In vivo, *ex vivo* and *in vitro* dasatinib activity in chronic lymphocytic leukemia

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Abstract. Dasatinib inhibits the breakpoint cluster region-Abelson murine leukemia 1 (*BCR-ABL1*) gene along with other kinases known to be overexpressed and abnormally active in patients with chronic lymphocytic leukemia (CLL). The current study used primary leukemic cells obtained from 53 patients with CLL that were treated with dasatinib. A 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxanilide (XTT) assay and Annexin V staining was performed to assess the cytotoxic effects of dasatinib treatment. The XTT assay revealed that the median cytotoxicity of dasatinib was 8.30% (range, 0.00-77.89%). Due to high dispersion of dasatinib activity, patients were divided into sensitive (n=27; 50.94%; median cytotoxicity, 22.81%) and resistant groups (n=26; 49.06%; median cytotoxicity, 0.00%). A median cytotoxicity of 8.30% was selected as a cut off value. Using Annexin V staining and flow cytometry on exemplary sensitive and resistant CLL samples, it was revealed that 17.71 and 1.84% of cells were apoptotic, respectively. The current study presented a case of a patient with concomitant occurrence of CLL and chronic myeloid leukemia (CML) with a major molecular response after dasatinib treatment. A simultaneous reduction of circulating CLL cells indicated *in vivo* anti-CLL activity induced by dasatinib. After an *in vitro* culture of the patient's mononuclear cells with subsequent dasatinib treatment, a higher percentage of CLL cells undergoing apoptosis was obseveed when compared with untreated samples (38.19 vs. 21.99%, respectively). Similarly, the percentage of CLL apoptotic cells ($\Delta\Psi m^{low}$) measured by chloromethyl-X-rosamine was higher after incubation with dasatinib (7.28%) than in the negative control (2.86%). In conclusion, dasatinib induced antileukemic effects against CML and CLL cells. The results of the current study indicated that dasatinib may induce apoptosis *ex vivo*, *in vitro* and *in vivo* in CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with a number of markers used for patient risk stratification. The most commonly utilized in clinical practice are: i) deletion of chromosome arms17p and 11q, ii) *TP53* and immunoglobulin heavy chain variable region (*IGHV*) genes mutations as well as iii) protein expression of the zeta chain associated protein-70 (ZAP-70) and CD38 (1-3).

Since CLL is a malignancy of B lymphocytes, the role of signaling through B-cell receptor (BCR) and microenvironment stimulation seems to be crucial in the pathogenesis of the disease (4,5). The inhibition of BCR signaling by targeting Bruton's tyrosine kinase (BTK) or phosphatidylinositol 3 kinase (PI3K) led to durable remissions and prolonged progression-free and overall survival even in CLL patients with negative prognostic features. Despite those advances in CLL therapy, there is still a group of relapsed and/or refractory patients, therefore, the need to investigate novel treatment options still exists (6). CLL cell survival is driven by overexpression and abnormal activity of several non-receptor tyrosine kinases, members of Src family kinase (SFK)-LYN, SYK, and c-ABL tyrosine kinase (4,7,8). It was proven that inhibition of c-ABL or LYN/SYK kinases with specific tyrosine kinase inhibitors (TKI) induces apoptosis of CLL cells. Thus targeting particular kinases might be a promising option for CLL therapy (4,9-12).

Dasatinib is a second generation inhibitor of breakpoint cluster region-Abelson murine leukemia 1 (BCR-ABL1) kinase approved in 2006 by the Food and Drug Administration in the treatment of chronic myeloid leukemia (CML) and chromosome Philadelphia-positive acute lymphoblastic leukemia (ALL) patients (13). Dasatinib is a multiple kinases

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inhibitor with significant activity against tyrosine kinases that are known to be important in the pathogenesis of several hematological malignancies and solid tumors. Low concentrations of dasatinib have been shown to inhibit not only BCR-ABL1, but also c-KIT, platelet-derived growth factor receptor (PDGFR), BTK, TEC, LYN, and other SFK. In CLL, increased SFK and c-ABL kinases activity was reported, giving thereby a rationale to evaluate dasatinib antileukemic activity in CLL (6,14,15). *In vitro* studies showed that dasatinib also inhibits anti-apoptotic proteins that are overexpressed in CLL, namely Bcl-xL, Bf1/A1 and Mcl-1 (16). Furthermore, this inhibitor contributes to a reduction of cytoskeletal activity by its interaction in the LYN/HS1 pathways (17,18). Moreover, we found earier, that dasatinib might target similar genes as thalidomide-antiangiogenic agent with proven antileukemic activity in CLL (19).

It was previously reported that dasatinib at a concentration of 5 μM was able to induce apoptosis in ZAP-70 positive CLL patients with unmutated *IGHV* genes (4). Moreover, Amrein *et al* (15) showed that CLL lymphocytes with del17 were highly sensitive to dasatinib when compared with CLL lymphocytes without del17 (median IC_{50} values of 0.1 and 34 μM, respectively). Meanwhile, other groups demonstrated that dasatinib in nanomolar concentrations induced 50% inhibition of SFK kinases causing significant apoptosis of CLL cells disregard of prognostic markers (8,9,20-22).

Dasatinib activity in CLL remains unclear. Moreover, there is a lack of consistent data regarding which group of patients could benefit most from therapy with this TKI. Therefore, we aimed to evaluate the effect of dasatinib *in vitro* in a cohort of 53 CLL patients. Moreover, as a proof of concept, we present the detailed *ex vivo* and *in vivo* analyses of dasatinib activity in a patient with concomitant CML and CLL who is being successfully treated with dasatinib until date.

Materials and methods

Patients. Peripheral blood and bone marrow samples were obtained from 53 patients diagnosed with CLL and a patient diagnosed with CLL and CML at the Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin, Poland. Table I summarizes the clinical characteristics of CLL patients. The study was approved by the Ethics Committee of the Medical University of Lublin (nos. KE-0254/269/2019 and KE-0254/305/2019).

Cell isolation. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were isolated by Ficoll (Biochrom AG) density gradient centrifugation. The viability of cells was evaluated by Trypan blue staining (Sigma-Aldrich; Merck KGaA) and quantified in a Neubauer chamber (Zeiss GmbH).

RNA extraction and reverse transcription. Total RNA was extracted using QIAamp RNA BloodMini Kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of isolated RNA were determined using spectrophotometer BioSpec-nano Micro-volume UV-Vis (Shimadzu). From each sample, 2 μg of total RNA was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System

for RT-PCR (Thermo Fisher Scientific, Inc.) and Veriti Dx 96-Well Thermal Cycler (Thermo Fisher Scientific, Inc.).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for BCR-ABL1. Quantitative analysis of *BCR-ABL1* gene expression was performed according to European Leukemia Net and EUTOS standards for monitoring a TKI therapy in CML (23). Two separate reaction mixtures providing amplification of *BCR-ABL1* fusion gene and Abelson (*ABL*) control gene were prepared. The following primers and probes were used: 5'-TCCGCTGACCATCAATAAGGA-3', forward primer *BCR-ABL1*; 5'-CACTCAGACCCTGAGGCTCAA-3', reverse primer *BCR-ABL1*; 5'FAM-CCCTTCAGCGGCCAGTAGCATC TGA-3'TAMRA, probe *BCR-ABL1*; 5'-TGGAGATAACACTCT AAGCATAACTAAAGG-3', forward primer *ABL*; 5'-GATGTA GTTCTTGGGACCCA-3', reverse primer *ABL*; 5'FAM-CCATTT TTGGTTTGGGCTTCACACCATT-3' TAMRA, probe *ABL*. The qRT-PCR reaction was performed on the 7500 Fast Dx Real-Time PCR instrument (ThermoFisher Scientific, Inc.), using 5 μl cDNA, gene-specific primers, molecular probe and TaqMan® Universal PCR Master Mix (ThermoFisher Scientific, Inc.) in 25 μl end volume. Thermocycling program was set for 50 cycles of 2 min at 50°C, 10 min at 95°C, 15 sec at 95°C, 60 sec at 60°C. Quantitative analysis of *BCR-ABL1* and *ABL* gene expression was performed using a calibration curve prepared by serial dilutions of BCR-ABL1 Mbcr Standards and *ABL* Control Gene 3 Standards for a known *BCR-ABL1* and *ABL* copy number. *BCR-ABL1* copy number was normalized to *ABL*. *BCR-ABL1*/*ABL* ratio expressed as a percentage (%) was calculated. The result was multiplied by laboratory-specific conversion factor (CF) and expressed using International Scale (IS).

Analyses of SF3B1, NOTCH1, MYD88 L265P and IGHV mutations. Genomic DNA was isolated from PBMCs using QIAamp DNA BloodMini Kit (Qiagen) according to the manufacturer's recommendation. Detection of *SF3B1*, *NOTCH1* and *MYD88* L265P mutation, and *IGHV* mutation status were assessed as described earlier in detail (24). A cutoff of 98% germline homology was used to assess *IGHV* mutation status. The sequences with a germline homology of 98% or higher were considered unmutated and those with a homology <98% were considered mutated.

Immunophenotypic analysis. The immunophenotypic analysis was performed by flow cytometry. In our study, the standard diagnostic flow cytometric analysis included monoclonal antibodies (MoAbs) anti-CD5-FITC/CD19-PE, anti-CD19-FITC and anti-CD23-PE (BD Biosciences). A standard, whole-blood assay with erythrocyte cell lysis was used for preparing the peripheral blood specimens. The samples were analyzed by flow cytometry directly after preparation. For data acquisition and analysis, a FACSCalibur instrument (Becton-Dickinson and Company) with CellQuest software (Becton Dickinson and Company) was used. For each analysis, 10,000 events were acquired and analyzed. The percentage of positive cells was measured from a cut-off set using isotype-matched nonspecific control antibody. Evaluation of ZAP-70 expression in CD19+/CD5+ leukemic cells in CLL samples was performed as previously described (25). Flow cytometric analysis of CD38 on leukemic cells was performed on PB samples using monoclonal antibodies: anti-CD19 FITC,

Table I. Clinical characteristics of patients.

Characteristic	N (%)
Age (years)	
Median	65
Range	47-84
Sex	
Female	33 (62.26)
Male	20 (37.74)
Rai stage	
0	22 (41.51)
I	13 (24.53)
II	8 (15.09)
III	3 (5.66)
IV	3 (5.66)
Not available	4 (7.55)
WBC (x10^9/l)	
Median	28.8
Range	3.70-144.00
LDH (IU/l)	
Median	384
Range	258-961
B2M (mg/l)	
Median	2.76
Range	1.61-9.49
ZAP-70 (cut-off 20%)	
Positive	30 (56.60)
Negative	12 (22.64)
Not available	11 (20.76)
CD38 (cut-off 30%)	
Positive	11 (20.75)
Negative	35 (66.04)
Not available	7 (13.21)
IGHV mutational status	
Mutated	15 (28.30)
Unmutated	15 (28.30)
Not available	23 (43.40)
Cytogenetics	
del11q	2 (3.77)
del13q	8 (15.10)
del17p	2 (3.77)
Normal karyotype	12 (22.64)
Not available	29 (54.72)
NOTCH1 mutational status	
Mutated	2 (3.77)
Unmutated	20 (37.74)
Not available	31 (58.49)
SF3B1 mutational status	
Mutated	0 (0.00)
Unmutated	28 (52.83)
Not available	25 (47.17)
MYD88 L265P mutational status	
Mutated	0 (0.00)
Unmutated	24 (45.28)
Not available	29 (54.72)

WBC, white blood cells count; LDH, lactate dehydrogenase; ZAP-70, zeta-chain-associated protein kinase 70; CD, cluster of differentiation; IGHV, immunoglobulin heavy chain variable region; B2M, β2-mikroglobulin; Del, deletion; NOTCH1, neurogenic locus notch homolog protein 1; MYD88, myeloid differentiation primary response gene 88; SF3B1, splicing factor 3B subunit 1.

anti-CD38 PE and anti-CD5 PE-Cy5 (BD Pharmingen). The cut-off for positivity of leukemia cells for ZAP-70 expression was ≥20%, while for CD38 was ≥30%.

Routine laboratory results such as white blood cell count (WBC), lactate dehydrogenase level (LDH) and B2M (β2-mikroglobulin), and cytogenetic and FISH analyses of CLL patients were accessed from the hospital laboratory at the first admission.

In vitro cytotoxicity assay with XTT dye. The cytotoxic effect of dasatinib was measured using *in vitro* 2,3-bis-(2-m ethoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxani-lide (XTT)-based method (Sigma-Aldrich; Merck KGaA). PBMCs were suspended in X-VIVO w/o Phenol red and Genatamycin (Lonza) and added on a 96-well plate at a concentration of 5x10^5 cells/100 μl/well. Dasatinib at a final concentration of 180 nM, reflecting plasma drug concentrations observed in a clinical setting, was added to experimental wells. As a negative control, only live cells were used, positive control consisted of cells treated with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA). Twenty-five microliters of XTT reagent was added to all samples. Plates were incubated for 24 h in a humidified atmosphere with 5% CO_2 at 37°C. Optical densities (OD) were measured at 450 nm with a VICTOR3 1420 multilabel counter (PerkinElmer, Inc.), as a background wavelength at 690 nm was used. Each sample was performed in triplicates. Cytotoxic effect was calculated as below: cytotoxicity = [1-(OD_s-OD_b)/(OD_c-OD_b)] x 100%, where: ODs is an OD of assayed sample, ODb is an OD of positive control and ODc is an OD of live cells (negative control).

Cell culture. The PBMCs of a patient diagnosed with CLL and CML were incubated at a concentration of 2x10^6 cells/ml for 24 h with 180 nM of dasatinib and without dasatinib in a control sample. Cells were incubated in a standard medium consisting of RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin and 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. After cell culture, the percentage of apoptotic cells within CD19$^+$ cells was measured using flow cytometry.

Apoptosis analyses. The percentage of apoptotic cells was measured after 24 h incubation with 180 nM of dasatinib and compared to non-treated samples. Two methods were used for apoptosis assessment:

i) Annexin V staining of CLL samples and CLL/CML patient. The analysis was performed using Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. PBMCs were washed with PBS (Biochrom AG), suspended in a binding buffer provided and stained with 5 μl of Annexin V-FITC. The PBMCs were incubated for 10 min in the darkness and immediately analyzed on a FACSCalibur (BD Biosciences).

ii) Mito Tracker Red CMXRos (Molecular Probes) technique used on CLL/CML patient. Chloromethyl-X-rosamine (CMXRos) is a cationic lipophilic fluorochrome that does not accumulate in depolarised mitochondria and thus can be used to detect disruptions in the mitochondrial membrane potential (ΔΨm). PBMCs were incubated with CMXRos for 30 min
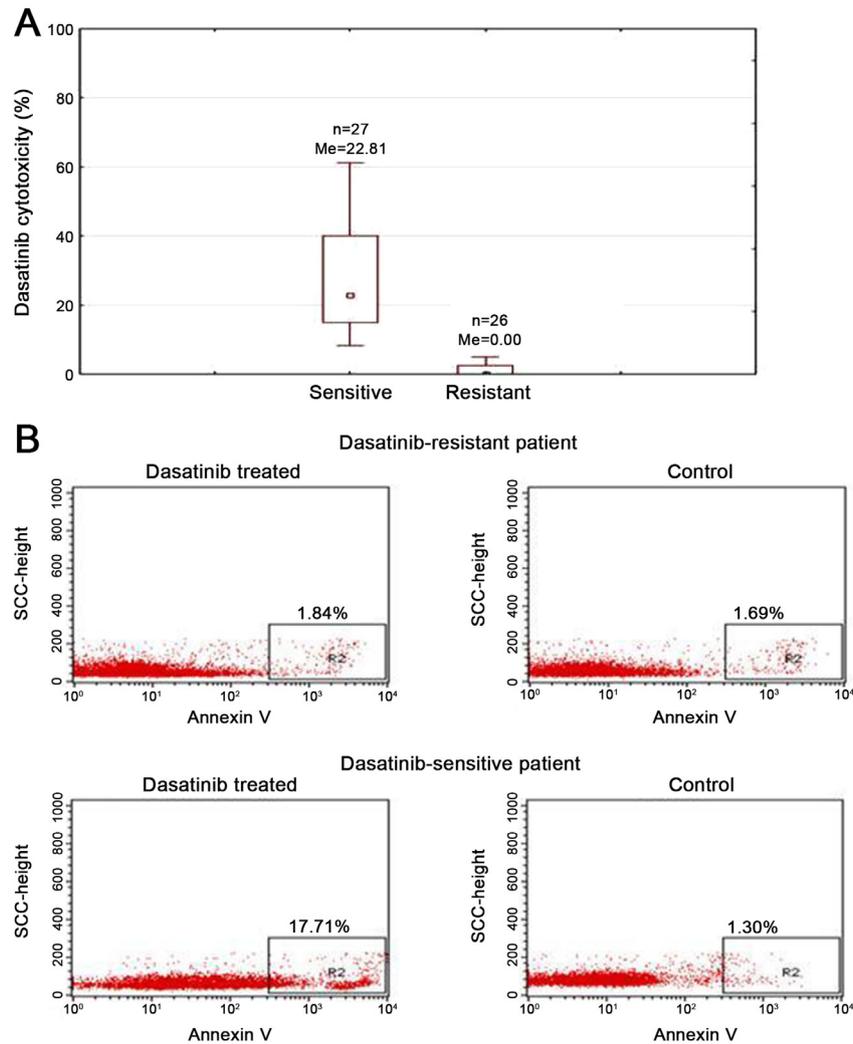
Figure 1. Response of patients with chronic lymphocytic leukemia after treatment with 180 nM dasatinib for 24 h. (A) Dasatinib cytotoxicity among sensitive and resistant patients evaluated using a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide test. (B) Apoptosis analysis of dasatinib resistant and sensitive patients. In each graph, the R2 quadrant represents apoptotic cells. Me, median; SSC, side scatter.

at 37˚C and analyzed on a FACSCalibur. Cells considered to be apoptotic displayed a decrease in mitochondrial membrane potential in CMXRos staining ($\Delta\Psi m^{low}$).

The collapse of $\Delta\Psi m$ is a marker of early apoptosis, preceding other hallmarks of cell death, such as DNA fragmentation or phosphatidylserine externalisation (detected by Annexin V).

Statistical analysis. Statistical analysis was performed using STATISTICA 12 program (StatSoft Polska Sp. z o. o.). All results are presented as median values with range. The Mann-Whitney U test was used to evaluate the differences between subgroups of patients. Correlations of variables were calculated with the Spearman rank correlation coefficient.

Results

Dasatinib induces cytotoxic effect of CLL cells. To assess the cytotoxic effect of dasatinib on primary CLL cells, we performed XTT test. Freshly isolated PBMCs of 53 CLL patients were incubated with 180 nM of dasatinib for 24 h. Observed median cytotoxicity of dasatinib was 8.3% and

it varied between individual cases (range: 0-77.89%). In 10 patients (18.87%) obtained dasatinib cytotoxicity was exceeding 30%. The cytotoxicity between 20-30% was observed in 7 patients (13.21%). In 9 patients (16.99%) dasatinib cytotoxicity ranged from 10 to 20%. In 27 cases (50.93%) the cytotoxicity was <10%. Due to high dispersion of dasatinib activity, we have divided analyzed group into sensitive (n=27, 50.94%, median cytotoxicity: 22.81%) and resistant patients (n=26, 49.06%, median cytotoxicity: 0%) using median cytotoxicity of 8.3% as a cut off value (Fig. 1A).

Dasatinib cytotoxicity correlates with β2-microglobulin levels. To identify patients who will benefit most from dasatinib therapy, we correlated the dasatinib activity with CLL prognostic factors. Statistical analysis revealed a positive correlation of dasatinib cytotoxicity with β2-microglobulin serum levels (R=0.317; P=0.041). We observed no correlations between dasatinib activity and white blood cell count, lactate dehydrogenase level or age of patients.

Dasatinib cytotoxicity in different prognostic subgroups. We observed a lack of differences in dasatinib cytotoxicity

in different stages of CLL, according to Rai classification (P=0.823). Rai stage 0 patients displayed the median cytotoxicity of dasatinib of 14.87% (range: 0-59.18%, n=22). In Rai stage I the median cytotoxicity of dasatinib was 7.46% (range: 0-36.90%, n=13). A similar result was observed in cases with Rai stage II-median: 7.36% (n=8; range: 0-77.89%). In groups with Rai stage III (n=3) and IV (n=3) the median dasatinib cytotoxicities were 10.92% (range: 0-46.22%, n=3) and 10.59% (range: 0.51-49.31%, n=3), respectively. We also found no significant differences of dasatinib activity depending on the sex of patients: Among women dasatinib cytotoxicity was 14.73% (range: 0-61.14%, n=33), while in group of men 6.61% (range: 0-77.89%; n=20, P=0.442). The effect of dasatinib on CLL primary cells was independent of CD38 (P=0.837) as well as ZAP-70 expression (P=0.404). Among CD38 and ZAP-70 positive cases median TKI cytotoxicity was 10.59% (range: 0-77.89%, n=11) and 18.93% (range: 0-77.89%, n=12), in cohorts assigned as CD38 and cases ZAP-70 negative we observed median cytotoxicity of 14.27% (range: 0-61.14%, n=35) and 9.58% (range: 0-61.14%, n=30), respectively. We also observed no differences in dasatinib response depending on the mutation status of *IGHV* genes (P=0.901). In the group with unmutated *IGHV* genes median dasatinib cytotoxicity was 17.76% (range: 0-77.89%, n=15), likewise to patients with mutated *IGHV* genes the median of 15.02% (range: 0-61.14%, n=15). Similarly, there were no statistical differences in the dasatinib effect in subgroups with different cytogenetic abnormalities (P=0.826). Mean cytotoxicity in cases with del11q was 4.29% (range: 4.13-4.46%, n=2) and in patients with del17p was 2.66% (range: 0-4.13%, n=2). In patients with del13q median cytotoxicity of dasatinib was 10.05% (range: 0-61.14%, n=8). Among patients with normal karyotype dasatinib cytotoxicity was 5.68% (range: 0-77.89%, n=12). Moreover, we analyzed dasatinb response in regard to the new prognostic factors of CLL, namely mutations in *NOTCH1*, *SF3B1* and *MYD88* genes. All tested samples did not have *SF3B1* (n=28) nor *MYD88* mutation (n=24). Of 22 examined cases 2 had mutation in *NOTCH1* and 20 were unmutated with median cytotoxicity of 29.85 and 2.74%, respectively, but the difference in dasatanib cytotoxicity between these subgroups was not of statistical significance (P=0.074).

Dasatinib induces apoptosis of CLL cells in vitro. To analyze induction of apoptosis after 24 h incubation with dasatinib Annexin V staining by flow cytometry on the representative sensitive or resistant to dasatinib cases (based on XTT results) was performed. We observed 17.71 and 1.84% of apoptotic cells, respectively for sensitive or resistant samples (Fig. 1B). In non-treated control cells the percentage of Annexin V positive cells was 1.30 and 1.69%, respectively (Fig. 1B).

Case report. In January 2008, a 76-year-old man was diagnosed with CLL in Rai stage II. Bone marrow aspiration showed 70-90% infiltration by small mature-appearing lymphocytes carrying a clonal immunophenotype of CD19+/CD20+/CD23+/CD5+. A flow cytometry analysis of the patient's peripheral blood revealed a monoclonal B-cell population (92%) positive for CD5, CD19, CD23. CD38 and ZAP-70 expressions were classified as negative (i.e. CD38 expression below cut-off value of 30% and ZAP-70 below 20%). The

IGHV gene mutation status was mutated. No chromosomal abnormalities were detected in the peripheral blood cells. At that time, disease was not active with no indications to start the treatment. In February 2009, the patient was referred to our institution with symptoms of active disease and was qualified to start therapy. He was treated with 6 cycles of fludarabine and cyclophosphamide (FC) chemotherapy. After completion of therapy, the patient had achieved a complete response.

In October 2013, leukocytosis with neutrocytosis was detected in peripheral blood. The white blood cell count (WBC) was 19.62x10⁹/l (61% of neutrophils, 6% of eosinophils, 1% of monocytes, 2% of band cells, 19% of lymphocytes, 9% of myeloblasts, 2% of promyelocytes), hemoglobin was 14.9 g/dl and platelets were 163x10⁹/l. Bone marrow examination revealed hypercellularity, increased percentage of granulocytic lineage and the percentage of lymphocytes was 5%. Karyotype was as follows: 46,XY, t(9;22)(q34;q11.2) (19). The cytogenetic analysis showed a Philadelphia chromosome in 100% of cells. Fluorescence *in situ* hybridization (FISH) analysis was performed on the same cytogenetic sample. It showed a standard *BCR-ABL1* rearrangement in 96% of interphase nuclei. RT-PCR showed a *BCR-ABL1* b3a2 transcript. The level of transcript [*BCR-ABL1* x CF] was 80%. Based on these findings, the diagnosis of the second malignancy-Ph+CML was made. In November 2013 imatinib at a dose of 400 mg/day was started. After two weeks, imatinib was stopped due to muscle and osteoarticular system pains. Based on the literature suggesting the effectiveness of dasatinib in CLL, we proposed to replace imatinib with dasatinib in an attempt to control both diseases with monotherapy. Dasatinib was introduced at a dose of 100 mg/day in December 2013. After 2 weeks, dasatinib treatment was suspended for 3 weeks due to pleural effusion. Steroids and diuretics have been used for pleural effusion management. After 1 month of dasatinib treatment, normalization of the peripheral blood hematological values was noticed. After seven months of therapy, the pleural effusion occurred again. Dasatinib dose reduction (from 100 to 50 mg) was required. In January 2015, after 12 months of dasatinib treatment, the patient exhibited major molecular response. This response has been maintained until the time of this report, with continued dasatinib therapy. Patient required no subsequent therapy for CLL. Fig. 2 displays changes in *BCR-ABL1* transcript, WBC and CLL cells (CD23+/CD19+/CD5+) from January 2008 to November 2019 (last observation).

Antileukemic effect of dasatinib in CLL. In this study, we assessed whether dasatinib could induce apoptosis of leukemic cells *ex vivo* of the abovementioned CLL/CML patient. To assess apoptosis, we used both Annexin V and CMXRos stainings. In the present assay, the mononuclear cells did not exhibit of *BCR-ABL1* transcript.

We found that the percentage of apoptotic cells (CD19+/Annexin V+) after *in vitro* incubation with dasatinib in a 24 h culture was higher than that in the culture without dasatinib (38.19 vs. 21.99%, respectively). Dot plots, illustrating the analysis method for the identification of CD19+/Annexin V+ cells are shown in Fig. 3A. Similarly, the percentage of apoptotic cells ($\Delta\Psi m^{low}$/CD19+) measured by CMXRos was higher after incubation with dasatinib (7.28%) than in negative control (2.86%) (Fig. 3B).
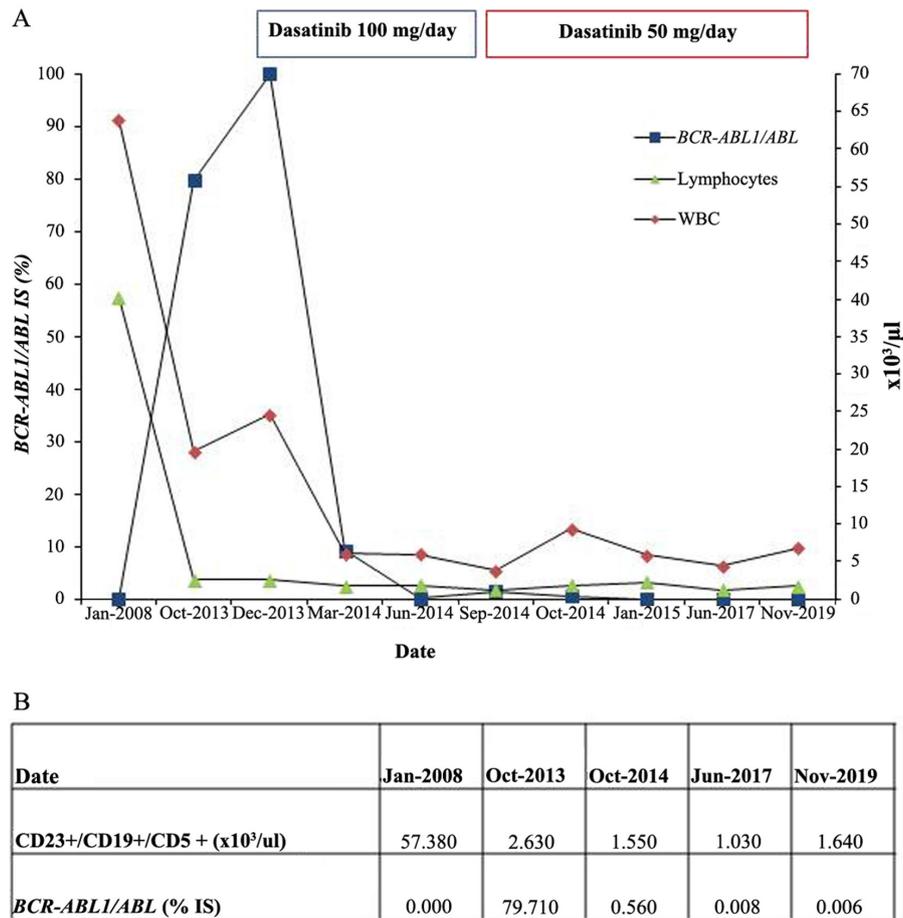
Figure 2. Laboratory test results performed during the observation of a patient with chronic lymphocytic leukemia/chronic myeloid leukemia. (A) White blood cell, absolute lymphocyte and the *BCR-ABL1* transcript levels were determined using the International Scale during dasatinib treatment. (B) The absolute CD23^{+}/CD19^{+}/CD5^{+} count and the *BCR-ABL1* transcript level before and during dasatinib treatment was also calculated. WBC, white blood cell; BCR-ABL1, breakpoint cluster region-Abelson murine leukemia 1; IS, international scale.

Discussion

Over the past years, highly active novel therapies, including kinase inhibitors targeting BTK or PI3K, were implemented in the treatment of CLL patients. This approach is of high specificity, although inhibition of these kinases affects the whole signaling via BCR and, in consequence, multiple cellular processes (26).

The mechanism underlying CLL development remains unclear, but it is thought that some stimulus (unknown antigen: exogenous, autologous or antigen-independent cell-autonomous signaling) of BCR in combination with genetic, cytogenetic and epigenetic abnormalities leads to abnormal activation of multiple signaling pathways (27). *In vitro* activity of dasatinib on the apoptosis of CLL cells has been reported in patients who were ZAP-70 positive, with unmutated *IGVH* genes, as well as with del17p or del11q (9,28,29). Our *in vitro* results point to the preferential effectiveness of dasatinib in CLL cells, regardless of theses prognostic factors, though its cytotoxicity correlates with β2-microglobulin serum levels. Moreover, Amrein *et al* (9) showed that addition of dasatinib at a concentration of 0.1 μmol/l sensitizes CLL cells to chlorambucil and fludarabine. While preclinical reports were encouraging, in phase 2 clinical trials dasatinib demonstrated moderate activity in relapsed CLL. Of 13 relapsed CLL

patients, who received 50 mg of dasatinib twice daily in phase 2 clinical study, 3 presented a decrease of lymphocyte count and regression of nodal disease (30). In another trial dasatinib was administered in a dose of 150 mg once a day to patients refractory to fludarabine-based therapies, partial responses were observed in 3 out of 15 cases and 9 patients showed a nodal response. These findings confirmed the modest activity of dasatinib in monotherapy with acceptable toxicity (22). In a phase 2 study combining fludarabine (40 mg/m^2/day, days 1-3 every 28 day) and dasatinib (100 mg/day, days 1-28) in fludarabine-refractory CLL patients, the overall response rate was 18%, but the clinical outcome of patients was significantly improved. Interestingly, temporary lymphocytosis in 61% of patients was observed early after introducing dasatinib (17). It is a similar phenomenon observed also after therapy with BTK and PI3K inhibitors, suggesting their shared mechanism of action. Kater *et al* (17) speculated that lymphocytosis is a result of dasatinib influence on BCR signaling and chemokine-controlled integrin-mediated retention and homing of malignant B cells in lymph node, what explains nodal responses of CLL patients after treatment. The preclinical efficacy of dasatinib is due to inhibition of LYN and SFK kinases in CLL cells, overcoming pro-survival signals from BCR and leading to apoptosis (8,20,31,32). Moreover, several SFK kinases contribute to the regulation of NK and NK/T cells.
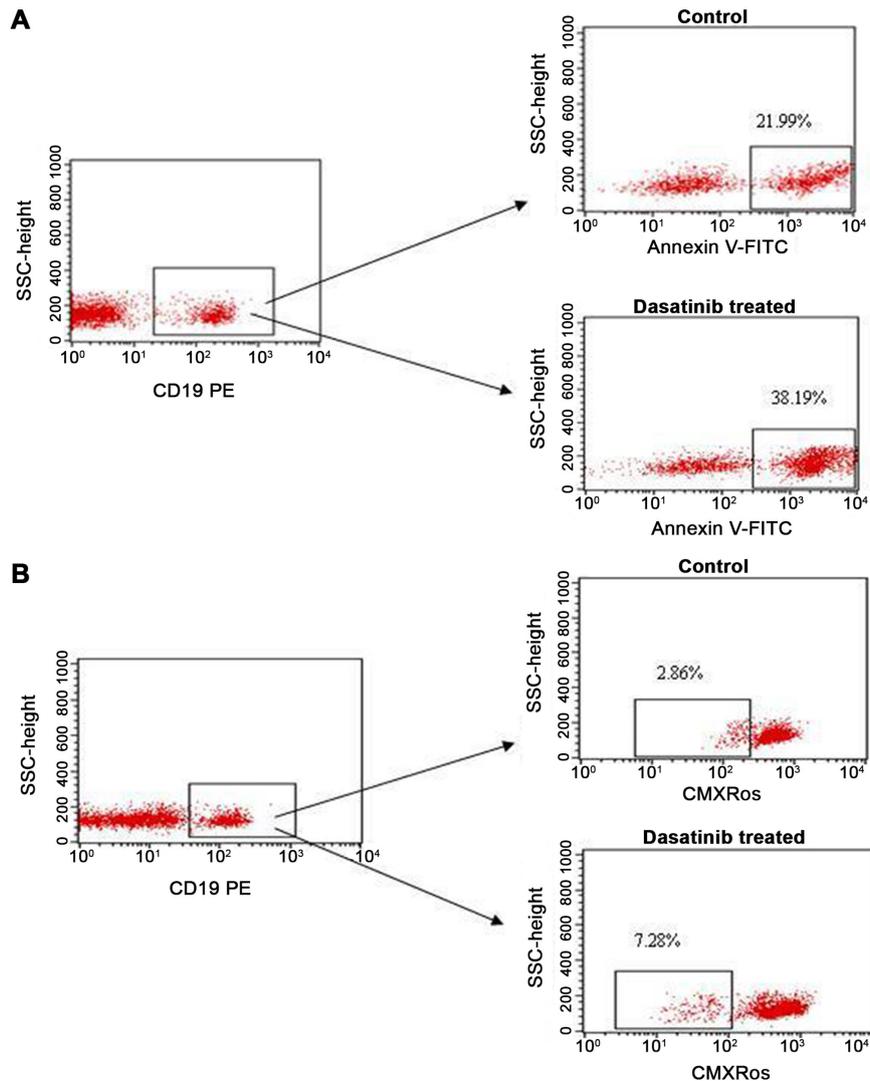
Figure 3. *In vitro* percentages of apoptotic cells were evaluated by (A) Annexin V and (B) CMXRos in negative control and after dasatinib treatment. A sunsequent analysis on the CD19+ cell population was performed. CMXRos, chloromethyl-X-rosamine; SSC, side scatter.

In CLL/CML patients responding to dasatinib, cytotoxic T and NK large granular lymphocytes were increased (33-35). Although the apoptotic effect of this inhibitor on CLL cells might be diminished by the presence of stromal cells and blood-derived 'nurse-like cells' (16,28).

CLL patients have more than twice the risk of second malignancies development, regardless of treatment status, when compared to the general population (36). In an analysis of patients treated with fludarabine-based protocols in front-line therapy, the risk of other cancers was 2.38 times higher than expected in the general population. Alkylating agents, like cyclophosphamide, did not show evidence of higher risk of second malignancies. Although cyclophosphamide demonstrates weaker carcinogenicity than other alkylating drugs, high doses of this agent might increase the risk of second cancers in CLL and Non-Hodgkin's lymphoma patients (37).

CML coexisting with CLL is a very rare phenomenon, reported in over 20 cases until date. Usually, as in the patient presented here, CML diagnosis follows CLL. Rarely the opposite situation or simultaneous occurrence of CLL and CML was reported. Until date, there is no consensus on the treatment approach for these patients (38). Typically, if CLL was in remission the treatment targeted against CML was introduced. Here, we reported on the patient initially treated with FC, who at the onset of CML was in remission for CLL. Although the patient was in a good prognostic group as characterized by *IGVH* mutational status, he was not treated optimally with immunochemotherapy including rituximab, we, therefore, might anticipate relapse within next months. In CLL8 clinical trail of German CLL Study Group, patients treated with FC, as compared to those with FCR, have significantly shorter progression-free survival (PFS) of 32.0 vs. 56.8 months (39).

Tecchio *et al* (40) reported a case of previously untreated CLL patient with trisomy of chromosome 12, a mutated *IGVH* gene and negative expression of ZAP-70 who developed CML nine years after CLL diagnosis. This patient received 400 mg/day of imatinib. After 2 months, imatinib was discontinued due to skin toxicity and treatment with dasatinib at 100 mg daily was started. Both diseases were monitored after 3 months of therapy, showing a complete hematologic remission and deep molecular response for *BCR-ABL1*. Similar data concerning a case of CLL patient with a del13q with coexisting CML was published by Nagao *et al* (41). After 3 months of treatment with dasatinib at a dose of 80 mg/day as the

first-line therapy, the patient demonstrated a major molecular response for CML and a partial response for CLL characterized by the significant reduction of lymphocyte count (41). Similarly, favorable outcome was observed in our patient with concomitant CML and CLL after 12 months of dasatinib treatment, as the patient exhibited major molecular response. We also observed a reduction of circulating CLL cells pointing to *in vivo* anti-CLL activity of dasatinib. In 2010, Serpa *et al* (33) documented a case of CML patient who developed CLL with del11q and positive expression of CD38 in 4th month of imatinib treatment. Based on the literature data suggesting the effectiveness of dasatinib on CLL, the authors changed imatinib to dasatinib at a dose of 100 mg/day. After 6 months, the patient obtained a partial response, with a reduction of lymphocytosis, the disappearance of enlarged lymph nodes, and the maintenance of major molecular responses. Moreover, Pitini *et al* (29) published case of CLL responsive to treatment with dasatinib at a dose of 140 mg daily in a patient coexisting with relapsed gastrointestinal stromal tumor (GIST).

Results of our study, extended clinical observation with functional analyses of dasatinib induced apoptosis.

In conclusion, dasatinib proved to induce durable antileukemic effects against CML and CLL cells. Its activity could be found in some sensitive CLL cases. Results of this study prove that dasatinib might induce apoptosis *in vivo*, *ex vivo* and *in vitro* in CLL and should represent a preferential therapeutic option for CML/CLL cases.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KG conceived the current study, discussed results and wrote the manuscript. AK and MKa performed the experiments, analyzed and interpreted the data, and wrote the manuscript. ABJ, KK and MM performed the experiments. MKo, WT and MH recruited patients and provided clinical data. TS designed the research and discussed results. KG, AK and MKa confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of the Medical University of Lublin (Lublin, Poland; approval nos. KE-0254/269/2019 and KE-0254/305/2019). All patients enrolled in the present study provided their signed informed consent. All forms together with medical history are enclosed in the Experimental Hematooncology Department's database.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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