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

Jacqueline M. Tromp1,2, Christian R. Geest2, Esther C.W. Breij3, Judith A. Elias1,2, Jacoline van Laar2, Dieuwertje M. Luijks1, Arnon P. Kater1, Tim Beaumont3, Marinus H.J. van Oers1, and Eric Eldering2

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-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 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 microenvironmment-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.
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-X<sub>L</sub>, 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-X<sub>L</sub> 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 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 Ficoll 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-acetylcyesteine (NAC) were purchased from Sigma Chemical Co. ABT-737 (13) was obtained under contract from 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 (AD1; Alexis), polyclonal anti-Bcl-XL (catalog #620211; BD Biosciences), antiserum to β-actin (clone I-19; Santa Cruz Biotechnology), polyclonal antibodies against A1/Bfl-070-E; Alexis), polyclonal anti-Bcl-2 (ADI-AAS-4572; Cell Signaling), monoclonal anti-Noxa (catalog #4572; Cell Signaling), and retrovirally transduced with Bcl-6 linked to a truncated form of the nerve growth factor receptor (NGFR) and GFP using the FACS Aria cell sorter.

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 data was set at P < 0.05, with 1 asterisk (*) representing 0.01 < P < 0.05; 2 asterisks (**) 0.001 < P < 0.01; 3 asterisks (***) 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; the majority (n = 23) was resistant to ABT-737 (right, solid lines), whereas a minority (n = 5) was shown to be sensitive to ABT-737 (right, dotted lines). CD40-stimulated CLL cells which were sensitive to ABT-737 are labeled according to the numbers in the table. Bars represent the mean ± SEM. B, CLL cells were stimulated with CD40L for 24, 48, and 72 hours. After detachment, cells were treated with 0.1 μmol/L ABT-737 for 24 hours. 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 hours). Protein lysates were probed for Mcl-1, Noxa, Bfl-1, Bcl-X<sub>L</sub>, 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. *P < 0.05; **P < 0.001.
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

<table>
<thead>
<tr>
<th>ID#</th>
<th>ABT sensitive</th>
<th>Age</th>
<th>Rai stage</th>
<th>%CD5</th>
<th>CD19&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IgVH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Chromosomal aberrations&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Previous therapy&lt;sup&gt;d&lt;/sup&gt;</th>
<th>P53&lt;sup&gt;e&lt;/sup&gt;</th>
<th>ZAP70%&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62</td>
<td>0</td>
<td></td>
<td>99.9</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>13,3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>85</td>
<td>0</td>
<td></td>
<td>90.4</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>8,8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>85</td>
<td>0</td>
<td></td>
<td>73.2</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>13,9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>1</td>
<td></td>
<td>90</td>
<td>UM</td>
<td>Trisomy 12</td>
<td>None</td>
<td>F</td>
<td>70,4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>1</td>
<td></td>
<td>87.4</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>9,0</td>
<td></td>
</tr>
<tr>
<td>ID#</td>
<td>ABT resistant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>0</td>
<td></td>
<td>82</td>
<td>UM</td>
<td>Trisomy 12</td>
<td>Clb</td>
<td>F</td>
<td>39,0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>76</td>
<td>1</td>
<td></td>
<td>95.7</td>
<td>M</td>
<td>Del 11q22</td>
<td>None</td>
<td>F</td>
<td>15,2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>76</td>
<td>2</td>
<td></td>
<td>91.1</td>
<td>UM</td>
<td>Del 13q14</td>
<td>ClbP</td>
<td>F</td>
<td>60,2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>63</td>
<td>2</td>
<td></td>
<td>89.6</td>
<td>M</td>
<td>None</td>
<td>None</td>
<td>F</td>
<td>52,8</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>52</td>
<td>0</td>
<td></td>
<td>88.2</td>
<td>UM</td>
<td>Trisomy 12</td>
<td>None</td>
<td>F</td>
<td>72,7</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>66</td>
<td>0</td>
<td></td>
<td>86.2</td>
<td>UM</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>64,1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>72</td>
<td>0</td>
<td></td>
<td>99.8</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>10,2</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>78</td>
<td>2</td>
<td></td>
<td>92.9</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>61</td>
<td>0</td>
<td></td>
<td>99.7</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>27,8</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>49</td>
<td>3</td>
<td></td>
<td>99.7</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>74</td>
<td>0</td>
<td></td>
<td>96.2</td>
<td>M</td>
<td>Del 13q14</td>
<td>None</td>
<td>F</td>
<td>17,2</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>80</td>
<td>0</td>
<td></td>
<td>93.4</td>
<td>M</td>
<td>None</td>
<td>None</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>64</td>
<td>4</td>
<td></td>
<td>94.6</td>
<td>M</td>
<td>Del 13q14</td>
<td>Clb</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>74</td>
<td>4</td>
<td></td>
<td>99.8</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>2,9</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>53</td>
<td>2</td>
<td></td>
<td>81,8</td>
<td>UM</td>
<td>Del 17p13</td>
<td>Clb, FCO</td>
<td>F</td>
<td>19,8</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>69</td>
<td>1</td>
<td></td>
<td>86,8</td>
<td>UM</td>
<td>ND</td>
<td>FCR</td>
<td>F</td>
<td>8,8</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>71</td>
<td>0</td>
<td></td>
<td>95,9</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>60</td>
<td>0</td>
<td></td>
<td>94.7</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>64</td>
<td>0</td>
<td></td>
<td>83.2</td>
<td>M</td>
<td>Del 13q14</td>
<td>None</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>62</td>
<td>0</td>
<td></td>
<td>99.9</td>
<td>M</td>
<td>Del 13q14</td>
<td>None</td>
<td>F</td>
<td>9,73</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>77</td>
<td>0</td>
<td></td>
<td>94.8</td>
<td>M</td>
<td>ND</td>
<td>None</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>76</td>
<td>0</td>
<td></td>
<td>99.8</td>
<td>UM</td>
<td>Trisomy 12</td>
<td>None</td>
<td>F</td>
<td>71,8</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>36</td>
<td>3</td>
<td></td>
<td>56.4</td>
<td>M</td>
<td>None</td>
<td>FCR, RCHOP, RDHAP</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>41</td>
<td>2</td>
<td></td>
<td>89</td>
<td>M</td>
<td>Del 13q14</td>
<td>Clb, F</td>
<td>F</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Percentage of cells positive for CD5/CD19 surface expression measured by FACS analysis.

<sup>b</sup>Mutated IgVH gene (+) denotes more than 2% mutations compared with germline sequence.

<sup>c</sup>As 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.

<sup>d</sup>Clb = 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.

<sup>e</sup>P53 functional status was measured via radiation-induced RNA expression of p53 target genes Puma and Bax as described previously F = p53 Functional.

<sup>f</sup>For 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. After detachment, cells were incubated for 24 hours with indicated concentrations of SB203580 (0.1, 1, and 10 μmol/L). 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. D, synergistic/additive effects of ABT-737 (0.1 μmol/L) and SB230580 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. F, 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.
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 Bcl-1 levels was observed and Bcl-X_l 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 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-X_l 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 and β-actin are shown. C, densitometric analysis of the Noxa/Mcl-1 ratio of CD40-stimulated CLL cells ± fludarabine and QVD is shown (n = 4). The Noxa/Mcl-1 ratio in CD40L-stimulated with CD40L in the presence of fludarabine and QVD 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. *, 0.01 < P < 0.05. D, after 48 hours of CD40 stimulation, CLL cells were detached from the feeder layer cells and incubated with different concentrations of ABT-737 (0.001, 0.01, 0.1, and 10 μmol/L) for 48 hours. Apoptosis was measured by MitoTracker analysis. As a control, CLL cells were stimulated on 3T3 cells without the expression of CD40L. E, synergistic effects of ABT-737 (0.1 μmol/L) and fludarabine 10 μmol/L (open circles) and 100 μmol/L (solid circles) were assessed. 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.

Figure 5. Increased Noxa levels in fludarabine-treated CD40-stimulated CLL cells results in synergistic apoptotic effects with ABT-737. A, CLL cells were stimulated with CD40L for 48 hours. After detachment, cells were incubated with ABT-737 (0.1 μmol/L) and/or fludarabine (10 μmol/L). RNA was isolated and a Noxa reverse transcription PCR was done. 18S RNA RT-PCR was used as a loading control. B, CLL cells were stimulated for 48 hours with CD40L. After detachment, cells were incubated with fludarabine (10 μmol/L) in the presence or absence of Q-VD. Western blot analysis of Noxa, Mcl-1, and β-actin are shown. C, densitometric analysis of the Noxa/Mcl-1 ratio of CD40-stimulated CLL cells ± fludarabine and QVD is shown (n = 4). The Noxa/Mcl-1 ratio in CD40-stimulated with CD40L in the presence of fludarabine and QVD 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. *, 0.01 < P < 0.05. D, after 48 hours of CD40 stimulation, CLL cells were detached from the feeder layer cells and incubated with different concentrations of ABT-737 (0.001, 0.01, 0.1, and 10 μmol/L) for 48 hours. Apoptosis was measured by MitoTracker analysis. As a control, CLL cells were stimulated on 3T3 cells without the expression of CD40L. E, synergistic effects of ABT-737 (0.1 μmol/L) and fludarabine 10 μmol/L (open circles) and 100 μmol/L (solid circles) were assessed. 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.

Tromp et al.
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.

Acknowledgments

The authors thank Sanne Tonino for conducting the Noxa siRNA experiments in CLL cells and the patients for donating blood samples. ABT-737, a BH3 mimic, was obtained under MTA from Abbott (Abbott Park, IL, courtesy Dr S. Rosenberg).

Grant Support

This work was supported by the Dutch Cancer Society, grant no UvA 2007–3856 (J.M. Tromp, J.A. Elias, M.H.J. van Oers, E. Eldering), and the Landsteiner Foundation for Bloodtransfusion Research grant no 831 (C.R. Geest, E. Eldering).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 5, 2011; revised November 16, 2011; accepted November 16, 2011; published OnlineFirst November 29, 2011.

References

18. Zall H, Weber A, Besch R, Zardt N, Hacker G. Chemotherapeutic drugs sensitize human renal cell carcinoma cells to ABT-737 by a mechanism...
involving the Noxa-dependent inactivation of Mcl-1 or A1. Mol Cancer 2010;9:164.
Tipping the Noxa/Mcl-1 Balance Overcomes ABT-737 Resistance in Chronic Lymphocytic Leukemia


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-1440

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/11/29/1078-0432.CCR-11-1440.DC1

Cited articles
This article cites 38 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/2/487.full.html#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
/content/18/2/487.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.