Effective targeting of primitive AML CD34+ cells by the second-generation proteasome inhibitor carfilzomib

Lieke H. van der Helm¹, Matthieu C.J. Bosman¹, Jan Jacob Schuringa¹, and Edo Vellenga¹

¹Department of Hematology, Cancer Research Center Groningen (CRCG), University Medical Center Groningen (UMCG), University of Groningen, Groningen, The Netherlands

Published as letter in British Journal of Haematology 2015; 171: 652-655
ABSTRACT

In the present study the sensitivity of AML cells to the second-generation proteasome inhibitors carfilzomib and oprozomib was investigated in comparison with the first-generation proteasome inhibitor bortezomib. In patient-derived AML CD34+ cells and AML cell lines, bortezomib and carfilzomib induced a similar reduction in survival and proteasome activity in short-term cultures, whereas the effect of oprozomib was less pronounced. Importantly, carfilzomib was more effective than bortezomib in targeting the more primitive leukemic cells, as reflected by a significant reduction in quiescent CD34+CD38- cells and stem cell frequency by carfilzomib. In contrast, normal bone marrow (NBM) CD34+ cells were only mildly affected. In parallel with the increased sensitivity, proteasome activity tended to be higher in AML cells compared to NBM and transcriptome analysis showed an increased expression of several proteasome subunits in AML CD34+ cells. Anti-apoptotic MCL-1 was upregulated upon proteasome inhibition, and the effect of the proteasome inhibitors on the survival of primary AML CD34+ cells could be further enhanced by inhibition of MCL-1. Our results indicate that the proteasome inhibitor carfilzomib is more effective in reducing the long-term survival of AML cells as compared to bortezomib and oprozomib, and might be a promising agent for the treatment of AML.
INTRODUCTION

According to the cancer stem cell model, acute myeloid leukemia (AML) is maintained by rare populations of (preleukemic and) leukemic stem cells that are relatively quiescent, resistant to therapy, and causing frequent relapses after intensive chemotherapy.\(^1\)\(^2\) These relapses contribute to 5-year survival rates of only 5-55% in adults, dependent on the cytogenetic risk group.\(^3\) Moreover, with a median age of about 70 years, a large group of AML patients is not eligible for intensive chemotherapy.\(^4\) Therefore, new treatment strategies with lower toxicities which target leukemic stem cells are warranted.

The ubiquitin-proteasome system plays an essential role in protein homeostasis of eukaryotic cells through selective degradation of abnormal and regulatory proteins. The proteasome is a barrel-like complex composed of two outer α-rings and two central β-rings, each containing seven subunits, and two regulatory caps that recognize ubiquitinated proteins. Proteins are cleaved at the proteolytic sites on the β5, β1, and β2 subunit, encoded by the *PSMB5*, *PSMB6*, and *PSMB7* gene, respectively. The proteasome is involved in the regulation of various critical cellular processes including cell proliferation, apoptosis, and DNA repair, and is important for the activation of the pro-survival transcription factor NF-κB by degradation of p-IκBα.\(^5\)\(^6\) In hematopoietic cells, a proteasome variant known as the immunoproteasome containing distinct catalytic subunits (β5, *(PSMB8)*, β1, *(PSMB9)*, and β2, *(PSMB10)*) is present besides the constitutive proteasome.\(^7\)\(^8\) The immunoproteasome is associated with processes of antigen presentation on top of constitutive proteasomal functions.

Several abnormalities of the ubiquitin-proteasome system have been described in leukemic cells including a higher expression of the proteasome in leukemic cells compared to normal peripheral blood cells as determined by immunohistochemistry and elevated proteasome activity in the plasma of AML patients. Moreover, NF-κB activity is shown to be increased in stem cell-enriched AML subpopulations as compared to normal bone marrow (NBM) CD34\(^+\) cells\(^5\)\(^10\), which might be related to the increased activity of various components upstream of NF-κB, such as IRAK1 and TAK1.\(^11\)\(^-\)\(^13\) Inhibition of NF-κB with the proteasome inhibitor MG-132 (which is only applicable *in vitro*) induced apoptosis in AML CD34\(^+\) cells but not in normal CD34\(^+\) cells.\(^14\) Therefore, proteasome inhibition may be a promising treatment strategy in AML.

The first-in-class proteasome inhibitor bortezomib shows clinical effectiveness in multiple myeloma and mantle cell lymphoma.\(^15\) In AML, bortezomib reduces NF-κB activity in particular in the more mature CD34\(^+\) AML cell fraction *in vitro* whereas the AML CD34\(^+\) cells are less sensitive to bortezomib, which can be ascribed to upregulation of the anti-apoptotic protein MCL-1 and improper inhibition of NF-κB.\(^16\)\(^-\)\(^18\) The second-generation proteasome inhibitor carfilzomib and its orally bio-available derivate oprozomib may be more effective since these inhibitors bind irreversibly and more specifically to the proteasome and the immunoproteasome.\(^19\)\(^-\)\(^21\) In clinical trials, carfilzomib was active in bortezomib-refractory and -relapsed multiple myeloma patients.
and induced fewer side effects than bortezomib.\textsuperscript{22-24} Although limited data is currently available on the effect of second-generation proteasome inhibitors in AML, they suggest that AML cells are sensitive to carfilzomib.\textsuperscript{25,26} However, the effect on primitive AML CD34\(^+\) cells is unclear. In the present study we demonstrate that carfilzomib was more effective in reducing the long-term survival of AML CD34\(^+\) cells as compared to bortezomib and oprozomib, whereas normal CD34\(^+\) cells were less affected by carfilzomib. Moreover, addition of an MCL-1 inhibitor increased the cytotoxic effects on AML CD34\(^+\) cells.

**MATERIALS AND METHODS**

**Reagents**

Carfilzomib (lot #6012-85) and oprozomib (lot #1262-071) were kindly provided by Onyx pharmaceuticals. Bortezomib (lot #9EZT500) was obtained from Janssen-Cilag. Obatoclax mesylate (Cat. #S1057) was obtained from Selleckbio.

**Cell lines, patient material and healthy controls**

The human leukemia cell lines HL-60, OCI-AML3, MOLM13, and THP-1 were cultured in RPMI 1640 supplemented with 10% FCS. Bone marrow and peripheral blood samples from AML patients (See Table SI for patient characteristics) and healthy controls were obtained after informed consent in accordance with institutional guidelines and the Declaration of Helsinki. Normal bone marrow (NBM) was obtained from potential donors for allogeneic bone marrow transplantation and patients who underwent elective total hip replacement. Mobilized peripheral blood cells were obtained from healthy donors who underwent apheresis for allogeneic bone marrow transplantation. Neonatal cord blood was obtained from healthy full-term pregnancies from the obstetrics departments of the UMCG and the Martini Hospital Groningen, the Netherlands. Mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Stem Cell Technologies), and CD34\(^+\) cells were selected by MicroBeads on the AutoMACS Pro Separator (Miltenyi Biotec).

**Short-term culture of primary cells**

AML, NBM, and normal peripheral blood CD34\(^+\) cells were expanded for 2-3 days prior to analysis on mouse stromal (MS5) cells in LTC medium (α-minimum essential medium supplemented with heat-inactivated 12.5% fetal calf serum, heat-inactivated 12.5% horse serum, penicillin and streptomycin, 2 mM glutamine, 57.2 µM β-mercaptoethanol and 1 µM hydrocortisone (all from Sigma, Zwijndrecht, The Netherlands) supplemented 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).\textsuperscript{27}
Cord blood CD34+ cells were expanded for 2-3 days in hematopoietic progenitor cell growth medium (HPGM; Lonza, Leusden, The Netherlands) supplemented with c-Kit ligand, Flt-3 ligand (both from Amgen, Thousand Oaks, CA), and TPO (100 ng/mL each) prior to analysis. Cultures were kept at 37°C and 5% CO2.

**Cell viability measurements**

Cell viability was assessed using MTS assays (Promega, Madison, USA). Cells were seeded in 96 wells plates and incubated with bortezomib, carfilzomib, or oprozomib for 24 hrs at 37°C after which MTS reagent was added. Cell viability was determined after 2-4 hrs by measuring the absorption at 490 nm using an iMark microplate reader (Bio-Rad Veenendaal, the Netherlands).

**Flow cytometry analysis and cell sorting**

Fluorescence activated cell sorting (FACS) analyses were performed on an LSR II flow cytometer, FACScalibur (Becton Dickinson (BD), Alpen a/d Rijn, The Netherlands), or MacsQuant (Miltenyi Biotec). Cell sorting was performed by MoFLo (Dako Cytomation, Carpinteria, CA, USA) after staining with CD34-APC (Cat. #555824, Lot #3191690, BD). For analysis of apoptotic cells, cells were stained with AnnexinV-FITC (IQP-120F, Miltenyi Biotec) and PI (IQP-121, IQ Products, Groningen, the Netherlands). For analysis of quiescent cells, cells were re-suspended in hematopoietic progenitor cell growth medium (HPGM, Lonza) and stained with 5 μg/mL Hoechst 33342 (Lot #458868, Invitrogen, Bleiswijk, the Netherlands) at 37°C for 45 min, followed by addition of 1 μg/mL Pyronin Y (Sigma) for 30 min. Cells were washed in the solution containing Hoechst and Pyronin Y, followed by staining with CD34-APC and CD38-Alexa700 (Cat. #303524, Lot #B169984, Biolegend, San Diego, CA) at 4°C for 20 min and FACS analysis. Samples with a maximum of 50% cell death in untreated controls after 24 hrs incubation were analyzed using FlowJo V10 software.

**Chymotrypsin-like proteasome activity measurements**

After 4 hrs incubation at 37°C with proteasome inhibitors, cells were lysed in lysis buffer (50 mM HEPES [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 2mM ATP). Lysis was performed on ice for 30 minutes with vortexing every 10 minutes. After 15 minutes centrifugation at maximum speed, the supernatant was collected and transferred in 96 wells plates containing assay buffer (115 mM NaCl, 1 mM KH2PO4, 5 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, 25 mM sodium HEPES buffer [pH 7.4]. Suc-LLVY-aminomethylcoumarin (AMC; Enzo Life Sciences) was added to each well in a final concentration of 50 mM at the start of the assay. This substrate is cleaved by chymotrypsin-like (proteasome) activity, releasing fluorescent AMC. The rate of fluorescence was measured for 60 minutes at 5 minute-intervals by a Synergy 2 plate reader (Miltenyi Biotek) using 360 nm excitation- and 460 nm emission filters. Non-proteasome background activity was
measured by total inhibition of the proteasome using 1 uM bortezomib and was distracted from all measurements.

**Long-term culture initiating cell (LTC-IC) assays**

CD34\(^+\) cells from AML, NBM, or CB samples were sorted and plated in limiting dilution in 96-well plates pre-coated with MS5 stromal cells and cultured for five weeks in LTC medium. For AML samples, wells containing cobblestone-area forming cells (CFCs) were scored as positive. For NBM and CB samples, wells containing CFCs two weeks after addition of methylcellulose (MethoCult H4230; StemCell Technologies) were scored as positive. The stem cell frequency was calculated using L-Calc Limiting Dilution Software (StemCell Technologies).

**Colony forming cell assays**

NBM CD34\(^+\) cells were incubated in RPMI with the different proteasome inhibitors. After 24 hrs, methylcellulose supplemented with 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL G-CSF, 20 ng/mL c-kit ligand, and 6 U/mL erythropoietin (Cilag Eprex) was added. After two weeks, CFCs were counted.

**Lentiviral transduction**

The pRRL-CMVd2EGFP-SFFV-tdTomato vector was made by inserting the XhoI-Xbal fragment (containing the CMV-d2EGFP cassette) from pCMV-d2EGFP\(^{33}\) (kindly provided by Hiroshi Harada, Radiation and Tumor Biology, Kyoto University, Kyoto, Japan), into the XhoI-NheI cut pRRL-SFFV-tdTomato vector. Lentiviral particles were produced by transient transfection of 293T cells. HL-60 cells were transduced in one round with lentiviral supernatant supplemented with polybrene (4 \(\mu\)g/mL; Sigma, Zwijndrecht, The Netherlands). Tomato positive cells were sorted and used for measurement of proteasome activity.

**Micro-array analysis**

Gene expression profiling of 66 AML CD34\(^+\) samples, 51 AML CD34\(^-\) samples, and 22 NBM CD34\(^+\) samples was performed previously using the Illumina HumanHT-12 Expression BeadChips as published.\(^{34,35}\) We selected from this dataset the seventeen proteasome subunit-coding genes and compared the expression rates by the Mann–Whitney U test. To assess the degree of multiple testing, we performed this analysis within a multivariate permutation test with 1000 permutations, a false discovery rate of 5% and a confidence level of 80%. This resulted in a list of significantly upregulated genes, which, based on permutations, contains no more than 5% false positive associations.
Figure 1. Survival of AML cells was equally reduced by bortezomib and carfilzomib and was less reduced by oprozomib after short-term incubation.

(A) Representative example of apoptosis measurements. MOLM13 cells were incubated with 8 nM bortezomib, carfilzomib, or oprozomib for 24 hrs.

(B) Apoptosis in AML cell lines and CB CD34+ cells after 24 hrs of incubation ($n = 3$ each).

(C) Cell viability in AML cell lines was measured after 24 hrs of incubation by MTS assays ($n = 3$ each).
Immunoblotting

Antibodies against MCL-1 (Calbiochem, Darmstadt, Germany, AM50 (RC13),) and β-Actin (C4) (Santa Cruz Biotechnology, CA, USA) were used in dilutions of 1:300 and 1:1000, respectively. Secondary fluorescent antibodies were obtained from Invitrogen (1:10000). Binding of antibodies was detected by an Odyssey infrared imager (Li-Cor Biosciences, Lincoln, NE).

Statistical analysis

All values are expressed as means ± SE. The Student t test and Wilcoxon signed-rank test were used for comparisons. P-values below 0.05 were considered to be significant.

RESULTS

After short-term incubation, carfilzomib and bortezomib induce an equal reduction of survival in AML cell lines whereas oprozomib induces less cell death.

To investigate whether AML cells and normal CD34⁺ cells are sensitive to the second-generation proteasome inhibitors, the AML cell lines MOLM13, THP1, OCI-AML3, HL-60, and cord blood CD34⁺ cells were incubated with carfilzomib and oprozomib and effects on apoptosis and survival were compared with effects of the first-generation proteasome inhibitor bortezomib. After 24 hrs, we observed a concentration-dependent reduction in viability and induction of apoptosis in all four cell lines (Fig 1A-C). The most sensitive were the MLL-AF9 translocation-bearing cell lines MOLM13 and THP-1, as was in accordance with previous reports on MLL-fusion leukemia cells.36,37 The effects of carfilzomib were comparable to bortezomib, e.g. the IC50 in MOLM13 cells was 7.1 nM for carfilzomib versus 6.2 nM for bortezomib, as measured by MTS viability assays (Fig 1C). Annexin V staining showed comparable results (Fig 1A-B). Cord blood CD34⁺ cells were also affected upon treatment with bortezomib and carfilzomib in this short-term culture setting, but they were less sensitive than MOLM13 and THP1 cells (e.g. 75% ± 23% apoptotic cells in MOLM13 versus 19% ± 14% in cord blood CD34⁺ cells upon 24 hrs incubation with 8 nM carfilzomib; p < 0.001; Fig 1B). At equimolar concentrations, oprozomib induced less cytotoxicity than carfilzomib and bortezomib in all four cell lines. Nevertheless, cord blood CD34⁺ cells were less sensitive to oprozomib than MOLM13 and THP-1 cells (e.g. 94% ± 1.2% apoptotic cells in MOLM13 vs. 29% ± 10% in cord blood CD34⁺ cells upon incubation with 128 nM carfilzomib; p < 0.001).

Proteasome activity is reduced in AML cell lines after incubation with carfilzomib, oprozomib and bortezomib.

To verify whether the reduction in cell survival after 24 hrs of exposure was preceded by a reduction in proteasome activity, we used the fluorogenic substrate SSLVY-AMC to measure the chymotrypsin-like activity as an indicator of the effect on the proteasome enzymatic activities.
Figure 2. Proteasome activity was reduced after incubation with carfilzomib, bortezomib, and oprozomib with prolonged effectivity of carfilzomib.

(A) Chymotrypsin-like proteasome activity was measured in cell lines after 4 hrs of incubation with 5 nM or 20 nM bortezomib, carfilzomib, or oprozomib, and was quantified with fluorescence produced upon cleavage of the proteasome substrate SLLVY-AMC (n = 4 each). (B) Basal chymotrypsin-like proteasome activity in untreated cell lines. (C - D) HL-60 cells were transduced with d2EGFP, incubated for 8 hrs with 20 nM bortezomib or carfilzomib and then washed thoroughly three times with PBS. The mean fluorescence intensity (MFI) of d2EGFP was measured after incubation and 16, 20, and 24 hrs after washing. A representative example (C) and the MFI relative to the untreated control (D) are shown (n = 3).
Indeed, we observed a reduction of chymotrypsin-like proteasome activity in all AML cell lines after 4 hrs of exposure to the three proteasome inhibitors (Fig 2A). Again, carfilzomib and bortezomib showed similar effects (e.g. 90% and 97% reduction, respectively, in HL60 after 4 hrs 20nM; $p = 0.019$ and $p = 0.009$), whereas the effect of oprozomib on chymotrypsin-like proteasome activity was smaller (e.g. 68% reduction in HL60; $p = 0.013$). Basal chymotrypsin-like proteasome activity was higher in the more sensitive MOLM13 and THP-1 cells compared to the less sensitive HL-60 and OCI-AML3 cells (Fig 2B). Because carfilzomib binds irreversibly to the proteasome in contrast to bortezomib, we assessed whether carfilzomib was able to reduce the proteasome activity for a longer period compared to bortezomib. For this purpose HL-60 cells were transduced with short-lived d2EGFP which is rapidly degraded by the proteasome in time, but will accumulate upon proteasome inhibition. Cells were incubated for 8 hrs with bortezomib or carfilzomib, after which the accumulation of d2EGFP was measured at different time points. Directly after incubation, carfilzomib and bortezomib induced similar accumulation of d2EGFP (MFI relative to the untreated control was 1.85 vs. 1.86, respectively; $p = 0.79$), suggesting that both compounds equally inhibited the proteasome activity. However, 16 hrs after removal of the inhibitors, the MFI declined in bortezomib-treated cells but not in carfilzomib-treated cells (relative MFI 1.27 vs. 1.78, respectively; $p = 0.007$), suggesting that carfilzomib indeed had a longer-lasting inhibitory effect on proteasome activity (Fig 2C-D), in line with the described irreversible binding of carfilzomib to the proteasome.

Figure 3. Survival and chymotrypsin-like proteasome activity were reduced in primary AML CD34$^+$ cells upon proteasome inhibition.
(A) Primary AML CD34$^+$ cells ($n = 14$) were incubated for 24 hrs with 50 nM bortezomib, carfilzomib, or oprozomib, after which survival was measured by flow cytometry. The three most sensitive samples towards carfilzomib are shown in grey and the three least sensitive samples are shown in white.
(B) Chymotrypsin-like proteasome activity was measured in primary AML CD34$^+$ cells after 4 hrs of incubation with 5 or 20 nM bortezomib, carfilzomib, or oprozomib ($n = 12$).
Survival and proteasome activity of primary AML CD34+ cells are also equally affected by carfilzomib and bortezomib after short-term incubation.

Next, we investigated the sensitivity of the patient-derived AML CD34+ cell subfraction to the proteasome inhibitors (n = 20, Table SI). After 24 hrs of incubation, we observed a cytotoxic effect of bortezomib and carfilzomib in most of the samples (69% ± 19% survival, p < 0.0001; and 56% ± 20% survival, p < 0.0001, respectively), whereas AML cells were relatively resistant to oprozomib (93% ± 9% survival, p = 0.020) (Fig 3A). Although the variation between the AML samples was distinct, we observed again a similar reduction of cell survival after short-term incubation with carfilzomib and bortezomib. The survival reduction was again associated with a decrease in chymotrypsin-like proteasome activity in primary AML CD34+ cells (Fig 3B).

Carfilzomib slightly reduced the percentage of quiescent AML CD34+CD38- cells and reduced the stem cell frequency of primary AML cells.

We have previously shown that bortezomib primarily affects the more mature AML CD34- cell fraction and has limited impact on AML CD34+ cells.18 Therefore, we assessed the effects of carfilzomib and oprozomib on the AML CD34+ and CD34- cell fractions, and focused in particular on the quiescent cell population. Following 24 hrs of incubation with carfilzomib and oprozomib, we noticed that the CD34- and CD34+CD38- cells were also in this case more affected than the relatively immature CD34+CD38- cells in this short-term assay. For example, upon carfilzomib treatment there was an 81% reduction in CD34- cells (p = 0.04), a 47% reduction in CD34+CD38- cells (p = 0.003), and a 10% reduction in CD34+CD38- cells (p = 0.19; Fig 4A-B). However, we observed with carfilzomib, but not with bortezomib and oprozomib, a slight but significant reduction of quiescent AML CD34+CD38- cells (41% quiescent CD34+CD38- cells vs. 52% in untreated control; p = 0.03; Fig 4C-D). To examine whether the AML stem cell-enriched cell fractions are also functionally affected upon addition of carfilzomib, AML CD34+ cells (n = 10) were cultured on an MS5 stromal layer in limiting dilution for five weeks to determine the long-term culture-initiating cell (LTC-IC) frequency. Importantly, upon a single treatment for 24 hrs with carfilzomib, the LTC-IC frequency was reduced to 47% of the untreated control (± 24%; p = 0.003), while bortezomib and oprozomib did not affect the LTC-IC frequency (97% ± 36%, p = 0.44; and 89% ± 39%, p = 0.21, respectively) (Fig 4E). To address the variable sensitivity of AML CD34+ cells to carfilzomib, we assessed the proteasome activity in three sensitive and two less-sensitive AMLs. We did not detect a difference in basal proteasome activity between these different AMLs. However, following 4 hrs of incubation with carfilzomib, we did not demonstrate any proteasome activity in the sensitive AMLs, while in the insensitive AMLs remaining proteasome activity could still be demonstrated, i.e. 37% and 60% of the starting value (data not shown).
Figure 4. Carfilzomib reduced the percentage of quiescent cells and the stem cell frequency of primary AML cells.

(A-D) AML CD34⁺ cells were expanded on MS5 stromal cells for two days and were incubated in liquid with proteasome inhibitors (50 nM) for 24 hrs. Survival of the different cell populations was determined by flow cytometry. (A) Representative example of CD34/CD38 stainings. (B) Survival percentages of CD34⁺CD38⁻, CD34⁺CD38⁻, and CD34⁻cell populations (n = 9). (C) Representative example of the analysis of quiescent CD34⁺CD38⁻cells. (D) Percentage of AML CD34⁺CD38⁻cells in the G0 phase of the cell cycle (n = 10). The average quiescent cell percentage of the untreated control cells was 52% ± 17%. (E) AML CD34⁺ cells were incubated for 24 hrs on MS5 stromal cells with proteasome inhibitors (20 nM) and then semi-populated weekly in LTC-IC assays (n = 10). The average LTC-IC frequency of the untreated controls was 1/43 ± 1/29. The two most sensitive samples towards carfilzomib are shown in grey and the two least sensitive samples are shown in white.
Figure 5. Normal control cells were only mildly affected by proteasome inhibition. 

(A) Normal bone marrow (n = 9) and normal peripheral blood (n = 2) CD34+ cells were treated with the proteasome inhibitors (20 nM) for 24 hrs and then put in methylcellulose. CFCs were scored after two weeks. The average colony count of the untreated controls was 225 ± 112 per 1000 cells. (B) NBM (n = 3) and cord blood (n = 4) CD34+ cells were incubated for 24 hrs on MSS stromal cells with proteasome inhibitors (20 nM) and then demipopulated weekly in LTC-IC assays. The average LTC-IC frequency of the untreated controls was 1/145 ± 1/177 (C) Percentage of NBM CD34+CD38- cells in the G0 phase of the cell cycle after 24 hrs incubation with the proteasome inhibitors (50 nM; n = 10). The average quiescent cell percentage of the untreated control cells was 64% ± 25%. (D) Chymotrypsin-like proteasome activity in primary AML CD34+ cells (n = 6) and NBM or peripheral blood CD34+ cells (n = 8).

Carfilzomib does not affect NBM CD34+ cells.

To evaluate the effect of carfilzomib on NBM CD34+ cells, we assessed the colony forming potential and LTC-IC frequency of NBM cells upon treatment with carfilzomib. Both the colony forming potential and the LTC-IC frequency were not significantly affected by carfilzomib (i.e. 68% ± 51% in the carfilzomib group compared to controls normalized to 100% (p = 0.053); LTC-IC frequency 91% ± 19% in the carfilzomib group compared to the untreated control (p = 0.11); Fig 5A-B). Furthermore, the frequency of quiescent NBM CD34+CD38- cells was not significantly altered upon exposure to carfilzomib (55% ± 30% vs. 64% ± 25% in controls; Fig 5C). Together, these data suggest that NBM CD34+ cells are less sensitive to carfilzomib than AML CD34+ cells. A difference in sensitivity of AML CD34+ and normal CD34+ cells might be explained by a difference in proteasome activity in AML cells compared to normal cells. By measuring the chymotrypsin-like activity of AML CD34+ (n=6) and NBM CD34+ cells (n=8), we indeed observed a trend towards
increased proteasome activity in AML CD34+ cells (Fig 5D). Higher proteasome activity in AML cells might be the result of higher levels of proteasome complexes due to increased expression of proteasomal subunits. To study this, we compared the expression levels of proteasome subunit coding genes in primary AML CD34+ cells of 66 patients and NBM CD34+ cells of 22 donors from a recently performed microarray.34,35 We observed significantly increased expression levels of nine out of 17 subunit-coding genes and a trend towards increased expression of all subunits in AML CD34+ versus NBM CD34+ cells (Fig S1A). We also observed increased expression of seven proteasome subunit-coding genes in AML CD34+ compared to AML CD34- cells, suggesting that the more primitive AML cells might have increased proteasome activity compared to the more mature cell population (Fig S1B).

**MCL-1 is upregulated in AML CD34+ cells after incubation with bortezomib, carfilzomib, and oprozomib and MCL-1 inhibition sensitizes AML CD34+ cells to proteasome inhibitor-induced cell death.**

The cytotoxic effects of proteasome inhibitors may be limited by the induction of anti-apoptotic signaling. We and others previously observed that treatment of AML CD34+ cells with bortezomib is hampered by upregulation of the anti-apoptotic protein MCL-1.18,38,39 Here, we observed that MCL-1 is also upregulated in HL-60 cells upon treatment with carfilzomib and oprozomib (Fig 6A). Whereas MCL-1 is required for the survival and maintenance of leukemic cells, and inhibition of MCL-1 abrogates leukemic outgrowth, we wondered whether inhibition of MCL-1 further sensitizes primary leukemic cells to carfilzomib and oprozomib. AML CD34+ cells were treated for

Figure 6. MCL-1 was upregulated in AML cells after incubation with bortezomib, carfilzomib, and oprozomib and MCL-1 inhibition sensitized AML CD34+ cells to proteasome inhibitor-induced cell death.

(A) HL-60 cells were incubated with proteasome inhibitors for 24 hrs. The anti-apoptotic MCL-1 isoform 1 was detected by western blot.

(B) Primary AML CD34+ cells (n = 10) were incubated with proteasome inhibitors (50 nM), the MCL-1 inhibitor obatoclax (5 μM), or both for 24 hrs. Survival was measured by flow cytometry.
24 hrs with carfilzomib or oprozomib in combination with obatoclax, a pan-BCL-2 family member inhibitor which is currently under clinical investigation. Co-treatment with obatoclax increased the sensitivity of primary AML cells to the proteasome inhibitors. Survival of AML CD34⁺ cells after 24 hrs of incubation with 50 nM bortezomib, carfilzomib, or oprozomib was 73%, 49%, and 96%, respectively, in the absence of obatoclax, versus 46% \( (p = 0.03) \), 27% \( (p = 0.03) \), and 65% \( (p = 0.04) \), respectively, when combined with obatoclax (Fig 6B).

**DISCUSSION**

The results of the present study demonstrate that carfilzomib and bortezomib are equally effective in targeting AML cells in short-term assays, but, more importantly, that carfilzomib was more effective in targeting the stem cell-enriched CD34⁺ AML cell fraction. The higher sensitivity of primitive AML cells to carfilzomib could be related to the irreversible binding of carfilzomib to the proteasome, which does not result in an advantage in short-term readouts, but results in a decreased viability in long-term assays due to prolonged proteasome inhibition.

NBM CD34⁺ cells were only mildly affected upon carfilzomib treatment. The variation in sensitivity between AML CD34⁺ cells and NBM CD34⁺ cells, and also within different AML cells, is likely related to differences in proteasome- and NF-κB activity. We and others indeed observed an increased chymotrypsin-like proteasome activity and increased proteasome subunit expression in AML CD34⁺ cells compared to NBM cells (Fig 5D, Fig S1A, and). Furthermore, previous studies have shown that AML CD34⁺ cells frequently gain constitutive NF-κB activity, in contrast to NBM CD34⁺ cells. As a consequence of the increased proteasome- and NF-κB activity, the stem cell-enriched AML CD34⁺ cells might be more dependent on these pathways for their survival, which provides a therapeutical window. In addition, the higher sensitivity of cell lines bearing the MLL-AF9 fusion (MOLM13 and THP-1) might be due to an accumulation of the MLL fusion protein upon proteasome inhibition, which is at higher levels detrimental to leukemia cell survival and triggers latent tumor suppression programs.

In the present study, we measured the chymotrypsin-like proteasome activity as an indicator of the effect on proteasome activity. Chymotrypsin-like activity is shown to be a biomarker for clinical response on standard therapies in AML and multiple myeloma patients in contrast to trypsin-like and caspase-like activities. The measurement of chymotrypsin-like proteasome activity covers both the constitutive proteasome- and the immunoproteasome activity which are both inhibited by bortezomib, carfilzomib and oprozomib. It has recently been suggested that higher ratios of the immunoproteasome correlate with sensitivity of AML cells to proteasome inhibition. However, the effects were only determined in AML blasts in short-term read-outs.

Our data suggest that AML cells were less sensitive to oprozomib compared to carfilzomib and bortezomib *in vitro* at equimolar concentrations. Lower efficacy of oprozomib was previously also observed in head and neck cancer cells and various cell lines. Nevertheless, oprozomib largely
inhibited tumor growth *in vivo*. In addition, the limited toxicity to cord blood and normal bone marrow CD34+ cells might provide the opportunity to apply higher doses of oprozomib.

To optimally eradicate AML cells, a combination of anti-cancer agents targeting different oncogenic pathways is presumably the most successful. An interesting target in combination with proteasome inhibitors is the anti-apoptotic protein MCL-1. MCL-1 is required for the maintenance of early hematopoietic progenitors and is highly expressed in AML CD34+CD38- cells as compared to AML progenitors and normal CD34+ cells. In this study, we showed that MCL-1 is upregulated upon incubation with carfilzomib and oprozomib and that simultaneous inhibition of MCL-1 by obatoclax has an additive cytotoxic effect on AML CD34+ cells.

In summary, our data indicate that the second-generation proteasome inhibitor carfilzomib might be more effective in reducing the long-term survival of AML CD34+ cells as compared to bortezomib and oprozomib. Addition of an MCL-1 inhibitor increased the cytotoxic effects on AML CD34+ cells. Therefore, carfilzomib in combination with MCL-1 inhibition is a promising therapeutic option for the treatment of AML patients.

**ACKNOWLEDGEMENTS**

The authors would like to thank Bart-Jan Wierenga for cloning pCMV-d2EGFP in the pRRL vector, Rudolf Fehrmann for the statistical analysis of micro-array data, Jeanet Dales for the preparation of primary material, and Geert Mesander, Henk Moes, and Roelof Jan van der Lei for assistance on cell sorting.
REFERENCES


Table SI. Clinical characteristics of studied patients

<table>
<thead>
<tr>
<th>AML</th>
<th>Diagnosis (WHO)</th>
<th>% CD34(^+)</th>
<th>NPM</th>
<th>FLT3</th>
<th>Cytogenetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AML with cytogenetic aberrations</td>
<td>29%</td>
<td>n.a.</td>
<td>wt</td>
<td>-Y, t(8;21)</td>
</tr>
<tr>
<td>2</td>
<td>AML without maturation</td>
<td>30%</td>
<td>wt</td>
<td>ITD</td>
<td>NK</td>
</tr>
<tr>
<td>3</td>
<td>Acute biphenotypic leukemia</td>
<td>57%</td>
<td>wt</td>
<td>ITD</td>
<td>NK</td>
</tr>
<tr>
<td>4</td>
<td>Acute monocytic leukemia</td>
<td>10%</td>
<td>mut</td>
<td>ITD</td>
<td>NK</td>
</tr>
<tr>
<td>5</td>
<td>Acute monocytic leukemia</td>
<td>85%</td>
<td>n.a.</td>
<td>wt</td>
<td>n.a.</td>
</tr>
<tr>
<td>6</td>
<td>AML with dysplasia</td>
<td>31%</td>
<td>wt</td>
<td>wt</td>
<td>NK</td>
</tr>
<tr>
<td>7</td>
<td>AML without maturation</td>
<td>86%</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>8</td>
<td>AML with minimal differentiation</td>
<td>90%</td>
<td>n.a.</td>
<td>n.a.</td>
<td>del 5q</td>
</tr>
<tr>
<td>9</td>
<td>AML with maturation</td>
<td>57%</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>10</td>
<td>Acute myelomonocytic leukemia</td>
<td>16%</td>
<td>wt</td>
<td>ITD</td>
<td>NK</td>
</tr>
<tr>
<td>11</td>
<td>Acute monocytic leukemia</td>
<td>67%</td>
<td>wt</td>
<td>ITD</td>
<td>NK</td>
</tr>
<tr>
<td>12</td>
<td>AML with dysplasia</td>
<td>25%</td>
<td>n.a.</td>
<td>wt</td>
<td>n.a.</td>
</tr>
<tr>
<td>13</td>
<td>AML without maturation</td>
<td>70%</td>
<td>wt</td>
<td>wt</td>
<td>NK</td>
</tr>
<tr>
<td>14</td>
<td>AML with genetic aberrations</td>
<td>87%</td>
<td>n.a.</td>
<td>n.a.</td>
<td>inv(3), -7, -10</td>
</tr>
<tr>
<td>15</td>
<td>Acute basophilic aberrations</td>
<td>34%</td>
<td>wt</td>
<td>wt</td>
<td>t(9;22), inv(16)</td>
</tr>
<tr>
<td>16</td>
<td>AML with maturation</td>
<td>29%</td>
<td>wt</td>
<td>ITD</td>
<td>NK</td>
</tr>
<tr>
<td>17</td>
<td>AML with dysplasia</td>
<td>58%</td>
<td>wt</td>
<td>ITD</td>
<td>NK</td>
</tr>
<tr>
<td>18</td>
<td>AML with maturation</td>
<td>39%</td>
<td>mut</td>
<td>ITD</td>
<td>t(3;5), +8</td>
</tr>
<tr>
<td>19</td>
<td>AML with genetic aberrations</td>
<td>76%</td>
<td>wt</td>
<td>wt</td>
<td>inv(16)</td>
</tr>
<tr>
<td>20</td>
<td>AML with dysplasia</td>
<td>18%</td>
<td>wt</td>
<td>wt</td>
<td>t(9;22), t(4;11)</td>
</tr>
</tbody>
</table>

AML was classified according to WHO classification. AML, acute myeloid leukemia; NPM, nucleophosmin; n.a., not available; wt, wildtype; mut, mutated; FLT3-ITD, Fms-like tyrosine kinase 3-internal tandem duplication; NK, normal karyotype.
Figure S1. Proteasome subunit-coding genes were higher expressed in AML CD34+ cells (n = 66) versus NBM CD34+ cells (n = 22) or AML CD34− cells (n = 51).

Seventeen subunit-coding genes from a micro-array were compared by the Mann–Whitney U test corrected by a multivariate permutations test. (A) Expression levels of proteasome subunit-coding genes in AML CD34+ cells versus NBM CD34+ cells. (B) Expression levels of proteasome subunit-coding genes in AML CD34+ cells versus AML CD34− cells.