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AT514, a cyclic depsipeptide from *Serratia marcescens*, induces apoptosis of B-chronic lymphocytic leukemia cells: interference with the Akt/NF-κB survival pathway

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Clinical treatment of B-cell chronic lymphocytic leukemia (B-CLL) is limited by the progressive drug resistance and nonselectivity of most drugs towards malignant cells. Depsipeptides are present in certain bacteria and display potent antitumor activity. We have studied the effect of the novel cyclodepsipeptide AT514 (serratamolide) from *Serratia marcescens* on B-CLL cell viability. AT514 induced apoptosis of B-CLL cells from the 21 patients studied, as confirmed by Annexin-V binding and nuclei condensation, with an average IC_{50} of 13 μM. AT514 was effective in those B-CLL cases resistant to fludarabine, but had no effect on normal PBL. AT514 preferentially activated the intrinsic apoptotic pathway, as evidenced by loss of mitochondrial membrane potential, release of cytochrome *c* and activation of caspase-9 and -3, but not of caspase-8. Importantly, AT514 interfered with phosphatidylinositol-3 kinase and protein kinase C survival signals since it increased the apoptotic effect of LY294002 and Bisl inhibitors, and induced Akt dephosphorylation at Ser 473. AT514 also decreased NF-κB activity by dramatically reducing the levels of p65 in B-CLL. This was confirmed on functional assays using NF-κB-luc-transfected Raji cells and transgenic mice. Our results establish that AT514 induces apoptosis of primary B-CLL cells and could be useful for clinical treatment of this malignancy.
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Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the progressive accumulation of monoclonal $CD5^+$ B lymphocytes arrested in the G_0/G_1 phase of the cell cycle.[1,2] Malignant cell accumulation is mainly due to inhibition of apoptosis rather than to increased proliferation.[3] Indeed, protein kinases involved in survival pathways, such as phosphatidylinositol-3 kinase (PI3-K), protein kinase C (PKC) and Akt/protein kinase B, are constitutively activated in B-CLL.[4–6] Likewise, the activity of the NF-κB family of transcription factors is also constitutively high in B-CLL.[6,7] Consequently, the expression of many genes, including those that regulate apoptosis such as the Bcl-2 family, is altered in this malignancy[3,8] and is modulated *in vitro* during spontaneous and drug-induced apoptosis.[9,10] Chemotherapeutic drugs, such as fludarabine, chlorambucil, prednisone, and certain monoclonal antibodies directed to specific cell surface proteins, also induce B-CLL apoptosis *in vivo*, although complete remission is difficult to attain and all patients eventually relapse.[11] It is therefore important to search for new agents which may be useful as novel therapies for B-CLL, alone or in combination with already known drugs.

Depsipeptides are naturally present in certain bacteria strains and have been shown to display antitumor activity.[12] The depsipeptide FR901228, for example, is a histone deacetylase inhibitor that induces cell death in many solid tumors, T cell leukemias and multiple myeloma.[13] FR901228 also induces apoptosis of B-CLL cells and is currently under clinical trials for treatment of this malignancy.[14,15] Depsipeptides may therefore be potent therapeutic agents for B-CLL.

During the search for new potential anticancer agents, we isolated the compound AT514 from cultures of *Serratia marcescens* and identified it as the water-insoluble cyclic depsipeptide serratamolide.[16] We have investigated the activity of this compound and recently found that AT514 inhibits cell growth and induces apoptosis of several cell lines derived from breast, lung and colon human tumors, as well as from T-cell leukemia or Burkitt lymphoma (Soto-Cerrato *et al.*, submitted for publication). In the present report, we have studied the effect of AT514 on primary B-CLL cells. We show that AT514 induces apoptosis of these cells by affecting the Akt survival pathway and this involves reduction in NF-κB activity, modulation in expression of Bcl-2 family members and caspase activation.

Materials and methods

Patients, B-CLL cell purification and normal peripheral blood lymphocytes (PBL)

A total of 21 patients with B-CLL diagnosis according to established clinical and laboratory criteria were studied; 19 of them had not received treatment at the time of this study. $CD5^+$ B-lymphocytes were purified from the peripheral blood of these patients after informed consent, by Ficoll–Hypaque (Nycomed, Oslo, Norway) centrifugation. PBL from healthy donors were purified from buffy coats by Ficoll–Hypaque centrifugation, passage through anti-CD14-conjugated microbeads and MACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) to remove monocytes.

Analysis of mitochondrial membrane potential ($\Delta\psi_m$) and cytochrome c release

For $\Delta\psi_m$ measurements, B-CLL cells were incubated for 24 h with or without 20 μM AT514 and treated for 20 min with 20 nM $DiOC_6$ (Cabiochem) at room temperature in the dark. Cells were washed, resuspended in PBS and analyzed by flow cytometry. For analysis of cytochrome *c* release into the cytosol, 30×10^6 cells were incubated for 24 h with or without 20 μM AT514. Cells were washed once in cold PBS and gently lysed in 200 μl

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ice-cold lysis buffer (25 mM Tris pH 6.8, 80 mM KCl, 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 0.1% digitonin, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride). After centrifugation at 12 000*g*, 4°C, 5 min, the cytosolic fraction was recovered in the supernatant and its protein content determined by the BCA assay (Pierce, Rockford, IL, USA). Equal amounts of protein were analyzed on 15% polyacrylamide SDS-PAGE and by Western blotting.

Analysis of NF-κB activity in transgenic mice and transfected Raji cells

Transgenic mice containing the NF-κB luciferase reporter gene were obtained from Dr Mercedes Rincón (University of Vermont, Burlington, USA).[17] Lymphocytes isolated from the spleen of these mice or Raji cells transfected with the NF-κB-luc or D3005 plasmids were cultured for 24 h in the absence or presence of 20 μM AT514. Incubation with anti-CD40 mAb was for 6 h. 10×10^6 cells for each condition were lysed in 40 μl passive lysis buffer (Promega Co., Madison, WI, USA) for 20 min at room temperature. After centrifugation, 20 μl of each supernatant was added to 50 μl luciferase substrate (Promega) and luciferase activity was determined on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) and normalized with respect to the total amount of protein in each supernatant and transfection efficiency, if applicable.

Results

AT514 induces apoptosis of B-CLL cells

To determine the effect of AT514 on B-CLL cell viability, cells from the 21 patients studied were incubated with various concentrations of AT514 for 24 h and their viability was measured by the MTT assay. Table 1 (Supplementary Information) lists the results obtained for each individual patient and Figure 1a represents the mean viability for the 21 cases. As can be observed, control cells, which received no drug, were highly viable (86% average) after 24 h. However, AT514 clearly induced B-CLL cell death in a dose-dependent manner, with an IC_{50} of 13 μM (Figure 1a). For comparison, we also treated the 21 cell samples with fludarabine, a drug known to induce apoptosis of B-CLL cells *in vitro* and commonly used for clinical treatment of these patients.[11] While most samples were sensitive to fludarabine after 48 h (not shown), patients 4, 10, 11, 12 and 18 were resistant to this drug (5–15% cell death, not shown), but were clearly sensitive to AT514 (Supplementary Table 1). Therefore, in these cases, AT514 appeared to be a more efficient agent in inducing B-CLL cell death. We also measured the effect of AT514 on normal PBL. As shown in Figure 1b, AT514 had a very limited effect on these cells decreasing their viability only to 75% after 24 h of treatment.

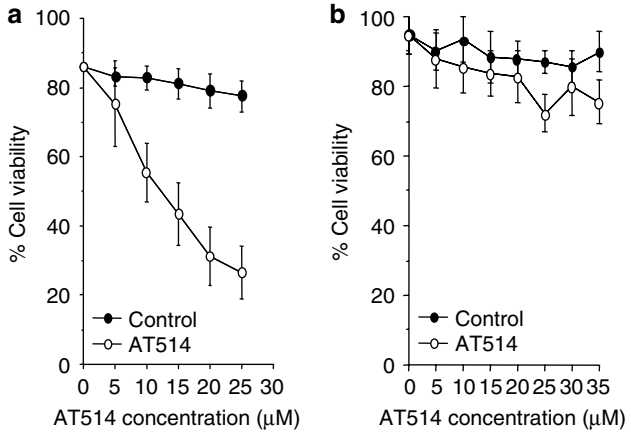

Figure 1 (a) Effect of AT514 on B-CLL cell viability. B-CLL cells were incubated in 96-well plates (2×10^5 cells/100 μl) with or without the indicated concentrations of AT514. After 24 h, cell viability was determined by the MTT method. All determinations were carried out in triplicate and values represent the average of the 21 cases studied. (b) PBL from four different healthy individuals were incubated as above and the viability was measured after 24 h. Determinations were carried out in triplicate and values are the average of the four cases studied.

To confirm that the cell death induced by AT514 was due to apoptosis, we first measured by flow cytometry the exposure of membrane phosphatidylserine using FITC-labelled Annexin V, and of cellular DNA using propidium iodide. As shown in Figure 2a for patient 4, AT514 had very little effect after 6 h of exposure as only 4.9% of cells were early apoptotic (Annexin-V+, PI−). However, after 15 h of AT514 treatment, the percentage of early and late (Annexin-V+, PI+) apoptotic cells increased (Figure 2a). After 24 h of exposure to AT514, 66.7% of cells were late apoptotic (Figure 2a). At this time, 4.2% of control cells were early apoptotic and 22.9% late apoptotic (Figure 2a). We also studied whether AT514 induced nuclei condensation and/or fragmentation. B-CLL cells were incubated with or without 20 μM AT514 for 24 h and stained for actin with TRITC-phalloidin (not shown) and with Hoechst to visualize the nucleus. As shown in Figure 2b, control cells contained an intact and uniformly stained nucleus, while cells treated with AT514 had condensed nuclei, characteristic of apoptotic cells. These results therefore indicated that AT514 induced apoptosis of B-CLL cells.

AT514 disrupts the mitochondrial membrane potential, induces release of cytochrome c *and activates caspase-9 and -3*

To determine the role of mitochondria in AT514-induced apoptosis, we first studied whether AT514 affected the mitochondrial membrane potential. B-CLL cells from patients 4 and 13 were incubated for 24 h in the absence or presence of 20 μM AT514, treated with $DiOC_6$ and analyzed by flow cytometry. As shown in Figure 3a, AT514 induced a loss of the mitochondrial membrane potential, increasing the number of $DiOC_6$-negative, apoptotic cells in both cases. We then analyzed by Western blotting the release of cytochrome *c* to the cytosolic fraction after treatment of B-CLL cells with AT514. As shown in Figure 3b for patients 4 and 13, cytochrome *c* was clearly increased in this fraction compared to the control. To determine if this resulted in activation of the initiator caspase 9, B-CLL cells were cultured for 24 h with or without 20 μM AT514, lysed and analyzed by Western blotting. As shown in Figure 3c for five different patients, AT514 effectively induced cleavage of 46 kDa procaspase-9 to the active form of 35 kDa. Likewise, PARP, a substrate of the effector caspase-3, was processed in these lysates and in lysates from cases 6, 9 and 16 (data not shown), from the native 116 kDa form to the 85 kDa product, indicating that caspase-3 had been activated by caspase-9.[18] Indeed, immunoblotting analyses confirmed the processing of caspase-3 from the native 32 kDa form to the 17 kDa active

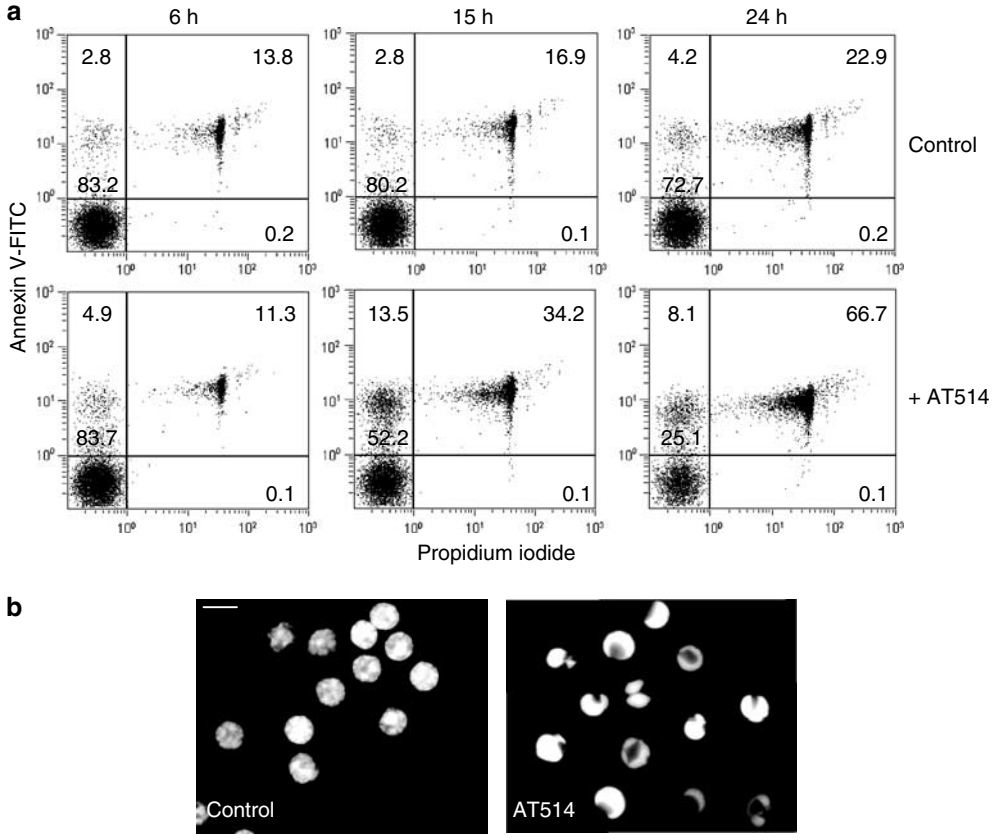

Figure 2 AT514 induces apoptosis in B-CLL cells. (a) B-CLL cells from patient 4 were treated with or without 20 μM AT514 for the indicated times, washed and incubated with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. Numbers represent the percentage of cells in each compartment. (b) Cells from patient 4 were treated with 20 μM AT514 for 24 h, placed in poly-D-lysine-coated coverslips and stained with Hoechst. Bar, 10 μm.

subunit (shown in Figure 3c for patients 4, 13 and 20). To further characterize the apoptotic pathway involved, we analyzed the possible activation of caspase-8, another initiator caspase which can also activate caspase-3. As shown in Figure 3d for patients 4, 13 and 20, no significant decrease in procaspase-8 levels was observed. Consequently, cleavage of the caspase-8 substrate Bid was not observed (Figure 3d). Altogether, these results suggested that AT514 primarily activated the intrinsic apoptotic pathway in B-CLL.

AT514 decreases the Bcl2/Bax ratio in B-CLL cells

To establish whether AT514 regulated the Bcl-2/Bax ratio, we measured the levels of both proteins in lysates of B-CLL cells from seven different patients, treated with or without 20 μM AT514 for 24 h. We found that AT514 consistently increased the levels of Bax (proapoptotic) and generally decreased the levels of Bcl-2 (antiapoptotic) (Supplementary Figure 1). Consequently, the Bcl-2/Bax ratio was greatly reduced in samples treated with AT514 compared to controls for the seven patients studied (Table 2, Supplementary Information).

AT514 affects the PI3-K/Akt survival pathway

Previous studies have shown that PI3-K and PKC are constitutively activated in B-CLL cells and contribute to the survival of these cells.[4–6] To determine whether AT514 was affecting this pathway, we first studied if AT514 enhanced the reported apoptotic effect of LY294002 and BisI, inhibitors of PI3-K and PKC, respectively.[4,5] To this end, we used suboptimal concentrations of AT514 (10 μM) and of both inhibitors (20 and 5 μM, respectively). Cells were incubated with inhibitors for 1 h prior to adding AT514. Figure 4a shows representative results for two patients out of the four studied with identical results. When used individually, AT514, LY294002 and BisI produced partial B-CLL apoptosis. However, when AT514 was combined with either inhibitor, apoptosis increased in all four cases, and the combination of AT514 with both LY294002 and BisI further reduced cell viability to 10–15% (shown in Figure 4a for patients 13 and 18), suggesting that AT514 was cooperating with both kinase inhibitors. Incubation with the ERK inhibitor U0126 (5 μM) had no effect on B-CLL viability, in agreement with previous reports[4,5] and the combination of U0126 with AT514 did not modify this effect (Figure 4a).

We next examined if AT514 affected the phosphorylation of Akt, the key effector of PI3-K-dependent survival signaling. As shown in Figure 4b for patients 13 and 18, Akt was phosphorylated in control cells after 24 h of culture. However, treatment with AT514 clearly inhibited Akt phosphorylation in a dose-dependent manner. As a control, incubation with LY294002 also inhibited Akt phosphorylation in both cases (Figure 4b), confirming that Akt activation was PI3-K dependent. These results suggested that AT514 was inhibiting the PI3-K/Akt survival pathway in B-CLL cells.

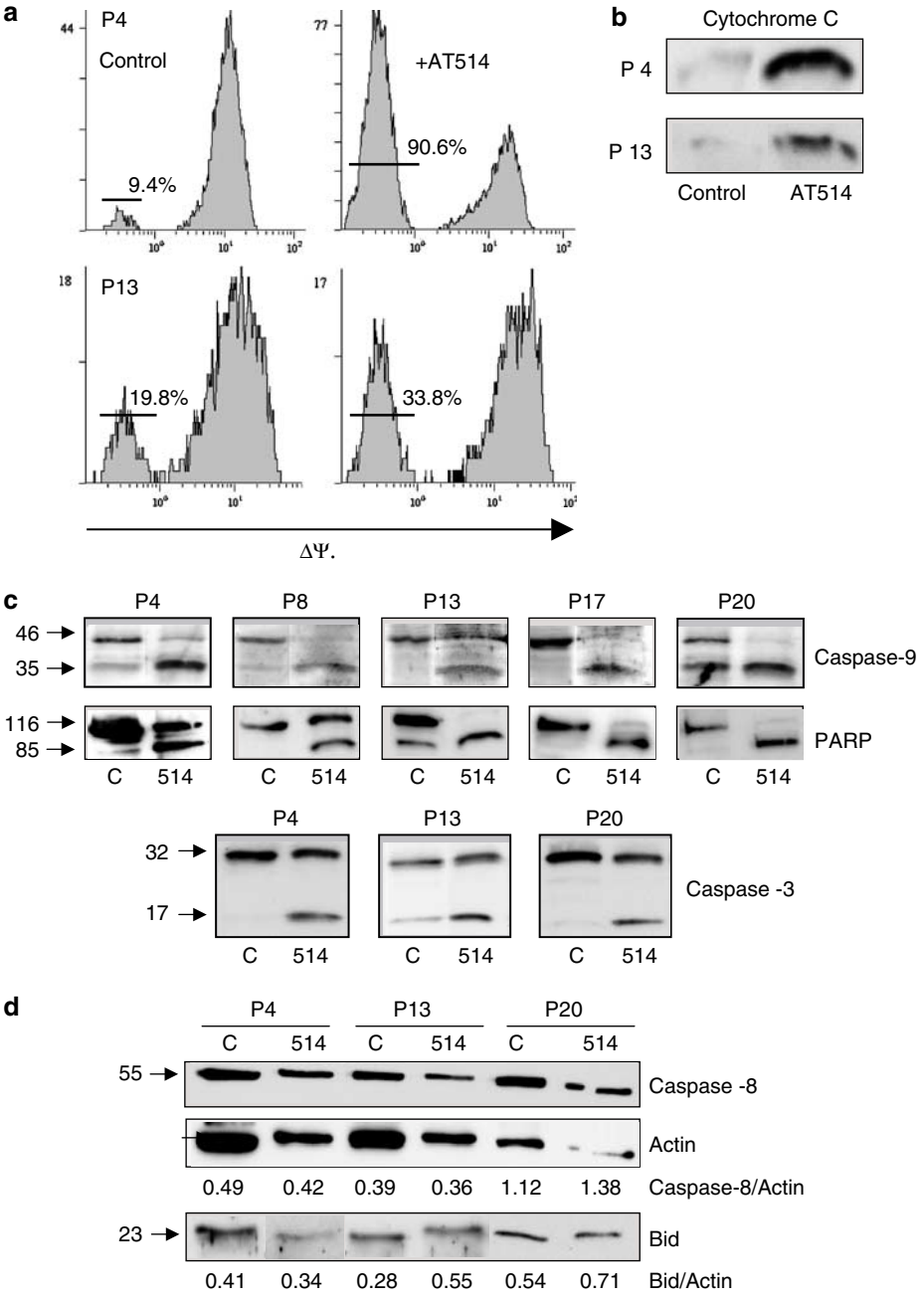

Figure 3 AT514 primarily activates the mitochondrial apoptotic pathway in B-CLL. (a) Flow cytometric analysis of the loss of mitochondrial membrane potential ($\Delta\psi_m$) after incubation of B-CLL cells with 20 μM AT514 for 24 h. (b) 30×10^6 B-CLL cells were treated or not with 20 μM AT514 and lysed. Protein (100 μg) from the cytosolic fractions was analyzed by Western blotting using an anti-cytochrome *c* Ab. (c) AT514 induces activation of caspase-9 and -3 and PARP cleavage. B-CLL cells from the indicated patients were incubated with or without (C, control) 20 μM AT514 for 24 h. Cells were then lysed and analyzed by Western blotting with specific antibodies. Conversion from the proactive forms of caspase-9 (46 kDa) and caspase-3 (32 kDa) to the active enzymes of 35 and 17 kDa, respectively, as well as the cleaved product (85 kDa) of PARP is indicated. (d) Lysates were also analyzed for caspase-8 activation and Bid cleavage using specific antibodies. Quantitation of protein bands was performed by the ECL method and values were corrected using actin as an internal control. Reduction in the levels of pro-caspase-8 (55 kDa) and Bid (23 kDa) was not observed.

AT514 decreases NF-κB activity

Akt suppression of apoptosis has been shown to involve the NF-κB transcription factor.[19,20] Consequently, NF-κB activity is constitutively high in B-CLL cells.[6,7] To determine if NF-κB was affected by AT514, we first analyzed by Western blotting the levels of NF-κB in the nuclear and cytosolic fractions of lysates of B-CLL cells treated with AT514. Figure 5a shows that in control cells NF-κB was present in both fractions, indicating a certain basal activity (NF-κB in the nucleus), as reported.[6,7] Treatment with AT514 dramatically reduced the levels of NF-κB in both fractions (Figure 5a), suggesting that AT514 was blocking the biosynthesis and/or transcription of NF-κB. To establish that this resulted in a reduced NF-κB activity, we took advantage of

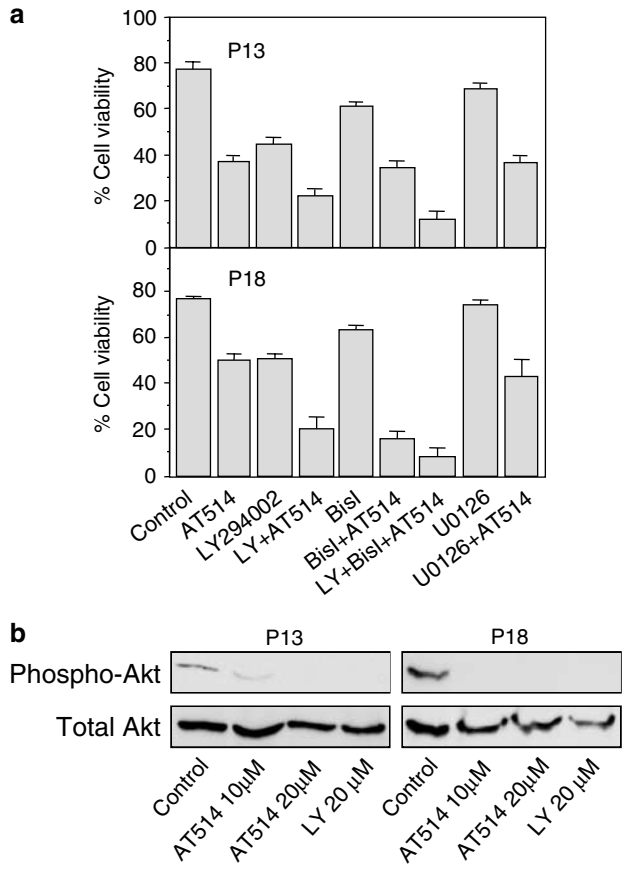

Figure 4 AT514 interferes with the PI3K/Akt survival pathway. (a) B-CLL cells from two representative patients were incubated for 1 h in the presence or absence of 20 μM LY294002, 5 μM BisI, or 5 μM U0126, prior to the addition of 10 μM AT514. After 24 h, cell viability was analyzed by flow cytometry using Annexin V and propidium iodide. Values represent the average of duplicate determinations. (b) B-CLL cells ($10–20 \times 10^6$) were incubated with or without 10 and 20 μM AT514 or 20 μM LY294002 for 24 h and lysed. Akt phosphorylation was analyzed by Western blotting using specific antibodies against total Akt or the phosphorylated form (Ser 473) of this kinase.

the NF-κB-luc reporter plasmid.[21] Owing to the difficulty of transfecting primary B-CLL cells, we used the B lymphoma cell line Raji for these experiments. AT514 effectively induced apoptosis of Raji cells in a dose-dependent manner (Figure 5b). These cells were transfected with NF-κB-luc or the control D3005 plasmid, incubated with or without 20 μM AT514 for 24 h, lysed and their activity was analyzed on a luminometer. Figure 5c shows that AT514 clearly reduced the luciferase activity displayed by untreated cells. This activity was also reduced to similar levels by LY294002, confirming that the NF-κB activity was regulated by PI3-K. NF-κB activity was effectively enhanced by an anti-CD40 mAb (Figure 5c), in agreement with a previous report.[7] Control cells transfected with D3005 had very low luciferase activity and this was not affected by treatment with AT514, LY29002 or anti-CD40 mAb (Figure 5c).

To further confirm that AT514 was inhibiting NF-κB activity, we purified lymphocytes from the spleen of transgenic mice containing the NF-κB luciferase reporter gene.[17] As shown in Figure 5d, these lymphocytes were partially sensitive to AT514, which decreased their viability to 55% after 24 h. Incubation of spleen lymphocytes from three different mice with AT514 clearly reduced the luciferase activity exhibited by untreated cells (Figure 5e). The control LY294002 produced a similar effect. These three sets of results clearly indicate that NF-κB plays an important role in the apoptotic mechanism induced by AT514.

Discussion

In this report, we show that the novel cyclodepsipeptide AT514 (serratamolide), naturally occurring in *S. marcescens*, is an efficient inducer of apoptosis in B-CLL cells. The present results expand our previous studies on established cancer cell lines (Soto-Cerrato *et al*., submitted) and represent the first evidence that AT514 induces apoptosis in human primary cancer cells.

The viability of B-CLL cells from the 21 patients studied here clearly diminished when exposed to AT514. Our results show that this was due to induction of apoptosis since cell death was accompanied by Annexin-V uptake, nuclei condensation, mitochondrial damage and caspase activation. Interestingly, AT514 had very little effect on normal PBL and was effective in the B-CLL cases that showed resistance to fludarabine, a drug commonly used in the treatment of these patients. This suggests that AT514 may be a very useful therapeutic agent for patients who are totally or partially resistant to fludarabine. Other differences between the mode of action of the two drugs include the p53 pathway. It is well established that fludarabine induces p53 expression[22] and we have shown that interfering with this expression by crosslinking $\alpha4\beta1$ integrin induces cell survival.[23] In results not shown, we did not observe induction of p53 in the present study, thus ruling out a role for this protein in the AT514 apoptotic pathway.

Both the intrinsic and extrinsic apoptotic pathways have been shown to operate in B-CLL. Thus, while some cytotoxic drugs (chlorambucil, fludarabine, rolipram) and γ-radiation activate caspase-8 and subsequent effector caspases,[24,25] induction of apoptosis by anti-CD22 inmunotoxins mainly involved the caspase-9 pathway.[26] Concomitant activation of both initiator caspases was also observed when apoptosis was induced by acadesine[27] or the histone deacetylase inhibitor MS-275.[28] Our present results indicate that AT514 preferentially activates the intrinsic, mitochondria-mediated, apoptotic pathway in B-CLL, since cytochrome *c* release and activation of caspase-9, but not of caspase-8, was clearly evident. This could be an important mechanistic difference with respect to the previously described effect of depsipeptide FR901228, which activates the extrinsic, caspase-8 mediated, apoptotic pathway in B-CLL.[15] Another major difference between depsipeptides FR901228 and AT514 is that FR901228 is a histone deacetylase inhibitor and was shown to acetylate histones H3 and H4 concomitant with induction of apoptosis.[15] In the present report, we did not observe increased acetylation of H3 and H4 histones upon B-CLL treatment with AT514 (results not shown), indicating that both depsipeptides activate different mechanisms for induction of apoptosis.

Several reports have recently shown that protein kinases such as PI3-K/Akt and PKC as well as the transcription factor NF-κB are constitutively activated in B-CLL and contribute to the defective apoptosis of these cells.[4–6,29] Our present results clearly show that AT514 interfered with this survival pathway, since it significantly increased the apoptotic effect of specific inhibitors for PI3-K and PKC. Moreover, we show that B-CLL cells had constitutively phosphorylated Akt, in agreement with a previous study,[6] and AT514 induced Akt dephosphorylation at

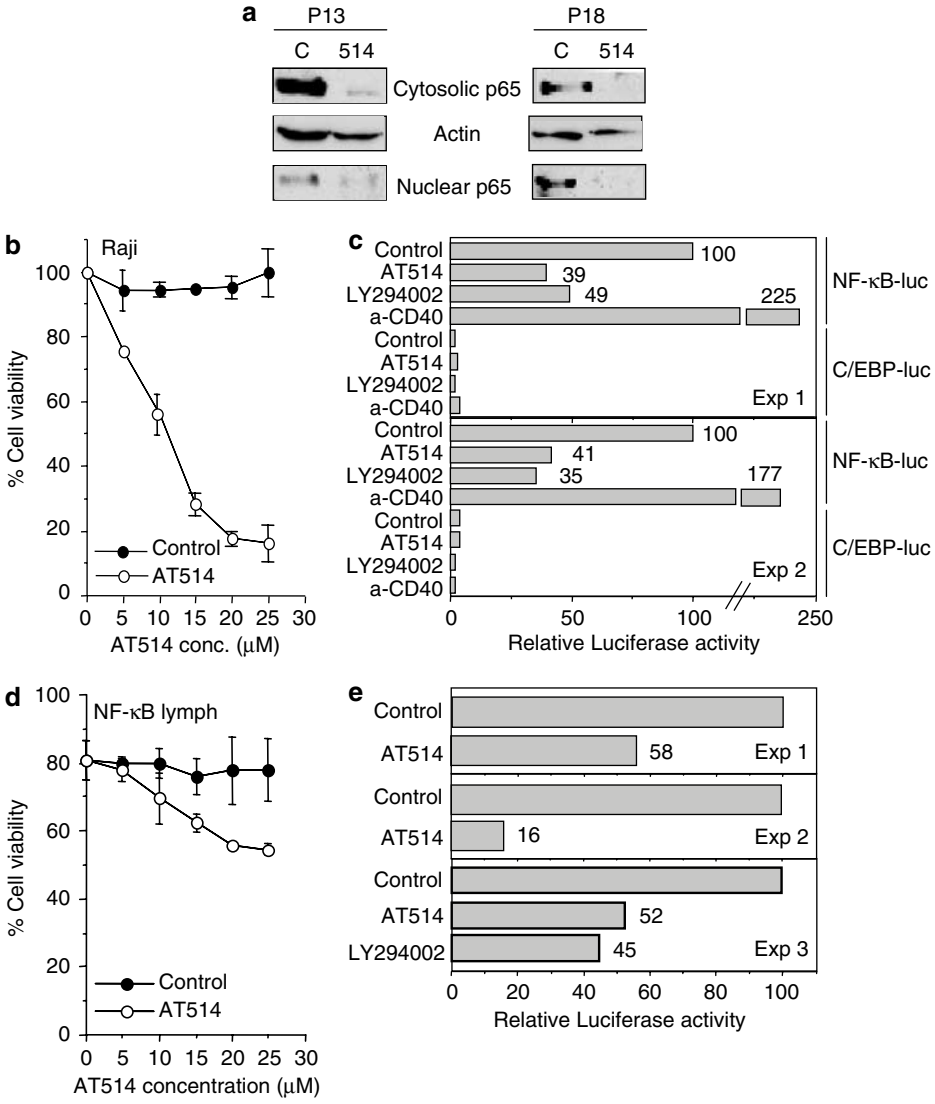

Figure 5 AT514 inhibits NF-κB activity. (a) B-CLL cells ($10–20 \times 10^6$) from two representative patients were incubated for 24 h with or without AT514; nuclear and cytosolic extracts were prepared and equal amounts of total protein were analyzed by Western blotting using an anti-p65 antibody. Actin was used as a loading control. (b) Raji cells were incubated with the indicated concentrations of AT514 and their viability determined after 24 h by the MTT method. (c) NF-κB-luc- or C/EBP-luc-transfected Raji cells were incubated with AT514 for 24 h, lysed and the luciferase activity determined on a luminometer. The effect of LY294002 and an anti-CD40 mAb is also indicated. The results from two independent experiments are shown. Values were corrected for transfection efficiency and total protein content on each lysate. (d) Spleen lymphocytes from NF-κB transgenic mice were incubated with the indicated concentrations of AT514 and their viability determined after 24 h by the MTT method. (e) Luciferase activity of these lymphocytes after treatment with AT514 for 24 h. Three different mice were studied (exps 1, 2 and 3) and values are normalized according to the total protein content on each lysate.

Ser 473. Constitutively activated Akt in B-CLL was not observed in another report[5] and this discrepancy remains to be explained. Akt controls cell survival by inducing phosphorylation and inactivation of proteins involved in apoptosis,[30] but also by activating NF-κB and thus the expression of survival genes.[20] In agreement with this pathway, we show in the present study that AT514 dramatically reduced the total levels of the p65 NF-κB component, thus directly affecting the activity of this transcription factor in B-CLL. We used two independent functional approaches, consisting of NF-κB-luc-transfected Raji cells and NF-κB-luc transgenic mice, to confirm that AT514 inhibited NF-κB activity. Our results clearly demonstrate that AT514 treatment induced a reduction in the activity of NF-κB in both cases.

The present findings provide a mechanism for AT514 induction of apoptosis in B-CLL cells, primarily involving the mitochondria-mediated apoptotic pathway and interference with Akt/NF-κB survival signals. To our knowledge, this is the first evidence showing a direct inhibition of Akt and NF-κB activation by a depsipeptide in B-CLL. A previous report[31] has shown that FR901228 (another depsipeptide known to induce apoptosis in B-CLL) diminished Akt activity of *ras*-transformed 10T1/2 cells, by reducing the total levels of this kinase. As we show in our study, AT514 inhibited Akt phosphorylation in B-CLL without affecting total Akt levels.

NF-κB controls the expression of several genes involved in apoptosis, including members of the Bcl-2 protein family.[32] Accordingly, we have found a highly consistent downregulation

of the antiapoptotic protein Bcl-2 concomitant with induction of apoptosis by AT514. In contrast, the levels of the proapoptotic protein Bax were dramatically increased by AT514 treatment. Although Bax is not under NF-κB control, it plays an important role in B-CLL apoptosis by determining the Bcl-2/Bax ratio, an important survival marker on these cells,[2,3] and we show in this report that AT514 consistently decreased this ratio. Bax may also be playing a crucial role in the mitochondrial-mediated apoptotic pathway initiated by AT514. It was recently shown that induction of B-CLL apoptosis by proteasome inhibitors produces a conformational change and mitochondrial translocation of Bax, which does not require caspase activation.[33] Although the initial stimulus that leads to these events is not known yet, it is interesting that PI3-K and Akt activities prevent Bax conformational change and translocation to mitochondria.[34,35] It is tempting to speculate that inhibition of PI3-K/Akt by AT514 initiates Bax-mediated mitochondria perturbation and subsequent caspase-9 and caspase-3 activation and apoptosis.

In conclusion, we show in this study that cyclodepsipeptide AT514 is a novel apoptotic agent for primary B-CLL cells, which directly blocks the PI3-K/Akt/NF-κB survival pathway and activates the mitochondria-mediated apoptotic cascade. It is noteworthy that several current cancer therapies are aimed at the inhibition of this survival pathway.[36] AT514 may therefore constitute an efficient drug for the clinical treatment of B-CLL, alone or in combination with conventional protocols.

Acknowledgements

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Supplementary Information

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu).

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