Tipping the Noxa/Mcl-1 Balance Overcomes ABT-737 Resistance in Chronic Lymphocytic Leukemia

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Abstract

Purpose: Chronic lymphocytic leukemia (CLL) cells in lymph nodes (LN), from which relapses are postulated to originate, display an antiapoptotic profile in contrast to CLL cells from peripheral blood (PB). The BH3 mimetic ABT-737 antagonizes the antiapoptotic proteins Bcl-XL 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 antiapoptotic proteins determine resistance to ABT-737 in CLL and whether combination of known antileukemia 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 carried out overexpression or knockdown of pro- and antiapoptotic 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-kB 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 to eradicate CLL in chemoresistant niches.

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 prosurvival signals which can result in microenvironment-induced resistance to cytotoxic agents (4–6). CLL cells originating from LNs show an altered expression of apoptotic genes, high Bcl-XL and Mcl-1 and low Noxa levels, compared with CLL cells from peripheral blood (PB; refs. 7, 8). CD40 stimulation of CLL cells also results in an upregulation of Bcl-XL and Mcl-1 and a downregulation of Noxa (8–11) and leads to resistance to various drugs (9, 12). Targeting antiapoptotic proteins such as Bcl-XL, 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 anticancer drugs. The BH3 mimetic ABT-737 is a potent small-molecule antagonist that binds with high affinity to the antiapoptotic molecules Bcl-XL, 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 seems to be crucial in inducing cell death by targeting Mcl-1 for proteasomal degradation.

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In contrast to proapoptotic proteins such as Bim, Bid, and Puma, which can bind to all of the antiapoptotic 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-XL 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 antiapoptotic 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 this 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 pharmacologic means to influence the expression of Bcl-2 family members in conjunction with ABT-737 treatment. We here show 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 mitogen-activated protein kinase (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, to eradicate CLL cells in the protective LN microenvironment.

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 antiapoptotic proteins such as Bcl-XL, Bcl-2, Mcl-1, and Bfl-1. In vitro, the LN microenvironment is mimicked via CD40 stimulation of primary CLL cells. BH3 mimetics have been developed as a new class of anticancer drugs and antagonize the antiapoptotic proteins Bcl-XL and Bcl-2 but not Mcl-1 or Bfl-1. This study shows that upon CD40 stimulation patient-specific variations in ABT-737 sensitivity correlate with differences in the relative levels of Mcl-1 and its antagonist Noxa. Increasing the Noxa/Mcl-1 ratio, with known antileukemia 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.

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 and conducted in agreement with the Helsinki Declaration of 1975, revised in 1983. PB mononuclear cells of patients with CLL, obtained after Ficol density gradient centrifugation (Pharmacia Biotech) were frozen and stored as described (9). Expression of CD5 and CD19 (both Beckton Dickinson (BD) Biosciences) 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 PBS containing 0.5% bovine serum albumin. For intracellular ZAP70 staining CLL cells were fixed and permeabilized (eBioscience) and subsequently stained for ZAP-70 (Alexa Fluor 488, clone 1E7.2; Invitrogen) and PerCP-conjugated CD19 (BD).

Reagents

Fludarabine (F-Ara-A), roscovitine, propidium iodide (PI), and N-acetylcysteine (NAC) were purchased from Sigma Chemical Co. ABT-737 (13) was obtained under MTA from Abbott (Abbott Park, Illinois, USA, courtesy Dr. S. Rosenberg). Bortezomib was obtained from Janssen-Cilag. Dasatinib was obtained from Novartis. A panel of chemicals was used to block different intracellular signaling pathways: BAY-11-7082, SB203580, SP600125, SB216763, and LY294002 (all from Calbiochem). The pan-caspase inhibitor Q-VD was purchased at R&D systems.

Cell culture and detection of apoptosis

PB lymphocytes of CLL patients were stimulated by coculture with NIH3T3 fibroblasts stably transfected with human CD40L, as described previously (9). After 24, 48, or 72 hours 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 hours. When indicated, CLL cells were treated with ABT-737 in the presence of CD40-expressing feeder cells. Previous studies in our group have shown that drug resistance lasts for 0 to 120 hours after detachment of CLL cells from CD40L-expressing fibroblasts (unpublished observation). CLL cells were preincubated with the different signaling inhibitors for 30 minutes (10 μmol/L). BAY-11-7082 was used in a concentration of 1 μmol/L. CLL cells were preincubated with dasatinib (0.1–10 μmol/L) for 30 minutes. The pan-caspase inhibitor Q-VD was used in a concentration of 12.5 μmol/L and preincubated for 30 minutes. The reactive oxygen species (ROS) scavenger NAC was used in a concentration of 5 mmol/L and added 30 minutes prior to fludarabine. Cells...
were treated with 1 to 100 μmol/L fludarabine (48 hours), 30 nmol/L bortezomib (24 hours), 25 μmol/L roscovitine (24 hours), and 0.001 to 10 μmol/L ABT-737 (24 hours) and stained with 200 nmol/L MitoTracker Orange (Molecular Probes) for 30 minutes at 37°C and analyzed by fluorescence-activated cell sorting (FACS; ref. 9). In addition, apoptotic and viable cells were discriminated via flow cytometry of cells stained with Annexin V APC (IQ Products) and PI (Sigma) 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).

Western blot and antibodies

Western blotting was done 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-Xi (catalog #620211; BD Biosciences), antisera to β-actin (clone I-19; Santa Cruz Biotechnology), polyclonal antibodies against A1/Bfl-0070 (Cruz Biotechnology), polyclonal anti-Bcl-2 (catalog #620211; BD Biosciences), antiserum to NGFR, and GFP using the FACS Aria cell sorter (BD Biosciences). All experiments were done using sorted transfected with CD40L, in the presence of interleukin (IL)-2 and retrovirally transduced with Bcl-6 linked to a truncated antiapoptotic protein of interest or Noxa RNAi linked to retroviral transduction reported.

Blots were scanned on the Odyssey imager (LI-COR Biosciences) and subjected to densitometry with the Odyssey software (Odyssey Application software version 3.0). Per sample relative expression compared with β-actin is reported.

Retroviral transduction

Transduction of memory B cells was done as described by Kwakkenbos and colleagues (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 (ANGFR) and an antiapoptotic protein of interest or Noxa RNAi linked to green fluorescent protein (GFP). Transduced cells were cultured on a feeder layer of mouse L fibroblasts stably transfected with CD40L, in the presence of interleukin (IL)21. Double transduced B cells were sorted based on coexpression of ANGFR and GFP using the FACS Aria cell sorter (BD Biosciences). All experiments were done 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 μmol/L ABT-737, 10 μmol/L 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 RT-PCR (Nterminus CTC TCG AGC CCG GGA AAA AGG CGG and Cerminus GGAATTCTCAGGTACTAAATGAGACGT), and an 18S RNA (loading control) reverse transcriptase PCR (RT-PCR) was done. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

siRNA and nucleofection

CLL cells were transfected using the Amaxa nucleofection technology (Amaxa), according to the manufacturers’ recommendations and as described previously (8). siRNA (Noxa Catalog #4392420 and Silencer Select Negative Control #1 Catalog #4390843) was obtained from Ambion and 1.5 to 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 done to analyze Gaussian distributions. If there was a Gaussian distribution, a 2-sided t test was used to analyze differences between the groups. If there was no Gaussian distribution, a 2-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 significant of the data was set at P < 0.05, with 1 asterisk (*) representing 0.01 < P < 0.05; 2 asterisks (**) 0.001 < P < 0.01; 3 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 × 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 (Dmax)].

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 PB (9, 26, 27, 32; Fig. 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; Fig. 1A, right panel, solid lines). Interestingly, we consistently observed that a small subset of CD40-stimulated CLL samples remained relatively sensitive to ABT-737 (n = 5; Fig. 1A, right panel, dotted lines). For each patient, this was a reproducible phenomenon.
observed in at least 2 independent experiments, as well as in 2 different samples. ABT-737–sensitive CLL cells were defined as those showing 50% or more apoptosis when treated with 0.1 μmol/L ABT-737, when compared with CLL cells stimulated with CD40L only. The EC_{50} calculated for ABT-737 “sensitive” CLL cells was 10-fold lower (0.07 ± 0.003 μmol/L; \( P = 0.0001 \)) than for ABT-737 “resistant” CLL cells (0.7 ± 0.003 μmol/L; \( P = 0.0001 \)). In these experiments, CLL cells were removed from CD40-expressing feeder cells prior to ABT-737 treatment, which might affect the outcome. However, control experiments in which CLL cells remained in contact with the feeder cells during ABT-737 exposure, showed similar levels of apoptosis, and the distinction between sensitive and resistant CLL cells remained (Supplementary Fig. S1). The characteristics of ABT-737 sensitivity in CD40-stimulated CLL cells were investigated with respect to differentially expressed pro- or antiapoptotic proteins in time-course experiments. After 24 hours of CD40 stimulation no differences in ABT-737 sensitivity were observed (Fig. 1B). Notably, 48 and 72 hours of CD40 stimulation resulted in a significant difference in apoptosis induced by ABT-737 between the 2 subgroups (48 hours \( P = 0.0025; 72 \text{ hours } P = 0.0025; \) Fig. 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. No differences in Bcl-X, Bcl-2, and Puma levels were observed in ABT-737–resistant versus ABT-737–sensitive CLL cells (Fig. 1C, and data not shown). In addition, Bfl-1 showed a peak in expression at 48 hours after CD40 stimulation in both subgroups (Fig. 1C and...
Supplementary Fig. S2). Densitometric analysis of various patient samples was done for expression of prosurvival Bcl-2 family members versus Noxa. A significantly higher Noxa/Mcl-1 ratio in ABT-737 sensitive compared with resistant CLL cells at 72 hours of CD40 stimulation was observed (Fig. 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

Table 1. Patient characteristics

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aPercentage of cells positive for CD5/CD19 surface expression measured by FACS analysis.
bMutated IgVH gene (+1) denotes more than 2% mutations compared with germline sequence.
cAs 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 more than 10% aberrant signals were considered abnormal. ND = not determined.
dCib = chlorambucil, CibP = 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.
eP53 functional status was measured via radiation-induced RNA expression of p53 target genes Puma and Bax as described previously.
fFor intracellular ZAP70 staining CLL cells were fixed and permeabilized (eBioscience) and subsequently stained for ZAP-70 (Alexa fluor 488, clone 1E7.2; Invitrogen), CD3 and CD19.
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 done. 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 (Supplementary Fig. S3). Moreover, siRNA for Bcl-Xl and Bfl-1 also nonspecifically 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. Because 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. PB-derived CD27\(^+\) memory B cells were immortalized by retroviral transduction with Bcl-6 in combination with antiapoptotic proteins Mcl-1, Bfl-1, Bcl-Xl, or Bcl-2, or combined with Noxa knockdown.

Overexpression or knockdown was confirmed by Western blot analysis for Bcl-Xl, Bcl-2, Bfl-1, Noxa, and Mcl-1 (Fig. 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 antiapoptotic 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, whereas overexpression of Bcl-Xl and Bcl-2 hardly affected sensitivity (Fig. 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 with Bcl-Xl and Bcl-2–transfected B cell lines (Fig. 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 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 pharmacologic inhibitor SB203580 diminished
Noxa levels but also resulted in decreased Mcl-1 levels (Fig. 3A). Therefore, no significant alteration of the Noxa/Mcl-1 ratio in CD40-stimulated CLL cells was observed upon p38 inhibition (Fig. 3B). Combination of SB203580 with ABT-737 resulted in a modest increase in apoptosis (Fig. 3C).

Calculations of the combined effects (30, 31) indicated additive effects on apoptosis when SB203580 (1 and 10 μmol/L) was combined with 0.1 μmol/L ABT-737 (Fig. 3D). In CD40-stimulated CLL cells sensitive for ABT-737 p-p38 levels were similar compared with p-p38 levels observed in 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 hours 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 10 μmol/L. Protein lysates were probed for Noxa, Mcl-1, and β-actin. Blots from one representative CLL sample shown, of a total of 4 analyzed. B, Western blots of Noxa and Mcl-1 were quantified with Odyssey software from 4 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, 1, and 10 μmol/L). After detachment, cells were incubated for 24 hours with indicated concentrations of ABT-737 (0.001–10 μmol/L). D, synergistic/additive effects of ABT-737 (0.1 μmol/L) and SB203580 1 μmol/L (open circles) and 10 μmol/L (solid circles) were assessed as described in the Materials and Methods section. 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. Each dot represents an individual CLL sample. E, CLL cells were stimulated with CD40L in the presence or absence of BAY-11-7082 (n = 4) for 24 hours. Western blots of Noxa and Mcl-1 were quantified with Odyssey software. The Noxa/Mcl-1 ratio in CLL cells stimulated with CD40L was set at 1 for each individual patient. Bars represent the mean ± SEM. **, 0.001 < P < 0.01. F, CD40-stimulated CLL cells were treated with BAY-11-7082 (0.5–1 μmol/L; n = 6) for 24 hours. After detachment, cells were incubated for 24 hours with indicated concentrations of ABT-737 (0.001–10 μmol/L). G, synergistic and additive apoptotic effects were assessed of ABT-737 (0.1 μmol/L) and the NF-κB inhibitor, BAY-11-7082 (open circles 0.5 μmol/L, solid circles 1 μmol/L) in CD40-stimulated CLL cells.
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 (Fig. 3A and data not shown). A significant alteration of the Noxa/Mcl-1 ratio and increased resistance to ABT-737 in CD40-stimulated CLL cells was observed upon NF-κB inhibition (Fig. 3E and F). In addition, synergistic apoptotic effects were observed when 1 μmol/L BAY-11-7082 was combined with 0.1 μmol/L of ABT-737 (Fig. 3G). Together, these data further underlined 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 antileukemia 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 Fig. 4A. A modest decline in Bfl-1 levels was observed and Bcl-X₇ levels remained equal when CD40-stimulated CLL cells were treated with dasatinib (Fig. 4A, left panel). Densitometric analysis showed a significant increase in the Noxa/Mcl-1 ratio upon dasatinib treatment (Fig. 4A, right panel). We next investigated whether concurrent treatment with dasatinib would sensitize CD40-stimulated CLL cells to ABT-737. Notably 10 μmol/L of dasatinib resulted in 100-fold increased ABT-737 sensitivity in CD40-stimulated CLL cells and synergy between ABT-737 and dasatinib (Fig. 4B and C). Lower concentrations of dasatinib (1 μmol/L) in combination with simultaneous CD40 stimulation and sequential treatment of 0.1 μmol/L of ABT-737 resulted in additive effects (Fig. 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 (Fig. 4D–F).

Fludarabine is a well-known antileukemia 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 (Fig. 5A and B). Although high doses of fludarabine (100 μmol/L) resulted in a ROS-dependent upregulation of Noxa (31), as shown by abrogation of Noxa induction by the addition of the ROS scavenger NAC, Noxa upregulation at a lower concentration of fludarabine (10 μmol/L) was independent of ROS (Supplementary Fig. S5). A minor decrease in Mcl-1 levels was observed in fludarabine-treated cells, but this was prevented by adding the pan-caspase inhibitor QVD, suggesting that the Mcl-1 degradation was caspase dependent (Fig. 5B). ABT-737 treatment as a single agent did not alter Noxa expression at mRNA (Fig. 5A) or protein level (Supplementary Fig. S5). Densitometric analysis of Western blots of CD40-stimulated CLL cells treated with fludarabine revealed a significantly higher Noxa/Mcl-1 ratio compared with controls (Fig. 5C). Importantly, addition of fludarabine resulted in a dose-dependent enhancement of ABT-737 sensitivity in CD40-stimulated CLL cells (Fig. 5D). Synergistic effects were observed with 10 and 100 μmol/L of fludarabine in combination with 0.1 μmol/L ABT-737 in CD40-stimulated CLL cells (Fig. 5E).

Collectively, these data showed 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**

This study shows 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- to 1,000-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 show 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-X₇ 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 and colleagues 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 proapoptotic factors, for example, Noxa that could counterbalance the effects of Mcl-1 upregulation. Second, owing to technical difficulties, our experiments using a siRNA approach toward Mcl-1 in CLL were not conclusive because nonspecific 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 ABT-737–sensitive CLL cells upon CD40 stimulation (Fig. 1C).

Our data are in line with previous studies using various cancer cell lines, which show that alterations in the Noxa/Mcl-1 ratio determine sensitivity to ABT-737 (18–20, 22, 23). Yecies and colleagues 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 (Supplementary Fig. S4). Basal levels of Noxa after 72 hours 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 (Supplementary Fig. S4).

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 with 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 CLL cells (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 because 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 with 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 were 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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