

Antiproliferative and Proapoptotic Effects of Proteasome Inhibitors and their Combination with Histone Deacetylase Inhibitors on Leukemia Cells

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Abstract: New chemotherapeutic agents are still required to further optimise treatment of leukemia patients. Proteasome inhibition by bortezomib, PR-171 (carfilzomib) and NPI-0052 (salinosporamide A) has been successfully used for the treatment of multiple myeloma and mantle cell lymphoma and is considered also as novel treatment strategy in leukemia. Combination of proteasome inhibitors bortezomib and NPI-0052 induces synergistic anti-multiple myeloma activity both *in vitro* using multiple myeloma cells and *in vivo* in a human plasmacytoma xenograft mouse model. Cell death resulting from proteasome inhibition requires caspase activation and increased levels of reactive oxygen species. While bortezomib induces several caspases, NPI-0052 activates predominantly caspase-8-dependent pathway. We studied the effect of bortezomib (10 nM) on DNA synthesis and apoptosis in human acute myeloid cell lines KASUMI-1, ML-1, ML-2 and CTV-1 cells. Bortezomib was potent inhibitor of DNA synthesis in all four types of leukemia cells and induced apoptosis in KASUMI-1, ML-2 and CTV-1 cells but not in ML-1 cells. Other research groups showed that histone deacetylase inhibitors (valproic acid or benzamide derivative MS-275) in combination with NPI-0052 or PR-171 induced greater levels of acute leukemia cell death than in combination with bortezomib. Proteasome inhibition as monotherapy and its combination with many conventional therapies as novel treatment strategies in leukemia are promising. Malignant cells are more sensitive to this treatment than normal hematopoietic cells.

Key Words: Proteasome, proteasome inhibitors, histone deacetylase inhibitors, leukemia, apoptosis.

INTRODUCTION

Ubiquitin-dependent proteolysis regulates the stability and function of key regulatory proteins that regulate the cell cycle, gene transcription, receptor endocytosis, intracellular trafficking, response to extracellular signal, signal transduction, antigen presentation, and control cell growth. Ubiquitin is a highly conserved protein of 76 amino acids that is covalently attached to substrate proteins through an energy-dependent enzymatic mechanism and polyubiquitinated proteins are degraded by a multicompartimentalized protease called the 26S proteasome [1-4]. For the discovery of ubiquitin and its function in non-lysosomal pathway of protein degradation, the 2004 Nobel Prize in Chemistry was awarded to Drs. Avram Hershko, Aaron Ciechanover and Irwin Rose [5-8].

Schematic representation of the ubiquitin conjugation (ubiquitination, also referred to as ubiquitylation or ubiquitylation) and of the the ubiquitin-proteasome system is shown in Fig. (1). Ubiquitination is a posttranslational modification of proteins. Ubiquitin is activated in an ATP-dependent manner by a ubiquitin-activating enzyme known as an enzyme-1 (E1). Subsequently, ubiquitin is transferred to a ubiquitin-conjugating enzyme-2 (E2). E2, with the help of a ubiquitin-protein ligase (E3) and in some cases in the

presence of an accessory factor (E4) [9], specifically attaches ubiquitin to the protein substrate. Only ten E1 enzymes, but about 100 E2 enzymes and 1000 E3 enzymes exist in human cells [10]. E3 ubiquitin ligases determine the specificity of protein substrates and are targets for pharmaceutical intervention. There are two major types of E3 ligases: the RING (really interesting new gene) domain-containing E3s and the Hect (homologous to E6-associated protein carboxyl terminus) domain-containing E3s. RING E3s bring the E2 enzyme in close proximity of the target protein, allowing the E2 to directly ubiquitinate the substrate. However, in the case of Hect E3s, ubiquitin is first transferred onto a conserved cysteine in the Hect domain. Consequently, Hect E3 enzyme ubiquitinates the substrate protein. Polyubiquitin chain formation results from a linkage between the C terminus of one ubiquitin and a lysine side chain in another. Generated polyubiquitin chain (at least four attached ubiquitins) functions as signal for the subsequent degradation of protein substrates in the 26S proteasome.

Eukaryotic proteasomes, model of a barrel-like structure in Fig. (2), consist of two outer α -rings and of two inner β -rings, each assembled from seven similar, but distinct, subunits. 19S regulatory complex caps both ends of the 20S proteasome to form 26S proteasome. Each 19S regulatory complex contains at least seventeen different subunits and is assembled from two main subcomplexes-a base that contains six ATPases (Rpt) plus three non-ATPase subunits (Rpn), and a lid subcomplex that sits on top of the base and consist at least eight non-ATPase regulatory particles (Rpn). 19S

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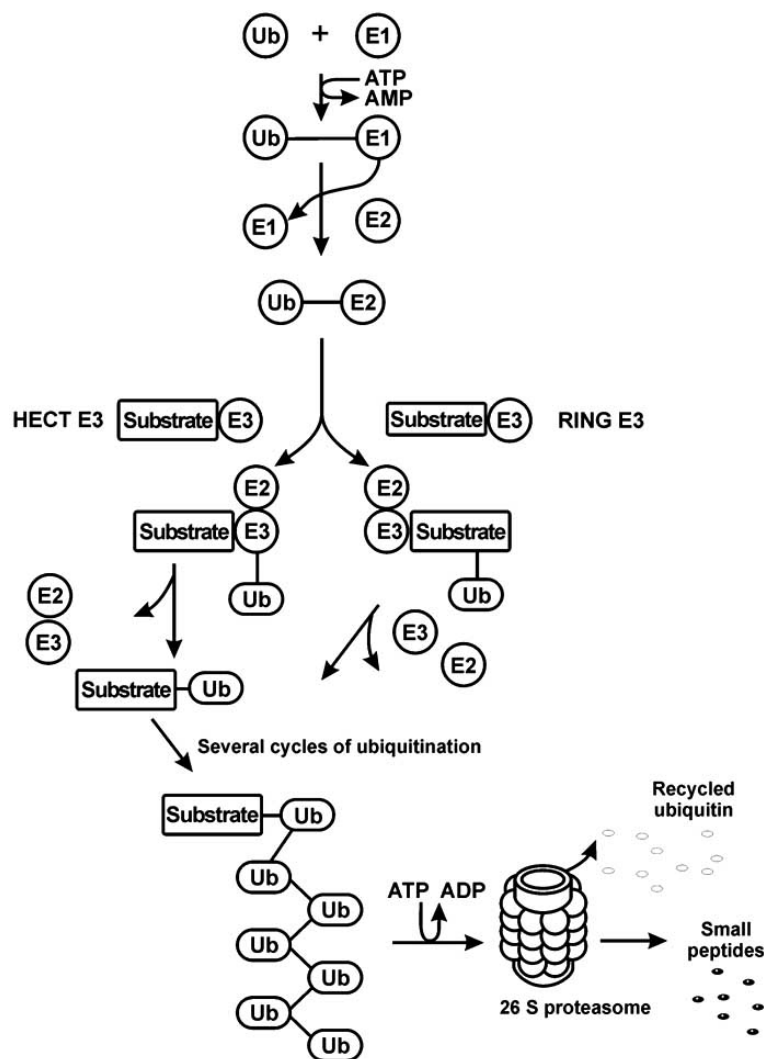


Fig. (1). The ubiquitin-proteasome system. Attachment of ubiquitin to the target protein requires three enzymatic steps. Ubiquitin-activating enzymes activate ubiquitin by forming a high energy thiol ester bond between an E1 active site-located cysteine residue and the C-terminal glycine residue of ubiquitin. This reaction requires energy provided by the hydrolysis of ATP and forms an activated thiol ester bond to ubiquitin-conjugating enzymes that serve as carrier proteins. Ubiquitin-protein ligases catalyze the covalent attachment of ubiquitin to the target protein by the formation of isopeptide bonds. Multiple cycles of ubiquitination finally result in the synthesis and attachment of polyubiquitin chains that serve as a recognition signal for the degradation of the target protein by the 26S proteasome.

regulatory complex recognizes the polyubiquitin proteolytic signal and unfolds substrates [4]. The 19S regulatory complex also takes a role in recycling ubiquitin as it contains deubiquitinating enzymes. The 20S core exhibits three enzymatic activities (chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing also named caspase-like) in the inner β -rings. Many proteins degraded by proteasome are implicated in important cell processes: cell-cycle-regulatory proteins (cyclins A,B,C,D and E, inhibitors of cyclin-dependent kinases -proteins p21^{WAF1/CIP1} and p27^{KIP1}, the tumor suppressor p53, inhibitor of NF- κ B- protein I κ B, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).

PROTEASOME INHIBITORS

Proteasomes belong to proteolytic enzymes called threonine proteases. Proteasome inhibitors were first synthesized

as tools to probe the function and proteolytic activity specificity of the proteasome [11,12]. Most of the proteasome inhibitors address the chymotryptic activity of the 20S proteasome core *via* adduct formation with N-terminal threonine hydroxyl group as part of the catalytically active center. Studies with proteasome inhibitors treated leukemia cell lines showed that proteasome inhibitors were good inducers of leukemia cells apoptosis [13,14]. Transformed cells are much more sensitive to proteasome inhibition than normal cells [15,16]. Thus, the possibility that proteasome inhibitors could be drug candidates appeared as a new hope for cancer therapy. Several classes of proteasome inhibitors were developed and several proteasome inhibitors are shown in Fig. (3). There are six major classes of proteasome inhibitors: peptide aldehydes, peptide semicarbazones, peptide vinyl sulfones, peptide boronates, peptide epoxyketones (epoxomycin and eponomycin) and β -lactones (lactacystin and its

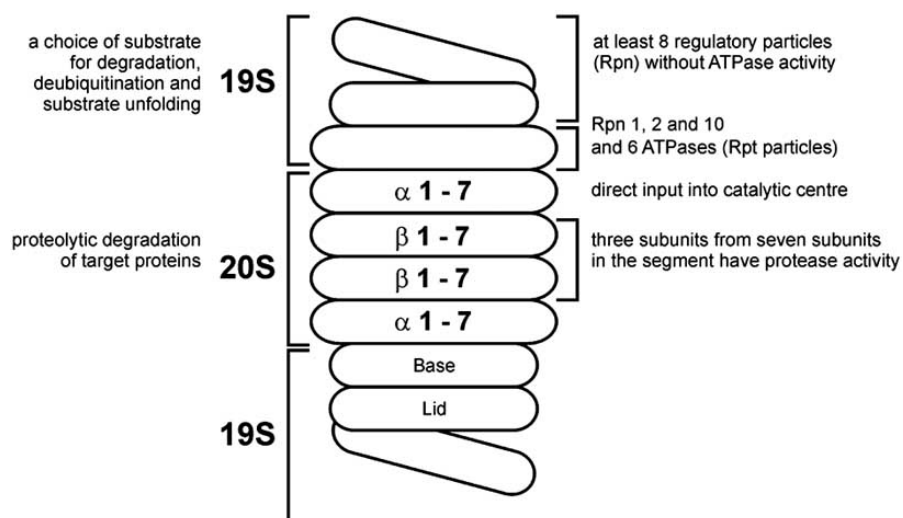


Fig. (2). The structure of the 26S proteasome. The 26S proteasome consists of the 20S catalytic core complex and two 19S regulatory complexes capping the 20S complex at both ends. The 20S complex is composed of four axially stacked rings. Each of the outer rings consists of seven polypeptide α subunits that serve as the gates through which proteasome substrates enter, whereas each of the two inner rings is formed by seven proteolytic β subunits, and only three of them, $\beta_1, \beta_2,$ and $\beta_5,$ are proteolytically active and harbor proteolytic sites that face the central cavity of the 20S complex. The 19S complex consists of the base and lid subcomplex. The base subcomplex contains six non-redundant ATPases. The lid subcomplex contains at least eight subunits including deubiquitinating enzymes and receptors for ubiquitinated proteins. The polyubiquitinated target protein enters the 19S regulatory complex and is recognized, deubiquitinated, unfolded, and translocated into the central cavity of the 20S catalytic core complex, where it is degraded by different hydrolytic activities. Ubiquitin is recycled by the ubiquitin carboxy terminal hydrolase. Peptides as a product of degradation are released from the 26S proteasome by diffusion and are used for major histocompatibility class I antigen presentation or are further degraded to single amino acids by cytosolic peptidases.

derivatives), based on the pharmacophore that reacts with the threonine residue in the active site of the proteasome. In leukemia, three classes of proteasome inhibitors entered clinical trials (peptide boronates /bortezomib/, β -lactones /NPI-0052/ and epoxomycin derivatives /PR-171/). Toxicity profiles of other three classes of proteasome inhibitors (peptide alde-

hydes, peptide semicarbazones and peptide vinyl sulfones) prevent their use in clinical trials.

Resistance to apoptosis and enhanced proliferation is characteristic feature of cancer cells. Proteasome inhibitors induce cell cycle arrest by interfering with timely degradation of cyclins and cyclin-dependent kinases inhibitors. Pro-

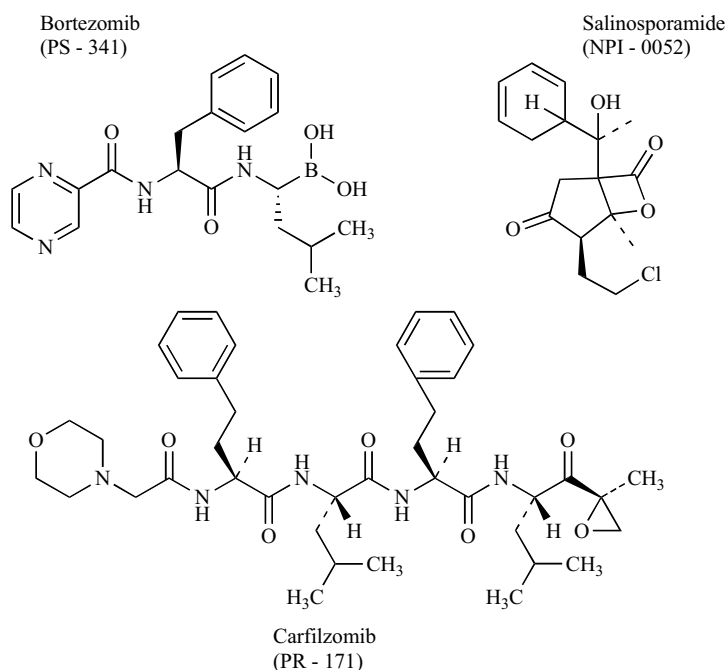


Fig. (3). Chemical structures of several selected proteasome inhibitors.

teasome inhibitors function as apoptosis inducers by inhibition of transcription factor NF-κB and by endoplasmic reticulum stress and subsequent generation of reactive oxygen species. Proteasome inhibitors stabilize proapoptotic proteins, such as p53, Bax, Bik and Bim while reduce levels of some antiapoptotic proteins, such as Bcl2.

The first developed proteasome inhibitors were peptide aldehydes, which mimic a protein substrate [17-19]. The peptide aldehydes form a reversible covalent hemiacetal intermediate between the aldehyde group of the inhibitor and the hydroxyl group of the amino terminal threonine. MG115 (N-benzoyloxycarbonyl(Z)-1-L-leuciny-L-leuciny-L-norvalinal), MG132 (N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal) and PSI (N-benzoyloxycarbonyl (Z) -Ile-Glu(O-t-Bu)-Ala-leucinal) are members of this group of proteasome inhibitors. However, many peptide aldehydes, for example MG132 and others, cause significant neurotoxicity and therefore they were not used in clinical trials.

Bortezomib

Boronate inhibitors of the proteasome are more potent than structurally similar peptide aldehydes [20]. Bortezomib (VELCADE, formerly known as PS-341, pyrazinylcarbonyl-Phe-Leu-boronate) inhibits proteasome by binding reversibly to the chymotrypsin-like site in the 20S core of the proteasome [21]. Bortezomib is the first proteasome inhibitor approved by the US Food and Drug Administration for the treatment of relapsed or relapsed and refractory multiple myeloma (MM) and some forms of non-Hodgkin’s lymphoma, mantle cell lymphoma [22-32]. Cellular mechanisms responsible for the clinical efficacy of bortezomib include inhibition of tumor cell adhesion to stroma and disruption cytokine-dependent survival pathways, in part through suppression of the transcription nuclear factor-κB (NF-κB) activity, inhibition of angiogenesis, induction of aggresome (aggregates of ubiquitin-conjugated proteins) formation, en-

doplasmic reticulum stress, and the unfolded protein response [33-39].

Bortezomib represses NF-κB function by stabilizing IκB, which binds NF-κB and prevents its nuclear translocation [40]. The association of NF-κB activation with tumor promotion, progression and metastasis is well documented and has been demonstrated in several mouse models [41,42]. NF-κB plays a role in angiogenesis, cell invasion, oncogenesis, proliferation, and suppression of apoptosis. Moreover, NF-κB inhibition induces chemosensitization and radiation sensitivity because many chemotherapeutic agents, and ionizing radiation activate antiapoptotic NF-κB functions [43-47].

Bortezomib induces DNA hypomethylation and silenced gene transcription by disruption of the NF-κB and the transcription factor Sp1, leading in turn to downregulation of DNA methyltransferase *DNMT1* gene transcription [48]. DNMT1 plays a critical housekeeping role in maintaining established patterns of DNA methylation in dividing cells [49]. DNA methyltransferases are overexpressed in AML and solid tumors and play an important role in the development and maintenance of the neoplastic phenotype [50]. DNA hypomethylation has a significant antitumor activity because it causes reactivation of tumor suppressor genes in leukemia cells and restores normal patterns of cell proliferation, differentiation, and apoptosis [51].

Bortezomib-mediated proteasome inhibition affect multiple signaling pathways, including cell cycle, growth arrest, stress response, microenvironment and apoptosis. Disruption of multiple cellular signaling by bortezomib initiates and maintains an active cell death pathway and causes apoptosis as we show in Fig. (4).

Bortezomib-mediated programmed cell death is connected with JNK (c-Jun-NH₂ terminal kinase) induction, generation of reactive oxygen species, transmembrane mito-

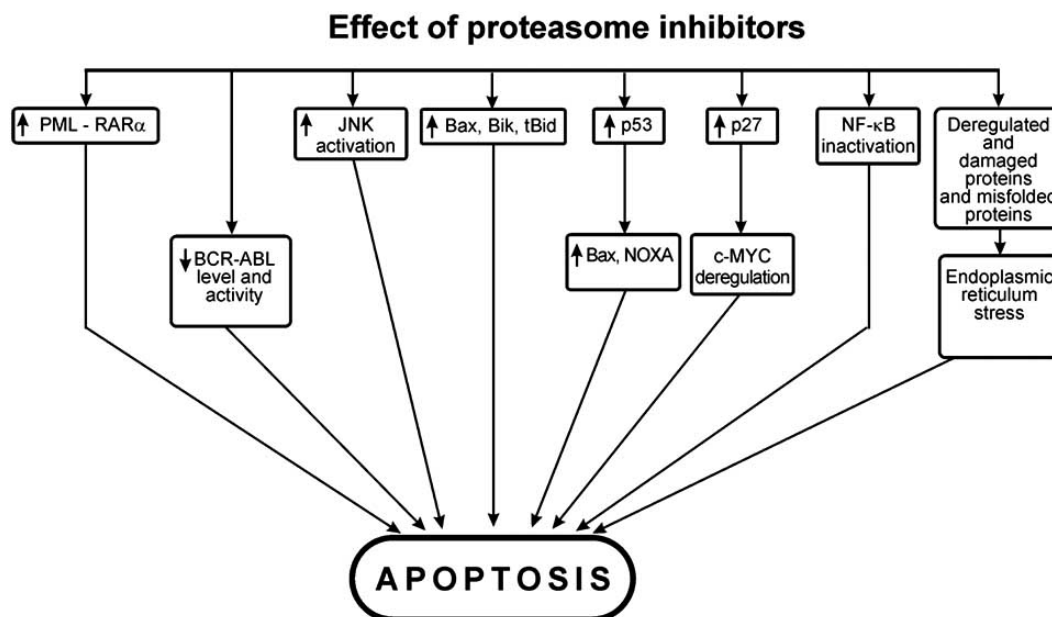


Fig. (4). Molecular targets of proteasome inhibitors in the process of apoptosis induction.

chondrial potential gradient collapse, release of proapoptotic mitochondrial proteins, such as cytochrome c, and activation of intrinsic, caspase-9- mediated and extrinsic, caspase-8 – mediated apoptosis. Bortezomib responses are also linked to the upregulation of the proapoptotic Bcl-2 protein family member NOXA and this effect is independent of constitutive activity of the phosphoinositide 3-kinase/AKT (protein kinase B) and NF- κ B pathways.

Bortezomib works best in multiple myeloma and mantle cell lymphoma with overexpression of cyclin D isoforms. Clinical studies with bortezomib in solid tumors are disappointing, but are also better in cyclin D-dependent cancer models [52]. Activation of NF- κ B has been noted in many tumor types, however only rarely has this been linked to an underlying genetic mutation [53]. Activation of the non-canonical NF- κ B pathway in multiple myeloma is important for the highest response rate to bortezomib. Sometimes, bortezomib may paradoxically activate NF- κ B and in these cases, bortezomib is the inefficient drug [54]. In addition to primary resistance, secondary or acquired resistance was observed and was ascribed to efflux through P-glycoprotein [55]. Furthermore, a number of toxicities including painful peripheral neuropathy, thrombocytopenia, anemia, fatigue, diarrhea, and neutropenia have restricted dosing schedule of bortezomib [56-59]. Therefore, clinical evaluation of additional proteasome inhibitors is warranted.

Second-Generation Irreversible Proteasome Inhibitors

Two second-generation irreversible proteasome inhibitors have entered phase I and phase II trials: 1) salinosporamide A (NPI-0052), a natural product derived from the fermentation of the marine Gram-positive actinomycete *Salinospora tropica* and related to lactacystin [60-66] and 2) carfilzomib (PR-171), a modified peptide related to the natural product epoxomicin [67-70].

Schematic representation of the mechanism of action of salinosporamide A is shown in Fig. (5). Inhibition of the chymotrypsin-like, trypsin-like, and caspase-like proteolytic activities of the 20S proteasome in leukemia cells by salinosporamide A (NPI-0052) results in activation of caspase-8. This leads to proapoptotic protein Bid cleavage and to the considerable decrease of mitochondrial transmembrane potential [65]. Mitochondrial membrane permeabilization, release of cytochrome c and activation of caspase-9 follow. Apoptosis proceeds then by activation of effector caspase-3 and consequent DNA fragmentation and increased levels of intracellular reactive oxygen species were detected [65].

Compared with bortezomib, MG-132, N-acetyl-leucyl-leucyl-norleucinal (ALLN), and lactacystin, salinosporamide A was found to be the most potent suppressor of NF- κ B activation [71]. Salinosporamide A (NPI-0052) potentiated the apoptosis induced by tumor necrosis factor alpha (TNF α), bortezomib, and thalidomide, and this correlated with down-regulation of gene products that mediate cell proliferation, (cyclin D1, cyclooxygenase-2, and c-Myc), cell survival (Bcl-2, Bcl-xL, cFLIP, TRAF1, IAP1, IAP2, and survivin),

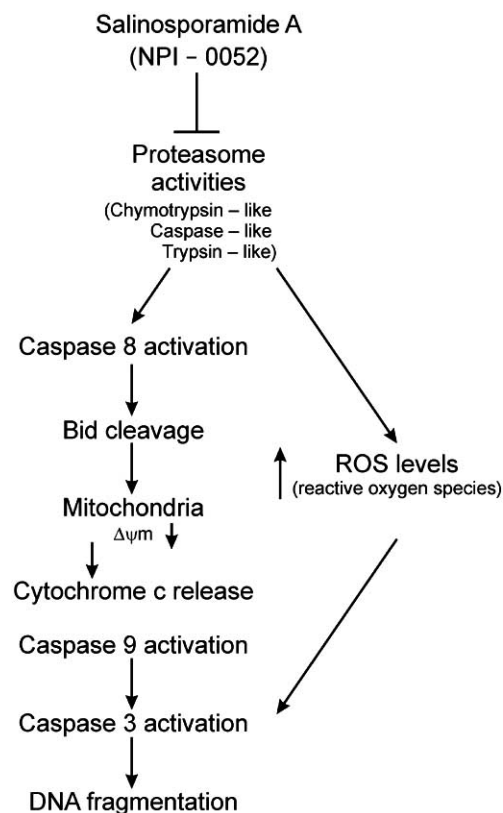


Fig. (5). Schema of the mechanism of inhibition of all three proteasome proteolytic activities (the chymotrypsin-like, caspase-like, and trypsin-like activities) by salinosporamide A (NPI-0052) in leukemia cells. NPI-0052 induces caspase-8, which initiates Bid cleavage and drops of mitochondrial membrane potential ($\Delta\psi_m$). Mitochondrial perturbations lead to the release of cytochrome c from the mitochondria, which then activates the mitochondrial – mediated proapoptotic pathway (activation of caspases-9 and -3 and consequent DNA fragmentation). Parallel pathway of cell death induction by NPI-0052 is connected with reactive oxygen species generation.

invasion (matrix metalloproteinase-9 and ICAM-1), and angiogenesis (vascular endothelial growth factor). Salinosporamide A also suppressed TNF-induced tumor cell invasion and receptor activator of NF- κ B ligand (RANKL)-induced osteoclastogenesis. NPI-0052 also inhibited both constitutive and inducible NF- κ B activation. Further studies showed that salinosporamide A inhibited TNF-induced inhibitory subunit of NF- κ B α ($I\kappa$ B α) degradation, nuclear translocation of p65, and NF- κ B-dependent reporter gene expression but had no effect on $I\kappa$ B α kinase activation, $I\kappa$ B α phosphorylation, or $I\kappa$ B α ubiquitination. Salinosporamide A enhances apoptosis, suppresses osteoclastogenesis, and inhibits invasion through suppression of the NF- κ B pathway. NPI-0052 induces apoptosis in multiple myeloma (MM) cells resistant to conventional and bortezomib therapies [71].

NPI-0052 is distinct from bortezomib in its chemical structure, effects on proteasome activities, mechanisms of action, and toxicity profile against normal cells. Moreover, NPI-0052 is orally bioactive. In animal tumor model studies,

NPI-0052 is well tolerated and prolongs survival, with significantly reduced tumor recurrence. Combining NPI-0052 and bortezomib induces synergistic anti-MM activity [66]. These studies therefore provide the rationale for clinical protocols evaluating NPI-0052, alone and together with bortezomib, to improve patient outcome in MM. Similar antitumor activity by NPI-0052 has been described in chronic lymphocytic leukemia and colon cancer cells [72,73].

Carfilzomib (PR-171) is a structurally- and mechanistically-novel proteasome inhibitor that exhibits a high level of selectivity for a single active site in the proteasome, as well as minimal cross reactivity to other protease classes. In models of MM, carfilzomib potently bound and specifically inhibited the chymotrypsin-like proteasome and immunoproteasome activities, resulting in accumulation of ubiquitinated substrates. Carfilzomib induced a dose- and time-dependent inhibition of proliferation, ultimately leading to apoptosis. Programmed cell death was associated with activation JNK, mitochondrial membrane depolarization, release of cytochrome c, and activation of both intrinsic and extrinsic caspase pathways. This agent also inhibited proliferation and activated apoptosis in patient-derived MM cells, as well as neoplastic cells from patients with other hematologic malignancies. Importantly, carfilzomib showed increased efficacy compared to bortezomib, and was active against bortezomib-resistant MM cell lines, and samples from patients with clinical bortezomib resistance. Carfilzomib also overcame resistance to other conventional agents, and acted synergistically with dexamethasone to enhance cell death. In addition to the Phase II trial in solid tumors, carfilzomib is currently being evaluated in two Phase II single-agent trials in multiple myeloma and a Phase I study in lymphoma. Phase I clinical studies have shown that patients with hematologic malignancies (MM and Waldenström's macroglobulinemia) who have relapsed or progressed following multiple therapies can achieve durable anti-tumor responses with carfilzomib [70].

Stapnes *et al.* [68] tested antiproliferative and proapoptotic effects of carfilzomib (10 nmol/l) in comparison with bortezomib (25 nmol/l) on primary AML cells derived from a total of 6 AML patients *in vitro*. An increased percentage of apoptotic cells accompanied by a statistically significant reduction of AML cells viability were detected mainly after 18 hours of culture. The antiproliferative effect of both proteasome inhibitors was also assayed for colony formation. Both, carfilzomib and bortezomib reduced AML colony formation and thus also affect the more immature clonogenic subset within the hierarchically organised AML cell population [68]. AML cells derived from 15 unselected patients were cultured together with normal bone marrow stem cells (BMSC) in the presence or absence of both proteasome inhibitors. Both drugs inhibited AML cell proliferation and decreased AML cell viability, even in the presence of BMSC. BMSC remained viable and adherent. The antiproliferative and proapoptotic effects of both proteasome inhibitors are not counteracted by the presence of non-leukemic stromal cells [68]. Both proteasome inhibitors showed similar antiproliferative effects for primary acute lymphoblastic leukemia (ALL) blasts from 8 unselected patients [68].

HISTONE DEACETYLASE INHIBITORS AND THEIR COMBINATION WITH PROTEASOME INHIBITORS

Histone deacetylase (HDAC) inhibitors are compounds that inhibit HDACs, enzymes that, in conjunction with histone acetylases, regulate the acetylation state of histones and of a growing number of nonhistone proteins including many transcription factors. There are several structural classes of histone deacetylase (HDAC) inhibitors (HDIs), including hydroxamic acids such as suberoylanilide hydroxamic acid (SAHA; vorinostat; Zolinza), belinostat (PXD101) or LAQ824/LBH589; cyclic peptides such as romidepsin (depsipeptide; FK-228; FR901228) or a potent immunosuppressant FR23522; short chain fatty acids such as phenylbutyrate (Buphenyl) and valproic acid (Depakote; Depakene); and benzamides such as MS-275 (Benzamidinone) or an isotype-specific aminophenylbenzamide MGCD0103 [74-77].

Coadministration of HDAC inhibitors with the proteasome inhibitors induce a marked increase in mitochondrial injury and apoptosis in many types of cancer cells. These events are associated with transcription factor NF- κ B inactivation, c-Jun NH₂-terminal kinase activation, p63 induction, and caspase-dependent cleavage of p21^{CIP1}, p27^{KIP1}, and Bcl-2, as well as antiapoptotic protein myeloid cell leukemia sequence 1 (Mcl-1), X-linked inhibitor of apoptosis, and cyclin D1 downregulation. Coadministration of HDAC inhibitors with the proteasome inhibitors induce also reactive oxygen species (ROS) generation and further apoptosis.

Histone Deacetylases (HDACs)

HDACs can be grouped into four distinct classes. Class I HDACs (HDAC1,2,3, and 8 with molecular weights of 22-55 kDa and homology in their catalytic sites) are ubiquitously expressed. Class II HDACs (HDAC4,5,7, and 9 with molecular weights between 120 kDa and 135 kDa) exhibit a tissue-specific pattern of expression. A subclass IIa HDACs (HDAC6 and 10) contain two catalytic sites. Both class I and II HDACs are zinc-binding enzymes, which are inhibited by SAHA at nanomolar concentrations. Class III HDACs are not inhibited by SAHA and do not have histones as primary targets. Class I and II HDACs, in addition to histones, have many nonhistone protein targets, including transcription factors and proteins that regulate cell proliferation, migration and death. Class IV or IIb HDACs includes HDAC11, which is distinct from the other classes, has conserved residues in the catalytic core region that are shared by both class I and II enzymes.

Aberrant expression of HDACs and their presence in complexes of fusion transcription factors complexes has been found in many leukemias, lymphomas and solid tumors [78,79]. Inhibitors of HDACs (HDIs) induce differentiation and/or apoptosis of transformed cells *in vitro* and inhibits tumor growth *in vivo* and they potentiate the proapoptotic effect of proteasome inhibitors.

Histone Deacetylase Inhibitors

Acetylation of nucleosomal histones in part regulates gene transcription in most cells. Differential acetylation of

nucleosomal histones results in either transcriptional activation (hyperacetylation and an open chromatin configuration) or repression (hypoacetylation and compacted chromatin) [80,81]. The role of chromatin remodeling in carcinogenesis was studied with the help of HDIs. HDIs induce the hyperacetylation of nucleosomal histones in cells resulting in the expression of aberrantly repressed genes (eg, tumor suppressor genes) that produce growth arrest, terminal differentiation, and/or apoptosis in carcinoma cells, depending on the HDI and dose used, and the cell type [82-85]. The inappropriate recruitment of HDACs provides at least one mechanism by which oncogenes could alter gene expression in favor of excessive proliferation. Thus, orally active HDIs with low toxicity towards normal cells and tissues, which would effectively inhibit tumor growth are needed for epigenetic anticancer therapy. In October 2006, the US Food and Drug Administration approved the first drug of this new class, vorinostat (SAHA, Zolinza) for treatment of cutaneous T-cell lymphoma. Several further HDIs are in clinical trials. HDIs have shown significant activity against a variety of hematological and solid tumors at doses that are well tolerated by patients, both in monotherapy as well as in combination therapy with other drugs.

Induction of Apoptosis by Histone Deacetylase Inhibitors

An almost universal effect of HDI treatment is the upregulated expression of the cyclin dependent kinase (CDK) inhibitor p21^{WAF1/CIP1}, a direct consequence of hyperacetylation of its promoter region and increased transcription of the gene [86]. No single mechanism for HDI-induced cell death has been identified. HDIs affect both G1 and G2/M cell cycle progression. G1 phase cell cycle block connected with p21^{WAF1/CIP1} and p27^{KIP1} upregulation was observed in most cell lines treated with HDIs [87]. HDI treatment causes cells to undergo aberrant mitosis and results in catastrophic mitotic failure triggering apoptosis [88,89]. Inhibition of HDAC activity was found to cause the improper kinetochore localization of the mitotic checkpoint proteins, and to prolong mitotic arrest, and thus to lead to chromosomal instability due to aberrant exit from the mitotic cell cycle arrest. In addition, treatment with HDI attenuated the activations of p38 and extracellular signal-regulated kinases, and increased the expression levels of cIAP-1 protein (a member of the inhibitor of apoptosis protein family), suggesting that the observed increased adaptation and chromosomal instability induced by inhibiting HDAC activity might be directly connected with the activations of cell survival and/or antiapoptotic signals. Moreover, the treatment of cells with mitotic defects with HDI increased their susceptibility to chromosomal instability. HDAC activity plays an important role in the regulation of mitotic checkpoint activation, and thus the aberrant control of HDAC activity contributes to chromosomal instability.

HDI treatment disrupt the integrity of the mitochondrial membrane. This is accompanied by loss of mitochondrial membrane potential and release into the cytoplasm of cytochrome c and Smac/DIABLO which potentiate caspase activation. Overexpression of the antiapoptotic protein Bcl-2

reduces HDI-induced cell death. The cleavage and activation of the proapoptotic BH3-only protein Bid by HDI treatment was also detected. HDI-induced proteolytic activation of caspase-2, -7 and -8 was strictly dependent on caspase-9. Translocation of protein Bid into the mitochondria also depends on caspase-9 [90,91]. Although HDIs trigger the mitochondrial pathway of apoptosis, HDIs also activate the extrinsic apoptosis pathway, including increased Fas (also known as CD95 and APO-1) and Fas ligand expression, activation of caspase-8, and cleavage of Bid [92-96]. Death receptors are cell surface receptors belonging to the tumor necrosis factor (TNF) super family, which trigger apoptosis upon ligand binding. One from the best characterised death receptors is Fas.

Synergistic Induction of Oxidative Injury and Apoptosis by Coadministration of HDAC Inhibitors with the Proteasome Inhibitors in Hematological Malignancies Cells

NF- κ B activation is reciprocally regulated by RelA/p65 acetylation and deacetylation, which are mediated by histone acetyltransferases (HATs) and deacetylases (HDACs). In leukemia cells, NF- κ B activation by the HDAC inhibitors (HDI) MS-275 and suberoylanilide hydroxamic acid was associated with hyperacetylation and nuclear translocation of RelA/p65. Interference with these events by either pharmacological or genetic means leads to a dramatic increase in HDI-mediated lethality through enhanced oxidative damage, downregulation of NF- κ B-dependent antiapoptotic proteins, and stress-related c-Jun N-terminal kinase 1 (JNK1) activation [97]. Bortezomib inhibits NF- κ B activation through inhibition of I κ B proteasome degradation, stabilizes I κ B and markedly potentiates apoptosis induced by HDI. Bortezomib works synergistically to enhance the proapoptotic effect of HDI and overcomes the overexpression of the antiapoptotic protein Bcl-2. Bortezomib induces Bcl-2 phosphorylation and its cleavage associated with arrest of the cell cycle at G₂ – M phase [98].

Synergistic induction of oxidative stress and apoptosis in human multiple myeloma cells was observed after sequential exposure to bortezomib with HDAC inhibitors [99]. These agents combination potently induces mitochondrial dysfunction and apoptosis of multiple myeloma cells through a radical oxygen species-dependent mechanism.

Inhibition of Aggresome Formation by HDAC-6 Inhibitors Potentiate the Efficacy of Bortezomib

Bortezomib triggers apoptosis in pancreatic cancer cells, but the mechanisms involved have not been fully elucidated. Nawrocki *et al.* [100] described that pancreatic cancer cells exposed to bortezomib formed aggregates of ubiquitin-conjugated proteins ("aggresomes") *in vitro* and *in vivo*. Bortezomib-induced aggresome formation was determined to be cytoprotective and could be disrupted using histone deacetylase (HDAC)-6 small interfering RNA or chemical HDAC inhibitors, which resulted in endoplasmic reticulum stress and synergistic levels of apoptosis *in vitro* and in an orthotopic pancreatic cancer xenograft model *in vivo*. Interestingly, bortezomib did not induce aggresome formation in

immortalized normal human pancreatic epithelial cells *in vitro* or in murine pancreatic epithelial cells *in vivo*. In addition, these cells did not undergo apoptosis following treatment with bortezomib, suberoylanilide hydroxamic acid, or the combination, showing tumor selectivity. Taken together, this study shows that inhibition of aggresome formation can strongly potentiate the efficacy of bortezomib and provides the foundation for clinical trials of bortezomib in combination with HDAC inhibitors for the treatment of pancreatic cancer or multiple myeloma [101] and possibly in other hematological malignancies.

CLINICAL TRIALS USING PROTEASOME INHIBITORS OR THEIR COMBINATION WITH HISTONE DEACETYLASE INHIBITORS AS A NOVEL TREATMENT STRATEGY IN LEUKEMIA

Although the treatment of patients suffering from leukemia has considerably improved throughout the last years, new chemotherapeutic agents are urgently required to further improve and optimise treatment protocols, minimise side-effects and prolong overall survival rates. Relapsing patients usually develop resistance to standard chemotherapeutics and new agents are needed. As far as concerns acute myeloid leukemia (AML), bortezomib has been tested in three small clinical trials, in one it was used as a single agent [102] and in two others, bortezomib was combined with conventional agents [103, 104].

Bortezomib as a Single Agent in Refractory or Relapsed AML

In the phase I study [102], authors investigated the maximum tolerated dose and dose-limiting toxicity of bortezomib as a single agent in patients with acute leukemias refractory to or relapsing after prior therapy. Fifteen patients were treated with 0.75 (n = 3), 1.25 (n = 7), or 1.5 (n = 5) mg/m² bortezomib administered twice weekly for 4 weeks every 6 weeks. Dose-limiting toxicity included orthostatic hypotension (n = 2), nausea (n = 2), diarrhea (n = 1), and fluid retention (n = 1), all at 1.5 mg/m² bortezomib. Proteasome inhibition was dose dependent and reached 68% at 1.5 mg/m² bortezomib. Peak inhibition was observed 1 h after treatment and returned to near baseline levels by 72 h after treatment. Incubation of blast cells with bortezomib *in vitro* showed induction of apoptosis in three of five patients investigated. Authors conclude that the maximum tolerated dose of bortezomib in patients with acute leukemia is 1.25 mg/m², using a twice-weekly for 4 weeks every 6 weeks schedule. The *in vitro* evidence of antileukemia and transient hematological improvements observed in some patients warrants further investigation of bortezomib in acute leukemias, probably in combination with other agents.

Combination of Bortezomib with Conventional Agents

In the first study [103], authors determined the maximum tolerated dose (MTD) and dose-limiting toxicities of bortezomib and pegylated liposomal doxorubicin (PegLD) but they used only 2 patients with AML. Bortezomib was given on days 1, 4, 8, and 11 from 0.90 to 1.50 mg/m² and PegLD

on day 4 at 30 mg/m² to 42 patients with advanced hematologic malignancies. Grade 3 or 4 toxicities in at least 10% of patients included thrombocytopenia, lymphopenia, neutropenia, fatigue, pneumonia, peripheral neuropathy, febrile neutropenia, and diarrhea. The MTD based on cycle 1 was 1.50 and 30 mg/m² of bortezomib and PegLD, respectively. However, due to frequent dose reductions and delays at this level, 1.30 and 30 mg/m² are recommended for further study. Pharmacokinetic and pharmacodynamic studies did not find significant drug interactions between these agents. Antitumor activity was seen against multiple myeloma, with 8 of 22 evaluable patients having a complete response (CR) or near-CR, including several with anthracycline-refractory disease, and another 8 having partial responses (PRs). One patient with relapsed/refractory T-cell non-Hodgkin lymphoma (NHL) achieved a CR, whereas 2 patients each with acute myeloid leukemia and B-cell NHL had PRs.

Bortezomib/PegLD was safely administered in this study with promising antitumor activity, supporting further testing of this regimen.

In the further study [104], bortezomib was given on days 1, 4, 8, and 11 at doses of 0.7, 1.0, 1.3, or 1.5 mg/m² with idarubicin 12 mg/m² on days 1 to 3 and cytarabine 100 mg/m²/day on days 1 to 7. A total of 31 patients were enrolled. The median age was 62 years, and 16 patients were male. Nine patients had relapsed AML (ages, 18-59 years, n = 4 and > or = 60 years, n = 5). There were 22 patients of > or = 60 years with previously untreated AML (eight with prior myelodysplasia/myeloproliferative disorder or cytotoxic therapy). All doses of bortezomib, up to and including 1.5 mg/m², were tolerable. Nonhematologic grade 3 or greater toxicities included 12 hypoxia (38%; 11 were grade 3), 4 hyperbilirubinemia (13%), and 6 elevated aspartate aminotransferase (19%).

Overall, 19 patients (61%) achieved complete remission (CR) and three had CR with incomplete platelet recovery. Pharmacokinetic studies revealed that the total body clearance of bortezomib decreased significantly (P < 0.01, N = 26) between the first (mean +/- SD, 41.9 +/- 17.1 L/h/m²) and third (18.4 +/- 7.0 L/h/m²) doses. Increased bone marrow expression of CD74 was associated with CR. The combination of bortezomib, idarubicin, and cytarabine showed a good safety profile. The recommended dose of bortezomib for phase II studies with idarubicin and cytarabine is 1.5 mg/m² [104].

Experimental Studies Comparing Bortezomib and Conventional Agents Activity in AML Cells

AML is a genetically heterogeneous clonal disorder characterized by the accumulation of acquired somatic genetic alterations in hematopoietic progenitor cells that alter normal mechanisms of self-renewal, proliferation and differentiation. There is the great problem of AML heterogeneity due to wide array of genetic lesions and immunophenotypic profiles. AML can be divided into immature (CD34⁺) and mature (CD34⁻) forms. Immature AML forms are associated with drug resistance and poorer outcome [105,106]. Colado

et al. [107] carried out a detailed analysis of the *in vitro* activity and mechanism of action of bortezomib on AML cells using both cell lines and fresh cells from patients with immature or mature AML. Activity of bortezomib was compared with that of conventional agents. Cells were incubated with 50 nM bortezomib, 1 μ M doxorubicin or 1 μ M cytarabine, or without any drug (control) for 18 h. The number of apoptotic cells was measured in each sample. Bortezomib induced caspase-3-dependent apoptosis in HEL cells because caspase-3 inhibitor blocked this apoptosis. The average percentage of apoptosis induced by bortezomib in the fresh total blast AML cell samples was $48 \pm 22\%$ (mean \pm SD). In 14 from 28 samples, bortezomib induced apoptosis in $\geq 50\%$ of leukemic cells. The apoptotic activity of bortezomib on CD34⁺ blast cells was similar to that observed in CD34⁻ blast cells.

Gil *et al.* [108] analysed *in vitro* drug resistance to bortezomib and other anticancer drugs in de novo and relapsed adult AML cells of 46 patients. AML cells were sensitive to bortezomib and the *in vitro* activity of this drug was not influenced by the presence of drug resistance proteins, which was also observed by other investigators [109]. Thus bortezomib is insensitive to multidrug resistance proteins and this is important for treatment of relapsed/refractory AML patients with overexpression of multidrug resistance proteins.

Riccioni *et al.* [110] explored *in vitro* the sensitivity of leukemic blasts derived from 30 AML patients to bortezomib. Bortezomib induced the apoptosis of primary AML blasts. 18 AMLs were highly sensitive to the proapoptotic effect of bortezomib. Remaining AML cases were moderately sensitive to bortezomib. The majority of AMLs sensitive to bortezomib showed immunophenotypic features of the M4 and M5 French-American-British classification subtypes and displayed myelomonocytic features. All AMLs with mutated *FLT3* were in the bortezomib-sensitive group. Bortezomib activated caspase-8 and caspase-3 and decreased cellular FLICE (Fas-associated death domain-like interleukin-1 β -converting enzyme)-inhibitory protein (c-FLIP) levels in AML blasts.

These results strongly suggest that proteasome inhibition should be considered in AML therapy.

Experimental Studies Using HDAC Inhibitors and Their Combination with Bortezomib

Modulation of gene expression through HDAC inhibition is considered a possible therapeutic strategy in AML [111,112]. *In vitro* effects and basal gene expression of structurally different HDAC inhibitors (HDIs) were examined. Primary human AML cells were derived from 59 consecutive patients. The HDIs valproic acid, PXD101, trichostatin A and sodium butyrate inhibited leukemic and clonogenic cell proliferation and increased apoptosis in a dose-dependent manner when tested at high concentrations. However, at lower concentrations proliferation increased for a subset of patients. This divergence was also observed in the presence of all-trans retinoic acid, theophylline and decit-

abine, and in cocultures with bone marrow stromal cells. Levels of IL-1beta, IL-6, GM-CSF and TNFalpha increased.

Based on the basal expression of 100 genes the patients with growth enhancement at intermediate HDAC inhibitor concentrations and those without this response were clustered into two mutually exclusive groups. Functional characterization and gene expression analyses identify AML patient subsets that differ in their response to HDAC inhibitors. These observations may explain why HDAC inhibitor therapy affects only a subset of AML patients [111-113].

The effect of bortezomib (10 nmol/l) and HDAC inhibitor valproic acid (1.2 mmol/l) on cytokine-dependent AML cell proliferation was tested [65]. Bortezomib caused 62% growth inhibition when tested alone and 79% inhibition in combination with valproic acid, whereas valproic acid alone caused no significant inhibition. HDAC inhibitors are promising candidates for molecular-targeted therapy for leukemia. Mechanisms of cytotoxic effects of depsipeptide were markedly enhanced by bortezomib in HL-60 and K562 cells [114]. Caspase-9 was activated, the mitochondrial membrane was permeabilized and cytochrome c was released and this event is almost completely blocked by the overexpression of Bcl-2. The mitochondrial damage caused the translocation of Bax to the mitochondria, mitochondrial depolarization and activation of caspases.

Antitumor Activity of Bortezomib in Combination with Trail in AML

Conticello *et al.* [115] examined the sensitivity of bone marrow cells from AML patients (34 patients; 25 newly diagnosed, 4 relapsed, 5 refractory) to bortezomib alone or in combination with TRAIL, a member of the tumor necrosis factor (TNF) family that induces apoptosis in tumor cells while sparing normal cells. Bortezomib induced cell death in blasts from all 34 AML patients. Apoptosis was connected a downregulation of antiapoptotic proteins Bcl-xL and Mcl-1, an upregulation of TRAIL-R1, TRAIL-R2, p21^{CIP1}, activation of executioner caspases and a loss of the mitochondrial membrane potential. Bortezomib primed also TRAIL-resistant AML cells for enhanced TRAIL-mediated killing. These results suggest that a combination of bortezomib or other proteasome inhibitors and TRAIL could be effective for treating AML patients, even patients who are refractory to conventional chemotherapy.

Our Experimental Studies on Induction of Apoptosis in AML Cells

We [116] studied the effect of TGF- β 1 and of bortezomib on induction of apoptosis in human acute myeloid leukemia cell lines ML-1, ML-2, CTV-1 and KASUMI1. Leukemia cells were preincubated for 24-96 h without addition (control group) or with TGF- β 1 (5 ng/ml or 10 ng/ml) or with bortezomib (4 nM or 10 nM). Apoptosis was detected by flow cytometry using annexin V-FITC/propidium iodide assay and by cell cycle analysis. Caspase-3-like enzyme activity was measured by enzymatic cleavage of fluorogenic peptide substrate, Ac-DEVD-7-amino-4-methyl-coumarin. Statistical

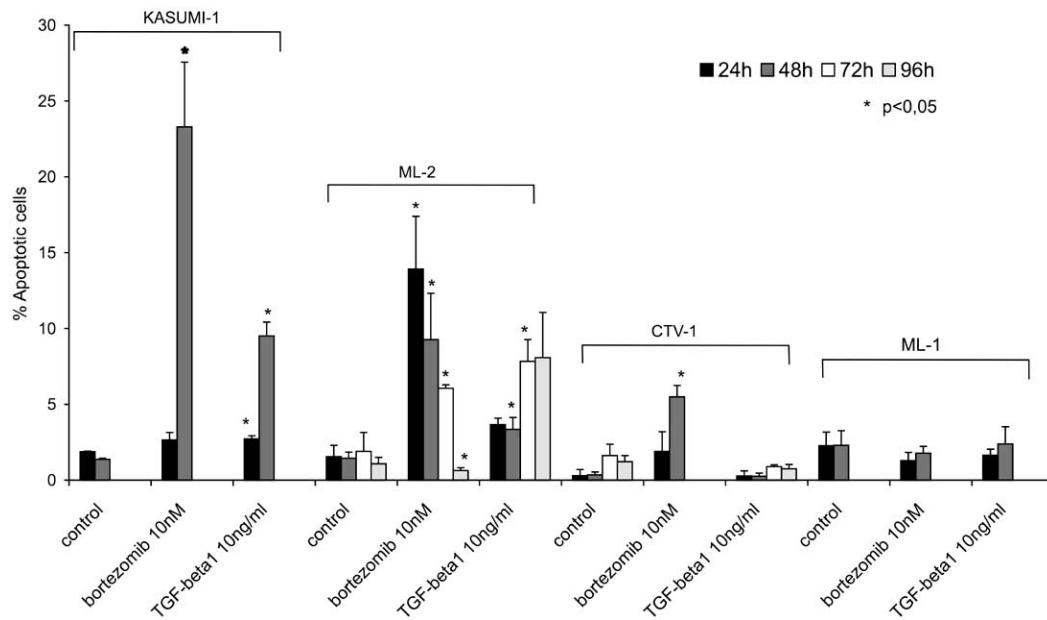


Fig. (6). Induction of apoptosis by bortezomib in comparison with TGF-β1 in human leukemia cells of lines KASUMI-1, ML-2, CTV-1 and ML-1. Leukemia cells were preincubated for 24-96 h without addition (control group) or with bortezomib (10 nM) or with TGF-β1 (10 ng/ml). Apoptosis was detected by flow cytometry using annexin V-FITC/propidium iodide assay. Statistical significance of results was analyzed by Student’s paired t-test.

significance of the experimental results was analyzed by Student’s paired t-test.

TGF-β1 inhibited DNA synthesis only in KASUMI-1 cells but not in other leukemia cells used. Bortezomib (10 nM) was potent inhibitor of DNA synthesis, measured by incorporation of [6-³H]thymidine into DNA, in all four types of leukemia cells and induced apoptosis in KASUMI-1, ML-2 and CTV-1 cells but not in ML-1 cells as is shown in Fig. (6). KASUMI-1 and ML-2 cells were also sensitive to induc-

tion of apoptosis by TGF-β1 but in lesser extent than by bortezomib. Kinetics of apoptosis was different in individual cell lines and was slower in induction by TGF-β1 than by bortezomib. Bortezomib (4 nM or 10 nM) induced caspase-3 in ML2 cells after 24 h treatment as is presented in Fig. (7) but only 10 nM bortezomib induced caspase-3 after 48 h treatment in KASUMI1 cells as is demonstrated in Fig. (8).

Our results show antiproliferative and proapoptotic effects of bortezomib in human AML cell lines. Only ML-1

caspase-3 activity, ML2 cells

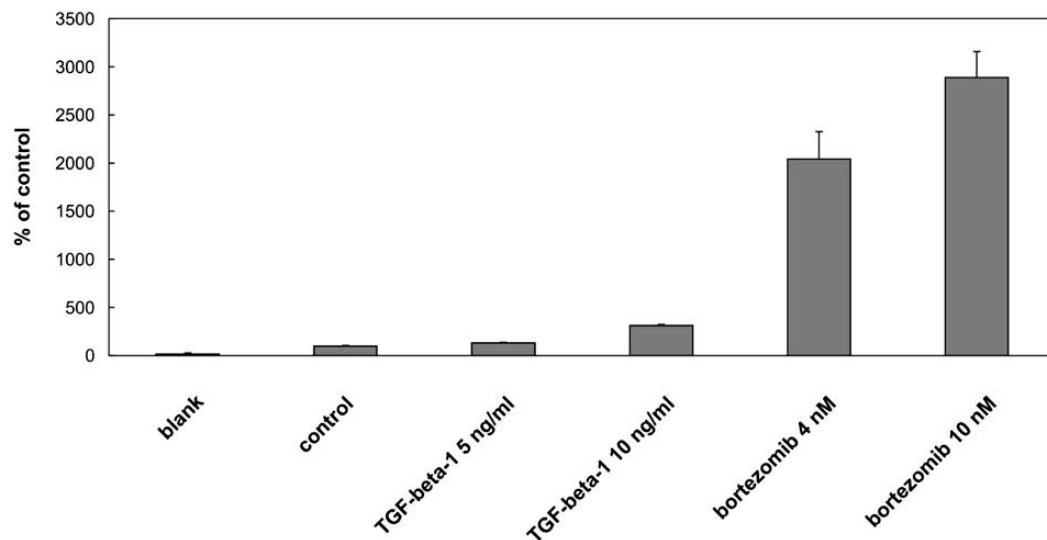


Fig. (7). Caspase-3 activity in ML-2 cells treated for 24 h without addition (control group) or with bortezomib (4nM or 10 nM) or with TGF-β1 (10 ng/ml or 20 ng/ml). Caspase-3-like enzyme activity was measured by enzymatic cleavage of fluorogenic peptide substrate, Ac-DEVD-7- amino-4-methyl-coumarin. Statistical significance of results was analyzed by Student’s paired t-test.

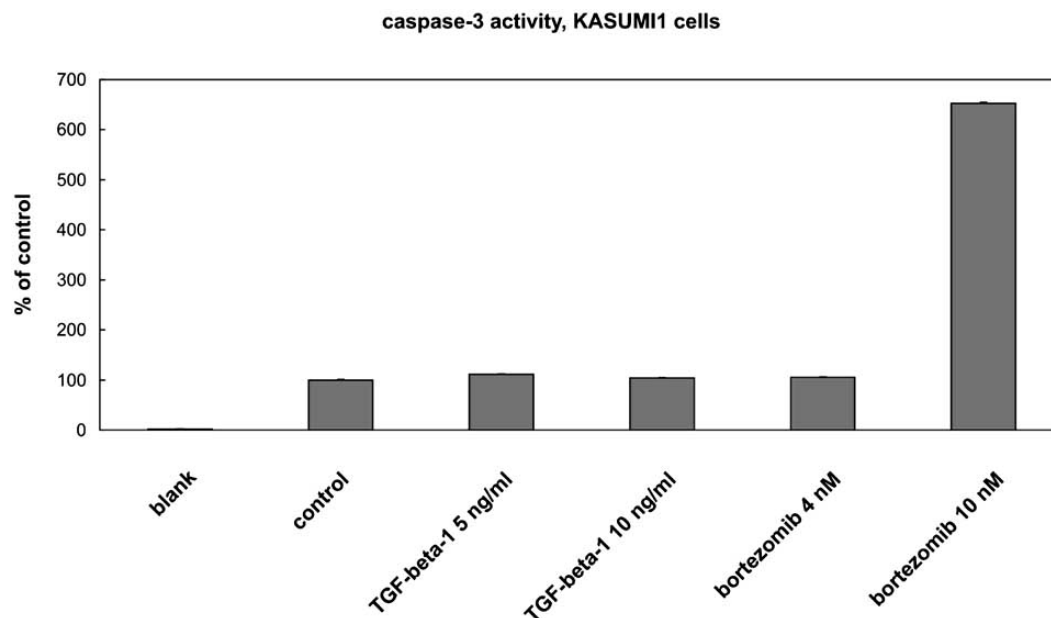


Fig. (8). Caspase-3 activity in KASUMI-1 cells treated for 48 h without addition (control group) or with bortezomib (4nM or 10 nM) or with TGF- β 1 (5 ng/ml or 10 ng/ml). Caspase-3-like enzyme activity was measured by enzymatic cleavage of fluorogenic peptide substrate, Ac-DEVD-7- amino-4-methyl-coumarin. Statistical significance of results was analyzed by Student's paired t-test.

cells were resistant to the induction of apoptosis by bortezomib. We confirmed also great heterogeneity of AML cells.

Effect of Bortezomib as a Single Agent or in Combination with HDAC Inhibitors or with Other Conventional Agents in Further Types of Leukemia

Bortezomib as a single agent or combined with HDAC inhibitors or with other drugs inhibits growth and induces apoptosis also in Bcr/Abl positive chronic myeloid leukemia cells sensitive or resistant to imatinib mesylate [117-119], chronic lymphocytic leukemia cells [120-125] and adult T-cell leukemia [126-131].

CONCLUSION AND PERSPECTIVES

Proteasomal proteolysis relies on the activity of six catalytically active proteasomal subunits (β 1, β 2, β 5 and three immunosubunits β 1i, β 2i, and β 5i) of the 20S proteasome or immunoproteasome core. These subunits have proteolytic activities (β 1-caspase like activity, β 2-trypsin-like activity, and β 5- chymotrypsin-like activity). Activities and constitutive subunits of proteasome vary in individual cases of AML and also between leukemia types [132]. This influences the sensitivity of the leukemias to proteasome inhibitors. Bortezomib inhibits the β 5 subunit activity and to a lesser extent the β 1 subunit activity. At higher concentrations, bortezomib inhibits also β 2 subunit activity. The sensitivity of cells to bortezomib depends on the β 2 to β 5/ β 1 activity ratio. When this ratio is relatively low, cells are sensitive to bortezomib. However, the relatively high ratio means resistancy of cells to bortezomib [132]. Recently, molecular basis of bortezomib resistance has been described [133]. An Ala49Thr mutation residing in a highly conserved bortezomib-binding pocket in the proteasome β 5 subunit (PSMB5) protein and a

dramatic overexpression of this mutated PSMB5 protein cause bortezomib resistance [133]. Bortezomib sensitivity in bortezomib-resistant cells can be restored by specific siRNA-mediated silencing of *PSMB5* gene expression [129]. In future, it is possible to tailor proteasome inhibitors to individual leukemia cases where the predominant proteolytic activities of the proteasomes are determined using labeled probes which bind the catalytic sites.

Bortezomib is an excellent example of a novel highly effective agents that had been quickly translated to clinical practice. Preclinical and clinical data demonstrate synergistic activity with other agents in relapsed and refractory multiple myeloma and mantle cell lymphoma, and also in newly diagnosed patients with these diseases. Preliminary results suggest that response rates may be improved by this combination therapy. Further studies are necessary to find the best sequence and combination of effective agents in order to improve the prognosis of patients in the near future. Adverse effects related to bortezomib are predictable, manageable, and reversible and if necessary, can be solved by dose reductions.

Bortezomib as single agent or combined with HDAC inhibitors or with other drugs inhibits growth and induces apoptosis also in leukemia cells sensitive or resistant to conventional chemotherapeutics. Results of studies in this field strongly suggest that proteasome inhibition should be considered in antileukemic therapies [134,135]. Moreover, a new generation of irreversible proteasome inhibitors (salinosporamide A and carfilzomib) that in preclinical studies at least partially overcome bortezomib resistance *in vitro* have been developed. These inhibitors are more potent, well tolerated, and have less neurotoxicity than bortezomib.

Hematopoietic cell transplantation (HCT) offers potentially curative therapy for patients with MDS and leukemia. The incidence of acute graft-versus-host disease (GVHD) is an undesirable complication of allogeneic bone marrow transplantation (BMT). Bortezomib, administered immediately following murine allogeneic BMT resulted in marked inhibition of acute GVHD with retention of graft-versus-tumor effects [136]. However, delayed bortezomib administration (5 or more days after BMT) increased GVHD susceptibility and significantly accelerated GVHD-dependent morbidity [137].

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