Dysregulation of survival and apoptosis pathways in acute myeloid leukemia
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CHAPTER

BORTEZOMIB SENSITIVITY OF ACUTE MYELOID LEUKEMIA CD34+ CELLS CAN BE ENHANCED BY TARGETING THE PERSISTING ACTIVITY OF NF-κB AND THE ACCUMULATION OF MCL-1

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Abstract – Sustained NF-κB activation is often observed in Acute Myeloid Leukemia (AML) and proteasome inhibition has therefore been proposed to efficiently target AML cells. In the present study, we questioned whether leukemic stem cell enriched CD34+ cells are sensitive to the proteasome inhibitor bortezomib. Surprisingly, we observed in short-term and long-term culture assays that CD34− AML cells were more sensitive to bortezomib treatment compared to the CD34+ AML cells at a clinical relevant dosage. Co-treatment with the apoptosis inducing cytokine TRAIL did not further enhance cell death in CD34+ AML cells in contrast to the effects in AML cell lines. The better survival of CD34+ AML cells upon bortezomib treatment was due to a persisting NF-κB activity which could be overcome by the IKK inhibitor BMS-345541. This difference in sensitivity might be related to differences in NF-κB activation in AML CD34+ versus CD34− cells as suggested by a gene expression profiling study. Besides NF-κB, MCL-1 strongly determines the effectiveness of bortezomib. MCL-1 accumulated in CD34+ AML cells upon bortezomib treatment and inhibition of MCL-1 by shRNA or obatoclax, significantly improved the sensitivity of CD34+ AML cells to bortezomib. These results demonstrate that combining bortezomib with specific NF-κB or MCL-1 inhibitors might potentially target the leukemic stem cells.

3.1 Introduction

Acute myeloid leukemia (AML) patients still have an unfavourable prognosis due to high incidence of relapse following intensive chemotherapy. It has been suggested that a small population of leukemic stem cells survives the treatment and that these cells are responsible for the sustained leukemic outgrowth and relapse of the disease [1, 2]. Therefore, new therapeutic options have to be examined in order to target these leukemic stem cells, and investigating drugs that induce cell death by different cellular pathways might be worth pursuing. The proteasome pathway plays an important role in the process of apoptosis [3–6]. The proteasome complex is involved in the degradation of ubiquitinated proteins such as IκBα, leading to NF-κB activation. Because NF-κB is frequently constitutively activated in AML (stem) cells [7, 8], proteasome inhibition has been proposed to efficiently target leukemic stem cells. The proteasome inhibitor MG-132 induces cell death in patient AML cells but this agent has a poor stability and bioavailability and is less specific than the proteasome inhibitor bortezomib [6, 7, 9]. Bortezomib has an inhibitory effect in AML cells and is able to efficiently target chronic myeloid leukemic stem cells in vitro as well as in vivo [10–14]. Whether AML stem cells can also be targeted with bortezomib is currently unclear, although clinical studies in patients have been initiated [15–17]. However, the presence of (activated) NF-κB is not a guarantee for the effectiveness of bortezomib; in mantle cell lymphoma and chronic lymphocytic leukemia limited cytotoxicity has been observed despite the expression and activation of NF-κB in these cells [18–21]. This might be the result of stabilization of the anti-apoptotic protein MCL-1, thereby limiting the effectiveness of bortezomib [21, 22]. Therefore it is likely that the efficacy of bortezomib can be improved when it is combined with agents that trigger alternative cell death
pathways. It has been demonstrated that TRAIL (Tumor necrosis factor-related apoptosis inducing ligand) is able to induce apoptosis by interacting selectively with death receptors 4 and 5 (DR4 and DR5) [23, 24]. Upon TRAIL binding, the death inducing signaling complex (DISC) is assembled which leads to the activation of the caspase cascade and eventually to cell death. Combining TRAIL with bortezomib has been proposed to efficiently target AML cells [12, 23, 25, 26]. In the present study we investigated the cytotoxic effects of bortezomib and TRAIL on primary AML cells by studying separately the stem cell enriched CD34+ cells versus the more mature CD34− AML cells. The results demonstrated that the cytokine TRAIL did not induce cell death in primary AML cells. Furthermore, CD34− AML cells were most sensitive to bortezomib compared to CD34+ AML cells due to inadequate inhibition of NF-κB activity in CD34+ AML cells. Effectiveness of bortezomib was further limited in AML CD34+ cells by accumulation of the anti-apoptotic protein MCL-1. Combining bortezomib with NF-κB or MCL-1 inhibitors strongly inhibited the survival of the stem cell enriched CD34+ AML cell fraction, suggesting a new opportunity for the treatment of AML patients.

3.2 Material and methods

3.2.1 Cell culture

The human promyelocytic leukemia cell line HL-60, the human monocytic leukemia cell line MOLM13 and the erythromyeloblastoid leukemia cell line K562 were cultured in RPMI 1640 supplemented with 10% FBS. The human monocytic leukemia cell line Oci-AML3 was cultured in RPMI 1640 supplemented with 20% FBS. Cell viability was assessed using WST-1 assay (Roche Applied Science). In short, cells were seeded in 96 wells plates and incubated with rhTRAIL (produced and purified as described) [27], 20 nM bortezomib (Velcade, Jansen-Cilcag) or the combination of both. After 24 hrs of incubation at 37°C and 5% CO2, 20 µL of WST-1 reagent was added. Cell viability was determined after 2 hrs by measuring the absorption at 490 nm using a microplate reader (Thermo Labsystems).

3.2.2 Primary AML cells and long term cultures on stroma

AML blasts from peripheral blood cells were studied after informed consent in accordance with the Declaration of Helsinki; the protocol was approved by the Medical Ethics Committee. AML mononuclear cells were isolated by density gradient centrifugation, and CD34+ cells were selected by MiniMacs (Miltenyi Biotec, Amsterdam, the Netherlands) or cell sorting. In general, the purity of the CD34+ and CD34− cell fractions was 90-95%. Cells were incubated for 24 hrs with 100 ng/mL rhTRAIL, 20 nM bortezomib, 5 µM BMS-345541 (Sigma-Aldrich) and/or 5 µM obatoclax mesylate (Selleckbio). Cell viability was assessed by microscopical analysis using trypan blue exclusion and/or by WST-1 assay. Thereafter 40.000 cells were expanded on MS5 stromal cells in long-term culture (LTC) medium (αMEM supplemented with heat-inactivated 12.5% FCS, heat-inactivated 12.5%
horse serum [Sigma, Zwijndrecht, the Netherlands], penicillin and streptomycin, 2 mM glutamine, 57.2 μM β-mercaptoethanol [Sigma] and 1 μM hydrocortisone [Sigma]) with interleukin 3 (IL-3; Gist-Brocades, Delft, the Netherlands), granulocyte colony-stimulating factor (G-CSF; Rhone-Poulenc Rorer, Amstelveen, the Netherlands), and thrombopoietin (TPO; Kirin, Tokyo, Japan) (20 ng/mL each) as described previously [28, 29]. Cultures were kept at 37°C and 5% CO2. Cultures were demipopulated weekly for analysis. The leukemic origin of the expanding cultures was established by performing polymerase chain reaction (PCR) analysis for the presence of genetic marker FLT3-ITD and by morphological analysis of the cells by May-Grünwald Giemsa staining of cytopsins.

### 3.2.3 FLOW CYTOMETRY ANALYSIS

Antibodies were obtained from Beckton Dickinson (Alphen a/d Rijn, The Netherlands). Cells were incubated with antibodies at 4°C for 30 minutes. All fluorescence-activated cell sorter (FACS) analyses were performed on a FACS calibur (Becton Dickinson) and data was analyzed using FlowJo 7.6.1. Cells were sorted on a MoFlo-XDP or Astrios (DakoCytomation, Carpinteria, CA, USA).

### 3.2.4 LENTIVIRAL TRANSDUCTIONS

Oligos containing the MCL-1 hairpin (5’-GCTAAACACTTGAAGACCATA-actcgaga-TATGGTCTTCAAGTGTTTAGCTTTTT-3’, Invitrogen) were annealed and cloned into the pTRIP vector, containing GFP, using the AscI and SbfI restriction sites [30]. The 2.5 × 10⁶ 293T Human Embryonic Kidney cells were transduced with 3 μg plasmid cytomegalovirus Δ8.91 (pCMV d8.91), 0.7 μg pVSV-G and 3 μg pTRIP MCL-1 shRNA. After 24 hrs medium was changed to hematopoietic progenitor growth medium (HPGM) (Cambrex) and after 12 hrs supernatant containing lentiviral particles was harvested and stored at −80°C. Oci-AML cell lines were transduced in one round. AML blasts were transduced in three consecutive rounds of 8 to 12 hours with lentiviral supernatant supplemented with 10% fetal calf serum, 20 ng/ml IL-3, G-CSF and TPO, and Polybrene (4 μg/mL). Transduction efficiency was measured by FACS analysis. Knockdown was investigated by quantitative reverse-transcription-polymerase chain reaction and western blot.

### 3.2.5 LTC-IC ASSAYS IN LIMITING DILUTIONS

Leukemic LTC-IC assays were performed as described previously [29]. Briefly, LTC-IC assays were done by plating rhTRAIL, bortezomib or combined treated AML CD34⁺ cells in limiting dilutions in the range of 10 to 10000 cells per well on MS5 stromal cells in 96-well plates in LTC medium. Cultures were semi-depopulated weekly, and after 5 weeks, the presence of cobblestone-area forming cells was analyzed in each well. The frequency of leukemic stem cell was calculated based on the total amount of cells plated in each well and the frequency of cobblestone area forming cells per well.
3.2.6 CFSE VIABILITY ASSAY

10 × 10⁶ HL60 cells were washed with PBS and labeled with 10 μM CFSE (Molecular Probes Europe, Leiden, the Netherlands) for 10 minutes at 37°C. The reaction was stopped by the addition of 5 volumes of FBS, followed by a 2-minute incubation at ice. After 3 consecutive washes the CFSE-labeled cells were resuspended in RPMI supplemented with 10% FBS and incubated for 2 days at 37°C and 5% CO₂. CFSE<sup>high</sup> and CFSE<sup>low</sup> cells were sorted and resuspended in RPMI supplemented with 10% FBS. After 2 hours, cell viability was assessed by performing a WST-1 assay (Roche).

3.2.7 NF-κB ASSAY

NF-κB activity was measured by ELISA using the TRANS-AM NF-κB p65 Transcription Factor Assay Kit (Active Motif, North America, Carlsbad, CA) following the manufacturer's recommendations. In short, nuclear extracts were prepared from AML CD34<sup>+</sup> and AML CD34<sup>−</sup> cells before and after treatment with 20 nM bortezomib. Protein concentration was measured by Bradford and equal amount of extracts were incubated in a 96 wells plate coated with oligonucleotides containing a p65 binding site. Binding was quantified by addition of p65 antibody and visualized by addition of anti-IgG HRP. Absorbance was measured at 450 nm using a microplate reader (Thermo Labsystems).

3.2.8 REAL TIME POLYMERASE CHAIN REACTION

For RT-PCR of MCL-1, total RNA was isolated from 1 × 10⁶ cells using the RNeasy kit from QIAGEN (Venlo, The Netherlands) according to the manufacturer's recommendations. 250 ng of RNA was reverse transcribed with MMLV reverse transcriptase (Biorad). Obtained cDNA was real-time amplified using primer sequences CCAAGAAAGCTGCATCGAAC (MCL-1 forward) and TATGCCAAACCAGCTC-CTAC (MCL-1 reversed) in iQ SYBR Green supermix (Biorad) with the MYIQ thermocycler (Biorad). RPL27 was used as a housekeeping gene with the primer sequences TCCGGACGCAAAGCTGTCATCG (RPL27 forward) and TCTTGCC-CATGGCAGCTGTCAC (RPL27 reversed).

3.2.9 WESTERN AND ANTIBODIES

The following antibodies were used for Western blotting: MCL-1 (Calbiochem, AM50 (RCI3)) and ACTIN (C4) (Santa Cruz Biotechnology).

3.2.10 MICRO-ARRAY ANALYSIS

Gene expression profiling of in total 112 samples was performed previously using the Illumina HumanHT-12 Expression BeadChips [31]. These 112 samples were divided as follows: 62 AML CD34<sup>+</sup>, and 50 AML CD34<sup>−</sup> samples. All samples were corrected for background using Illumina GenomeStudio and then jointly forced to
Figure 3.1: Bortezomib sensitized AML cell lines to TRAIL-induced cell death
A) HL60 cells, B) MOLM13 cells, C) K562 cells and D) OCI-AML3 cells were incubated with TRAIL alone or combined with bortezomib (5 nM) and after 24 hrs cell survival was measured. * p < 0.05
positive values, normalized and transformed using the R packages Bioconductor and Lumi [32, 33]. Probes with a detection p-value larger than 0.01 in all samples, as provided by GenomeStudio, were deleted. Log2 transformation and quantile normalization were applied. As a measure of quality control we performed a principal component analysis (PCA) on the correlation matrix of all 112 samples [34]. The first component was removed from the data [35]. To ensure reliability and reproducibility of the results we used multivariate permutations (MP) to determine the significance of our results.

3.2.1.1 STATISTICAL ANALYSIS

All values are expressed as means ± SE. The Student t test was used for all other comparisons. Differences were considered statistically significant at \( P \leq 0.05 \).

3.3 RESULTS

3.3.1 THE CYTOTOXIC EFFECTS OF BORTEZOMIB IN PRIMARY AML CELLS CANNOT BE PROMOTED BY TRAIL AND IS PREDOMINANTLY OBSERVED IN THE CD34+ AML CELL FRACTION

The leukemic cell lines HL60, MOLM13, K562 and Oci-AML3 were incubated with bortezomib, TRAIL or the combination to investigate whether the cytotoxic effects of bortezomib can be improved by combining it with TRAIL which makes use of alternative cell death pathways. The HL60, MOLM13 and K562 cell lines were susceptible for the cytotoxic effects of TRAIL in a concentration dependent manner, which could further be promoted by combining it with a low concentration of bortezomib (Figure 3.1A-3.1C). The Oci-AML3 cells were insensitive to TRAIL treatment alone but the AML cells could be sensitized by co-exposure to bortezomib (Figure 3.1D). In view of the efficacy of bortezomib and TRAIL in all the cell lines tested, their effects were also studied on primary AML patient cells in short-term and long-term cultures \((n = 19\), Supplementary Table 3.2\). Here, we used a concentration of 20 nM of bortezomib, which is a more clinically relevant dosage; a concentration 5 fold lower than the maximum tolerated dose in humans [25, 36]. The short-term cultures demonstrated that bortezomib (20 nM) induced a cytotoxic effect in 74% of samples (> 25% cell death compared to untreated samples) while the addition of TRAIL (100 ng/mL) did not further promote the bortezomib-mediated effects in the majority of cases, in contrast to the observed effects in cell lines (Figure 3.2A). However, it appeared that not all the primary AML cells had the same susceptibility to bortezomib treatment. By using the mononuclear cell fraction \((n = 5\), we observed that upon bortezomib addition the CD34+ percentage within the total population of AML cells increased by 16% after 24 hrs. In addition, by sorting the AML cell fraction \((n = 6\) in CD34+ versus CD34− AML cells, it was observed that particularly the CD34+ AML cell fraction was less sensitive for the cytotoxic effects of bortezomib (survival of CD34+ AML versus CD34− AML cells was 75% ± 18% versus 45% ± 18%, \( p = 0.03\), Figure 3.2B). We also determined leukemic stem cell
Figure 3.2: Cytotoxic effects of bortezomib were observed in majority of primary AML samples but CD34+ AML cells were less sensitive for bortezomib treatment

A) Primary AML samples (n = 19) were incubated with TRAIL (100 ng/mL), bortezomib (20 nM) or the combination and cell survival was determined. B) Primary AML samples (n = 6) were purified by column for CD34 expression and incubated with bortezomib (20 nM) for 24 hrs and cell survival was quantified.
Figure 3.3: Bortezomib resistance could not be assigned to bortezomib being less effective in non-dividing cells
HL60 cells were incubated with CFSE and sorted in CFSE$^{\text{bright}}$ and CFSE$^{\text{dim}}$ cell populations. Bortezomib was added for 24 hrs and cell survival was determined and demonstrated no significant difference between CFSE bright and dim cells.

frequencies in AML LTC-IC assays in limiting dilution ($n = 10$). In line with the limited sensitivity of AML CD34$^+$ cells to bortezomib in short-term culture assays, we observed also that the AML LTC-IC frequencies were not changed by the short term bortezomib treatment. (relative frequencies: untreated = 1 ± 0.36, TRAIL = 0.99 ± 0.38, $p = 0.95$, bortezomib = 0.76 ± 0.27, $p = 0.11$). Similarly, no difference was observed when bortezomib was combined with TRAIL. The difference in susceptibility of CD34$^+$ versus CD34$^-$ AML cells for bortezomib might be related to a difference in cell quiescence. To investigate this possibility, HL60 cells were used as model system and stained with CFSE. CFSE$^{\text{high}}$ cells are the non-dividing cell population, which did not divide within 6 days but were still able to form colonies (data not shown). Interestingly, these cells were as effectively targeted as the dividing CFSE$^{\text{low}}$ cells (Figure 3.3).

3.3.2 NF-κB ACTIVITY IS PREDOMINANTLY INHIBITED BY BORTEZOMIB IN PRIMARY CD34$^+$ AML CELLS
Since the cytotoxicity of bortezomib was different in CD34$^+$ and CD34$^-$ AML cells, we evaluated whether bortezomib affected NF-κB activity in AML cells in distinct ways. Nuclear extracts were prepared from CD34$^+$ and CD34$^-$ AML cells ($n = 6$) before and after addition of bortezomib and active p65 was measured. The NF-κB activity was equally highly elevated in CD34$^+$ and CD34$^-$ AML cells before addition of bortezomib. Reduction in NF-κB activity upon bortezomib treatment was most pronounced in CD34$^-$ AML cells (1.77-fold reduction versus 1.18-fold reduction in the CD34$^+$ and CD34$^+$ AML cells respectively, $p = 0.05$, Figure 3.4A). It appeared that the remaining NF-κB activity in the CD34$^+$ AML contributed to the higher survival of the CD34$^+$ AML population. The IKK inhibitor BMS-345541 (5 µM)
Figure 3.4: NF-κB activity is predominantly inhibited by bortezomib in primary CD34+ AML cells

A) Primary AML CD34+ and CD34− cells were treated with bortezomib (20 nM) during 24 hrs. Nuclear extracts were prepared from both cell populations and NF-κB activity was measured using p65 ELISA B) Primary AML cells were treated with bortezomib (20 nM), the IKK-inhibitor BMS-345541 (5 µM) or the combination of both in combination with bortezomib reduced the cell survival of CD34+ AML cells to similar levels seen in CD34− AML cells while the survival of these cells was not affected by co-addition of BMS-345541 to the bortezomib treatment (Figure 3.4B).

The discrepancy in response of NF-κB activity upon bortezomib addition in CD34+ and CD34− AML cells might be due to a difference in NF-κB activation in these cells. To study this in more detail we analyzed the results of a recent performed micro-array study by comparing CD34+ and CD34− AML cells with normal bone marrow CD34+ cells. The results showed that a panel of cytokines, chemokines or cytokine-receptors are strongly upregulated (1.4-8.4 fold) in CD34− AML cells compared to CD34+ AML cells (Table 3.1). 13 out of 14 of these ligands/receptors can induce NF-κB activation, suggesting alternative NF-κB activation pathways in CD34+ and CD34− AML cells.
Table 3.1: Gene array data comparing CD34+ and CD34− AML cells [31]

Represented cytokines which are significantly upregulated in CD34− AML cells versus CD34+ AML cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>fold change (AML CD34+/CD34− cells)</th>
<th>p-value</th>
<th>Definition</th>
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<tr>
<td>IL32</td>
<td>8.44</td>
<td>6.63 × 10−27</td>
<td>Interleukin 32</td>
</tr>
<tr>
<td>IL18RAP</td>
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<td>2.60 × 10−22</td>
<td>Interleukin 18 receptor accessory protein</td>
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<tr>
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<td>7.61 × 10−15</td>
<td>Chemokine (C-C motif) ligand 5</td>
</tr>
<tr>
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<td>7.69 × 10−15</td>
<td>Chemokine (C-C motif) ligand 5</td>
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<td>1.25 × 10−13</td>
<td>CXC chemokine receptor 1</td>
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<tr>
<td>IL6</td>
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<td>9.05 × 10−12</td>
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<tr>
<td>TNF</td>
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<td>3.29 × 10−4</td>
<td>Tumor necrosis factor</td>
</tr>
</tbody>
</table>

3.3.3 MCL-1 inhibition sensitizes primary AML cells to bortezomib-induced cell death

The moderate cytotoxic effects of bortezomib on the total AML cell population, and in particular on AML CD34+ cells, may also be caused by alternative anti-apoptotic programs initiated or stabilized following bortezomib treatment thereby limiting the effectiveness of bortezomib. As depicted in Figure 3.5A MCL-1 protein accumulated significantly upon bortezomib treatment in primary CD34+ AML cells (n = 2, Supplementary Table 3.2, patient 4 and 17). Interestingly, we also observed increased accumulation of MCL-1 in the AML CD34− cells (n = 2, unpublished data), suggesting that MCL-1 is a common target of the proteasome in both AML CD34+ as well as AML CD34− cells. To determine whether accumulation of MCL-1 has an anti-apoptotic function, Oci-AML3 cells were transduced with MCL-1 shRNA and exposed to bortezomib. In SCR transduced cells, again an accumulation of MCL-1 protein was observed after bortezomib addition, whereas the MCL-1 hairpin significantly reduced MCL-1 RNA and protein expression (Figure 3.5B/3.5C). Moreover, MCL-1 downmodulation resulted in a more than 2-fold increased sensitivity to bortezomib compared to control transduced cells (IC₅₀ = 5.5 nM versus 12.2 nM) (Figure 3.5D). These effects were specific for bortezomib since Oci-AML3 cells treated with TRAIL did not induce a change in cell survival when combined with the MCL-1 shRNA. Similarly, primary CD34+ AML cells (n = 5) were transduced...
Figure 3.5: MCL-1 is stabilized upon bortezomib addition and targeting MCL-1 enhanced bortezomib induced cell death

A) Primary CD34⁺ AML cells (n = 2) were incubated with bortezomib (20 nM) and after 24 hrs cells were harvested for western blot B) Quantitative PCR for MCL-1 of MCL-1 RNAi-transduced Oci-AML3 cells C) MCL-1 RNAi transduced Oci-AML3 cells were incubated with bortezomib (5 nM) and after 24 hrs cells were harvested for western blot D) MCL-1 RNAi transduced Oci-AML3 were incubated with bortezomib and cell survival was analyzed after 24 hrs E) Primary CD34⁺ AML cells (n = 5) were transduced with MCL-1 RNAi and incubated with bortezomib (20 nM) and cell survival was detected after 24 hrs F) Primary CD34⁺ AML cells (n = 4) were treated with bortezomib, obatoclax and the combination of both for 24 hrs and cell survival was measured
with the MCL-1 hairpin and demonstrated a 2 fold decrease in cell survival following bortezomib treatment compared to SCR hairpin transduced cells (Figure 3.5E). Comparable results were also obtained with obatoclax which targets BCL-2 family members, including MCL-1. Primary CD34+ AML patient cells (n = 4) exposed to bortezomib and obatoclax (5 μM) showed a significantly enhanced cell death compared to each agent separately (cell survival after bortezomib 71% ± 7%, obatoclax 52% ± 13%, combination 33% ± 7%, p < 0.05) (Figure 3.5F).

3.4 Discussion

The treatment of AML patients consists of different courses of chemotherapy thereby targeting distinct cell cycle and cell death pathways to optimize the cytotoxicity for the leukemic compartment. In the present study we studied whether the cytotoxic effects of bortezomib on the stem cell-enriched AML CD34+ cell fraction could be enhanced by targeting the anti-apoptotic protein MCL-1 or by co-treatment with TRAIL.

Bortezomib, used at a clinically relevant dosage, induced cell death in a large panel of primary AML cells in accordance with recent data from Rushworth et al. [14]. Residual resistance in some AML samples was proposed to be attributed to high levels of NRF2, which prevented bortezomib induced accumulation of ROS [14]. However, we observed that particularly the CD34− AML fraction was sensitive to bortezomib treatment, while the effects on the leukemic-stem cell-enriched CD34+ AML fraction were not significant. It appeared that NF-κB activity and MCL-1 accumulation but not the cell cycle status, are important determinants for this resistance of CD34+ AML cells towards bortezomib. High NF-κB activity is a distinct property of AML cells and can be induced by the autocrine or paracrine production of cytokines or due to activating mutations such as FLT3-ITD [37, 38]. We also demonstrated that NF-κB was highly activated in AML cells, but the basal NF-κB activity was not different between the CD34+ versus CD34− AML cells. Interestingly, the degree of downregulation upon bortezomib treatment was less pronounced in CD34+ AML cells compared to CD34− AML cells. This suggests that separate regulatory NF-κB pathways are involved in both cell types which are efficiently blocked in CD34− AML cells compared to CD34+ AML cells. It has indeed been suggested that multiple NF-κB pathways exist in different cell types and that the recently identified ‘proteasome inhibitor’-resistant (PIR) pathway might prevent degradation of NF-κB upon bortezomib addition [20, 39, 40].

Here, gene array studies demonstrated that cytokines and chemokines that trigger NF-κB activation are significantly higher expressed in CD34+ AML cells versus CD34− AML cells. This cytokine/chemokine profile might represent and activate the canonical NF-κB pathway in CD34− AML cells, while the PIR pathway might be more present in CD34+ AML cells.

Since increasing the dosage of bortezomib is from a clinical perspective not possible, we investigated alternative options to improve the effectiveness of bortezomib. A strong accumulation of the anti-apoptotic protein MCL-1 was noticed
following treatment of bortezomib. MCL-1 belongs to the family of mitochondrial BCL-2-family and is highly expressed in various subtypes of AML, which is in accordance with the data in our gene array set comparing CD34+ AML and CD34+ normal bone marrow cells [31, 41]. In normal hematopoiesis MCL-1 is required for the survival of hematopoietic stem cells and is involved in the self-renewal of these cells [42, 43]. Several studies have also shown that MCL-1 is necessary for the survival and outgrowth of mouse and human AML cells [41, 44–47]. It has recently been suggested that upregulation of MCL-1 upon proteasome inhibition is due to the activation of the unfolded protein response (UPR). Upon addition of bortezomib to multiple myeloma cells, expression of ATF4 was increased which directly enhanced transcription of the MCL-1 gene and blockage of MCL-1 transcription significantly enhanced the cytotoxic effects of bortezomib in these cells [22]. In accordance, we show that inhibition of bortezomib-induced MCL-1 accumulation strongly enhanced the cytotoxic effects of bortezomib in primary CD34+ AML cells providing evidence that MCL-1 protein plays a significant role in resistance towards bortezomib treatment. Differences in sensitivity to bortezomib of AML CD34+ and AML CD34− cells is most likely not due to variety in MCL-1 accumulation, as MCL-1 accumulation was observed in both AML CD34− and CD34+ cells.

Although promising results have been described on the chemosensitivity of AML cell lines upon TRAIL addition, no significant cytotoxic effect was observed as single agent or in combination with bortezomib in primary AML cells. A large panel of primary AML cells was largely resistant to TRAIL treatment which could not be ascribed to differences in the expression of cell death receptors. The microarray study demonstrated that in particular the DR4 receptor is significantly higher expressed in AML CD34+ cells compared to normal bone marrow CD34+ cells [31]. Despite these findings, neither of these AML cells did respond to DR4 specific variants (unpublished observation) [48].

In summary the results demonstrate that CD34− AML cells are most sensitive to bortezomib treatment at a clinically relevant dosage due to inadequate inhibition of NF-κB activation in CD34+ AML cells and to the accumulation of the anti-apoptotic protein MCL-1. Combining bortezomib with IKK- or MCL-1 inhibitors strongly inhibited the survival of the stem cell-enriched AML CD34+ cell fraction. Therefore, it will be interesting to further investigate these combinations for the treatment of AML patients in the future.


**BIBLIOGRAPHY**


Table 3.2: Clinical characteristics of studied patients
AML was classified according to French American British (FAB) classification. AML = acute myeloid leukemia, n.a. = not available, int = intermediate, wt = wildtype, FLT3-ITD = Fms-like tyrosine kinase 3-internal tandem duplication, NPM = nucleophosmin, NK = normal karyotype

<table>
<thead>
<tr>
<th>AML</th>
<th>FAB</th>
<th>Risk</th>
<th>FLT3</th>
<th>NPM</th>
<th>% CD34</th>
<th>Cytogenetics</th>
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<td>1</td>
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<td>trisomy 4</td>
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<td>2</td>
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<td>wt</td>
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<tr>
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<td>wt</td>
<td>wt</td>
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<td>inv (16)</td>
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<tr>
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<td>ITD</td>
<td>wt</td>
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<td>n.a.</td>
</tr>
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</tr>
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<td>wt</td>
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<tr>
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