'From the cradle to the grave': novel therapeutic approaches to attack the microenvironment in chronic lymphocytic leukemia

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Cross-talk among Bcl-2 family members in B-CLL: seliciclib acts via the Mcl-1/Noxa axis and gradual exhaustion of Bcl-2 protection

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ABSTRACT

Seliciclib (R-roscovitine) is a cyclin-dependent kinase inhibitor in clinical development. It triggers apoptosis by inhibiting de novo transcription of the short-lived Mcl-1 protein, but it is unknown how this leads to Bax/Bak activation that is required for most forms of cell death. Here, we studied the effects of seliciclib in B cell chronic lymphocytic leukemia (B-CLL), a malignancy with aberrant expression of apoptosis regulators. Although Seliciclib induced Mcl-1 degradation within 4 hrs, Bax/Bak activation occurred between 16 and 20 hrs. During this period, no transcriptional changes in apoptosis-related genes occurred. In untreated cells, pro-survival Mcl-1 was engaged by the pro-apoptotic proteins Noxa and Bim. Upon drug treatment, Bim was quickly released. The contribution of Noxa and Bim as a specific mediator of seliciclib-induced apoptosis was demonstrated via RNAi. Significantly, 16 hrs after seliciclib treatment, there was accumulation of Bcl-2, Bim, and Bax in the ‘mitochondria-rich’ insoluble fraction of the cell. This suggests that after Mcl-1 degradation, the remaining apoptosis neutralising capacity of Bcl-2 is gradually overwhelmed, until Bax forms large multimeric pores in the mitochondria. These data demonstrate in primary leukemic cells hierarchical binding and cross-talk among Bcl-2 members, and suggest that their functional interdependence can be exploited therapeutically.
INTRODUCTION

B cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous disease, typified by the accumulation of long-lived monoclonal B cells arrested at the G0/G1 phase of the cell cycle. B-CLL is considered a malignancy involving deregulated apoptosis. High levels of pro-survival Bcl-2 and Mcl-1 proteins can be detected in B-CLL, which are associated with aggressive disease and refractoriness to chemotherapy. Contrasting with these clinical observations, CLL cells succumb easily to ‘spontaneous’ apoptosis during in vitro culture. So it remains unclear whether overexpression of these anti-apoptosis proteins is strictly required to block imminent apoptosis, or affords protection during stress or drug treatment. This aspect is particularly relevant in light of the concept of ‘oncogene addiction’, which holds that each cancer type may strictly depend on just a few particular oncogenes, like Bcl-2.

A pro-apoptotic subgroup within the Bcl-2 family consists of so called BH3-only proteins and includes Bid, Bim, Noxa, Puma, Bad, Bmf, Bik and Hrk. By forming heterodimeric complexes at the mitochondria, the interplay between BH3-only proteins and pro-survival Bcl-2-like counterparts determines the activation of pro-apoptotic Bax-like members and subsequent cell death. Paradoxically, Noxa and Bmf are overexpressed in circulating B-CLL cells although the functional significance of these observations remains unclear. Puma, a bona-fide p53 target gene, is rapidly induced in B-CLL cells obtained from the majority of patients upon treatment with therapeutic drugs such as fludarabine and chlorambucil. Still, it has been difficult to demonstrate an improvement in overall survival upon treatment with these chemotherapeutic drugs and disease relapse is inevitable in virtually all patients. Up to 30% of B-CLL tumors have a defect in the p53 pathway, which is often associated with resistance to therapy. Therefore, there is an urgent need for novel drugs that work independently of p53.

Recently, the cyclin-dependent kinase (CDK) inhibitor seliciclib (CYC202, R-roscovitine), has emerged as a potential drug for treatment of B cell malignancies including B-CLL. Another CDK inhibitor, flavopiridol has recently been tested in CLL with encouraging results. Seliciclib induces apoptosis, most likely by inhibiting RNA polymerase II dependent transcription and thereby resulting in loss of the short-lived pro-survival Mcl-1 protein. Yet, it remains unresolved how the decrease in Mcl-1 levels is coupled to apoptosis induction. Despite the notion that Bax and Bak are indispensable for apoptosis, the molecular cascade that mediates their activation is presently a matter of debate. New developments have shed light on the interaction between members of the Bcl-2 family. Specifically, the pivotal pro-survival Mcl-1 interacts, amongst others, with Noxa, Bim, Puma and Bak in model cell lines.

These findings may be relevant in connection with our observation of elevated
expression of Noxa in B-CLL\textsuperscript{6}, as well as in light of the above mentioned concept of ‘oncogene addiction’.

In the present study, we investigated the molecular mechanism underlying apoptosis elicited by seliciclib in B-CLL, with emphasis on protein interactions between Bcl-2 family members. We show for the first time that Mcl-1 is associated with Noxa in primary leukemic cells and that Noxa is required to trigger the mitochondrial apoptosis pathway upon seliciclib treatment. Furthermore, despite rapid proteasome-dependent degradation of Mcl-1 (within 4 hrs), Bax/Bak activation and apoptosis are substantially delayed (16-20 hrs). During this time Bim and Bax become associated with Bcl-2 and these proteins eventually translocate to a mitochondria-rich insoluble fraction. Our data suggest that in absence of Mcl-1, the remaining apoptosis neutralizing capacity of Bcl-2 is gradually overwhelmed, resulting in Bax/Bak multimerisation and pore formation in the mitochondria.

**MATERIALS AND METHODS**

**B-CLL patients and isolation of leukemic B cells**

Peripheral blood samples were obtained from B-CLL patients from the outpatient clinic of the department of Hematology of the Academic Medical Center, Amsterdam; the department of Internal Medicine, Meander Medical Center, Amersfoort and the department of Internal Medicine, The Netherlands Cancer Institute, Amsterdam. Informed consent was obtained according to the guidelines of the local Ethical Review Board. Clinical characteristics of patients are presented in Table 1. This study was conducted in accordance with the ethical standards in our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983. Peripheral blood mononuclear cells (PBMC), obtained via density-gradient centrifugation were frozen in 15\% fetal calf serum (FCS) containing 10\% dimethyl sulphoxide (DMSO; Sigma Chemical Co., St Louis, MO, USA) and stored in liquid nitrogen. Expression of CD5, CD19 and CD23 (all Becton Dickinson (BD) Biosciences, San Jose, CA, USA) on leukemic cells was assessed by flow cytometry (FACScalibur, BD Biosciences) and CellQuest software (BD Biosciences)\textsuperscript{6}.
Seliciclib acts via the Mcl-1/Noxa axis

Cell lines, retroviral constructs and transduction

Cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Gibco Life Technologies, Paisley, Scotland), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (ICN Biomedicals GmbH, Meckenheim, Germany), 5 mg/l gentamycin and 5 mM L-glutamine (Gibco). The Burkitt lymphoma Ramos FSA clone with enhanced response to CD95, the caspase-9-DN (C9DN) overexpressing Ramos FSA cell line, Ramos 2G6 clone and the Bcl-2 overexpressing Ramos 2G6 cell line have been described previously\textsuperscript{15,16}. The B-CLL cell line Mec-\textsuperscript{17} was a kind gift of Dr. Caligaris-Cappio (Milano, Italy). Mock and two Noxa siRNA sequences (N7 or N8) were retrovirally transduced into the Ramos FSA, Mec-1 and J16 cell lines as described previously\textsuperscript{18}. Furthermore, siRNA targeting Bim (pSuper-Bim) was kindly provided by Andreas Villunger (Biocenter, Innsbrück, Austria) and subcloned in the EcoRI/Xho sites of pRetroSuper. After retroviral transduction of J16 cell line, the B18 and B22 subclones were obtained after limiting dilution. The pcDNA3.1 vector encoding Myc-hMcl-1 was obtained from Professor J. Borst (Netherlands Cancer Institute). After digestion with BamH1 and Xba the product was inserted into the BamH1 and SnaB1 blunt ended sites of the LZRS-IRES-GFP vector, resulting in LZRS-Myc-hMcl-1-IRES-GFP.

Reagents

Seliciclib was obtained from Cyclacel Ltd (Dundee, UK). Fludarabine, staurosporine, etoposide, carbonyl cyanide m-chlorophenylhydrazone (CCCP), Taxol and propidium iodide (PI) were purchased from Sigma Chemical Co. Anti-human Fas\textsuperscript{10} (agonistic antibody to the CD95 receptor) and anti-human IgM mAbs (CLB/MH15) were a kind gift from Prof. Dr. L. Aarden (Sanquin, Amsterdam, The Netherlands). The pan-caspase inhibitor zVAD-fmk was obtained from Alexis Biochemicals (Läuferlingen, Switzerland). APC-labeled Annexin V was from IQ Products (Groningen, The Netherlands) and MitoTracker Orange was from Molecular Probes (Leiden, The Netherlands). Proteasome inhibitors MG132 and bortezomib, were obtained from Calbiochem, (Amsterdam, The Netherlands) and Janssen-Cilag, (Tilburg, The Netherlands), respectively.

Analysis of apoptosis

For cell stimulations, B-CLL cells (>90% CD19\textsuperscript{+}CD5\textsuperscript{+} PBMC suspensions) were used at a concentration of 1.7×10\textsuperscript{6} cells/ml in 24-well plate, Ramos and Mec-1 cell lines at a concentration of 5×10\textsuperscript{5} cells/ml. Cells were harvested and washed in ice-cold HEPES buffer (10 mM HEPES, 150 mM KCl, 1 mM MgCl\textsubscript{2} and 1,3 mM CaCl\textsubscript{2}, pH 7.4),
and incubated with APC-labeled Annexin-V (BD Biosciences) for 20 minutes. Prior to analyses, PI was added (final concentration 5 μg/ml). Viable cells were defined by Annexin V/PI staining. Alternatively, cells were incubated with 200 mM MitoTracker for 30 mins at 37°C. To assess activation of Bax and Bak, B-CLL cells (1×10^6) were washed in PBS prior to fixation in 4% formaldehyde for 5 min at room temperature. Cells were washed in PBS and incubated in the presence of 1 μg/ml of anti-Bax (Ab-6, clone 6A7, Calbiochem) or anti-Bak (Ab-1, Calbiochem), diluted in permeabilisation buffer (0.5% BSA + 500 μg/ml digitonin in PBS) for 30 min at room temperature. Cells were washed in PBA (PBS + 0.2% BSA + 0.02% NaN3) and incubated with goat anti-mouse PE conjugated secondary antibody (Jackson ImmunoResearch Europe Ltd., Suffolk, England), diluted 1/200 in permeabilization buffer for 30 min at room temperature. Cells were washed with PBA, resuspended in PBS and analysed using the FACS.

Western blotting and immuno-precipitation

Cell lysates for Western blotting were separated by 13% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting as described previously. Blots were probed with the following antisera: monoclonal anti-Mcl-1 (BD Pharmingen), monoclonal anti-Bax (BD Pharmingen), Bak monoclonal antibody (mAb) (Upstate Biotechnology, Inc. Lake Placid, NY, USA), monoclonal anti-Bim (Fig. 2 and Fig. 5A; Chemicon, Temecula, CA, USA), polyclonal anti-Bim (Fig. 3AIII and Fig. 5B; Nventa, San Diego, CA, USA) monoclonal anti-Noxa from Imgenex (San Diego, CA, USA), polyclonal anti-Bcl-2 (Alexis Biochemicals), polyclonal cleaved caspase 3 (Asp175) antibody, polyclonal anti-p44/42-MAP Kinase (Cell signaling Technology Inc., Danvers, MA, USA) antibody and antiserum to β-actin was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

For immuno-precipitation (IP) of Mcl-1, 50×10^6 cells were washed twice in ice-cold PBS (supplemented with 10 mM Na_3VO_4) and lysed in 500 μL ice-cold Triton X-100 lysis buffer (1% Triton-X100, 20 mM Tris-Cl (pH=7.4), 135 mM NaCl, 1.5 mM MgCl_2,10% Glycerol, 2 μg/ml Leupeptin, 1 mM PMSF and 2 μg/ml Trypsin inhibitor). Whole cell lysates were precleared with normal rabbit serum precoupled to protein A-sepharose and incubated for 3 hr with 5 μg polyclonal anti-Mcl-1 (BD Pharmingen) precoupled to protein A-sepharose. Beads were pelleted, washed six times with lysis buffer and boiled in SDS sample buffer, and further processed for Western blotting.

CHAPS lysates were used in certain experiments to avoid detergent-induced conformational changes of Bax, by lysing 50×10^6 cells (for IP) or 15×10^6 cells (for Western Blot) for 30 min on ice in lysis buffer (1% Chaps, 20 mM Tris-Cl (pH=7.4),
135 mM NaCl, 1.5 mM MgCl$_2$, 10% Glycerol, 1 mM EGTA, 2 mM Na$_2$VO$_4$, 10 mM NaF, 2 μg/ml Leupeptin, 1 mM PMSF, 0.1 mM TLCK and 2 μg/ml Trypsin inhibitor). After centrifugation (15 min; 13,000 × g; 4°C), the supernatant was used for IP or as ‘CHAPS soluble fraction’ on Western Blot. Pellets were solubilized in SDS sample buffer and used in Western Blotting as the ‘CHAPS insoluble fraction’. Immuno-precipitation of Bim (monoclonal anti-Bim (Chemicon)) was performed as described above in 500 μL ice-cold CHAPS and lysates were precleared with normal rat serum precoupled to protein G-sepharose.

Immunocytochemistry and confocal analysis
B-CLL cells were incubated with 200 nM MitoTracker for 24 hrs at 37°C and treated as described above (intracellular flow cytometry procedure). Cells (1 × 10$^6$) were resuspended in 25 μl vectashield (Hardset with DAPI; Vector Laboratories, CA, USA) and were spotted on cover slips. Images were independently captured by confocal laser microscopy (model TCS 4D, Leica, Heidelberg, Germany) at the wavelengths 488 and 594 nm lines of the krypton/argon laser used for the excitation of FITC and MitoTracker (Alexa594), respectively. Excitation of DAPI was by 594 nm.

Statistical analyses
The student’s t-test was used for analysis of differences between 2 groups. P-values < 0.01 were considered statistically significant.

RESULTS
Seliciclib induces apoptosis and rapid proteasome-dependent degradation of Mcl-1
The effect of seliciclib on cell viability was investigated in 20 B-CLL samples. B-CLL cells were >90% pure as determined by flow cytometry. Consistent with previous reports$^{20}$, in vitro culture of B-CLL cells was accompanied by moderate spontaneous increase of apoptosis. Increasing doses (10, 25 and 50 μM) of seliciclib resulted in dose-dependent increase of apoptosis (Fig. 1A). In comparison, cells treated with high dose of fludarabine (100 μM) showed a lower level of apoptosis at 24 hrs of treatment (Fig. 1A), but this increased over time (data not shown). IgV$_H$ mutated and unmutated B-CLL patients (n=8 vs. n=12) showed no difference in apoptosis induction by seliciclib (data not shown).
Seliciclib was reported to result in rapid proteasomal breakdown of Mcl-1 protein.$^{11}$
Mcl-1 is also described as a substrate for caspase-mediated degradation \(^{21}\). We tested which of these pathways predominates in B-CLL. Seliciclib was applied in combination with zVAD-fmk and the proteasome inhibitors MG132 and bortezomib. As shown in Figure 1B, the degradation of Mcl-1 in B-CLL cells was at best partially affected by zVAD-fmk (lane 2 vs. 3). In contrast, the proteasome inhibitors MG132 and bortezomib almost fully prevented Mcl-1 degradation (lane 2 vs. 4 and 5). Thus, in B-CLL cells Mcl-1 is largely degraded already 4 hrs after addition of seliciclib, predominantly via the proteasome.

In B-CLL, Mcl-1 associates with Noxa and Bim but not with Bak

Prior studies in model cell lines indicate that Mcl-1 selectively interacts with Bim, Bak, Puma and Noxa, whereas Noxa has a strong preference for Mcl-1 and possibly Bfl-1\(^{13,14,22}\). In primary B-CLL cells the majority of Noxa protein was associated with Mcl-1 (Fig. 2, compare IP with cleared lysate lanes). As also reported for normal tonsil B cells\(^{23}\), Bim was clearly but not exclusively associated with Mcl-1. Cells were then analysed after 2 hrs of Seliciclib treatment, i.e. before extensive degradation of Mcl-1 and zVAD-fmk or MG132 were added to block caspases or the proteasome. Rapid dissociation of Bim from Mcl-1 could be observed, while Noxa was still largely bound (Fig. 2, left panels).

Previous studies in HeLa cells indicated that Mcl-1 directly sequesters Noxa, Bim and Bak, and upon UV treatment, Bak is displaced by Noxa\(^{24}\). In B-CLL cells, Mcl-1 was
associated with Noxa and Bim, but we consistently could not detect association of Mcl-1 with Bak, irrespective of seliciclib treatment, although Bak was present in CLL lysates (Fig. 2, right panel). As expected, proteasome inhibition by MG132 resulted in increased Mcl-1 protein, but also increased Noxa levels (Fig. 2, right panels). Bax protein levels were unaffected by either seliciclib or MG132 treatment. In summary, in primary B-CLL cells undergoing seliciclib treatment Noxa remained bound to Mcl-1 while Bim dissociated.

**Figure 2. In seliciclib treated B-CLL cells, Mcl-1 engages Noxa and Bim but not Bak.**

B-CLL cells were treated with 25 μM seliciclib for 2 hrs. The pan-caspase inhibitors zVAD-fmk (100 μM) and proteasome inhibitor MG132 (10 μM) were added as indicated. Cell lysates were subjected to immunoprecipitation (IP) assays using control Ab (CAb) or anti-Mcl-1 antibody. Bound (IP) and unbound fractions (cleared lysate = 5% supernatant of lysate after IP) as well as total cell fractions (5% of total lysate) were analysed by Western blotting to evaluate protein associations with Mcl-1. The blotting membrane was rehybridized with anti-Bim, anti-Bak, anti-Bax and anti-β-actin antibodies as indicated. In the case of Bim, the data derive from a separate experiment. Experiments were repeated three times with similar results.

**Noxa deficient cells exhibit resistance to seliciclib-induced apoptosis**

To test whether the association between Noxa and Mcl-1 was reflected at a functional level, the endogenous levels of Noxa and Mcl-1 were manipulated in model systems via RNAi and overexpression. A significant reduction of Noxa in Ramos FSA cells was achieved with two different siRNA sequences (N7 and N8; Fig. 3Ai). Functionally, Noxa RNAi conferred a significant resistance to apoptosis induced by seliciclib compared to the mock transduced cells (Fig. 3B), which correlated with the knock-down in Noxa protein. These data were confirmed in the Mec-1 cell line (Fig. 3C), originally derived from a B-CLL patient, and the Jurkat T cell line18 (Fig. 3D). In contrast, upon several other death stimuli like fludarabine, staurosporine, CD95 and BCR stimulation, no significant effect of reduced Noxa levels was observed (Fig. 3B). Conversely,
increased Mcl-1 levels (Fig. 3Aii) conferred resistance to seliciclib, but also to several other apoptotic stimuli (Fig 3E). In agreement with the Mcl-1 association studies in B-CLL presented in figure 2, knock-down of Bim also resulted in increased resistance against seliciclib. As might be expected from its broader interaction range compared to Noxa, Bim knock-down conferred resistance against diverse apoptotic stimuli, among which taxol, in agreement with earlier studies. In conclusion, these data indicate that Noxa functions specifically in case of Mcl-1 degradation, such as after seliciclib treatment.

Figure 3. Noxa RNAi selectively affords resistance to seliciclib, compared to Bim RNAi and Mcl-1 overexpression.

Noxa knock down was obtained using two different Noxa RNAi constructs (N7 and N8) in Ramos and Mec-1 cells. Ramos FSA was transduced with LZRS-Mcl-1-iRES-GFP vector to obtain overexpression of Mcl-1. Bim knock down in Jurkat T cells was achieved with a Bim RNAi construct, yielding two independent clones B18 and B22. Transduction with empty vectors (M) served as control. Total cell lysates derived from GFP+ cells were separated by SDS-PAGE.

(A) Noxa, exogenous Mcl-1 and Bim protein levels were monitored by Western blotting in Ramos FSA, Mec-1 and Jurkat T cells (J16). Mcl-1 was c-myc tagged, which could be characterized with an anti-c-myc antibody. β-actin and a nonspecific band (*) served as a loading control.

(B) Ramos FSA (M) (black), N7 (gray) and N8 (white) were cultured 24 hrs in the absence or presence of 25 μM seliciclib, 100 μM fludarabine (fluda), 0.25 μM staurosporine (stauro), 5 μg/ml α-CD95 or α-BCR antibodies.

(C) Mec-1 (M) (black) and N8 (gray) were cultured 24 hrs in the absence or presence of 50 μM seliciclib, 0.25 μM staurosporine (stauro) and 5 μg/ml α-CD95.
Delay in Bax/Bak activation upon seliciclib treatment

Most forms of apoptosis ultimately require activation of Bax and/or Bak, which can be monitored with conformation-specific antibodies. Surprisingly, we observed that at 4 hrs after seliciclib treatment, when Mcl-1 degradation was almost complete as shown above, activation of Bax/Bak was still minimal (see Fig. 4, upper panel). At 12 hrs, Bax/Bak activation was still modest (middle panel). Only after 20 hrs substantial conformational changes of Bax/Bak occurred. At all timepoints the mitochondrial membrane potential as measured via MitoTracker staining mirrored the pattern of
Bax/Bak activation, and substantial damage occurred only at 20 hrs. These data were obtained in various B-CLL samples tested (n=3) and demonstrate that there was a substantial delay between Mcl-1 degradation and the actual onset of apoptosis. During this period, there were no significant changes in apoptosis-regulatory genes, such as p53-responsive Puma, as detected by RT-MLPA (data not shown).

Figure 4. Seliciclib induced cell death in B-CLL involves loss of the mitochondrial membrane potential and Bax/Bak conformational change. B-CLL cells were incubated in the absence (control, gray peak) or presence of 25 μM seliciclib and monitored every 4 hrs up to 24 hrs. Only 4, 12 and 20 hr time points are shown. Bax/Bak conformational changes were determined by intracellular flow cytometry using anti-Bax and anti-Bak monoclonal antibodies (solid black lines). For analysis of the mitochondrial membrane potential (ΔΨm), cells were incubated with MitoTracker Orange for 30 minutes.

Translocation of Bim, Bax and Bcl-2 to the mitochondria-rich insoluble fraction subsequent to Mcl-1 degradation
In order to elucidate events occurring between Mcl-1 degradation and the onset of apoptosis, we monitored candidate proteins to be potentially involved in this process. This was done by co-immunoprecipitation of CHAPS-soluble lysates taken 0, 4 and 16 hrs after seliciclib treatment. The data shown in figure 2 suggested early dissociation of Bim from Mcl-1. Since Bim can also bind Bcl-2 which is in general highly expressed in B-CLL, we studied Bim associations over time. Bim was bound to Bcl-2 at 0 hrs, and this association declined following prolonged seliciclib treatment (Fig. 5A, left panel). These findings were confirmed in the reverse experiment by immunoprecipitation of Bcl-2 (data not shown). Significantly, at 16 hrs of treatment, Bim, Bcl-2 and Bax were detected in the insoluble fraction of the cell (right panel). Moreover, we could not detect association between Bim and Bax or Bak (the latter is generally expressed at low levels in B-CLL cells) in viable nor in apoptotic cells. In accordance with published findings, we could not detect association between Bcl-2 and Noxa. The transition of Bim, Bax and Bcl-2 to the CHAPS-insoluble cell
fraction was confirmed by Western blotting of 4 additional B-CLL samples taken after 0, 4 and 16 hrs of seliciclib treatment (Fig. 5B). Together, these data demonstrate that Bim, Bcl-2 and Bax associations change during drug treatment, and this correlates with transition to an insoluble state. The mitochondrial membrane protein porin/VDAC2 was predominantly expressed in the ‘mitochondria rich’ insoluble fraction. Presumably, after release from Mcl-1 and subsequently Bcl-2, Bim is responsible for activation of Bax, although this is not detectable via direct interaction in the soluble cell fraction.

As Bax is translocated to the membrane fraction during seliciclib-induced apoptosis, we assessed its subcellular distribution using confocal microscopy. Figure 5C shows that Bax is localized throughout the cytoplasm in untreated B-CLL cells. Following 16 hrs seliciclib treatment (+ pretreatment with zVAD), Bax showed a punctate distribution and co-localization with the mitochondria, suggesting that Bax multimerizes at the mitochondria.

Figure 5. Bim associates with Bcl-2 and Mcl-1 and shifts to the mitochondria-rich insoluble fraction over time in B-CLL cells. See color figures.

B-CLL cells were treated with 25 μM seliciclib for 0, 4 and 16 hrs.

(A) Cell lysates were subjected to immunoprecipitation (IP) assays using a Bim antibody. The bound (IP) fraction (left panel) was analysed by Western blotting to evaluate the association of Bcl-2, Mcl-1, Bak and Bax with Bim over time. Total cell fractions (5% of total lysate; middle panel) and insoluble fractions (right panel) were monitored by Western blotting. The blotting membrane was hybridized with Bcl-2, Mcl-1, Bak, Bax, Bim, β-actin and Porin antibodies as indicated. (* Immunoglobulin heavy chain; ** Immunoglobulin light chain)

(B) CHAPS- soluble and -insoluble fractions from 4 other B-CLL patients were analysed by Western blotting to evaluate the localization of Bim, Bax and Bcl-2 over time.

(C) Immunocytochemical staining for Bax of B-CLL cells incubated in the absence or presence of 25 μM seliciclib for 24 hrs. MitoTracker Orange was used for localization of the mitochondria.
Bcl-2 confers a temporary block to selicilib-induced apoptosis

To directly determine the role of Bcl-2 in the delayed onset of selicilib-induced apoptosis, we used a Ramos model cell line overexpressing Bcl-2\(^1\). In parental Ramos cells treated with selicilib, Mcl-1 degradation (Fig. 6A; upper panel) occurred within 4 hrs, similar to B-CLL cells (Fig. 1B). The decline in Mcl-1 levels occurred independently of caspase activation, as demonstrated by overexpression of C9DN. In contrast to B-CLL, Mcl-1 degradation was almost simultaneous with apoptosis induction, shown by cleavage of effector caspase 3 (Fig. 6A; panel 3), and annexin-V exposure (Fig. 6B). Overexpression of Bcl-2 in Ramos cell lines recapitulated the delayed onset of apoptosis after selicilib treatment observed in B-CLL cells. Clearly, apoptosis was blocked after 4 hrs of treatment, whereas at 24 hrs virtually all cells were apoptotic, despite the presence of Bcl-2 (Fig. 6B). In comparison, time course analysis of Ramos cells with Noxa knock-down or Mcl-1 overexpression also showed that the block in selicilib induced apoptosis was not absolute. Here, the fraction of apoptotic cells increased more gradually over time up to the last point measured (48 hrs; Fig. 6C and 6D). The effect of selicilib on Mcl-1 produced from a recombinant vector may be less than on the endogenous transcript. However, the recently described phosphorylation of Mcl-1 at Ser64 which enhances its apoptotic activity is apparently also prevented by selicilib\(^2\), therefore a complete block of selicilib-induced apoptosis of overexpressed Mcl-1 is not expected. Together, our data indicate that manipulation of individual Bcl-2 family members is insufficient to warrant full protection to selicilib, which suggest they are subject to an intricate functional interplay.
Figure 6. Bcl-2 overexpression in Ramos cell line recapitulates delayed onset of apoptosis.
(A) Ramos 2G6 and Ramos FSA C9DN (DN9c) cells were treated with 25 μM seliciclib. At the indicated time points samples were analyzed by Western blotting for the presence of Mcl-1 (top panel), Noxa (second panel), activated caspase-3 (third panel) and β-actin (bottom panel) as loading control.
(B) Ramos 2G6 and Bcl-2 cells (5×10⁵/ml) were left untreated or treated with 50 μM seliciclib for 4 and 24 hrs.
(C) Ramos FSA and N8 cells (5×10⁵/ml) were left untreated or treated with 25 μM seliciclib for 4, 24 and 48 hr.
(D) Ramos FSA and Mcl-1 cells (5×10⁵/ml) were left untreated or treated with 25 μM seliciclib for 4, 24 and 48 hr. Viability in B, C and D was assessed by means of MitoTracker staining. Data represent mean ± SD from three independent experiments.
In hematological cells, the primary apoptotic trigger ascribed to the novel cyclin-dependent kinase inhibitor seliciclib is a rapid decrease in Mcl-1 levels independent of p53 function\textsuperscript{9,11}. This is of clinical importance in view of the poor prognosis of B-CLL with functional deficiency in the p53 pathway\textsuperscript{26} and the outgrowth of p53-deficient clones during treatment\textsuperscript{1}. Furthermore, there is broad interest in the exact mechanism of action of the various Bcl-2 family members, as it has become clear that their protective function can be targeted also directly by pharmacological compounds\textsuperscript{29}. However, in the absence of a functional link to Bax and/or Bak activation, drug-induced Mcl-1 decrease is in itself not sufficient to explain cell death.

Our findings emphasize two new aspects in the chain of events triggered in B-CLL cells upon seliciclib treatment and Mcl-1 degradation. We found previously that the BH3-only protein Noxa is expressed at high levels in B-CLL\textsuperscript{6}, and here we establish that the molecular effects of seliciclib rely on Noxa. Secondly, subsequent to Mcl-1 degradation, we observed a gradual deterioration of the protection afforded by Bcl-2, which was most probably caused by liberated Bim.

Biochemical studies have shown that distinct BH3-only proteins have differential binding affinities to pro-survival Bcl-2 proteins\textsuperscript{13,14,30}. Reportedly, Noxa has a strong preference for binding to Mcl-1 which we confirm here in primary B-CLL cells (Fig. 2). At present, the precise role of Noxa is not entirely understood, and our data point to a new and specific role in drug-induced cell death. This was demonstrated by a resistance to seliciclib in Noxa knock-down leukemic cell lines (Fig. 3). Difference in Noxa expression did not affect apoptosis by other stimuli (fludarabine, staurosporine, CD95 and BCR). Together, these observations imply that the lethal effect of seliciclib in B-CLL cells specifically involves Noxa function. In agreement with their broader interaction range, Mcl-1 overexpression or Bim knock-down had an inhibitory effect on multiple apoptotic stimuli.

Although Noxa levels are 5-10 times higher in B-CLL than in normal B cells\textsuperscript{6}, this is apparently in itself not a direct trigger for apoptosis, which fits well with the current view of its function as an indirect apoptosis mediator\textsuperscript{13,14}. The available evidence indicates that Noxa acts by displacing pro-apoptotic Bcl-2 members from Mcl-1\textsuperscript{24,30}. We have studied potential involvement of two pro-apoptotic candidates Bak and Bim in seliciclib-mediated apoptosis in B-CLL. In agreement with studies in HeLa cells\textsuperscript{24}, we found that Noxa can liberate Bak from Mcl-1 in leukemic cell lines (data not shown). However, we consistently could not detect Mcl-1 associated with Bak in primary B-CLL cells. Instead, we observed that the level of Bim associated with Mcl-1 rapidly declined upon seliciclib treatment, while Noxa remained bound (Fig. 2). This is consistent with
the differential binding affinities among the BH3-only members reported by others\textsuperscript{13,22}. Once liberated from Mcl-1, Bim is reportedly capable of directly activating Bax and/or Bak\textsuperscript{14,30}. Direct association between Bax or Bak with purported direct BH3-only activators such as Bid and Bim in model systems has been described\textsuperscript{31,32}. Yet, direct association of endogenous proteins under physiological conditions is difficult to observe. We were also unable in various co-immunoprecipitation approaches to find Bim complexed with Bax or Bak in early or late apoptotic B-CLL cells. The aspect of direct vs. indirect activation of Bax (or Bak) by BH3-only members such as Bim is currently the subject of intense research and debate\textsuperscript{19,30,33}. Although it is in fact still unknown what actually causes transition of cytosolic Bax\textsuperscript{34}, its activation is known to result in multimerization and pore formation in mitochondria\textsuperscript{35}. Therefore, we looked for shifts from soluble to insoluble fraction upon apoptosis initiation, and could observe transition of both Bim and Bax to the mitochondria-rich insoluble cell fraction (Fig. 5). Our findings in primary B-CLL cells suggest that any direct interaction between Bim and Bax is presumably short-lived, and/or induces rapid multimerization and insertion of Bax in the mitochondrial membrane. At this point the interacting proteins become insoluble and can no longer be detected by standard co-immunoprecipitation approaches. This possible sequence of events upon seliciclib treatment of B-CLL cells is schematically depicted in figure 7.

The proposed sequence of events from Mcl-1 degradation, Noxa-dependent Bim displacement and liberation, and finally Bax activation is compatible with recent data and interpretations concerning the hierarchical involvement of BH3-only members in initiation of the mitochondrial apoptosis pathway\textsuperscript{13,30}. Moreover, a functional linkage between Noxa and Bim was very recently reported in cell lines\textsuperscript{36}. During the period between Mcl-1 degradation and eventual Bax/Bak activation in B-CLL cells, there were no significant changes in gene transcription of Bcl-2 family members (data not shown). The available data suggest that after Mcl-1 degradation, Bcl-2 is temporarily capable of withstanding the pro-apoptotic action of Bim and Bax, but is progressively overwhelmed. In support of this, overexpression of Bcl-2 in a model system could indeed delay but not prevent apoptosis induced by seliciclib treatment (Fig. 6). The gradual undermining of Bcl-2 function could either be a direct effect of seliciclib by an as yet unknown pathway, or indirectly result from shifts in the distribution of and interactions between Bcl-2 family members.

The findings reported here agree with and complement recent findings concerning the mechanism of action of the BH3-mimetic ABT-737\textsuperscript{37,38}. This compound can counteract the protective function of Bcl-2, Bcl-X\textsubscript{L} and Bcl-w and has potential as a cancer therapeutic\textsuperscript{29}. Cells overexpressing Mcl-1 are however resistant to ABT-737, and strategies to target Mcl-1 levels enhance its efficacy\textsuperscript{37,38}. Based on our findings
with B-CLL cells reported here and elsewhere, both direct targeting of Mcl-1 by drugs such as seliciclib as well as indirect targeting by means of bortezomib to increase Noxa levels, may be a promising combination with ABT-737 or similar compounds. Indeed, it has been shown very recently that seliciclib treatment dramatically increased ABT-737 lethality in human leukemia cell lines. Overall, the functional cross-talk between Bcl-2 family members suggest that the ‘addiction’ of leukemic cells to overexpression of anti-apoptosis proteins such as Bcl-2 and Mcl-1 could be specifically targeted and exploited in a therapeutic setting.

Figure 7. Model for the steps in apoptosis triggered by seliciclib in B-CLL cells. In the initial phase (< 4hrs) Mcl-1 is degraded rapidly in a proteasome dependent manner. As Mcl-1 levels fall, Bim is released while Noxa remains bound. Liberated Bim can be absorbed by Bcl-2 for a while, but gradually there is a transition to a situation where Bcl-2 is no longer capable of binding excess Bim. What is actually causing this transition is unknown. Both disrupting the balance between Bcl-2 family members or unknown signaling events might eventually lead to Bcl-2 dysfunction. From that point on, Bim is able to activate Bax, leading to multimerization and apoptosis. As indicated in the main text, whether Bim activates Bax directly, indirectly or via transient interactions is currently the subject of controversy. Therefore, this scheme only emphasizes that all three direct participants (Bcl-2, Bim and Bax) shift to an insoluble fraction of the cell, which in the case of multimeric Bax is presumably mitochondrial.
Table 1. Patient’s characteristics.

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Of 24 patients the following characteristics were determined at time of inclusion in the study: sex (M= male, F= female); age (in years); Rai stage: 0 (good prognosis), I-II (intermediate prognosis), and III-IV (poor prognosis); IgV\(\text{\textsubscript{H}}\) mutation status (UM = unmutated IgV\(\text{\textsubscript{H}}\) genes, M = mutated IgV\(\text{\textsubscript{H}}\) genes) and treatment. ND = not determined
ACKNOWLEDGEMENT

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Chapter 2 • Seliciclib acts via the Mcl-1/Noxa axis

REFERENCE LIST


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