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ANEXO 1

de la tesis doctoral con el título:

STAT1 EN LA APOPTOSIS INDUCIDA POR FLUDARABINA E INHIBIDORES DE JAK KINASAS EN LAS CELULAS DE LLC-B.
PAPEL DE LAS CELULAS ADHERENTES EN LA APOPTOSIS INDUCIDA POR FLUDARABINA

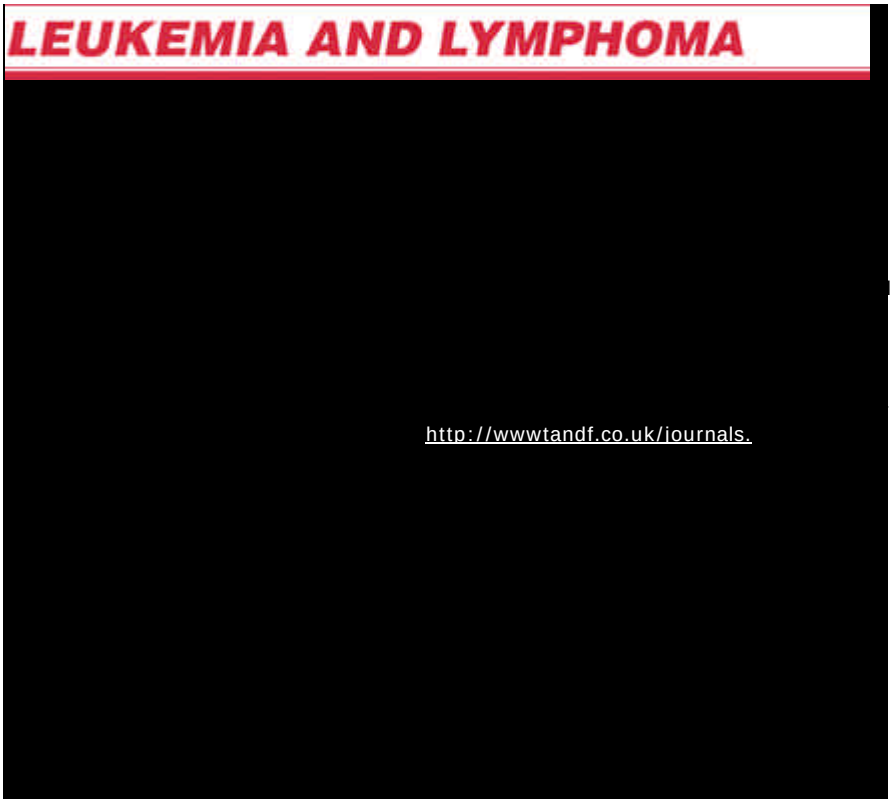
Presentada para la obtención del título de Doctor en Inmunología por la Universitat Autònoma de Barcelona

Luis Martinez Lostao
Barcelona, Diciembre del 2004

ANEXO 1

ANEXO 1

Recientemente ha sido aceptado para su publicación el manuscrito que se encontraba en revisión en la revista *Leukemia & Lymphoma*:



A continuación, se incluye la pruebas de imprenta del mencionado artículo tal y como han sido enviadas por la Editorial *Taylor & Francis* para su próxima publicación en la revista *Leukemia & Lymphoma*.

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Role of the STAT1 pathway in apoptosis induced by fludarabine and JAK kinase inhibitors in B-cell chronic lymphocytic leukemia

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Abstract

Signal transducers and activators of transcription (STAT) proteins comprise a family of transcription factors that have been implicated in tumoral transformation, especially in hematological malignancies. Because of this, the JAK/STAT pathway is attractive as a therapeutic target in these tumors. In the present study, we analyzed the ability of fludarabine and two JAK kinase inhibitors, AG490 and WHI-P131, to block STAT1 activation and induce apoptosis on B-cell chronic lymphocytic leukemia (B-CLL) cells. All drugs were able to induce a high percentage of apoptosis on B-CLL cells from all patients studied. However, only AG490 and WHI-P131 were able to strongly suppress the STAT1 activation of B-CLL cells. In conclusion, our data show that JAK kinase inhibitors, such as AG490 and WHI-P131 are able to inhibit the STAT1 pathway on B-CLL cells and are strong inductors of apoptosis on these cells.

Keywords: *STAT1, B-CLL, apoptosis, fludarabine, AG490, WHI-P131*

Introduction

Signal transducers and activators of transcription (STAT) proteins comprise a family of transcription factors involved in normal cellular events, such as differentiation, proliferation, cell survival, apoptosis, normal hematopoiesis and regulation of hematopoietic cell function [1]. However, aberrant activation of STAT signaling gives rise to various pathological events, such as cell transformation and oncogenesis, specially in hematological malignancies [2]. Implication of the JAK/STAT pathway in these disorders has been widely reported. JAKs and STATs are constitutively activated in hematopoietic cells transformed by oncogenic tyrosine kinases, and also in a variety of lymphomas and leukemias [3–6]. Direct evidence implicating deregulation of the JAK/STAT pathway in hematological malignancies was the identification of the TEL-JAK2 fusion protein causing constitutive activation in lymphoid and myeloid leukemias in mice and humans [7,8].

B-cell chronic lymphocytic leukemia (B-CLL), the most common leukemia in the Western world, is characterized by the accumulation of mature CD5+ B-cells arrested in the G0/early G1 phase of the cell cycle. There is strong evidence that the B-CLL is primarily related to defective apoptosis [9–11]. Although serine phosphorylation of STAT1 and STAT3 has been described in cells from untreated B-CLL patients [12], no constitutive tyrosine phosphorylation of STATs has been seen in B-CLL. However, it has been reported that fludarabine was able to inhibit STAT1 signaling in lymphocytes and other cell types causing a specific depletion of this protein and, therefore, suppressing the ability of the cells to respond to interferons and others cytokines and growth factors that mediate their action through the activation of STAT1 [13]. This observation raises the possibility that, although tyrosine phosphorylation of STAT1 has not been observed in unstimulated cells from B-CLL patients, STAT1 could be a key factor in the transmission of antiapoptotic signals in B-CLL. It also supports

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further studies on drugs able to block the STAT1 activation for use as therapeutic drugs on B-CLL patients. In line with this observation, the JAK kinase inhibitor AG490 suppresses the growth of acute lymphoblastic leukemia (ALL) cells in an in vitro and in vivo model without affecting normal hematopoiesis [14]. Another JAK kinase inhibitor, WHI-P131, induces apoptosis on several leukemic cell lines and ALL cells [15,16].

In the present study, we analyzed the ability of fludarabine, a well known inductor of apoptosis in B-CLL cells, and two JAK kinase inhibitors, AG490 [17,18] and WHI-P131 [19], to block STAT1 activation on B-CLL cells. We also studied the ability of these drugs to induce apoptosis on these cells. Our data show that while all drugs were able to induce apoptosis on the B-CLL samples analyzed, only AG490 and WHI-P131 were able to strongly suppress STAT1 activation by interferon-gamma (IFN-γ) on B-CLL cells.

Materials and methods

Patients and cell samples

This study included 18 patients with B-CLL diagnosed according to standard criteria. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples from B-CLL patients by density-gradient centrifugation over Ficoll-Hypaque (Lymphoprep™; Nycomed, Oslo, Norway). All B-CLL samples selected for this study contained more than 90% of CD19+/CD5+ tumoral cells as determined by flow cytometry analysis. Table I summarizes the clinical features of the 18 patients included in the study. In some cases studies with all drugs were not done because of unavailability of cells.

Reagents

Fludarabine phosphate (Beneflur; Schering, Madrid, Spain) was used in cell cultures at a final concentration of 50 μM. AG490 (Tyrphostin A42; Calbiochem, San Diego, CA) and WHI-P131 (Calbiochem, San Diego, CA) were solubilized in DMSO (Dimethyl sulfoxide, Merck, Hoberbrunn, Germany), stored at -20°C and protected from light. AG490 and WHI-P131 were used in cell cultures at a final concentration of 170 μM. Human interferon-gamma (hIFN-γ: Roche, Mannheim, Germany) was used at a final concentration of 100 U ml^{-1}.

Cell cultures

PBMC from B-CLL patients isolated by density-gradient centrifugation were resuspended at concentrations of 4×10^6 cells ml^{-1} in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 10% Fetal Bovine Serum (Bio Whittaker, Verviers, Belgium), penicillin 100 U ml^{-1}, streptomycin 100 μg ml^{-1} (Bio Whittaker, Verviers, Belgium) and 2 mmol l^{-1} glutamine (Gibco BRL, Rockville MD) and cultured at 37°C and 5% of CO_2.

PBMC were incubated with drugs or maintained untreated for 24 h. Because AG490 and WHI-P131 were solubilized in DMSO, cells treated only with

Table I. Clinical features of the 18 B-CLL patients included in the study.

ID patients	Age (years)	Age/Sex	Duration of disease	Leukocyte count ($\times 10^9$/l)	Lymphocyte count ($\times 10^9$/l)	Rai staging	Previous *treatment
6A	54	F	3	14.15	11.28	II	None
7A	72	M	6	16.41	10.68	II	None
11A	71	F	3	31.92	23.62	0	None
13D	75	M	3	73.33	64.53	II	None
14C	91	F	7	7.45	4.09	I	None
15A	71	M	2	14.69	10.66	0	None
22A	53	M	11	25.08	23.32	I	None
27B	45	F	7	33.92	28.15	0	None
30A	67	M	7	11.78	6.73	0	None
31A	61	M	3	84.11	77.38	II	None
32A	77	M	1	10.48	6.21	0	None
33A	90	F	14	27.04	22.82	0	None
34A	71	M	1	20.36	16.08	II	None
36A	75	M	2	18.47	13.55	0	None
38A	63	M	5	32.05	27.27	0	None
39A	78	M	2	25.83	15.91	0	None
40A	86	M	2	38.40	28.42	0	None
41A	78	F	4	6.19	5.69	IV	None

*Previous treatment in the last 9 months.

DMSO for 24 h were used as a control. After treatment, PMBC were activated with hIFN-γ for 30 min. Cells were then used to perform cell extracts for EMSA.

Analysis of apoptosis by annexin V binding

Exposure of translocated phosphatydilserine in apoptotic CD19+/CD5+ B-CLL cells was quantified by surface annexin V binding staining as previously described [20]. In brief, 2×10^5 aliquots of controls and drug-treated mononuclear cells were collected, washed in PBS, and incubated with phycoerythrin (PE)-conjugated anti-CD19 monoclonal antibody (Immunotech, Marseille, France), and phycoerythrin-cyanin 5 (Pc5)-conjugated anti-CD5 monoclonal antibody (Immunotech, Marseille, France) for 15 min in the dark. Then, cells were washed and resuspended in 200 μl of binding buffer (10 mmol l^{-1} HEPES, pH 7.4, 2.5 mmol l^{-1} $CaCl_2$, 140 mmol l^{-1} NaCl), and incubated with 0.5 μg ml^{-1} of annexin V-fluorescein isothiocyanate (FITC) (Bender MedSystems, Vienna, Austria) for 15 min in the dark. Samples were analyzed on an Epics XL-MCL (Coulter, Miami, Florida). Flow cytometry data were analyzed using XL2 software (Coulter, Miami, Florida).

Apoptosis in B-CLL samples was analyzed at 0, 24, 48 and 72 h of treatment with the corresponding drugs (fludarabine, AG490 and WHI-P131).

Cell extract

Cell extracts from PBMC from B-CLL samples were obtained as previously described [21]. Briefly, after treatment with the different drugs and hIFN-γ, cells were placed on ice and washed twice with cold Tris-buffered saline (TBS: 10 mM Tris-HCl pH 7.4, 150 mM NaCl). They were extracted in buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EGTA and 5 mM $MgCl_2$ 5 with 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg ml^{-1} protease inhibitor cocktail Complete Mini EDTA-free (Roche, Mannheim, Germany). Insoluble material was removed by centrifugation at 15,000 *g* for 1 min. Soluble material was considered as cytoplasmatic extract and stored at − 80°C.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed according to Kanno *et al.* [22] with some modifications [23]. Briefly, cell extracts were incubated with 50,000 c.p.m. (equivalent to approx. 1 ng) ^{32}P-labeled Fcγ oligonucleotide (5′-GATCGTATTTCCCAGAAAAGGAAC-3′ and its complement) in 15 μl of binding buffer (20 mM HEPES pH 7.9, 50 mM KCl, 10% glycerol, 0.1 mM EDTA pH 7.9, 0.5 mM DTT) containing 4 μg of DNA calf thymus (Pharmacia Biotech, Uppsala, Sweden). All extracts were normalized for protein concentration using the Bio-Rad protein assay reactive (BIO-RAD, Munchen, Germany) according to the manufacturer's instructions. Incubation was performed at room temperature for 20 min. The products of the binding reaction were then separated on a 5% acrylamide gel in running buffer (Tris-base 90 mM, boric acid 90 mM, Na_2EDTA 2.5 mM) at 200 V for 1.5 h. The gel was dried, autoradiographed at − 80°C and the band shift intensities were assessed with Fluor-S MultiImager (BIO-RAD, Hercules, CA) using software Quantity One 4.3.1. (BIO-RAD, Hercules, CA).

Three EMSA independent assays were performed for each sample. Extract from B-CLL cells activated with hIFN-γ was considered as the positive control of the activation of the STAT1 pathway. STAT1 activation of the drug-treated extracts for each leukemia was calculated in percentage values taking the mean EMSA signal from its positive control as 100% of activation.

Data analysis

EMSA results are shown as mean ± SD of three experiments each. Statistical significance was evaluated using Student *t* test. A P value < 0.05 was considered significant. Associations between the inhibition of STAT1 activation induced by the drugs on B-CLL cells and drug-induced apoptosis were determined using Pearson's correlation coefficient. A P value of < 0.05 was considered significant.

Results

Analysis of drug-induced apoptosis

Flow cytometry analysis with annexin V-FITC staining was performed to measure drug induced-apoptosis in B-CLL cells (Figure 1A). The effect of the drugs on the viability of B-CLL cells from all patients at several time points (0, 24, 48 and 72 h) is shown in Figure 2. The data of drug induced apoptosis of each B-CLL patient at 72 h is shown in Table II. Samples treated with fludarabine, AG-490 or WHI-P131 at 72 h showed high percentages of annexin V positive B-CLL cells. As expected, fludarabine induced apoptosis in all samples (mean = 77.8%; range: 44.5–93.1%; $P < 0.00001$). AG490 and WHI-P131 also induced apoptosis in high percentages in B-CLL cells (AG490: mean = 85.3%; range: 52.7–96.5%; $P < 0.00001$;

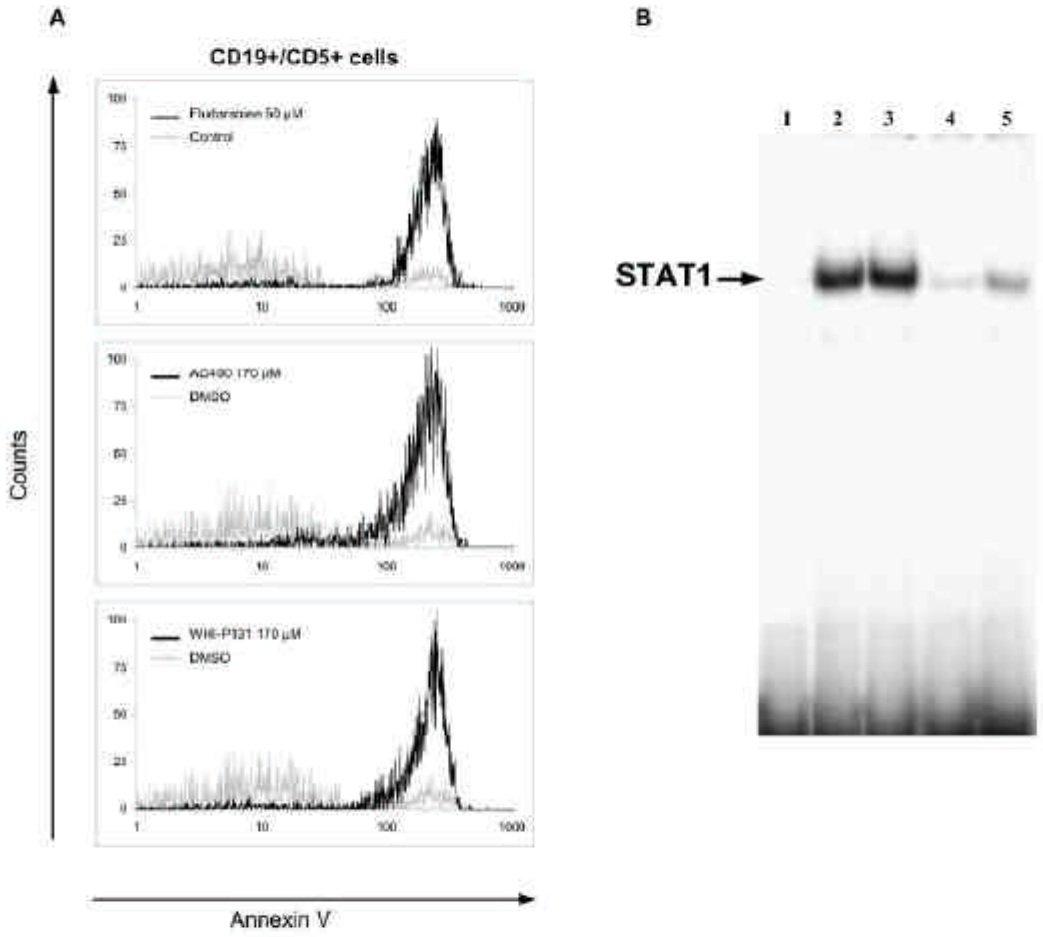

Figure 1. Flow cytometry and EMSA analysis in a representative B-CLL patient. Flow cytometry was performed to analyze apoptosis in B-CLL cells in vitro in a B-CLL patient (CLL 40A) after 72 h of treatment with fludarabine, AG490 and WHI-P131.(A) Apoptosis in B-CLL cells was expressed as percentage values of CD19 + /CD5 + B-CLL cells annexin V positive. The histogram shows apoptosis with the drugs (black) and apoptosis on controls (grey). (B) EMSA was performed to analyze drug-induced inhibition of the hIFN-γ-induced STAT1 activation in the same patient (CLL 40A). (1) untreated B-CLL cells; (2) B-CLL cells treated with hIFN-γ; (3) B-CLL cells treated with fludarabine 50 μM and then with hIFN-γ; (4) B-CLL cells treated with WHI-P131 170 μM and later with hIFN-γ; (5) B-CLL cells treated with AG490 170 μM and then with hIFN-γ. EMSA was performed using cell extracts from hIFN-γ-stimulated B-CLL cells previously treated or untreated with the different drugs (fludarabine, AG490 and WHI-P131) for 24 h and a ^{32}P-labeled Fcγ oligonucleotide probe. DNA-STAT1 complexes were separated by 5% acrylamide gel, and the results were analyzed by Fluor-S MultiImager. Detailed methodology is given under Materials and methods. In this representative B-CLL sample, only the previous treatments with AG490 and WHI-P131 were able to inhibit the activation of STAT1 induced by hIFN-γ (black arrow).

WHI-P131: mean = 84.4%; range: 67.3–96.3%; $P < 0.00001$) and was comparable to apoptosis induced by fludarabine (AG490 vs. fludarabine: $P = 0.287$; WHI-P131 vs. fludarabine: $P = 0.251$).

Analysis of drug-induced inhibition of STAT1 activation

Analysis of the inhibition of STAT1 activation induced by the drugs on B-CLL samples was performed by EMSA. Figure 1B shows cell extracts from a representative B-CLL sample treated in vitro with fludarabine, AG490 and WHI-P131 for 24 h and analyzed to evaluate inhibition of hIFN-γ-induced STAT1 activation. In this sample, previous treatment with fludarabine did not inhibit STAT1 activation by hIFN-γ whereas treatment with WHI-P131 and AG490 inhibited hIFN-γ-induced STAT1 activation (Figure 1B).

In the case of fludarabine, when B-CLL samples were treated with 50 μM dose for 24 h, a decrease (mean = 22.6%; range: 0–75.2%; $P = 0.279$; Figure 3A) in the amount of STAT1 activated by IFN-γ was

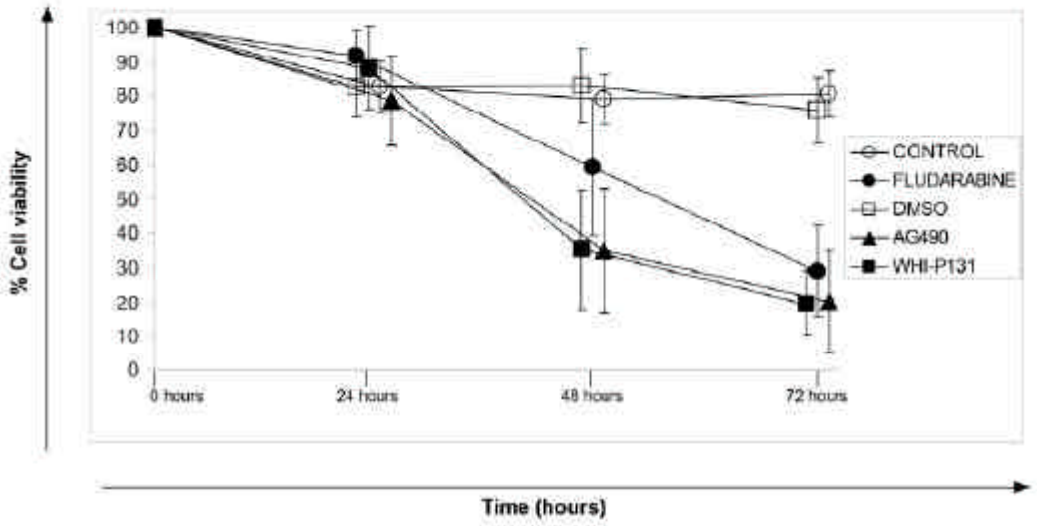

Figure 2. Viability of tumoral cells from all B-CLL patients treated with the drugs at several time points. Treatment with fludarabine at 50 μM was normalized against control (untreated cells). Treatment with AG490 at 170 μM and WHI-P131 at 170 μM was normalized against DMSO. B-CLL cell viability is shown as mean percentage ± standard deviation.

Table II. Drug-induced apoptosis of the 18 B-CLL patients included in the study.

ID patients	Control	Fludarabine 50 μM	DMSO 10 μg ml^{-1}	AG490 170 μM	WHI-P131 170 μM
6A	16.6	54.3	18.9	52.7	52.1
7A	25.2	88.4	ND	ND	ND
11A	ND	ND	39.3	95.3	ND
13D	30.9	46.5	40.0	95.3	86.4
14C	14.8	75.0	ND	ND	ND
15A	ND	ND	26.3	92.2	ND
22A	ND	ND	36.1	80.0	ND
27B	14.4	46.6	17.5	64.2	84.7
30A	8.8	77.6	ND	ND	ND
31A	31.8	66.8	28.1	ND	90.5
32A	21.7	90.8	ND	ND	ND
33A	21.3	81.8	12.5	96.5	96.3
34A	19.0	67.0	22.8	96.5	ND
38A	18.7	27.7	9.3	ND	67.3
36A	5.6	49.4	ND	ND	ND
39A	19.4	41.6	28.1	87.1	ND
40A	17.7	51.0	22.9	93.5	85.2
41A	23.6	65.9	18.5	74.5	ND

observed. Only in two samples the inhibition of STAT1 activation was significant (CLL 7A, $P=0.007$; CLL 41A, $P=0.043$).

When B-CLL samples were treated with AG490, a JAK kinase inhibitor, a strong inhibition of STAT1 signal was found in 10 of 11 B-CLL samples (mean = 70.2%; range: 34.7–100%; $P=0.004$; Figure 3B).

WHI-P131 has been described as a selective JAK3 kinase inhibitor [24], and it should not affect the activation of the STAT1 pathway by IFN-γ. Surprisingly, when we analyzed inhibition of STAT1 activation by WHI-P131 (Figure 3C), a strong inhibition was observed in 6 of the 7 samples (mean = 78.7%; range: 36.5–96.4%; $P=0.043$). Remarkably, a strong correlation between inhibition of STAT1 activation and apoptosis was found with WHI-P131 ($R^2=0.7116$, $P=0.017$).

Discussion

The results of this study clearly show that while fludarabine, AG490 and WHI-P131 were able to induce a high percentage of apoptosis in all analyzed

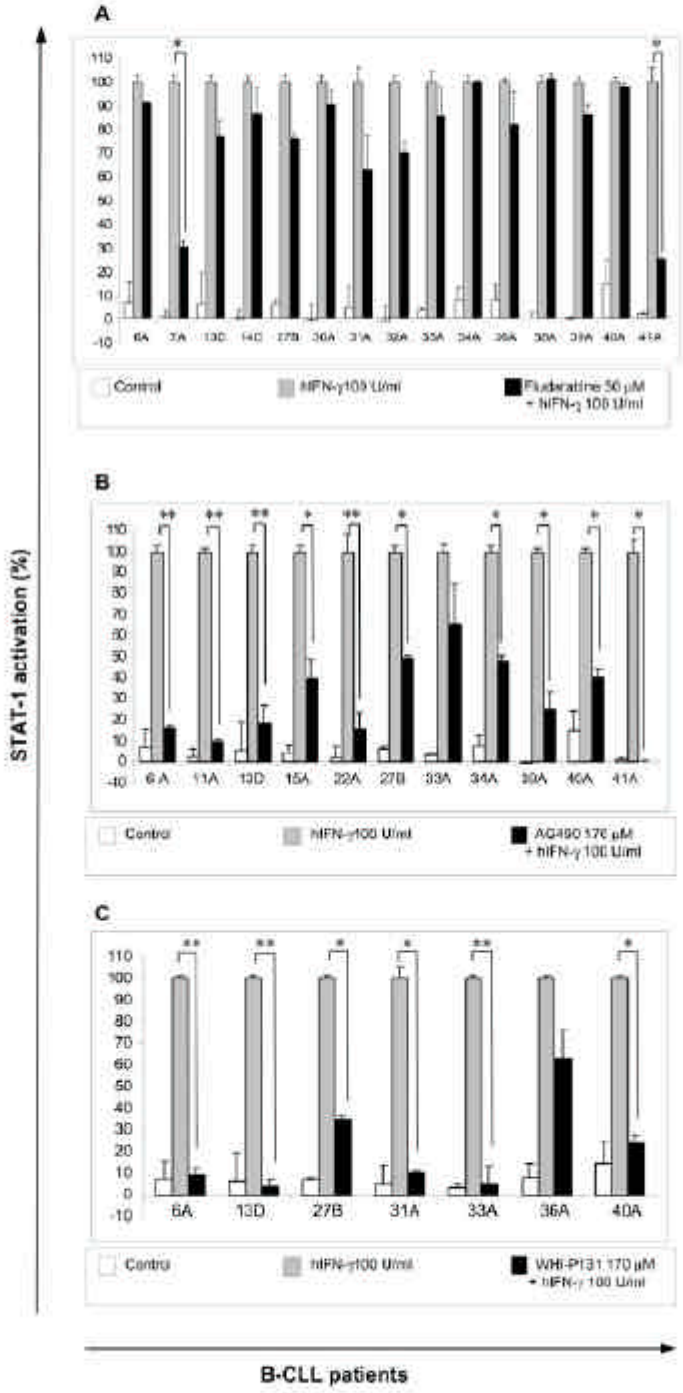

Figure 3. Effect of drugs in the STAT1 activation on B-CLL cells. (A) EMSA analysis of the samples from 15 B-CLL patients treated in vitro with 50 μM fludarabine for 24 h; (B) 11 B-CLL patients treated in vitro with 170 μM AG490 for 24 h; (C) 7 B-CLL patients treated in vitro with 170 μM WHI-P131 for 24 h. This figure shows mean percentage $\pm$ standard deviation of three independent EMSA experiments performed for each B-CLL sample. Extract from B-CLL cells activated with hIFN-γ was considered as the positive control of the activation of the STAT1 pathway. STAT1 activation of the drug-treated extracts for each leukemia sample shown as percentage values taking the EMSA signal from its positive control as 100% of activation. *Significant decrease in the activation of STAT1 on B-CLL cells treated with AG490 compared to control value at $P < 0.05$. **Significant decrease in the activation of STAT1 on B-CLL cells treated with AG490 compared to control value at $P < 0.01$.

B-CLL samples, only AG490 and WHI-P131 were able to strongly suppress the activation of the STAT1 pathway. The role of fludarabine as inductor of apoptosis on B-CLL cells is well known [25]. However, to our knowledge this is the first time that apoptotic activity of AG490 and WHI-P131 is showed on B-CLL cells.

The role of fludarabine in STAT1 inhibition is unclear. Although a previous study [13] had shown a strong inhibition of STAT1 on lymphocytes treated with fludarabine, in a recent study by the same group, only a slight inhibition of STAT1 on fludarabine-treated B-CLL cells was found [26]. In line with this, we found a not significant inhibition of STAT1 activation in the group of B-CLL samples treated with fludarabine.

AG490, a tyrphostin family molecule, was included in this study because it was first described as a selective inhibitor of JAK2 tyrosine kinase [27], and therefore able to interfere with the activation of STAT1 by IFN-γ. In this paper we have shown that AG490 inhibits STAT1 activation, and importantly, induces apoptosis of B-CLL cells. Nevertheless, since AG490 has also been described as a JAK3 inhibitor [18,28], a role of this pathway in apoptosis of B-CLL cells induced by AG490 can not be ruled out.

The other JAK kinase inhibitor used in this study, WHI-P131, has been described as a specific inhibitor of JAK3 [19], and abolished the thrombin-induced tyrosine phosphorylation of STAT1 and STAT3 dependent on activity of this JAK kinase [24].

Moreover, since the STAT1 activation by IFN-γ is dependent of JAK1 and JAK2 kinases, as previously reported [29], our data support that WHI-P131 is able to inhibit JAK1 and/or JAK2 kinases on B-CLL cells. It is worth mentioning that when B-CLL cells were treated with WHI-P131, a significant correlation was observed between the suppression of STAT1 activation and the apoptosis on B-CLL cells.

It has been described that several cytokines, such as IFN-γ [30], IL-4 [31] and IL-8 [32], inhibit in vitro apoptosis in a large percentage of B-CLL cells; and that B-CLL cells produce IL-7, and express IL-7 receptor which could have a key role in the leukemic process [33]. Therefore, drugs like AG490 and WHI-P131, able to suppress JAK kinase signaling, could block the antiapoptotic signals provided by these or other cytokines and growth factors, explaining the strong induction of apoptosis by these drugs.

In conclusion, our study shows, for the first time, that drugs that block the JAK/STAT pathway, such as AG490 and WHI-P131, induce apoptosis on B-CLL cells. Since apoptosis induced by fludarabine does not seems to be related with inhibition of the STAT1 pathway, combination of this drug with JAK kinase inhibitors could be synergistic for induction of apoptosis on B-CLL cells. Based in these data, combination of these drugs offers a potential new way to treat patients with B-CLL.

Acknowledgments

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