Molecular mechanisms of bortezomib resistance in acute leukemia

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Chapter 2

Proteasome inhibitors in leukemia

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INTRODUCTION

Although treatment of patients suffering from leukemia improved throughout the last decades, new chemotherapeutic agents are still required to minimize side effects and increase event free survival rates. Many leukemia patients still suffer from a relapse following initial therapy.[2-5] Since patients with a relapse often prove more resistant to chemotherapeutics[3,6], it is important to develop new drugs that act through other cellular pathways to minimize cross-resistance and increase response. In this context, the use of proteasome inhibitors might prove a huge step forward since these inhibitors not only act on a very powerful regulatory target, but also influence several cellular pathways simultaneously. Moreover, these drugs may sensitize malignant cells to conventional anticancer drugs.

Proteasomes are among the most ingenuous key regulators of the functioning cell. The proteasome is responsible for degradation of many intracellular proteins, thereby helping to maintain the cellular homeostasis during biological processes such as cell cycle, signal transduction, response to stress and gene transcription. Among other functions, the proteasomal complex rapidly turns over misfolded proteins to avoid accumulation of dysfunctional proteins.[7-9] Furthermore, the proteasome, in particular the immunoproteasome, generates small peptides to initiate immune responses.[10] These peptides bind to major histocompatibility complex (MHC) class I molecules and are transported to the plasma membrane.[11-15] If the immune system does not tolerate the displayed peptide, cytolytic CD8 T-lymphocytes will eradicate the cell.[16]

In multiple myeloma (MM), proteasome inhibitors have been shown to be very successful.[17-25] Not only do these inhibitors act on MM cells themselves, but they also downregulate protective interactions with bone marrow stromal cells and inhibit blood vessel development.[26,27] Proteasome inhibitors can be more effective than traditional drugs such as glucocorticoids when used as a single drug, and interact in an additive or even synergistic way when combined with these drugs.[28-30]

The cancer cell selectivity for proteasome inhibitors is favorable. Multiple myeloma cells and leukemic cells are significantly more sensitive to proteasome inhibition than $CD34^+$ bone marrow progenitor cells or lymphocytes from healthy persons.[20,31-35] Furthermore, proteasome inhibitors inhibit leukemic stem cells very specifically.[36] Finally, proteasome inhibition increases sensitivity of cancer cells to several anti-cancer treatments such as glucocorticoids, anthracyclines, gemcitabine, cisplatin, immunomodulatory drugs (IMiDs), inhibitors histone-deacetylase inhibitors (HDACi), kinase inhibitors, farnesyltransferase, inhibitors, Bcl-2 family inhibitors and heat-shock protein inhibitors and radiation.[28,37-43]

This review describes the knowledge of proteasome inhibitors at the start of this thesis project with a focus on leukemia. In addition, an updated overview of published and ongoing clinical trials with proteasome inhibitors in leukemia is presented.

UBIQUITIN-PROTEASOME PATHWAY

More than 80% of all eukaryotic protein degradation is controlled by the ubiquitin-proteasome pathway.[1,8,10,44] This pathway regulates protein ubiquitination, and subsequent recognition and degradation by the proteasome (Figure 1).

The proteasome is present in both the cytoplasm and nucleus of cells.[45,46] The 26S proteasome is a large intracellular protease (1,500-2,000kDa) that consists of a 20S core catalytic complex and two 19S regulatory subunits.[47-49] The 20S proteasome complex is a macromolecule of 700 kDa, made up of four stacked rings. The two outer rings contain seven α-subunits, while the two inner rings consist of seven β-subunits. The β-1, -2, and -5 subunits contain the postglutamyl peptidyl hydrolytic-, tryptic-, and chymotryptic-like proteolytic activities of the proteasome, respectively.[47,48,50,51] Together, these three can hydrolyze almost all peptide bonds of proteins, thus forming smaller polypeptide

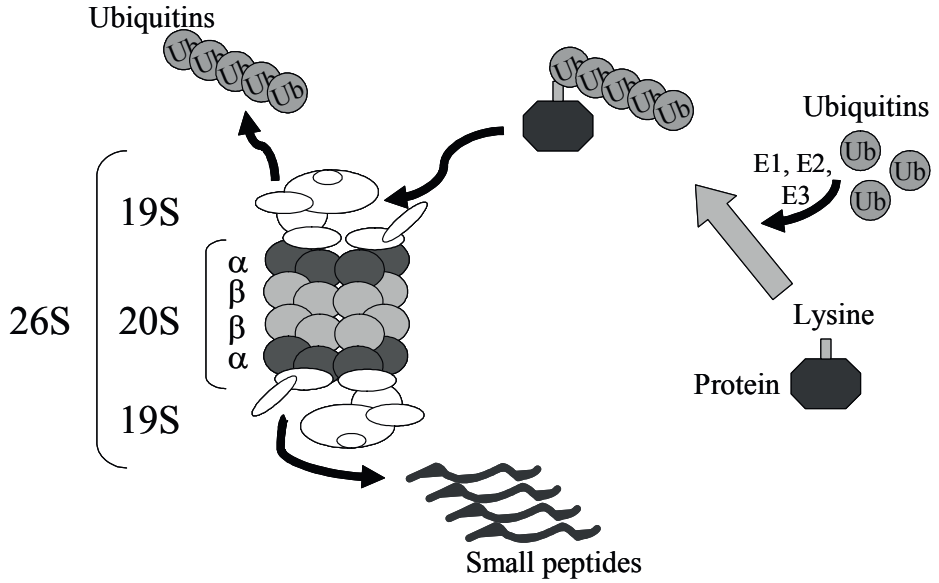


Figure 1. Constellation and functional representation of the mechanism of action of the proteasome. Upon degradation, proteins become ubiquitinated by enzymes E1, E2 and E3. After ubiquitination, the protein is targeted to the 19S complex of the proteasome, where it is de-ubiquitinated and unfolded. Subsequently, the protein is processed to the 20S complex, where it is further degraded into peptides. The ubiquitin components can be recycled.

units. When combined with the two 19S regulatory units, the 26S proteasome is formed. This form of the proteasome is the most important mediator of protein degradation.

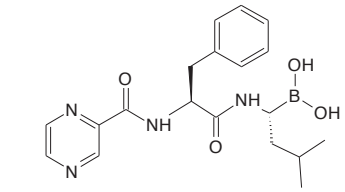
In addition, upon γ interferon exposure, immuno β-type variants (β1i/LMP2, β2i/MECL-1 and β5i/LMP7) are incorporated instead of the constitutive β-subunits leading to the formation of the immunoproteasome (reviewed in Tanaka *et al.*[52-54]). This proteasome variant plays a import role in MHC I mediated antigen presentation[55] and prevention of IFN-triggered oxidative stress induced protein aggregates formation[56].

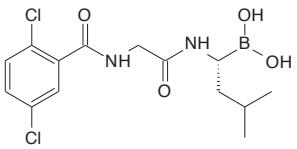
The ubiquitin-conjugating system targets proteins for degradation by attachment of poly-ubiquitin (Ub) chains.[57] This ubiquitination is mediated by three enzyme families: E1, E2 and E3. The Ub-activating E1 enzyme binds and activates ubiquitin. The E2 and E3 families consist of many members. One of the Ub-conjugating enzymes E2 transfers the activated ubiquitin to an E3 family member, after which this E3 Ub-ligase can mediate the attachment of Ub to the desired protein. By repeating this step, a Ub chain is formed.[8,58] After attachment of Ub chains to a protein, this protein binds to the subunits of the 19S complex, where it is de-ubiquitinated and subsequently unfolded. The Ub-components can then be recycled. Following unfolding, the protein is processed to the 20S complex, where peptides of various lengths (3-22 amino acids) are formed and trimmed by aminopeptidases for antigen presentation[59,60] or complete hydrolysis to amino acids for recycling in protein synthesis[61].

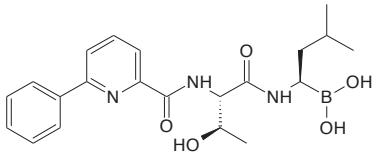
PROTEASOME INHIBITORS

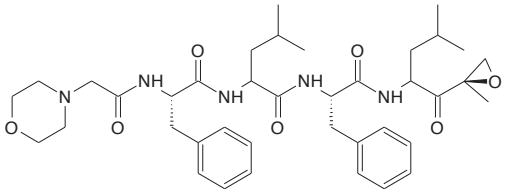
Proteasome inhibitors block cancer progression by interfering with the degradation of regulatory proteins. It is assumed that the ratio of pro- and anti-apoptotic proteins within a cell becomes disturbed, thereby resulting in an increased sensitivity to drug induced apoptosis.[62] Additionally, proteasome inhibition can cause apoptosis by directly affecting the levels of various specific proteins like inhibitory protein IκB, thereby inactivating the survival protein nuclear factor κB (NF-κB).[63,64] Proteasome inhibition can also lead to increased activity of p53 and pro-apoptotic Bax protein, and accumulation of cyclin-dependent kinase inhibitors like p27 and p21.[48,65-68]

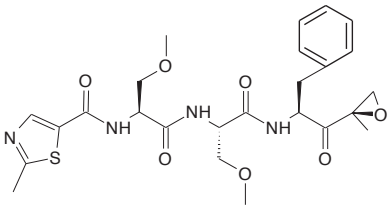
Currently, many proteasome inhibitors have been described, including MG-132, ALLnL, lactacystin, epoxomycin, bortezomib, NPI-0052/marizomib, PR-171/carfilzomib, PR-047/ONX 0912/oprozomib, PR-957/ONX 0914 and MLN9708/ixazomib.[65,69-80,80,81] Features of the most well-described proteasome inhibitors are summarized in Table I and chemical structures are depicted in figure 2. These inhibitors can be classified into five major groups: peptide aldehydes, peptide vinyl sulfones, peptide boronates, peptide epoxyketones, and β-lactones.[74,82-85] Peptide aldehydes, peptide vinyl sulfones and β-lactones lack enzyme specificity, are metabolically instable, or bind irreversible to the proteasome.[65]

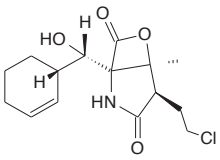


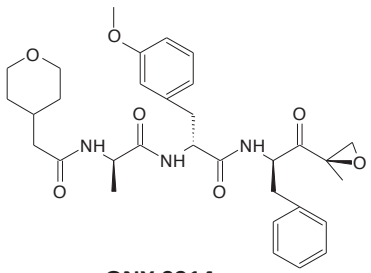












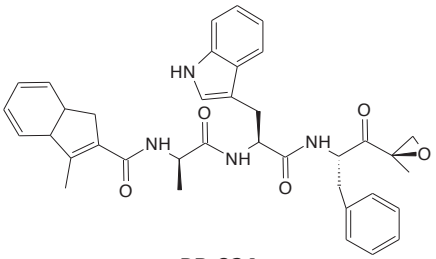


Figure 2. Chemical structures of proteasome inhibitors

Peptide boronic acids were the first suitable group for clinical usage. They dissociate in a slower rate from the proteasome, and have up to 1,000-fold higher potency than peptide aldehydes, are selective and bind reversibly to the proteasome.[65,86-88] Epoxyketones are quite specific and irreversible inhibitors of the proteasome. In addition to inhibitors that target the constitutive proteasome, immunoproteasome inhibitors are available and possibly effective in leukemia.[53,89] Several proteasome inhibitors are emerging to clinical trials with promising results in treatment of several malignancies.[20,90] Currently, several members of this group are emerging to clinical trials with promising results.[20]

Table I. Proteasome inhibitors

Class	Compounds	Binding to proteasome	Binding to other targets	Specificity and mechanisms
Peptide aldehydes	MG-132, ALLnL, ALLnM, LLnV, PSI.	Reversible	Calpain I, Cathepsins	Interact with the catalytic threonine residue of the proteasome.
Peptide boronates	Bortezomib, MG-262, PS273 CEP-18770 (delanzomib) MLN9708/MLN2238 (ixazomib citrate / ixazomib)	Reversible	Thus far none known	Selective proteasome inhibitors. Interact with the catalytic threonine residue of the proteasome.
Peptide vinyl sulfones	NLVS, YLVS	Irreversible	Cathepsins	Interact with β-subunits of the proteasome.
Peptide epoxyketones	Dihydroeponemycin Epoxomycin, PR-171 (carfilzomib) PR-047 (ONX 0912, oprozomib)	Irreversible	DHEM: Cathepsin B (weak)	Selective proteasome inhibitors. Bind specifically to β5-subunit of the proteasome.
	PR-957 (ONX 0914) PR-924			Selective immune proteasome inhibitors. Bind to immune β-subunits of the proteasome.
β-lactones	Lactacystin	Irreversible	Cathepsin A, Tripeptidyl peptidase II	Relatively specific but weak proteasome inhibitors. Binds to β-subunits of the proteasome.
	NPI-0052 (marizomib)	Irreversible	Salinosporamide A	Binds to β-subunits of the proteasome

Abbreviations, MG-132: Carbobenzoxy-L-leucyl-L-leucyl-leucinal; ALLnL: N-acetyl-L-leucyl-L-leucyl-L-norleucinal; ALLnM, N-acetyl-L-leucyl-L-leucyl-Lmethioninal; LLnV: N-Carbobenzoxy-L-leucyl-L-norvalinal; PSI: N-carbobenzoxy-L-isoleucyl-L-γ-t-butyl-L-glutamyl-L-alanyl-L-leucinal; Leu-Leu-vinyl sulfone; MG-262: N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucyl boronic acid. Refs: [9,48,65,70,74,81,86,134-143]

Bortezomib

The most frequently described and well-known proteasome inhibitor is bortezomib (Velcade, PS-341), a dipeptide boronic acid analogue with a broad anti-tumor activity in several cell lines and murine and human tumor models.[37,65,72,91-96] It is the first proteasome inhibitor that has been approved by the US Food and Drug Administration (FDA) and by the European Medicines Agency (EMEA) for use in MM.[97] Bortezomib specifically inhibits the proteasome pathway rapidly and in a reversible manner by binding directly to the β-5 subunit of the 20S complex, thereby blocking its enzymatic activity.[98] Exposure to bortezomib *in vitro* leads to stabilization of several intracellular protein levels such as cyclin-dependent kinase inhibitors (e.g. p21) and pro-apoptotic Bik/NBK.[99,100] Cells accumulate in the G2-M phase of the cell cycle and subsequently undergo apoptosis.

In MM, bortezomib could inhibit growth of dexamethasone- and doxorubicin-resistant myeloma cell lines, and induce apoptosis in dexamethasone-resistant primary cells.[27,101] Synergistic interactions were found with doxorubicin and melphalan in MM cells, and with dexamethasone in leukemia cells.[28,96,102] Clinically, approximately one third of patients with relapsed and refractory MM showed significant clinical benefit in a large clinical phase II trial.[103] These findings were confirmed in several subsequent studies and currently, additional clinical trials for MM are ongoing focusing on optimal schedules.[20]

Several (pre)clinical studies have evaluated the anti-cancer role of bortezomib (and other proteasome inhibitors) in other hematological neoplasias and solid tumors as well, including mantle cell lymphoma and diffuse large B-cell lymphoma.[104,105] In a LOVO xenograft model studying colon cancer, bortezomib has demonstrated increased anti-tumor effect in combination with several standard chemotherapy agents, including CPT-11, cisplatin, docetaxel, fluorouracil, gemcitabine, irinotecan and paclitaxel.[92] In a PC-3 prostate xenograft model, bortezomib does not seem to enter the brain, spinal cord, testes or the eye, thereby avoiding treatment-related side effects on these tissues. Pre-clinical studies showed that the effect of bortezomib was independent of p53 status, and not overlapping with other chemotherapeutic agents.[65]

PROTEASOME INHIBITORS AND LEUKEMIA

Already in 1990, it was shown that human leukemic cells expressed abnormally high levels of proteasomes compared to normal peripheral blood cells.[106] Both protein and mRNA proteasome expression were, in comparison to normal monocytes, higher in several lymphoid and myeloid cell lines (Daudi, DG75, CCRF-CEM, MOLT-10, U937, HL-60 and K562). Furthermore, an increase of proteasome expression was shown both in leukemic cells from patients with acute lymphoblastic leukemia (ALL), adult T-cell leukemia, and acute myeloid leukemia (AML), as well as in bone marrow cells from patients with chronic lymphocytic leukemia (CLL) and chronic myelocytic leukemia (CML). The latter increase of proteasome expression seemed to be related to cellular proliferation, presumably in a cell-cycle dependent manner.

The results mentioned above seem to indicate that dividing cells in particular are sensitive to proteasome inhibition. It has also been shown that induction of differentiation of chronic and acute leukemic cell lines results in rapid and marked down-regulation of ubiquitin expression [107]. Moreover, human leukemia cells that had been induced to differentiate were significantly less sensitive to proteasomal inhibition than their dividing precursors.[108,109]

Leukemic stem cells have many characteristics of normal hematopoietic stem cells, including a highly similar immunophenotype, and a predominantly G0 cell-cycle sta-

tus.[110,111] Therefore, preferential proteasome inhibition of only dividing cells might be insufficient when applied for clinical use. However, it has been shown that proteasome inhibitors can also induce apoptosis in leukemic stem cells, and that furthermore these stem cells are more susceptible to proteasome inhibition than normal stem cells.[36] Since leukemic stem cells have a high NF-κB expression, it is thought that the downregulation of NF-κB by proteasome inhibitors is of relevance for this specificity, although direct inhibition of NF-κB does not induce the same degree of apoptosis.

Overall, the benefits of using proteasome inhibitors in leukemia are promising.[96]

In vitro studies of proteasome inhibitors in leukemia

Due to the success of proteasome inhibition in MM, studies have been set up to investigate the benefit of proteasome inhibitors in the treatment of leukemia. A selection of several *in vitro* studies of these inhibitors in leukemia is summarized in Table II.

Not only the effect of proteasome inhibitors alone, but also the combination with other cytostatics has been investigated.[20,96] Although many proteasome inhibitors are known, the specificity of bortezomib, in combination with the particular achievements of this drug in MM, resulted in an increased use of this inhibitor in the more recently published studies.

Proteasome inhibitors seem very successful in inducing apoptosis in leukemic cells. As shown in Table II, in cell lines (both of myeloid and lymphoid origin), as well as in primary chronic and acute leukemia cells, inhibitors such as PSI and bortezomib successfully induced cell death. Moreover, normal, non-leukemic cells seemed less sensitive to these inhibitors, suggesting a favorable therapeutic index.[32,33,112]

Proteasome inhibitors already effectively induce apoptosis in leukemic cells as single drug. A number of studies have also investigated the combination of proteasome inhibitors with other chemotherapeutics, such as taxol, flavopiridol and glucocorticoids.[28,33,113] All studies showed enhanced sensitivity upon use of proteasome inhibitors.

In these studies, drugs were added simultaneously to the cells. Two studies also investigated the importance of sequential addition of the drugs. In one study, the additional effect was only seen after pre-treatment with the proteasome inhibitor. Upon co-incubations, no enhanced cytotoxic effects were seen.[35] The second study showed the opposite; the interactions were synergistic when drugs were given simultaneously, but only additive when given sequentially. [28] Since only two studies described the effect of sequential administration, and since these studies result in opposite conclusions, further investigations on this subject are warranted.

Several molecular interactions have been investigated to obtain further insights in the pathways that are affected by proteasomal inhibition. Numerous studies show that the mitochondrial apoptotic pathway is affected, including SMAC activation, cytochrome c release and caspase activation. Furthermore, the survival protein NF-κB is downregu-

Table II. Selection of pre-clinical studies of proteasome inhibitors (PI) in leukemia.

Proteasome inhibitors	Leukemic cells	Study results and mechanisms involved	Refs
Several	AML cell line HL60	Induction of apoptosis. Increase of $p27^{Kip1}$. Activation of cysteine proteases.	[108]
PSI	CML, AML, ALL cell lines	Induction of apoptosis in all cell lines. Enhanced taxol and cisplatinum cytotoxicity. PSI was more active on leukaemic than on normal $CD34^+$ bone marrow progenitors.	[33]
Lactacystin	AML cell line U937	Lactacystin combined with PKC activator bryostatin enhanced apoptosis.	[144]
Lactacystin, MG-132	Primary CLL cells	Induction of apoptosis in both GC sensitive and –resistant cells. Activation of cysteine proteases. Apoptosis is blocked by caspase antagonist zVADfmk. Inhibition of NF-κB.	[114]
MG-132, LLnL, lactacystin	AML, ALL cell lines, primary AML cells	Synergistic interactions between PI and cyclin-dependent kinase inhibitors flavopiridol and roscovitine. Downregulation of XIAP, $p21^{CIP1}$, and Mcl-1.	[113]
Bortezomib	Primary CLL cells	Induction of apoptosis associated with release of SMAC and cytochrome c.	[115]
Bortezomib	CML, AML, ALL Cell lines	Synergistic with flavopiridol. Blockade of the IκB/NF-κB pathway. Activation of the SAPK/JNK cascade. Reduction in activity of STAT3 and STAT5.	[42]
Bortezomib	Primary CLL cells	Dose-dependent cytotoxicity of bortezomib. Additive effect with purine nucleoside analogues cladribine and fludaribine. CLL cells more sensitive than normal lymphocytes.	[145]
Bortezomib	AML, ALL cell lines, primary paediatric AML, ALL cells	Lymphoblastoid, CML and AML cell lines. Bortezomib induced apoptosis and acted at least additive with dexamethasone, vincristine, asparaginase, cytarabine, doxorubicin, geldanamycin, HA14.1 and trichostatin A.	[28]
Bortezomib	AML cell lines	Synergistic with tipifarnib. The combination overcomes cell adhesion-mediated drug resistance.	[146]
Bortezomib	Pediatric ALL xenocraft model	*In vitro* and *in vivo* activity of bortezomib against primary pediatric ALL cells in a xenocraft mouse model.	[147]
Bortezomib, PSI	CML, AML cell lines	PSI enhanced toxicity of daunoblastin, taxol, cisplatinum and bortezomib. PSI and bortezomib suppressed clonogenic potential of AML and CML more than that of normal bone marrow (NBM) progenitors. Bortezomib inhibited the clonogenic potential of CML and NBM more effectively.	[35]
Carfizomib	Primary AML and ALL cells	Inhibits proliferation and induces apoptosis AML, inhibits proliferation in ALL	[112]
Carfilzomib, bortezomib	AML cell lines and primary AML cells	Synergistic effect on proteotoxic stress together with the protease inhibitors ritonavir, nelfinavir, saquinavir and lopinavir.	[148]
Carfilzomib, bortezomib	ALL cell lines *in vitro* and in xenograft model	Proteasome inhibitors evoke latent tumor suppression programs in pro-B MLL leukemias through MLL-AF4.	[149]

Table II. Selection of pre-clinical studies of proteasome inhibitors (PI) in leukemia. (continued)

Proteasome inhibitors	Leukemic cells	Study results and mechanisms involved	Refs
Carfilzomib	MM, AML, burkitt lymphoma cell lines	Induces proapoptotic sequelae, including proteasome substrate accumulation, Noxa and caspase 3/7 induction, and phospho-eIF2α suppression.	[141]
Marizomib	ALL, AML and CML cell lines and in xenograft model	Induces caspase-8 and ROS-dependent apoptosis alone and in combination with HDAC inhibitors	[150,151]
Marizomib, bortezomib	AML and ALL cell line	Anti-leukemic activity, synergistic in combination with bortezomib.	[140]
ONX 0914	AML and ALL cell lines	Growth inhibition, proteasome inhibitor-induced apoptosis, activation of PARP cleavage and accumulation of polyubiquitinated proteins.	[152]
PR-924	AML and ALL cell lines	Growth inhibition, immune proteasome inhibition, apoptosis, activation of PARP cleavage.	[139]
Ixazomib	Primary CLL cells	Annexin-V staining, PARP1 and caspase-3 cleavage and an increase in mitochondrial membrane permeability, apoptosis was only partially blocked by the pan-caspase inhibitor z-VAD.fmk	[153]

Abbreviations, PSI: N-carbobenzoxy-L-isoleucyl-L-γ-t-butyl-L-glutamyl-L-alanyl-L-leucinal; LLnV: N-Carbobenzoxy-L-leucyl-L-norvalinal; LLnL: N-acetylleucylleucylnorleucinal; MG-13 2: Carbobenzoxy-L-leucyl-L-leucyl-leucinal; GC: glucocorticoid; PKC: protein kinase C.

lated and there is an increase of activation of cysteine proteases.[108,114,115] Although it is still not known how many pathways, directly or indirectly, are disturbed by proteasome inhibitors, it is clear that these inhibitors can overcome resistance to other cytostatics. Some examples have already been given in the studies described in Table II.[32,114]

In vivo studies of proteasome inhibitors in leukemia

Many of the initial studies regarding the effect of proteasome inhibition have been performed in *in vitro* systems. The first *in vivo* anti-tumor activity of proteasome inhibitors was demonstrated in a human Burkitt's lymphoma xenograft mouse model.[116] In 2002, a pre-clinical study was published in which bortezomib was combined with humanized anti-Tac in a murine model of adult T-cell leukemia.[117] In this study, bortezomib alone did not result in prolongation of the survival of the tumor-bearing mice, which was ascribed to a limited dosing schedule. However, in combination with humanized anti-Tac, bortezomib therapy was associated with complete response (CR) in several mice, whereas anti-Tac alone only resulted in a partial response (PR).

Clinical studies of proteasome inhibitors in leukemia

The last years several clinical trials with proteasome inhibitors have been performed in patients. Table III summarizes such studies that included leukemia patients.

Table III. Clinical studies of bortezomib in leukemia.

Study drugs	Cohort	N	Phase	Study results and mechanisms involved	Refs
BTZ	Several haematologic malignancies	27	I	Bortezomib was given twice weekly for 4 weeks every 6 weeks. The MTD was 1.04mg/m^2. CR in 1 MM patient. PR in 1 patient with MCL and 1 with FL.	[118]
BTZ	Refractory or relapsed acute leukemia	15	I	Bortezomib was given twice-weekly for 4 weeks every 6 weeks. The MTD was 1.25mg/m^2. No ³grade 3 toxicities. 5 patients showed haematological improvement. No CR achieved.	[119]
BTZ, PegLD	AML, MM and NHL	42	I	Bortezomib was given on days 1, 4, 8, 11 and PedLD on day 4. MTD of BTZ 1.3mg/m^2. No significant pharmacokinetic and pharmacodynamic interactions between bortezomib and PegLD. 16 of 22 MM patients achieved CR, near-CR or PR. 1 CR and 1 PR in NHL patients. 2 of 2 AML patients achieved a PR.	[120]
BTZ	recurrent childhood ALL, AML, blastic phase CML, M3	12	I	Bortezomib was administered twice weekly for 2 weeks followed by a 1-week rest,. MTD of bortezomib was 1.3 mg/m^2/dose. 5 patients were fully evaluable. DLT's occurred in 2 patients at the 1.7 mg/m^2 dose level. No OR achieved.	[124]
BTZ, IDA, AraC	AML	31	I	Addition of BTZ to AML induction chemotherapy. Bortezomib added on days 1, 4, 8 and 11. 19 CR, 3 CRp, 2 PR and 7 no response. BTZ was well-tolerated up to 1.5 mg/m^2.	[121]
BTZ,VCR, DEX, PegAspa, DOX	recurrent childhood ALL	10	I	Combination of bortezomib (1.3 mg/m^2) with ALL induction therapy is active with acceptable toxicity. 6 patient achieved CR.	[125]
BTZ, VCR, DEX, PegAspa, DOX	recurrent childhood ALL	22	II	14 patients achieved CR, and 2 achieved CRp, 3 patients died from bacterial infections, 2 of 2 included T-cell ALL patients did not respond.	[126]
BTZ, tipifarnib	Relapsed or refractory ALL(26) or AML (1)	27	I	Combination well tolerated. 2 patients achieved CRp and 5 SD.	[154]
BTZ, DNR, AraC	AML (age >65)	95	I/II	Combination was tolerated. 62 patients achieved CR and 4 patients CRp.	[122]
BTZ, 17-AAG	Relapsed or refractory AML	11	I	The combination of 17-AAG and BTZ led to toxicity without measurable response in patients with relapsed or refractory AML	[123]
BTZ, DAC	poor-risk AML	19	I	Combination was tolerable and active in this cohort of AML patients; 7 of 19 patients had CR or CRi. 5 of 10 patient > 65 years had CR	[155]
BTZ, LEN	14 MDS/CMML 9 AML	23	I	MTD of BTZ 1.3mg/m^2 was tolerable in this regimen. Responses were seen in patients with MDS and AML. Two fatal infections occurred	[156]

Table III. Clinical studies of bortezomib in leukemia. (continued)

Study drugs	Cohort	N	Phase	Study results and mechanisms involved	Refs
BTZ IDA	Relapsed AML (7) or AML > 60 year (13)	20	I	4 patients achieved complete remission. 1 treatment-related death. Overall the combination was well tolerated.	[157]
BTZ, AZA	Relapsed or refractory AML	23	I	Dose of 1.3mg/m2 BTZ was reached without dose limiting toxicities. 5 out of 23 patients achieved CR	[158]
BTZ,MIDO vs BTZ ,MIDO, DHAD, VP16 , AraC	Relapsed/ refractory AML	21	I	56.5% CR rate and 82.5% overall response rate (CR + CR with incomplete neutrophil or platelet count recovery). Combination is active but is associated with expected drug-related toxicities. DLTs were peripheral neuropathy, decrease in ejection fraction and diarrhea.	[159]

Abbreviations, *Study outcome:* MTD: maximum tolerated dose; DLT: dose limiting toxicities; CR: complete response; CRi: incomplete remission; CRp: CR with incomplete platelet recovery; PR: partial response; OR: objective response; SD: stable disease; PFS: progression-free survival; EFS: event-free survival; OS: overall survival. *Malignancies:* MCL: mantle cell lymphoma; FL: follicular lymphoma; NHL: Non-Hodgkin lymphoma; *Drugs:* 17-AAG: 17-N-Allylamino-17-Demethoxygeldanamycin; AraC: cytarabine; AZA: azacitidine; BTZ: bortezomib; DAC: decitabine; DEX: dexamethasone; DHAD: mitoxantrone; DNR: daunorubicin; DOX: doxorubicin; IDA: idarubicin; LEN: lenalidomide; PegLD: pegylated liposomal doxorubicin; PegAspa: pegylated L-asparaginase; VCR: vincristine, VP16: etoposide.

Thus far, majority of the published clinical leukemia studies regarding proteasome inhibition have been performed using bortezomib, as this drug showed a unique toxicity profile in the NCI pre-clinical assay and is approved for MM.[65] Bortezomib was shown to act in a dose-dependent manner, and recovery of normal proteasome function was seen within 72 hours after the last dose.[118] In the two single-drug studies described, patients suffering from leukemia showed hematological improvements, but in these phase I studies no CRs were reached.[118,119] Overall, although bortezomib seemed to have biological activity, the clinical benefits were limited when given as a single-drug agent.

These results might appear somewhat disappointing, however in 2005 the first phase I combination study in several hematological malignancies including leukemia was published, in which bortezomib was combined with pegylated liposomal doxorubicin.[120] Bortezomib was given on days 1, 4, 8, 11 and pegylated liposomal doxorubicin on day 4. Forty-two patients were included, with an overall response rate of 73% in MM patients. Grade 3 or 4 toxicities in this study included thrombocytopenia, lymphopenia, neutropenia, fatigue, pneumonia, peripheral neuropathy, febrile neutropenia and diarrhea. Both evaluable AML patients in this study achieved a PR.

In another study bortezomib was combined with AML induction chemotherapy (idarubicin and cytarabine). Bortezomib was added on days 1, 4, 8 and 11. The overall response rate was 77%, with 61% of the AML patients reaching a CR. The highest dose used was 1.5mg/m^2 bortezomib and was well tolerated.[121] A similar combination, bortezomib together with daunorubicin and cytarabine, was studied in a phase I/II in older

patients with AML (age > 65 year) and showed a comparable CR rate of 65% with a MTD of 1.3mg/m^2.[122] Subsequently, several phase I trials have been published with varying response rate (summarized in table III). Noteworthy, an pre-clinical promising combination of bortezomib with the heat shock inhibitor 17-AAG showed only toxicity without measurable responses in a phase I trial.[123]

Table IV. Ongoing and unpublished clinical trials of bortezomib in acute leukemia which include pediatric patients.

Study drugs	Time period	N	Phase	Cohort	Age	Sponsor	Clinical trial identifier
BTZ + intensive reinduction chemotherapy	Mar 2009 Sept 2014	60	II	Relapsed ALL	1–31	National Cancer Institute (USA)	NCT00873093
BTZ , DEX, VCR, MTX	Sep 2009 Jul 2014	24	II	Relapsed/ refractory ALL	0.5–19	Erasmus Medical Center (Rotterdam, The Netherlands)	NTR1881 †
BTZ, ATO	May 2013 May 2018	30	II	Relapsed Acute Promyelocytic Leukemia (APL)	1-75	Christian Medical College, Vellore, India	NCT01950611
Standard leukemia chemotherapy ± BTZ	Apr 2014 Feb 2019	1400	III	T-Cell ALL or Stage II-IV T-Cell Lymphoblastic Lymphoma	2-30	National Cancer Institute (USA)	NCT02112916
BTZ , SAHA + reinduction chemotherapy	Apr 2015 Apr 2019	30	II	Refractory or relapsed MLL rearranged leukemia	<21	St Jude Children's Research Hospital (Memphis, TN,USA)	NTC 02419755
BTZ , PANO + reinduction chemotherapy	Dec 2015 Apr 2019	40	II	Relapsed T-cell leukemia or lymphoma	<21	St Jude Children's Research Hospital (Memphis, TN,USA)	NCT02518750
BTZ + induction chemotherapy	Oct 2015 Oct 2020	50	I/II	Infant leukemia and lymphoblastic lymphoma	<1	St Jude Children's Research Hospital (Memphis, TN,USA)	NCT02553460
BTZ + reinduction chemotherapy	July 2015 Apr 2019	20	II	Refractory or relapsed leukemia and lymphoblastic lymphoma	1-39	Children's Mercy Hospital Kansas City	NCT02535806
BTZ + HR reinduction chemotherapy	Aug 2015 Aug 2018	250	II	High Risk (HR) relapsed ALL	< 18	Charité - Universitätsmedizin (Berlin, Germany)	EudraCT Number: 2012-000810-12 †

Abbreviation, *Drugs:* ATO: arsenic trioxide; BTZ: bortezomib; DEX: dexamethasone; MTX: methotrexate; PANO: panobinostat SAHA: vorinostat; VCR: vincristine.
Source: www.clinicaltrials.gov and www.skion.nl (†)

Bortezomib was also tested in pediatric ALL cohorts. In a phase I study bortezomib was administered twice weekly for 2 consecutive weeks at either 1.3 or 1.7 mg/m^2 dose followed by a 1-week rest in pediatric patients with relapsed ALL. The treatment was well tolerated and the optimal dose was set at 1.3 mg/m^2. No objective clinical responses were obtained in this small group of heavily pretreated patients.[124] In contrast, a phase I and a subsequent phase II trial in a similar pediatric cohort of relapsed ALL patients combining bortezomib with other drugs showed promising results. Combining bortezomib with vincristine, dexamethasone, pegylated-asparaginase and doxorubicin, resulted in a CR response of 60% and 63% respectively.[125,126] Three patients in the phase II trial died from severe infection; after addition of vancomycin, levofloxacin, and voriconazole prophylaxis, no further infectious mortality occurred in the last 6 patients. Recently, BTZ was combined with dexamethasone, mitoxantrone, and vinorelbine (BDMV) in children with relapsed ALL which were unable to receive vincristine-prednisone-L-asparaginase-doxorubicin secondary to asparaginase intolerance. 7 out of 10 patients showed complete remission after 1 cycle of BDMV with expectable toxicity.[127] In a pediatric cohort with relapsed or secundary AML addition of BTZ to induction chemotherapy regime consisting of either idarubicin and cytarabine or etoposide and cytarabine, did not show additive value. Although well tolerated with chemotherapeutics, the study did not exceed preset minimum response criteria to allow continued accrual. [128]

Currently ongoing clinical studies in leukemia are focusing on the combination of bortezomib with multiple cytotoxic agents. In addition, studies with second generation proteasome inhibitors have started. An overview of the clinical trials in leukemia is presented in Table III. Ongoing clinical studies and studies of which results are not published yet, is given in table V. In addition, an overview of clinical and unpublished studies using second generation proteasome inhibitors, is given in table VI. Table IV summarizes the studies in pediatric cohorts. Although the first results of the use of bortezomib in combination studies are very promising, it seems too early to speculate on the final impact of proteasome inhibitors for treatment of leukemia.

RESISTANCE MECHANISMS; STATUS AT THE START OF THE THESIS PROJECT

Despite of promising results from clinical studies using bortezomib, acquired and intrinsic resistance to treatment with bortezomib have been reported.[84,129,130]. Since conventional mechanisms of drug resistance mediated by efflux pumps like MDR1, BRCP1 and MRP's, only MDR1/P-glycoprotein seemed to play a modest role in conferring bortezomib resistance[131,132], several studies have focused further on the etiology of bortezomib sensitivity and resistance.

Table V. Ongoing and unpublished clinical trials of proteasome inhibitors in acute leukemia.

Study drugs	Time period	N	Phase	Cohort	Age	Sponsor	Clinical trial ID
BTZ , DHAD, VP16, AraC	Jan 2006 Sept 2016	55	I/II	Relapsed/ refractory acute leukemias	>18	Thomas Jefferson University (PA, USA)	NCT00410423
BTZ , FLAG, IDA	Apr 2008 Jan 2013	40	I/II	Refractory or relapsed AML	>18	PETHEMA Foundation	NCT00651781
BTZ , SAHA, SFN	Feb 2010 Sept 2016	38	I/II	Poor risk AML	>18	Indiana University (IN, USA)	NCT01534260
BTZ, BEL	May 2010 Feb 2014	24	I	Relapsed/ refractory acute leukemias	>18	Virginia Commonwealth University (VA,USA)	NCT01075425
BTZ , NFV	July 2010 Mar 2013	18	I	Relapsed or progressive advanced hematologic cancer	>18	Swiss Group for Clinical Cancer Research (Switzerland)	NCT01164709
BTZ , DHAD, VP16, AraC	July 2010 May 2014	34	I	Relapsed/ refractory AML	18–70	Case Comprehensive Cancer Center (OH, USA)	NCT01127009
Several drugs in randomization arms ± BTZ	June 2011 June 2017	1250	III	Initial AML	>29	National Cancer Institute (USA)	NCT01371981
DAC vs BTZ, DAC	Nov 2011 June 2015	172	II	AML	>60	National Cancer Institute (USA)	NCT01420926
BTZ , DOX, PegAspa , VCR, DEX, AraC, MTX	Mar 2013 July 2017	17	II	Relapsed/ refractory ALL	>18	National Cancer Institute (USA)	NCT01769209
BTZ , SFN, DAC	July 2013 Dec 2016	30	I	AML	>60	National Cancer Institute (USA)	NCT01861314
BTZ, DOX	Mar 2015 Mar 2017	30	II	AML	18–80	University of California, Davis (CA, USA)	NCT01736943
BTZ, LEN	Mar 2015 Aug 2018	24	I	Relapsed AML and MDS after Alllo SCT	>18	Massachusetts General Hospital (MA,USA)	NCT023121

Abbreviations, *Drugs:* 17-AAG: 17-N-Allylamino-17-Demethoxygeldanamycin; AraC: cytarabine; BEL: belinostat; BTZ: bortezomib; DAC: decitabine; DEX: dexamethasone; DHAD: mitoxantrone; DNR: daunorubicin; DOX: doxorubicin; IDA: idarubicin; FLAG: fludarabine, cytarabine (Ara-C) and granulocyte-colony stimulating factor (G-CSF); LEN: lenalidomide; MTX: methotrexate; NFV: nelvinavir; PegLD: pegylated liposomal doxorubicin; PegAspa: pegylated L-asparaginase; SAHA: vorinostat; SFN: sorafenib; VCR: vincristine; VP16: etoposide. Source: www.clinicaltrials.gov

Table VI. Ongoing clinical trials of second generation proteasome inhibitors in acute leukemia.

Study drugs	Time period	N	Phase	Cohort	Age	Sponsor	Clinical trial ID
CFZ	Sept 2010 Jul 2015	18	I	Relapsed/ refractory ALL and AML	>18	Washington University School of Medicine (MO, USA)	NCT01137747
IXA, DHAD, VP16, AraC	May 2014 Nov 2017	30	I	Relapsed / refractory AML	18 - 70	Case Comprehensive Cancer Center; National Cancer Institute (NCI)	NCT02070458
IXA	Mar 2014 Mar 2016	16	II	Relapsed / refractory AML	> 18	Stanford university / National Cancer Institute (NCI)	NCT02030405
IXA, DHAD, VP16, AraC	Oct 2014 Nov 2018	30	I	Relapsed / refractory AML	18-70	Case Comprehensive Cancer Center (USA)	NCT 02070458
CFZ , DEX, DHAD, PegAspa, VCR	Dec 2014 Jul 2017	39	I/II	Relapsed / refractory AML	<18	Onyx Therapeutics Inc. (CA, USA)	NCT02303821
CFZ , CYCLO, VP16	Jul 2015 Dec 2017	50	I	Relapsed leukemia and solid tumors	6-29	Phoenix Children's Hospital (AZ, USA)	NCT 02512926
IXA + induction and consolidation chemotherapy	Nov 2015 Feb 2022	54	I	AML	>60	Massachusetts General Hospital (MA,USA)	NCT02582359

Abbreviations, *Drugs:* AraC: cytarabine; CFZ: carfilzomib; CYCLO: cyclophosphamide; DEX: dexamethasone; DHAD: mitoxantrone; IXA: ixazomib; VCR: vincristine; VP16: etoposide. Source: www.clinicaltrials.gov.

Most of the studies have focused on the proteasome subunit composition in relation to bortezomib sensitivity and resistance. The ratio between β2-type and (β1+ β5)-type catalytic subunits has been correlated with bortezomib response *in vitro* and *ex vivo* in primary patient hematological malignant cells.[95] The importance of the proteasome subunit composition in bortezomib sensitivity is confirmed by studies in two bortezomib resistant cell-lines. The bortezomib resistant AML cell line HL-60 showed upregulation of the β1and β5 subunits, and the bortezomib resistant Burkitt lymphoma cell line showed upregulation of the β1, β2 and β5 catalytic domains of the proteasome.[94,95] The pan proteasome inhibitor NPI-0052 might be useful in overcoming this resistance. When treating bortezomib-resistant multiple myeloma cells *ex vivo* with NPI-0052, apoptosis could still be induced.[73]

Mechanisms distinct of the proteasome itself have also been suggested to be involved in bortezomib sensitivity and resistance. A microarray study has shown that overexpression of activating transcription factor (ATF) 3, ATF4, ATF5, c-Jun, JunD and caspase-3 is correlated with bortezomib sensitivity in B-cell lymphoma cells.[80] Furthermore, overexpression of Cyclin D1 increased bortezomib sensitivity *in vitro* and *in vivo* in a breast

cancer model.[133] In contrast, overexpression of heat shock protein (HSP)27, HSP70, HSP90 and T-cell factor 4 is associated with bortezomib resistance in B-cell lymphoma cells.[80] These data together suggest that although the proteasome conformation is very important in bortezomib sensitivity, other factors are involved in intrinsic and acquired bortezomib resistance.

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