Research Paper

Apoptosis of B-cell chronic lymphocytic leukemia cells induced by a novel BH3 peptidomimetic

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B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in human adults of the Western world and no definitive cure is yet available. The disease is characterized by accumulation of clonal malignant B lymphocytes resistant to apoptosis. Strategies to hit the anti-apoptotic drift of the Bcl-2 family in B-CLL cells are being explored. A novel peptidomimetic based on the BH3 domain of the pro-apoptotic protein Bim and recently shown to exert significant apoptotic activity on acute myeloid leukemia cells, both in vitro and in vivo, was assayed on ex-vivo derived leukemic cells from untreated B-CLL patients (n = 7). We found that this peptide, named 072RB, induced apoptosis of B-CLL samples at a concentration that does not affect viability of peripheral and bone marrow derived lymphocytes from healthy donors. Apoptosis was demonstrated by activation of Bak and Bax, externalization of plasma membranes phosphadydilserines, appearance of hypodiploid events in DNA flow cytometry histograms and was accompanied by dissipation of the mitochondrial transmembrane potential. Before the onset of marked apoptotic signs a progressive decline of the relevant anti-apoptotic proteins Bcl-XL and Mcl-1 could be observed. The negative control peptide 072RBL94A was ineffective for B-CLL cells, supporting the sequence specificity of 072RB activity. No relationship was found between responsiveness to 072RB and Mcl-1/Bcl-XL basal levels or decrease magnitude, possibly because of the limited sample size of the study. Altogether, we demonstrate that 072RB induces significant apoptosis of B-CLL cells subsequent to Bcl-XL and Mcl-1 downregulation.

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common B-cell malignancy in the Western world and is characterized by a clonal accumulation of apoptosis-resistant malignant CD5+ CD19+ CD23+ B lymphocytes.1 It is a remarkably heterogeneous disorder, since some patients present an indolent clinical course, whereas others display an aggressive disease rapidly progressing and refractory to current chemoimmunotherapeutic regimens. Heterogeneity also emerges from the analysis of IgV gene rearrangements, demonstrating that B-CLL clones bear germline or somatically hypermutated rearrangements and stereotyped B-cell receptors.2,5 A few molecular prognostic factors have been so far identified that predict early relapse and rapid progression of disease, that include unmutated immunoglobulin heavy-chain variable-region (IgVH) status4,5 and CD38 expression.5,6 For these high-risk patients allogeneic bone marrow transplantation can be adopted, as long as they are young and able to tolerate the procedures; otherwise, no cure or maintenance programs are effective.

Key factors for B-CLL cell resistance to apoptosis and disease progression is a marked sensitivity to survival stimuli received from the tissue microenvironment associated to a misbalanced Bcl-2 cell death machinery that is shifted towards protection from apoptosis.7 The Bcl-2 family comprises anti-apoptotic members (e.g., Bcl-2, Bcl-XL, Mcl-1), multi-domain pro-apoptotic proteins (e.g., Bak and Bax) and single-domain pro-apoptotic members, the BH3-only proteins (e.g., Bim, Bad, Noxa, Puma).8 Proteins of the first two classes share at least three of the four conserved structural motifs known as Bcl-2 homology domains (BH1-BH4), while the BH3-only proteins contain only the single BH3 motif (9–16 aminoacids).8 Generally pro-death proteins bind to multidomain proteins by inserting their BH3 domain, an amphipathic α-helix, into a hydrophobic cleft made up by the folding of BH1, BH2 and BH3 domains.8 The cell’s fate is governed by a complex and finely tuned network of this kind of protein-protein interactions, that can be either apoptosis-promoting or -blocking. In lymph nodes and bone marrow, where B-CLL leukemic cells accumulate before
being released in the peripheral blood for their journey to and from infiltrated tissues, cells are subjected to survival stimuli that mediate high levels of the anti-apoptotic protein Bcl-X\textsubscript{L}\textsuperscript{9} and low levels of the pro-apoptotic BH3-only molecule Bim\textsuperscript{10}. Moreover, Bcl-X\textsubscript{L} expression of peripheral blood B-CLL cells is further increased when the cells are subjected in vitro to microenvironment stimuli such as ligation of CD40 by CD40L (CD154)\textsuperscript{10,11} or sustained engagement of the B-cell receptor.\textsuperscript{12} Concomitantly to Bcl-X\textsubscript{L} upregulation, the expression of the BH3-only pro-apoptotic protein Bim is downregulated in peripheral blood B-CLL cells subjected to in vitro CD40 stimulation.\textsuperscript{10}

Bim is one of the most critical Bcl-2 family proteins for the regulation of apoptosis and homeostasis in the hematopoietic system.\textsuperscript{13} Bim (like Bid and Puma) is a “promiscuous” pro-apoptotic protein since it avidly binds, in addition to Bcl-X\textsubscript{L}, all anti-apoptotic proteins,\textsuperscript{14} while other BH3-only proteins bind only to subsets. In addition to inactivate anti-apoptotic protein activity, a very recent study demonstrates that Bim is able to directly activate Bax by binding to an interaction site that is distinct from the canonical binding groove characterized for anti-apoptotic proteins.\textsuperscript{15} Activation of Bim can be observed after different cellular stresses\textsuperscript{13,16} and repression of Bim is associated with mantle cell\textsuperscript{17} and Burkitt\textsuperscript{18} lymphomas. Antitumor chemotherapies kill neoplastic cells through Bcl-activated pathways, in acute myeloid,\textsuperscript{19} chronic lymphocytic leukaemia and myeloma cell lines.\textsuperscript{20} Altogether, Bim can be classified as a powerful tumour suppressor\textsuperscript{13,16,21} and represents a good BH3-mimetic candidate for counteracting the anti-apoptotic pool of Bcl-2 family members, with particular regard to Bcl-X\textsubscript{L}.

A Bim-derived BH3 mimetic drug was recently synthesized by replacing specific residues of the wt Bim-BH3 sequence with natural and non-natural aminoacids to increase the affinity for Bcl-X\textsubscript{L}.\textsuperscript{22} The peptide was further modified to improve its stability in serum and increase its ability to penetrate cell membranes. The resulting peptide was named 072RB. 072RB was shown to induce dose-dependent apoptosis of leukemic cell lines and ex-vivo derived leukemic cells from acute myeloid leukaemia (AML) patients, but not of normal peripheral blood mononuclear cells and bone marrow cells from normal donors.\textsuperscript{22} Xenografts of human AML cells in NOD/SCID mice displayed a significant delay of leukemic cell growth upon treatment with 072RB administered intravenously, without gross systemic and tissue toxicity.\textsuperscript{22} Conversely, no effect was observed when B-CLL cells were treated with the negative control peptide, 072RBL94A, that bears a point mutation (Leu94Ala) capable of decreasing the binding affinity between the BH3-domain of human Bim and Bcl-X\textsubscript{L} by 42 times.\textsuperscript{22,23}

In this study we investigate the effects of 072RB on ex-vivo derived B-CLL cells. We ask whether 072RB is capable of inducing apoptosis of B-CLL cultured in vitro and how the expression of relevant anti-apoptotic Bcl-2 family members is affected by the treatment. The dependence of B-CLL responsiveness to 072RB on the pattern of anti-apoptotic protein level or other molecular markers is also addressed.

**Results**

Samples from seven previously untreated B-CLL diagnosed patients that contained not less than 95% leukemic cells were utilized for this study. Surface CD38 immunofluorescence and sequencing of the IgVH gene rearrangement were performed, since CD38 expression\textsuperscript{9} and the IgVH mutational status,\textsuperscript{2} independently, define two subgroups of B-CLL cases that follow different clinical courses.\textsuperscript{4,5} As can be seen in Table 1, three are the patients belonging to the unfavorable molecular prognostic subgroup and four the patients with better prognosis.

The cytotoxic effect of 072RB on these B-CLL samples was investigated. A concentration of 10 μM was first assayed based on previous data on leukemic cell lines and ex-vivo AML cells.\textsuperscript{22} Figure 1A displays data on flow cytometric measurements of Propidium Iodide (PI) fluorescence, Forward (FSC) and Side Light Scatter (SSC) from one representative B-CLL sample treated with the peptide and assayed for PI exclusion to assess cellular viability. A significant proportion of cells displaying positive PI fluorescence, i.e., damaged plasma membrane, can be observed upon 072RB treatment after 72 h. It is of note that two subpopulations can be identified on the FSC-SSC dot plots. As observed on other cellular models, the “high FSC-low SSC” (gate R1) and the “low FSC-high SSC” (gate R2) subpopulations might provide a gross and approximate representation of live and death cells, respectively. By analyzing the PI exclusion features of cells residing in the two gates we confirmed the viability characteristics of the two subpopulations, albeit some cells with “low FSC-high SSC” pattern still display plasma membrane integrity, as demonstrated by their negative or dimly positive PI fluorescence. This may suggest that cells undergoing early apoptotic events shift from the “high FSC-low SSC” to the “low FSC-high SSC” cluster before plasma membrane rupture.

The time course of cellular viability for all patient samples treated with 10 μM 072RB is reported in Figure 1B and demonstrates an important dead cell fraction at 72 h if compared with the level of spontaneous apoptosis, known to occur when B-CLL are kept in culture. According to the non-parametric statistics of binomial distributions the difference between spontaneous and drug-induced apoptosis is significant at a p = 0.57 = 0.0078 level, and analyzed by the two-sided paired Wilcoxon signed-rank test the viable cell fraction difference between control and treated cultures is significant at p = 0.00042.

**Table 1** CD38 expression and IgVH mutational status for the B-CLL patients of this study

<table>
<thead>
<tr>
<th>B-CLL sample</th>
<th>CD38 expression (positive cells)\textsuperscript{a}</th>
<th>Heavy and light chain isotype\textsuperscript{b}</th>
<th>VH gene\textsuperscript{c}</th>
<th>Mutational status (% of identity)\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>G034</td>
<td>Pos (37%)</td>
<td>μ/κ</td>
<td>IGHV4-39*01</td>
<td>U (100)</td>
</tr>
<tr>
<td>G035</td>
<td>Neg</td>
<td>μ/κ</td>
<td>IGHV4-34*12</td>
<td>M (92.3)</td>
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<tr>
<td>G062</td>
<td>Pos (60%)</td>
<td>μ/κ</td>
<td>IGHV3-23*01</td>
<td>M (97.9)</td>
</tr>
<tr>
<td>G063</td>
<td>Pos (60%)</td>
<td>μ/κ</td>
<td>IGHV5-51*01</td>
<td>U (100)</td>
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<tr>
<td>G065</td>
<td>Neg</td>
<td>μ/κ</td>
<td>IGHV3-30*03</td>
<td>M (94.8)</td>
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<tr>
<td>G066</td>
<td>Neg</td>
<td>γ/κ</td>
<td>IGHV3-23*04</td>
<td>M (91.7)</td>
</tr>
<tr>
<td>G067</td>
<td>Pos (40%)</td>
<td>μ/κ</td>
<td>IGHV1-69*01</td>
<td>U (100)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Evaluated by surface immunofluorescence and flow cytometry (FITC conjugated-CD38 antibody from BD Biosciences). The cut-off percentage for defining the CD38 negative/positive status was set to 30%, according to a previous study where two clinically distinct subgroups of patients were identified based on this percentage.\textsuperscript{5} \textsuperscript{b}Evaluated by PCR amplification using specific primers as described.\textsuperscript{2} \textsuperscript{c}Variable region of the Ig Heavy Chain. \textsuperscript{d}Somatic mutations evaluated as described in Materials and Methods. Samples are classified as mutated (M) or unmutated (U) depending on a 2% mutation threshold.\textsuperscript{5}
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control peptide, 072RB, bearing a point mutation (Leu94Ala) that decreases the binding affinity between the BH3-domain of human Bim and Bcl-XL by 42 times without altering its α-helicity, does not affect B-CLL cellular viability.

We asked whether 072RB kills B-CLL cells by apoptosis. Flow cytometric DNA histograms reveal a significant increase of the sub-G1 cell fraction in 072RB treated cultures compared to control cultures (Fig. 2A). Externalization of phosphatidylserines (assessed by annexin V-FITC fluorescence) and dissipation of the mitochondrial transmembrane potential (by the drop of DiOC6 fluorescence) shown for one highly responding B-CLL sample after 48 h treatment, were observed for all B-CLL samples at 72 h (Fig. 2B and C). In particular, the ratio between the annexin V positive cell fraction in 072RB treated B-CLL samples and in untreated control cells is significantly higher than the ratio between 072RBL94A treated cultures and controls (Fig. 2B, right). Similar results were achieved for DiOC6 negative cells (Fig. 2C, right). Notably, DiOC6 negative cells display a biunivocal correspondence to cells with “low FSC-high SSC” light scatter features, as clearly observed on bivariate FSC-DiOC6 dot plots (not shown). This suggests that the subpopulation of “low FSC-high SSC” cells that still display intact plasma membrane, as discussed above in relation to Figure 1A, corresponds to cells undergoing mitochondrial depolarization.

Activation of the pro-apoptotic effector molecules Bax and Bak in B-CLL samples exposed to 072RB was demonstrated. Cells were processed for intracellular immunofluorescence utilizing antibodies specific for their active conformational state and measured by flow cytometry (not shown) and confocal microscopy (Fig. 1S in Suppl. Materials). Altogether, these observations confirm that the cytotoxic activity of 072RB observed at 10 μM concentration on B-CLL cells is due to apoptotic cell death induction.

We next wondered whether 072RB affects protein levels of the anti-apoptotic Bcl-2 family members Bcl-XL and Mcl-1. To this end, intracellular immunofluorescence was performed and cells were measured both by flow cytometry and confocal microscopy. Flow cytometry revealed that the basal protein level in B-CLL cells was significantly higher than in normal peripheral blood B cells from healthy donors by a fold increase of the median value for Mcl-1 and Bcl-XL in B-CLL samples of 1.5 and 1.9 with respect to normal B cells, respectively (p = 0.004) (not shown). Figure 3 displays the expression of Mcl-1 and Bcl-XL for one representative B-CLL patient sample after 48 h cell culture without or with peptides. It is of note that only the viable cell fraction within the “high FSC-low SSC” window in flow cytometric FSC-SSC plots (see gate R1 on Fig. 1A)
is reported and analyzed, in order to gain information on early molecular events occurring before mitochondrial depolarization and well before the onset of plasma membrane damage. The treatment with 072RB was able to reduce the expression of both anti-apoptotic proteins (Fig. 3). As can be evinced from Mcl-1 and, to a lesser extent, Bcl-XL both anti-apoptotic proteins (Fig. 3), the decline of protein expression was continuous down to a threshold level and then dropped discretely to negative values. This pattern of protein decrease was also confirmed by observations at different times (histograms not shown). To account for the two subsequent steps of protein reduction, we summarized the data obtained for all B-CLL patient samples on two graphs, obtained by plotting the mean anti-apoptotic protein expression (normalized to its isotype-matched negative control) either for cells within the “positive” fluorescence compartment only, or for all cells (the latter obtained by the weighted mean from the two “positive” and “negative” compartment and therefore accounting both for the decrease of “positive” fluorescence intensity and the increase of “negative” cell fraction). As mentioned, only cells within the “high FSC-low SSC” window were evaluated. Interestingly, according to confocal microscopy observations the cells with reduced anti-apoptotic protein expression were found to be the cells that displayed Bax and Bak activation (Suppl. Material, Fig. 1S). Altogether, the data demonstrate a significant overall decrease of anti-apoptotic protein levels that takes place before the onset of marked cellular apoptotic features. Noteworthy, the decline of anti-apoptotic protein levels is observed at 48 h when only few 072RB-treated B-CLL cells display damaged plasma membrane.

**Discussion**

The features of BH3-only proteins are being exploited in the last years for the development of therapeutic anti-cancer approaches that specifically target anti-apoptotic proteins overexpressed in tumors. We show in this report that a recently developed peptidic BH3 mimetic, 072RB, exerts apoptotic function on ex-vivo derived leukemic cells from B-CLL patients. Cell death is associated to externalization of phosphatidylserines, appearance of hypodiploid events in DNA flow cytometry histograms, dissipation of mitochondrial transmembrane potential, activation of Bak and Bax and decrease of Bcl-XL and Mcl-1 protein expression. The degree of apoptotic response to 072RB is variable among the different patients, but does not correlate with basal levels of Mcl-1 and Bcl-XL nor with the magnitude of their decrease upon peptide treatment. Also, B-CLL samples from patients with unfavorable molecular prognostic markers (i.e., with unmutated IgVH and/or positive CD38 expression) were not significantly less responsive to 072RB than the other set of patients. Whether this is due to the still limited patient sample size has to be further explored.

072RB causes dissipation of the mitochondrial transmembrane potential of B-CLL cells. It has to be determined whether it represents an upstream event, as recently described for chronic lymphocytic leukemia cells where the mitochondrial inner membrane permeabilization induced by the Bad-like BH3 mimetic ABT-737 resulted in subsequent mitochondrial matrix swelling and rupture of the outer mitochondrial membrane. Alternatively, as previously observed in tumor cell lines, the collapse of mitochondrial transmembrane potential could be an indirect consequence of the permeabilization of the outer mitochondrial membrane caused by the 072RB-induced Bax and Bak activation. Nevertheless, it can be inferred from our data that...
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are highly sensitive to antagonists of the anti-apoptotic protein function, because of the consequent massive release of pro-death proteins. Instead, nonmalignant cells survival does not normally depend on anti-apoptotic proteins—unless they are exposed to exogenous death signaling. Inactivation of anti-apoptotic protein function by BH3 mimetics, therefore, is not expected to exert dramatic effects on healthy cells. Although this hypothesis57,58 has not been yet fully demonstrated, we feel that it might provide an attractive model that accounts for the differential sensitivity of cancer and normal cells to 072RB.

We have no data yet about in vivo effects of 072RB on B-CLL. Xenograft models of B-CLL using SCID mice have been hampered by inefficient engraftment.31,32 A recent xenograft model was obtained by transplanting primary B-CLL cells into NOD/SCID mice,33 but engraftment was successful only with advanced stage B-CLL cells. Another in vivo model was developed, the hTCL1 transgenic mouse.34 However, it has been questioned for its B-CLL specificity,35 since overexpression of TLC1 is not unique to this disease and mice made transgenic for TCL1 using different tissue-specific promoters/enhancers do not develop a B-cell-CLL-like disease, but rather develop other types of lymphoid malignancies.35 These limitations are affecting the design of studies that extend the present data to specific in vivo models.

A progressive decline of Bcl-XL and Mcl-1 levels was observed when B-CLL cells were treated with 072RB. To exclude that this decrease was an unspecific consequence, rather than a cause, of apoptotic cell death, all flow cytometric analyses were performed on still alive cells, after gating out cells that displayed apoptotic features. Accordingly, we had the demonstration that Bcl-XL and Mcl-1 started their decline well before overt apoptotic cell damage.
Decreased levels of Bcl-X	extsubscript{L} and/or Mcl-1 expression had been already observed by biochemical assays on B-CLL cells (e.g., Western blots) in response to some cytotoxic drugs.\textsuperscript{36-38} However, it is difficult, in such experimental settings that account for all B-CLL cells irrespec-
tive of their integrity status, to state whether the reduction of protein expression predisposes to cell death or is the result of apoptosis.

The mechanisms that govern the decline of Bcl-X	extsubscript{L} and Mcl-1 upon exposure of B-CLL cells to 072RB are to be fully elucidated. We may envisage that 072RB liberates BH3-only molecules held in check by anti-apoptotic proteins by competing for their hydrophobic groove and allowing BH3-only proteins to exert their pro-apoptotic activities. However, it is not clear how the binding of 072RB to Bcl-X	extsubscript{L} and Mcl-1 affects their half-life and/or their new synthesis. As far as the BH3-only protein Noxa is concerned, it is believed that its binding to Mcl-1 triggers proteasome-mediated Mcl-1 degradation, possibly by the binding of the E3 ubiquitin ligase Mule to the complex.\textsuperscript{39} Conversely, the wt Bim BH3 sequence was shown in a recent study to stabilize Mcl-1 levels, perhaps by precluding the binding of Mule.\textsuperscript{40} Interestingly, when the discrete C-terminal sequence—T\textsuperscript{103}AYYARR\textsuperscript{108}—from Bim is substituted into Noxa in place of the respective Noxa sequence, Mcl-1 degradation is no longer observed.\textsuperscript{40} Our Bim-BH3-derived peptide 072RB lacks this sequence and bears instead one non-natural amino acid to increase the peptide stability followed by a 16 aa Antennapedia-derived internalizing vector.\textsuperscript{22} Whether this different C-terminal sequence may have a role in making the Mcl-1-072RB complex available to the E3 ubiquitin ligase Mule or some other adapter molecule should be explored.

072RB was designed to maximize the affinity of the Bim-BH3 domain for Bcl-X	extsubscript{L}.\textsuperscript{22} However, we observed, previously on leukemic cell lines\textsuperscript{22} and here on peripheral blood B-CLL cells, that marked cellular expression of Bcl-2 and Mcl-1 does not impair cell killing by our Bcl-X	extsubscript{L} inhibitor. Bim is a promiscuous BH3-only protein, targeting all multi-domain proteins of the Bcl-2 family, and in silico alignments revealed that, even after optimization of the interaction with Bcl-X	extsubscript{L}, all interaction hot spots between 072RB and Bcl-X	extsubscript{L} are conserved in Bcl-2 and Mcl-1 sequences.\textsuperscript{22} Therefore, it is possible that 072RB still interacts with the BH3-binding cleft of Bcl-2 and Mcl-1, and thus kills cells via interaction with all Bcl-2-like anti-apoptotic proteins.

Induction of Mcl-1 decline, in addition to Bcl-X	extsubscript{L}, represents an attractive feature for 072RB. Like Bcl-X	extsubscript{L}, Mcl-1 as well represents a critical survival factor for B-CLL and contributes to chemoresistance and disease relapse. In particular, high levels of Mcl-1 are associated with poor treatment response and high risk of B-CLL disease progression.\textsuperscript{41,42} Specific downregulation of Mcl-1 by small interfering RNAs elicits apoptosis in B-CLL cells in vitro.\textsuperscript{12,43} A short sequence insertion in the Mcl-1 promoter that upregulates the protein levels identifies high-risk B-CLL patients.\textsuperscript{4} Altogether, a compound such as 072RB that downregulates both Bcl-X	extsubscript{L} and Mcl-1 may represent a valuable tool for effectively counteracting apoptosis-resistance of B-CLL cells.

BH3 mimetics often lack overall single-agent apoptogenic ability, and are mostly effective when utilized in conjunction with other therapeutic compounds, so that they can target the whole spectrum of Bcl-2 anti-apoptotic proteins. To date, the most studied and promising synthetic small molecule BH3-mimetic is ABT-737.\textsuperscript{28} However, ABT-737, like its second generation orally bioavailable compound ABT-263,\textsuperscript{45} is effective in unleashing cell killing only if Mcl-1 activity is impaired,\textsuperscript{28,29,46,47} due to its BAD-like BH3 mimetic structure. Nevertheless, this compound was able to induce apoptosis as single agent on peripheral blood B-CLL cells\textsuperscript{20,28} and the authors justify this result by showing that their samples, in contrast to literature data, display low Mcl-1 levels.\textsuperscript{20} However, upon stimulation of CD40 the sensitivity to ABT-737 is reduced approximately 100-fold.\textsuperscript{10}

B-CLL cells are particularly susceptible to survival stimuli present in lymphoid tissues, such as interaction of CD40 with the CD40 ligand (CD154) molecule expressed by CD4\textsuperscript{+} T cells. Therefore, it is important to determine whether CD40 engagement increases the apoptotic threshold to 072RB. We obtained preliminary results from experiments carried out on B-CLL cells cultured over monolayers of a stable CD40 ligand-expressing fibroblasts cell line, and found that the expression of both Mcl-1 and Bcl-X	extsubscript{L} was increased if compared to CD40-unstimulated B-CLL cells. Moreover, the level of 072RB-induced apoptosis was reduced, and the decline of Mcl-1, but not Bcl-X	extsubscript{L}, was less marked (unpublished data of ongoing research). If these data will be confirmed, the single-agent efficacy of 072RB should be revisited and co-treatment regimens with drugs that specifically inactivate Mcl-1 should be explored. As an example, a Bim-BH3-like mimetic drug modified to be highly selective for Mcl-1,\textsuperscript{38} was recently published. This peptide cannot be utilized as single agent either, since it promotes cell death only when also Bcl-X	extsubscript{L} is neutralized.\textsuperscript{48}

The time of apoptosis onset after exposure of B-CLL cells to 072RB is longer than expected, also in the light of the short time (1–3 hours) required for 072RB to internalize and colocalize with mitochondria.\textsuperscript{22} We feel that for a Bim-like BH3 mimetic to bind its anti-apoptotic partners, thereby freeing the sequestered pro-apoptotic molecules to activate Bax and Bak, a timing shorter than three days should be expected. We could speculate that 072RB binds only to newly synthesized anti-apoptotic molecules, and not to anti-apoptotic molecules once they are already complexed to their pro-apoptotic targets. However, it has to be mentioned that the half-life of anti-apoptotic molecules is rather low (in the order of a few hours).\textsuperscript{49,50} Thus, this hypothesis does not entirely support our observation. Nevertheless, we exclude cellular toxicity by unspecific unknown factors associated to the antennapedia internalizing sequence or other structural features of 072RB that are unrelated to the specific binding of 072RB with its targets. In fact, one aminoacid substitution that does not alter the structure of the molecule and its α-helicity (L94A) completely abolishes the apoptotic effect of 072RB, both in AML and B-CLL leukemic cells. The exact mecha-
nism of apoptotic elicitation by 072RB has to be elucidated.

Materials and Methods

B-CLL cells and cultures. B-CLL cells were obtained from the peripheral blood of B-CLL patients after routine diagnostic procedures. All patients provided informed consent according to the regulation of the Declaration of Helsinki. Mononuclear cells were separated by Ficoll density gradient centrifugation and assayed by flow cytometry (FACSCalibur, BD Biosciences, San Diego, CA) for standard diagnostic immunophenotyping (CD19, CD5, CD23, CD79B, CD22, CD38) and identification of heavy chain (IgM, IgG or, very rarely, IgA) and light chain (kappa or lambda) isotype.
Cells were then resuspended in freezing solution (10% DMSO and 90% Fetal Bovine Serum (FBS)) and cryopreserved in liquid nitrogen. Samples from previously untreated B-CLL patients and containing at least 95% leukemic cells were considered eligible for the study. B-CLL cells cultures were obtained by seeding thawed cells into RPMI (InVitrogen S.r.l., Milan, Italy) culture medium supplemented with 10% FBS (InVitrogen) at a cell density of 2 x 10^3/ml. Cells were kept in fresh culture medium for three hours before drug addition. Only samples displaying a low level of spontaneous apoptosis in culture were utilized (i.e., after 3 days in vitro culture the percentage of viable B-CLL cells had to be at least 60%). After 48 h from the first drug addition, the peptides were re-added to the culture medium due to their limited half-life (36 hours for 072RB and >32 hours for 072RBL94A).}

**RNA extraction, PCR amplification and analysis of IgVH sequences.** Total RNA was extracted from peripheral blood mononuclear cells of B-CLL patients using the Rneasy mini kit (Qiagen S.p.A, Milan, Italy), as previously described. Two μg RNA were reverse-transcribed into cDNA using M-MLV reverse transcriptase (RT, InVitrogen) primed by an oligo dT (16mer) primer. Reactions were carried out in a 25 μl volume using 20 pmol of primer, and 200 U of RT at 42°C for 1 hr. The reaction volume was increased up to 100 μl by addition of distilled water. To determine the nucleic acid sequence of IgVH, 2 μl of cDNA from each B-CLL sample were amplified using a sense VH leader, family-specific, primer in conjunction with the appropriate antisense IgM, IgG, IgA. Sequence of primers and PCR conditions have been described previously. The VH PCR products were sequenced directly, after ethanol precipitation, using an automated sequenator (310 Genetic Analyzer, Applied Biosystems, Monza, Italy). Two independently generated PCR products were sequenced for each sample. Sequences obtained were compared with Ig germline gene database at the international ImMunoGeneTics information system http://imgt.cines.fr (Initiator and coordinator: Marie-Paule Lefranc, Montpellier, France) using the IMGT/V-QUEST tool (http://imgt.cines.fr/IMGT_vquest/share/textes/).

**DNA flow cytometry for apoptosis evaluation.** Cells were permeabilized with 0.05% Triton X-100 and incubated with 30 μg/ml PI (Propidium Iodide) (Sigma Chemical Co.) and 0.5 mg/ml RNase for 30 min at room temperature and in the dark. Flow cytometric measurements of excitation laser Forward Light Scatter (FSC), Side Light Scatter (SSC), and PI emission fluorescence, were performed on a FacsCalibur (Becton Dickinson, San José, CA) flow cytometer. At least 10,000 events were acquired on a linear scale from each sample. Frequency distributions of DNA content were analysed for the evaluation of proliferation and apoptosis as previously described. \(^{53}\)

**PI exclusion and annexin V assays.** Plasma membrane damage was tested by PI exclusion assays. Briefly, cellular uptake of PI was monitored by flow cytometric measurements of PI fluorescence approximately 5 minutes after incubation of the cells with 1 μg/ml PI at room temperature. Cellular externalization of phosphatidylserine molecules was assessed by flow cytometric measurements of annexin V binding on cells stained with annexin V-FITC, according to standard manufacturer instruction (BioVision, Mountain View, CA, USA). Cells were run on a FacsCalibur (Becton Dickinson) flow cytometer. For each sample 10,000 events were collected on a logarithmic scale, both for annexin V-FITC and PI fluorescence.

**Dissipation of mitochondrial transmembrane potential.** Loss of mitochondrial transmembrane potential (MTP) was evaluated by decreased fluorescence of the cationic dye 3,3′-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>). Live cells were incubated for 15 min at 37°C with 40 nM DiOC<sub>6</sub> and analysed by the FacsCalibur flow cytometer equipped with a 488 nm laser suitable for DiOC<sub>6</sub> excitation and cut-on optical filters suitable for DiOC<sub>6</sub> emission-fluorescence (peak at 552 nm) (channel FL-1). At least 10,000 events were acquired per sample on a logarithmic scale.

**Flow cytometric evaluation of Bcl-2 family member expression.** Cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% saponin, incubated for 30 min at room temperature in the presence of the primary antibody, washed and incubated with fluorochrome-conjugated secondary antibodies for 30 min at room temperature. After washing, cells were counterstained with Dapi and either mounted with Mowiol on coverslips (for microscopy imaging on the laser scanning spectral confocal microscope TCS SP2 AOS from Leica (Heidelberg, Germany)) or directly measured on the FacsCalibur flow cytometer (Becton Dickinson), after verifying that no DAPI contribution was collected by the photomultiplier FL-1. In several experiments, where only flow cytometry was adopted, no DAPI staining was performed. Primary antibodies specific for anti-apoptotic proteins were: mouse anti-Bcl-X<sub>L</sub> (H-5) and rabbit anti-Mcl-1 (H-260) antibodies from Santa Cruz Biotechnology (San Diego, CA) both used at 8 μg/ml. Conformation-specific antibodies for the detection of Bax and Bak activation were anti-Bax clone 3 (BD Biosciences, San José, CA) and anti-Bak AB-1 (clone TC-100, Calbiochem, San Diego, CA) both used at 5 μg/ml. Secondary antibodies, from Molecular Probes (InVitrogen, Eugene, OR), were Goat Immunoglobulins, either anti-Rabbit or isotype-specific anti-Mouse, and conjugated to Alexa fluorochromes (Alexa Fluor 488 or Alexa Fluor 546).

**Statistical analysis.** Kruskal-Wallis test was used for analyzing differences between more than 2 groups, after which two-sided Mann Whitney U test was employed for further analyze significance of differences between two groups. Paired samples were analyzed by the sign and the Wilcoxon signed-rank test. The level of correlation between drug sensitivity and anti-apoptotic protein expression and variation, or expression of prognostic molecular markers were determined using non-parametric Spearman correlation analysis.

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**Note**

Supplementary materials can be found at: www.landesbioscience.com/supplement/GhiottoCBT8-3-Sup.pdf
References


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