

Excessive amount and activity of μ -calpain affects apoptotic machinery in chronic B-cell leukemia cells and influences the course of the disease

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B-cell Chronic Lymphocytic Leukemia (B-CLL) is the most common hematological disorder among middle-aged/elderly people in the Western countries. We have shown earlier that B-CLL cells exhibit elevated total amount and available activity of μ -calpain, belonging to a family of ubiquitous, strongly Ca-dependent proteases, involved in the control of proliferation and apoptosis. In this study we attempted to estimate a potential clinical value of μ -calpain in relation to B-CLL clinical staging in patients with extremely high lymphocytosis and studied the molecular mechanisms associating calpain activity with clinical progress of the disease. We observed significant correlations between the amounts of intracellular μ -calpain and clinical staging of the disease, with Rai stage 1 corresponding to the highest calpain amounts in the leukemic cells. There was also a positive, statistically significant correlation between the amount of μ -calpain and phosphorylated (p)ZAP-70 in B-CLL lymphocytes. Calpain activity in the B-CLL cells is associated with decreased activities of pro-apoptotic caspases -3 and -9, and reciprocally with an increased amount of anti-apoptotic Bcl-2. Together, all of these findings make calpain activity in B-CLL cells a promising target modifying the properties of these cells and facilitating therapy. Finally, the proportion of CD19⁺ B cells with elevated μ -calpain and pZap-70 was markedly reduced in patients after successful therapy.

Key words: Calpains, Zap-70, caspases, Bcl-2, chronic B cell leukemia

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Abbreviations: AICD, activation-induced cell death; CBA, B-cyto-metric bead array CLL, chronic lymphocytic leukemia; IgVH, B-cell-receptor heavy chain gene; MFI, mean fluorescence intensity; PARP, poly-ADP-ribose polymerase

INTRODUCTION

Chronic lymphocytic leukemia (B-CLL) occurs mostly in older middle-aged and elderly people (median age 60 years). The disease is characterized by accumulation of malignant B lymphocytes with the general immunophenotype CD19⁺CD23⁺CD5⁺. More than 99% of circulating B-CLL cells are arrested in the G0/G1 phase of the cell cycle, therefore their accumulation *in vivo* appears to result from inhibition of apoptosis rather than from increased proliferation (Rai *et al.*, 1975; King *et al.*, 1998; Kitada *et al.*, 1998; Rai *et al.*, 2004). Dysregulation of apoptotic cell death mechanisms is therefore thought to have a profound effect on pathogenesis and progression of this malignancy (Kitada *et al.*, 1998; Jewell, 2002; Danilov *et al.*, 2006).

Natural history of B-CLL seems to be heterogeneous, with clinically distinct subgroups characterized by gross differences in survival time, i.e. up to 25 years after diagnosis with relatively slow disease progression, and 8 years on average, characterized by rapid progression of the disease. These subgroups are genetically distinguished by the presence of somatic mutations in the variable region of the B-cell-receptor heavy chain gene (IgVH), as its wild, non-mutated status directly determines the disease aggressiveness (Damle *et al.*, 1999; Hamblin *et al.*, 1999; Krober *et al.*, 2002). Although it is possible to distinguish groups of patients more prone to an unfavorable course of this disease, our understanding of molecular mechanisms underlining differences in the clinical course of B-CLL is still elusive. Moreover, the exact pathogenesis of the disease is not entirely clear despite decades of studies (Chiorazzi & Ferrarini, 2011; Podhorecka *et al.*, 2016). The two most commonly approved surrogate biomarkers for IgVH gene mutational status (which is not routinely tested in clinical practice) are ZAP-70 (Zeta-associated protein of 70 kDa, a member of the tyrosine kinase family, physiologically expressed in T and NK cells and not found in normal, mature B cells) (Crespo *et al.*, 2003; Wiestner *et al.*, 2003; Kim *et al.*, 2004; Munoz *et al.*, 2006; Mansour *et al.*, 2007) and the p38 protein (Damle *et al.*, 1999) not studied in this work. Both are correlated with a more aggressive disease course (surrogate markers of the IgVH status), while ZAP-70 is thought to be a more reliable marker (Crespo *et al.*, 2003; Gachard *et al.*, 2008; Rassenti *et al.*, 2008; Amaya-Chanaga & Rassenti, 2016). Concordance between ZAP-70 and IgVH gene status reaches over 80%, making it a very useful, although imperfect, alternative (Gachard *et al.*, 2008). Un-

fortunately, ZAP-70 is not a relevant factor in determining the risk of drug resistance presented by malignant B cells (Hoellein *et al.*, 2010). Detecting new, reliable, prognostic and predictive markers determining survival time and therapeutic response appears vital for increasing the efficacy of treatment of this leukemia.

Our previously reported data show that one of the characteristics of B-CLL cells is the marked overexpression of μ -calpain, a ubiquitous calcium-dependent cysteine protease, as well as its total available activity, which we also suggested to be involved in the apoptotic arrest of B-CLL leukemic cells due to caspase-3 destruction (Witkowski *et al.*, 2002; Lopatniuk & Witkowski, 2011). We have also demonstrated a similar mechanism of escape from apoptosis in the case of acute lymphoblastic leukaemia in children (preB-ALL) and have proven that apoptosis of these blasts can be induced (at least in vitro) by calpain inhibition (Mikosik *et al.*, 2015). Furthermore, we have recently shown that also in non-malignant human lymphocytes (both T and B) the activity of μ -calpain, as well as of m-calpain (a form of the enzyme requiring higher levels of Ca^{2+} for its activation), is constitutive and maintained as such thanks to an equally constitutive transcription of their genes, CANP-1 and CANP-2, respectively (Mikosik *et al.*, 2016). In the same work we have shown that in fact this constitutive calpain activity is necessary for normal lymphocyte proliferation and secretion of cytokines; both functions were abolished when calpains were inhibited (Mikosik *et al.*, 2016). Calpains are well known for their involvement in preventing cellular death mechanisms of multiple neoplastic diseases (Lopatniuk & Witkowski, 2011), and identification of their role in B-CLL might not only be of academic value but may also prove useful for the understanding of pathogenesis and clinical course of this leukaemia. We have recently proposed a continuum of change of intracellular calpain activities in normal human lymphocytes and related malignancies. Thus, low constitutive calpain activity in resting lymphocytes would control protein activation/inactivation, maintain readiness for proliferation and secretion of biomolecules; on the other hand, transiently increased activity of the enzyme in antigen- or mitogen-activated normal lymphocytes would stimulate divisions by control over levels of signal transduction-, division- and movement-associated molecules; this transient elevated calpain activity might also participate in the induction of activation-induced cell death (AICD) and cessation of excessive divisions of normal lymphocytes. Finally, in the transformed (lymphoid leukemias, lymphomas) blasts, constantly high calpain activity would shift the balance towards excessive proliferation and reduced apoptosis, promoting growth of neoplastic cell numbers (Witkowski, 2018).

At the time of writing of the first of our above mentioned papers no techniques were available for a reliable determination of ongoing calpain activity in the living cells. Therefore, our paper compared the total available activity of the enzyme assessed by casein zymography, then a relatively novel technique (Witkowski, *et al.*, 2002). We have since developed a western blot technique based on detection of specific cleavage products of the constitutively expressed intracellular calpain inhibitor – calpastatin – which reports the most recent calpain activity in the living cells just before their processing (Mikosik *et al.*, 2007; Mikosik *et al.*, 2015). The questions, which we aim to address here, are as follows:

First, is μ -calpain more active in resting, living peripheral BCLL cells as compared to non-malignant peripheral B lymphocytes? Second, what are the molecular

mechanisms by which μ -calpain activity protects BCLL cells from apoptosis? And finally, in the light of the abovementioned role of ZAP-70 in B-CLL biology, is it possible to determine a correlation between ZAP-70 and μ -calpain amount and/or activity in leukemic cells which would establish the utility of calpain as an alternative for this marker? In order to fully understand the clinical connotations of μ -calpain activity in B-CLL cells, we decided to verify the prognostic and predictive value of μ -calpain in the studied leukaemia. Specifically, the possibility of μ -calpain hyper-activity being a predictive factor was studied in relation to the standard B-CLL staging system based on leukemic cell burden proposed by Rai *et al.*, with defined early (Rai 0), intermediate (Rai I/II) and advanced (Rai III/IV) stages of the disease estimated clinically (Rai & Han, 1990; Zwiebel & Cheson, 1998).

MATERIALS AND METHODS

Patients. Peripheral blood of 43 patients (mean age 66 ± 8 years, range 53–80 years, 10 women and 31 men), with clinically and immunophenotypically diagnosed B-cell Chronic Lymphocytic Leukemia (B-CLL), (based on the immunophenotype: $\text{CD}5^+ \text{CD}19^+ \text{CD}20^+ \text{CD}22^+ \text{CD}23^+$) was supplied by the Department of Hematology, Medical University of Gdansk. According to Rai classification, stage 0 was found in 17 patients, stage I in 8, stage II in 2, stage III in 2 and stage IV in 11 patients. 28 patients were not treated and the remaining 13 were given standard chemotherapy protocols involving up to 6 rounds of fludarabine and cyclophosphamide. For the latter, the therapy-free period before current calpain analyses was between 6 and 18 months (average 11.6 months). The control group consisted of 15 healthy individuals (mean age 71 ± 7 years, range 67–82 years, 5 women and 10 men). None of the participants in the study suffered from acute infectious or inflammatory diseases at the time blood was drawn. Peripheral blood samples were obtained following diagnosis or during routine follow-up visits with written informed consent of all participants. The project was approved by the Local Independent Committee for Ethics in Scientific Research at the Medical University of Gdansk, as certified by statements: NKEBN/748/2003; NKEBN/221/2007; NKEBN/186/2011; NKEBN/2777/2011; and NKBBN/321/2014. All patients gave written informed consent for participation in this study. The applied methods and procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2008.

PBMC and B cell isolation. Fasting peripheral blood was drawn into Vacutainer™ tubes containing EDTA and peripheral blood mononuclear cells (PBMCs) were purified by floatation over Histopaque® (Sigma, Deisenhofen, Germany), counted and suspended in complete culture medium (RPMI 1640+10% BSA+2 mM L-glutamine+100 U/ml penicillin+100 $\mu\text{g}/\text{ml}$ streptomycin) at 1×10^6 cells/ml. Blood from individuals diagnosed with B-CLL, whose PBMCs consisted of 93 to 98% of leukemic $\text{CD}19^+ \text{CD}5^+$ lymphocytes, was not subjected to further enrichment of leukemic cells. The rationale for this was an extremely low percentage of normal lymphocytes among the leukemic ones and the possibility that aggressive separation may affect the cell membranes and calcium fluxes in the B-CLL cells, leading to changed calpain activities. Non-malignant B cells from healthy individuals were enriched using the immunomagnetic separation

technique and Dynabeads™ CD19 Pan B beads and DETACH-a-BEAD™ CD19 (Invitrogen), following the manufacturer's protocol. Purity of thus obtained B cells was established using flow cytometry and the proportion of viable CD19⁺ lymphocytes was maintained at a level of 92–97%. Viability of the studied cells was always above 95%, as determined by trypan blue exclusion test.

Intracellular μ -calpain and ZAP-70 expression.

In order to identify normal and malignant B cells, cell suspensions which were obtained as stated in the section above (PBMC and B cell isolation) were stained for 30 minutes on ice with RPE-Cy5-conjugated anti-CD19 monoclonal antibody (Dako Cytomation) at 1 μ g/10⁵ cells, then washed twice with ice-cold PBS and further processed.

Relative amounts of intracellular μ -calpain and phospho-ZAP-70 were determined after fixation and permeabilization of the anti-CD19 – stained cells using 2% paraformaldehyde and 0.25% saponin in PBS, and then by staining with PE-conjugated anti- μ -calpain and Alexa Fluor®488-conjugated anti-ZAP-70 (Anti-Phospho-Zap70) (both from Becton Dickinson Biosciences, USA), at concentrations recommended by the manufacturer. Flow cytometry analysis was then performed using the FACScan™ flow cytometer (BD Biosciences, San Diego, USA) and the CellQuest™ software. Appropriate isotype controls were applied. CD19⁺ lymphocytes were gated in order to exclude cell doublets and debris. Data from at least 10000 CD19⁺ lymphocytes were collected from each sample. Results were shown as mean fluorescence intensity (MFI) after subtraction of MFI of the CD19⁺ cells stained with appropriate isotype controls (corrected MFI, cMFI). As we have demonstrated earlier, all of human peripheral blood lymphocytes contain some calpain, so there are no calpain-negative lymphocytes (Mikosik *et al.*, 2013; Mikosik *et al.*, 2016). However, we hypothesized that the amounts of calpain “per cell” may vary especially when leukemic cells are concerned. Thus, proportions of B-CLL cells containing high levels of calpain in the total CD19⁺ population were determined as proportion of cells in which the measured relative amount of calpain (cMFI) was higher than mean average calpain content + 2 standard deviations seen in the normal B cells. The cytometer was calibrated on the daily basis using the CaliBRITe™ 3-color bead kit (BD Biosciences) and manufacturer's protocol, and the CV for the beads was maintained below 2%.

Activity of calpains in B-CLL cells and non-malignant B cells. Establishing the actual level of calpain activity in circulating B-CLL cells and normal B cells was crucial for the project. This was achieved using a previously developed technique, based on immunodetection and quantification of products resulting from specific cleavage of calpastatin by calpains active in cells (Mikosik *et al.*, 2007, Mikosik *et al.*, 2015). Briefly, cells were lysed in EDTA-free Complete Lysis-M™ buffer (Roche) containing the protease inhibitor cocktail (leupeptin, aprotinin, iodoacetamide and PMSF – all at 10 μ g/ml) according to the manufacturer's protocol, and total protein lysates corresponding to 1 million cells were separated by SDS-PAGE. For a method control, a separate B-CLL lysate sample was depleted of calpastatin and its detectable fragments by overnight incubation with 1:100 anti-calpastatin mouse monoclonal antibody (Abcam); the immune complexes between calpastatin or its immunoreactive fragments and the antibody were then removed by incubation with Protein A-Sepharose™ beads which were then removed by centrifugation (Braun *et al.*, 1998); clear sample was then processed for WB with the other

samples. In order to demonstrate that cleavage of calpastatin was performed by endogenous calpains, we incubated the parallel B-CLL cell samples with 4 μ M (IC50) calpain inhibitor II for 24 hours prior to lysis. Following electrotransfer (TRANS-BLOT™ Semi Dry™, BioRad, USA) to a nitrocellulose membrane (PROTRAN NITROCELLULOSE, 0.45 μ m, Schleicher & Schuell, Germany), nonspecific antibody binding was blocked in 3% solution of skimmed milk powder in Tris-buffered saline (TBS) with 0.05% Tween-20 for 1 hour. Membranes were then carefully washed and incubated overnight at 4°C with either 1:1000 mouse anti-human calpastatin monoclonal antibody or 1:2000 mouse anti-human actin monoclonal antibody (both from Abcam, Great Britain). After another washing step, the membranes were incubated with 1:2000 HRP-conjugated rabbit polyclonal antibody against mouse IgG for 2 hours. Bound antibodies were detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate™, Thermo Scientific, USA) visualized on the x-ray film. Developed and fixed films were digitalized using the GDS-8000 System and the Labworks Image Acquisition and Analysis Software Version 4.0 (UVP Bioimaging System, UK). Relative *in vivo* calpain activity was semi-quantitatively assessed by the detection and densitometric quantification of protein bands representing the individual degraded fragments accompanied by either the reduced density or lack of bands which represent native calpastatin. The fact that this is a direct result of calpain proteolysis was confirmed by the presence of WB-detectable native calpastatin in the lysates of B-CLL cells treated with calpain inhibitor as described above. Densitometric analysis was performed using the open source ImageJ 1.46r program.

Influence of specific calpain inhibition on B-CLL apoptosis *in vitro*. B-CLL cells obtained as described above were suspended in complete culture medium and treated with either 10 μ M calpain inhibitor II (Ac-Leu-Leu-Met-aldehyde; Calbiochem) or 2 μ M calpain inhibitor IV (Z-Leu-Leu-Tyr-aldehyde; Calbiochem) for 24h at 37°C in 5% CO₂ (Zhu & Uckun, 2000, Mikosik *et al.*, 2015). Calpain inhibitors used in these experiments are likely to also exhibit inhibitory activity against other cysteine proteases, notably including the cathepsins B and L. Published reports indicate that the levels of both cathepsins are lower in malignant cells than in normal B lymphocytes and that they actually have a pro-apoptotic effect on B-CLL cells (Stroev & Boriskina, 1996; Yoon *et al.*, 2013). Still, in order to confirm that the observed pro-apoptotic effect of peptide calpain inhibitors is in fact the effect of specific calpain inhibition, we performed specific knockdown of the μ -calpain gene by RNA interference. In these experiments, B-CLL cells were suspended in AIM-V serum-free 1x medium (GIBCO) at a density of 120,000 cells per 30 μ l of medium. Transfection was carried out following a modified protocol for transfection of suspension cell lines (Qiagen, USA). Each experiment included: cells transfected with a siRNA mixture of 4 types of siRNAs targeting different regions within the mRNA for the large subunit of the human μ -calpain (5' UUG UGU UUG CUG AUG AUC CUA 3', 5' UUC CAU UUG CGG AUG GUC CGG 3', 5' UUU GAC AGC AGU UCC GUG GGA 3', 5' UAA CCA GUA CAC AAG UCC CUG 3') (Qiagen, USA), cells transfected with a non-targeting negative control siRNA (AllStars Negative Control siRNA, Qiagen), as well as non-transfected cells. Dilutions of the negative control siRNA or the μ -calpain targeting siRNA mixtures were prepared from 125 ng (1 μ l; in order to reach a final concentration of 50 nM of siRNA in 200

μ l of cell suspension) of siRNA and 29 μ l of the AIM medium combined in separate wells by gentle pipetting. Either 0.5, 0.75 or 1 μ l of HiPerFect Transfection Reagent liposomes (Qiagen, USA) was then added to the diluted siRNA, the mixtures were gently pipetted and incubated at room temperature for 10 minutes in order to enable the formation of transfection complexes. Next, the transfection complexes were added drop-wise onto selected cells, while 30 μ l of the AIM medium was added to the non-transfected cells and the cell suspensions were gently pipetted up and down approximately 6 times. Cells were incubated with the transfection complexes for 6 hours at 37°C, then 140 μ l of the AIM medium was added to each well and cells were further incubated for 24 or 48 hours.

The proportion of early apoptotic, late apoptotic and necrotic cells in samples which underwent calpain inhibition was assessed by flow cytometry as mitochondrial depolarization (staining with 2 mg/ml 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1, Molecular Probes; (Bedner, Li *et al.*, 1999)) which we have successfully used in our recent study of lymphocyte apoptosis in the bipolar disease (Pietruczuk *et al.*, 2018), as well as by binding of annexin V and 7-AAD (Annexin V PE Apoptosis Kit, BD), respectively.

Influence of calpain inhibition on caspase-3 and -9 activities and on the amount of Bcl-2. The amounts of active caspase-3, caspase-cleaved poly-ADP-ribose polymerase (PARP) and Bcl-2 were measured by the PE-Conjugated Monoclonal Active Caspase-3 Antibody Apoptosis Kit 1, as well as by the quantitative cytometric bead array (CBA) technique (CBA™ Human Apoptosis Kit), both from BD, USA. Active caspase-9 was similarly detected using the Caspase 9 Staining Fluorescent Kit™ (BioGene, UK). All reagents were used according to manufacturers' protocols. In the case of both caspases, the relative amounts of their active forms were calculated as differences between total fluorescence (MFI) of B-CLL cells stained with relevant, active caspase-specific antibody and that of the cells stained with adequate isotype control.

Cytometric comparison of Ca²⁺ levels and calcein signals in resting and IgM-stimulated B-CLL and control B cells. Resting and stimulation-dependent concentrations of Ca²⁺ were assessed by flow cytometry. Resting B-CLL and normal B cells (present simultaneously in samples of patient PBMC and distinguished by the FSC/SSC difference and different surface CD19 staining intensity), were loaded with 3 μ M of fura-3/AM for 30 min at 37°C in the dark, washed in order to remove excess of unbound probe, once again suspended in medium brought to 37°C and fluorescence corresponding to the resting cytoplasmic Ca²⁺ concentration was recorded by FACS for the following 60 seconds. B lymphocytes and B-CLL cells were then stimulated using the anti-IgM antibody (BD) at 1 μ g/ml after which the calcium signal (expressed as the change of FURA-3 fluorescence over time) was recorded over the next 10 minutes. The sample tube was maintained at 37°C throughout the analysis. In order to calculate the actual concentration of Ca²⁺ in the cytoplasm, the maximum and minimum intracellular Ca²⁺ concentration was calibrated by measuring Fura-3 fluorescence of normal and leukemic B cells treated with 0.5 μ M ionomycin (Calbiochem) in the medium containing 1 mM Ca²⁺ and in the same medium containing no added Ca and 1 mM EDTA, respectively.

Statistical analysis. Data were analyzed using Statistica 7.0 (Stat-soft Inc., USA). Student T, Wilcoxon or

Mann-Whitney U tests were used to analyse the statistical significance of the experimental results, depending on the data distribution. Statistical significance was accepted when $p < 0.05$.

RESULTS

Calpain amounts in B-CLL cells relate to B-CLL therapy and staging

We have previously shown, both by Western blot and flow cytometry, that the amounts of μ - and m-calpains are significantly higher in B-CLL cells than in normal circulating B lymphocytes (Witkowski *et al.*, 2002; Mikosik *et al.*, 2007). Here, we compared cell proportions according to μ -calpain content between untreated B-CLL patients and those undergoing standard chemotherapy. Analysis showed that untreated patients generally present similar percentages of CD19⁺CD5⁺ B-CLL cells containing high amounts of intracellular μ -calpain compared to patients treated with chemotherapy (mean \pm S.D.: 86.01 \pm 3.43 *vs* 83.49 \pm 6.45, $p = 0.58$). Moreover, we observed the significantly highest amounts of μ -calpain in untreated patients staged RAI 1 compared to either RAI 0 or cumulated RAI 2-4 (Fig. 1). Interestingly, calpain amounts in the B-CLL cells of chemotherapy-treated patients at RAI 1 stage of the disease were significantly lower than these observed in untreated patients at the same stage of the disease and similar to those observed for other stages of the leukemia.

Interestingly, we have also found a significant ($r = 0.676$, $p < 0.05$) correlation between the proportion of leukemic CD19⁺CD5⁺ cells among peripheral blood leucocytes and the proportion of CD19⁺ lymphocytes containing high (exceeding the mean + 2SD of its value for normal B cells) amounts of μ -calpain in the untreated patients. While most of these patients with high (>50%) proportion of CD19⁺CD5⁺ cells showed also high (>60%) proportion of leukemic B cells containing large amounts of calpain defined as above, in a very few

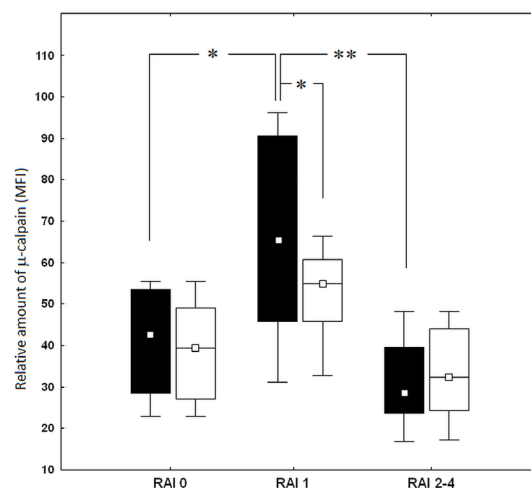


Figure 1. The amounts of μ -calpain in leukemic B-CLL cells differ depending on RAI stage in untreated BCLL patients (■), but not in patients undergoing chemotherapy (□).

Differences in calpain content (shown as corrected MFI, see Materials and Methods for details) between RAI 0 and RAI 1 and between RAI 1 and RAI 2-4 in group A are statistically significant (Mann-Whitney U test, * $p < 0.05$ and ** $p < 0.025$, respectively). The graphs present medians, 1st and 3rd quartiles, and Min/Max.

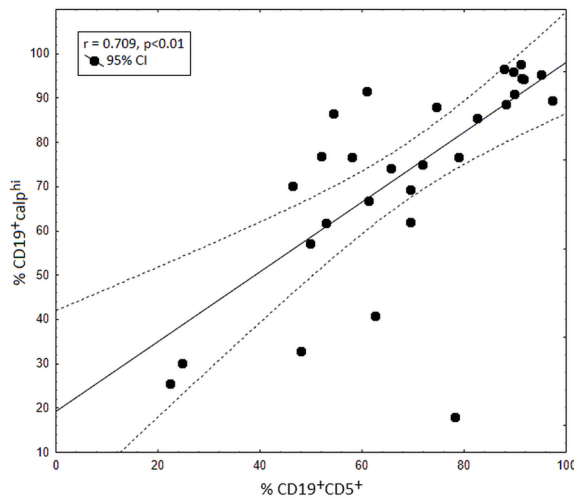


Figure 2. Statistically significant correlation between the proportion of untreated patients' peripheral blood B-CLL CD19⁺CD5⁺ lymphocytes and the percentage of leukemic CD19⁺ cells which contain high amounts of μ -calpain. These CD19⁺calp^{hi} cells were defined as in Materials and Methods. CI – confidence interval.

(3/28) the correlation was negligible and even while the proportion of CD19⁺CD5⁺ cells in their blood exceeded 90%, the proportions of leukemic B cells containing high amounts of calpain did not rise (Fig. 2). Both proportions were higher in Rai 0 and Rai 1 than in Rai 2-4 patients, but the differences had not reached a statistical significance (not shown).

Resting calpain activity is higher in B-CLL cells than in non-malignant B cells

In our paper which first suggested the role of calpains in the resistance of B-CLL cells to apoptosis, we demonstrated an elevated amount of μ -calpain corresponding to a higher *total available* activity of the enzyme in these

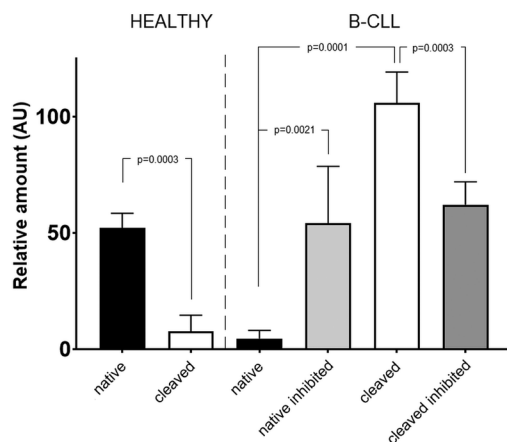


Figure 3. Circulating B-CLL cells exhibit calpain activity *ex vivo* which decreases native calpastatin levels and leads to its degradation.

Calpain activity was assessed by Western blot (WB) detecting the endogenous calpastatin cleavage patterns with calpastatin-specific antibody, as described in Materials and Methods and in the Supplement. Analysis of relative amounts of native calpastatin (native, black bars) and its cleaved fragments (cleaved, white bars) in healthy and B-CLL cells; the effect of calpain inhibition in the B-CLL samples on the relative amounts of native protein (native inhibited) and cleaved fragments (cleaved inhibited) is shown as gray bars. Means \pm S.D. are shown; N=12, Student's *t*-test.

leukemic cells measured by the casein zymography technique (Witkowski *et al.*, 2002). However, this approach does not allow for the detection (and comparison) of the activities of the enzyme in living, circulating B-CLL cells. Thus, in this work we used our previously developed technique for the visualization of products of specific, calpain-dependent proteolysis of calpastatin (Mikosik, Zaremba *et al.*, 2007; Mikosik *et al.*, 2015) to demonstrate that the enzyme is constitutively highly active in B-CLL cells circulating in peripheral blood *in vivo*, although not so much in normal B cells of healthy individuals (Fig. 3). Calpain inhibition with tripeptide calpain inhibitor II led to a significant increase in the amount of native (not cleaved) form of calpastatin, at the expense of its cleaved fragments which, in turn, decreased when the cells were treated with calpain inhibitor prior to lysis.

We have shown earlier that in the normal B cells of healthy elderly (and even centenarians) the relative amounts of calpastatin and μ -calpain measured by flow cytometry are roughly at a stoichiometric 1:1 ratio (Mikosik *et al.*, 2013). We assessed this ratio for B-CLL cell samples and found it to be on average 0.15:1, which compared to control samples is a difference of strong statistical significance ($p < 0.0001$, N=10).

Interestingly, as already mentioned above, we have observed relatively higher amounts of the μ -calpain in the B-CLL cells of patients at the clinical Rai 1 stage of the disease; however, we did not see a parallel increase in the observable intracellular activity of the enzyme, which was uniformly increased in the B-CLL compared to normal B cells (not shown).

Correlation between calpain and phospho – ZAP70 (pZAP-70) expression in B-CLL cells

We have shown before that constitutive calpain activity is associated with and possibly responsible for maintaining significant levels of phosphorylation of NF κ B, PLC γ 1 and p56Lck in resting T cells (Mikosik *et al.*, 2016). In the T cells, ZAP-70 is a member of signaling pathway between the TCR/CD3 complex and PLC γ ; its levels were also shown to correlate with increased phosphorylation of PLC γ and with higher calcium fluxes in the B-CLL cells (Chen *et al.*, 2008). Therefore we investigated if there is a possible correlation between the

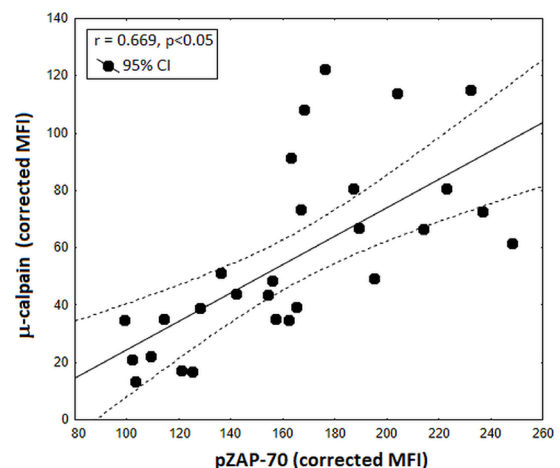


Figure 4. Statistically significant correlation between the amounts of μ -calpain and the phosphorylated form of ZAP-70 (pZAP-70) protein in B-CLL cells of untreated patients (both expressed as corrected MFI, see Materials and Methods for details). CI – confidence interval.

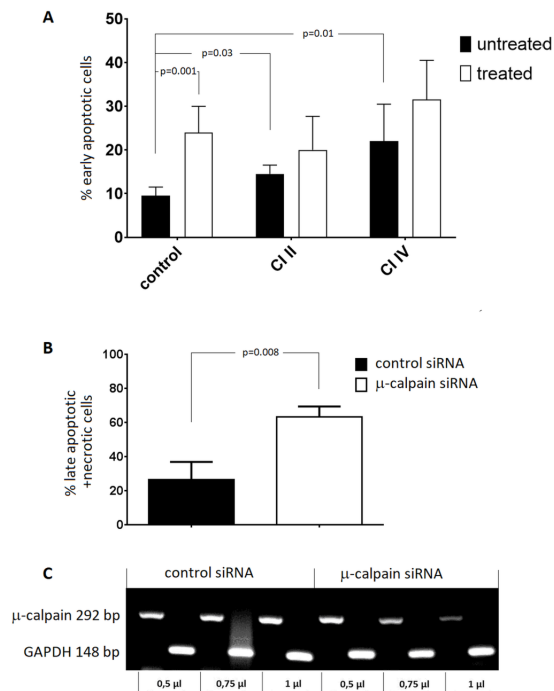


Figure 5. Specific calpain inhibition increases the proportion of early apoptotic B-CLL cells.

(A) B-CLL cells of untreated (black bars, N=28) or treated patients (white bars, N=13) were incubated with calpain inhibitor II (CI II) or calpain inhibitor IV (CI IV) and the proportion of early apoptotic cells (those with depolarized mitochondria) was estimated by flow cytometry of JC-1-stained cells, as described in Materials and Methods and the online Supplement. Means and S.D. values are shown. (B, C) Specific knockdown of the μ -calpain gene with siRNA results in increased apoptosis of B-CLL cells 24 hours after transfection – (B). Both specific and negative control siRNAs were used at 50 nM, and were combined with either 0.5, 0.75 or 1.0 μ l of the liposome solution, the latter being most effective (a representative result of such experiment (one of 3) is shown in (C)). See Materials and Methods for details.

expression of pZAP-70 and μ -calpain in B-CLL cells of the untreated patients and in fact found a strongly significant positive correlation (Fig. 4).

Calpain inhibition affects B-CLL cell apoptosis

Our next goal was to determine whether the observed calpain hyperactivity may be a direct pro-survival and/or anti-apoptotic factor in the B-CLL cells. To this end, we have first assessed how inhibition of calpain activity *in vitro* influences the proportion of apoptotic CD19⁺CD5⁺ cells. Incubation of B-CLL cells *in vitro* with the calpain inhibitors II or IV for 1 hour resulted in significantly increased proportion of early apoptotic B-CLL cells of untreated patients (exhibiting mitochondrial depolarization) (Fig. 5A). Interestingly, the same proportion of B-CLL cells showing early apoptosis was significantly higher in samples obtained from treated patients, and was not further increased by calpain inhibition (Fig. 5A). We noted a statistically significant, negative correlation between the intracellular calpain levels in B-CLL cells and early apoptotic reaction intensity in untreated patients ($r=-0.649$, $p<0.05$, N=28). High initial amounts of calpain in leukemic lymphocytes correlated with stronger mitochondrial depolarization also after 24 hours of incubation with calpain inhibitor, and therefore with a higher final apoptosis level (not shown), suggesting protective, anti-apoptotic effect of the protease activity.

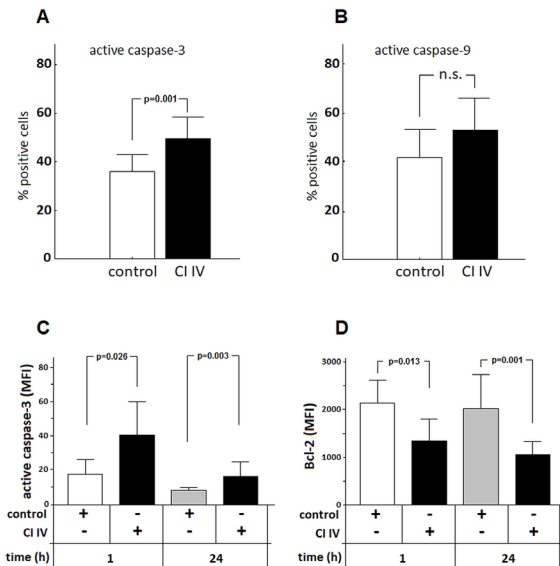


Figure 6. Calpain inhibition in B-CLL cells affects the expression of crucial molecules participating in apoptosis and its prevention.

(A) calpain inhibition with CI IV significantly increases proportions of B-CLL cells expressing active caspase-3; (B) calpain inhibition increases proportions of B-CLL cells expressing active caspase-9. Also, calpain inhibition reciprocally affects the amounts of active caspase-3 (C) and Bcl-2 (D) in B-CLL cells treated with the inhibitor for 1 and 24 hours. Bars indicate means and SD. Exact values of p are shown for statistically significant differences (N=15). See Materials and Methods for details.

The two calpain inhibitors used here are capable of inhibiting both, the conventional μ -calpain as well as m-calpain, with the former being less specific while the latter has been recently described as more specific for m-calpain. We described the expression and activity of m-calpain in the peripheral blood leukocytes and B-CLL cells, although in quantities and total activities similar to those exhibited by normal B cells (Witkowski *et al.*, 2002). Still, in order to ascertain that the pro-apoptotic effect of calpain inhibition is at least in a considerable degree related to the inhibition of μ -calpain, we used gene knockdown by RNA interference involving the use of siRNA specific for CANP-1 (μ -calpain) mRNA. We found that this extremely specific inhibition of μ -calpain resulted in the significantly increased apoptosis of B-CLL cells, despite the knockdown effect being partial, as usually observed in such experiments (Fig. 5B, C).

It is relatively well documented that the decreased propensity of B-CLL cells to undergo apoptosis is associated with over-expression of anti-apoptotic proteins of the Bcl-2 family in these cells (Danilov *et al.*, 2006) and the related decreased activation of caspases, including caspase-3 and -9 (Kitada *et al.*, 1998, Furlan *et al.*, 2010). Calpains have been tentatively described as modifiers of both, the Bcl-2 proteins and these caspases (Łopatniuk & Witkowski, 2011). Thus, it seemed possible that at least one of the mechanisms leading to increased apoptosis of B-CLL cells with inhibited calpains is the modified amount and/or activity of bcl2 or the caspases. In order to verify this hypothesis we looked at the proportions of B-CLL cells expressing detectable amounts of active caspase-3 or active caspase-9, as well as assessed the amounts of Bcl-2, caspase-3 and caspase-9 in B-CLL cells treated with calpain inhibitors. We have found that

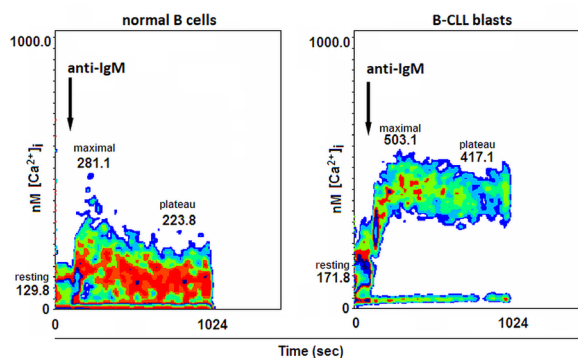


Figure 7. Both the resting levels of cytosolic Ca^{2+} and the anti-IgM-induced calcium signals are increased in BCLL cells compared to normal B lymphocytes.

FACS plots recording Ca^{2+} fluxes from normal $\text{CD}19^{+}$ B lymphocytes (left panel) and $\text{CD}19^{+}$ B-CLL cells (right panel) of a single patient are representative for 3 experiments yielding very similar results. See Materials and Methods for details.

calpain inhibition significantly elevated the proportions of B-CLL cells containing active caspase-3; a similar, but non-significant trend was also seen for caspase-9 (Fig. 6A, B). Calpain inhibition resulted in the significant increase of the amount of active caspase-3, accompanied by the reduction of the amount of cellular Bcl-2, both of which were visible 1 hour, as well as 24 hours after onset of calpain inhibition (Fig. 6C, D). A negative correlation was noted between the initial amounts of μ -calpain and changes in the levels of active caspase-3 and -9 induced by calpain inhibition. This correlation was statistically significant for caspase-3 ($r = -0.9$, $p < 0.05$) but not for caspase-9.

Calpain activity in B-CLL cells may be enhanced by higher amounts of Ca^{2+} as well as increased calcium signals in these cells

Both μ - and m -calpain are capable of proteolysis only in the presence of sufficient amounts of ionized calcium, which is then bound by the EF-hand domains of the enzyme (Lopatniuk & Witkowski, 2011). We have shown that at least some (functionally necessary) calpain activity is detectable in resting human T and B cells. Several reports suggest that even resting B-CLL cells may generate “cell-autonomous” Ca signals, independently of stimulation (e.g. Duhren-von Minden *et al.*, 2012). We have studied possible differences in resting and/or anti-IgM-stimulated concentrations of cytosolic Ca^{2+} from the B-CLL cells and non-malignant B cells present in the same blood samples of untreated patients. Resting Ca^{2+} was on average 30% higher in B-CLL cells than in normal B cells (Fig. 7). Also, anti-IgM stimulation of B-CLL cells resulted in a considerably higher maximum level of cytosolic Ca^{2+} than parallel stimulation of the normal B lymphocytes from patients’ samples. Moreover, extending the stimulation time to 10 minutes retained an average of 89.7 % of the maximum cytosolic Ca^{2+} concentration in leukemic cells (this being 2.5 times the resting Ca^{2+} concentration for these cells), while normal B lymphocytes retained 79.6% of their maximum cytosolic Ca^{2+} (1.7 times the resting value). Thus, calcium signal in B-CLL cells was stronger and sustained longer than that observed in normal B cells, which may be responsible for higher calpain activity in the former.

DISCUSSION

The pathomechanisms of chronic B-lymphocytic leukemia (B-CLL) still remain rather obscure despite decades of study. It is now recognized that in its early, non-lymphomatous form, this relatively mild malignancy results in the characteristic large accumulation of malignant ($\text{CD}19^{+}\text{CD}5^{+}\text{CD}23^{+}$) cells in the periphery, not due to their extreme proliferation, but rather to their avoidance of apoptosis (Danilov *et al.*, 2006). To a variable extent this characteristic is associated with mutations in genes encoding the B-cell receptor BCR and the variable region of the B-cell-receptor heavy chain IgVH (Grevet *et al.*, 2007). Still, the mechanism of apoptosis avoidance in B-CLL cells remains unclear. In our earlier studies we demonstrated that circulating B-CLL cells contain high amounts of the enzyme μ -calpain – a cytosolic, neutral, ubiquitous, strongly Ca-dependent cysteine protease – due to the increased transcriptional activity of its gene, and that inhibition of the enzyme might increase the intracellular availability of caspase-3 which suggested that it may be involved in the protection of B-CLL cells from apoptosis (Witkowski *et al.*, 2002). μ -calpain is implicated in mechanisms controlling cellular proliferation and apoptosis in various cells. However, due to the limited proteolytic cleavage of such important biomolecules as caspase-3, the X-linked inhibitor of apoptosis protein (XIAP) or the mitochondrial Apoptosis Inducing Factor (AIF), this enzyme is better known for its pro-apoptotic properties (Lopatniuk & Witkowski, 2011). We hypothesized that hyperactivation of calpain may in fact protect B-CLL cells from apoptosis, e.g. by caspase-3 or p53 degradation as well as by the release of $\text{NF}\kappa\text{B}$ from I κB and its activation, leading to the induction of genes coding for anti-apoptotic proteins (Lopatniuk & Witkowski, 2011; Mikosik *et al.*, 2016). The latter effect of calpain could, at least in theory, lead to elevated proliferation rates of bone marrow precursors of the mature $\text{CD}19^{+}\text{CD}5^{+}$ B-CLL cells. However, this possibility is hard to study as these peripheral, mature leukemic cells do not proliferate and rather undergo spontaneous apoptosis *in vitro*. Interestingly enough, a recent paper had demonstrated a link between higher expression of ZAP-70 and activation of $\text{NF}\kappa\text{B}$ (Pede *et al.*, 2013); we could speculate that excessive calpain activity (that we show here for B-CLL cells) may promote (or link) both cellular events, as we have earlier demonstrated the role of calpain activity in the phosphorylation (i.e., activation) of $\text{NF}\kappa\text{B}$ (Mikosik *et al.*, 2016).

We clearly demonstrate here that proteolytic activity of μ -calpain present in excess in circulating B-CLL is evident in these malignant, but otherwise unstimulated cells, and is significantly elevated as compared to normal B cells. This activity was measured towards calpastatin, the natural intracellular inhibitor and substrate of μ -calpain. We may conclude this observation by stating that the balance between activation and inhibition of the enzyme, which is typical for non-neoplastic cells (Lopatniuk & Witkowski, 2011), is therefore derailed by practically complete obliteration of its native, intracellular inhibitor. Apart from concerns regarding possible consequences and clinical associations of this activity, this observation introduces the fundamental question how is it possible for μ -calpain to be active in B-CLL cells since according to the classical description of this enzyme it requires at least micromolar concentrations of Ca^{2+} for performing proteolysis. A possible explanation may be found in a recent report of the so-called “cell-autonomous” (stimulation-independent) calcium signals

in B-CLL cells (Duhren-von Minden *et al.*, 2012). Our observations (Fig. 7) seem to corroborate and confirm this report, showing higher average resting levels of Ca^{2+} in B-CLL cells, while additionally showing that BCR stimulation of B-CLL cells yields a significantly higher maximum rise of cytosolic Ca^{2+} and that this signal is prolonged compared to normal B lymphocytes. In fact, “calcium spikes” with high local Ca^{2+} concentrations had already been described for some malignant cell types, including B-CLL cells, where the required machinery in the form of STIM-ORAI calcium channels similar to these seen in normal B and T cells exists (Debant *et al.*, 2015; Mikosik *et al.*, 2016). This suggests that conditions required for the spontaneous, sustained, and in consequence anti-apoptotic activity of μ - and possibly even m-calpain in circulating B-CLL cells may in fact be obtained. Using an alternative method demonstrating cleavage of a fluorescent calpain substrate, we have demonstrated such constitutive calpain activity even in the nonmalignant, resting human peripheral blood T and B cells (Mikosik *et al.*, 2016). Lack (or at most very little) of visible product of calpain activity (cleaved calpastatin) in the control B cells of age-matched healthy individuals (Fig. 3) may reflect lower actual spontaneous activity of the enzyme in the resting B cells compared to B-CLL cells.

Furthermore, in this work we have confirmed that the expression and/or activity of calpain in B-CLL cells may be directly responsible for their reluctance to undergo apoptosis. The mechanisms responsible for this phenomenon most likely include the proteolytic activity of calpain against caspase-3, -9, and Bcl-2, as calpain inhibition affects the levels of all three of these proteins, notably reducing expression of Bcl-2 and elevating the expression of active caspases in the B-CLL cells in which calpain was inhibited even for just one hour. Observed correlations between intracellular calpain levels/activities and the reduced levels of active caspases -3 and -9 suggest a direct relation between the hyperactivity of μ -calpain and the inhibition of the apoptotic pathway observed in B-CLL cells. Interestingly, this effect was not observed in the group of patients undergoing chemotherapy which may indicate that chemotherapy and calpain inhibition both affect the same proapoptotic pathways in a non-synergistic way. Some data indicate that m-calpain (which we have shown to be active in the B-CLL cells, but not at the amount and activity levels different from these observed in healthy B cells) can be inhibited by Gas2, and in some cells this may increase their susceptibility to apoptosis (Benetti *et al.*, 2001; Niapour *et al.*, 2012; Sun *et al.*, 2015). Interestingly, relation between Gas2 and m-calpain was shown for both, the acute myeloid and lymphoid leukemias, pointing at a possibility that targeting Gas2 would increase the leukemic cells’ apoptosis. So far, there are no published data suggesting the involvement of Gas 2 in B-CLL leukemogenesis and specifically in the apoptosis escape of B-CLL cells, and in the light of our findings (mainly on the role of μ -calpain, and not m-calpain in B-CLL cell biology) we believe this to be rather unlikely until proven otherwise.

The anti-apoptotic activity of calpains in B-CLL cells is all the more interesting when put in the context of clinical aspects of the disease, especially RAI staging and chemotherapy. As shown in Fig. 1A, the highest calpain expression is observed in cells of untreated patients at the RAI 1 stage. However, this high amount of calpain was not accompanied by significantly higher endogenous activity of the enzyme at this disease stage. The mecha-

nism of this phenomenon is unclear. In our opinion, the main reason behind discrepancy between calpain amount and activity in the B-CLL cells from various clinical stages, may be the well-known lack of direct relation between the calpain amount in the cytoplasm and its actual activity. Thus, it is possible that B-CLL cells from some cases would contain high amounts of the enzyme (e.g. due to increased transcription and synthesis, which we described already in 2002; Witkowski *et al.*, 2002), but the enzyme may be either highly active or not, depending on the local conditions in the cells. These conditions include first of all the availability of ionized calcium (Ca^{2+}) and we show here that both, the resting and stimulated levels of Ca^{2+} in the cytoplasm of B-CLL cells are higher than these observed in normal peripheral blood B cells, potentially promoting the endogenous activity of the enzyme. High amounts of detectable calpain in the RAI 1 cells would then be the consequence and illustration of two factors. One would be likely its correlation with amounts of pZAP-70 (Fig. 4); active (phosphorylated) ZAP-70 may stimulate pro-survival and anti-apoptotic pathways in the B-CLL cells, possibly including the stimulation of calpain expression (although this latter possibility would require further studies). On the other hand, it is known that ZAP-70 itself is a substrate degraded by calpain, at least in activated T cells. High and correlated amounts of both, as shown here, would suggest that either the enzyme is not very active in these cells (which is unlikely, as we did show uniformly high calpain activity in the B-CLL cells from all cases studied) or that its phosphorylation may depend on the calpain activity. In fact, we have shown such a relation between the calpain activity and amounts of other phosphorylated signaling molecules in the normal T cells, so the latter possibility seems plausible (Mikosik *et al.*, 2016). Another reason for high levels of calpain in RAI 1 cells may paradoxically be associated with its high turnover: higher than normal production associated with relatively lower self-degradation due to activation of the enzyme. An interesting possibility for explaining the deregulation of the calpain-calpastatin system in B-CLL cells has recently emerged from the studies of the role of different micro-RNAs (miRNAs) in the development of B-CLL. Papers reporting the results of such studies also mention (among many others) miRNA17 and miRNA150 as increasing and decreasing in the B-CLL cells, respectively (Ntoufa *et al.*, 2016; Szurian *et al.*, 2017). While studying changes in the miRNAs in human blood lymphocytes in aging, we have found that levels of miRNA 17-3p increase with aging and correlate with increasing levels of μ -calpain mRNA; we see the opposite for miRNA 150-5p, which targets the CAST gene encoding miRNA for the endogenous calpain inhibitor – calpastatin (Frackowiak *et al.*, in prep). We could speculate that modified amounts of miRNA 17 and 150 in B-CLL cells may act *via* modifying the ratio of calpain to calpastatin. We could further hypothesize that there is an evolution of the calpain amount with disease progression, possibly serving to maintain high endogenous calpain activity, which in turn would be responsible for decreasing the B-CLL cells’ susceptibility for apoptosis by removing crucial caspases (as we have first shown for caspase 3 (Witkowski *et al.*, 2002), and repeated and extended for other elements of the pro- and antiapoptotic pathways in the current work). We recently proposed such a scenario for the role of calpain activity in lymphomas (Witkowski, 2018). However, based on our observation of the necessary role of spontaneous calpain activity for the proliferation of normal lymphocytes, we may hypothesize that

its increased activity in the B-CLL cells promotes their early proliferation while in the BM, more evident at the RAI 1 stage. Interestingly, when comparing patients at the RAI 1 stage of the disease, the calpain levels are significantly lower in the chemotherapy-treated cohort compared to the untreated patients.

This may be explained by the apoptotic response induced by chemotherapeutic agents in the B-CLL cells of treated patients. It may be possible that the population of B-CLL lymphocytes with high calpain content (characteristic for the RAI 1 stage) is more susceptible to chemotherapy-induced apoptosis and that therapy directly influences metabolic pathways of μ -calpain synthesis in B-CLL. Presumably, chemotherapeutics somehow reduce calpain activity or expression and, consequently, decrease the effect of inhibitors of this enzyme sought in vitro. These results may shed light on the mechanism of the effect observed in vivo regarding certain antileukemic drugs, as the actual role of some of them and the exact mechanism(s) through which they decrease the survival of B-CLL cells in patients still remain to be investigated.

The statistically significant relationship between μ -calpain content and either RAI stages or ZAP-70, suggests its utility as an alternative for IgVH mutational status. Although various other factors, such as ZAP and p38, prove to be a useful tool, none are thought to be fully reliable, and ZAP70 alone raised great concerns as to its accuracy due to its variability rate ranging between 77% and 94% of concordance with the Ig gene status (Crespo *et al.*, 2003; Wiestner *et al.*, 2003; Wiestner, 2005; Rassenti *et al.*, 2008; Doubek *et al.*, 2011; Hamblin, 2011). Interestingly, there are reports demonstrating that ZAP-70 is actually cleaved and degraded by calpains both intracellularly, as well as *in vitro* (in cell-free experiments) (Penna *et al.*, 1999). The same report also suggests that phospho-ZAP-70 may undergo calpain-dependent degradation, although it does not present relevant results. On the other hand, Zhang and others (Zhang *et al.*, 2011) had recently reported that in murine B cells, calpain may lead to their hyper-responsiveness resulting from the increased level of pZAP-70 due to cleavage of the Lyp phosphatase (Zhang *et al.*, 2011). Our results, demonstrating a positive correlation between pZAP-70 and calpain in B-CLL cells, seem to confirm the latter observation. As already mentioned above, even in resting lymphocytes calpain activity is promoting the phosphorylation of NF κ B, the transcription factor which is also important for the proliferation of B-CLL cells and functionally associated with the activity of ZAP-70 (Ruiz-Lafuente *et al.*, 2014; Seda & Mraz, 2015). That way, excessive calpain activity in the B-CLL cells may not only prevent their apoptosis, but actively promote their proliferation by amplifying the pro-proliferative signals, as suggested above. On the other hand, elevated amounts of ZAP-70 may be themselves conducive to increased activity of calpain in B-CLL cells, via their association with greater intracellular calcium influx (Chen *et al.*, 2008). Recently, the notion of calpain activity involvement in protection of chronic leukemia/lymphoma cells from apoptosis has been lent support by a paper, where such relation was demonstrated in Burkitt's lymphoma cell line BL2 and the transgenic murine E μ -myc lymphoma model (Li *et al.*, 2012). This suggests again, that the anti-apoptotic role of excessive calpain activity may be more universal in the hematological malignancies.

The two forms of the disease identified on the basis of ZAP-70 expression/IgVH mutational status analysis also differ with regard to the postulated place of origin

and maturation in lymph nodes (Chiorazzi & Ferrarini, 2011). The first B-CLL type presumably rises from a pregerminal, immature cell characterized by unmutated variable heavy chain genes, while the second, postgerminal type exhibits somatically mutated VH genes (Damle *et al.*, 1999; Hamblin *et al.*, 1999; Chiorazzi & Ferrarini, 2011). Calpains have been long known to play an important role in B-lymphocyte maturation (Ruiz-Vela *et al.*, 1999). Our results might possibly explain the physiological link between calpain and B-CLL development.

Summarizing, our data presented here provide sound evidence for the involvement of μ -calpain activity in the pathomechanism of B-CLL, propose molecular mechanisms of this involvement and prove this enzyme to be a potential target in the treatment of this malignancy, especially at its early Rai stages.

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Authorship

JMW authored the project hypothesis and aims, designed the experiments, coordinated the studies, analyzed data, formulated the conclusions, wrote the final version of the manuscript. PL, ZP, AM and AD performed most of the experiments and analyzed data. AM and AH recruited and characterized the patients and healthy controls and provided clinical (staging) data. JF designed and performed the siRNA experiments. EB and PL analyzed data and participated in drafting the manuscript. TF critically participated in the writing and revision of the final version. All authors reviewed and accepted the manuscript.

Conflict of Interest Disclosure

The author(s) declare no competing interests.

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