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BCL-2 PROTEIN EXPRESSION AND p53 GENE MUTATION IN CHRONIC LYMPHOCYTIC LEUKEMIA: CORRELATION WITH IN VITRO SENSITIVITY TO CHLORAMBUCIL AND PURINE ANALOGS

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ABSTRACT

Background and Objective. Bcl-2 oncogenic protein expression plays a major role in blocking the apoptotic mechanism. p53 gene mutations have also been suggested to account for the chlorambucil resistance in CLL. Thus we studied the relationship between bcl-2 protein expression, p53 gene mutations and *in vitro* drug sensitivity in CLL.

Methods. Fifty-three samples from untreated CLL patients in early disease stages were tested *in vitro* for chemosensitivity to chlorambucil (CLB), fludarabine (FAMP) and 2-chlorodeoxyadenosine (2-CDA) using the MTT assay. Intracellular bcl-2 protein expression was evaluated by flow cytometry analysis. p53 gene mutations were detected by using polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis.

Results. The median LD50 values were 1.55 μM, 4.41 μM and 58.2 μM for 2-CDA, FAMP and CLB, respectively. About 23%, 41% and 11% of samples were defined as being sensitive to FAMP, 2-CDA and CLB, respectively, when samples were clustered for LD50 threshold values corresponding to the plasmatic levels of the drug. No statistically significant difference in bcl-2 protein expression was noted between sensitive and resistant samples for each drug. A p53 gene mutation was detected in 4 of the 30 cases studied and all of them were among samples resistant to CLB.

Interpretation and Conclusions. Bcl-2 expression is not an indicator of *in vitro* response to drugs in CLL; similarly, although the four cases showing a p53 gene mutation were associated with CLB resistance, drug resistant samples were also observed in the group of patients showing wild type p53, suggesting multiple mechanisms of drug resistance in CLL.

Key words: CLL, MTT assay, bcl-2, p53, chemosensitivity

B-cell chronic lymphocytic leukemia (CLL) is a disorder characterized by clonal expansion of $CD5^+/CD23^+$ B-lymphocytes.[1-3] Patients requiring therapy are commonly treated with chlorambucil (CLB);[1-6] fludarabine (FAMP) and 2-chloro-deoxyadenosine (2-CDA)[1-3,5] have also shown potent anti-tumor activity in CLL. Experimental evidence has indicated that CLB[7] and the active triphosphate metabolites of both purine analogs[8] exert their cytotoxic effects by inducing apoptosis in CLL. It is well known that bcl-2 oncogenic protein expression plays a major role in blocking apoptotic mechanisms.[9] Overexpression of the bcl-2 gene product has been shown to be related to drug-induced apoptosis and associated with a poor response to chemotherapy in acute leukemia.[10] Moreover, DNA fragmentation is reduced in bcl-2 transfected human pre-B leukemic cell lines cultured *in vitro* in the presence of chemotherapeutic agents.[11] CLL cells express the bcl-2 protein[12,13] and a bcl-2-mediated form of drug resistance might therefore exist in CLL. An alternative mechanism provided by p53 gene mutations has also been suggested to account for the CLB resistance in CLL.[14] It is still unclear to what extent different levels of bcl-2 protein expression and p53 gene mutations may affect *in vitro* sensitivity to cytotoxic drugs in CLL.

In this study we pursued two aims. First, we evaluated the *in vitro* chemosensitivity of peripheral CLL cells to CLB, FAMP and 2-CDA using the MTT assay,[15-18] defining a sample as sensitive or resistant, as previously reported,[18] based on the lethal dose (LD)50 cut-off values corresponding to the plasma drug levels achievable during therapy with them.[19-21] Second, we tried to determine whether higher bcl-2 levels or the presence of p53 gene mutations could confer some degree of *in vitro* drug resistance to CLL cells.

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Materials and Methods

Patient population, cell separation and immunophenotype analysis

Fifty-three samples from untreated $CD5^+/CD19^+/CD23^+$ CLL patients in early stages of disease (forty-seven in Binet stage A and 6 in stage B) were studied after obtaining the patients' informed consent. Mononuclear cells (PBMC) were separated from fresh venous blood samples using a Ficoll-Hypaque gradient centrifuge (J-Bio, Les Ulis Cedex, France). After isolation, the cells were washed twice and re-suspended in RPMI 1640 medium (Gibco, BRL, Life Technologies LTD, Paisely, Scotland, UK) supplemented with 15% heat-inactivated FCS (Gibco), 100 IU/mL penicillin and 100 mg/mL streptomycin, 0.125 µg/mL fungizone (Squibb Inc., New York, NY, USA), and 2 mmol/L l-glutamine (J-Bio). A total of 0.5×10^6 PBMC were labelled with either the FITC-conjugated monoclonal antibodies (MoAbs) CD5 and CD19 (Ylem, Italy), or the purified MoAb CD23 (Becton Dickinson, Mountain View, CA, USA), and subsequently with a FITC-labelled secondary reagent [$F(ab')_2$ fragments of a rabbit anti-mouse Ig](Ylem). The leukemic cell percentages ranged from 80% to 95%, as assessed using an anti-CD19 (MoAb). For intracellular detection of the bcl-2 oncoprotein, a purified anti-Bcl-2 MoAb (DAKO-Bcl-2, 124 DAKO A/S, Denmark) was used after permeabilizing the cells with a cell fixative (ORTHO Permeafix™, ORTHO Diagnostic System, Raritan, NJ, USA).[22] Between 5,000 and 10,000 cells were examined for fluorescence using an EPICS Profile II flow cytometer (Coulter Electronics, Inc., Hialeah, FL, USA). The lymphocytic population was gated on a two-parameter forward angle versus 90° light scatter histogram. The percentage of cells expressing the surface antigen of interest was determined by establishing a cursor position channel using an irrelevant FITC-conjugated mouse IgG subclass (Becton Dickinson) or an FITC-labelled secondary reagent as a negative control.

Drug preparation

CLB (Sigma, St Louis, MO, USA) was dissolved in absolute ethanol. 2-CDA was kindly provided by Dr. D. Lutz on behalf of the International Society for Chemo- and Immunotherapy (IGCI, Vienna, Austria) and purchased from Dr. Zygmunt Kazimierczuk (The Foundation for the Development of Diagnostics and Therapy, Warsaw, Poland). Commercially available FAMP (Fludara, Berlex, Alameda, CA, USA) was diluted in the medium. Stock solutions of the drugs were diluted in RPMI 1640 and aliquoted at 100%.

In vitro culture assay and assessment of chemosensitivity

The MTT assay for cell viability was used to estimate *in vitro* drug sensitivity as previously described.[15-18] One hundred µL of a 10×10^6/mL cell suspension were plated in 96 round-bottom wells (Nunc, Roskilde, Denmark). Different concentrations of CLB, FAMP or 2-CDA were plated in the appropriate well with the CLL cells in a volume of 25 µL. Four replicate wells were used as control for each drug dose. The plates were incubated in a humidified atmosphere for four days at 37°C in 5% CO_2. Fifty µL of 5 mg/mL MTT (Sigma) in PBS were added to each well and the plates were re-incubated for another four hrs. Fifty µL of 100% dimethylsulfoxide (DMSO) (Calbiochem, La Jolla, CA, USA) were added to each well to solubilize the MTT formazan. The plates were kept in the incubator for 1 hr and then their spectrophotometric adsorbance at 540 nm was determined using a multi-well scanning spectrophotometer (EL 310, Biotek Instruments, Burligton, VT, USA). The dose-dependent cytotoxic effects of the drugs were studied in each sample by culturing CLL cells with 437-0.00437 µM 2-CDA, 270-0.027 µM FAMP and 328-0.0328 µM CLB. Drug dose-response curves were drawn and their LD50 values (i.e. the drug dose capable of killing 50% of the cells) were determined using previously employed software.[17,18]

Detection of p53 mutations

p53 mutations were detected using polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis. The genomic DNA was prepared from peripheral lymphocytes by protein K digestion, extraction with phenol/chloroform and precipitation with ethanol. The oligonucleotides used to amplify sequences of the p53 gene exons 5 through 9 have already been reported.[23] SSCP analysis was performed using a modified form of a previously described method.[24] Briefly, 100 ng of genomic DNA, 5 pmoles of the primer, 2.5 µM dNTPs, 1 µCi of (α-^{32}P)dCTP, 10 mM TRIS· HCl (pH 8.8), 50 mM KCl, 1 mM $MgCl_2$, 0.01% gelatine and 0.5U Taq polymerase (Boehringer Mannheim, Germany) were used in a final volume of 10 µL. Thirty cycles of denaturation (94°C), annealing and extension (72°C) were carried out on an automated heat block (Perkin-Elmer Cetus, Norwalk, CT, USA). The reaction mixture (4 µL) was diluted 1:25 in 0.1% $NaDodSO_4$- 10 mM EDTA and mixed 1:1 with a sequencing stop solution containing 20 mM NaOH. The samples were heated at 95°C for 5 minutes, chilled on ice and immediately loaded (4 µL) onto a 6% acrylamide-TBE gel containing 10% glycerol. The gels were run at 8W for 12-15 hrs at room temperature, fixed in 10% acetic acid and air dried. Autoradiography was performed overnight at –80°C using an intensifying screen.

Statistical analysis

The Wilcoxon rank-sum test, Pearson correlation coefficients and all statistical calculations were performed using the SAS/STAT software package, release 6.06 of SAS Institute Inc.,1993.

Results

MTT assay results and definition of drug resistance

Table 1 shows the LD50 values for 2-CDA, FAMP and CLB in samples obtained from early stage CLL patients. The lowest median LD50 value was reached after exposure of CLL cells to 1.55 µM 2-CDA versus 4.41 µM FAMP and 58.2 µM CLB. As previously reported,[18] we referred to plasma drug levels achievable *in vivo* during therapy for the cut-off values in order to define a sample as sensitive or resistant to each drug. These values corresponded to 0.3 µM for 2-CDA,[19]), 3 µM for FAMP[20] and 7 µM for CLB.[21] Thus, 12/51 (23.5%), 21/51 (41.2%) and 6/53 (11.3%) CLL samples proved to be sensitive to 2-CDA, FAMP and CLB, respectively.

Expression of bcl-2 protein, p53 gene mutations and pattern of chemosensitivity

Expression of bcl-2, evaluated both in terms of percentages of positive cells and of mean channel of fluorescence intensity (MIF), was not significantly different between sensitive and resistant samples to 2-CDA, FAMP and CLB (Table 2). Moreover, Pearson coefficient analysis failed to demonstrate any significant correlation between the LD50 results and both the percentage and the mean fluorescence intensity of bcl-2 protein expression on CLL cells (data not shown).

To investigate the presence of p53 alterations, DNA from 30 CLL samples was amplified for p53 exons 5 to 9 and analyzed by SSCP assay. Figure 1 shows the results in 10 representative cases, one of which demonstrates a single nucleotide change in exon 5 with a missense mutation resulting in amino acid substitution (Val-Met). The mutations, detected in 4 of the 30 cases (13.3%), occurred at exon 5 in case #10, at exon 7 in case #15, at exon 8 in case

Table 1. *In vitro* cytotoxic effect of 2-CDA, FAMP and CLB on peripheral cells of CLL samples by the MTT assay. Samples were defined as sensitive on the basis of LD50 cut-off values corresponding to 0.3 μM, 3 μM and 7 μM for 2-CDA, FAMP and CLB, respectively.

LD50 (μM)	*2-CDA*	*FAMP*	*CLB*
*mean+sem	33.3±13.9	45.2±13.3	102±15.5
median	1.55	4.41	58.2
No. of samples sensitive/total (%)	12/51 (23.5)	21/51 (41.2)	6/53 (11.3)

**In some cases, the LD50 values were higher than the highest concentration of the drug tested or lower than the lowest concentration. In calculating the means, the highest and lowest concentrations were taken as the LD50 values for these samples.*

Table 2. Expression of the bcl-2 protein among sensitive and resistant samples to 2-CDA, FAMP and CLB.

	% of positive cells	*^p =*	*°MIF*	*^p =*
2-CDA				
Sensitive	*73.8±4.0	ns	24.8±5.5	ns
Resistant	79.8±2.7		24.9±2.9	
FAMP				
Sensitive	75.6±3.8	ns	22.4±4.2	ns
Resistant	80.1±2.8		22.7±3.3	
CLB				
Sensitive	65.9±6.7	ns	20.0±4.8	ns
Resistant	77.6±3.0		22.3±2.7	

**Data are expressed as mean values±SE. °MIF = mean channel of fluorescence intensity. ^Wilcoxon rank-sum test.*

Table 3. MTT assay results and p53 mutation study in 30 samples from CLL patients in the early stages of disease. The CLB, FAMP and 2-CDA data from the MTT assay are reported as LD50 values (μM).

Case #	*CLB*	*FAMP*	*2-CDA*	*p53 gene mutations*
1	39.54	12.9	13.6	NO
2	26.03	0.51	3.01	NO
3	0.032	0.035	0.004	NO
4	7.82	0.035	0.004	NO
5	7.72	0.035	0.004	NO
6	90.9	6.29	2.17	NO
7	1.47	0.28	32.5	NO
8	16.07	3.57	0.32	NO
9	12.78	14.6	1.96	NO
10	82.44	19.1	2.03	Exon 5, cod. 143; GTG(ATG, Val-Met)
11	118.8	3.37	4.75	NO
12	0.755	1.45	0.111	NO
13	35.31	0.021	0.445	NO
14	25.78	0.15	0.74	NO
15	47.86	0.393	0.162	Exon 7, cod. 248; CGG(CAG, Arg-Glm)
16	11.77	39.17	3.71	NO
17	4.54	9.64	8.38	NO
18	27.01	5.1	0.001	NO
19	15.0	0.62	3.2	NO
20	25.17	10.72	3.57	Exon 8, cod. 267; CGG(TGG, Arg-Trp)
21	7.83	1.59	0.081	NO
22	60.49	0.912	0.377	NO
23	3.58	0.168	4.83	NO
24	17.72	38.75	6.015	NO
25	15.17	4.72	3.57	NO
26	27.16	1.60	0.26	Exon 6, cod. 212; TTT(TGT, Phe-Cys)
27	2.17	0.864	0.136	NO
28	51.62	4.62	2.82	NO
29	3.34	1.36	0.366	NO
30	1.86	3.8	0.042	NO

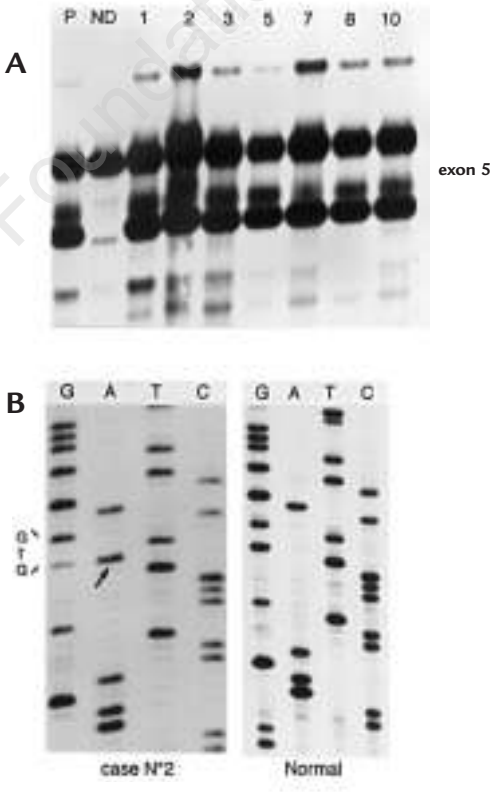

Figure 1. Analysis of p53 mutations in CLL. A. PCR/SSCP analysis of exon 5 of the p53 gene in some representative cases. The band corresponding to the undenatured normal fragment is shown (ND); placental human DNA was used as normal control (P). B. Direct sequencing of the PCR-amplified fragment corresponding to exon 5 from CLL case no. 2 (codon 143, GTG-ATG, Val-Met) and placental human DNA (normal). The 5' primer used for PCR amplification was also used as sequencing primer. The mutated codon is indicated. The band corresponding to the mutated base pair is indicated by an arrow.

#20 and at exon 6 in case #26 (Table 3). Conversely, p53 mutations were counted in both sensitive and resistant samples to purine analogs. These cases were all CLB resistant *in vitro*, while purine analog sensitivity was variable.

Discussion

In this study, the tetrazolium salt (MTT)-based assay[15-18] was used to investigate the *in vitro* cytotoxic effect of 2-CDA, FAMP and CLB in 53 untreated CLL patients in early stages of disease. Several *in vitro* assays have been used to measure drug sensitivity; in particular, the *differential staining cytotoxicity* (DiSC) assay methodology can aid in identifying new anticancer agents and in choosing a less empirical therapeutic approach in CLL.[25] The MTT assay also works well when a high number of cells are available and few non-neoplastic cells *contaminate* the specimens. These conditions are easily achievable in CLL. Moreover, the MTT assay has successfully provided a good correlation with clinical results.[26]

The median LD50 value of 2-CDA was lower than that of FAMP and CLB (1.55 μM versus 4.41 μM and 58.2 μM, respectively), but MTT assay values cannot be used crudely to predict *in vivo* clinical response. In a recent paper dealing with *in vitro* chemosensitivity to CLB, FAMP and 2-CDA,[18] we defined drug sensitivity on the basis of LD50 cut-off values of 0.3 μM, 3 μM, and 7 μM, corresponding to the plasma levels achievable after standard therapy with 2-CDA,[19] FAMP[20] and CLB.[21] Thus clustered, about 41%, 23% and 10% of the samples from CLL patients in early stage of disease in the present study, proved to be sensitive to FAMP, 2-CDA and CLB, respectively. In order to validate this method of defining *in vitro* drug sensitivity we are conducting a prospective *in vitro/in vivo* study with a large cohort of CLL patients. Our preliminary data indicate that predictive accuracy with the MTT assay was at least 30-40% for those achieving, and 100% for patients failing to achieve a complete clinical response (paper in preparation). According to these results, the MTT assay is useful not only in identifying patients whose lymphocyte counts should be reduced after treatment, as previously reported,[27] but also in predicting clinical complete responders. These findings could be helpful for knowing in advance the likelihood of a clinical response and thus preventing unnecessary toxicity in the elderly CLL population.

Overexpression of the bcl-2 protein occurs in a significant portion of CLL samples,[13] a condition which contributes to leukemic cell survival through the loss of apoptotic function.[28] During culture, the bcl-2 content of the cells is reduced and they die by apoptosis.[29] Interleukin (IL)-4, as well as α- and γ-interferon are capable of preventing apoptotic death in some cases[29-33] by maintaining cell bcl-2 protein levels. Conversely, IL-10 downregulates bcl-2 protein content,[34] and this finding could introduce a new concept to therapeutic strategy in CLL. In our study, expression of the bcl-2 oncoprotein, as evaluated by flow cytometry analysis, failed to correlate with the sensitivity pattern to 2-CDA, FAMP and CLB, drugs capable of inducing apoptosis in CLL cells.[7,8] Western blotting was employed to evaluate a possible correlation between bcl-2 protein content and FAMP-induced DNA fragmentation in CLL.[35] The results of that analysis are in line with our finding; in fact, the amount of the oncoprotein, as determined by this more accurate method, did not correlate with the level of FAMP-induced DNA fragmentation,[35] while its higher expression was inversely related with survival in untreated CLL patients. In an ECOG study, Kitada demonstrated that the expression of bcl-2 and its related proteins Bax, Mcl-1 and bcl-x, as evaluated by immunoblotting, did not correlate with either FAMP- or 2-CDA-induced apoptosis *in vitro*.[36] In addition, our preliminary data suggest that CLL cells, whose bcl-2 content was downregulated by IL-10, did not show any significant improvement in their initial *in vitro* drug chemosensitivity (paper in preparation), confirming a limited role, at least *in vitro*, for bcl-2 in protecting CLL cells from drug-induced cytotoxicity. Overall, these data suggest that bcl-2 expression seems to have an impact on disease progression, but not on treatment response.

Although inactivation of the p53 gene by mutation or allelic deletion has been found in about 10-15% of CLL patients[14,23,27,37] and therefore it may be defined a relatively uncommon event, evidence of a role for p53 in conferring pharmacological resistance to CLL cells has been documented.[14,27,37] In this respect, a correlation between p53 gene mutations and resistance to CLB has been demonstrated.[14,27] More recently, it was reported that none of the CLL patients showing a p53 gene deletion, compared with 56% of the cases without a deletion, responded to therapy with purine analogs.[37] In our study, p53 gene mutations were detected in 4/30 samples, which, in turn, proved to be resistant to CLB *in vitro* in all cases, and to both purine analogues in 2 cases. As in El Rouby's report,[14] both drug resistant and sensitive cases were observed in the group of patients showing wild type p53, suggesting that p53 inactivation is not a major mechanism of drug resistance in CLL. Therefore alternative mechanisms which exhibit drug-protection capability should be considered. Together with the well-known mechanisms of resistance, such as variable cellular levels of sulfydryl groups,[38] deoxycytidine kinase, 5'-nucleotidase[39] and DNA repair-enzyme,[40] cytokines and growth factors were said to play a role in this field,[17,41] In particular, high intra-

cellular levels of basic fibroblastic growth factor were reported to be associated with a delay in FAMP-induced cell death.[42]

In conclusion, the study of drug resistance mechanisms, together with *in vitro* chemosensitivity assays, although not capable of mimicking the complexity of *in vivo* pharmacological resistance mechanisms, could be extended to prospective trials aimed at showing their usefulness in predicting clinical response in CLL.

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