Proteasome: an emerging target for cancer therapy

Ivana Zavrski[a], Christian Jakob[a], Peter Schmid[a], Holger Krebbel[a], Martin Kaiser[a], Claudia Fleissner[a], Marleen Rosche[a], Kurt Possinger[a] and Orhan Sezer[a]

Proteasome inhibitors represent novel anti-cancer drugs which interact with the proteasome–ubiquitin pathway. The 26S proteasome is a multicatalytic threonine protease with three distinct catalytic activities. It is responsible for intracellular protein turnover in eukaryotic cells, including the processing and degradation of short- and some long-living proteins required for regulation of various cellular functions. Subsequently, the inhibition of the proteasomal function results in stabilization and accumulation of its substrates, which notably include cyclins, cyclin-dependent kinase inhibitors, transcriptional factors, tumor suppressor proteins and proto-oncogenes. This results in confounding signals in the cell inducing cell cycle arrest and activation of apoptotic programs. Acting on transcriptional factor NF-κB, which is upregulated in some tumors undergoing chemotherapy or irradiation and downregulated by proteasome inhibition, a significant chemosensitization and consequently synergistic effects concerning the anti-tumor activity could be achieved. Bortezomib is the first proteasome inhibitor that has entered clinical trials. In multiple myeloma, both the US Food and Drug Administration and European Medicine Evaluation Agency granted approval for the use of bortezomib (Velcade) for the treatment of multiple myeloma patients who have received at least two prior therapies and have demonstrated disease progression on the last therapy. At present, other trials examine the activity in a variety of solid tumors and hematological malignancies. This paper reviews preclinical and clinical results. *Anti-Cancer Drugs* 16:475–481 © 2005 Lippincott Williams & Wilkins.

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[a]Department of Oncology and Hematology, University Hospital Charité, Berlin, Germany.

Correspondence to O. Sezer, Department of Oncology and Hematology, University Hospital Charité, 10117 Berlin, Germany.
Tel: +49 30 450613105; fax: +49 30 450527907;
e-mail: sezer@charite.de

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Structure of the proteasome

The 26S proteasome is a highly conserved, multicatalytic enzyme complex expressed in the cytoplasm and nucleus of eukaryotic cells [1,2]. It is responsible for extralysosomal degradation of ubiquitinated proteins, which are predominantly involved in the regulation of cell differentiation, cell cycle control, stress response, antigen presentation and removal of misfolded proteins. Using electron microscopy, proteasomes appear to be symmetrical ring-shaped particles (Fig. 1a) [1,3]. The 26S enzyme complex consists of two major components: the catalytically active 20S core and two ATP-dependent 19S regulatory subunits [1,4]. The catalytic core shows a four-striated cylindrical structure with a 7-fold rotational symmetry. The two outer rings are composed of seven non-proteolytic α subunits; both inner rings are built from seven β subunits, which express post-glutamyl peptide hydrolase-like (β1 subunit), trypsin-like (β2 subunit) and chymotrypsin-like (β5 subunit) activities formed by N-terminal threonine residues (Fig. 1b) [5–7]. The outer α rings interact with the regulatory 19S subunits, which are responsible for recognition of ubiquitinated substrates and cleavage of the ubiquitin chains prior to translocation into the core. For appropriate hydrolysis of proteins, a conformational plasticity of the complex must be provided for the interaction between the substrates, core and the regulatory complex [8,9].

Biological functions of the proteasome

The observation of high proteasome levels in human leukemia cells and the increase of proteasome concentration in normal human mononuclear cells during blastogenic transformation induced by phytohemagglutinin, their oscillation during the cell cycle and parallel increase with induction of DNA synthesis led to the hypothesis that proteasomes may be involved in transformation and proliferation of cells [10]. Further studies revealed that many cell cycle and cell survival regulatory proteins, like cyclins, cyclin-dependent kinase inhibitors (p19, p21, p27 and p57), tumor suppressor proteins (p53 and Rb), proto-oncogenes (c-*myc*, c-*fos* and c-*jun*), topoisomerases, pro-apoptotic molecules (Bax, MDM2 and IAPs), transcriptional factors (E2A, E2F and STAT) and inhibitor of NF-κB, I-κB, are degraded by the proteasome (Table 1) [11–31]. The disruption of the balance of exactly coordinated levels of pro- and anti-proliferative signals leads to confounding signals in cells, which result

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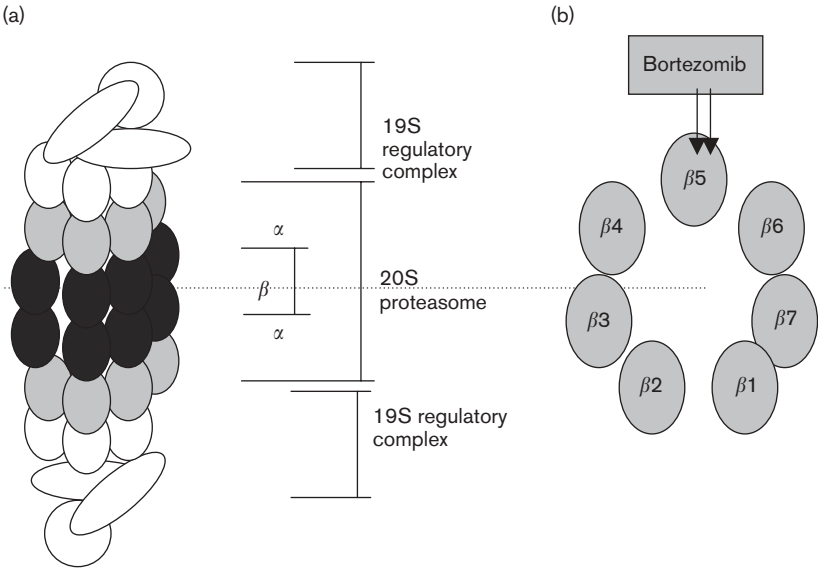
Fig. 1



The profile of the 26S proteasome. (a) The 26S proteasome is a 2000-kDa protein complex which consists of one 20S core (gray) and two regulatory 19S subunits (white). The core consists of two inner β rings (dark gray), carrying the catalytic sites faced to the central cavity. The outer α rings (light gray) arbitrate the interaction between the catalytic sites and the regulatory subunits. (b) Bortezomib interacts with threonine residues located on the β5 subunit that confers chymotryptic proteolytic activity.

Table 1 Examples of proteasome substrates and their physiological functions

	Protein	Function	Reference
Transcriptional factors	E2A	cellular growth and differentiation	Kho 1997 [27]
	E2F	cell cycle regulation by gene expression control	Campanero 1997 [28]
	STAT	transcription of cytokine-inducible genes	Kim 1996 [29]
Inhibitors of transcriptional factors	I-κB	inhibition of transcriptional factor NF-κB, which is responsible for the transcription of numerous growth factors, cell adhesion molecules, angiogenetic factors and anti-apoptotic proteins	Palombella 1994 [30]; Traenckner 1994 [31]
Cyclins	cyclin A	cell cycle regulation during S phase and mitosis	Sudakin 1995 [11]
	cyclin B	cell cycle regulation during mitosis	Zhang 1998 [12]
	cyclin C	cell cycle re-entry	Cooper 1999 [13]
	cyclin D	cell cycle regulation during G_1 phase	Diehl 1997 [14]
	cyclin E	cell cycle regulation during G_1 phase and S phase	Clurman 1996 [15]
Cyclin-dependent kinase inhibitors	p19	G_1/S progression	Thullberg 2000 [16]
	p21	Cell cycle regulation	Blagosklonny 1996 [17]
	p27	Cell cycle regulation	Pagano 1995 [18]
	p57	cell cycle regulation and differentiation	Urano 1999 [19]
Oncogenes	c-Myc/c-Fos/c-Jun	promotion of proliferation, control of transcriptional factor AP-1	Stancovski 1995 [23]
Tumor suppressor proteins	p53	cell cycle arrest, senescence, apoptosis	Maki 1996 [20]
	Rb	repressor of progression towards S phase by inactivation of the transcription factor E2F which is required for activation of S phase genes	Boyer 1996 [21]
Apoptosis	topoisomerase II	promotion of cell survival, DNA replication	Nakajima 1996 [24]
	Bax	promotion of apoptosis	Chang 1998 [25]
	MDM2	promotion of apoptosis	
	IAPs	inhibition of apoptosis	Yang 2000 [26]

in cell cycle arrest, activation of caspases and induction of apoptosis. Furthermore, previous research revealed a crucial role of NF-κB in cancer progression. When liberated from I-κB, NF-κB translocates into the nucleus, where it binds to DNA and controls transcription of several genes involved in growth, cell differentiation and apoptosis. Moreover, it promotes oncogenesis and is constitutively activated in some malignant cells [32,33], or in response to chemotherapeutic drugs, radiation, cytokines or oxidants [34,35]. Consistent with this, inhibition of NF-κB is a critical point in inducible chemoresistance and it seems to increase the susceptibility of malignant cells to chemotherapeutics or irradiation (for review, see [36,37]). Targeting NF-κB by using inhibitors of I-κB phosphorylation could not completely prevent cell proliferation, which indicates that effects of proteasome inhibitors are not only due to NF-κB blockade alone, but also result from targeting other signaling pathways [38].

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Proteasome inhibitors

The first compound with documented effects on proteasomal function was identified as non-peptidic *Streptomyces* metabolite lactacystin. Its intermediate clasto-lactacystin β-lactone interferes with the β5 (chymotrypsin-like) subunit irreversibly by selective modification of N-terminal threonine residues, and reversibly with the β1 (peptidylglutamyl) and β2 (trypsin-like) subunits [39,40]. In the course of further characterization of proteasome structure using X-ray crystallography, more selective and potent proteasome inhibitors have been synthesized [5,41]. They include tripeptide aldehydes PSI and MG-132, which inhibit reversibly β2 and β5 subunits by forming hemiacetal adducts with the active site of β subunits. In addition, they also inhibit thiol proteases like calpains and cathepsin B, and display metabolic instability due to their configuration, so that poor specificity and bioavailability limit their utility *in vivo* [5]. The next generation of proteasome inhibitors includes boronic acid dipeptides MG-262 and bortezomib, which are capable of building stable, but reversible tetrahedral intermediates with the amino-threonine residues of the catalytically active core complex. This explains their slow dissociation from the proteasome in comparison to the aldehydes, the 1000-fold potency and an extremely high sensitivity for the chymotrypsin-like activity of the proteasome. The reversibility of the proteasome inhibition and high stability allowed bortezomib to enter clinical trials.

Preclinical experience with proteasome inhibitors

The first evidence of broad anti-tumor potential of proteasome inhibitors was given in 1999 by the National Cancer Institute screen of 60 tumor cell lines [42]. Mean IC_{50} values for bortezomib, a reversible boronic acid dipeptide proteasome inhibitor, were found to be in the nanomolar range. For further evaluation of mechanisms of proteasome inhibitor-mediated cytotoxicity, the authors used the prostate tumor PC-3 cell line. In an *in vitro* assay, the induction of apoptosis was independent of p53 status, but was accompanied by p21 stabilization, G_2/M arrest and PARP cleavage. Using a PC-3/nude mouse model, treatment with bortezomib significantly suppressed tumor growth at well-tolerated doses. The pharmacodynamics of i.v. administered drug were studied in white blood cells, brain, colon, liver, muscle, prostate and testes. In contrast to brain and testes, where no proteasome inhibition was observed, in other tissues the 20S activity significantly decreased 1 h after treatment, but returned to baseline levels after 24 h [42].

Further investigations showed pro-apoptotic effects in the MCF-7 and in EMT-6 murine mammary carcinoma *in vivo/in vitro* assay [43], Lewis lung carcinoma [43], squamous cell carcinoma [44,45], pancreatic cancer cell lines [46], ovarian cell lines HEY, A2780, SKOV3 and OVCA 429 [47], human glioma cell lines U-87MG and T98G [48], myeloid leukemia lines, and adult B and T cell leukemia cell lines [49–51]. In multiple myeloma, it was demonstrated that bortezomib induced apoptosis directly and additionally by abrogating paracrine growth stimulation [52]. Furthermore, the adhesion of myeloma to bone marrow stromal cells was diminished by treatment with proteasome inhibitors, which resulted in reduced mitogen-activated protein kinase growth signaling and protection against apoptosis induced by dexamethasone [52]. Acting on NF-κB, subtoxic dosages of bortezomib could sensitize both drug-susceptible [37] and primarily drug-resistant myeloma cell lines to doxorubicin and melphalan [53]. Interestingly, the extent of apoptosis induction in primary myeloma cells by proteasome inhibitors was independent of chromosome 13 deletion status, a subgroup with a dismal prognosis [54]. The combination therapy with gemcitabine followed by bortezomib showed the greatest induction of apoptosis in MIA-PaCa-2 pancreatic cancer cell line, when compared with simultaneous incubation or preincubation with bortezomib. When the agents were given in sequence or in combination, no correlation with the modified levels of p21, p27 and BCL-2 have been observed, which might indicate that bortezomib could block endogenous cell survival response following exposure to chemotherapy [46]. In human colon cancer cell lines CCD841, KM12L4, LOVO and WiDr, pre-treatment with bortezomib for 1 h prior to addition of camptothecin suppressed camptothecin induced NF-κB activation markedly, resulting in enhanced anti-tumor effects, reflected in significantly improved growth inhibition when compared with treatment with bortezomib or camptothecin alone. When camptothecin and bortezomib at the maximum tolerated dose (MTD) of 1.0 mg/kg were given systemically to mice bearing a LOVO cancer xenograft, a significant improvement of tumoricidal response was observed and reached more than 90% decrease in tumor size. The authors hypothesized that the camptothecin/topoisomerase I complex would be degraded by the proteasome under physiological conditions, since it was shown that destruction of topoisomerase I could be prevented in cells treated with proteasome inhibitors MG-132 and lactacystin [55]. The inhibition of the proteasomal degradation of the complex might prolong the half-life time of the complex and result in enhanced activity of the topoisomerase inhibitors [56].

Clinical experience with bortezomib

Phase I trials

To determine MTDs, dose-limiting toxicities (DLTs) and pharmacodynamics (PD), several studies concerning hematological malignancies and solid tumors, including prostate and non-small cell lung carcinoma (NSCLC), have been performed (Table 2). The MTDs were found

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Table 2 Phase I trials with bortezomib

Tumor	Treatment schedule	No. of patients	MTD and response	DLTs/adverse events	References
Refractory hematologic malignancies	2 × w × 4 q6[a]	27	MTD 1.04 mg/m^2; major anti-tumor activity in myeloma with one CR, some in MCL and FL	DLT: thrombocytopenia, electrolyte disturbances, malaise, fatigue; adverse events: neutropenia, diarrhea, constipation	Orlowski 2002 [57]
AML/ALL/MDS high risk	2 × w × 4 q6	15	MTD 1.25 mg/m^2; transient hematological improvement in 33% of patients	DLT: orthostatic hypotension	Cortes 2004 [58]
Solid tumors	2 × w × 2 q3[b]	43	MTD: 1.56 mg/m^2; one major response in a patient with refractory NSCLC	DLT: diarrhea, neuropathy; adverse events: fever, fatigue, nausea, vomiting, rash, pruritus, headache	Aghajanian 2002 [60]
Solid tumors	1 × w × 4 q5[c]	53	MTD 1.6 mg/m^2; biologic and anti-tumor activity in androgen independent prostate carcinoma	DLT: diarrhea, hypotension, syncope	Papandreou 2004 [59]
NSCLC	2 × w × 2 q3 + gemcitabine + carboplatin	16	recommended dose for phase II trial for first-line treatment: bortezomib 1.0 mg/m^2 2 × w × 2 q3, gemcitabine 1000 mg/m^2, carboplatin AUC 5.0; some partial remission and stable disease	DLT: thrombocytopenia, neutropenia	Davies 2004 [61]
Unresectable HCC	2 × w × 2 q3	18 so far	MDT 1.3 mg/m^2	grade 2–3: thrombocytopenia, hypotension, fatigue, neuropathy, abdominal cramps, loss of appetite; grade 4: thrombocytopenia	Hegewisch-Becker 2004 [62]

[a]Treatment schedule twice weekly for 4 weeks, followed by a 2-week rest.
[b]Treatment schedule twice weekly for 2 weeks all 3 weeks for the initial treatment period.
[c]Treatment schedule once weekly for 4 weeks all 5 weeks.

between 1.04 and 1.6 mg/m^2, whereas the most common DLTs were hypotension, syncope, diarrhea, fatigue, peripheral neuropathy, electrolyte disturbances and thrombocytopenia. Orlowski *et al.* observed one complete remission and some partial remissions in patients with plasma cell dyscrasias; two of 10 patients with a non-Hodgkin's lymphoma achieved a partial remission; no response was seen in the four patients with a Hodgkin's lymphoma. Pharmacodynamics studies revealed a time- and a dose-dependent inhibition of the 20S proteasome. The activity seen in patients with multiple myeloma established the rationale for a phase II trial [57]. In other phase I trials, there was some evidence of biological activity among the patients with acute leukemias [58], prostate cancer [59] and NSCLC [60]. Trials concerning unresectable hepatocellular carcinoma and NSCLC are ongoing [61,62].

Phase II and III trials

Based on the results of phase I trials, a large multicenter, open-label, non-randomized phase II trial was performed in 202 patients with relapsed or refractory multiple myeloma who had received at least three prior therapies. Patients were treated with 1.3 mg/m^2 bortezomib as an i.v. bolus twice weekly for 2 weeks, followed by 1 week rest, for up to 8 cycles. In patients with stable disease after 4 cycles or progressive disease after 2 cycles, oral dexamethasone on the day of and the day after each dose of bortezomib was added to the regimen. Thirty-five percent of patients achieved a minimal, partial or complete remission (responders); the median time to first response was 1.3 months, the median duration of response for all responders was 12 months, for patients in complete or near-complete response 15 months. Median survival among all 202 patients was 16 months. The most frequent grade 3 adverse events were thrombocytopenia (28% of patients), fatigue (12%), peripheral neuropathy (12%) and neutropenia (11%) [63]. In a parallel phase II trial, the patients with relapsed or refractory multiple myeloma were randomized to receive 1.0 or 1.3 mg/m^2 bortezomib with a permission of dexamethasone in patients with progressive or stable disease after 2 or 4 cycles of single agent therapy. The response rates (clinical + partial responses) were 30 and 38%, respectively, and after addition of dexamethasone, the response rates for all patients rose to 37 and 50% in the 1.0 and 1.3 mg/m^2 cohorts, which suggested some synergy between the both drugs [64]. Results from those studies formed the basis for the approval of bortezomib by the US Food and Drug Administration (FDA) and the European Medicine Evaluation Agency (EMEA) for patients with relapsed multiple myeloma after two prior standard therapy regimes.

Two other ongoing trials examine the efficacy of bortezomib alone and in combination with doxorubicin and dexamethasone in untreated multiple myeloma patients, including the feasibility of peripheral blood stem cell collection and high-dose chemotherapy [65,66]. The partial and (near) complete remission rates achieved were 75% for single-agent therapy and 100% for the combination therapy.

The first phase III trial was performed in patients with multiple myeloma relapsed after one to three prior therapies. In total, 669 patients were randomized in two

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cohorts. The first cohort was treated with 1.3 mg/m^2 bortezomib twice weekly for 2 weeks all 3 weeks for 8 cycles, followed by a treatment once weekly for 4 weeks all 5 weeks for 3 cycles. The second cohort was treated with dexamethasone 40 mg on days 1–4, 9–12 and 17–20 all 5 weeks for 4 cycles, followed by a treatment on days 1–4 all 4 weeks for 3 cycles. Patients who received bortezomib demonstrated a highly significant benefit in time to progression ($p < 0.0001$) and overall survival ($p = 0.038$). Furthermore, the rate of grade 3 or higher infections was lower in the bortezomib cohort (6.7 versus 11%, $p = 0.096$), but no difference was found regarding the time to skeletal events [67].

Further phase II studies concerning metastatic neuroendocrine tumors [68] and renal cell carcinoma [69,70] found only little evidence for clinically significant activity of bortezomib in single-agent use. Other trials for patients with refractory colorectal cancer, breast cancer, advanced NSCLC, small cell lung cancer and relapsed or refractory indolent or aggressive non-Hodgkin's lymphoma are ongoing [71–74]. An overview of phase II/III trials is given in Table 3.

Conclusions

Proteasome inhibitors represent a novel class of potent anti-tumor agents with documented activities among a broad range of tumors *in vitro*. The acceptable therapeutic index in animal models was explained by greater susceptibility and enhanced induction of apoptosis in fast proliferating, malignant cells and complete restoration of proteasome function in non-malignant, quiescent cells. Acting on NF-κB, bortezomib abrogates several chemotherapy-induced resistance mechanisms and may act synergistically with a broad range of cytostatic agents. Results of early clinical studies in hematological malignancies and solid tumors provided the basis for the approval of bortezomib by the FDA and EMEA for the therapy of refractory and relapsed multiple myeloma, and for further combination trials in a wide variety of neoplastic diseases.

Table 3 A selection of phase II and III trials with bortezomib

Tumor	Treatment schedule	No. of patients	Remission rates	Adverse events	References
Relapsed or refractory multiple myeloma	1.3 mg/m^2 2 × w × 2 q3[a] optionally plus dexamethasone	202	CR/PR/MR 35%, NC 24%	gastrointestinal symptoms, fatigue, thrombocytopenia, sensory neuropathy	Richardson 2003 [63]
Relapsed or refractory multiple myeloma	1 versus 1.3 mg/m^2 2 × w × 2 q3 optionally plus dexamethasone	54	CR + PR for bortezomib alone: 30 versus 38%; CR + PR for patients receiving bortezomib alone or in combination with dexamethasone 37 versus 50%	grade 3: thrombocytopenia, neutropenia, lymphopenia, peripheral neuropathy; grade 4: 9%, thrombocytopenia and peripheral neuropathy	Jagannath 2004 [64]
Relapsed or refractory multiple myeloma phase III	1.3 mg/m^2 bortezomib versus dexamethasone	669	Mean TTP 5.7 (bortezomib) versus 3.6 months, OS $p = 0.038$ for bortezomib		Richardson 2004 [67]
Untreated multiple myeloma	1.3 mg/m^2 2 × w × 2 q3 dexamethasone 40 mg doxorubicin 0–4.5–9 mg/m^2 d1–4	15	PR 100%, 2/15 CR; 8/8 successful PBSC collection	grade 1–2 only	Cavenagh 2004 [65]
Untreated multiple myeloma	1.3 mg/m^2 2 × w × 2 q3 optionally dexamethasone	42 planned	PR, near CR in 75%	grade 1–3: gastrointestinal, peripheral neuropathy, syncope, neutropenia; no grade 4 toxicity observed	Jagannath 2004 [66]
Aggressive or indolent refractory and relapsed non-Hodgkin's lymphomas	1.5 mg/m^2 2 × w × 2 q3	45	remarkable activity especially in MCL, 11 responders	grade 3–4: gastrointestinal, hypotension, neuropathy, thrombopenia and neutropenia, 2 pneumonia, 2 meningitis/ encephalitis	Goy 2004 [74]
Renal cell carcinoma	1.5 mg/m^2, de-escalation to 1.7 mg/m^2 2 × w × 2 q3	23	PR 5%	grade 3: thrombocytopenia, neutropenia, anemia, gastrointestinal toxicity, neuropathy, electrolyte disturbances; grade 4: arthralgia, diarrhea, vomiting	Davis 2004 [69]
Renal cell carcinoma	1.5 mg/m^2, de-escalation to 1.3 mg/m^2 2 × w × 2 q3	37	PR 11%, SD 38%	grade 2/3: sensory neuropathy in 53%	Kondagunta 2004 [70]
Refractory colorectal cancer	bortezomib versus bortezomib + irinotecan	175 planned	NA	grade 3–4 in 35%: gastrointestinal, fatigue, neuropathy, neutropenia and thrombocytopenia	Dragovich 2004 [71]
Metastatic breast cancer	1.5 mg/m^2 2 × w × 2 q3	12	no objective response	no grade 4	Brown 2004 [72]
Metastatic neuroendocrine tumors	1.5 mg/m^2 2 × w × 2 q3	16	69% SD, no PR/CR	peripheral neuropathy, diarrhea, vomiting, ileus	Shah 2004 [68]
NSCLC	bortezomib versus bortezomib + docetaxel	155 planned	NA	grade 3–4 in 53%: neutropenia, respiratory, gastrointestinal, nausea, fatigue	Fanucchi 2004 [73]

CR, complete remission; PR, partial remission; SD, stable disease.
[a]Treatment schedule twice weekly for 2 weeks all 3 weeks for the initial treatment period.

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