Akt inhibitors induce apoptosis in chronic lymphocytic leukemia cells

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ABSTRACT

Background

The phosphatidylinositol-3-kinase/Akt pathway has been described to be critical in the survival of chronic lymphocytic leukemia cells. In this study we analyzed the effect of two selective chemical inhibitors of Akt (Akti-1/2 and A-443654) on the survival of chronic lymphocytic leukemia cells.

Design and Methods

Using cytometry we studied the cytotoxic effects of Akt inhibitors on peripheral B and T lymphocytes from patients with chronic lymphocytic leukemia and from healthy donors. We studied the changes induced by Akti-1/2 and A-443654 at the mRNA level by performing reverse transcriptase multiplex ligation–dependent probe amplification. We also studied the changes induced by both Akt inhibitors in some BCL-2 protein family members on chronic lymphocytic leukemia cells by western blotting. Moreover, we analyzed the cytotoxic effect of Akt inhibitors in patients' cells with deleted/mutated *TP53*.

Results

Both inhibitors induced apoptosis in chronic lymphocytic leukemia cells in a dose-dependent manner. Moreover, B cells from patients with chronic lymphocytic leukemia were more sensitive to Akt inhibitors than T cells from leukemic patients, and B or T cells from healthy donors. Survival factors for chronic lymphocytic leukemia cells, such as interleukin-4 and stromal cell-derived factor-1α, were not able to block the apoptosis induced by either Akt inhibitor. Akti-1/2 did not induce any change in the mRNA expression profile of genes involved in apoptosis, while A-443654 induced some changes, including an increase in *NOXA* and *PUMA* mRNA levels, suggesting the existence of additional targets for A-443654. Both inhibitors induced an increase in PUMA and NOXA protein levels, and a decrease in MCL-1 protein level. Moreover, Akti-1/2 and A-443654 induced apoptosis irrespective of *TP53* status.

Conclusions

These results demonstrate that Akt inhibitors induce apoptosis of chronic lymphocytic leukemia cells and might be a new therapeutic option for the treatment of chronic lymphocytic leukemia.

Key words: Akt, chronic lymphocytic leukemia, apoptosis.

Introduction

The phosphatidylinositol-3-kinase (PI3K) pathway has been described to be critical in the survival of chronic lymphocytic leukemia (CLL) cells.[1-11] PI3K phosphorylates the D-3 position of phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-diphosphate. The cellular levels of PI3K products are controlled by the balance between PI3K activity and the phosphatase activity of PTEN (phosphatase and tensin homolog deleted on chromosome ten).[12] Interestingly, PTEN protein is reduced or not detected in 48% of patients with CLL.[13]

One of the most important targets of PI3K products is the serine-threonine kinase Akt, also known as protein kinase B (PKB).[14] Akt resides in the cytosol in a low-activity conformation, and it is activated through recruitment to cell membranes by PI3K lipid products and phosphorylation at Thr308 and Ser473. Once Akt is activated, it is able to promote cell survival through phosphorylation and inactivation of key components in the apoptotic cascade. Akt substrates include the members of the Forkhead family of transcription factors[15] and glycogen synthase kinase-3 (GSK-3),[16] which are inhibited by Akt. Furthermore, GSK-3 inhibition induces the up-regulation of the anti-apoptotic protein MCL-1.[16] Activation of the PI3K/Akt pathway in CLL cells induces the phosphorylation of the Forkhead family member FoxO3a and GSK-3, and an increase in MCL-1 protein, while inhibition of PI3K induces a loss of cell viability, dephosphorylation of FoxO3a and GSK-3, and a decrease in the level of MCL-1 protein.[3,4,11] Importantly, a stronger activation of Akt pathway has been related to a higher capacity for cell cycle progression in CLL cells from patients with progressive disease.[17] All together, these studies suggest that Akt plays a prominent role in the survival of CLL; however, the effect of selective chemical Akt inhibitors on the survival of CLL cells has not been reported yet.

A-443654 is a potent, ATP competitive and reversible inhibitor of Akt catalyzed phosphorylation activity. It is a pan-Akt inhibitor and has equal potency against Akt1, Akt2 or Akt3.[18,19] Together with the decrease in phosphorylation of Akt targets, a concomitant increase in the phosphorylation of Ser473 and Thr308 Akt residues has been observed. This increase is PI3K-dependent, as demonstrated by the fact that incubating the cells with LY294002 blocks it.[20] Recently, it was reported that this inhibitor also inhibits other protein kinases, albeit with slightly lower potency, such as PKA, PRK2, MSK1 and DYRK1A.[21] It was also reported that Akti-1/2, an ATP non-competitive Akt inhibitor, was a highly selective Akt inhibitor, blocking Akt1 and Akt2 but not Akt3 activity; the pleckstrin homology domain is required for the activity of this inhibitor.[22,23]

In this study we examined the effect of these two Akt inhibitors on CLL cells.

Design and Methods

Patients with chronic lymphocytic leukemia, healthy donors and cell isolation

Samples from patients with CLL (Table 1) or healthy donors were studied. CLL was diagnosed according to standard clinical and laboratory criteria. Blood samples were obtained from the Hospital de Bellvitge, L'Hospitalet de Llobregat, Spain. Written informed consent was obtained from all patients in accordance with the Hospital de Bellvitge Ethical Committee's requirements.

Mononuclear cells from peripheral blood samples were isolated by centrifugation on a Ficoll-Hypaque (Seromed, Berlin, Germany) gradient and cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide (Sigma-Aldrich, Steinheim, Germany), and are referred to as CLL cells throughout this article. B-cells (CD19+ cells) were purified from samples from three patients (n. 49, 50 and 51) by using the RosetteSep Human B Cell Enrichment Cocktail (StemCell Technologies, Vancouver, Canada). Blood samples or isolated mononuclear cells were incubated with RosetteSep Human B Cell Enrichment Cocktail at 50 µL/mL for 20 min prior to centrifugation on a Ficoll-Hypaque, according to the manufacturer's protocol.

Immunological and genetic analyses

CD38 and ZAP-70 were determined by flow cytometry in fixed cells with conjugated antibodies (PE clone HB7, Becton Dickinson, Franklin Lakes, NJ, USA and Alexa-Fluor 488, Caltag Laboratories, Burlingame, CA, USA, respectively). CD19 was determined by flow cytometry with a conjugated antibody [phycoerythrin (PE)–conjugated anti-CD19; Becton Dickinson].

Genomic alterations were detected by fluorescence *in situ* hybridization (FISH). Fluorescent-labeled DNA probes were used in interphase cytogenetic analyses. Locus-specific probes (LSI P53/Spectrum-Orange, LSI ATM/Spectrum-Green, LSI 13S319/Spectrum-Orange, LSI 13q34/SpectrumAqua) were used to determine loss of these genetic regions within interphase nuclei. Trisomy 12 was detected in interphase nuclei using a chromosomal centromere enumeration probe (CEP) labeled with Spectrum-Green. These five probes are packaged together in a commercially available kit (Vysis Chronic Lymphocytic Leukemia Multicolor Kit) and were used in accordance with the manufacturer's specifications. Cells were fixed with fresh fixative before placement onto slides. The probe mixture was applied directly to slides. These slides were denaturated at 74°C for 2 min and incubated overnight at 37°C. Slides were then washed with 0.4 x saline sodium citrate-0.3% nonidet P-40 (NP-40) at 73±1°C for 2 min and 2 x saline sodium citrate-0.1% nonidet P-40 at room temperature for 1 min. 4'6-diamidino-2-phenylindole (DAPI) II counterstain was applied to the target area. Slides were stored at 20°C in the dark. Two hundred nuclei were analyzed for each probe, using a NIKON fluorescent microscope. Cut-off levels used were 5% for CEP 12 and 7% for locus-specific probes. The karyotype of all samples was determined.

Table 1. Characteristics of the chronic lymphocytic leukemia patients studied.

Patient	Age/Sex	WBC (x10⁹/L)	Lymphocytes (%)	CD19⁺ (%)	CD38⁺ (%)	ZAP70⁺ (%)	Genomic alterations	F/T	EC₅₀ (μM) Akti-1/2	EC₅₀ (μM) A-443654
1	75/F	41	78	77	2	ND	ND	F		
2	63/F	71	84	72	28	53	ND	F	7.5	
3	64/F	100	76	96	87	40	del13q, del17p	T		
4	72/M	49	96	84	2	0.56	del13q	F		
5	76/F	27	84	75	15	3	ND	F	9	0.75
6	71/M	94	94	88	16	26	normal	F		0.7
7	61/M	76	84	82	2	6	ND	F		0.6
8	70/M	98	94	86	2	5	del17p	F	8	0.6
9	77/F	137	68	90	10	14	normal	F		0.45
10	73/F	82	88	80	20	5	ND	F		0.65
11	74/F	31	93	89	ND	14	ND	F		0.4
12	65/M	16	93	83	91	38	normal	F		0.5
13	57/M	31	84	79	50	2	del11q	F		0.8
14	61/F	90	96	80	9	5	normal	T	12.5	0.65
15	50/M	60	94	80	30	6	del13q	T	10	0.85
16	71/F	77	84	86	6	12	normal	T	13	0.75
17	66/F	91	95	73	20	28	del13q	F	8.5	0.55
18	66/F	44	86	80	4	14	ND	T	11	0.75
19	81/F	76	92	85	51	27	ND	T	8	0.45
20	70/M	58	94	70	12	10	del13q	F	12	
21	82/M	67	80	80	78	0.05	ND	F	13	
22	75/F	93	84	90	4	0,64	del13q	F	15	
23	85/F	100	80	80	12	22	del13q	F	7	
24	63/F	56	91	83	13	1,5	normal	T		0.4
25	75/M	83	89	82	35	15	del13q	F		
26	53/M	113	94	94	51	10	del13q	T		0.5
27	59/F	89	90	80	7	34	normal	F	5	0.75
28	81/F	22	92	72	5	3	ND	F	6	0.65
29	86/M	22	89	71	28	42	ND	F		0.7
30	69/M	53	87	74	ND	ND	del13q	T		0.5
31	56/M	55	95	72	70	53	del13q, del11q	T	12	
32	54/M	30	73	48	13	54	ND	T	12.5	0.8
33	71/F	54	74	80	9	44	del13q	T	10	0.85
34	81/F	31	83	79	7	7	normal	T		
35	72/M	37	86	74	2	1	normal	F		
36	74/M	590	97	ND	24	52	tris12	T		
37	85/M	31	87	80	30	50	ND	F		0.4
38	71/M	48	87	87	18	4	normal	F		
39	54/M	132	93	88	19	14	normal	T		0.5
40	70/M	131	98	86	93	39	del17p, del11q	T		
41	73/F	18	86	ND	24	29	del17p	T	5	
42	81/M	24	95	85	60	31	tris12, del11q	T		
43	66/F	44	86	80	4	14	ND	T	12	0.8
44	80/M	96	93	80	2	0.48	del13q, del11q	T		
45	74/F	43	94	87	10	8	del13q	T		
46	58/M	74	89	90	24	6	del13q	T		
47	70/M	79	84	91	ND	ND	del13q	T		
48	78/M	85	95	84	ND	ND	tris12	T		
49	64/M	88	95	97	ND	10	del13q	F		
50	67/F	88	90	96	20	28	del13q	F		
51	72/M	51	92	97	7	26	ND	F		

WBC: white blood cell count; F: female; M: male; del: deletion; tris: trisomy and ND: not determined; F: fresh cells; T: frozen-thawed cells.

Reagents

Akti-1/2 (previously known as Akt-I-1/2) was purchased from Calbiochem-Novabiochem (San Diego, CA, USA), A-443654 was kindly provided by Abbott (North Chicago, IL, USA), recombinant human interleukin-4 (IL-4) and stromal cell-derived factor-1α (SDF-1α) were purchased from Immunotools (Friesoythe, Germany), annexin V–fluorescein isothiocyanate and propidium iodide were from Bender MedSystems (Vienna, Austria). Nutlin-3a was provided by Hoffmann-La Roche. Z-VAD.fmk was purchased from Bachem (Bubendorf, Switzerland). Ethanol and RNase A were from Sigma-Aldrich.

Cell culture

Lymphocytes were cultured immediately after thawing or isolation in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U penicillin and 100 ng/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. To avoid differences in cell viability due to the cell concentration, flow cytometry experiments were performed at a concentration of $1{\times}10^6$ cells/mL, whereas the concentration used for reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) and the experiments to obtain cell extracts to perform western blotting was 2.5 to $3{\times}10^6$ cells/mL.

Analysis of apoptosis by flow cytometry

Apoptosis was assessed by exposure of phosphatidylserine and membrane integrity. This was determined by annexin V-fluorescein isothiocyanate and propidium iodide double staining. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (Becton Dickinson), as described previously.[24] Cell viability was measured as the percentage of annexin V and propidium iodide double-negative cells. The results of flow cytometric analysis of three representative samples are shown in *Online Supplementary Figure S1A*.

To analyze apoptosis in T cells and B cells from the samples, $5{\times}10^5$ cells were incubated for 24 h with the indicated factors. Cells were then washed in annexin-binding buffer, and incubated in 50 μL annexin-binding buffer with allophycocyanin-conjugated anti-CD3 and phycoerythrin-conjugated anti-CD19 from Becton Dickinson, for 10 min in the dark. Cells were then diluted with annexin-binding buffer to a volume of 150 μL and incubated with 1 μL annexin V-fluorescein isothiocyanate for 15 min in the dark. Cells were analyzed using FACSCalibur and CellQuest software. Results of flow cytometric analysis of one CLL sample and one sample from a healthy donor are shown in *Online Supplementary Figure S1B*.

Apoptosis in T cells and B cells was also assessed by subdiploid DNA analysis. Briefly, $1{\times}10^6$ cells were harvested, washed twice in phosphate-buffered saline containing 1% fetal bovine serum (PBS/1% FBS) and fixed in 70% ethanol. The cells were centrifuged, washed in PBS/1% FBS, and resuspended in 0.5 mL PBS/1% FBS, containing allophycocyanin–conjugated anti-CD3 or allophycocyanin–conjugated anti-CD19. Tubes were incubated for 20 min at room temperature in the dark and then propidium iodide (50 μg/mL) and RNase A (100 μg/mL) were added and incubated for 30 min at room temperature in the dark before flow cytometry analysis to identify the sub-G_0 peak corresponding to apoptosis in $CD3^+$ or $CD19^+$ cells. Cells were analyzed using FACSCalibur and CellQuest software.

Western blot analysis

Cells were lysed with Laemmli sample buffer, and western blotting was performed as described previously.[2] We used antibodies against p53 (Ab-5, Neomarkers, Fremont, CA, USA), MCL-1 and Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BCL-2 (Dako, A/S, Glostrup, Denmark), PUMA and NOXA (Abcam, Cambridge, UK), P-Ser473-Akt, P-GSK-3α/β and P-FoxO1/FoxO3a (Cell Signaling Technologies, Beverly, MA, USA), and β-actin (Sigma-Aldrich). Antibody binding was detected using a secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK).

Reverse transcriptase multiplex ligation-dependent probe amplification

RNA was analyzed by RT-MLPA using a SALSA MLPA KIT R011 Apoptosis mRNA from MRC-Holland (Amsterdam, The Netherlands) for the simultaneous detection of 38 mRNA molecules.[25] In brief, RNA samples (200 ng total RNA) were first reverse transcribed using a gene-specific probe mix. The resulting cDNA was annealed overnight at 60°C to the MLPA probe mix. Annealed oligonucleotides were ligated by adding Ligase-65 (MRC-Holland, Amsterdam, The Netherlands) and incubated at 54°C for 15 min. Ligation products were amplified by polymerase chain reaction (PCR; 35 cycles, 30 s at 95°C; 30 s at 60°C, and 1 min at 72°C) with one unlabeled and one FAM labeled primer. The final PCR fragments amplified were separated by capillary electrophoresis on a 48-capillary ABI-Prism 3730 Genetic Analyzer (Applied Biosystems/Hitachi, Foster City, CA, USA). Peak area and height were measured using GeneScan analysis software (Applied Biosystems). The sum of all peak data was set at 100% to normalize for fluctuations in total signal between samples, and individual peaks were calculated relative to the 100% value.

Statistical analysis

Results are shown as the mean ± standard error of the mean (SEM) of values obtained in independent experiments. The paired Student's *t* test was used to compare differences between paired samples. Data were analyzed using the SPSS 14.0 software package (Chicago, IL, USA).

Results

Akti-1/2 and A-443654 inhibit Akt in chronic lymphocytic leukemia cells

To assess the effect of Akti-1/2 and A-443654 on Akt activity in CLL cells, we examined the phosphorylation status of Akt or Akt substrates which are used to assess the activation status of Akt. Akti-1/2 inhibited Ser473 phosphorylation in a dose-dependent manner (Figure 1A). As previously described for other cell types,[18,20] A-443654 induced an increase in Ser473 phosphorylation. In order to confirm that A-443654 was inhibiting Akt, we analyzed the status of the Akt substrates GSK3α/β and FoxO1/FoxO3a. Both inhibitors reduced the phosphorylation of GSK3α/β and FoxO1/FoxO3a (Figure 1B), demonstrating that they inhibited Akt activity.

Akt inhibitors induce apoptosis in chronic lymphocytic leukemia cells

To examine the ability of Akt inhibitors to induce apoptosis in CLL cells, we incubated CLL cells with a range of concentrations of Akti-1/2 (0.5-20 μM) and A-443654 (0.1-1 μM) for 24 h and measured cell viability. Both inhibitors induced apoptosis in a dose-dependent manner (Figure 2A and 2B) and in a time-dependent manner (Online Supplementary Figure S2). The half-maximal effective concentration (EC_{50}) was 9.85 ± 0.67 μM (range, 5 to 15 μM) for Akti-1/2, and 0.63 ± 0.03 μM (range, 0.40 to 0.85 μM) for A-443654. Similar results were obtained in purified $CD19^+$ CLL cells (Online Supplementary Figure S3). Akti-1/2 and A-443654 induced apoptosis in all the analyzed samples, independently of sex, ZAP70 status, CD38 status or genomic alterations (data not shown).

Differential effect of Akt inhibitors on B and T cells from patients with chronic lymphocytic leukemia and healthy donors

Next, we analyzed the sensitivity of normal B and T cells to Akt inhibitors, in terms of induction of apoptosis. The number of apoptotic T cells ($CD3^+$ cells) was measured in CLL samples and blood samples from healthy donors exposed to several doses of Akti-1/2
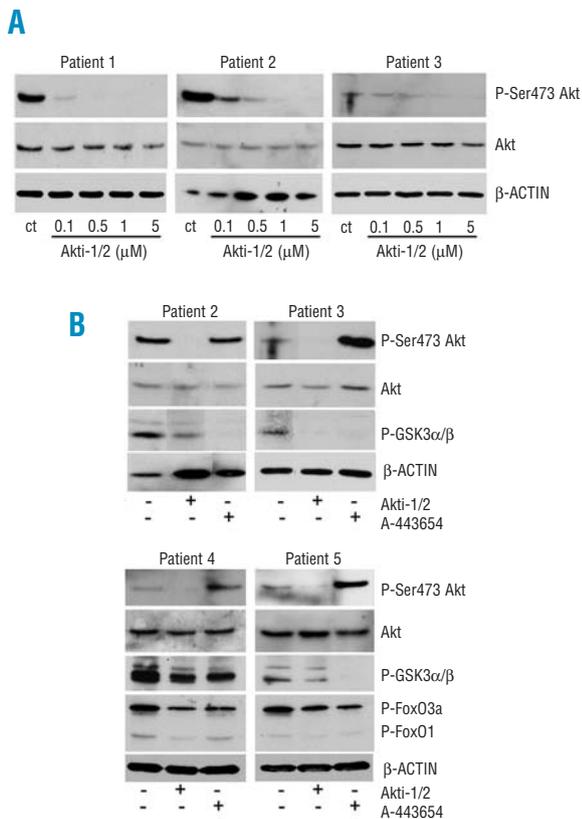
Figure 1. Akti-1/2 and A-443654 effects on the phosphorylation of Akt and Akt substrates. CLL cells were incubated with or without a range of doses of Akti-1/2 for 2 h. (A) Cells were lysed and whole extracts were analyzed by western blot as described in the Design and Methods section. Results from three patients are shown (n=3). (B) CLL cells were incubated with or without 5 μM Akti-1/2 and 0.5 μM (patients 2, 4 and 5) or 1 μM (patient 3) A-443654 for 2 h. Cells were lysed and whole extracts were analyzed by western blot as described in the Design and Methods section. Results from four representative patients are shown (n=9).
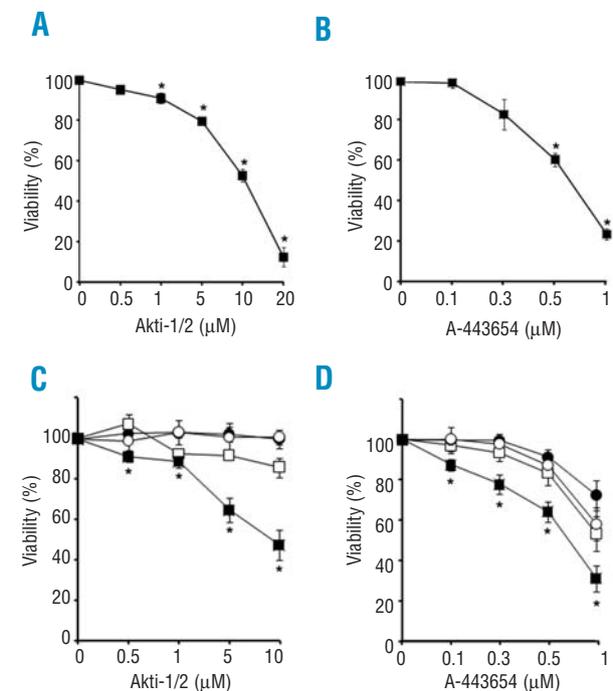
Figure 2. Cytotoxic effect of Akt inhibitors on CLL cells and on peripheral blood lymphocytes (PBL) from healthy donors. Cells from CLL patients were incubated for 24 h with or without various doses of Akti-1/2 (A, n=15) and A-443654 (B, n=15). Viability was measured by analysis of phosphatidylserine exposure and PI uptake as described in the Design and Methods section. Cells from CLL patients and healthy donors were incubated for 24 h with or without various doses of Akti-1/2 (C, n=9 and n=3, respectively) and A-443654 (D, n=13 and n = 8, respectively). Viability was measured as non-apoptotic $CD3^+/CD19^-$ T cells from PBL (○) and CLL (●) or $CD3^-/CD19^+$ B cells from PBL (□) and CLL (■) as described in the Design and Methods section. Viability is expressed as the percentage of the viability of untreated cells. Data are shown as the mean value ± SEM. *$p<0.005$, treated versus untreated cells (A, B), or B cells versus T cells from patients with CLL (C, D).

(up to 10 μM) and A-443654 (up to 1 μM) for 24 h. Incubation with 5 μM Akti-1/2 reduced the percentage of viable CLL cells to 64.64±6.12%. In contrast, the percentage of viable T cells from CLL samples was 102.4±3.46%. Interestingly, B and T cells derived from healthy donors were resistant to Akti-1/2-induced apoptosis. Thus, after incubation with 5 μM Akti-1/2 the percentage of viable B cells and T cells were 91.6 ± 2.1% and 100.7±7.2%, respectively (Figure 2C). Similar results were obtained in the treatment of CLL cells and cells from healthy donors with A-443654, but the difference between CLL cells and B or T cells from healthy donors was less pronounced than in the case of Akti-1/2. Incubation with 0.5 μM A-443654 reduced the percentage of viable CLL cells to 64.2±4.9%. In contrast, the percentage of viable T cells was 91.3±3.7%. Furthermore, B and T cells derived from healthy donors were less sensitive than cells from CLL samples to A-443654-induced apoptosis. Thus, after incubation with 0.5 μM A-443654 the percentages of viable B and T cells were 83.4±5.8% and 87.1±5.9%, respectively (Figure 2D). Similar results were obtained when CLL cells and cells from healthy donors were treated for 48 h with both Akt inhibitors (*Online Supplementary Figure S4*). We confirmed this differential induction of apoptosis between CLL cells and normal lymphocytes by performing an analysis of the DNA content. We observed that CLL cells were more sensitive than T cells and normal B cells to the Akt inhibitor-induced increase in sub-G_0 peak (*Online Supplementary Figure S5*). These results indicate that B cells from CLL samples are more sensitive than normal B and T cells to Akt inhibitor-induced apoptosis.

Effect of survival factors in combination with Akt inhibitors

We studied the effect of two well-known survival factors in CLL cells, IL-4[26] and SDF-1α,[27] in combination with Akt inhibitors. We used selected CLL samples in which these factors induced a survival effect. Thus, we treated CLL cells with 10 ng/mL IL-4 (Figure
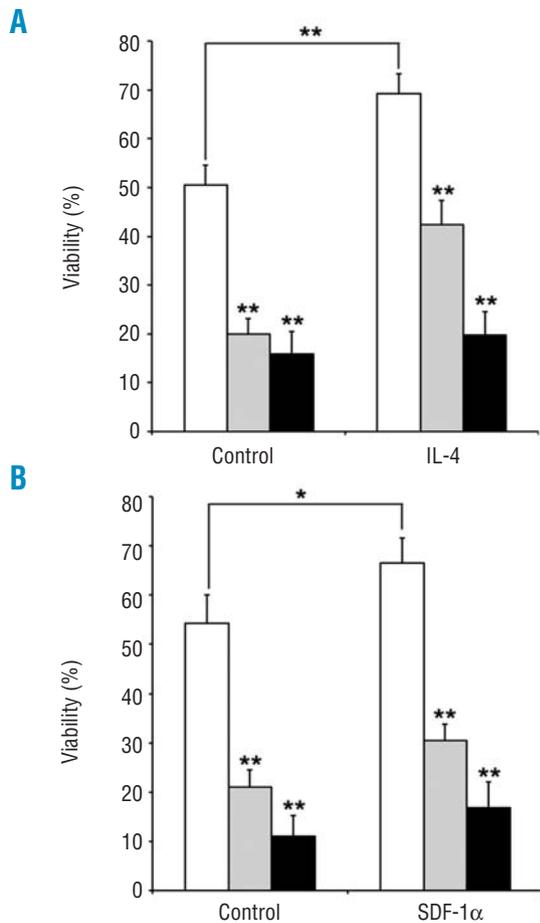
Figure 3. Effect of survival signals on the apoptotic activity of Akt inhibitors. CLL cells were untreated or treated with 10 ng/mL IL-4 (A, n = 10) or 50 ng/mL SDF-1α (B, n = 7) without (white filled bars) or with 10 μM Akti-1/2 (gray filled bars) or 0.5 μM A-443654 (black filled bars) for 48 h. Viability was measured by analysis of phosphatidylserine exposure and propidium iodide uptake as described in the Design and Methods section. Data are shown as the mean value±SEM. **p<0.001 control *versus* IL-4 and Akt inhibitors treated cells *versus* control, IL-4 or SDF-1α; *p<0.01 control *versus* SDF-1α-treated cells.
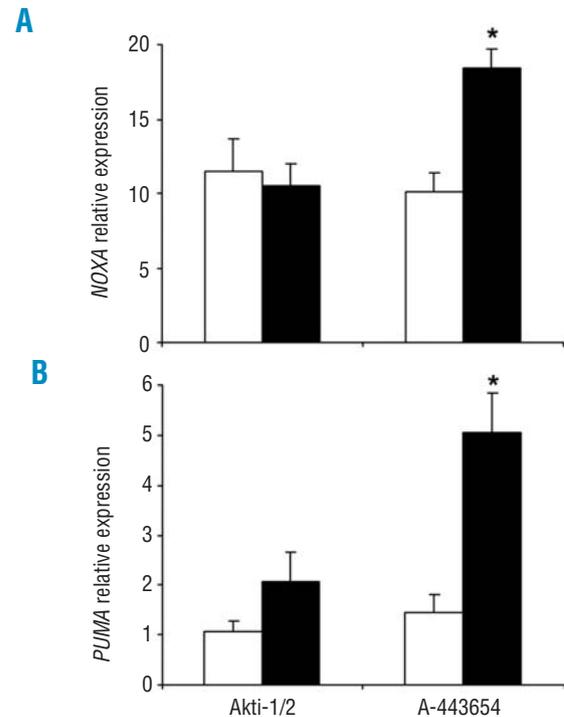
Figure 4. Apoptosis-related gene expression profile induced by Akti-1/2 and A-443654. Cells from CLL patients were untreated (open bars) or treated with 5 μM Akti-1/2 or 0.5 μM A-443654 (black filled bars) for 24 h. Cells were lysed, and *NOXA* (A) and *PUMA* (B) mRNA expression was analyzed by RT-MLPA as described in the Design and Methods section. The results are shown as the mean value ± SEM of four and eight different experiments for Akti-1/2 and A-443654, respectively. *p<0.005 treated *versus* untreated cells.

3A) or 50 ng/mL SDF-1α (Figure 3B) and with or without 10 μM Akti-1/2 or 0.5 μM A-443654 for 48 h, and then measured cell viability. Neither IL-4 nor SDF-1α was able to inhibit the apoptosis induced by both Akt inhibitors. These results show that Akt inhibitors can induce apoptosis in CLL cells even in the presence of survival signals.

Characterization of Akt inhibitor-induced apoptosis in chronic lymphocytic leukemia

We investigated the effect of Akt inhibitors on the overall apoptosis mRNA expression profile by performing RT-MLPA.[25] Treatment of CLL cells with 5 μM Akti-1/2 for 24 h did not induce any significant change in the RT-MLPA profile (Figure 4 and *Online Supplementary Figure S6A*). The results indicated that Akt inhibition has minimal effects on the apoptosis mRNA expression profile. Surprisingly, incubation of CLL cells with 0.5 μM A-443654 for 24 h induced an increase in the mRNA levels of *NOXA* and *PUMA* and a decrease in the mRNA levels of *BMF, BID*, one *BAX* probe, *BCLw, NAIP, AIF, APAF* and *APAF_L* (Figure 4 and *Online Supplementary Figure S6B*). Similar results were obtained in purified CD19+ CLL cells (*Online Supplementary Figure S7*). Thus, A-443654-induced mRNA changes are likely independent of its activity of inhibiting Akt.

We then investigated the effect of Akt inhibitors on the expression of BCL-2 family proteins. Western blot analysis revealed that Akti-1/2 and A-443654 increased
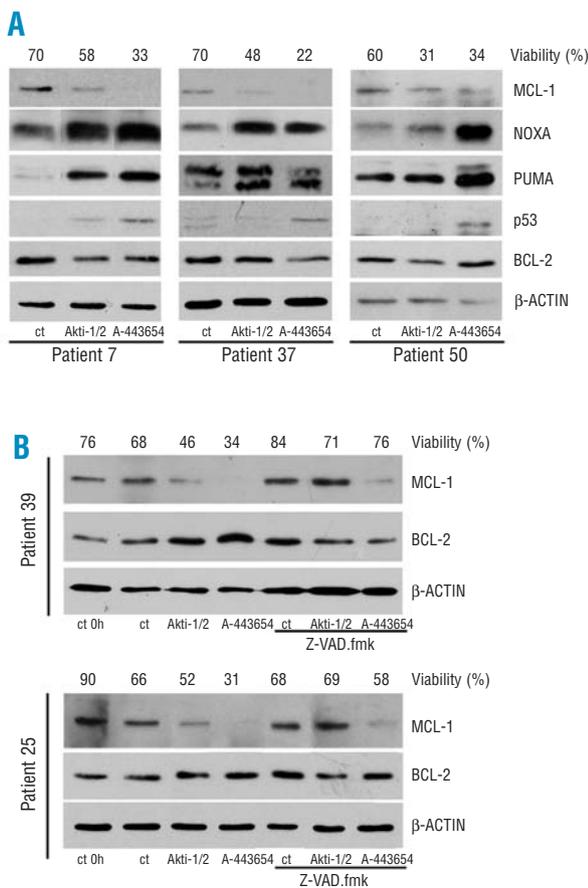
Figure 5. Apoptosis profile induced by Akti-1/2 and A-443654. (**A**) Apoptosis-related protein expression profile induced by Akti-1/2 and A-443654. Cells were untreated (ct) or treated with 5 μM Akti-1/2 (n = 10) and 0.5 μM A-443654 (n = 13) for 24 h, and MCL-1, NOXA, PUMA, p53 and BCL-2 expression was determined by western blot. Cell viability is expressed at the top of the figure. β-actin was used to standardize protein levels. Results from three representative patients are shown, two corresponding to Ficoll isolated cells (patients 7 and 37) and one corresponding to purified CD19+ CLL cells (patient 50). (**B**) Effect of Z-VAD.fmk on Akti-1/2 and A-443654-induced MCL-1 decrease. Cells were pretreated with or without 200 μM Z-VAD.fmk for 30 min and then treated with 5 μM Akti-1/2 and 0.5 μM A-443654 for 24 h (results from two representative patients are shown, n = 3), and MCL-1 levels were determined by western blot. BCL-2 and β-actin were used to standardize protein levels. Cell viability is expressed at the top of the figure.
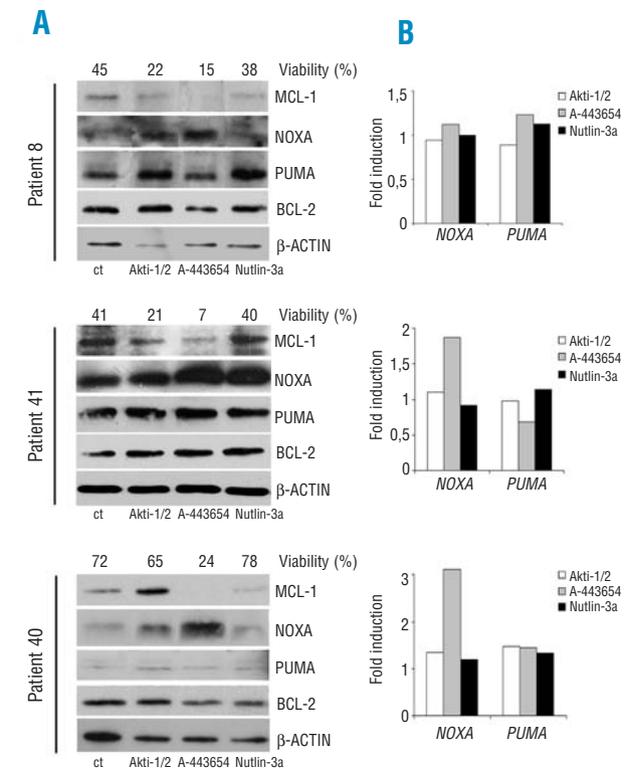
Figure 6. Apoptosis profile induced by Akti-1/2 and A-443654 in *TP53* deleted/mutated samples. (**A**) Protein expression profile and cytotoxic effect of Akti-1/2, A-443654 and Nutlin-3a. Cells were untreated (ct) or treated with 5 μM Akti-1/2, 0.5 μM A-443654 or 5 μM Nutlin-3a for 24 h, and MCL-1, NOXA and PUMA expression was determined by western blot. BCL-2 and β-actin were used to standardize protein levels. Viability was measured by analysis of phosphatidylserine exposure and propidium iodide uptake as described in the Design and Methods section. (**B**) Apoptosis-related gene expression profile induced by Akti-1/2 and A-443654 in *TP53* deleted/mutated CLL samples. Cells were untreated or treated with 5 μM Akti-1/2 (white filled bars) or 0.5 μM A-443654 (gray filled bars) and 5 μM Nutlin-3a (black filled bars) for 24 h. Cells were lysed, and the expression of apoptosis-related genes was analyzed by RT-MLPA as described in the Design and Methods section. The results are shown as fold induction relative to untreated cells.

NOXA protein levels and decreased MCL-1 protein levels in all the samples analyzed. PUMA protein levels were also increased in 50% of the samples after treatment with Akt inhibitors (Figure 5A). Furthermore, p53 protein was increased by A-443654 but not by Akti-1/2 in all the samples analyzed.

We further analyzed the apoptosis-related protein expression profile induced by Akti-1/2 and A-443654 at different times. We did not observe any change in MCL-1, NOXA and PUMA protein levels after 3 h of treatment with Akt inhibitors (*data not shown*). NOXA protein was increased by Akt inhibitors at 6 h while PUMA protein levels were increased at 12 h. Moreover, MCL-1 protein was decreased after incubation with A-443654 for 6 h (*Online Supplementary Figure S8*). Finally, pretreatment with 200 μM Z-VAD.fmk for 30 min blocked the decrease in MCL-1 induced by Akti-1/2 but not that induced by A-443654 (Figure 5B). These results demonstrate that the decrease in MCL-1 induced by Akti-1/2 is caspase-dependent whereas that induced by A-443654 is caspase-independent and precedes the activation of caspases.

Akti-1/2 and A-443654 induce apoptosis irrespective of TP53 status in chronic lymphocytic leukemia cells

To study the role of p53 in Akti-1/2 and A-443654-induced apoptosis we analyzed the effect of these compounds on CLL samples with deleted/mutated *TP53*. Patients' samples with deleted/mutated *TP53* or altered expression have been described previously.[28-30] Patient 8 had a 17p deletion in one allele in 43% of peripheral blood lymphocytes, patient 41 had a 17p deletion in one allele in 94% of peripheral blood lymphocytes, and patient 40 had a frame-shift mutation in one allele (nucleotide deletion in codon 272) and a 17p deletion in the other allele in 86% of peripheral blood lymphocytes. First, we incubated these CLL cells with 5 μM Akti-1/2 or 0.5 μM A-443654 for 24 h. Interestingly, we observed a decrease in viability in two of the three samples with Akti-1/2 treatment and in all three samples with A-443654 treatment (Figure 6A).

Western blot analysis revealed an increase in NOXA levels in the three samples when treated with both inhibitors, while PUMA levels increased in only one sample (patient 8) with 5 μM Akti-1/2 treatment. MCL-1 levels decreased with Akti-1/2 in patients 8 and 41, where Akti-1/2 induced apoptosis. In patient 40, MCL-1 levels increased with Akti-1/2 treatment, and the inhibitor did not induce apoptosis. A-443654 treatment induced a decrease in MCL-1 levels in the three samples (Figure 6A). As a control of *TP53* status we used 5 μM Nutlin-3a, which has been described to induce apoptosis and p53 accumulation in cells with wild-type *TP53* but not in those with deleted/mutated *TP53*.[28]

Next, we examined the apoptosis mRNA expression profile by performing RT-MLPA. Incubation with 5 μM Akti-1/2 or 0.5 μM A-4436545 induced almost the same mRNA expression profile as that in CLL cells with the wild-type *TP53*, except that *PUMA* mRNA

levels did not increase after treatment with A-443654 (Figure 6B).

Discussion

In this study we investigated the effect of two Akt inhibitors on the viability of CLL cells. Both Akt inhibitors induced apoptosis in primary CLL cells. These results are in agreement with a recent report describing that introduction of constitutively active myr.Akt increases the viability of CLL cells.[31]

The mechanism of action of the two Akt inhibitors is somewhat different (*Online Supplementary Figure S9*). Surprisingly, Akti-1/2 inhibits Akt but does not induce changes in the RT-MLPA profile. Akt modulates the transcriptional activity of at least one transcription factor, FoxO3a,[15] which would induce changes in the mRNA levels of its transcriptional targets *BIM*[32] and *PUMA*.[33] However, RT-MLPA experiments suggest that dephosphorylation of FoxO3a induced by Akti-1/2 is not sufficient to induce the transcription of *BIM* and *PUMA* in CLL cells. As A-443654 is a less specific Akt-inhibitor,[21] the simplest model would be to consider that inhibition of Akt (common to both inhibitors) does not induce changes in the RT-MLPA profile, and that A-443654 has additional targets to explain its effects on the expression of genes. Thus, A-443654 induces an increase in the levels of p53 protein and the induction of *PUMA* mRNA, a transcriptional target of *TP53* in CLL.[28,34] In agreement with these data, the induction of *PUMA* mRNA by A-443654 was decreased in CLL cells with deleted/mutated *TP53*. Interestingly, Akti-1/2 induced PUMA and NOXA proteins without affecting *PUMA* and *NOXA* mRNA. The mechanism for this effect is unknown and could be explained by increased translation or decreased proteolysis of these proteins.

A common effect of both Akt inhibitors is the modulation of NOXA/MCL-1 balance. It has been reported that in primary CLL cells, the majority of NOXA protein is associated with MCL-1.[35]

Thus, Akti-1/2 and A-443654 treatment induced an increase in NOXA protein levels and a down-regulation of the levels of MCL-1, a critical survival protein in CLL cells. Furthermore, inhibition of caspases prevents the down-regulation of MCL-1 induced by Akti-1/2, suggesting that MCL-1 cleavage participates in an amplification loop that increases cytochrome c release and apoptosis in CLL cells, as described for PKC inhibitors.[36] In agreement with our results, the introduction of constitutively active myr.Akt increases MCL-1 protein, and inhibition of MCL-1 by treatment with siRNA induces apoptosis in CLL.[31] The mechanism of regulation of MCL-1 protein by Akt in CLL cells is unknown. The Akt substrate GSK-3 has been reported to induce destabilization of MCL-1 protein.[16] However, inhibition of GSK-3 does not inhibit the apoptotic effect of PI3K inhibitors.[7] As GSK-3 is inhibited by PKC,[37] perhaps the over-expression of active PKC-βII[38] blocks this pathway in CLL cells.

We found that B cells from CLL samples were more

sensitive to Akt inhibitors than T cells from CLL samples, and B or T cells from healthy donors. Chemotherapeutic drugs, including fludarabine, chlorambucil, and doxorubicin induce apoptosis equally in both B and T cells, leading to immunosuppression.[39,40] Thus, the differential effect of Akti-1/2 and A-443654 in B and T lymphocytes is of interest. In conclusion, the results presented here suggest that clinically suitable small-molecule inhibitors of Akt alone or in combination with chemotherapeutic drugs might be a new therapeutic option for the treatment of CLL.

Authorship and Disclosures

MdeF performed the research and contributed to data analysis and manuscript writing. DIS, AMC, LCM, AFS and DMGG performed the research and contributed with analytical tools. EdelaB contributed with patients' samples and data. GP designed the research and contributed to data analysis. JG designed the research and contributed to data analysis and manuscript writing. The authors reported no potential conflicts of interest.

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