with low ZAP-70 expression (n = 90) were significantly enriched in genes related to regulation of apoptosis, the majority of them being 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 upregulated in high and low ZAP-70 groups, namely FKBP5, DDIT4, and TMEM2. In addition, 4 genes were commonly downregulated by dexamethasone in both ZAP-70 expression groups: KMO, PALM2-AKAP2, IFIT2, and SAMD9L. Of note, FKBP5 was the most upregulated gene in both ZAP-70 groups and was represented by 3 different probe sets. Interestingly, FKBP5 expression was also higher in the high ZAP-70 CLL group in both untreated (224840_at, log ratio = 0.958, FDR = 0.0129) and treated cells (probe sets: 224840_at, log ratio = 0.734, FDR = 0.0390 and 224856_at, log ratio = 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 2 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
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, 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 ± SD: 0.95AU ± 0.58 vs. 0.57 AU ± 0.22; P = 0.032; Fig. 4A). FKBP5 baseline levels correlated with an increased apoptotic cell death at 24 hours of treatment with dexamethasone (n = 38; P = 0.027; Fig. 4B). Interestingly, and in accordance with the GEP results, FKBP5 expression was induced 10-fold in mean after 6 hours of dexamethasone treatment [mean

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NOTE: Genes are ranked according to their log ratio values calculated as the difference in log expression value using the untreated cells group as baseline.

aCommon probe sets in high and low ZAP-70 expression groups.
FKBP5 mRNA expression ± SD: treated cells (n = 43) 3.04 AU ± 2.12 vs. untreated cells (n = 41) 0.36 AU ± 0.22; P < 0.001; Fig. 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: 4.31 AU ± 2.51 vs. 1.95 AU ± 0.65; P < 0.001; Fig. 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 1 of the top 10 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 2 ZAP-70 subgroups (Fig. 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: 40.45 AU ± 21.46 vs. 32.71 AU ± 22.05; P = 0.040; Fig. 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; Fig. 4F).

Moreover, we observed that this induction of GILZ correlated with cell viability (n = 40; P < 0.001; Fig. 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; Fig. 4H) determined after 24 hours of treatment with dexamethasone.

### Inhibition of FKB5 or GILZ expression by siRNA in primary CLL cells impairs response to dexamethasone treatment ex vivo

To investigate whether FKB5 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 48 hours of transfection with siRNA targeting FKB5 or GILZ in 4 CLL cases. As can be observed in Fig. 5, the percentage of live cells after 24 hours of treatment with 13.25 μmol/L dexamethasone is higher in CLL cells transfected with siRNA targeting FKB5 or GILZ than in cells transfected with nontargeting RNA. The mean downregulation of FKB5 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 FKB5 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. 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 acute lymphoblastic leukemia (ALL; refs. 26, 42–44), this protein seeming to be the unique pro-apoptotic protein involved in cell death induced by glucocorticoids in CLL (31). In our study, besides confirming the early upregulation 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. Because the pro-apoptotic mechanism of Bim has been shown to be independent of p53 (47), its upregulation 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 biologic 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 biologic relevance in the glucocorticoid pathway for further studies in larger series of patients. FKB5, 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 FKB5 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 FKB5 by siRNA decreased the sensitivity of CLL cells to dexamethasone. Our results are in line with previous studies conducted in ALLs, where the levels of glucocorticoid receptor have been correlated with the degree of induced apoptosis (48). FKB5 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 known 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 related with the induction of the downstream apoptotic pathway (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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