The microenvironment and treatment resistance in chronic lymphocytic leukemia
Tromp, J.M.

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Tipping the Noxa/Mcl-1 balance overcomes ABT-737 resistance in chronic lymphocytic leukemia


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Abstract

**Purpose:** Chronic lymphocytic leukemia cells in lymph nodes (LNs), from which relapses are postulated to originate, display an anti-apoptotic profile in contrast to CLL cells from peripheral blood (PB). The BH3 mimetic ABT-737 antagonizes the anti-apoptotic proteins Bcl-X\textsubscript{L} and Bcl-2, but not Mcl-1 or Bfl-1. Previously, it was shown that CD40-stimulated CLL cells were resistant to ABT-737. We aimed to define which anti-apoptotic proteins determine resistance to ABT-737 in CLL and whether combination of known anti-leukemia drugs and ABT-737 was able to induce apoptosis of CD40-stimulated CLL cells.

**Experimental Design:** To mimic the LN microenvironment, PB lymphocytes of CLL patients were cultured on feeder cells expressing CD40L and treated with ABT-737 with or without various drugs. In addition, we performed overexpression or knockdown of pro- and anti-apoptotic proteins in immortalized primary B cells.

**Results:** Upon CD40 stimulation patient-specific variations in ABT-737 sensitivity correlated with differences in levels of Mcl-1 and its antagonist Noxa. Knockdown of Noxa, as well as Mcl-1 overexpression, corroborated the importance of the Noxa/Mcl-1 ratio in determining the response to ABT-737. Inhibition of NF-κB resulted in increased Noxa levels and enhanced sensitivity to ABT-737. Interestingly, increasing the Noxa/Mcl-1 ratio, by decreasing Mcl-1 (dasatinib and roscovitine) or increasing Noxa levels (fludarabine and bortezomib), resulted in synergy with ABT-737.

**Conclusions:** Thus, the Noxa/Mcl-1 balance determines sensitivity to ABT-737 in CD40-stimulated CLL cells. These data provide a rationale to investigate the combination of drugs which enhance the Noxa/Mcl-1 balance with ABT-737 in order to eradicate CLL in chemoresistant niches.

**Translational Relevance:** Chronic lymphocytic leukemia (CLL) is an incurable disease despite the development of new treatment strategies. CLL cells originating from the lymph node (LN) microenvironment are postulated to be chemoresistant due to an upregulation of anti-apoptotic proteins such as Bcl-X\textsubscript{L}, Bcl-2, Mcl-1 and Bfl-1. In vitro, the LN microenvironment have been mimicked via CD40 stimulation of primary CLL cells. BH3 mimetics have been developed as a new class of anti-cancer drugs and antagonize the anti-apoptotic proteins Bcl-X\textsubscript{L} and Bcl-2, but not Mcl-1 or Bfl-1. This study shows that upon CD40-stimulation patient-specific variations in ABT-737 sensitivity correlated with differences in the relative levels of Mcl-1 and its antagonist Noxa. Increasing the Noxa/Mcl-1 ratio, with known anti-leukemia agents such as fludarabine and dasatinib, resulted in enhanced sensitivity to ABT-737. These data provide a rationale to investigate the combination of fludarabine or dasatinib with ABT-737 in a clinical setting as a novel treatment modality for refractory CLL.
Introduction

To date chronic lymphocytic leukemia (CLL) is an incurable disease despite new treatment strategies developed in the last decade. Prognosis is associated with mutation status of the immunoglobulin variable heavy chain (IGVH) genes and chromosomal aberrations such as 11q- and especially 17p- and mutations of p53 (1-3). The chemoresistant lymph node (LN) microenvironment is postulated as the source of relapses that invariably occur. In the LNs, CLL cells receive pro-survival signals which can result in microenvironment-induced resistance to cytotoxic agents (4-6). CLL cells originating from lymph nodes show an altered expression of apoptotic genes, high Bcl-X\textsubscript{L} and Mcl-1 and low Noxa levels, compared to CLL cells from peripheral blood (PB) (7, 8). CD40 stimulation of CLL cells also results in an upregulation of Bcl-X\textsubscript{L} and Mcl-1 and a downregulation of Noxa (8-11) and leads to resistance to various drugs (9, 12). Targeting anti-apoptotic proteins like Bcl-X\textsubscript{L}, Bcl-2, Mcl-1 and Bfl-1 may provide an important new therapeutic approach to overcome chemoresistance in the LN microenvironment.

BH3 mimetics have been developed as a new class of anti-cancer drugs. The BH3 mimetic ABT-737 is a potent small-molecule antagonist that binds with high affinity to the anti-apoptotic molecules Bcl-X\textsubscript{L}, Bcl-2 and Bcl-w, but not Mcl-1 or Bfl-1 (13). In various cell lines derived from solid tumors, it has been described that high levels of Mcl-1 and Bfl-1 result in resistance to ABT-737 (14-17). Furthermore, in many cancer cell lines Noxa can contribute to abrogation of ABT-737 resistance by antagonizing Mcl-1 (18-23). Noxa is considered as a weak inducer of apoptosis on its own, but appears to be crucial in inducing cell death by targeting Mcl-1 for proteasomal degradation (24, 25). In contrast to pro-apoptotic proteins like Bim, Bid and Puma, which can bind to all of the anti-apoptotic family members, Noxa is unique in binding specifically to Mcl-1 and Bfl-1 (24). Unexpectedly, for primary CLL it was reported that resistance to ABT-737 upon CD40-stimulation is mediated by Bcl-X\textsubscript{L} and Bfl-1 and is independent of Mcl-1 (26). Others have shown differences in ABT-737-sensitivity in unstimulated CLL cells, but no correlation between ABT-737 sensitivity and expression profiles of different pro- and anti-apoptotic proteins was observed (27). Thus, currently there is a discrepancy regarding the role of Mcl-1 in ABT-737 resistance in primary CLL cells versus cancer cell lines.

The aim of the current study was to investigate whether patient-specific variations in expression of apoptosis regulating proteins occur in CD40-stimulated CLL cells and to what extent they correlate with sensitivity or resistance to ABT-737. Second, we set out to identify pharmacological means to influence the expression of Bcl-2 family members in conjunction with ABT-737 treatment. We here demonstrate that the Noxa/Mcl-1 ratio strongly correlates with sensitivity to ABT-737 in CD40-stimulated CLL cells. In addition, we show that both the NF-κB and p38 MAPK signaling pathways are involved in regulating Noxa and Mcl-1 levels in CD40-stimulated CLL cells and can therefore modulate sensitivity for ABT-737. Furthermore, our data provide a rationale to combine...
the oral analog of ABT-737, ABT-263, with currently approved drugs that increase the Noxa/Mcl-1 ratio, such as dasatinib and fludarabine, in order to eradicate CLL cells in the protective LN microenvironment.

**Material and Methods**

**Patient material**
After informed consent patient material was obtained during diagnostic or follow-up procedures at the departments of Hematology and Pathology of the Academic Medical Center Amsterdam. This study was approved by the AMC Ethical Review Board (ERB) and conducted in agreement with the Helsinki Declaration of 1975, revised in 1983. Peripheral blood (PB) mononuclear cells of patients with CLL, obtained after Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, The Netherlands) were frozen and stored as described (9). Expression of CD5 and CD19 (both Beckton Dickinson (BD) Biosciences, San Jose, CA, USA) on leukemic cells was assessed by flow cytometry (FACScalibur, BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

**Flow cytometry**
CLL cells were washed and resuspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). For intracellular ZAP70 staining CLL cells were fixed and permeabilized (eBioscience, San Diego, CA) and subsequently stained for ZAP-70 (Alexa fluor 488, Invitrogen, clone 1E7.2) and PerCP-conjugated CD19 (Beckton Dickinson, San Jose, CA).

**Reagents**
Fludarabine (F-Ara-A), Roscovitine, Propidium Iodide (PI) and N-acetylcysteine (NAC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ABT-737 (13) was obtained under MTA from Abbott (Abbott Park, Illinois, USA, courtesy Dr. S. Rosenberg). Bortezomib was obtained from Janssen-Cilag (Tilburg, The Netherlands). Dasatinib was obtained from Novartis (Basel, Switzerland). A panel of chemicals was used to block different intracellular signaling pathways: BAY-11-7082, SB203580, SP600125, SB216763 and LY294002 (all from Calbiochem, Gibbstown, NJ, USA). The pan-caspase inhibitor Q-VD was purchased at R&D systems (Minneapolis, USA).

**Cell culture and detection of apoptosis**
Peripheral blood lymphocytes of CLL patients were stimulated by co-culture with NIH3T3 fibroblasts stably transfected with human CD40L, as described previously (9). After 24, 48 or 72 hrs of culture at 37°C, CLL cells were carefully detached by pipetting and subsequently incubated in medium with or without ABT-737 and/or additional drugs where indicated for an additional 24/48 hrs. When indicated, CLL cells were treated
with ABT-737 in the presence of CD40L-expressing feeder cells. Previous studies have shown that drug resistance lasts for 0 to 120 hrs after detachment of CLL cells from CD40L expressing fibroblasts (Hallaert, unpublished). CLL cells were preincubated with the different signaling inhibitors for 30 minutes (10 μM). BAY-11-7082 was used in a concentration of 1 μM. CLL cells were preincubated with Dasatinib (0.1-10μM) for 30 minutes. The pan-caspase inhibitor Q-VD was used in a concentration of 12.5 μM and preincubated for 30 minutes. The Reactive Oxygen Species (ROS) scavenger NAC was used in a concentration of 5 mM and added 30 minutes prior to fludarabine. Cells were treated with 1-100μM fludarabine (48 hrs), 30nM bortezomib (24 hrs), 25μM roscovitine (24 hrs) and 0.001-10 μM ABT-737 (24 hrs) and stained with 200 nM MitoTracker Orange (Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37°C and analyzed by FACS (9). Additionally, apoptotic and viable cells were discriminated via flow cytometry of cells stained with 200 nM MitoTracker Orange (Molecular Probes, Leiden, The Netherlands) and propidium iodine (PI; Sigma, St Louis, MO) as described (28). Specific apoptosis is defined as % cell death in ABT-737 treated cells - % cell death in medium control. The concentration of ABT-737 to induce 50% apoptosis (EC50) was calculated by nonlinear regression analysis by fitting a sigmoidal dose-response curve to ABT-737-induced apoptosis using the Graphpad Prism software (Graphpad Prism 5.0, La Jolla, USA).

Western Blot and antibodies
Western blotting was performed as described previously (9). Blots were probed with polyclonal anti-Mcl-1 (catalog #4572 Cell Signaling), monoclonal anti-Noxa (catalog #IMG-349A Imgenex), polyclonal anti-Bcl-2 (ADI-AAS-070-E Alexis), polyclonal anti-Bcl-XL (catalog #620211, BD Biosciences), antiserum to β-actin (clone I-19; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal antibodies against A1/Bfl-1 were a kind gift of Prof. Dr. J. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). IRDye 680 donkey anti-rabbit IgG, IRDye 800 donkey anti-goat IgG or IRDye 800 donkey anti-mouse IgG (Westburg, Leusden, the Netherlands) were used as secondary antibodies. Western blots were scanned on the Odyssey imager (LI-COR Biosciences, Lincoln, NE), and subjected to densitometry with the Odyssey software (Odyssey Application software version 3.0). Per sample relative expression compared to β-actin is reported.

Retroviral Transduction
Transduction of memory B cells was performed as described by Kwakkenbos et al (29). In short, CD27+ memory B cells were isolated from a buffy coat from a healthy donor and retrovirally transduced with Bcl-6 linked to a truncated form of the nerve growth factor receptor (ΔNGFR) and an anti-apoptotic protein of interest or Noxa RNAi linked to GFP. Transduced cells were cultured on a feeder layer of mouse L fibroblasts stably transfected with CD40L, in the presence of IL-21. Double transduced B cells were sorted based on
co-expression of ΔNGFR and GFP using the FACS Aria cell sorter (BD Biosciences). All experiments were performed using sorted double transduced cells that had been kept in culture in presence of CD40L+ L cells and IL-21.

**Noxa RT-PCR**
Primary CLL cells were treated with 0.1 μM ABT-737, 10 μM fludarabine, or ABT-737 and fludarabine. After 48 hours RNA was isolated with the ‘GenElute Mammalian Total RNA Miniprep Kit’ (Sigma-Aldrich) according to the manufacturer’s protocol. A Noxa Reverse-Transcription PCR (Nterminus CTC TCG AGC CCG GGA GAA AGG CGC and Cterminus GGGAATTCTCAGGTACTAAATTGAAGAGCT) and an 18S RNA (loading control) RT-PCR was performed. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

**siRNA and nucleofection**
CLL cells were transfected using the Amaxa nucleofection technology (Amaxa, Koln, Germany), according to the manufactures’ recommendations and as described previously (8). siRNA (Noxa Cat#4392420 and Silencer Select Negative Control#1 Cat#4390843) was obtained from Ambion and 1.5-3 μg was used for the transfection experiments. Cell suspensions mixed with siRNA were transferred to the provided cuvette and nucleofected with an Amaxa Nucleofector apparatus using program X-05. Thereafter cells were immediately transferred into pre warmed 6 –well and cultured for 24 hours before protein lysates were obtained.

**Statistics and calculation of synergistic and additive effects**
The Shapiro-Wilk normality test was performed to analyze Gaussian distributions. If there was a Gaussian distribution a two-sided t test was used to analyze differences between the groups. If there was no Gaussian distribution, a two-tailed Mann-Whitney U test was used to analyze differences between the groups and a Wilcoxon matched paired test to analyze differences between paired samples. Statistically significance of the data was set at P < 0.05, with one asterisk (*) representing 0.01 < P < 0.05; two asterisk (**) 0.001 < P < 0.01; three asterisk (***) P<0.001. To assess synergistic and additive effects, drug interactions were analyzed as described before (30, 31). In short, observed survival corrected for baseline apoptosis of the sample is plotted against expected survival, calculated from the fraction of surviving cells of samples treated with the individual drugs and ABT-737 (Expected survival = survival drug x survival ABT-737). The diagonal line (XY line) represents the situation in which observed survival = predicted survival. Dots beneath this line indicate synergistic interactions (as observed survival < expected survival). Dots above the XY line represent additive interactions (observed survival > expected survival, but < survival of most active single drug (D_{max})).
Results

Sensitivity to ABT-737 is associated with a high Noxa/Mcl-1 ratio in CD40-stimulated CLL samples

ABT-737 is a potent inducer of cell death in CLL cells derived from peripheral blood (PB) (9, 26, 27, 32) (Figure 1A, left panel). To mimic the LN microenvironment, CLL cells from PB were cultured in vitro on feeder cells expressing human CD40L. In line with earlier studies (9, 26), CD40 stimulation of CLL cells resulted in strong resistance to ABT-737 in the majority of CLL samples (n=23) (Figure 1A, right panel, solid lines).

Figure 1. Time course analysis of pro- and anti-apoptotic proteins in ABT-737 resistant versus ABT-737 sensitive CLL cells upon CD40-stimulation. A. CLL cells were stimulated with 3T3 (n=13; left panel) or CD40L-expressing 3T3 cells (n=28) for 72 hrs (right panel). After detachment, cells were incubated with different concentrations of ABT-737 (0.001μM- 10μM) and analyzed for apoptosis by MitoTracker staining after 24 hrs. A heterogeneous response to ABT-737 was observed in CLL cells stimulated with CD40L; the majority (n=23) was resistant to ABT-737 (right panel, solid lines), whereas a minority (n=5) was shown to be sensitive to ABT-737 (right panel, dotted lines). CD40-stimulated CLL cells which were sensitive to ABT-737 are labeled according to the numbers in the table. B. CLL cells were stimulated with CD40L for 24, 48 and 72 hrs. After detachment cells were treated with 0.1μM ABT-737 for 24 hrs. The number of patient samples analyzed was 7 ABT-737 resistant CLL samples and 5 ABT-737 sensitive CLL samples. C. CLL cells were stimulated with CD40L for the indicated time (0, 24, 48 and 72 hrs). Protein lysates were probed for Mcl-1, Noxa, Bfl-1, Bcl-XL, Bcl-2 levels and β-actin as loading control. Blots from one representative ABT-737 resistant and one ABT-737 sensitive CLL sample is shown. D. Western blots of Noxa and Mcl-1 were quantified with Odyssey software in both ABT-737 sensitive (n=4) and ABT-737 resistant (n=3) CD40-stimulated CLL cells. Bars represent the mean ± SEM. * .01 < P < .05, ** .001 < P < .01.
### Table 1. Patient characteristics.

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1Percentage of cells positive for CD5/CD19 surface expression measured by facs analysis.
2Mutated IgVH gene (+) denotes >2% mutations compared to germline sequence.
3As determined by FISH. Probes for 11q22.3 (ATM), centromere 12 (CEP12), 13q14 (D13S319), 14q32 (IGH) and 17p13 (TP53) were obtained from Abbot-Vysis. Samples with > 10% aberrant signals were considered abnormal. ND = not determined
4Clb = chlorambucil, ClbP = chlorambucil+prednisone, F = fludarabine, FCR = fludarabine, cyclophosphamide, rituximab, FCO = fludarabine, cyclophosphamide, ofatumumab, P = prednisone, RCHOP = rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone, RDHAP = rituximab, fludarabine, endoxan, alemtuzumab
5P53 functional status was measured via radiation-induced RNA expression of p53 target genes Puma and Bax as described previously F=p53 Functional.
6For intracellular ZAP70 staining CLL cells were fixed and permeabilized (eBioscience, San Diego, CA) and subsequently stained for ZAP-70 (Alexa fluor 488, Invitrogen, clone 1E7.2), CD3 and CD19.
Interestingly, we consistently observed that a small subset of CD40-stimulated CLL samples remained relatively sensitive to ABT-737 (n=5) (Figure 1A, right panel, dotted lines). For each patient, this was a reproducible phenomenon observed in at least two independent experiments, as well as in two different samples. ABT-737 sensitive CLL cells were defined as those showing ≥50% apoptosis when treated with 0.1 μM ABT-737 when compared to CLL cells stimulated with CD40L only. The EC50 calculated for ABT-737 “sensitive” CLL cells was 10-fold lower (0.07 ± 0.007 μM) than for ABT-737 “resistant” CLL cells (0.7 ± 0.003 μM) (p=0.0001). In these experiments, CLL cells were removed from CD40L-expressing feeder cells prior to ABT-737 treatment, which might affect the outcome. However, control experiments where CLL cells remained in contact with the feeder cells during ABT-737 exposure, demonstrated similar levels of apoptosis and the distinction between sensitive and resistant CLL cells remained (Supplemental Figure 1).

The characteristics of ABT-737 sensitivity in CD40-stimulated CLL cells were investigated with respect to differentially expressed pro- or anti-apoptotic proteins in time-course experiments. After 24 hours of CD40 stimulation no differences in ABT-737 sensitivity were observed (Figure 1B). Notably, 48 hrs and 72 hrs of CD40 stimulation resulted in a significant difference in apoptosis induced by ABT-737 between the two subgroups (48 hrs p=0.0025; 72 hrs p=0.0025) (Figure 1B). These differences in ABT-737 sensitivity in CLL cells could not be related to prognostic factors such as mutation status, chromosomal aberrations, p53 function and ZAP70 expression (Table 1). Notably, in ABT-737 sensitive CLL cells Noxa levels remained high during the course of CD40 stimulation whereas in ABT-737 resistant CLL cells Noxa levels declined after 24 hours of CD40 stimulation (Figure 1C). Both patient types showed gradually declining Mcl-1 levels after 24 hours of CD40-stimulation. No differences in Bcl-XL, Bcl-2 and Puma levels were observed in ABT-737 resistant versus ABT-737 sensitive CLL cells (Figure 1C, and data not shown). In addition, Bfl-1 showed a peak in expression at 48 hrs after CD40 stimulation in both subgroups (Figure 1C and Supplemental Figure 2). Densitometric analysis of various patient samples was performed for expression of prosurvival Bcl-2 family members versus Noxa. A significantly higher Noxa/Mcl-1 ratio in ABT-737 sensitive compared to resistant CLL cells at 72 hrs of CD40 stimulation was observed (Figure 1D). The results presented here did not show a correlation between Noxa/Bfl-1 or Noxa/Bcl-XL balance and ABT-737 sensitivity over time. In conclusion, the observed changes in Noxa expression and variation in the Noxa/Mcl-1 ratio correlated with increased sensitivity to ABT-737 in CD40-stimulated CLL cells.

Knockdown of Noxa, or overexpression of Mcl-1 and Bfl-1, results in resistance to ABT-737

Previous studies have shown that the Noxa/Mcl-1 balance plays an important role in ABT-737 sensitivity in various cancer cell lines (18-23). To confirm whether Noxa and Mcl-1 levels affected sensitivity to ABT-737 also in primary CLL cells, nucleofection
with siRNA against Noxa and Mcl-1 was performed. Unfortunately, these studies were hampered by off-target effects of various specific and control siRNAs especially on the Mcl-1 protein level. For example, CLL cells treated with siRNAs designed to target Noxa showed specific knockdown of Noxa but also Mcl-1 levels were decreased, resulting in an unaltered Noxa/Mcl-1 ratio (Supplemental Figure 3). Moreover, siRNA for Bcl-XL and Bfl-1 also non-specifically decreased Mcl-1 levels (data not shown). This precluded reliable interpretation of the contribution of the various Bcl-2 members in determining sensitivity for ABT-737. Since in our hands overexpression or knockdown of proteins using retroviral transduction have not yielded consistent results in primary CLL cells, we used primary human B cells to address the role of Noxa and other Bcl-2 members in ABT-737 sensitivity. Peripheral blood derived CD27+ memory B cells were immortalized by retroviral transduction with Bcl-6 in combination with anti-apoptotic proteins Mcl-1, Bfl-1, Bcl-XL or Bcl-2, or combined with Noxa knockdown.

Figure 2. Immortalized primary B cells with overexpression of Mcl-1 and Bfl-1 or knockdown of Noxa are resistant to ABT-737. A. Primary human memory B cells were immortalized by overexpressing Bcl-XL, Bcl-2, Bfl-1 and Mcl-1 or knockdown of Noxa as described in the Materials and Methods. Protein lysates were probed for Bcl-XL, Bcl-2, Bfl-1, Noxa, Mcl-1 and β-actin. Immortalized primary B cells were incubated with different concentrations of ABT-737 as indicated and analyzed for apoptosis by Annexin V APC/PI staining after 24 hrs. Specific apoptosis was calculated as described in Materials and Methods. Bars represent the mean ± SEM. * .01 < P < .05; ** .001 < P < .01. C. Primary human memory B cells were immortalized by overexpressing Bcl-XL, Bcl-2, Bfl-1 and Mcl-1 or knockdown of Noxa. Immortalized primary B cells were incubated with 25 µM roscovitine as indicated and analyzed for apoptosis by Annexin V APC/PI staining after 24 hrs. Specific apoptosis was calculated as described in Materials and Methods. Averaged results from six experiments are presented. Bars represent the mean ± SEM. ** .001 < P < .01.
Overexpression or knockdown was confirmed by Western blot analysis for Bcl-X\(_L\), Bcl-2, Bfl-1, Noxa and Mcl-1 (Figure 2A). Interestingly, lower levels of Noxa were observed in B-cells with Mcl-1 overexpression, but not in B-cells which overexpressed Bfl-1, suggesting that Noxa and Mcl-1 protein levels are (inversely) correlated, in agreement with previous reports (24). Of note, memory B-cells which were transfected only with Bcl-6 without overexpression of an anti-apoptotic protein did not remain viable and could not be studied. Overexpression of Mcl-1 or Bfl-1, as well as Noxa knockdown afforded protection against apoptosis induction by ABT-737, while overexpression of Bcl-X\(_L\) and Bcl-2 hardly affected sensitivity (Figure 2B). In contrast, when these B cell lines were incubated with roscovitine, a cyclin dependent kinase inhibitor resulting in Mcl-1 and Bfl-1 degradation, we observed significant higher apoptosis levels in cells overexpressing Mcl-1, Bfl-1 and B cells with low Noxa levels as compared to Bcl-X\(_L\) and Bcl-2 transfected B cell lines (Figure 2C).

Together, these results underscore the specific role for the Noxa/Mcl-1 balance in determining resistance to ABT-737.

**NF-κB and p38 MAPK signaling pathways are involved in the regulation of Noxa levels in CLL**

A role for p38 mitogen-activated protein kinase (MAPK) signaling in Noxa regulation has been described in various types of cancer (33, 34). Therefore, we investigated whether p38 MAPK signaling, or other signaling components known to be involved in regulating survival, such as NF-κB, JNK, Gsk-3 and PI3K, may play a role in the regulation of Noxa and/or Mcl-1 in CLL cells upon CD40 stimulation. Inhibition of p38 MAPK activity by the pharmacological inhibitor SB203580 diminished Noxa levels, but also resulted in decreased Mcl-1 levels (Figure 3A). Therefore, no significant alteration of the Noxa/Mcl-1 ratio in CD40-stimulated CLL cells was observed upon p38 inhibition (Figure 3B). Combination of SB203580 with ABT-737 resulted in a modest increase in apoptosis (Figure 3C). Calculations of the combined effects (30, 31) indicated additive effects on apoptosis when SB203580 (1 μM and 10 μM) was combined with 0.1 μM ABT-737 (Figure 3D). In CD40-stimulated CLL cells sensitive for ABT-737 p-p38 levels were similar compared to p-p38 levels observed in ABT-737 resistant CLL cells (data not shown), indicating that other signaling pathways than p38 MAPK signaling are involved in the differential regulation of Noxa in ABT-737 resistant versus ABT-737 sensitive CD40-stimulated CLL cells.

Interestingly, inhibition of NF-κB via BAY-11-7082 in CD40-stimulated CLL cells resulted in a clear upregulation of Noxa levels in both ABT-737 sensitive and ABT-737 resistant CLL cells (Figure 3A and data not shown). A significant alteration of the Noxa/Mcl-1 ratio and increased sensitivity to ABT-737 in CD40-stimulated CLL cells was observed upon NF-κB inhibition (Figure 3E and 3F). In addition, synergistic apoptotic effects were observed when 1μM BAY-11-7082 was combined with 0.1μM of ABT-737 (Figure 3G).
Figure 3. Noxa and Mcl-1 levels are regulated by p38 MAPK signaling pathway in CD40-stimulated CLL cells. 

A. CLL cells were stimulated with CD40L for 24 hrs in the presence or absence of a panel of signaling inhibitors as indicated. Dasatinib, NF-κB inhibitor (BAY-11-7082), p38 inhibitor (SB203580), JNK inhibitor (SP600125), Gsk-3 inhibitor (SB216763) and the PI3K inhibitor (LY294002) were used in a concentration of 10μM. Protein lysates were probed for Noxa, Mcl-1 and β-actin. Blots from one representative CLL sample shown, of a total of four analyzed. 

B. Western blots of Noxa and Mcl-1 were quantified with Odyssey software from four CD40-stimulated CLL cells. The Noxa/Mcl-1 ratio in CLL cells stimulated with CD40L in the presence of the p38 inhibitor was related to the Noxa/Mcl-1 ratio in CLL cells stimulated with CD40L alone, which was set at 1 for each individual patient. Bars represent the mean ± SEM. 

C. CLL cells were stimulated with CD40L for 24 hours (n=5) in the presence or absence of different concentrations of p38 inhibitor SB203580 (0.1 μM, 1μM and 10μM). After detachment, cells were incubated for 24 hours with indicated concentrations of ABT-737 (0.001μM- 10μM). 

D. Synergistic/additive effects of ABT-737 (0.1 μM) and the NF-κB inhibitor, BAY-11-7082 (open circles 0.5 μM, solid circles 1 μM) in CD40-stimulated CLL cells.
Together, these data further underline the importance of the role of the Noxa/Mcl-1 balance in ABT-737 sensitivity in CD40-stimulated CLL cells.

Drugs that increase the Noxa/Mcl-1 ratio synergize with ABT-737

Next, we investigated whether known anti-leukemia drugs which increase the Noxa/Mcl-1 ratio resulted in enhanced sensitivity to ABT-737. Previously, we have shown that treatment with dasatinib caused a clear decline in Mcl-1 levels in CD40-stimulated CLL cells (9), and this was confirmed in Figure 4A. A modest decline in Bfl-1 levels was observed and Bcl-X\textsubscript{L} levels remained equal when CD40-stimulated CLL cells were treated with dasatinib (Figure 4A, left panel). Densitometric analysis showed a significant increase in the Noxa/Mcl-1 ratio upon dasatinib treatment (Figure 4A, right panel). We next investigated whether concurrent treatment with dasatinib would sensitize CD40-stimulated CLL cells to ABT-737. Notably, 10μM of dasatinib resulted in 100-fold increased ABT-737 sensitivity in CD40-stimulated CLL cells and synergy between ABT-737 and dasatinib (Figure 4B, C). Lower concentrations of dasatinib (1μM) in combination with simultaneous CD40 stimulation and sequential treatment of 0.1μM of ABT-737 resulted in additive effects (Figure 4C). Next, we investigated whether other drugs, such as bortezomib and roscovitine, which are known to modulate the Noxa/Mcl-1 balance, resulted in synergistic effects with ABT-737. As expected, both roscovitine and bortezomib treatment resulted in an enhanced Noxa/Mcl-1 ratio in CD40-stimulated CLL cells and in synergistic apoptotic effects when combined with ABT-737 (Figure 4D, 4E and 4F).

Fludarabine is a well known anti-leukemia drug which is often used in the treatment of CLL. Therefore, we investigated whether fludarabine enhanced the Noxa/Mcl-1 ratio in CD40-stimulated CLL cells. Treatment of CLL cells with fludarabine resulted in upregulation of Noxa levels at both mRNA and protein level (Figure 5A and 5B). Although high doses of fludarabine (100μM) resulted in a reactive oxygen species (ROS) dependent upregulation of Noxa (31) as shown by abrogation of Noxa induction by the addition of the ROS scavenger N-acetyl cysteine (NAC), Noxa upregulation at a lower concentration of fludarabine (10μM) was independent of ROS (Supplemental Figure 5). A minor decrease in Mcl-1 levels was observed in fludarabine-treated CLL cells, but this was prevented by adding the pan-caspase inhibitor QVD, suggesting that the Mcl-1 degradation was caspase-dependent (Figure 5B). ABT-737 treatment as a single agent did not alter Noxa expression at mRNA (Figure 5A) or protein level (Supplemental Figure 5). Densitometric analysis of Western blots of CD40-stimulated CLL cells treated with fludarabine revealed a significantly higher Noxa/Mcl-1 ratio compared to controls (Figure 5C). Importantly, addition of fludarabine resulted in a dose-dependent enhancement of ABT-737 sensitivity in CD40-stimulated CLL cells (Figure 5D). Synergistic effects were observed with 10 μM and 100μM of fludarabine in combination with 0.1μM ABT-737 in CD40-stimulated CLL cells (Figure 5E).
Figure 4. Dasatinib induces an increased Noxa/Mcl-1 ratio and sensitizes CD40-stimulated CLL cells to ABT-737. A. Left panel: CLL cells were stimulated with CD40L in the presence and absence of dasatinib. Protein lysates were probed for Mcl-1, Noxa, Bfl-1, Bcl-X<sub>L</sub> and β-actin. Blots from one representative CLL sample shown, of a total of four analyzed. Right panel: Densitometric analysis of the Noxa/Mcl-1 ratio of CD40-stimulated CLL cells ± dasatinib is shown (n=4). The Noxa/Mcl-1 ratio in CLL cells stimulated with CD40L in the presence of dasatinib was related to the Noxa/Mcl-1 ratio in CLL cells stimulated with CD40L alone, which was set at 1 for each individual patient. Bars represent the mean ± SEM. * .01 < P < .05. B. CLL cells were stimulated with CD40L for 24 hours (n=6) in the presence or absence of different concentrations of dasatinib (0.1 μM, 1μM and 10μM). As a control CLL cells were stimulated on 3T3 cells without the expression of CD40L. After detachment, CLL cells were incubated with different concentrations of ABT-737 (0.01μM, 0.1μM, 1μM and 10μM) and analyzed for apoptosis by MitoTracker staining after 24 hrs. C. Synergistic or additive effects of ABT-737 (0.1 μM) and dasatinib 1 μM (open circles) and 10 μM (solid circles) were assessed as described in materials and methods. The diagonal line (XY line) represents the situation in which observed survival = predicted survival. Dots beneath this line indicate synergistic interactions and dots above the XY line represent additive interactions. D. CLL cells were stimulated for 72 hours with CD40L, after detachment CLL cells were treated with 25 μM roscovitine or 30 nM bortezomib for 24 hours. Protein lysates were probed for Noxa, Mcl-1 and β-actin. Synergistic and additive apoptotic effects were assessed of ABT-737 and roscovitine E. and ABT-737 and bortezomib F. in CD40-stimulated CLL cells.
Collectively, these data show that altering the Noxa/Mcl-1 ratio, either by targeting Mcl-1 levels or increasing Noxa levels, results in increased sensitivity of CD40-stimulated CLL cells to ABT-737.

**Discussion**

The present study demonstrates that the Noxa/Mcl-1 ratio is crucial in determining ABT-737 sensitivity in CD40-stimulated CLL cells. Previously, we and others have shown that CD40 triggering of CLL cells reduces sensitivity for ABT-737 100-1000 fold (9, 26). However, we here report that a minority of CD40-stimulated CLL cells retained sensitive
to ABT-737. This was associated with high Noxa protein levels. We used this property to establish that the Noxa/Mcl-1 ratio is important in determining the response to ABT-737. As expected, either decreasing Mcl-1 levels with dasatinib and roscovitine or increasing Noxa levels with bortezomib and fludarabine resulted in synergistic effects with ABT-737 in CD40-stimulated CLL cells. Together these data demonstrate the importance of the Noxa/Mcl-1 balance in determining ABT-737 sensitivity, and suggest ways to manipulate this therapeutically to counteract the protective microenvironment of CLL patients.

In previous studies using cell lines and lymphoma mouse models, Mcl-1 has been shown to play an important role in ABT-737 resistance (14-16, 35). Yet, in primary CLL cells a role for Mcl-1 in ABT-737 resistance has not been confirmed until now. Instead, Bcl-X<sub>L</sub> and Bfl-1 were pinpointed as determinants for ABT-737 sensitivity in CD40-stimulated CLL cells (26). We argue that this apparent discrepancy is due to differences in interpretation of data, not in actual inconsistencies or differences in experimental systems. First, the conclusion of Vogler et al that Mcl-1 does not contribute to resistance to ABT-737 is partially based on experiments in which CLL cells were stimulated with IL-4 and IFN-γ. This enhances Mcl-1, and in fact modestly increases resistance to ABT-737 (26). In light of our data, we argue that IL-4 and/or IFN-γ might affect pro-apoptotic factors, e.g. Noxa, that could counterbalance the effects of Mcl-1 upregulation. Second, due to technical difficulties, our experiments using a siRNA approach towards Mcl-1 in CLL were not conclusive, because non-specific and/or off-target effects also affected Mcl-1 levels, which precluded proper interpretation of the data. Therefore, we chose to overexpress various Bcl-2 members and to silence Noxa in immortalized primary human B cells and these experiments firmly established a role for the Noxa/Mcl-1 balance in ABT-737-mediated apoptosis. Bfl-1 overexpression in immortalized human B cells also induced resistance to ABT-737, however, in contrast to Mcl-1 and Noxa, no significant differences in Bfl-1 levels were observed in the ABT-737 resistant versus sensitive CLL cells upon CD40 stimulation (Figure 1C).

Our data are in line with previous studies using various cancer cell lines, which demonstrate that alterations in the Noxa/Mcl-1 ratio determine sensitivity to ABT-737 (18-20, 22, 23). Yecies et al. investigated the potential mechanisms of ABT-737 resistance in lymphoma cell lines (17) and showed that resistance to ABT-737 was induced after long term exposure to low doses of ABT-737. Increased levels of Mcl-1 alone, or in conjunction with Bfl-1, were observed in resistant lymphoma cell lines. In agreement with this study, we observed that, upon ABT-737 treatment, Mcl-1 levels were significantly increased in CD40-stimulated CLL cells (Supplemental Figure 4). Basal levels of Noxa after 72 hrs of CD40-stimulation were very low or absent and did not alter in the presence of ABT-737. An increase of Mcl-1 upon ABT-737 treatment could further explain resistance to ABT-737, although the mechanism of this apparent feedback is as yet unknown. Notably, we did
not observe an increase in Bfl-1 levels upon ABT-737 treatment in CD40-stimulated CLL cells (Supplemental Figure 4).

Various treatments in cancer cell lines including bortezomib, UVB irradiation and cisplatin have been reported to induce Noxa by activation of the p38 MAPK signaling pathway in a p53 dependent, as well as a p53 independent, manner (33, 34, 36). Our data with CD40-stimulated CLL cells also suggest a role for p38 MAPK signaling in Noxa regulation. However, both Noxa and Mcl-1 levels declined in the presence of the p38 inhibitor SB203580. This did not tip the Noxa/Mcl-1 balance in CD40-stimulated CLL cells sufficiently and therefore did not show synergistic apoptotic effects with ABT-737. Interestingly, we observed increased Noxa levels when the NF-κB pathway was inhibited, resulting in an enhanced Noxa/Mcl-1 balance and in synergistic apoptotic effects when combined with ABT-737. The mechanism by which the NF-κB pathway regulates Noxa levels in CD40-stimulated CLL cells is currently unknown.

Recently, Scielzo and collaborators showed a dichotomy in responsiveness to soluble CD40L in CLL cells in vitro (37). CLL cells which did not respond to soluble CD40L with upregulation of Mcl-1 and Bcl-2 showed a worse clinical outcome compared to CLL cells which responded to CD40 ligation. CD40-responsiveness was correlated with ZAP70 expression. We investigated whether ZAP70 expression was correlated with ABT-737 sensitivity, but no significant difference in percentage of ZAP70 positive cells was observed between ABT-737 sensitive (23.1% ± 26.6%; mean ± SD) and ABT-737 resistant CLL cells (33.7% ± 25.6%; mean ± SD) (p=0.3) (Table 1).

In our experiments dasatinib was present throughout stimulation with CD40L and showed synergy with ABT-737. This might seem counterintuitive in view of clinical application, since in the LN environment resident CLL cells are supposedly receiving CD40 stimulation prior to treatment with dasatinib. However, the number of (CD40L-expressing) T cells is much lower compared to the number of CLL cells in proliferation centers (7, 38) and CLL cells proliferate. Therefore, it can be expected that a continuous flux of CLL cells will exit the LN and new cells will arise which will be sensitized by dasatinib. Thus, we argue that the actual situation in LN is at least partially mimicked by simultaneous exposure in vitro.

Finally, p53 mutations are frequently observed in CLL and might affect induction of Noxa, and/or responses to combination regimens containing fludarabine. In preliminary experiments we observed that p53 dysfunctional CLL cells in fact also showed enhanced cell death when fludarabine and ABT-737 where combined (data not shown). In this limited sample set, no obvious correlation with Noxa or Mcl-1 expression was found, suggesting involvement of non-p53 pathways or additional sensitizing factor(s) for cell
death induced by ABT-737, and emphasizing that further studies are needed to investigate this important aspect.

In conclusion, we here show that the Noxa/Mcl-1 balance plays an important role in resistance to ABT-737 in CD40-stimulated CLL cells. In general, combining cytotoxic drugs, which influence the Noxa/Mcl-1 balance, with ABT-263 seems to be a promising therapy in the treatment of CLL by inducing apoptosis of CLL cells in the protective LN microenvironment.

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Reference List


**Supplemental Figure 1.** A. ABT-737 resistant CLL cells were stimulated with CD40L for 72 hours (n=4). The black line shows apoptosis levels from CLL cells detached from the feeder layer and subsequently treated with different concentrations of ABT-737 (0.001-10 μM). CLL cells treated with ABT-737 in the presence of CD40-expressing feeder cells are depicted in gray. B. ABT-737 sensitive CD40-stimulated CLL cells treated with ABT-737 after detachment from feeder cells (black line) and while continuously in contact with CD40-expressing feeder cells (gray line) (n=5).

**Supplemental Figure 2.** CLL cells were stimulated with CD40L for the indicated time (24, 48 and 72 hrs). Western blots of Bfl-1 were quantified with Odyssey software in both ABT-737 sensitive (n=4) and ABT-737 resistant (n=4) CD40-stimulated CLL cells. Bars represent the mean ± SEM.

**Supplemental Figure 3.** Nucleofection with Noxa siRNA or control siRNA was performed on CLL cells in the presence or absence of the proteasome inhibitor GSI. Protein lysates were probed for Mcl-1, Noxa and β-actin. As a control, lysates of untransfected CLL cells in the presence or absence of GSI are shown.
Supplemental Figure 4. A. CLL cells were stimulated with CD40L for 72 hrs. After detachment, cells were incubated with 0.1 μM of ABT-737. Protein lysates were probed for Mcl-1, Bfl-1, Noxa and β-actin. B. Densitometric analysis of Mcl-1/β-actin levels of fourteen CLL samples is shown. Bars represent the mean ± SEM, *** P < .001.

Supplemental Figure 5. CLL cells were stimulated with CD40L for 48 hrs. After detachment, cells were incubated with 10 μM or 100 μM fludarabine ± Q-VD (12.5 μM), ± NAC (5mM). Protein lysates were probed for Noxa, Mcl-1 and β-actin.