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CHAPTER

Proteasome inhibitors in acute leukemia

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Expert Review of Anticancer Therapy 2013

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ABSTRACT

Proteasome inhibition has been recognized as a novel treatment modality in hematologic malignancies. Initially, the reversible proteasome inhibitor bortezomib (BTZ) demonstrated efficacy in multiple myeloma (MM), which supported its approval for relapsed and refractory MM in 2003. Later on, carfilzomib, a next generation irreversible proteasome inhibitor was FDA approved in July 2012 for relapsed/refractory MM. Currently, several other proteasome inhibitors are undergoing pre-clinical and clinical evaluation. The successes of proteasome inhibitors in MM are now being translated to other hematologic malignancies including acute leukemia. The first clinical studies with BTZ in leukemia revealed promising clinical activity, in particular when combined with conventional chemotherapeutics. Here we summarize and discuss the position of proteasome inhibitors in acute leukemia treatment. Special focus is also attributed to immunoproteasome inhibitors. As a future perspective, it is anticipated that proteasome inhibitors may prove to be of added value in therapeutic interventions for acute leukemia.

INTRODUCTION

Survival of patients suffering from leukemia has improved enormously over the last decades. Nowadays, for pediatric acute lymphocytic leukemia (ALL) the 5-year overall survival is over 85% in high-income countries. This has been achieved by combination chemotherapy regimens including glucocorticoids, vincristine, and asparaginase for remission induction therapy, with an initial remission rate of >95%. However, for pediatric ALL relapse occurs in about 20% of patients and outcome after relapse remains poor with remission rates of 40% to as low as 10% for bad-prognostic subtypes¹. Children with acute myeloid leukemia (AML) treated with cytarabine and anthracyclin-based regimens, currently experience a probability of long-term survival of about 70%. However, 30-40% of pediatric AML patients will experience a relapse, and outcome from relapsed AML is poor². The age of diagnosis is a prognostic factor in acute leukemia. Adult patients with ALL have a substantial worse prognosis than younger patients with a 5-year survival ranging from 50% for younger adults, until 20% for patients older than 45. Similarly, adult AML has a substantial worse prognosis with a 5-year survival from around 40% which declines gradually with age until approximately 4% for patients older than 75³. Accordingly, for the treatment-refractory and relapsed patients with low chances of survival, there is still room for improvement of therapeutic options in conjunction with better supportive care. With respect to novel therapeutic opportunities, proteasome inhibitors are attractive candidates given their proven track record in the treatment of several hematological malignancies, including multiple myeloma (MM) and mantle cell lymphoma. This review aims to position the current and future status of proteasome inhibitors in leukemia treatment with special focus on acute leukemias.

UBIQUITIN-PROTEASOME SYSTEM

The ubiquitin-proteasome system (UPS) controls normal protein homeostasis in cells and thereby influences cellular processes such as intracellular protein processing and degradation, apoptosis, inflammation, antigen presentation, cell growth and survival, and

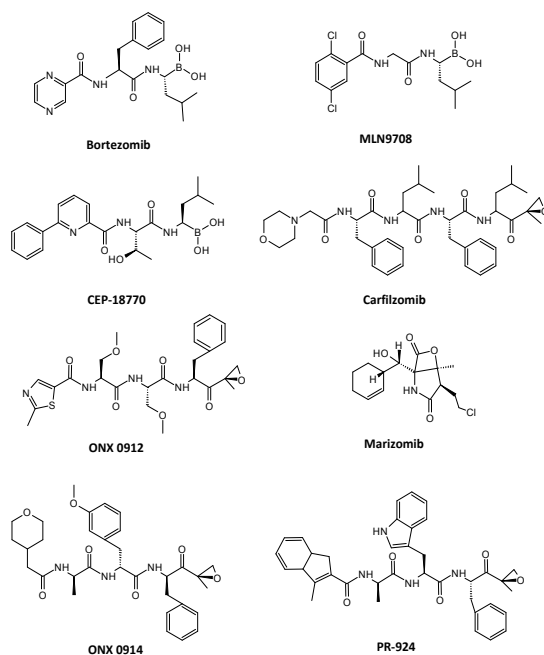


Figure 1. Proteasome inhibitors

further broken down by aminopeptidases to amino acids reutilized for protein synthesis.

cell cycle control. Proteins marked for degradation after ubiquitin conjugation will be degraded by the proteasome⁴. The constitutive 26S proteasome consists of two outer 19S regulatory particles and an inner 20S core particle with 2 identical rings of 7 α -subunits and 2 identical rings of seven β -subunits. The α -subunits are responsible for recognizing and unfolding the ubiquitin-bound proteins and the three catalytically active β -subunits; β 1 (PSMB6, caspase-like activity), β 2 (PSMB7, trypsin-like activity), and β 5 (PSMB5, chymotrypsin-like activity) facilitate proteolysis. Upon protein degradation, shorter peptides are generated that can either be trimmed to 8-9 mer peptides for presentation on major histocompatibility complex (MHC) class I molecules on the cell surface to initiate an immune response, or

IMMUNOPROTEASOMES

Beyond constitutive proteasomes, the immunoproteasome represents an additional variant that is dominantly expressed in hematopoietic cells⁵⁻⁷. The immunoproteasome differs from the constitutive proteasome in the 11S regulatory particles and the catalytically active β -subunits; β 1i (PSMB9), β 2i (PSMB10), and β 5i (PSMB8). Immunoproteasome expression is markedly induced upon stimulation of inflammatory cytokines such as IFN- γ and to a lesser extent TNF- α . For a long time the dominant function of immunoproteasomes was assigned for the provision of antigenic peptides for presentation on MHC class I molecules. Recently, an alternative function of the immunoproteasome was proposed in facilitating efficient clearance of protein aggregates that arise upon interferon-induced oxidative stress, thereby preventing cell death⁸.

PROTEASOME INHIBITION

Because deregulation of UPS-controlled processes is linked to diverse human diseases, the UPS has become an important therapeutic target. In MM cells, sensitivity to proteasome inhibition comes with the fact that these antibody producing cells are highly dependent on their protein turnover and rely on a tight balance between proteasome workload and catalytic capacity⁹. Disruption of proteasome activity results in rapid accumulation

of regulatory proteins in the cell which will cause endoplasmic reticulum stress and consequently lead to apoptosis. Moreover, the induction of the unfolded protein response, which would normally block protein translation and induction of alternative degradation pathways in stress situations, is probably disturbed in MM cells, leading to imbalanced protein homeostasis rendering these cells highly susceptible to proteasome inhibitors⁶. Given original observations that leukemia cells express higher levels of proteasomes¹⁰ and are more prone to undergo apoptosis after proteasome inhibition than normal cells¹¹, the proteasome has also gained interest as therapeutic target for leukemia.

CHARACTERISTICS OF PROTEASOME INHIBITORS

To date, at least eight major structural classes of proteasome inhibitors have been identified and explored in a (pre)clinical setting. These include peptide aldehydes, peptide boronates, peptide α,β -epoxyketones, peptide ketoaldehydes, β -lactones, peptide vinyl sulfones, syrbactins, and oxatiazol-2-ones (reviewed by Kisselev *et al*¹²). The first proteasome inhibitors developed were peptide aldehydes, being reversible inhibitors of the proteasome. However, since they are rapidly inactivated by oxidation and also target other proteases, their clinical development was not further pursued. The chemical structures of the proteasome inhibitors discussed in this review are depicted in Figure 1. Peptide vinyl sulfones and syrbactins did also not reach clinical evaluation due to their toxicity profile. The dipeptyl boronic acid Bortezomib (BTZ) is the first proteasome inhibitor that entered clinical practice and is now routinely used for the treatment of relapsed/refractory MM and mantle cell lymphoma. BTZ is a reversible proteasome inhibitor which mainly targets the CT-L activity (of both constitutive- and immunoproteasome) and to lesser extent the caspase-like (C-L) activity of the proteasome. Relevant clinical disadvantages of BTZ relate to its inability of oral administration, its toxicity profile, next to the emergence of resistance. One other drawback of BTZ is its binding to red blood cells and slow dissociation rate thereof. To overcome these limitations, the orally available boronate MLN9708 was designed, which is a reversible inhibitor of the proteasomal CT-L activity and at higher concentrations also the C-L and trypsin-like (T-L) activities. MLN9708 has a faster proteasome dissociation rate from red blood cells which will be an advantage for improved tissue distribution compared to BTZ¹³. MLN9708 is the first orally available inhibitor to enter clinical trials in MM. A third orally available boronate-based proteasome inhibitor is CEP-18770, which has entered clinical trials in patients with solid tumors and lymphoma. Just like BTZ and MLN9708, CEP-18770 inhibits both CT-L and C-L activities in the nanomolar range. Next to BTZ, the epoxyketone Carfilzomib (CFZ) is FDA approved since July 2012 for MM patients who received at least 2 prior therapies¹⁴. CFZ is an irreversible proteasome inhibitor, which is a potent and more selective inhibitor of the CT-L activity (both constitutive- and immunoproteasome) than BTZ. A beneficial property over BTZ is that CFZ has minimal off-target effects on other proteases, which may underlie fewer side effects. The orally available analog of CFZ is ONX 0912, which currently started clinical testing in hematologic malignancies¹⁵. Lastly, marizomib (Salinosporamide A/NPI-0052) was introduced, which represents a potent proteasome inhibitor with a natural β -lactone active group derived from the marine bacterium *Salinospora tropica*. Marizomib is an irreversible proteasome inhibitor that is different from all other proteasome inhibitors in that it predominantly inhibits the CT-L and T-L activities of the proteasome, and to some extent also the C-L activity¹⁶.

Table 1. Overview of active-site proteasome inhibitors in (pre)clinical development

Name	Class	Reversible/ irreversible	Subunit specificity	Phase	Cohort	Reference
Bortezomib	boronates	reversible	$\beta 5 > \beta 1 > \beta 2$	approved	relapsed MM	101
				phase III	mantle cell lymphoma	101
				phase III	initial MM leukemia	101
MLN9708	boronates	reversible	$\beta 5 > \beta 1 > \beta 2$	phase I/II	relapsed/refractory MM	101
CEP-18770	boronates	reversible	$\beta 5 > \beta 1 > \beta 2$	phase I/II	relapsed/refractory MM	101
Carfilzomib	epoxyketones	irreversible	$\beta 5$	approved	relapsed/refractory MM	101
				phase II	initial MM	33,34,35
ONX 0912	epoxyketones	irreversible	$\beta 5$	phase I	advanced hematologic malignancies	36
ONX 0914	epoxyketones	irreversible	$\beta 5i$	pre-clinical		38,39
PR-924	epoxyketones	irreversible	$\beta 5i$	pre-clinical		6,4
Marizomib	β -lactones	irreversible	$\beta 5 > \beta 2 > \beta 1$	phase I	relapsed/refractory MM solid tumors	101

MM; multiple myeloma

The above-mentioned proteasome inhibitors represent active-site inhibitors and are all currently undergoing clinical evaluation. Two other next generation proteasome inhibitors have recently started pre-clinical testing. ONX 0914 is the first $\beta 5i$ specific irreversible proteasome inhibitor with 40-fold greater specificity for $\beta 5i$ over $\beta 5$ ¹⁷. ONX 0914 is in pre-clinical evaluation for immunologic disorders, and recently also for hematologic malignancies. While ONX 0914 was selected based on efficiency of immunoproteasome inhibition in rats, PR-924 was identified as being more selective for human immunoproteasome. Notably, this epoxyketone tripeptide is 130-fold more selective for $\beta 5i$ than $\beta 5$ ⁶. All the above-mentioned proteasome inhibitors interact with the N-terminal Thr1 site of proteasomes, although by different mechanisms (reviewed by: Huber *et al*¹⁸). Next to these active site inhibitors, also non-competitive proteasome inhibitors were developed, which bind to structural non-active subunits or to regulatory particles^{19,20}. This review will focus on active-site proteasome inhibitors that are currently in pre-clinical or clinical evaluation for hematologic malignancies. An overview of the active site inhibitors is shown in Table 1.

(PRE)CLINICAL STUDIES IN HEMATOLOGIC MALIGNANCIES

Several pre-clinical and clinical studies involving proteasome inhibitors are ongoing to determine its position in the treatment of hematological malignancies. First, an overview will be presented on the recent developments in MM treatment as prototypical disease treated with proteasome inhibitors, followed by discussing recent advances in optimizing acute leukemia therapy.

MULTIPLE MYELOMA

Besides the approval of BTZ for relapsed/refractory MM, phase III studies have been performed in which combinations of dexamethasone (DEX) with BTZ, thalidomide, or lenalidomide (LEN) proved its efficacy as induction regimen²¹. More recent clinical

studies aim to improve induction regimen for newly diagnosed transplant-eligible MM with combining more agents. For example, a phase III study showing that combining BTZ, thalidomide and DEX was superior to thalidomide and DEX²², and another study illustrating the effectiveness of BTZ, LEN and DEX²³, supports the superiority of three-drug regimen. A recurrent theme with BTZ treatment of MM patients has been its association with side effects as symptomatic peripheral neuropathy^{22,24}. This neuropathy may be due to inhibition of HtrA2/Omi, a neuronal cell survival protease²⁵. To diminish peripheral neuropathy caused by intravenously administration of BTZ, subcutaneous administration was tested and showed a lower incidence of peripheral neuropathy²⁶. MLN9708 was the first oral proteasome inhibitor that entered clinical trials in 2009. To date, 12 clinical trials of MLN9708 are ongoing in hematologic malignancies [101]. Interim analysis of 3 dose-escalation phase I studies of (i) once-weekly intravenous MLN9708 in relapsed and refractory lymphoma patients²⁷ and (ii) twice-weekly oral²⁸ and (iii) once-weekly oral MLN9708 in relapsed and refractory MM patients²⁹ showed that the drug is generally well tolerated with infrequent peripheral neuropathy. A phase I/II study in previously untreated MM patients combined weekly oral MLN9708 with LEN and DEX up to twelve 28-day cycles, followed by MLN9708 maintenance therapy. The combination seemed well tolerated, with only 1 out of 65 patients experiencing grade 3 peripheral neuropathy at the recommended phase II dose (2.97 mg/m²)³⁰. Thus, these preliminary data suggest good activity of MLN9708 in relapsed, as well as previously untreated MM patients. As a further extension, recently the first phase III clinical trial (NCT01564537) was initiated in which the combination MLN9708, LEN, and DEX will be compared to a placebo, LEN, and DEX in 703 relapsed or refractory MM patients [101]. Single-agent CEP-18770 entered clinical evaluation in 2007 in patients with solid tumors and MM and was administered twice weekly intravenously. The maximum tolerated dose (MTD) was determined at 1.5 mg/m². At this dose in MM patients, the proteasome activity was inhibited for almost 50%. Fewer cases of neurotoxicity were observed than with BTZ (10% grade 1-2 peripheral neuropathy), although the treatment schedule needs further optimization to maintain optimal proteasome inhibition and to reduce skin rash as the main toxicity³¹. Two phase I/II studies in relapsed and refractory MM are currently ongoing; one study combining CEP-18770 with LEN and DEX in 85 patients (NCT01348919), the other study investigates single-agent CEP-18770 in 67 patients (NCT01023880) [101]. CFZ was the first α',β' -epoxyketone-based proteasome inhibitor to enter clinical trials in 2005. From July 2012 onwards, CFZ is approved for MM patients who progressed during or after therapy with BTZ and an immune-mediated inflammatory drug (IMiD). To date, 34 studies with CFZ are ongoing in hematological malignancies [101]. In the relapsed setting, results of 3 recently published phase II trials of single-agent CFZ in MM showed a lower toxicity profile and lower incidence of peripheral neuropathy than with BTZ³². CFZ has also been examined in phase II combination regimens with DEX and several IMiDs, and is currently being investigated in three phase III clinical trials; the first is randomizing patients between CFZ and DEX versus BTZ and DEX (ENDEAVOR study), the second comparing CFZ, LEN, and DEX versus LEN and DEX, and a third trial comparing CFZ with the best supportive care [101]. Next to its effectiveness in relapsed/refractory MM, CFZ has shown to be effective in phase II studies as treatment for initial MM, combined with LEN and DEX³³, with cyclophosphamide, thalidomide and DEX³⁴, or with thalidomide and DEX³⁵. The oral analog of CFZ, ONX 0912 (Oprozomib), entered clinical evaluation for hematologic malignancies in 2011. Interim analysis of the phase 1b dose-escalation study in 9 patients

with pre-treated hematologic malignancies showed that oral Oprozomib is generally well tolerated. Up to 80% inhibition of proteasome activity was reached at doses below the MTD. Dose-escalation will continue and a phase II expansion at the MTD is planned³⁶. The β -lactone marizomib is currently tested in phase I clinical trials as single agent or combined with DEX in patients with relapsed/refractory MM. A combined interim analysis of two dose-escalation studies showed a different toxicity profile than for BTZ, with no peripheral neuropathy reported. Furthermore, of 15 evaluable patients, 3 were BTZ-refractory and reached PR. Thus, marizomib may be attractive as proteasome inhibitor for further (combination) studies³⁷. The immunoproteasome inhibitor ONX 0914 has been pre-clinically evaluated in immune-competent cells and experimental models of arthritis³⁸, but its efficacy against MM cells is still relatively unexplored. *In vitro*, the MM cell line RPMI-8226 was 10-fold less sensitive for ONX 0914 than for BTZ. A highly BTZ-resistant MM cell line 8226/BTZ100 (resistant to 100 nM BTZ) showed cross-resistance to ONX 0914, but 8226 cells with low levels of BTZ-resistance (8226/BTZ7) retained full sensitivity to ONX 0914³⁹. Interestingly, the novel immunoproteasome inhibitor PR-924 did not induce a cytotoxic response in MM cells at the concentration specific for inhibition of β 5i, this was only achieved in conjunction with β 5 inhibition⁶. Consistently, when used at higher concentrations, PR-924 was able to initiate an antitumor response, also in MM patient samples *in vitro*⁴⁰.

LEUKEMIA

Based on the promising results in MM, BTZ also underwent clinical evaluation in patients with leukemia, with currently 28 studies recruiting patients [101,102]. A summary of ongoing clinical trials of proteasome inhibitors in acute leukemia is presented in Table 2. Studies published so far showed modest single-agent activity in children⁴¹ and adults⁴²⁻⁴⁴. However, phase I studies in which BTZ was combined with conventional chemotherapeutics showed promising clinical activity in adult AML patients⁴⁵⁻⁴⁷ and pediatric ALL patients^{48,49}. Conversely, a phase II study of BTZ combined with re-induction chemotherapy in relapsed pediatric AML patients did not show improved CR rate or overall survival⁵⁰. An *ex vivo* study of pediatric patient samples revealed that ALL cells were more sensitive to BTZ than AML cells and normal bone marrow. Interestingly, the majority of AML samples resistant to DEX showed sensitivity to the combination of BTZ and DEX⁵¹. Szczepanek et al⁵² showed that BTZ was even more potent in T-ALL patient samples compared to common/pre-B ALL. In this study BTZ appeared to be a potent drug for this relatively therapy-resistant subgroup of ALL, although the two T-ALL patients included in a phase II clinical trial with BTZ did not reach a CR⁴⁹. In this context, it should be taken into account that the recent clinical trials include heavily pre-treated patients. We and others observed sensitivity of primary human AML and ALL patient samples for BTZ and CFZ in the nanomolar range, in favour of CFZ^{53,54}. Additionally, pediatric ALL patient samples were significantly more sensitive than AML samples for BTZ, CFZ, ONX 0912, ONX 0914, and DEX⁵⁴. Single agent CFZ is currently tested in a dose-escalation phase I clinical trial (NCT01137747) in adult patients with relapsed AML and ALL [101]. ONX 0914 has recently been tested in a T-ALL cell line showing proteasome inhibition in the nanomolar range, although its cell growth inhibitory potency was 30-fold and 55-fold less than BTZ and CFZ, respectively. Moreover, BTZ-resistant ALL cell lines were cross-resistant to ONX 0914³⁹.

COMBINATION THERAPIES

One of the main challenges in chemotherapeutic interventions is to find optimal conditions for single drugs or drug combinations that selectively target malignant cells while sparing normal cells to limit drug-induced toxicities. For BTZ modest clinical activity was seen as single-agent in hematologic malignancies, whereas combination therapies were clearly superior. Notwithstanding this fact, the known toxicity profile of BTZ invites for further optimization of combination therapies to achieve a broader proteasome inhibition at lower and probably safer doses. The majority of the combinations involving BTZ in MM and lymphoma patients showed synergistic antitumor efficacy in pre-clinical studies and therefore provide the rationale for clinical trials. Ongoing combination clinical trials of BTZ include those with histone-deacetylase inhibitors (HDACi), kinase inhibitors, farnesyltransferase inhibitors, Bcl-2 family inhibitors, and heat-shock protein inhibitors (reviewed by Wright⁵⁵). Accumulating data indicated that most combinations were tolerable and supported interest for studying combinations with next generation proteasome inhibitors, both for MM and leukemia. Below an overview is presented of pre-clinical studies performed in MM and leukemia on combinations of proteasome inhibitors with conventional chemotherapeutics, with other proteasome inhibitors, and with HDACi.

PROTEASOME INHIBITOR + CONVENTIONAL CHEMOTHERAPEUTICS

The initial therapeutic benefits for MM by BTZ and CFZ combined with conventional chemotherapeutics for MM, set the stage for recently initiated use of the next generation proteasome inhibitors in the combination studied for MM as well as leukemia. CEP18770 combined with melphalan synergistically induced anti-MM effects *in vitro* and inhibited tumor growth, even in BTZ and melphalan-resistant MM mouse models⁵⁶. Subsequently, CEP-18770 was combined with DEX and LEN and was shown to significantly reduce tumor growth compared with LEN or DEX alone⁵⁷. Combinations of low concentrations of MLN9708 with LEN or DEX triggered synergistic activity in MM *in vitro*⁵⁸, while ONX 0912 combined with DEX or LEN were illustrated additive¹⁵. Also Marizomib showed synergistic apoptosis induction when combined to LEN in a series of MM cell lines and samples of relapsed MM patients⁵⁹. In leukemic cell lines, BTZ was shown to interact in an additive or synergistic way when combined with traditional anti-leukemic drugs, including glucocorticoids⁶⁰. Furthermore, BTZ and CFZ combined with idarubicin or cytarabine showed additive antiproliferative and proapoptotic effects on primary AML blasts⁵³. Together, these results demonstrate the potential of incorporating proteasome inhibitors in conventional chemotherapeutic regimens for hematologic malignancies, thereby paving the way to limit off-target effects and limit toxicity.

PROTEASOME INHIBITOR + PROTEASOME INHIBITOR

Combinations of BTZ and marizomib below their individual IC₅₀ showed synergistic effects in MM, leukemia, and lymphoma cell lines. Furthermore, the combination significantly decreased viability in tumor cells from 5 relapsed MM patients (2 being BTZ-resistant) and reduced tumor growth in a xenograft MM mouse model without any noticeable toxicity⁶¹. CEP-18770 in combination with BTZ synergistically induced apoptosis in two MM cell lines and significantly delayed tumor progression in MM mouse models compared to each agent

Table 2. Summary of ongoing clinical trials of proteasome inhibitors in acute leukemia

Study	time period	n	Phase	Cohort	Age-group	Sponsor	reference
Bortezomib + belinostat	May 2010 - February 2014	24	phase 1	relapsed/rafractory acute leukemias	>18y	Virginia Commonwealth University, Richmond, VA, USA	NCT01075425 [27]
Bortezomib + mitoxantrone + etoposide + cytarabine	January 2006 - January 2014	55	phase 1/2	relapsed/rafractory acute leukemias	>18y	Thomas Jefferson University, Philadelphia, PA, USA	NCT00404023 [27]
Decitabine vs. bortezomib + decitabine	November 2011 - November 2013	172	phase 2	AML	>60y	National Cancer Institute, USA	NCT01420926 [27]
Bortezomib	November 2011 - September 2013	35	phase 2	AML in remission	>18y	Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium, Seattle, WA, USA	NCT01465386 [27]
Bortezomib + mitoxantrone + etoposide + cytarabine	July 2010 - June 2012	24	phase 1	relapsed/rafractory AML	18-70y	Case Comprehensive Cancer Center, Cleveland, OH, USA	NCT01127009 [27]
Bortezomib + midostaurin vs. mitoxantrone + etoposide + mitoxantrone + vorinostat + sorafenib	August 2010 - August 2014	42	phase 1	relapsed/rafractory AML	>18y	Cancer Center, Columbus, OH, USA	NCT01174888 [27]
Bortezomib + vorinostat + sorafenib	February 2010 - February 2015	38	phase 1/2	AML	>18y	Indiana University, Bloomington, IN, USA	NCT01534260 [27]
Bortezomib +/- several chemotherapeutic drugs in randomization arms	June 2011 - February 2015	1250	phase 3	initial AML	>29y	National Cancer Institute, USA	NCT01371981 [27]
Bortezomib + Doxil (doxorubicin)	March 2013 - March 2017	30	phase 2	AML	18-80y	University of California, Davis, CA, USA	NCT01736943 [27]
Bortezomib + vorinostat + dexmethasone + methotrexate	June 2011 - June 2013	33	phase 2	relapsed/rafractory ALL	2-30y	Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA	NCT01312818 [27]
Bortezomib + intensive reinduction chemotherapy	March 2009 - September 2014	151	phase 2	relapsed ALL	1-31y	Children's Oncology Group, USA	NCT00873093 [27]
Bortezomib + Nelivnavir mesylate	July 2010 - March 2013	24	phase 1	relapsed or progressive advanced hematologic cancer	>18y	Swiss Group for Clinical Cancer Research, Switzerland	NCT01164709 [27]
Bortezomib + dexmethasone + vincristine + methotrexate	September 2009 - September 2012	24	phase 2	relapsed/rafractory ALL	0.5-19y	Erasmus Medical Center, Netherlands	NTR1881 [42]
Carfilzomib	October 2010 - August 2013	36	phase 1	relapsed/rafractory CLL, SLL (small), PLL (pro)	>18y	Ohio State University Comprehensive Cancer Center, Columbus, OH, USA	NCT01212380 [27]
Carfilzomib	September 2010 - April 2013	18	phase 1	relapsed/rafractory ALL and AML	>18y	Washington University School of Medicine, St. Louis, MO, USA	NCT01137747 [27]
NPI-0052 (marizomib)	July 2007 - December 2012	50	phase 1	advanced malignancies	>18y	Nereus Pharmaceuticals Inc, San Diego, CA, USA	NCT00629473 [27]

AML, acute myeloid leukemia, ALL, acute lymphocytic leukemia, CLL, chronic lymphocytic leukemia, SLL, small lymphocytic leukemia, PLL, prolymphocytic leukemia

alone⁵⁶. Lastly, ONX 0912 combined to BTZ also showed synergistic effects in a MM cell line¹⁵. Thus, combination regimens including two proteasome inhibitors seem effective in enhancing anti-MM activity. However, the molecular basis underlying these synergistic effects remain elusive and still needs confirmation in a clinical setting.

PROTEASOME INHIBITOR + HISTONE DEACETYLASE INHIBITOR

Since several HDACs are relevant for the formation of protein aggregates, the combination of proteasome inhibitors with HDACi may be highly advantageous. Several pre-clinical studies have been performed to explore the added value of HDACi in BTZ combination therapy for MM⁶². The most widely studied HDACi in this respect vorinostat (SAHA), revealing promising outcomes in relapsed/refractory MM³². Based on encouraging results with BTZ, also combination studies of next generation proteasome inhibitors were recently performed. Chauhan *et al*^{15,58} demonstrated that MLN2238 combined to vorinostat, and ONX 0912 combined with the HDACi MS-275 were synergistic in a MM cell line. Beyond MM, combinations of proteasome inhibitors and HDACi have also been tested in leukemic cell lines and patient samples. Combining BTZ with the HDACi Trichostatin A displayed synergistic or additive effects in ALL and AML cell lines⁶⁰. Furthermore, combining BTZ and valproic acid showed additivity in an AML cell line and synergism in a therapy-resistant AML variant⁶³. Consistently, greater inhibition of proliferation of AML blasts was noted when combining BTZ with vorinostat compared to each drug alone⁵³. Also marizomib was found to synergistically induce apoptosis with HDACi MS-275, even with greater potency than for BTZ combined with MS-275¹⁶. Altogether, these data strongly support the potential for combining proteasome inhibitors with HDACi in clinical trials in leukemia. Currently, 13 clinical trials are combining BTZ and a HDACi (mostly vorinostat) in hematologic malignancies, of which 2 in leukemia. The first phase II trial (NCT01312818) examines BTZ, vorinostat, and DEX in patients with relapsed or refractory ALL from 2-30 years of age. The second phase I/II study (NCT01534260) evaluates BTZ, vorinostat and sorafenib in a subgroup of adult patients with AML [101].

PROTEASOME INHIBITOR RESISTANCE

Primary and acquired resistance and the fact that duration of response decreases at subsequent therapies remains a recurrent theme for most chemotherapeutic drugs. This also holds true for proteasome inhibitors like BTZ in a MM treatment setting. Despite the fact that BTZ-retreatment was found to be effective, the response rates as well as the duration of response were reduced as compared to initial treatment, hence indicating the development of BTZ-resistant subgroups of patients⁶⁴. The mechanisms of BTZ resistance in the clinic are still poorly understood, but can have a multifactorial basis. It was reported that a subset of MM and mantle cell lymphoma patients harbor constitutive NF- κ B activity which did not respond to inhibition by BTZ and induction of apoptosis⁶⁵. Of further notice, patients with a mutation in the NF- κ B gene were more sensitive to BTZ-treatment than patients without such mutation⁶⁶, providing additional evidence that NF- κ B activity is an important determinant for BTZ-resistance. Another determinant is the microenvironment in bone marrow stroma, which contributes to MM cell survival and drug-resistance. By releasing selected cytokines (e.g. IL-6), these stromal cells trigger induction of NF- κ B activity⁶⁷. *In vitro* studies revealed

that prolonged exposure of MM and leukemia cell lines to BTZ provoked acquired resistance to BTZ associated with point mutation(s) in the *PSMB5* gene encoding the $\beta 5$ subunit. These mutations introduced substitutions for amino acids that are critical of optimal BTZ in the $\beta 5$ -binding pocket and may thus give rise to a decreased BTZ binding affinity³⁹. Conceivably, to compensate for this defect, upregulated expression of mutated $\beta 5$ was observed in BTZ resistant cell lines whereas expression of non-mutated immunoproteasome subunits was downregulated. Although *PSMB5* mutations were not yet identified in patients lacking BTZ response, upregulation of *PSMB5* gene expression has been reported in a MM patient resistant to BTZ-based therapy compared to pre-treatment expression⁶⁸. Other mechanisms of BTZ resistance are reviewed by McConkey and Zhu⁶⁹ and Kale and Moore⁷⁰. Next generation proteasome inhibitors were also designed for their ability to overcome BTZ resistance⁷⁰. Consistently, CFZ was shown to overcome resistance to BTZ⁷¹. Recently, however, epoxyketone-based proteasome inhibitors such as CFZ and ONX 0914 were found to be substrates for the drug efflux transporter P-glycoprotein (Pgp) and Pgp-overexpressing cells displayed resistance to these drugs⁷². Moreover, acquired resistance to CFZ can be conferred by upregulation of Pgp⁷³. Notably, BTZ and marizomib are no preferred Pgp substrates^{72,74}. Intriguingly, the marine bacterium *Salinospora Tropica* that produces marizomib, has developed a self-resistance mechanism to its own natural product in that it harbors a proteasome $\beta 5$ subunit with a single amino-acid substitution⁷⁵, identical to ones found in human MM and leukemia cell lines with acquired BTZ-resistance³⁹.

EXPERT COMMENTARY

Disease relapses remain a significant impediment for cure in acute adult and pediatric acute leukemia. Empirically, in pediatric ALL, a good response to glucocorticoids is a favorable prognostic factor for survival. Hence, GC-resistant and relapsed ALL patients may benefit from GC-sensitization strategies, for which proteasome inhibitors may be attractive candidates. Specifically, observations that leukemia cells express higher levels of proteasomes compared with normal cells, NF- κ B activity in leukemic stem cells is upregulated compared with normal hematopoietic stem cells, and non-overlapping mechanisms of action of proteasome inhibitors with other leukemic drugs, provides a sound rationale of incorporating proteasome inhibitors in the treatment of (relapsed) acute leukemia. Importantly, also the availability of orally formulated proteasome inhibitors is of clinical benefit. Successful clinical activity of BTZ in MM and lymphoma patients made its translation to leukemia an obvious choice although early clinical studies in leukemia with single-agent BTZ were somewhat disappointing. For single drug and/or prolonged (oral) proteasome inhibitor exposure emergence of acquired drug resistance, as observed in MM patients, is a potential limiting factor. Most optimal exploitation of proteasome inhibitors should therefore come from combination therapies with other conventional chemotherapeutic agents. In fact, combinations of proteasome inhibitors with GCs or HDAC inhibitors showed successful pre-clinical and clinical results. Thus, proteasome inhibitors can be a valuable extension to current chemotherapy of AML/ALL, especially in cases of drug-resistance. Importantly, BTZ therapy has been associated with toxic side effects as peripheral neuropathy. Schedule alterations allowed better handling of toxicity profiles, and could further be diminished when BTZ is used at lower concentrations in combination therapies or by the introduction of next generation proteasome inhibitors devoid of toxicities noted for BTZ. Finally, considering the abundant

expression and functional relevance of the immunoproteasome in hematological cells, novel opportunities for selective targeting of the immunoproteasome has gained considerable recent interest. Recent work indicated that the therapeutic index can be improved by specifically targeting the immunoproteasome. Intriguingly, however, initial studies revealed that rationally designed immunoproteasome inhibitors do not elicit an anti-leukemic effect as single agent at concentrations that maximally inhibit immunoproteasome activity. Therefore, their role in leukemia treatment awaits further mechanistic and clinical studies.

FIVE-YEAR VIEW

Although major improvements have been achieved in the treatment of pediatric and adult leukemia patients, the prevention of relapse and further improvement of survival of relapsed patients still leaves many clinically and laboratory-directed challenges. In the upcoming years, we anticipate further attempts in designing fine-tuned treatment protocols involving a proteasome inhibitor combined with classical and novel compounds with proven efficacy in leukemia treatment, such as HDACi and GCs. To diminish BTZ-induced toxicities, it will be possible to switch to the orally available next generation proteasome inhibitors e.g. MLN9708 or ONX 0912. MLN9708 has shown similar efficacy as BTZ in MM patients, but obviously its oral administration is more comfortable for patients and is therefore worthwhile to be tested in the clinical leukemic setting. Furthermore, there is an absolute need for randomized clinical trials to be able to compare the added value of proteasome inhibitors to the treatment of leukemia. Spin-off studies from these clinical trials may include laboratory-directed studies aimed to identify parameters of response and/or onset of drug resistance and unraveling the molecular basis thereof. Because of the increasing interest into the role of immunoproteasome, the impact of immunoproteasome-inhibitors deserves further evaluation from a perspective of their effect on the immune response and potential anti-inflammatory effects and alterations in cytokine environment of leukemia cells in relation to therapy response. Also, since targeting of the immunoproteasome alone did not elicit marked anti-leukemic effects, further testing of these inhibitors in combination regimen seems warranted. Lastly, our incomplete knowledge of the dynamics of constitutive- and immunoproteasome expression and their differential regulation in leukemic cell subtypes deserves further exploration to build better prediction models for therapy response. In this context, preliminary data from our laboratory (unpublished results) indicates that ratios of immunoproteasome/constitutive proteasome subunit expression may serve as a potential parameter for assessing proteasome inhibitor sensitivity in leukemic patient blast cells.

KEY ISSUES

- Disease relapses remain a significant impediment in acute leukemia, leaving opportunities for novel drug treatments to improve long term response and survival.
- Proteasome inhibition has become a topic of current interest as novel treatment strategy in hematologic malignancies, with BTZ as the first prototypical proteasome inhibitor approved for relapsed and refractory MM in 2003.

- BTZ-including therapies have been associated with toxic side effects, notably peripheral neuropathy. Next generation proteasome inhibitors, including ones available for oral administration, facilitate overcoming this problem.
- Carfilzomib is the first next generation irreversible proteasome inhibitor approved by the FDA (July 2012) for relapsed/refractory MM, setting the stage for several other proteasome inhibitors currently undergoing clinical or pre-clinical evaluation.
- The immunoproteasome has been recognized for targeted interventions. Rationally-designed immunoproteasome inhibitors are being examined preclinically and await clinical evaluation.
- Emergence of drug-resistance should be considered for repeated and long-term administration of (immuno) proteasome inhibitors. Several potential molecular mechanisms of resistance to proteasome inhibitors have been identified.
- Initial clinical studies with BTZ in leukemia revealed promising clinical activity, particularly when used in combination with conventional chemotherapeutics.
- Future research should include design of randomized clinical trials and fine-tuning on optimal combination therapies involving a proteasome inhibitor combined with conventional drugs, other proteasome inhibitor, or histone-deacetylase inhibitors, with the aim of improving efficacy, response and survival, and diminishing toxicity.

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