Relationship between *in vitro* drug sensitivity and clinical response of patients to treatment in chronic lymphocytic leukemia

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Abstract. To improve the efficacy of therapeutic options in chronic lymphocytic leukemia (CLL) an *in vitro* system to determine the response of mononuclear blood cells from blood of patients was elaborated. The study combines four approaches, i.e., cell viability, apoptosis rate, differential scanning calorimetry (DSC), and immunoblotting to develop personalized therapy protocols based on the cell sensitivity to drug exposure of individual CLL patients. The complementary analyses were performed on 28 peripheral blood samples from previously untreated CLL patients before therapy. The induction and progress of apoptosis in CLL cells exposed *in vitro* to purine analogs combined with mafosfamide, i.e., cladribine + mafosfamide (CM) and fludarabine + mafosfamide (FM) were assessed using the above approaches. The changes in thermal profiles (decrease/loss of transition at 95±5˚C) coincided with an accumulation of apoptotic cells, a decrease in the number of viable cells, and differences in the expression of the apoptosis-related protein PARP-1. No significant changes were observed in the thermal profiles of nuclei isolated from CLL cells resistant to the treatment. The complementary assays revealed a strong relationship between both the *in vitro* sensitivity of leukemia cells to drugs and the clinical response of the patients, determined usually after the sixth course of treatment (after ~6 months of therapy). As a summary of studies followed by complementary tests, our findings demonstrate the value of *in vitro* exposure of CLL cell samples to drugs intended to treat CLL patients, before their administration in order to recommend the most suitable and effective therapy for individual patients.

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Introduction

Chronic lymphocytic leukemia (CLL) belongs to the group of hematological neoplasms with unknown etiology. Great progress in cancer diagnostics and treatment has been made, but this type of leukemia remains incurable. The simultaneous coexistence of two populations of quiescent and cycling cells in CLL treatment represents a special challenge (1-3).

It is accepted that an accumulation of genetic aberrations or epigenetic modifications in neoplastic lymphocytes could be related to heterogeneity in the clinical course of CLL and transferred on response to therapy. Recently, a large number of studies have been focused on the identification and evaluation of factors which have an impact on treatment and reflect prognostic value, e.g., genomic aberrations, alterations in miRNA level, and epigenetic modifications (4-6). Differences in the expression of factors regulating apoptosis and cell signaling, as well as the microenvironment of malignant cells, may also be responsible for the heterogeneity of leukemic cells and their response to therapy (7-9).

At present, there is an increasing number of treatment options, and a large number of agents with anticancer potential are undergoing preclinical and clinical studies (10-12). Among the new therapies for CLL, much attention is paid to agents with the potential to turn on apoptosis (13). In light of the new therapeutic options (e.g., immunochemotherapy, immunomodulators, kinase inhibitors), and disease heterogeneity, one of the most pressing issues is an elaboration of effective methods for determining the individual sensitivity of CLL patients to potential therapeutic(s), and the selection of optimal treatment for each patient (14,15).

The special importance of such approaches has been confirmed by the data showing a profound immunological defect reflected by hypogammaglobulinemia and elevated vulnerability to patient infections associated with fludarabine and cyclophosphamide administration (16). Therefore, personalized

therapy needs to be particularly addressed towards 'refractory' CLL patients (17).

The results of two single cases given in our earlier report (18), together with the combined results of 28 other cases presented in the current report, confirm an association between the results of *in vitro* testing (cytotoxicity and pro-apoptotic ability) of the tested drugs and the subsequent clinical response to the applied treatment.

Differential scanning calorimetry (DSC) is a relatively fast thermal technique that provides data on physical and energetic properties of cellular structures/compounds (19-21). Earlier, it was documented that this method could be useful for the monitoring of native chromatin in cell nuclei (19).

It has been reported by our laboratory that an additional thermal transition occurs at 95±5˚C in the DSC profiles of nuclear fraction preparations obtained from the mononuclear cells of patients in an advanced stage of CLL, along with the main transition at 83±3˚C characteristic for healthy donors (22,23). Moreover, the obtained results also revealed that the decrease (or even loss) of thermal transition at 95±5˚C in thermal scans of nuclear preparations of CLL cells, after both *in vivo* and *in vitro* drug administration, are attributable to chromatin fragmentation during apoptosis (23,24).

In the current study, the comparative cytometric analysis of cell viability, apoptosis rate, DSC profiles of nuclear preparations, and PARP-1 expression by western blotting in the group of 28 patients were applied to examine the ability of leukemic cells to enter apoptosis after their exposure to cladribine or fludarabine combined with mafosfamide.

As described, a significant decrease or even complete loss of thermal transition at 95±3˚C was observed in DSC scans of nuclear preparations when therapy was effective (22,24). Moreover, our results reveal that a comparison of the DSC profiles of nuclear preparations with the results of cell viability, apoptosis rate, and proteolytic degradation of the PARP-1 display a good predictive value for CLL cell sensitivity to anticancer drugs. This prediction is of importance as it allows an opportunity to choose the optimal therapy for patient avoiding ineffective anticancer therapy.

Materials and methods

Ethics statement. The study was approved by the Local Ethics Committee of the Medical University of Lodz (Lodz, Poland) (no. RNN/143/10/KE); all patients signed a declaration of consent.

Patients, response criteria. In the current study the peripheral blood samples from 28 randomized, untreated previously progressive CLL patients (16 men, 12 women) with white blood cell counts ranging from 45 to 600x10^9/l were included (Table I). The immunophenotypic characteristics of leukemic cells ($CD5^+/CD19^+/CD23^+$, presence on cell surface immunoglobin κ or λ chains) were determined cytometrically. The diagnosis of CLL and clinical staging determination were established according to standard clinical, immunological and cytological IWCLL criteria (25). The group included eligible patients who underwent randomization (26).

Blood samples from the patients were collected before administration with cladribine + cyclophosphamide (CC), or fludarabine + cyclophosphamide (FC). The choice of therapeutic schedules was made on accepted ECOG standards and as a result of prognostic factor analysis. Drug combination was applied the next day. Clinical response to the treatment after six cycles of drug administration was evaluated by NCI-sponsored Working Group criteria (25). Complete response (CR), partial response (PR) or non-responder (NR) criteria have been explained before (24,26).

Isolation of CLL cells. Peripheral blood mononuclear cell (PBMC) samples of CLL patients were collected on EDTA. Mononuclear cells from blood samples were separated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. The CLL cell pellets were washed with phosphate-buffered saline (PBS), resuspended in RPMI medium and divided for the planned experiments.

Fluorescence in situ hybridization (FISH). FISH analysis was performed on the interphase nuclei of leukemic cells from the blood of CLL patients before the onset of treatment (27). The following commercially available probes were used: LSI D13S319 (13q14.3)/LSI 13q34/CEP 12 probe, LSI p53 (17p13.1), LSI ATM (11q22.3) probe (Vysis; Abbott Laboratories, Abbott Park, IL, USA). The estimated cut-off levels were as follows: 8% for del(13)(q14.3), del(11)(q22.3) and del(17)(p13.1), and 5% for trisomy 12. Signals were counted in 200 interphase nuclei for each sample.

In vitro treatment. PBMC samples from patients were resuspended at a final density of 2.5-3.5x10^6 cells/ml in RPMI-1640 medium with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and incubated with cladribine or fludarabine with an active form of cyclophosphamide-mafosfamide for 48 h or without any agents [controls (Ctr)] at concentrations described previously (28,29). Control leukemic cells, as well as the CLL cells were exposed to anticancer agents for 48 h at 37˚C in an atmosphere of 5% CO_2.

Cladribine (Biodrybin) was from the Bioton S.A./Institute of Biotechnology and Antibiotics (Warsaw, Poland), fludarabine from Bayer Schering Pharma AG (Berlin, Germany). The alkylating agent mafosfamide was donated by Baxter Oncology GmbH (Frankfurt, Germany) or purchased from Niomech IIT GmbH (Bielefeld, Germany).

Cell viability and determination of apoptotic cell number. PBMC samples were incubated in culture medium only (Ctr) or in drug supplementation as indicated. The level of viable, as well as apoptotic cells after 48 h of drug treatment, was assessed by flow cytometry using Vybrant Apoptosis Assay kit no. 4 (Molecular Probes, Inc., Eugene, OR, USA). PBMC samples were analysed on LSR II Becton-Dickinson cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Preparation of nuclear fraction and whole cell lysates. Both, the pelleted control and drug-treated CLL cell samples were rinsed with cold PBS and then suspended in isotonic sucrose solution containing 5 mM $MgCl_2$, 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4) and protease inhibitors as previously described (22). Cell samples were homogenized in a Potter

Table I. Patients' characteristic features, comparative results of *in vivo* and *in vitro* response to treatment.

No.	Gender	Age	Stage of disease	FISH	Vybrant		DSC		PARP-1 cleavage		Response *in vitro*	Treatment *in vivo*	Response to treatment
					CM	FM	CM	FM	CM	FM			
1	M	50	III	del(11)(q22), del(13)(q14)	↓↓	↓↓	↓↓	↓↓	+	+	SR	CC	PR
2	M	60	III	del(11)(q22), del(13)(q14)	↓↓	↓↓	↓	↓	+	+	SR	CC	PR
3	M	52	II	del(11)(q22), del(17)(p13)	→	→	→	↓	+/-	+	WR	CC	NR
4	F	57	IV	del(11)(q22), del(13)(q14)	→	→	→	↓	0	0	WR	CC	NR
5	F	56	IV	del(13)(q14)	→	↓	↓	↓↓	+/-	+/-	WR	CC	NR
6	M	69	I	del(11)(q22), del(13)(q14)	→	→	↓	→	nd	nd	MR	CC	CR
7	F	80	IV	normal	↓	↓	↓↓	↓↓	+	+/-	SR	CC	CR
8	F	80	I	del(17)(p13), +12	↓	↓	↓	↓↓	0	0	MR	CC	PR
9	M	58	III	del(13)(q14)	↓↓	↓↓	↓		+	+	SR	CC	PR
10	M	58	III	ND	↓↓	→	↓	→	+/-	+/-	SR	CC	CR
11	F	57	II	Normal	↓↓	→	↓↓	↓↓	nd	nd	SR	CC	CR
12	F	60	III	ND	↓	→	↓↓	↓	+	0	SR	CC	CR
13	F	51	IV	del(11)(q22)	↓	↓	↓	↓	+/-		MR	CC	CR
14	F	55	III	del(13)(q14)	↓↓	↓	↓↓	↓↓	+	+/-	SR	CC	CR
15	M	52	IV	Normal	↓	↓	↓↓	↓↓	+	+	SR	CC	CR
16	M	61	III	12	↓	↓	↓↓	↓↓	+	+	SR	CC	PR
17	M	61	IV	ND	↓↓	↓↓	↓	↓↓	+/-	+/-	MR	CC	NR
18	M	69	IV	ND	→	↓↓	→	↓↓	0	+/-	WR	CC	NR
19	M	76	I	del(13)(q14)	↓	↓	↓↓	↓↓	+	+/-	SR	CC	CR
20	M	64	I	del(11)(q22)	→	→	↓↓	→	+/-		MR	CC	CR
21	M	57	I	del(11)(q22)	↓	↓	↓	↓↓	0	+/-	MR	FC	CR
22	F	65	0	ND	↓	↓	↓↓	↓↓	+/-	+	MR	FC	PR
23	F	53	II	del(11)(q22), del(13)(q14)	↓↓	↓↓	↓	↓↓	+/-	+/-	SR	CC	CR
24	M	71	0	Normal	↓↓	↓↓	↓	↓	+/-	+/-	MR	FC	CR
25	F	66	I	del(11)(q22), del(17)(p13)	↓	↓↓	↓	↓↓	nd	nd	MR	CC	CR
26	F	71	IV	del(13)(q14)	↓	↓	↓	↓↓	+	+	SR	CC	PR
27	M	66	IV	del(11)(q22), del(13)(q14), +17	↓	↓	↓↓	↓	+/-	+/-	MR	CC	PR
28	M	58	IV	Normal	↓↓	↓↓	↓↓	↓↓	+	+	SR	CC	PR

DSC, differential scanning calorimetry; FISH, fluorescence *in situ* hybridization; CM, cladribine + mafosfamide; FM, fludarabine + mafosfamide; M, male; F, female; nd, no data; SR, strong response; WR, weak response; MR, medium response; CC, cladribine + cyclophosphamide; FC, fludarabine + cyclophosphamide; PR, partial response; NR, non-responder; CR, complete response.

homogenizer, and centrifuged at 800 x g for 8 min resulting in a crude nuclear pellet.

PBMC samples were lysed in buffer containing 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1% Triton X-100, 2 mM

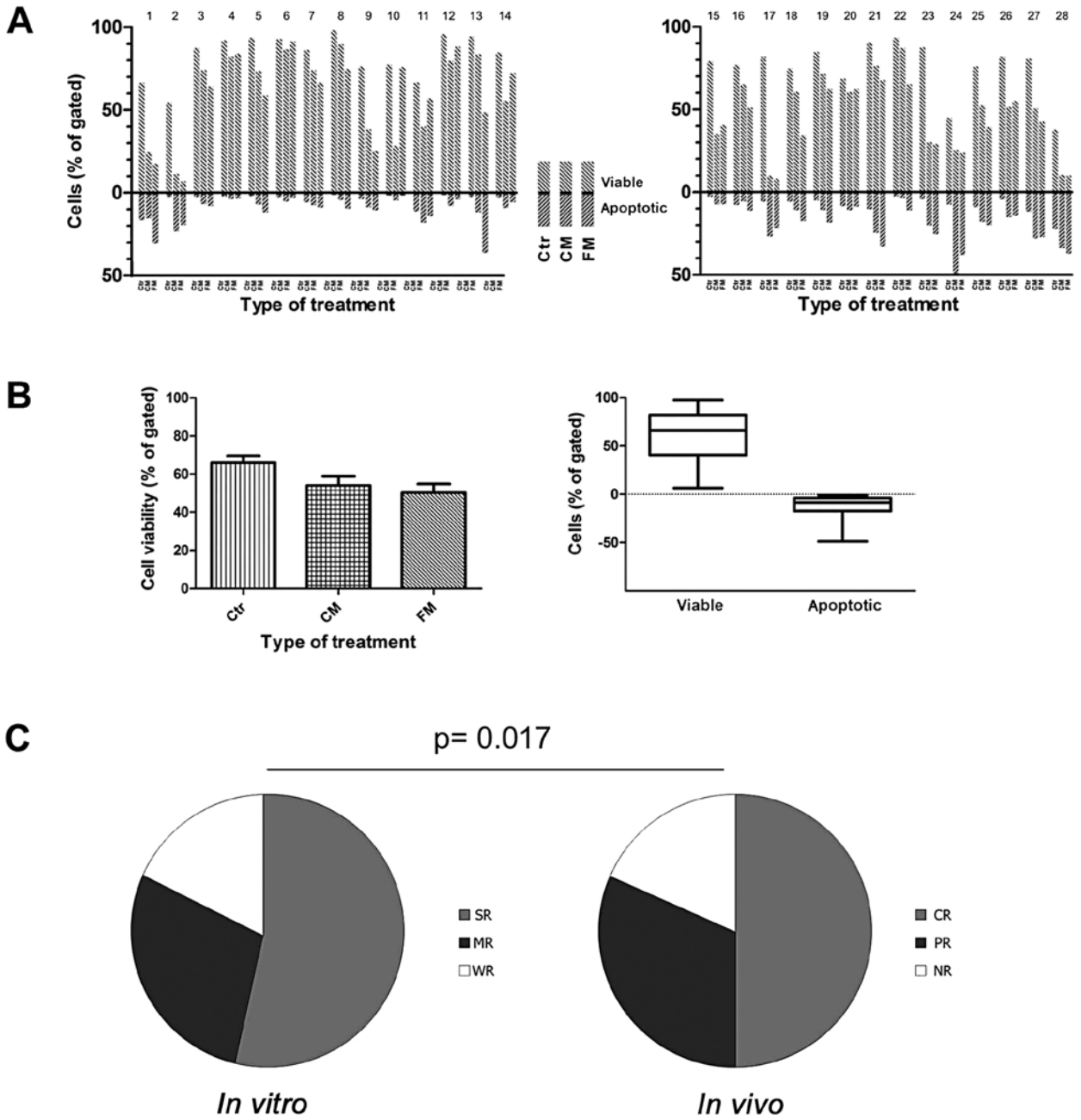

Figure 1. (A) Viability and (B) apoptosis rate of chronic lymphocytic leukemia (CLL) cells exposed to cladribine + mafosfamide (CM), fludarabine + mafosfamide (FM) or without drugs [controls (Ctr)] for 48 h. (C) Comparative statistical analysis using Fisher's exact test with Bonferroni correction of *in vivo* responses versus *in vitro* tests for CLL patients administered with drugs shows a strong correlation between results obtained *in vitro* and *in vivo* (p=0.017). The obtained results were divided into *in vivo* and *in vitro* treatment. *In vivo* response: complete response (CR), partial response (PR), and non-responder (NR); Results of *in vitro* analysis were characterized as: strong reaction (SR), median reaction (MR) and weak reaction (WR) to drugs.

$MgCl_2$, 0.1 M dithiothreitol and protease inhibitor cocktail (22).

Protein electrophoresis and immunoblotting. Protein concentration was determined colorimetrically (30). Protein samples (40 μg) were separated on 8.0% SDS-polyacrylamide gels and blotted onto Immobilon-P membrane. Equal protein loading and protein transfer were confirmed by Ponceau S staining. To avoid non-specific protein binding sites membranes were saturated with 5% non-fat dry milk in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for at least 1 h at room temperature. After extensive washing in TBS containing 0.05% Tween-20 (TBST) blots were incubated with primary antibodies specific to PARP-1 (sc-7150, 1:2,000), and actin (sc-7210, 1:1,000) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. The immune complexes were detected using alkaline phosphatase (AP)-coupled secondary antibodies as previously described (28).

DSC. Samples of the nuclear fraction isolated from PBMCs incubated with or without anticancer agents were prepared for calorimetric tests by the Almagor and Cole procedure (21,22). The probes were transferred into sample pans and hermetically sealed. Calorimetric experiments were performed on a

Setaram TG-DSC 111 calorimeter (Setaram Instrumentation, Caluire, France) from 20 to 120˚C at a scanning rate R (5˚C/min, 0.083˚C /sec) as reported previously (24).

Monitoring of apoptosis and necrosis. The appearance of apoptotic and necrotic cells was monitored under a fluorescence microscope (magnification, x400, Olympus IX70; Olympus, Tokyo, Japan). The cells after drug exposure were washed and suspended in PBS at the concentration $1x10^6$ cells/ml. Cell suspensions were incubated with YO-PRO-1 and propidium iodide, and transferred onto microscopic slides for examination. The fluorescent dyes vary in characteristics and ability to penetrate cells. YO-PRO-1 passes through the plasma membrane of apoptotic cells and labels them selectively with green fluorescence, while the red-fluorescent propidium iodide is permeant only to necrotic cells. Stained cells were classified on the basis of their morphological and staining characteristics as early, late apoptotic or necrotic (31).

Data analysis. Descriptive statistic analysis was used to summarise patients' clinical, laboratory and *in vitro* cladribine + mafosfamide (CM)/fludarabine + mafosfamide (FM) treatment data. The changes in cell viability, apoptosis rate, thermal transition at 95±5˚C or PARP-1 proteolytic cleavage in respect to the clinical response of patients were compared using Fisher's exact test with Bonferroni correction. The details of the statistical analyses are included in the text and Fig. 1C.

Results

The results of in vitro leukemic cell exposure to anticancer agents indicate their susceptibility to anticancer drugs. Leukemic PBMCs obtained from blood of 28 randomized patients scheduled for the first chemotherapy cycle were incubated for 48 h with cladribine or fludarabine combined with mafosfamide (CM or FM). The clinical characteristic of CLL patients, type of treatment and response *in vivo* and *in vitro* are presented in Table I.

Exposure of PBMC samples to both anticancer drug combinations reflects the differences in cell viability, as well as apoptosis rate. Comparative analysis of the above studies showed a different personal sensitivity and response of examined cells to the used drug combinations (Figs. 1 and 2). Moreover, the exposure of CLL cells to both investigated combinations usually decreased the viable cells number, which was associated with an elevation in apoptotic rate, but to a different extent. Among the investigated patient samples, we did not obtain the same results for a single patient. In leukemic cells isolated from blood of some patients, apoptosis at high rate was rapidly induced, whereas in other cases it was delayed and much weaker.

The comparison of the combined average results is illustrated in Fig. 1A and B. The diagrams in Fig. 2A and B show the combined data of *in vitro* tests for CLL patients nos. 14 and 18, respectively. Patient no. 18 did not respond (NR) to CC therapy applied in the clinic, but the results of *in vitro* tests suggest that for this patient, FC would display a chance for better response. The results for patient no. 14, who achieved a CR *in vivo*, also *in vitro* tests show that this type of treatment will be more profitable for this patient in comparison to FM/FC. The results of *in vitro* tests demonstrate leukemic patients' personal predispositions that could be transferred to treatment efficacy *in vivo* (compare Figs. 1 and 2).

Moreover, in the group of the studied blood samples from CLL patients with sensitive and reactive leukemic cells, the response to the treatment *in vivo* as well as to *in vitro* conditions usually occurred. The second group showed a weak response to the drugs used, which could be correlated with the unsatisfactory response or even resistance of patients to therapy *in vivo*. A third group of CLL cells displays a high sensitivity to *in vitro* condition, which was concomitant with high reduction of cell viability in control untreated cells incubated for 48 h, as well as PARP-1 cleavage in control untreated cells. For these cases, the additional analysis using supplementary techniques are suggested to demonstrate the course of apoptosis process.

Thermal transition at 95±5˚C is characteristic for nuclear samples from advanced stage of CLL patients. A study from our laboratory has shown that in advanced stages of CLL, the thermal profile of nuclei with an additional thermal transition at 95±3˚C is present in ~74% of cases in advanced/aggressive disease (22). In the results presented here, all control nuclear preparations of leukemic cells indicated thermal transition at ~95˚C. Moreover, in the majority of nuclear samples isolated from PBMCs of advanced leukemia patients qualified to drug administration (advanced stage of disease), this thermal transition was usually dominant. Interestingly, the changes in thermal profiles of nuclear preparations from leukemic PBMCs exposed to studied drug combinations were correlated with viable cell reduction, reflecting the potential efficacy of treatment.

To show that results of complementary tests closely adhere to DSC profiles, we selected for presentation two exemplary cases with different therapeutic outcomes: one illustrating a positive response to CM and potential resistance to FM (patient no. 14; Table I, Figs. 1 and 2A), and the second one reflecting a weak sensitivity to CM in comparison to FM (patient no. 18; Table I, Figs. 1 and 2B).

The thermal transition of exemplary nuclear fraction (patient no. 14) at 95±5˚C was reduced after leukemic cell exposure to drugs for 48 h; the cells of this patient were characterized by a higher apoptosis rate in respect to control cells (Fig. 2AI and II). No decrease of transition at 95±5˚C was observed in the cells treated with CM (patient no. 18) that could be a reason of chromatin hyper-condensation during PBMC cell exposure to drugs (Fig. 2BIII). While the changes in thermal profiles directed towards decrease of thermal transition at 95±5˚C seem to be attributable to a degradation of chromatin during apoptotic DNA fragmentation.

The comparative analyses of nuclear fraction DSC scans with cell viability and apoptosis marker PARP-1 expression reflect leukemic cell sensitivity to anticancer drugs. Using the complementary *in vitro* tests, we assessed some para-meters that simultaneously performed could predict a potent efficacy of anticancer drugs in leukemic PBMCs. It must be emphasized that the treatment response strongly varied between individual patients (Fig. 1A and B). Interestingly, for all studied 28 patients,

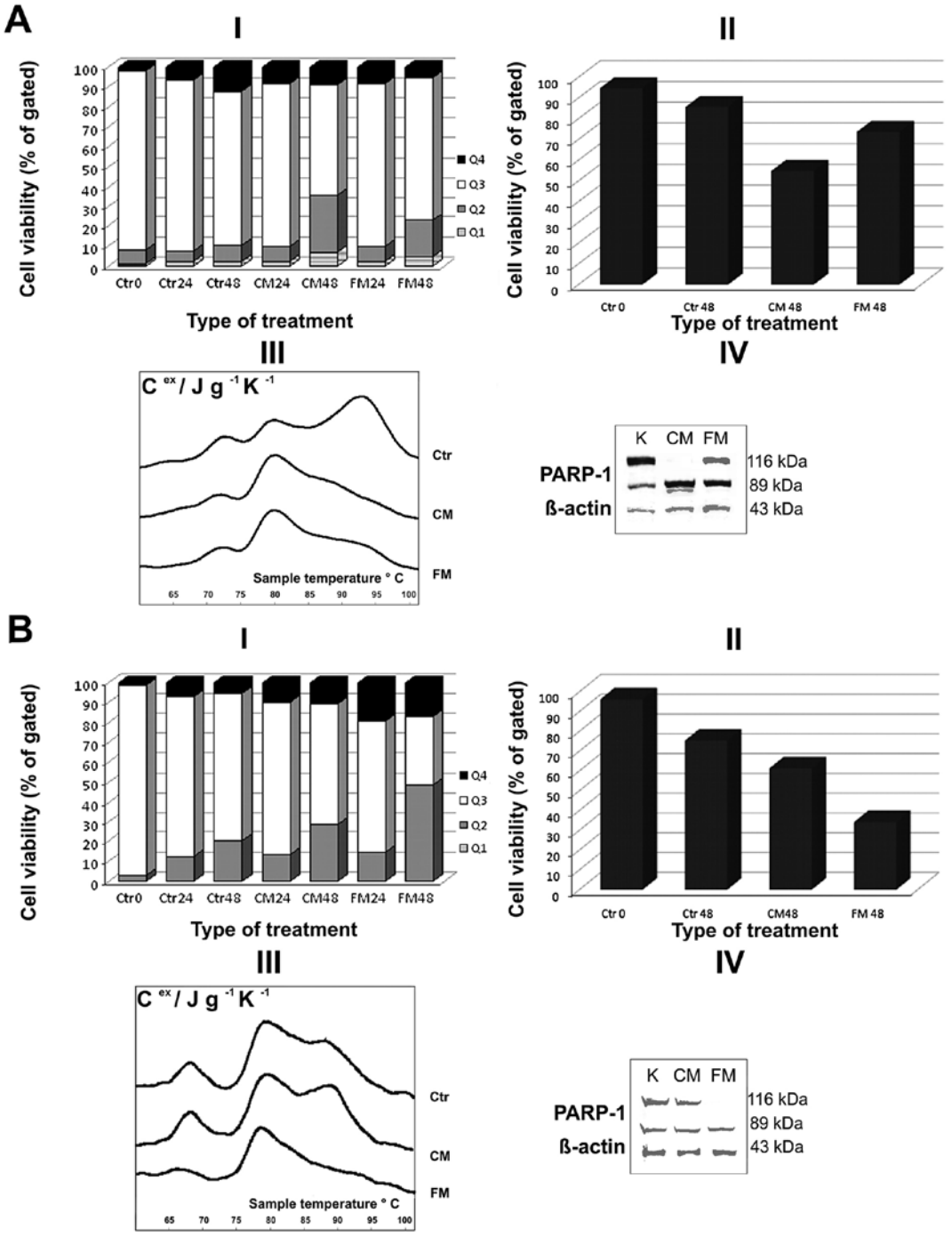

Figure 2. (I and II) Viable (Q3), apoptotic (Q4) and necrotic + late apoptotic cells (Q2) of chronic lymphocytic leukemia (CLL) cell population from blood of exemplary patients, i.e., (A) no. 14 who responded *in vitro* and *in vivo* to cladribine + mafosfamide (CM)/cladribine + cyclophosphamide (CC), and (B) no. 18 who did not respond *in vitro* and *in vivo* to CM, respectively. (III) Differential scanning calorimetry (DSC) profiles of peripheral blood mononuclear cells (PBMCs) nuclei from blood of exemplary patients were exposed to CM or fludarabine + mafosfamide (FM) or without drugs [controls (Ctr)] for 48 h, respectively. C^{ex}, excess heat capacity. (IV) Changes in expression of apoptosis marker PARP-1 in CLL cell samples from blood of two exemplary patients after their exposure to CM, FM or without drugs (Ctr).

the changes in thermal profiles at 95±5˚C of nuclear samples obtained from PBMCs exposed to anticancer drugs correlated with the reduction of viable cell percentage and elevation of apoptotic cell fraction accompanied by the proteolysis in apoptotic marker PARP-1 (Fig. 2). In the group of examined 28 patients, 8 of them display a positive reaction (CR) *in vivo* and strong reaction (SR) *in vitro*, while negative responses were observed for 5 patients (3 of them *in vivo* and *in vitro*). Similarly, *in vivo* and *in vitro* correlations were seen for 4 patients reflecting PR. It must be stated that sometimes patients did not respond the same *in vitro* and *in vivo*. For five cases, there was an SR *in vitro* followed by a PR *in vivo*, and for six others the reaction to drug administration *in vivo* (CR) was stronger than the reaction *in vitro* (MR) (Table I).

It must be underlined that we did not receive the same results of four complementary tests for two individuals among 28 studied CLL patients. From the obtained results we have selected two exemplary CLL patients, i.e., one responding to

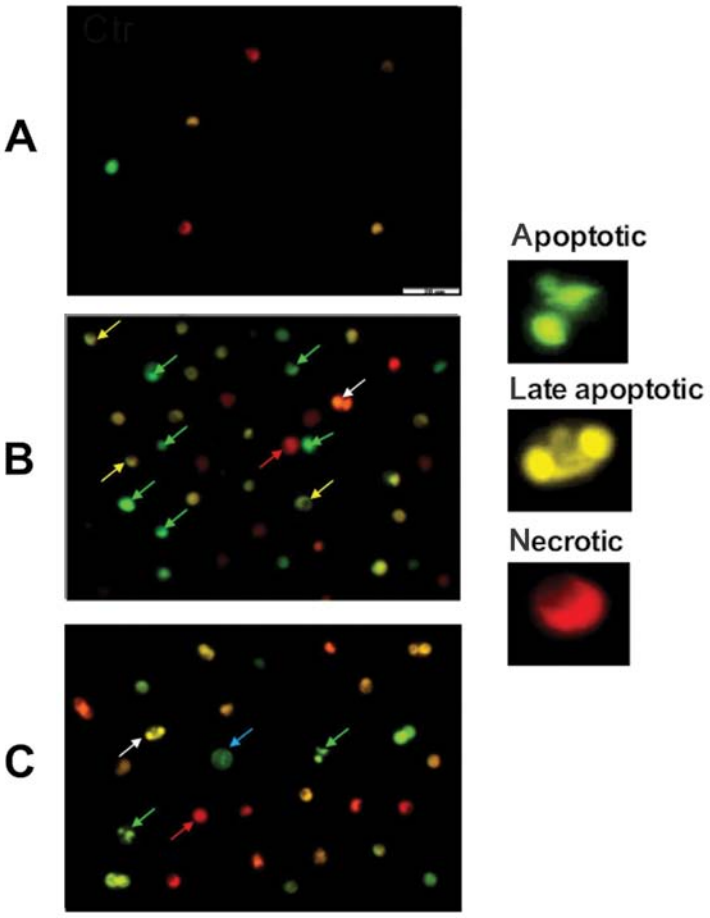

Figure 3. Morphological changes of chronic lymphocytic leukemia (CLL) cell samples (patient no. 14), after exposure to (B) cladribine + mafosfamide (CM), (C) fludarabine + mafosfamide (FM) or (A) without drugs for 48 h (magnification, x400).

CM and resistant to FM (no. 14), and the second one (no. 18) displaying a weak response to CM treatment. As demonstrated in Figs. 1A and 2, personal differences and distinct cell responses to *in vitro* CM and FM treatment in terms of cell viability (I), and apoptosis rate (II) were observed. These data were confirmed by the decrease of thermal transition at 95±5˚C after cell exposure to CM, and the even higher thermal profile in the case of cells incubated with FM (patient no. 14; Fig. 2AIII). The obtained data were also confirmed by strong proteolytic cleavage of PARP-1 in leukemic cells incubated with CM (Fig. 2AIV). For patient no. 18, only a slight decrease of the living cell numbers exposed to CM in comparison to control ones (Figs. 1A, B and 2BI, II) was observed (Figs. 1 and 2) and correlated with no changes in the thermal profiles of nuclear fraction preparations. A slight PARP-1 cleavage, even in control untreated cells of this patient, was observed (Fig. 2BIV).

The apoptotic and necrotic morphological changes in examined cells were monitored during cell incubations with anticancer drugs (Fig. 3). The cell suspensions were incubated with YO-PRO-1 and propidium iodide and analyzed under fluorescence microscope (Olympus IX70) at magnification x400. Numerous shrunken cells with the typical morphological features of early (green arrow) and late (yellow arrow) apoptosis, i.e., chromatin condensation, marginalization (semilunar shape) and nuclear fragmentation were seen after CM and FM treatment. Some swollen enlarged cells typical for necrosis (red arrow) and the cells with two nuclei (white arrow) were also observed. In some cells, the beginning of nuclear changes were visible as a condensed ring/sphere along the nuclear envelope (blue arrow).

The stained cell areas observed after leukemic cell incubations with drugs (patient no. 14) for 48 h chosen as an example of anticancer drugs effect monitoring on leukemic cell viability is illustrated in Fig. 3.

Finally, we addressed the question of whether evaluation of thermal profile changes upon drug treatment of leukemic cells might possess prognostic value. Interestingly, the results of the *in vitro* experiments suggest that patient no. 14 should respond better to CM/CC treatment. During randomized therapy option this patient was administered with CC, and after six cycles of chemotherapy he reached complete remission. The results for patient no. 18 indicated his very weak *in vitro* response to CM in comparison with FM. This patient was administered with CC and he did not respond (NR) to scheduled therapy (Table I).

The comparative statistical analysis using Fisher's exact test with Bonferroni correction between clinical patient response (after six courses of therapy) and *in vitro* test data reflects a statistical correlation ($p=0.017$) between *in vivo* and *in vitro* response to the applied treatment (Fig. 1C). Interestingly, we did not find such correlation between the response to treatment

versus stage of disease (Rai staging), or cytogenetic aberration on 11q and 13q chromosome.

As is shown in Table I, among the 28 patients for whom cytogenetic assay was performed, 25 underwent CC therapy and 12 reached CR (48%). The deletion within chromosome 13 (13q14) was the most common genetic abnormality in the investigated group (11/28, 39.28%). Among the studied samples of CLL patients, only three cases of chromosomal 17 abnormality (17p13.1) were detected, and one of them reached CR.

These results suggest that *in vitro* response of leukemic cells to anticancer agents display predictive value and could be helpful in the optimal therapeutic strategy selection for individual patients in order to avoid ineffective therapy based on purine analog combined with alkylating agent.

Discussion

Inherited genetic predispositions to CLL has directed attention towards extensive studies on genetic alterations which could disturb gene expression (6,32). Studies looking for new genetic aberrations and epigenetic modifications in chromatin structure have been performed (4-6). The diversities in response to CLL therapy occur in respect to personal genetic differences, having impact on cell signal transduction, cell cycle alterations and disturbance of apoptosis. Sometimes, because of personal variations in expression of some genes even directed therapy towards cancer-related or specific markers could not be fully effective. Personal diversities in response to treatment or retreatment that introduce toxicity could direct into myelodysplasia or secondary neoplasms (33,34).

More than 95% of leukemic cells in peripheral blood of patients display hyper-condensed heterochromatin and are between G0/G1 phase of the cell cycle. In more aggressive cases of disease, the population of cycling cells induce another request for CLL treatment directions. According to the theoretical predictions, activity of drugs and their capability to induce apoptosis should be the most effective manner for elimination of leukemic cells (34,35). Standard therapies used in clinical routine are based on the combination of purine analogs with alkylating agent(s), which induce DNA damage (36). In light of published data, a proportion of patients acquires resistance to the treatment (14,37,38). Thus, the optimization of drug(s) before their administration and elaborating tailored therapy is a need for the group of non-responding patients or those with high expression of unfavorable prognostic factors.

In the current study, we evaluated *in vitro* cytotoxicity, apoptosis induction potential and the changes in chromatin conformation caused by cladribine or fludarabine combined with mafosfamide to evaluate their potency for leukemic cell elimination by apoptosis. As the consequence of two CLL cases published recently (18), we extended our study up to 28 patients. We used four experimental approaches for monitoring, i.e., cell viability, rate of apoptosis, DSC analysis of nuclear preparations, and expression of apoptotic marker PARP-1. The obtained data revealed that *in vitro* exposure of leukemic PBMCs to drug combinations before patient treatment might indicate prognostic value. In the examined cases we observed that patients whose PBMCs were insensitive to given drug combinations *in vitro* did not respond to the treatment *in vivo* either. The positive responses to drug combinations *in vitro*, as the result of cell elimination by apoptosis, were in most cases followed by CR to therapy *in vivo*. It must be underlined that for some CLL patients whose cells indicate special sensitivity to *in vitro* conditions individualized tumor response testing suggested by Matutes *et al* (39) or cytotoxic tests performed previously by Nagourney *et al* (40) could not be fully effective (compare Fig. 1A and B).

Finally, the disappearance of some of the CLL cells as a consequence of apoptosis induction after their exposure to drugs was also confirmed by the changes in expression of certain apoptosis-related proteins, for example PARP-1. A recently published report concerning anticancer drug bioactivity on library of approved anticancer drugs confirms CLL cell exceptionality, but direct studies towards tailoring therapy for other types of malignances in general are needed (41).

In summary, the results of our studies revealed that determination of the *in vitro* CLL cell sensitivity based on purine analogs and their combinations with alkylating agent would be instrumental in the development of personalized therapy. It should be stated that from our point of view *in vitro* study gives the opportunity for CLL status analysis, before and during drug application (18,22-24). It reflects a special importance for a subset of patients resistant to therapy, as well as for those heavily pre-treated or those in weak condition.

Our current results for 28 patients confirm studies published previously as a case report (18) suggesting that *in vitro* incubations of leukemia cells with anticancer drugs is of predictive value and would help to select the optimal therapeutic strategy for individual patient in order to avoid ineffective treatment.

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References

1. Oscier D, Dearden C, Erem E, *et al*: Guidelines on the diagnosis, investigation and management of chronic lymphocytic leukaemia. Br J Haematol 159: 541-564, 2012.
2. Hallek M: Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. Am J Hematol 88: 803-816, 2013.
3. Klein U and Dalla-Favera R: Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol 8: 22-33, 2008.
4. Zenz T, Mertens D, Döhner H and Stilgenbauer S: Importance of genetics in chronic lymphocytic leukemia. Blood Rev 25: 131-137, 2011.
5. Rogalińska M and Kilianska ZM: Targeting Bcl-2 in CLL. Curr Med Chem 19: 5109-5115, 2012.
6. Martin-Subero JI, López-Otín C and Campo E: Genetic and epigenetic basis of chronic lymphocytic leukemia. Curr Opin Hematol 20: 362-368, 2013.
7. Chiorazzi N: Implications of new prognostic markers in chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program 2012: 76-87, 2012.
8. Rodriquez-Vicente AE, Díaz MG and Hernández-Rivas JM: Chronic lymphocytic leukemia: a clinical and molecular heterogenous disease. Cancer Genet 206: 49-62, 2013.
9. Oppezzo P and Dighiero G: Role of B-cell receptor and the microenvironment in chronic lymphocytic leukemia. Blood Cancer J 3: e149, 2013.
10. Robak T: Application of new drugs in chronic lymphocytic leukemia. Mediterr J Hematol Infect Dis 2: e2010011, 2010.
11. Rogalińska M and Kiliańska ZM: Potential new agents for chronic lymphocytic leukemia treatment. Anticancer Agents Med Chem 10: 666-682, 2010.

12. Qiu LN, Zhou YL, Wang ZN, Huang Q and Hu WX: ZGDHu-1 promotes apoptosis of chronic lymphocytic leukemia cells. Int J Oncol 41: 533-540, 2012.
13. Chen R and Plunkett W: Strategy to induce apoptosis and circumvent resistance in chronic lymphocytic leukemia. Best Pract Res Clin Haemat 23: 155-166, 2010.
14. Bosanquet AG, Richards SM, Wade R, *et al*: Drug cross-resistance and therapy-induced resistance in chronic lymphocytic leukemia by an enhanced method of individualised tumour response testing. Br J Haematol 146: 384-395, 2009.
15. Rogalinska M, Blonski J, Góralski P, *et al*: Usefulness of differential scanning calorimetry for monitoring ex vivo the changes in responses of CLL cells to anticancer drugs: development of personalized therapy. Blood (ASH Annual Meeting abstracts) 116: 4635, 2010.
16. Riches JC, Ramsay AG and Gribben JG: Immune dysfunction in chronic lymphocytic leukemia: the role for immunotherapy. Curr Pharm Des 18: 3389-3398, 2012.
17. Zenz T, Gribben JG, Hallek M, *et al*: Risk categories and refractory CLL in the era of chemoimmunotherapy. Blood 119: 4101-4107, 2012.
18. Rogalińska M, Franiak-Pietryga I, Błoński JZ, *et al*: Toward personalized therapy for chronic lymphocytic leukemia: DSC and cDNA microarray assessment of two cases. Cancer Biol Ther 14: 6-12, 2013.
19. Balbi C, Abelmoschi ML, Gogioso L, *et al*: Structural domains and conformational changes in nuclear chromatin: a quantitative thermodynamic approach by differential scanning calorimetry. Biochemistry 28: 3220-3227, 1989.
20. Allera C, Lazzarini G, Patrone E, *et al*: The condensation of chromatin in apoptotic thymocytes shows a specific structural change. J Biol Chem 272: 10817-10822, 1997.
21. Almagor M and Cole RD: Differential scanning calorimetry of nuclei as a test for the effects of anticancer drugs on human chromatin. Cancer Res 49: 5561-5566, 1989.
22. Rogalińska M, Góralski P, Kobylińska A, *et al*: Changes in leukemic cell nuclei revealed by differential scanning calorimetry. Leuk Lymphoma 46: 121-128, 2005.
23. Góralski P, Rogalińska M, Błoński JZ, *et al*: The differences in thermal profiles between normal and leukemic cells exposed to anticancer drug evaluated by differential scanning calorimetry. J Therm Anal Calorim 118: 1339-1344, 2014.
24. Rogalinska M, Goralski P, Wozniak K, *et al*: Calorimetric study as a potential test for choosing treatment of B-cell chronic lymphocytic leukemia. Leuk Res 33: 308-314, 2009.
25. Cheson BD, Bennett JM, Grever M, *et al*: National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood 87: 4990-4997, 1996.
26. Robak T, Blonski JZ, Gora-Tybor J, *et al*: Cladribine alone and in combination with cyclophosphamide or cyclophosphamide plus mitoxantrone in the treatment of progressive chronic lymphocytic leukemia: report of a prospective, multicenter, randomized trial of Polish Adult Leukemia Group (PALG CLL2). Blood 108: 473-479, 2006.
27. Kotkowska A, Wawrzyniak E, Blonski JZ, Robak T and Korycka-Wolowiec A: Chromosomal aberrations in chronic lymphocytic leukemia detected by conventional cytogenetics with DSP30 as a single agent: comparison with FISH. Leuk Res 35: 1032-1038, 2011.
28. Rogalińska M, Błoński JZ, Komina O, *et al*: R-roscovitine (Seliciclib) affects CLL cells more strongly than combinations of fludarabine or cladribine with cyclophosphamide: inhibition of CDK7 sensitizes leukemic cells to caspase-dependent apoptosis. J Cell Biochem 109: 217-235, 2010.
29. Kobylinska A, Bednarek J, Blonski JZ, *et al*: *In vitro* sensitivity of B-cell chronic lymphocytic leukemia to cladribine and its combinations with mafosfamide and/or mitoxantrone. Oncol Rep 16: 1389-1395, 2006.
30. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275, 1951.
31. Gasiorowski B, Brokos A, Kulma A, Ogorzałek A and Skórkowska K: A comparison of the methods applied to detect apoptosis in genotoxically-damaged lymphocytes cultured in the presence of four antimutagens. Cell Biol Mol Lett 6: 141-159, 2001.
32. Houlston RS, Catovsky D and Yuille MR: Genetic susceptibility to chronic lymphocytic leukemia. Leukemia 16: 1008-1014, 2002.
33. Abrisqueta P, Crespo M and Bosch F: Personalizing treatment for chronic lymphocytic leukemia. Expert Rev Hematol 4: 27-35, 2011.
34. Robak T, Jamroziak K, Gora-Tybor J, *et al*: Comparison of cladribine plus cyclophosphamide with fludarabine plus cyclophospahamide as a first-line therapy for chronic lymphocytic leukemia: a phase III randomized study by the Polish Adult Leukemia Group (PALG-CLL3 study). J Clin Oncol 28: 1863-1869, 2010.
35. Decker T, Hipp S, Ringshausen I, *et al*: Rapamycin-induced G1 arrest in cycling B-CLL cells is associated with reduced expression of cyclin D3, cyclin E, cyclin A, and survivin. Blood 101: 278-285, 2003.
36. Van den Neste E, Cardoen S, Offner F and Bontemps F: Old and new insights into the mechanisms of action of two nucleoside analogs active in lymphoid malignancies: Fludarabine and cladribine (Review). Int J Oncol 27: 1113-1124, 2005.
37. Wierda WG, Kipps TJ, Mayer J, *et al*: Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. J Clin Oncol 28: 1749-1755, 2010.
38. Sieklucka M, Pozarowski P, Bojarska-Junak A, *et al*: Apoptosis in B-CLL: The relationship between higher *ex vivo* spontaneous apoptosis before treatment in III-IV Rai stage patients and poor outcome. Oncol Rep 19: 1611-1620, 2008.
39. Matutes E, Bosanquet AG, Wade R, *et al*: The use of individualized tumor response testing in treatment selection: second randomization results from the LRF CLL4 trial and the predictive value of the test at trial entry. Leukemia 27: 507-510, 2013.
40. Nagourney RA, Evans SS, Messenger JC, Su YZ and Weisenthal LM: 2 Chlorodeoxyadenosine activity and cross resistance patterns in primary cultures of human hematologic neoplasms. Br J Cancer 67: 10-14, 1993.
41. Shen M, Zhang Y, Saba N, *et al*: Identification of therapeutic candidates for chronic lymphocytic leukemia from a library of approved drugs. PLoS One 8: e75252, 2013.