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The Effects of Pyrrolo[1,2-b][1,2,5]Benzothiadiazepines in MEC1 Cells

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ABSTRACT

Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia and is currently incurable. To expand the therapeutic armamentarium, we investigated antitumor activity of pyrrolo[1,2-b][1,2,5]benzothiadiazepine (PBTDs) in MEC1 cells. We found that PBTD (RS2778) treatment enhanced the activation of pro-apoptotic members, such as caspase-9, 3, poly (ADP-ribose) polymerase (PARP), and bax, but suppressed the activation of anti-apoptotic molecule BCL-2 in these cells. Furthermore, PBTD (RS2778)-induced autophagic cell death was verified by LC3-II conversion, and upregulation of Beclin-1 and ATG5. In addition, such compound impeded hyper phosphorylation of AKT as were determined by Western blot. In summary, PBTD (RS2778) inhibited viability and induced multiple cellular events including apoptosis, autophagic cell death, in human MEC1 cells. This distinct activity of PBTD (RS2778) against these cells suggests potential for PBTDs as a therapeutic agent for treatment of CLL. J. Cell. Biochem. 116: 339–349, 2015.

KEY WORDS: AKT; AUTOPHAGY; BAX/BCL-2 RATIO; B-CELL LEUKEMIA CELLS; PBTDs

Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia [Dores et al., 2007]. The median age at diagnosis is 72 years, and despite the availability of several treatments it remains an incurable cancer [Abbott, 2006; Wierda et al., 2010]. CLL is characterized by the accumulation of clonal B cells in peripheral blood, bone marrow, lymphnodes, and spleen due to a combined effect of deferred apoptosis and slow, but persistent proliferation [Rai et al., 1975; Chiorazzi and Ferrarini, 2003]. Clinically, CLL is a heterogeneous disease: patients with early stage disease (Rai stage 0, I or II) are usually asymptomatic and require no treatment, while those with advanced stage (Rai stage III or IV) or symptomatic disease have compromised survival (median 3 years) and need therapeutic intervention [Rai et al., 1975]. The exact cellular and molecular events responsible for the transition of CLL from an indolent to a clinically aggressive stage are not well defined. However, several genetic abnormalities are now recognized that predict clinically aggressive and chemotherapy resistant disease with compromised survival outcome [Shanafelt, 2009].

Progression of CLL is associated with changes in cellular signaling molecules, which not only can be used as prognostic markers, but also can be exploited as therapeutic targets. These include proteins, such as Akt and B-cell lymphoma 2 (BCL-2), whose up-regulation results in an increased survival of tumor cells. For patients with symptomatic and previously untreated CLL, the most commonly used therapeutic agents include fludarabine either alone or in combination with an anti-CD20 monoclonal antibody (mAb), such as rituximab, with or without cyclophosphamide [Byrd et al., 2003; Keating et al., 2005; Hillmen et al., 2007; Hallek et al., 2009]. Other effective therapeutics approved for the treatment of patients with CLL include: bendamustine, alemtuzumab (anti-CD52 mAb), and ofatumumab [Hillmen et al., 2007; Knauf et al., 2009]. Although the use of these agents can result in high rates of clinical remission, eventually all patients relapse and become refractory to therapy. Thus, there remains a continued need to develop new therapeutic stratagems for the treatment of patients with CLL.

Conflict of interest: The authors declare no conflict of interest.
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Pyrrolo[1,2- b][1,2,5]benzothiadiazepines (PBTDs) have been demonstrated to induce cell cycle arrest and cell death in both in long-term cultured leukemia cell lines and both in primary leukemia cells derived from Chronic Myelogenous Leukemia (CML) patients at onset and CML-chemoresistant patients [Silvestri et al., 2006; Marfe et al., 2007; Marfe and Di Stefano, 2010; Di Stefano et al., 2010].

Cell deaths include programmed cell death (PCD) and necrosis. Among them, apoptosis is the common form of PCD in multicellular organisms, which appears to be morphologically characterized by cell shrinkage, chromatin condensation, and formation of apoptotic bodies. These processes are influenced by the unbalance of pro- and anti-apoptotic signals regulated by BCL-2-family members [Adams, 2003]. The main biochemical features of apoptosis include caspase activation and DNA fragmentation [Ghavami et al., 2009]. Apoptosis is induced by various physiological or toxic stimuli such as chemotherapeutic drugs, DNA damage, ultraviolet irradiation, oxidative stress, and endoplasmic reticulum stress [Maddika et al., 2007]. Except for apoptosis, another cell death model, autophagy, is a bulk degradation system for cytoplasmic components including organelles through the lysosomal pathway, and is characterized by the formation of autophagosomes [Klionsky and Emr, 2000]. Autophagosomes ultimately fuse with lysosomes, thereby generating single-membrane autophagolysosomes and degrading their content. In addition to its basic role in the turnover of proteins and organelles, autophagy has multiple physiological and pathophysiological functions including roles in cell differentiation, immune defense, and cell death. Recent studies have demonstrated that there is a close relationship between autophagy and apoptosis in different cell types or under different pathological condition. We previously have demonstrated that PBTDs-induced cell death is connected with a down-regulation of BCL-2 and activation of caspase 3 in CML cells. Since, it has reported that targeting BCL-2 can be an effective therapeutic strategy for the treatment of CLL [Hallaert et al., 2007], we have hypothesize that PBTDs can be used as a potential therapeutic agent in CLL [Silvestri et al., 2006; Marfe et al., 2007; Marfe and Di, 2010; Di Stefano et al., 2010].

In addition, the Phosphoinositide 3-Kinase (PI3K) cascade is also a critical component of survival signaling including PI3K-activated AKT (phosphorylated AKT) which inhibits cell death pathways by inactivating pro-apoptotic proteins such as BAD, procaspase-9, and members of the Forkhead transcription factor family. Overexpression of PI3K appears to play a critical role in B-CLL cell survival [Cuni et al., 2004; Herman et al., 2010]. Here, we reported that PBTD (RS2778) induces apoptosis in MEC1 cells by activating pro-apoptotic signals including caspase-3 and BAX and by suppressing anti-apoptotic signal, BCL-2. Furthermore, autophagy was also observed in PBTD-(RS2778)-treated leukemia cells along with the down-regulation of AKT signaling.

MATERIALS AND METHODS

COMPOUNDS

In our studies, we have found that some PBTDs were able to induce apoptosis both in long-term cultured leukemia cell lines and in primary blast samples [Silvestri et al., 2006; Marfe et al., 2007; Marfe and Di Stefano, 2010; Di Stefano et al., 2010]. For this reason, we tested PBTDs (RS678 and RS2778) in MEC1 cells and they possess as common features the sulfur dioxide group at position 5 and the ethoxycarbonyl function at position 11 of the pyrrolo[1,2-b][1,2,5] benzothiadiazepines nucleus, while the PBTDs (RS2778) contains the naphthoyl group at position 10 (Fig. 1).

PBTDs TREATMENT AND CELL VIABILITY

PBTDs were dissolved in Dimethyl sulfoxide (DMSO) and diluted in RPMI-1640 culture medium at the required concentration. The final concentration of DMSO in cultures did not interfere with cell viability. All the procedures involving PBTDs treatment were performed in dim light and the culture plates were covered with aluminum foil. MEC1 cells were routinely cultured between 2×10^5 and 10^6 cells/ml in the same medium as above but supplemented with 1 mM sodium pyruvate, and experiments were carried out by seeding cells at 5×10^5/ml. Briefly, the MEC1 cells were incubated with PBTDs (RS678, RS2778) at 10 and 15 μM concentrations up to 48 h. After respective time points, cell viability was assessed by Annexin test. Cells (0.5×10^6) were resuspended in PBS and were analyzed by exposing to Annexin V-PE. Apoptotic cells were stained with a 7-aminoactinomycin D red fluorescent probe. Apoptotic cells were analyzed by using a FACScan cytometer (FACScan; Becton Dickinson, Oxford, UK).

DNA FRAGMENTATION ASSAY

Briefly, cells were washed twice with phosphate-buffered saline (PBS) and lysed by addition of a hypotonic solution (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl pH 7.5). After centrifugation at 1600*g* for 5 min, the supernatant was collected and the extraction was repeated with the same lysis buffer. The supernatants was brought to 1% SDS and treated with RNase A (final concentration 5 mg/ml) for 2 h at 56°C followed by digestion with proteinase K (final concentration 2.5 mg/ml) at 45°C for at least 6 h. Before hydrolysis, a further cleaning of DNA was performed by phenol–chloroform extraction, followed by three successive ethanol precipitation in 2 M ammonium acetate. Pellets were dried for 30 min and resuspended in 200 μl Tris–EDTA pH 8.0 [Herrmann et al., 1994]. Aliquots of 20 μl containing 10 μg DNA were electrophoresed in 1.5% agarose gel.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

The protein extracts, obtained from cell lysates, were electrophoresed on SDS–polyacrylamide gels and probed with the following primary antibodies: mouse monoclonal anti-caspase-9 (Santa Cruz), mouse monoclonal anti-caspase-8 (Santa Cruz), rabbit polyclonal anti-caspase-3 (Santa Cruz), mouse polyclonal anti-PARP (Santa Cruz), rabbit polyclonal anti-AKT-ser473 (Santa Cruz), rabbit polyclonal anti-AKT (Santa Cruz), rabbit polyclonal anti-LC3B (Abcam), rabbit polyclonal anti-Beclin-1, rabbit polyclonal anti-ATG5 (Abcam), mouse monoclonal anti-BAX (Santa Cruz), mouse monoclonal anti-BCL-2 (Santa Cruz), and rabbit polyclonal β–actin antibodies (Sigma). Immunoreactive proteins were visualized by enhanced chemiluminescence system (Amersham Life Science). The relative amount of protein expression was quantified

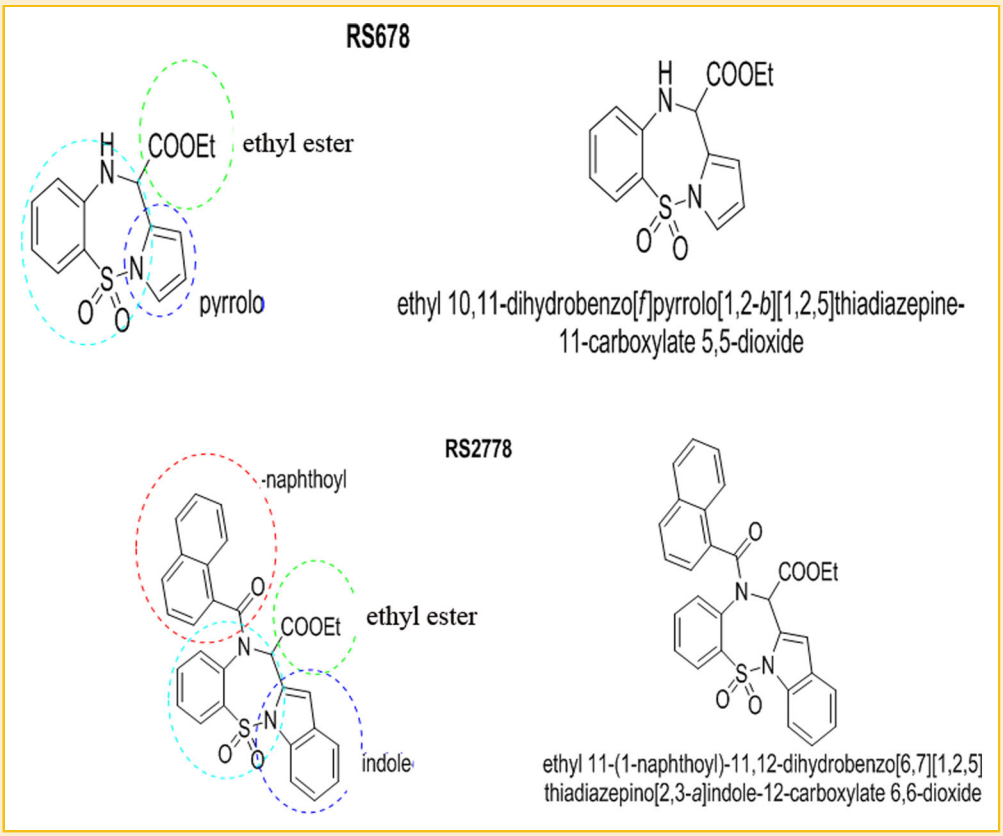

Fig. 1. The chemical structure of the compounds.

using a Gel-Doc phosphorimager and Quantity One software (Bio-Rad) and normalized by the intensity of β-actin.

STATISTICAL ANALYSIS

The results are presented as mean ± SEM of three experiments. One-way ANOVA with Turkey test was performed using GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). *P*-value <0.05 was considered statistically significant.

RESULTS

EFFECT OF PBTDs ON NUMBER OF MEC1 VIABLE CELLS IN CULTURE AND DNA FRAGMENTATION

MEC1 cells were shown to have normal growth characteristics expected under standard in vitro culture conditions. After the plating of MEC1 cells, they were subsequently incubated at 10 and 15 μM with two different PBTDs (RS678 and RS2778) at 24 and 48 h. We used the Annexin V test detection to monitor the cell viability (Fig 2A,B). After treatment for 48 h with PBTD (RS2778), a significant decrease in the number of viable cells was observed as compared to PBTD (RS678)-treated cells, reaching 39% at 10 μM and 60% reduction at 15 μM in MEC1 cells (Fig. 2C,D). Quantification of apoptosis can be a useful measure of cancer cell kinetics. Alteration of the balance between proliferation and apoptosis is associated with cancer. An increase in apoptosis was observed in MEC1 cells, treated with PBTD (RS2778) (Fig. 2). In fact, we examined the effect of PBTDs (RS678 and RS2778) on DNA fragmentation that was considered as a distinctive trait of apoptosis, and produced 180- to 200-base pair internucleosomal DNA ladder gel patterns. As shown in Figure 2E, PBTD (RS678) was not able to induce DNA fragmentation, while PBTD (RS2778) had this effect within 24 and 48 h at both concentrations.

PBTD (RS2778) ACTIVES THE INTRINSIC APOPTOTIC PATHWAY

Based on the observation that PBTD (RS2778) induced programmed leukemia cell death at 24 h, further investigations were performed to evaluate its mechanism of action. To ascertain whether caspase activation is involved, protein was extracted from PBTD (RS2778)-treated leukemic cells and probed for the activation of caspases 9, 8, and 3 by Western blot analysis. Such compound induced caspases 9 and 3 activation (Fig. 3A,B), but not caspase 8 (Fig. 3E) in a dose-dependent manner as observed by the presence of cleaved caspases 9 and 3, respectively (Fig. 3C,D). However, none of the mentioned above caspases were found to be active during the treatment with PBTD (RS678) as depicted in Figure 3. This indicates that PBTD (RS2778) promotes apoptosis through activation of the intrinsic apoptotic pathway in leukemia cells.

Since we observed that PBTD (RS2778) activated caspase-3 in the MEC1 cells, we investigated the cleavage of the caspase-3 substrate PARP during the compound treatment. Clearly, cleavage of PARP, as

indicated by a decrease in the full-length 113 kDa protein and appearance of the 85 kDa cleaved PARP product, was prominent in PBTD (RS2778)-treated cells, whereas it was almost completely absent in PBTD (RS678)-treated cells (Fig.4A). A small amount of cleaved PARP was already found after 24 h during the PBTD (RS2778) treatment at both concentrations, and this effect was much more pronounced at 48 h (Fig 4B).

PBTD (RS2778) INDUCES AUTOPHAGY IN LEUKEMIA CELLS

We subsequently examined whether PBTDs induce autophagy of leukemia cells after PBTDs (RS2778 and RS678) treatment. Thus, we investigated the cleavage of LC3-I, a marker of autophagy, by Western blot analysis. Under conditions of autophagy, LC3-I (18 kDa) is cleaved on its carboxyl end to LC3-II (16 kDa), which is

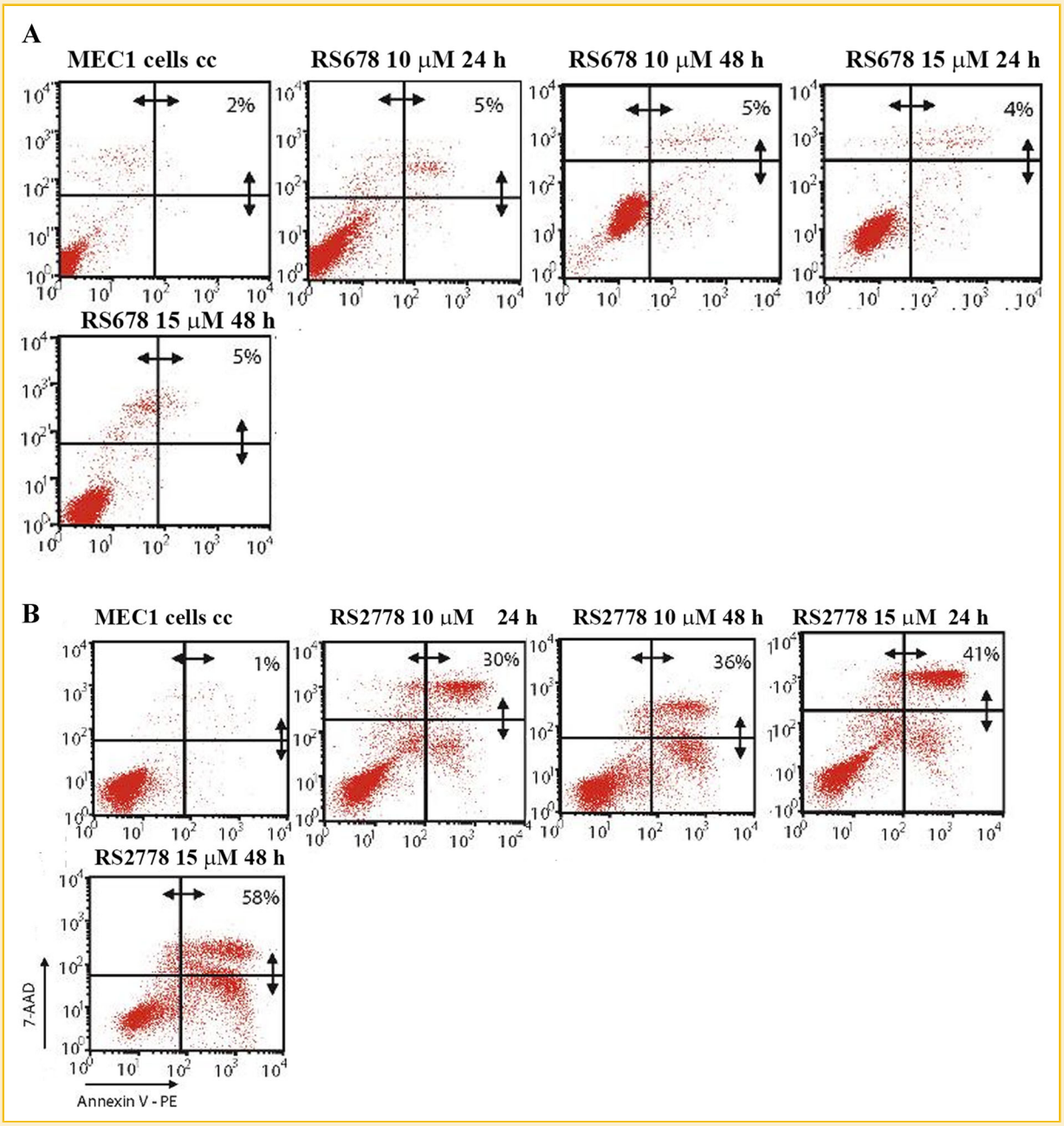

Fig. 2. Pyrrolo[1,2-b][1,2,5]benzothiadiazepines (PBTDS) effect in MEC1 cells examined for induction apoptosis and DNA fragmentation. A,B: Flow-cytometric analysis of apoptosis in control cells or cells, treated with RS678 and RS2778 at concentration 10 and 15 μM (24 and 48 h) as determined by using Annexin V-PE and 7-AAD . The percentage of cells in the upper right quadrant denote cells that stain positive for Annexin V and 7-AAD. The cells in the lower right quadrant stain positive for Annexin V only. C, D: Apoptosis induction was measured using an Annexin V-PE and 7-AAD detection kit and the data is presented as % apoptotic cells. E: DNAs were extracted from untreated (cc) and treated cells. Agarose gels are representative of at least three separate experiments.

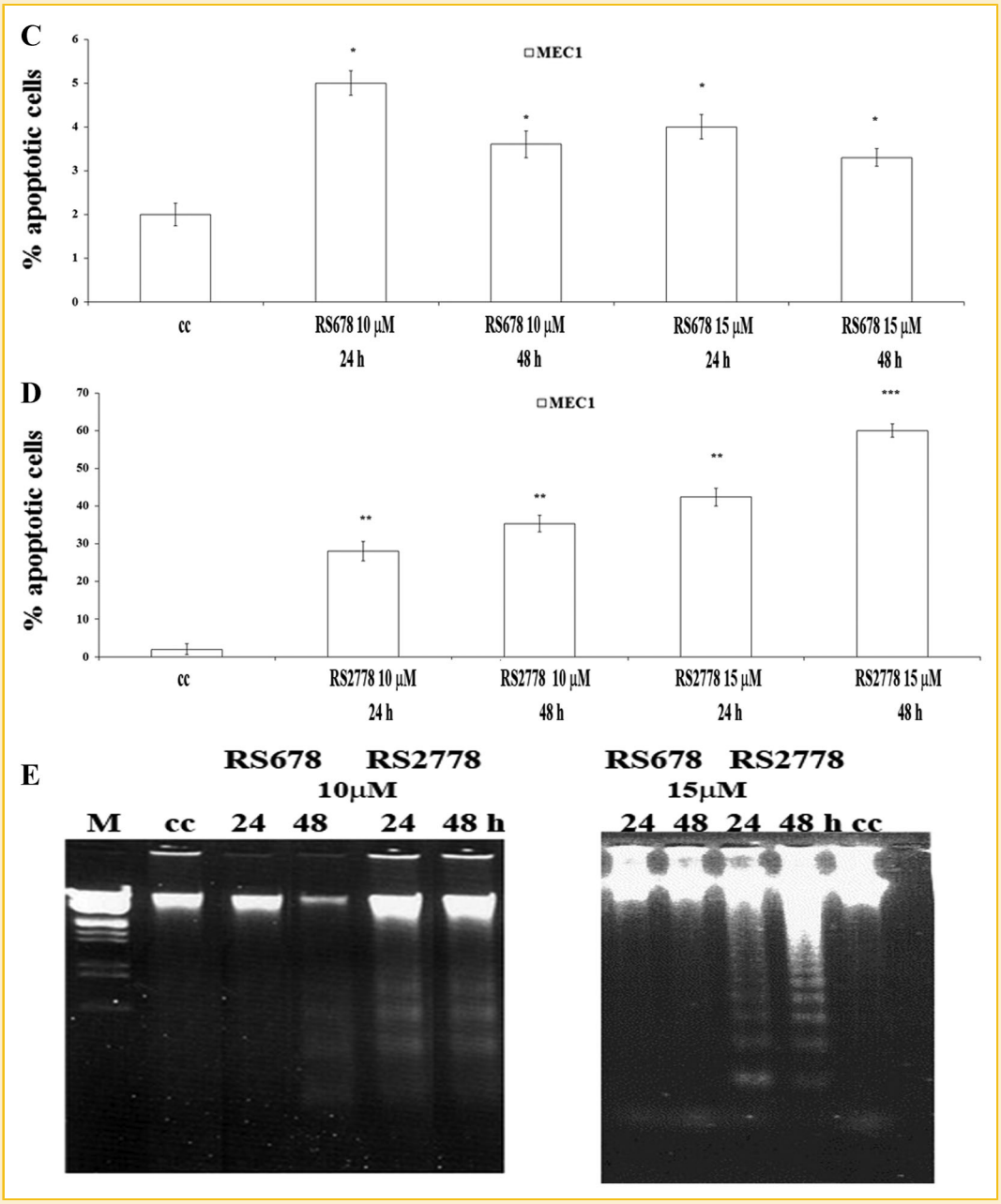

Fig. 2. (*Continued*)

inserted into autophagosomes that surround the intracellular organelles preceding autophagy [Noda et al., 2009; Glick et al., 2010]. Untreated MEC1 cells showed both LC3-I and LC3-II proteins at equal intensities, while PBTD (RS2778)-treated CLL cells showed a dose-dependent increase in LC3-II levels indicating cleavage of LC3-I (Fig.5A,B). We further examined the expression level of Beclin-1 (ATG6 protein) and ATG5 by immunoblot. The PBTD (RS2778) increased the Beclin-1 and ATG5 expression level in these cells in a dose-dependent manner (Fig.5A,C,D). Taken together, these results suggest that RS2778 is involved in leukemia cell death by autophagy indution

In addition, none of the mentioned above proteins were involved in same cells during the treatment with PBTD (RS678) as depicted in Figure 5A.

PBTD (RS2778) TREATMENT INFLUENCES THE EXPRESSION BCL-2 FAMILY

Previous investigations have suggested that PBTDs alters the expression levels of BAX and BCL-2 in vitro [Silvestri et al., 2006; Marfe et al., 2007; Marfe and Di Stefano, 2010; Di Stefano et al., 2010]. To elucidate the effect of PBTDs (RS2778 and RS678) on the expression of these proteins, leukemia cells were treated with

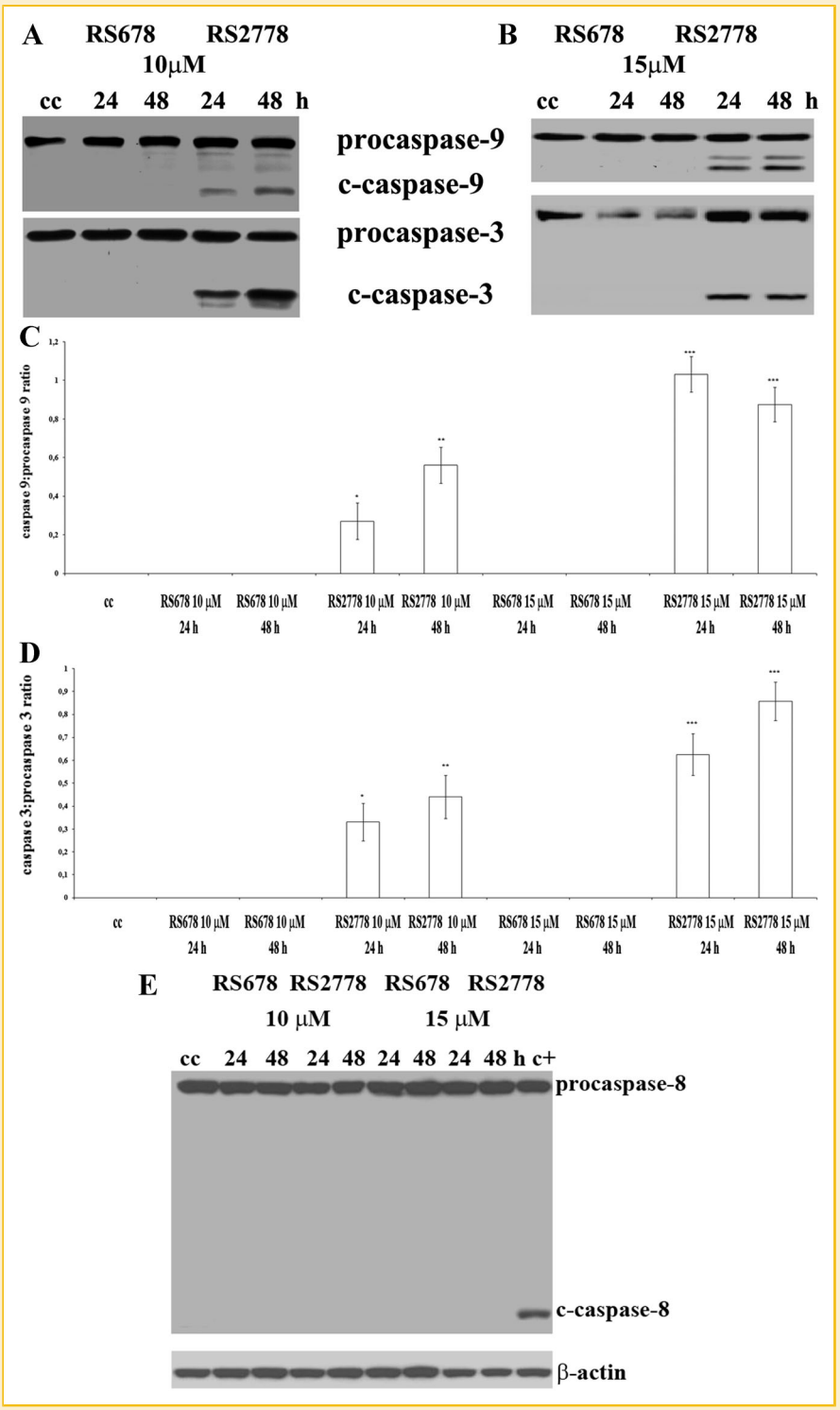

Fig. 3. Pyrrolo[1,2-b][1,2,5]benzothiadiazepines (PBTDS) effect on MEC1 cells examined for caspase activation. A,B: Whole cell lysates prepared from untreated (lane cc) and treated cells. Cellular extracts were analyzed by Western Blot using anti-caspase-9, and 3 antibodies. **β**-Actin was used as a control for protein loading. Blots are representative at of at least three separate experiments. C: Cleaved caspase-9/procaspase-9 ratio. Band signals from procaspase-9 and cleaved caspase-9 were measured using a Gel-Doc phosphorimager and Quantity One software and the ratio was calculated. Significant differences between the untreated and treated cells are indicated by probability *P*. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. D: Cleaved caspase-3/procaspase-3 ratio. Band signals from procaspase-9 and cleaved caspase-9 were measured using a Gel-Doc phosphorimager and Quantity One software and the ratio was calculated. Significant differences between the untreated and treated cells are indicated by probability *P*. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. E: Whole cell lysates prepared from untreated (lane cc) and treated cells. Cellular extracts were analyzed by Western Blot using anti-caspase-8 antibodies. **β**-Actin was used as a control for protein loading. Blots are representative at of at least three separate experiments.

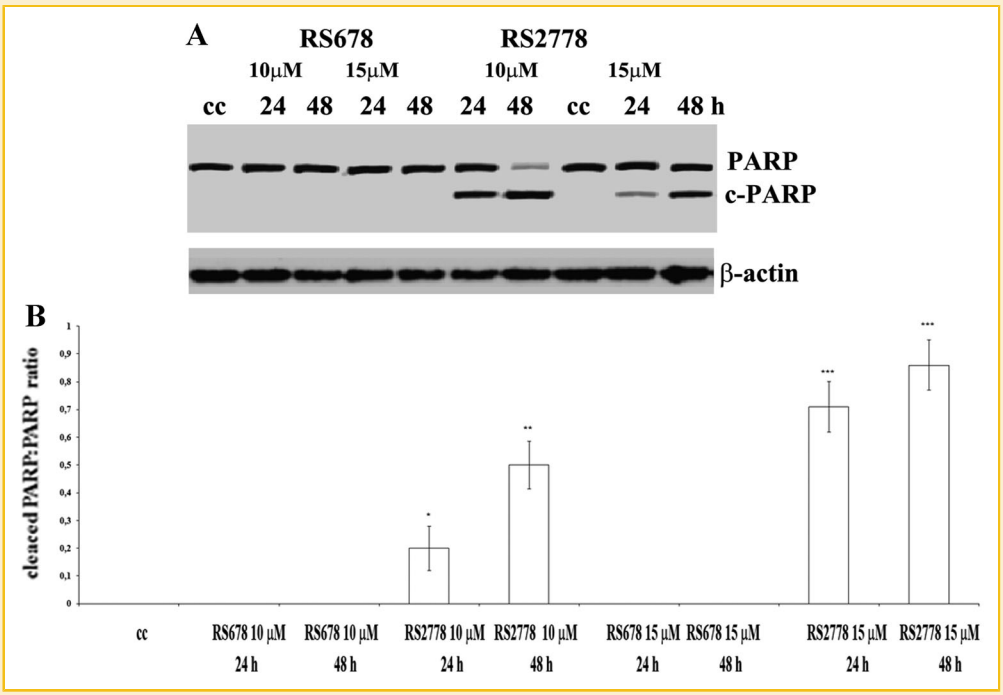

Fig. 4. Pyrrolo[1,2-b][1,2,5]benzothiadiazepines (PBTDS) effect on MEC1 cells examined for PARP cleavage. A: Whole cell lysates prepared from untreated (lane cc) and treated cells. Cellular extracts were analyzed by Western Blot using specific antibody. β-Actin was used as a control for protein loading. Blots are representative at of at least three separate experiments. B: Cleaved PARP/PARP ratio. Band signals from PARP and cleaved PARP were measured using a Gel-Doc phosphorimager and Quantity One software and the ratio was calculated. Significant differences between the untreated and treated cells are indicated by probability *P*. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

different concentrations of PBTDs (RS2778 and RS678) for 24 and 48 h. Protein extracts were made and immunoblot analyses were performed for the presence of BCL-2 and BAX. The treatment of PBTD (RS2778) alone up-regulated BAX and inhibited BCL-2 in a dose-dependent manner (Fig. 6A), thus altering the BAX/BCL-2 ratio in these cells (Fig. 6B).

PBTD (RS2778) INHIBITS AKT PATHWAY LEADING TO APOPTOSIS OF MEC1 CELLS

PI3K/AKT signaling is particularly critical in the survival of CLL and for this reason the its inhibition can induce apoptosis. In particular, AKT plays a critical role in controlling the balance between survival and apoptosis. Earlier work, with protease inhibitors showed inhibition of AKT in hematological malignancies [Witzig and Kaufmann, 2006]. To investigate whether the AKT pathway is involved in apoptosis induced by PBTDs, MEC1 cells were treated with PBTDs (RS2778 and RS678) (10 and 15 μM) for 24 and 48 h. Then, the expression levels of p-AKT were analyzed using Western Blot. As shown in Figure 7A,B, only PBTD (RS2778) down-regulated the expression levels of p-AKT in MEC1 cells. Taken together, these data indicate that PBTD (RS2778)-induced apoptosis in leukemia cells is associated with down-regulation of the PI3K/AKT signaling pathway.

DISCUSSION

In spite of recent therapeutic progresses, CLL is still an incurable malignancy [Dighiero and Hamblin, 2008; Tam and Keating, 2010]. Because the leukemic B cells accumulating in the blood are mostly quiescent but defective in their cell death program, a new strategy of treatment based on the induction of apoptosis has attracted much attention [Kolb et al., 2003; Pleyer et al., 2009].

Our previous studies have reported that PBTDs can induce apoptosis in hematological malignancies, including chemotherapy-refractory CML cells. Here, we planned the synthesis of new PBTD derivatives to investigate the effect of 1-naphthoyl group on MEC1 cells and we showed that PBTD (RS2778) induced significant apoptosis in MEC1 cells in a dose-dependent manner, which were examined by annexin V and PI double staining, but we will test the new synthetic compounds RS2778-like in order to better understand their mechanism action.

Increasing knowledge about apoptotic processes has identified several targets which can be used as specific cell death markers, including the changes in mitochondrial membrane potential, cytochrome C, caspase members and so on. The combination of released cytochrome C and apoptotic protease activating factor-1 will active caspase cascade and apoptosis [Slee et al., 1999]. Despite we did not directly examined the release of cytochrome C, our results demonstrated a dose-dependent increase in the numbers of annexin V positive or annexin V/PI double positive cells and the activation of caspase-9, 3 in PBTD (RS2778)-treated leukemia cells (Fig. 2). In addition, we found that the proteolytic cleavage of PARP was already observed at 24 and 48 h, when the typical DNA laddering was also evident. It is well known that the apoptosis is mainly determined by a defective balance among pro- and anti-apoptotic members of the BCL-2 family, often related to resistance of leukemia cells to

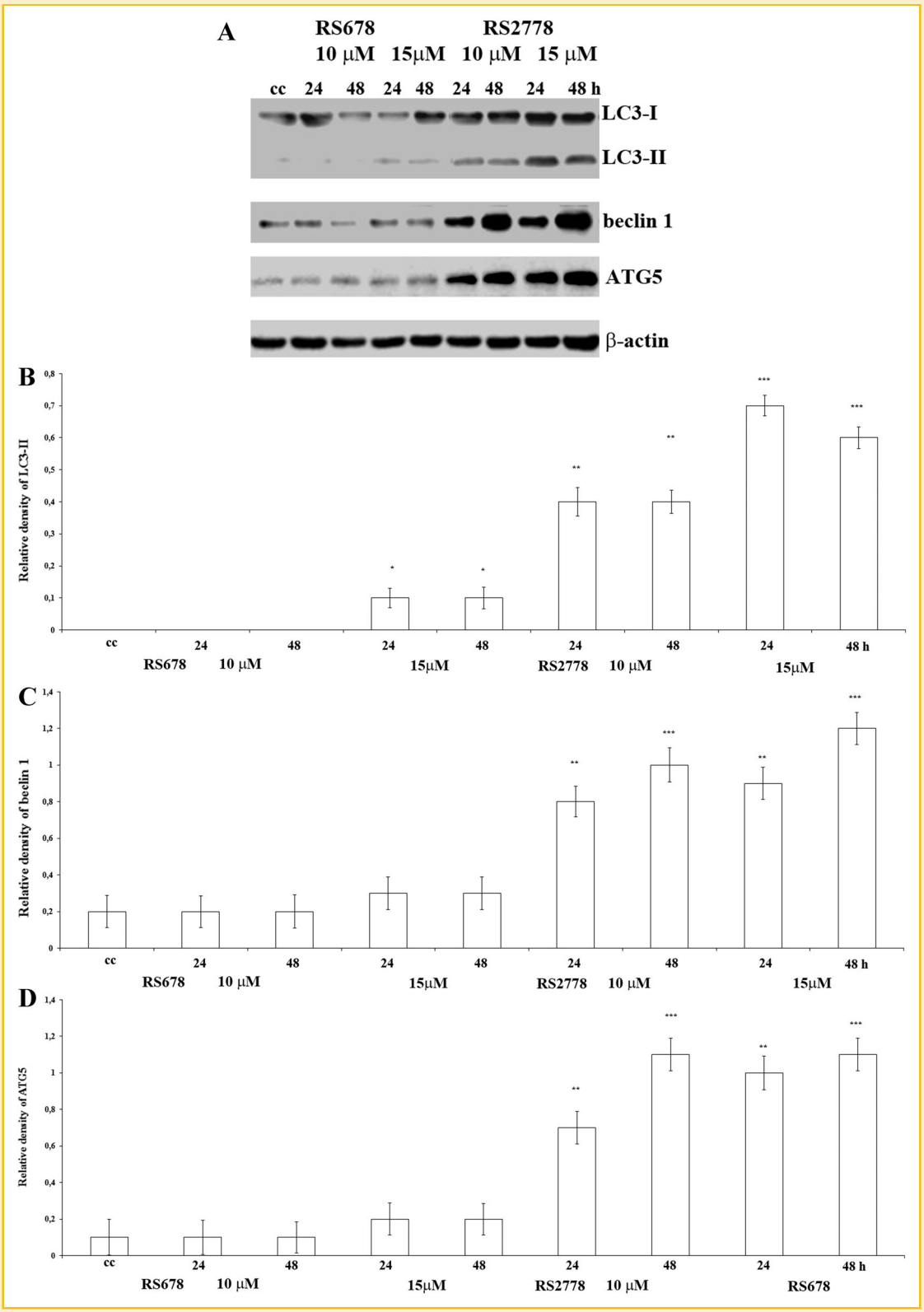

Fig. 5. Pyrrolo[1,2-b][1,2,5]benzothiadiazepines (PBTDS) effect in MEC1 cells examined for LC3 I–II, Beclin-1 and ATG5 proteins. A: Whole cell lysates prepared from untreated (lane cc) and treated cells. Cellular extracts were analyzed by Western Blot using specific antibodies. β-Actin was used as a control for protein loading. Blots are representative at of at least three separate experiments. B,C,D: PBTDs effect in cells examined for LC3 I–II conversion and Beclin-1 and ATG5 expression level. LC3 I–II conversion and Beclin-1 and ATG5 expression level in untreated (lane cc) and treated cells was obtained by densitometry analysis of the blots shown in (A). Significant differences between the untreated and treated cells are indicated by probability *P*. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

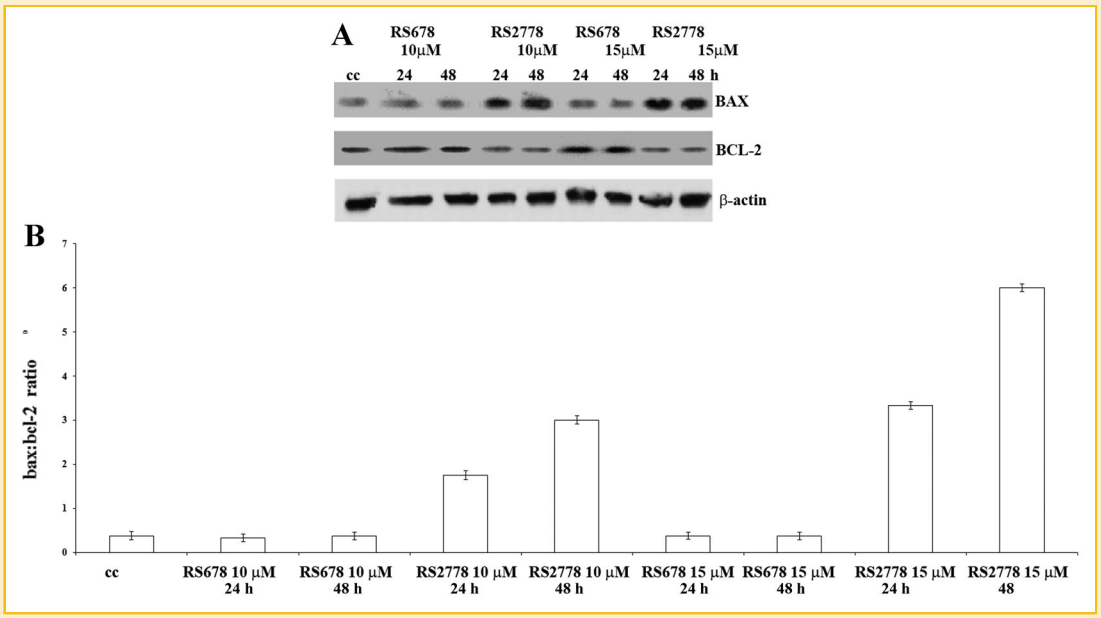

Fig. 6. Pyrrolo[1,2-b][1,2,5]benzothiadiazepines (PBTDS) effect on MEC1 cells examined for BCL-2 proteins family. A: Whole cell lysates prepared from untreated (lane cc) and treated cells. Cellular extracts were analyzed by Western Blot using specific antibodies. β-Actin was used as a control for protein loading. Blots are representative at of at least three separate experiments. B: PBTDs effect in cells examined for BAX/BCL-2 ratio. BAX/BCL-2 ratio in untreated (lane cc) and treated cells was obtained by densitometry analysis of the blots shown in (A). Significant differences between the untreated and treated cells are indicated by probability *P*. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

chemotherapy [Capitani and Baldari, 2010]. In our experimental condition, the expression ratio of BAX and BCL-2 was significantly increased in PBTD (RS2778)-treated leukemia cells, indicating that the pro-apoptotic signals were enhanced and the anti-apoptotic signals were suppressed.

It was previously suggested that autophagy and apoptosis were distinct forms of cell death, but more recent data seems to imply that there is a mechanistic overlap between the two archetypes that represent a continuous spectrum of different shades of physiological cell death [Dourmashkin et al., 1997]. Autophagy has been postulated

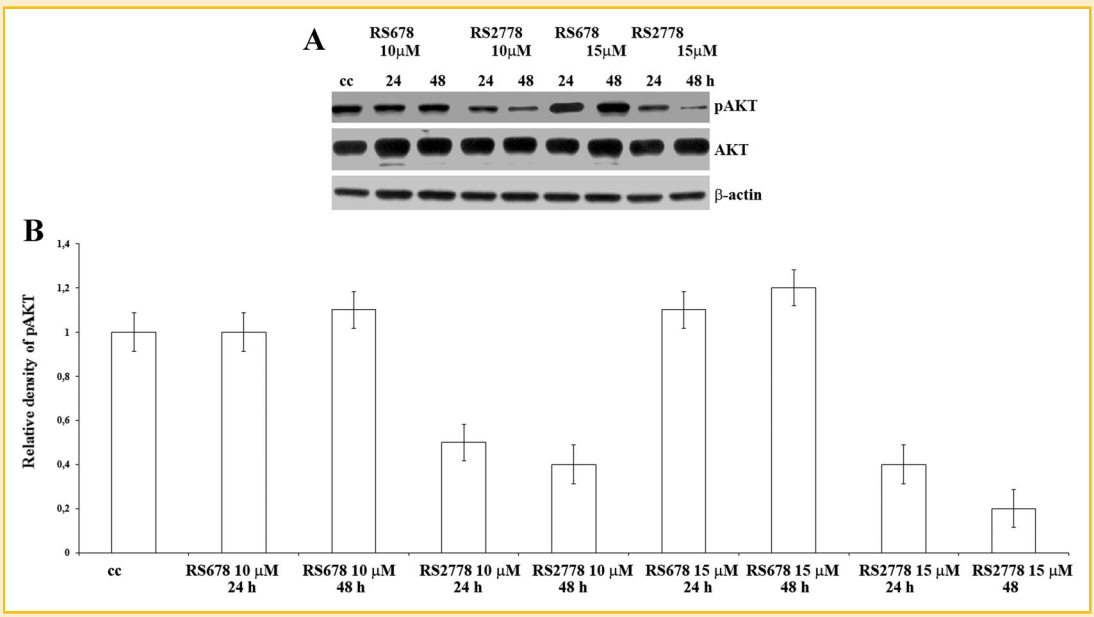

Fig. 7. Pyrrolo[1,2-b][1,2,5]benzothiadiazepines (PBTDS) effect on MEC1 cells examined for AKT signaling. A: Whole cell lysates prepared from untreated (lane cc) and treated cells. Cellular extracts were analyzed by western blot using specific antibodies. β-Actin was used as a control for protein loading. Blots are representative at of at least three separate experiments. B: PBTDs effects in cells examined for AKT phosphorylation. The AKT phosphorylatin in untreated (lane cc) and treated cells was obtained by densitometry analysis of the blots shown in Figure 6A. Significant differences between the untreated and treated cells are indicated by probability *P*. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

to be a primary cell response to apoptotic inducers and when the autophagic capacities are overloaded, mitochondrial pro-apoptotic factors would activate the death program [González-Polo et al., 2005]. Some observations indicate that autophagy has functions in helping cells to escape apoptosis through the sequestration of cytochrome c [Li et al., 2011]. Other evidences showed that active autophagy appeared to increase the tendency to undergo apoptosis, and both correlate with the mitochondrial permeability transition in certain cells. This is in agreement with other cases where autophagy is required for efficient apoptosis. For example, Peng and his colleagues reported inhibition of autophagy decreased the apoptosis induced by vincristine [Peng et al., 2004]. Thus, this process is utilized by the cells as a protective mechanism against cellular stress, such as nutrient deprivation, and conversely it can be disruptive, resulting in cell death [Klionsky and Emr, 2000]. In either of these cellular fates, the cytoplasmic constituents are sequestered by the formation of a double membrane structure called an autophagosome. It has been reported that a cytosolic protein, LC3-I, is proteolytically cleaved into a smaller LC3-II protein and forms the autophagosome [Noda et al., 2009; Glick et al., 2010]. Cleavage of the autophagy marker, LC3-I, into LC3-II is evident in MEC1 cells after 24 h of treatment with PBTD (RS2778), indicating the activation of autophagy. Furthermore, besides conversion of LC3-I to LC3-II, specific features of autophagic cell death also included involvement of autophagy-related proteins such Beclin-1 and ATG5.

Recent studies demonstrate that BCL-2 also protects cells against autophagy through binding to Beclin-1 [Levine et al., 2008]. In this regard, we observed that PBTD (RS2778) inhibits the BCL-2 protein in MEC1 cells in a dose-dependent manner. Thus, inhibition of BCL-2 by PBTD (RS2778) can also trigger both processes of apoptosis and autophagy. It is possible that PBTD (2778) treatment reduces the cellular pool of BCL-2, which simultaneously triggers both apoptosis and autophagy. Further work is required to clarify the molecular mechanism underlying the inhibition of BCL-2 by PBTD (RS2778).

Moreover, the serine/threonine kinase, AKT, is a downstream effector of PI3K and is constitutively activated in many B-CLL cases and contributes to the typical B-CLL cell antiapoptotic phenotype [Barragán et al., 2003; Escobar-Díaz et al., 2005]. 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 [Longo et al., 2007]. All together, these studies suggest that AKT plays a prominent role in the survival of CLL pathway and allows cells to survive by the inhibition of proapoptotic signals and the induction of survival signals [Cuni et al., 2004; Herman et al., 2010]. The function of PI3K-AKT pathway and its links to autophagy and apoptosis are disputing problems. Some reports described that PI3K-AKT activation suppresses autophagy in mammalian cells [Petiot et al., 2000; Arico et al., 2001]. However, emerging studies have pointed out that PI3K-AKT pathway positively regulates autophagy [Cui et al., 2006]. In this study, we showed that the AKT phosphorylation was down-regulated during the treatment with PBTD (RS2778).

In conclusion, the data presented in this report demonstrate that the compound PBTD (RS2778) induces apoptosis in leukemia cells by simultaneously invoking autophagy and apoptosis. The cross-talk between these mutually exclusive yet complementary cell death pathways is complex, and several molecular regulators, including BCL-2, play a critical role in the process. By inhibiting this protein, it may initiate the apoptotic process. As progression of asymptomatic CLL to its aggressive disease form involves accumulation of the bcl-2 family of proteins, treatment with PBTD (RS2778) could be a potential therapy.

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