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Targeting apoptosis in solid tumors: the role of bortezomib from preclinical to clinical evidence

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The ubiquitin–proteasome pathway is the main proteolytic system present in the nucleus and cytoplasm of all eukaryotic cells. Apoptosis activation induced by ubiquitin–proteasome pathway inhibition makes the proteasome a new target of anticancer therapy. Bortezomib is the first proteasome inhibitor to be approved by the US FDA; in 2003 as a third line and in 2005 as a second line therapy for the treatment of multiple myeloma only. This review focuses on the use of bortezomib, not only in its therapeutic role but also, more specifically, in its biologic role and discusses the most recent applications of the drug in solid tumors, both at a preclinical and clinical level.

Keywords: bortezomib, clinical studies, preclinical, ubiquitin–proteasome pathway

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1. Introduction

The ubiquitin–proteasome pathway (UPP) is the main proteolytic system present in the nucleus and cytoplasm of all eukaryotic cells. Although this ATP-dependent pathway was discovered in the 1979 [1], proteasome involvement was only demonstrated at the end of the 1980s [2].

Proteasome 26S is a multienzymatic complex found in all eukaryotic cells. The use of specific inhibitors has shown that the UPP is responsible for the cytoplasmatic turnover of a great variety of different cell proteins, thus, assuming an extremely important role in the regulation of not only a large number of physiologic processes, but also in the development of a great many human diseases. In fact, cyclins (A, B, C and D), cyclin-dependent kinase (CDK) inhibitors (p21 and p27), protein inhibitors (IκB), tumor suppressors (p53), regulatory proteins (MDM2), oncogenes (c-fos, c-jun, c-myc, N-myc and β-catenine), together with anti and proapoptotic factors (Bcl-2 and Bax) are all proteasome targets [3-5].

Preclinical studies have shown that the use of proteasome inhibitors in the treatment of several tumors might represent a new possibility of improving anticancer therapy because of their ability to induce the process of programmed cell death [6,7]. Therefore, whether natural or synthetic, they should be considered as a new class of potentially useful anticancer drugs [8].

Bortezomib (PS-341) is a dipeptide boronic acid able to inhibit, selectively and reversibly, the proteasome 26S [8], thus, leading to antiproliferative, proapoptotic and antiangiogenetic activity [9]. This induced inhibition leads to the stabilization of the CDK inhibitors p21 and p27, the tumor suppressor p53,

the proapoptotic proteins Bid and Bax and of the transcription factor NF-κB.

Bortezomib is generally well-tolerated by patients and, what is more, is able to increase the antitumoral effects of chemotherapy, radiotherapy and immunotherapy, without adding to their toxic effects [10,11].

Due to its high level of specificity, bortezomib was the first proteasome inhibitor to be approved by the US FDA; in 2003 as a third line and in 2005 as a second line therapy for the treatment of multiple myeloma (MM) only.

This review focuses on the use of bortezomib, not only in its therapeutic role but also, more specifically, in its biologic role and discusses the most recent applications of the drug in solid tumors, both at a preclinical and clinical level.

2. The ubiquitin–proteosome pathway

The UPP involves two events: first, ubiquitylation and then degradation brought about by the proteasome. The former process requires intervention by the enzymes E1, E2 and E3, which, as soon as they are activated, bind the ubiquitin chain step-by-step to the target protein, thus causing its degradation (**Figure 1**). All eukaryotes have many E2 and E3 enzymes, but most eukaryotes have only one or a small number of distinct E1 enzymes [12]. The ubiquitin-activating enzyme (E1), which binds ubiquitin by means of an ATP-dependent step, transfers a ubiquitin molecule to the ubiquitin-conjugating carrier protein (E2), which then interacts with the ubiquitin-protein ligase (E3) in order to bind the carrier protein covalently to a protein-target lysine. Several ubiquitin molecules are bound in this way, thus permitting the formation of a chain that is needed for recognition by the proteasome 26S. Ubiquitylation is a reversible process, due to the intervention of the deubiquitylation enzymes. The minimum length of an ubiquitin chain required for protein recognition is at least four molecules [13].

2.1 Structure of the proteasome

The proteasome 26S is a multienzymatic cylindrical complex made up of 2.5 kDa, which belongs to the N-terminal nucleophilic hydrolase family. It is present in the nucleus and in the cytoplasm of several eukaryotic cells [14]. This organelle consists of a 20S tubular core (CP), able to trigger off catalytic activity, with the 19S regulating subunits (RPs) linked to each end; these are responsible for the recognition of the ubiquitinized proteins and for the regulation of entry to the 20S core (**Figure 2**). More specifically, each subunit is made up of a base and a lid. The latter is made up of at least nine polypeptides and is responsible for binding and removing the polyubiquitin chains from the assigned proteins so that the chains can then be recycled. The base consists of eight polypeptides, six of which have ATPase activity, which reduce the polypeptide and catalyze their translocation to the 20S core [15,16].

The 20S (72 kDa) is made up of four stacked rings; two outer rings consisting of seven α-subunits (α1 – α7) and two inner ones of seven β-subunits (β1 – β7) [17]. Each inside ring presents three sites with chymotrypsin-like, trypsin-like and postglutamyl peptide hydrolase-like catalytic activity, arranged along the inside edge of the channel [17-19]. The β5 subunit, which possesses a chymotrypsin-like activity, cuts the polypeptides after hydrophobic residues; the β2 subunit, with trypsin-like activity, cleaves after basic amino acids and, finally, the β1 subunit, with its post-glutamyl peptide hydrolase-like, preferentially splits peptide bonds after acidic residues. Protein degradation occurs progressively by generating peptides of 3 – 25 amino acids, which are then dispersed on the outside and subsequently recycled by the cells [20,21].

The specific and reversible inhibition of the chymotrypsin-like activity of the proteasome is sufficient for the induction of cell cycle arrest and apoptosis, attributed to the inhibition of NF-κB. In particular, the inhibition of the chymotrypsin-like site, observed in patients treated with bortezomib, is sufficient to cause apoptosis of MM cells [22].

3. Proteasome and cancer

In normal conditions, UPP is involved in maintaining the stability of several proteins implicated in the regulation of cell cycle and division, DNA repair and transcription, the immune response and inflammation, antigen processing, differentiation, cell development and in apoptosis [23,24]. Among these, the proteins p21 and p27, belonging to the Cip/Kip family of the CDK inhibitors, prevent the formation of several different CDK–cyclin complexes and inhibit progression of the cell cycle. Low levels of p21 and p27, brought about by proteasome hyperactivity, represent a negative prognostic factor in several types of cancer. As a result, inhibiting action by the proteasome leads to upregulation of these proteins, which may lead to apoptosis in *in vitro* tumoral cells [25-27].

Another important proteasome substrate is the p53 tumor-suppressor protein, which play an important role in apoptosis induction. Proteasome inhibition gives rise to an increase in p53 expression and stability, thus, promoting the programmed death process [28].

The regulation of the NF-κB by the proteasome is particularly interesting (**Figure 3**). This consists of a heterodimeric transcription complex (p50/p65), involved not only in the regulation of immune and inflammatory response but also in tumorigenesis, due to cell-growth stimulation, angiogenesis induction and apoptosis blockage [29]. In normal conditions, NF-κB is kept inactive in the cytoplasm by the IκB, whereas in conditions of cell stress, the IκB becomes phosphorylated by the IκB kinase complex and subsequently ubiquitinized and degraded by the proteasome. IκB degradation leads to the release of NF-κB

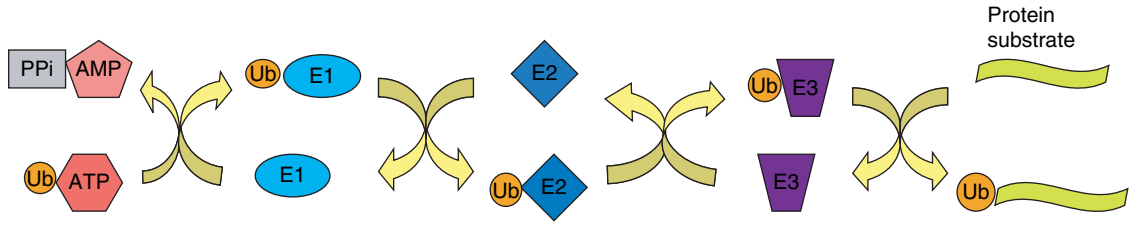

Figure 1. Ubiquitination of a target protein for its degradation by the proteasome. A ubiquitin molecule is transfered in an ATP-dependent manner to the ubiquitin-activating enzyme (E1) and then to the ubiquitin-conjugating carrier protein (E2), which then interacts with the ubiquitin-protein ligase (E3) in order to covalently bind the carrier protein to a protein-target lysine. Several ubiquitin molecules are bound in this way, thus permitting the formation of a chain, needed for recognition by the proteosome 26S.

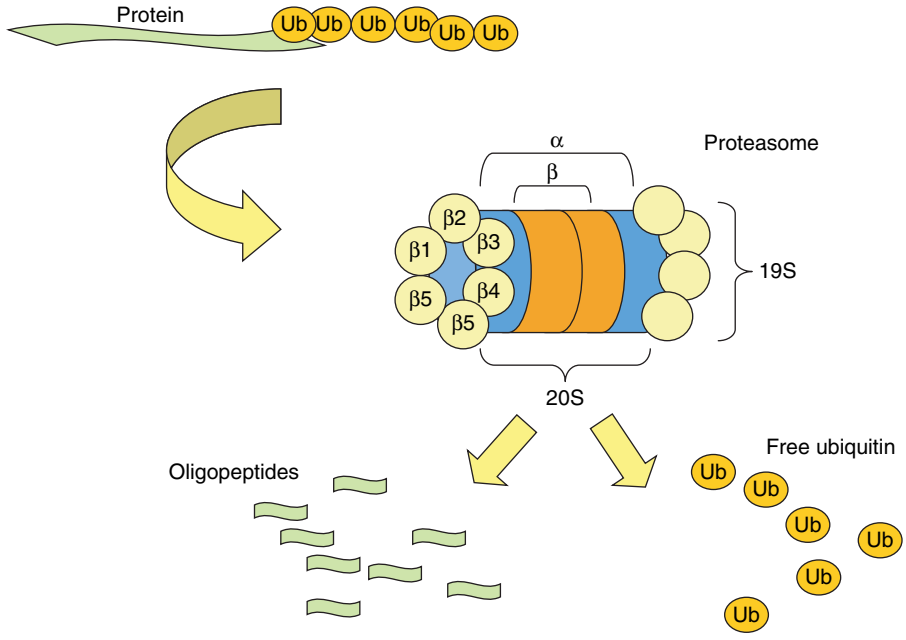

Figure 2. Protein degradation by the proteasome pathway.
β1: Post-glutamyl site; β2: Trypsin-like site; β5: Chymotrypsin-like site.

and its translocation into the nucleus. The interaction of NF-κB with the DNA gives rise to the expression of target genes such as cytokines (IL-6), survival factors (IAPa, Bcl-XI9), intracellular adhesion molecules, vascular adhesion molecules and E-selectins (**Figure 4**) [30]. The mechanism of action of NF-κB leads to cell resistance to chemotherapy [31,32]; proteasome inhibition strong enough to prevent IκB degradation, might, therefore, be useful in avoiding chemotherapy resistance because it would induce apoptosis. In fact, *in vitro* studies have shown that the presence of the super repressor IκB, which is a mutated form able to resist degradation by the proteasome, leads to the death of myeloma cells that have proven resistant to melphalan. Furthermore, the transfection of IκB leads to an increased response of colon cancer cells to 7-Ethyl-10-Hydroxycamptothecin (SN-38) compared with that of non-transfected cells [32,33].

Then the apoptosis activation, induced by UPP inhibition, makes the proteasome a new target of anticancer therapy [18].

4. Bortezomib

Bortezomib (PS-341) is a dipeptidyl boric acid approved by the US FDA in 2003 for the treatment of patients with relapsing MM. This compound has been tested by the National Cancer Institute (NCI) on 60 tumoral cell lines, demonstrating that it has a greater possibility of action in several of these compared with 60,000 other compounds. More specifically, it has been seen that low concentrations of bortezomib (7 nM) are able to reduce tumor growth by ~ 50%; the injection of bortezomib directly into human tumors implanted into mice has shown that in 40% of the

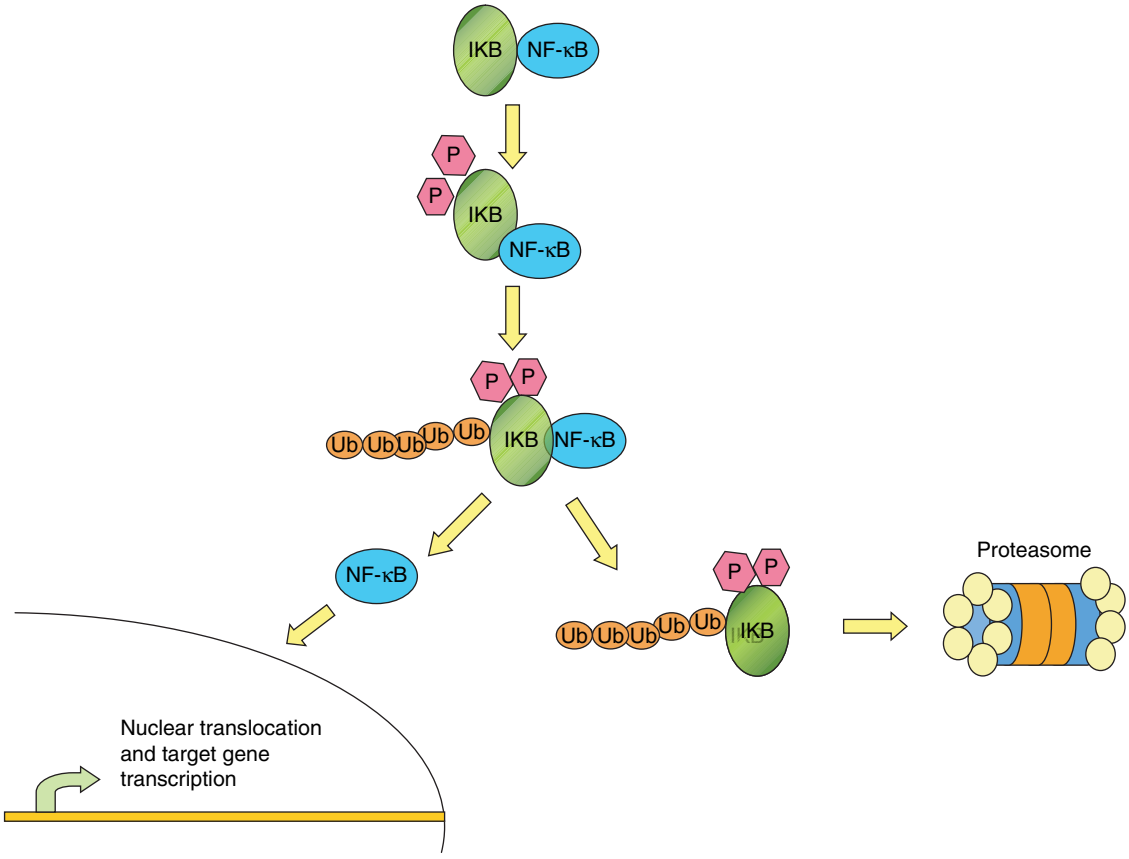

Figure 3. Proteasome action on the IKB-NF-κB pathway.
IKB: Inhibitor of NF-κB.

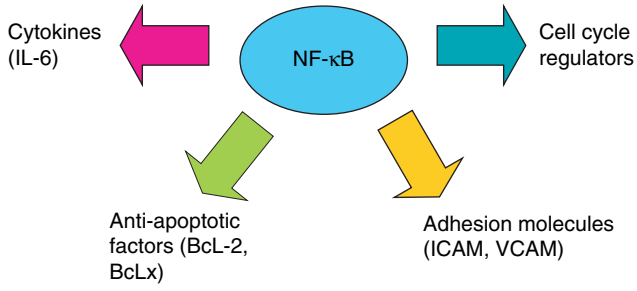

Figure 4. Principal NF-κB target genes.
ICAM: Intracellular adhesion molecules; VCAM: Vascular adhesion molecule.

cases there is a reduction of 70% in the volume of the tumoral mass [34].

Bortezomib inhibits the proteasome by interacting with a threonine residue present in each of the catalytic sites, but with an affinity that gradually decreases for the sites β5, β1 and β2 (β5 < β1 << β2) respectively [35].

4.1 Bortezomib and apoptosis

The proteasome inhibition brought about by bortezomib contributes to the accumulation of a series of proteic factors that play an important role in cell cycle regulation, such as the proapoptotic factors c-jun and p53, thereby inducing or increasing sensitivity to apoptosis (**Figure 5**). p53 is able to induce apoptosis through both the intrinsic, or mitochondrial, pathway and through the extrinsic death receptor pathway. In the former, p53 directly or indirectly modulates the expression of the protein targets, which include Bax, Bid, Puma and Noxa, involved in permeability regulation of the mitochondrial membrane. The subsequent emission of mitochondrial proteins causes the release of cytochrome C, caspase activation and subsequent cell death. The extrinsic pathway is needed in order to accentuate apoptotic response and, furthermore, is mediated by activation of the death receptors DR4, DR5, CD95, located on the plasma membrane, which inhibit the production of inhibitor of apoptosis proteins [36,37]. Moreover, among the proteins accumulated inside the cell subsequent to proteasome inhibition, there are the TNF-related apoptosis-inducing ligands (TRAIL), which are type II transmembrane proteins able to induce apoptosis in various tumor cell lines (**Figure 6**) [38]. The TRAIL trigger off the programmed cell death process by means of interaction with two receptors, death receptor 4 (DR4) and death receptor 5 (DR5). In more specific terms, the interaction of TRAIL-R1 with DR4 and

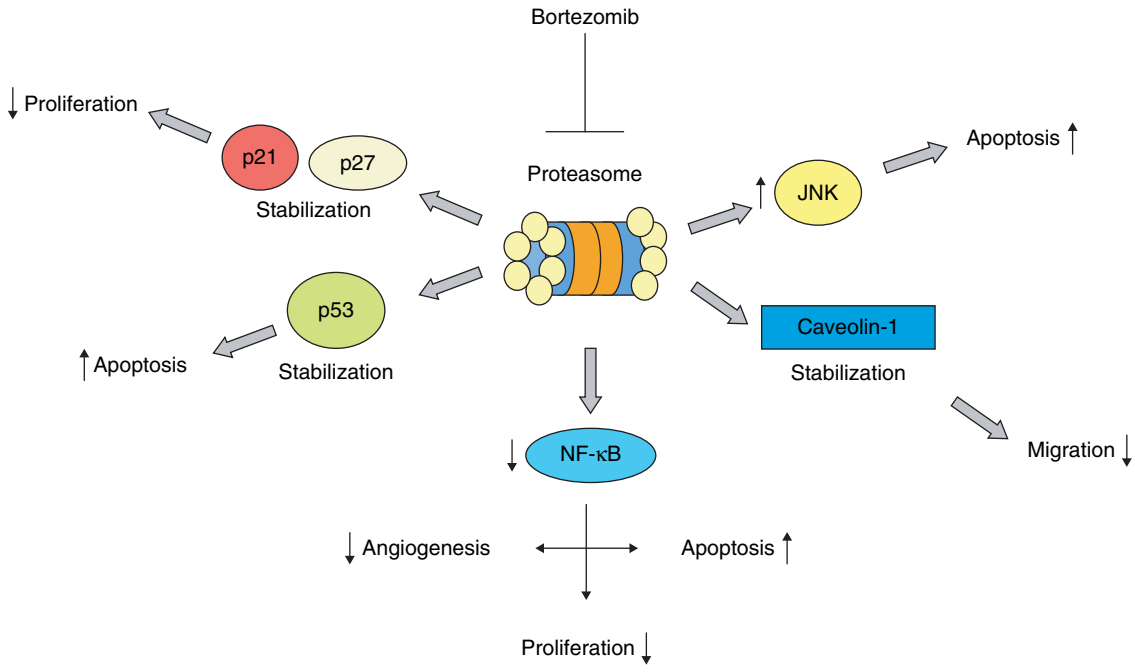

Figure 5. Effects of proteasome inhibition by bortezomib. Alteration of several proteins leads to apoptosis, reduction of angiogenesis, migration and cellular proliferation.

of TRAIL-R2 with DR5 leads to the formation of the death-inducing signaling complex, which is able to recruit the Fas-associated death domain adaptor molecule. The latter then recruits and activates caspases-8 and -10 in order to activate the proteic cascade, which is able to induce programmed cell death [39]. However, caspase-8 also activates Bid, a protein of the Bcl-2 family, which translocates to the mitochondria and, by giving rise to its permeation, induces apoptosis [40].

Proteasome inhibition also causes the oxidative stress responsible for the activation of Jun N-terminal kinase (JNK), which precedes the phosphorylation of c-jun. Once it is activated, c-jun will form, with c-Fos, the heterodimer AP-1; a transcription factor involved in the regulation of several cell processes, such as proliferation, differentiation, cell death and survival, which then induce the expression of various genes, including c-jun, Fasl and Bim. The latter two will be responsible for the activation of cell death, through both the intrinsic and extrinsic pathways [41].

Bortezomib also activates programmed cell death by means of other mechanisms, such as the activation of the heat-shock proteins (Hsp). The combined action of geldanamycin plus bortezomib disrupts Hsp90 and proteasome function, promotes the accumulation of aggregated, ubiquitinated proteins and results in enhanced antitumor activity [42-44].

In a sperimental model of C-26 colon carcinoma in mice, the combined action of bortezomib with TNF induced a dysregulated response to endoplasmic reticulum (ER) stress leading to apoptosis of cancer cells, evidenced by caspase-3 cleavage, p53 accumulation, increased stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) phosphorylation. Therefore, the combined treatment not only inhibited tumor growth, but also prolonged survival of animals bearing the C-26 tumors [45].

In breast cancer cell lines, bortezomib reduced the activity of 20S tubular core in a concentration-, time- and cell line-dependent manner. Proteasome inhibition causes nucleus-to-cytoplasm relocalization of the ER and plasma membrane to perinuclear lysosomes, relocalization of ErbB2, increased degradation and loss of ER and ErbB2 function, and induction of cellular apoptosis [46].

Proteasome inhibition with bortezomib may be used to prevent graft-versus-host disease (GVHD) and preserving, or possibly even promoting, graft-versus-tumor responses in cancer. Inflammatory cytokines produced by T cells and other immune cells have been shown to be critical for GVHD generation. Bortezomib is capable of inhibiting proliferation of alloreactive T cells in a dose-dependent manner. The suppression of GVHD may be due to a reduction of cytokine production concurrent with the induction of apoptosis of the alloreactive T cells [47].

Finally, in non-small cell lung cancer (NSCLC) cells, leukemia cells and head and neck squamous cell carcinoma (HNSCC) cells, bortezomib brings about the formation of species are reactive to oxygen, which, by determining the alteration of the mitochondrial membrane potential and the release of cytochrome C, are the primary cause of programmed cell death [48-50].

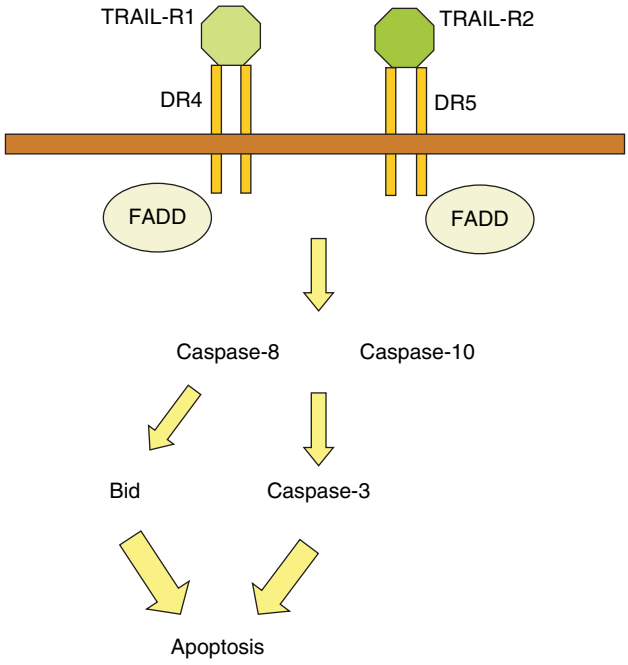

Figure 6. The extrinsic pathway of apoptosis.
DR: Death receptor; FADD: Fas-associated death domain; TRAIL: TNF-related apoptosis-inducing ligand.

5. Preclinical and clinical studies

5.1 Preclinical studies

5.1.1 Bortezomib as a single agent

Bortezomib can be used for the treatment of several types of cancer because of its ability to inhibit growth and induce apoptosis in various cell lines of solid tumors, for example, carcinoma of the lung, prostate, pancreas, colorectum, breast, ovary, kidney, head and neck (**Table 1**). Furthermore, *in vitro* studies have shown that apoptosis brought about by bortezomib depends on p53 expression in certain cell lines only, such as breast or lung cancer [51-53] but not in others, for instance, colorectal and prostate cancer and MM [34,54-57], as this process is not induced by a single protein, but rather, the percentage of pro- and antiaptotic factors present in the cell [11,58].

5.1.1.1 Non-small cell lung carcinoma

In vitro studies conducted on NSCLC H460 cells have shown that treatment with bortezomib causes cell cycle arrest at the G2–M phase and, subsequently, apoptosis resulting from caspase-3 activation. The exposure of this cell line to bortezomib is also responsible for the reduction in the number of cells in the G1 phase, which is dose- and time-dependent [57]. Moreover, H460 cells treated with bortezomib undergo a morphologic change similar to that of cells treated with antitubuline agents, such as paclitaxel and vinblastine. However, this fact is not due to the direct action of bortezomib on the polymerization and depolymerization of the microtubules, but rather to its specific action on the proteasome, as the inhibition of this leads to the accumulation of proteins which are generally short-lived [59]. However, other studies conducted on the same cell lines have shown that bortezomib has apoptotic action through its activation of the JNK [60].

Studies performed on lung cancer cells (H460, H358, H322), have shown that the action of Bortezomib is able to increase the number of G2–M phase cells, by stabilizing the proteins p53 and p21 cip/waf-1 and cyclin B1 [61,62].

When hypoxia is induced, *in vitro* studies have shown an increase in the proapoptotic effects of bortezomib. Low levels of oxygen lead to increased sensitivity to bortezomib of lung or colon cells compared with that of cells that have developed in standard concentrations of oxygen [63].

5.1.1.2 Renal cancer

Renal cancer cells treated with bortezomib have shown a decrease in the expression levels of the FLICE inhibitory protein, a negative regulator of the TRAIL proapoptotic pathway, together with an increase in apoptosis [64]. However, several other reports have shown that in RCC the cell death process is activated through the direct action of bortezomib on the NF-κB factor and is dose-dependent [65].

5.1.1.3 Multiple myeloma

The exposure of MM cells to bortezomib has shown that the drug is also able to prevent the activation of caveolin-1, a protein involved in cell motility and migration in several different tissues. Its activation requires phosphorylation by VEGF, a cytokine that promotes angiogenesis and is one of the transcriptional targets of NF-κB. This indicates that bortezomib is able to inhibit both cancerous cell migration and the process of tumor angiogenesis [66,67]. These various activities have also been confirmed by experiments conducted on solid tumor cell lines (breast, lung, pancreas and ovary) [65].

5.1.1.4 Breast cancer

In several breast cancer cell lines and in mice bearing EMT-6 breast cancers a 90% reduction of tumor survival has been described subsequent to either a single intraperitoneal or dose-dependently injection of bortezomib [68,69]. Several other studies of MDA-MB-231 breast cancer cells have shown an upregulation of p27 followed by proteasome inhibition after the administration of bortezomib [11,70].

5.1.1.5 Pancreatic cancer

In mouse xenografts of pancreatic BxPc3 tumors, an intravenous or intraperitoneal injection of bortezomib causes an increase of p21 expression and inhibits tumor growth [71].

5.1.1.6 Prostate cancer

In human LNCaP-Pro-5 prostate cancer cell lines, high doses (1 μM) of bortezomib bring about the reduction of

Table 1. Effect of bortezomib in solid tumors *in vitro* and *in vivo*.

Cancer	Effect
NSCLC	Cell cycle arrest at G2–M, activation of caspase-3, activation of JNK, increase in p53, p21 cip/waf-1, cyclin B1, MDM2, tumor growth delay
Renal	Decreased c-FLIP, Inhibits NF-κB pathway, increased tumor apoptosis
Multiple myeloma	Inhibits caveolin-1, Inhibits of VEGF secretion, decreased angiogenesis and tumor growth
Breast	Decreased tumor growth, up-regulation of p27
Colorectal	Up-regulation of p27, decreased tumor growth, increased tumor apoptosis, enhanced cytotoxic effects of irinotecan, camptothecins, CPT-11 and radiation treatment
Pancreatic	Up-regulation of p21, increased tumor apoptosis, enhanced cytotoxic effects of gemcitabine, CPT-11
Prostate	Decreased angiogenesis, decreased VEGF, increased tumor apoptosis
Head and neck	Decreased angiogenesis, inhibits NF-κB pathway, production of ER and ROS, activation of caspase-9, apoptosis
Ovarian	Cell–cell interaction destruction, Inhibits NF-κB pathway, increased tumor apoptosis, enhanced cytotoxic effects of docetaxel

c-FLIP: FLICE inhibitory protein; ER: Endoplasmic reticulum; JNK: c-Jun N-terminal kinase; MDM: Murine double minute; NSCLC: Non-small cell lung cancer; ROS: Reactive oxygen species; VEGF: Vascular endothelial growth factor.

the microcirculation density and of the number of growth factors of the vascular endothelium together with apoptosis activation. A reduction in microcirculation density induced by bortezomib has also been observed in prostate tumors [72,73]. While in human PC3M-Pro4 prostate cancer cell lines, whereas the drug does not cause any particular change in microcirculation density, it does, however, induce an increase in tumor cell death [73].

5.1.1.7 *Head and neck squamous cell carcinomas*

In HNSCC, bortezomib plays an antitumoral role due to its inhibition of the prosurvival NF-κB pathway; furthermore, the proteasome inhibition induced by bortezomib causes the release of the reactive oxygen species. These are responsible for the activation of apoptosis. In bortezomib-resistant HNSCC cells, the drug does not induce any destruction of cell adhesion in treated cells [74,75].

5.1.2 *Bortezomib and combined treatments*

Bortezomib brings about an increase in chemosensitivity to other chemotherapy drugs, thus permitting the administration of lower doses of these in combined treatments. Cells which have proven resistant to doxorubicin, mitoxantrone or melphalan, become more treatment-sensitive in the presence of bortezomib [76,77]. The administration of the drug when combined with irinotecan, a topoisomerase inhibitor, has antiproliferative and proapoptotic activity in murine xenograft models of colon cancer. This combined therapy causes a reduction in tumor size by 94% (69) and 89% (70) compared with controls. Furthermore, such tumors are smaller when compared with tumors treated with bortezomib or irinotecan as single agents. In xenograft models of pancreatic cancer, the use of bortezomib together with gemcytabine and with docetaxel in ovarian cancer brings about an increase in antiproliferative, proapoptotic, antitumoral and antiangiogenetic activity. *In vitro* studies performed on melanoma cells have shown that the combined action of bortezomib and temozolomide has cytotoxic and antiproliferative effects; moreover, in *in vivo* murine xenografts, a complete remission of tumors has been observed for > 200 days [78]. Preclinical studies have shown that bortezomib is able to sensitize tumor cells to radiotherapy. The combination of bortezomib with radiotherapy gives rise to a reduction in the growth of colon, prostate tumors and NSCLC [79-81].

5.2 Clinical studies in solid tumors

Considering the action of bortezomib in several tumor cell lines, many Phase I and Phase II clinical trials have explored its toxicity and activity in patients with different solid tumors. The most important results of these studies are summarized in **Table 2** and **Table 3**.

5.2.1 *Phase I clinical trials*

The first clinical trial exploring the role of bortezomib as a single agent in patients with advanced solid tumors was reported by Aghajanian *et al.* [82]. In this study, 43 patients were treated with bortezomib doses ranging from 0.13 – 1.56 mg/m^2 administered intravenously twice a week for 2 weeks, with a week rest period. The authors reported one partial response (PR) of 3 months duration in a patient with refractory NSCLC, whereas three patients had stable disease with a median response duration of 4 months. Grade 3 dose-limiting toxic effects were diarrhea and sensory neurotoxicity but the last one was observed only in patients with pre-existing neuropathy. The authors concluded that the treatment warranted further testing in a Phase II trial, but they cautioned that particular care should be taken when treating patients with pre-existing neuropathy.

Phase I studies evaluated the combination of bortezomib with antineoplastic agents. In the first study, Appleman *et al.* [83] administered bortezomib in combination with gemcitabine to patients with advanced solid tumors. Thrombocytopenia was the dose-limiting toxicity and the maximum tolerated dose regimen was determined to be 1.0 mg/m^2 for bortezomib and 1000 mg/m^2 for gemcitabine.

Table 2. Phase I clinical trials of bortezomib in patients with solid tumors.

Reference	Tumor type	Number of patients	Treatment schedule	Results	Adverse events
Aghajanian *et al.* (2002) [82]	Advanced solid tumors	43	Bortezomib (0.13 – 1.56 mg/m^2) twice weekly for 2 weeks and 1 week rest period	1 PR in NSCLC 2 SD	DLT: diarrhea, neuropathy AE: fever, fatigue, nausea, vomiting, rash, pruritus, headache
Appleman *et al.* (2003) [83]	Advanced solid tumors	31	Bortezomib (1 – 13 mg/m^2) days 1, 4, 8 and 11 of a 3-week cycle plus gemcitabine on days 1 and 8	1 PR in NSCLC	DLT: thrombocytopenia
Albanell *et al.* 2003 [84]	Anthracycline-pretreated breast cancer	17	Bortezomib (1 – 1.3 mg/m^2) days 1, 4, 8 and 11 of a 3-week cycle + docetaxel on day 1	6 PR	DLT: neutropenia, neuropathy, mucositis, emesis
Ryan *et al.* (2003) [85]	Advanced solid tumors	51	Bortezomib (1 – 1.3 mg/m^2) days 1, 4, 8 and 11 of a 3 week cycle plus Irinotecan	10 SD	DLT: nausea, emesis, diarrhea AE: fatigue, thrombocytopenia, neutropenia
Papandreou *et al.* (2004) [86]	Advanced solid tumors (AIPC)	53	Bortezomib (0.3 – 2 mg/m^2) days 1, 8, 15 and 22 every 35 days and 1 week rest period	2/47 (4%) ↓ > 50% PSA 9/47 (4%) stable PSA	DLT: diarrhea, hypotension, syncope
Blaney *et al.* (2004) [87]	Refractory solid tumors (pediatrics)	15	Bortezomib (1.2 – 1.6 mg/m^2) for 2 weeks and 1 week rest period	None	DLT: thrombocytopenia
Davies *et al.* (2004) [88]	Advanced NSCLC	16	Bortezomib (1 – 1.3 mg/m^2) days 1, 4, 8 and 11 of a 3-week cycle plus gemcitabine and carboplatin on day 1	4 PR 5 SD	DLT: thrombocytopenia
Aghajanian *et al.* (2005) [89]	Ovarian or primary peritoneal cancer	15	Bortezomib (0.75 – 1.5 mg/m^2) twice weekly for 2 weeks and 1 week rest period (cycles 2 – 6) plus carboplatin AUC 5 day 1 (cycles 1 – 6)	RR 47% 2 CR 5 PR	DLT: Diarrhea, neuropathy, rash, constipation
Voortman *et al.* (2005) [90]	Solid tumors (first line)	13	Escalating doses of bortezomib (from 0.7 to 1.6 mg/m^2) plus cisplatin and gemcitabine	4 PR in 10 evaluated patients (2 with bladder, 2 with NSCLC)	DLT: neutropenia
Hamilton *et al.* (2005) [91]	Advanced solid tumors and one cutaneous T cell lymphoma	40	Bortezomib (0.25 – 1.9 mg/m^2) days 1 and 4 of a 2 week cycle	None	DLT: neuropathy, diarrhea, fatigue

AE: Adverse event; AIPC: Androgen-independent prostate cancer; AUC: Area under the curve; CR: Complete response; DLT: Dose-limiting toxicity; NSCLC: Non-small-cell lung cancer; PR: Partial response; PSA: Prostate-specific antigen; RR: Response rate; SD: Stable disease.

Table 3. Phase-II clinical trials of bortezomib in patients with solid tumors.

Reference	Tumor type	Number of patients	Treatment schedule	Results	Adverse events
Price *et al.* (2004) [95]	AIPC	45	Bortezomib 1.3 (A) or 1.6 (B) mg/m^2 plus docetaxel 40 mg/m^2 once weekly for 2 weeks and 1 week rest period	A) 5/25 (24%) ↓ > 50% PSA B) 3/10 (30%) ↓ > 50% PSA 4/10 ↓ < 50% PSA	Neutropenia, neuropathy, mucositis, emesis
Shah *et al.* (2004) [96]	Metastatic neuroendocrine tumors	16	Bortezomib (1.5 mg/m^2) twice weekly and 10 days rest period	11 (69%) SD	Neuropathy, diarrhea, emesis, ileus
Davis *et al.* (2004) [97]	Advanced renal cell carcinoma	23	Bortezomib (1.5 mg/m^2) twice weekly for 2 weeks every 3 weeks	PR 5%	Thrombocytopenia, neutropenia, anemia, neuropathy, arthralgia, diarrhea, emesis
Kondagunta *et al.* (2004) [98]	Advanced renal cell carcinoma	37	Bortezomib (1.5 mg/m^2 reduced to 1.3 mg/m^2) twice weekly for 2 weeks and 1 week rest period	4 (11%) PR 14 (38%) SD	Neuropathy
Fanucchi *et al.* (2004) [99]	Advanced NSCLC	60	Bortezomib (1.5 mg/m^2) days 1, 4, 8 and 11 of a 3 week cycle plus/minus docetaxel on day 1	Bortezomib: PR: 10.3%, SD: 17.2% Bortezomib + docetaxel: PR: 16.1%, SD: 45.2% (interim analysis)	Neutropenia, gastrointestinal toxicity, nausea, fatigue
Stevenson *et al.* (2004) [100]	Advanced NSCLC	22	Bortezomib (1.3 – 1.5 mg/m^2) days 1, 4, 8 and 11 of a 3 week cycle	1 (4%) PR 9 (40%) SD (Ongoing trial)	Nausea, emesis, neuropathy, constipation, rash, thrombocytopenia.
Mackai *et al.* (2005) [101]	Metastatic colorectal cancer	19	Bortezomib (1.3 mg/m^2) twice weekly for 2 weeks and 1 week rest period	RR 0% 3 (18%) SD	Lymphopenia, dyspnea, pain, rash, hyponatremia, diarrhea, emesis, neuropathy, myalgia
Markovic *et al.* (2005) [102]	Metastatic malignant melanoma	27	Bortezomib (1.5 mg/m^2) twice weekly for 2 weeks and 1 week rest period	6 (22%) SD	Neuropathy, constipation, fatigue, thrombocytopenia, ileus, abdominal pain, infections
Alberts *et al.* (2005) [103]	Metastatic pancreatic adenocarcinoma	42 (ArmA) 39 (ArmB)	Bortezomib (1.5 mg/m^2) twice weekly for 2 weeks and 1 week rest period Bortezomib (1.5 mg/m^2) twice weekly for 2 weeks and 1 week rest period + gemcitabine on day 1 – 8	ArmA : RR 0% Arm B: RR 10% (PR)	Nausea, emesis, neuropathy, thrombocytopenia, fatigue, abdominal pain
Maki *et al.* (2005) [104]	Recurrent or metastatic sarcoma	23	Bortezomib (1.5 mg/m^2 escalated to 1.7 mg/m^2) twice weekly	1 PR	Constipation, abdominal pain, myalgia, neuropathy, fatigue
Yang *et al.* (2006) [106]	Metastatic breast cancer	12	Bortezomib (1.5 mg/m^2) twice weekly for 2 weeks and 1 week rest period	1 SD	Fatigue, rash
Ocean *et al.* (2007) [107]	Advanced gastric adenocarcinomas	44	Bortezomib (1.3 mg/m^2) twice weekly for 2 weeks and 1 week rest period plus/minus irinotecan on days 1 – 8	RR: 9 versus 44%	Cardiac arrest, leukopenia, diarrhea, nausea, emesis, thrombocytopenia, anemia, death

AIPC: Androgen-independent prostate cancer; NSCLC: Non-small-cell lung cancer; PR: Partial response; PSA: Prostate-specific antigen; RR: Response rate; SD: Stable disease.

In the study of Albanell *et al.* [84], bortezomib was administered with docetaxel to anthracycline-treated patients who had advanced breast carcinoma. The maximum tolerated dose had not been reached; however, dose-limiting toxicities of febrile neutropenia and neuropathy occurred in 14% of patients. In the irinotecan–bortezomib trial by Ryan *et al.* [85], the maximum tolerated dose was bortezomib 1.3 mg/m^2 and irinotecan 125 mg/m^2. Ten patients achieved stable disease. The most common grade ≥ 3 bortezomib-related nonhematologic adverse events were fatigue, diarrhea and nausea, whereas grade ≥ 3 bortezomib-related hematologic adverse events included neutropenia and thrombocytopenia but they were rarely dose-limiting. Although these studies were primarily designed as dose-finding studies to assess toxicities, antitumor activity was also demonstrated.

Papandreou *et al.* [86] reported the results of a trial exploring the role of single agent bortezomib in advanced solid tumors. Of the 53 patients assessed, 48 had androgen-independent prostate cancer and were treated with bortezomib at doses ranging from 0.13 to 2 mg/m^2, administered weekly for 4 weeks with a week rest period. Two patients had a prostate-specific antigen response and two had a PR in lymph nodes. The authors suggested further exploration of this compound in combination with chemotherapeutic agents as treatment for patients with advanced prostate cancer.

The role of bortezomib in the treatment of various pediatric solid malignancies has also been investigated by Blaney *et al.* [87]. Thrombocytopenia was the only dose-limiting toxicity but no objective responses were found.

Activity of bortezomib in combination with gemcitabine and carboplatin was reported in NSCLC by Davies *et al.* [88]. The maximum tolerated dose in this trial was bortezomib 1 mg/m^2, gemcitabine 1.000 mg/m^2 and carboplatin at an area under the concentration-time curve of five. Four patients experienced a PR and seven patients experienced stable disease.

Aghajanian *et al.* recently published the results of a study of bortezomib and carboplatin in recurrent ovarian or primary peritoneal cancer [89]. 15 patients were treated with six cycles of a fixed dose of carboplatin and increasing dose of bortezomib (from 0.75 to 1.5 mg/m^2), administered twice-weekly for 2 week cycles every 3 weeks. There was no hematologic dose-limiting toxicity at a dose of 1.5 mg/m^2. The overall response rate was 47%, with two complete responses (CRs) and five PRs, including one CR in a patient with platinum-resistant disease. The recommended dose of bortezomib administered in combination with carboplatin (AUC 5) was 1.3 mg/m^2.

At present, an on-going trial by Voortman *et al.* [90] is examining the efficacy of combination therapy of gemcitabine, cisplatin and bortezomib in advanced solid tumor patients in first-line treatment. In the first 10 patients evaluated, there were four PRs (two bladder, two NSCLC). The most common grade 3 and 4 toxicity was neutropenia.

Moreover, Hamilton *et al.* [91] treated 40 patients with solid tumors or lymphomas using an intermittent schedule with bortezomib doses ranging from 0.25 to 1.9 mg/m^2 on day 1 and 4, every 2 weeks. Dose-limiting toxicity was evident at the 1.75 and 1.9 mg/m^2 dose levels and was more frequent in patients receiving individual doses > 3 mg/m^2. Peripheral neuropathy represented the principal dose-limiting toxicity and occurred in patients who received higher dosages and in those previously treated with neurotoxic agents. Other dose-limiting toxic effects included diarrhea, fatigue and thrombocytopenia. Reversible inhibition of 20S proteasome activity was dose-dependent, and treatment was more beneficial when administered as total dose (mg) per fraction rather than in mg/m^2. Although the authors did not state whether CRs or PRs were obtained, they observed antitumor activity in patients with melanoma, NSCLC and RCC.

At the American Society of Clinical Oncology 2007 meeting, the results of a Phase I study combining vorinostat (a histone deacetylase inhibitor) with bortezomib were presented. Subjective and objective evidence of clinical activity has been observed in patients with refractory solid tumors [92]. The association of bortezomib with topotecan has been explored by GQ Chen *et al.* in a Phase I study including refractory cancer patients. This association has demonstrated to be feasible and well-tolerated. An expanded cohort at this dose will examine efficacy of this combination in patients with small cell cancers [93]. A Phase I study of bortezomib in combination with 5FU/LV plus oxaliplatin in patients with advanced colorectal cancer has been recently presented at last American Society of Clinical Oncology meeting. This combination showed a predictable toxicity profile and early evidence of clinical activity. The expanded Phase II study is ongoing [94].

5.2.2 *Phase II clinical trials*

Price *et al.* published a Phase I/II trial of bortezomib plus docetaxel in advanced androgen-independent prostate cancer [95]. In the Phase II part of the trial, patients were treated with docetaxel 40 mg/m^2 and two doses of bortezomib (1.3 or 1.6 mg/m^2) given on weeks 1 and 2 every 3 weeks. No dose-limiting toxicity was observed with the 1.6 mg/m^2 bortezomib dose. The preliminary results suggested a promising activity of this combination in some patients previously treated with chemotherapy.

Contrasting results were obtained in two Phase II trials at single institutions conducted in patients with advanced RCC [97,98]. Bortezomib was administered twice-weekly for 2 weeks of every 3 weeks in both the studies. In the first trial by Davis *et al.* [97], bortezomib was administered at the dose of 1.5 mg/m^2 escalated to 1.7 mg/m^2 because there were not observed grade 3 or 4 toxicity, but the study was closed because a planned analysis on 21 – 23

evaluable patients showed only one objective response. In the other study by Kondagunta *et al.* [98], the first 25 of the 37 patients enrolled were treated with a dose of bortezomib of 1.5 mg/m^2, subsequently decreased to 1.3 mg/m^2 because of toxicity. Of the 37 assessable patients, the best response was a PR in four patients (11%), lasting 8 – 20 months, whereas stable disease was achieved in 14 patients (38%).

Shah *et al.* [96] evaluated the use of single agent bortezomib in patients with metastatic neuroendocrine tumors but no objective responses were reached and the median period of stable disease observed was very short. Mackay *et al.* [101] also did not find any objective response in patients with metastatic colorectal cancer treated with bortezomib alone. Markovic *et al.* [102] evaluated patients with metastatic malignant melanoma treated with bortezomib alone. Response rate in this study was also 0% and only six patients achieved stable disease. Therefore, combination therapy was strongly recommended in all these studies.

Multiple clinical studies in patients with NSCLC were initiated based on promising preclinical and Phase I clinical data. Fanucchi *et al.* [99] investigated the safety and efficacy of bortezomib monotherapy compared with the combination of bortezomib and docetaxel in patients with advanced NSCLC. At interim analysis, bortezomib demonstrated activity both alone and combined with docetaxel. In fact 10.3% of patients treated with bortezomib alone reached PRs and stable disease was achieved in 17.2% of them. Combination therapy was also more effective with 16.1% of PRs and 45.2% of stable disease. Promising findings of bortezomib alone in patients with advanced NSCLC were also reported in an ongoing trial by Stevenson *et al.* [100]. Numerous other clinical trials are being conducted at present to investigate the potential of bortezomib in the treatment of NSCLC. Alberts *et al.* [103] investigated the efficacy of bortezomib monotherapy compared with the combination of bortezomib and gemcitabine in patients with metastatic pancreatic adenocarcinoma. The response rate for patients receiving combination therapy was 10%, the same as that expected for gemcitabine alone. For this reason, the authors concluded that there is not a role for bortezomib in metastatic pancreatic adenocarcinoma. In a multi-center Phase II study, Maki *et al.* [104] evaluated efficacy of bortezomib in 23 patients with recurrent or metastatic sarcoma. Bortezomib had minimal activity as a single agent in this setting of patients with only a PR. These researchers concluded that bortezomib should be investigated in combination with agents with demonstrated preclinical synergy. In a Phase II study, bortezomib has been studied in combination with gemcitabine and carboplatin in 114 chemonaive stage IV and selected stage IIIB NSCLC patients. A total of 20% had a PR and 66% a stable disease. At a median follow-up of 13 months, progression free and median survival times were 5 and 11 months respectively. These results are encouraging and deserve further randomized clinical trials [105]. Yang *et al.* [106] evaluated bortezomib monotherapy in patients with metastatic breast cancer. Also in this case, no objective responses were reached. For this reason, the future development of this agent for the treatment of breast cancer should be considered in combination with other antitumor agents.

Recently, Ocean *et al.* [107] presented a study enrolling patients with advanced gastric adenocarcinomas, randomized to bortezomib monotherapy or to the combination of bortezomib and irinotecan. Monotherapy with bortezomib reached a 9% response rate, whereas the combination reached a 44% of response rate. Considering that the response rate for irinotecan monotherapy is 14 – 20%, researchers concluded that further studies are warranted in gastric cancer patients. A Phase I/II trial was initiated to evaluate the combination of capecitabine and bortezomib in heavily pretreated patients with metastatic breast cancer (taxane and/or anthracycline resistant). Patients were treated with bortezomib (1.0 – 1.3 mg/m^2; days 1, 4, 8 and 11) and capecitabine (1.500 – 2.500 mg/m^2, days 1 – 14) in 3 week intervals. The maximum tolerated doses were bortezomib 1.3 mg/m^2 and capecitabine 2500 mg/m^2. Dose-limiting toxicities were Grade 3 stomatitis in 1 out of 6 patients and Grade 3 diarrhea in 1 out of 6 patients. In the 21 patients treated at the maximum tolerated doses, risk ratio was 17.6% and 47% of patients had stable diease. These activity results are promising [108].

Preclinical studies demonstrated that bortezomib exerts activity against several B cell malignancies by inducing apoptosis and sensitizing tumor cells to radiation or chemotherapy. Based on these results, clinical trials have been conducted with bortezomib in B cell non-Hodgkin's lymphoma. In these studies, bortezomib presented a good safety profile and showed promising clinical activity. Mantle cell lymphoma appeared significantly more sensitive to bortezomib than other non-Hodgkin's lymphomas. Bortezomib could, in the near future, play a promising role in the treatment of B cell non-Hodgkin's lymphoma [109-111]. On the other hand, bortezomib has no single agent activity in relapsed/refractory classical Hodgkin lymphoma [112].

6. Expert opinion

The proteasome plays a central role in regulation of the cell cycle, proliferation, cell death, angiogenesis, metastasis and resistance to chemotherapy and radiation therapy. Results from cell culture systems, animal tumor models and early clinical studies, suggest that proteasome inhibition represents a new therapeutic target for human cancers. Bortezomib is the first agent of this novel class to enter clinical trials and its antitumor activity has been reported from preclinical investigations in a variety of tumor models. Preclinical studies suggest that bortezomib has a unique mechanism of action and may display selective targeting of malignant cells over normal cells. Furthermore, these studies suggest

that bortezomib may overcome resistance of cancer cells to standard chemotherapy agents and radiation therapy in patients with relapsed or refractory disease, acting synergically with them. In some diseases, like MM, antitumor activity is significant even as a single agent, so bortezomib has been approved for the treatment of some patients with MM [113]. Results of clinical studies in solid tumors are mixed. The response rates of single agent use are very low and appropriate combination chemotherapy should be evaluated in the future. In conclusion, these data collectively suggest that proteasome inhibition is a novel approach to cancer therapy that might offer a response in patients where more conventional chemotherapeutics have failed. At present, several Phase II and Phase III trials in hematologic malignancies and solid tumors are ongoing. Several open issues are yet unexplored, such as the late toxicity profile, the right dosage when associated with cytotoxic and/or other targeted therapies, the synergistic action with biologic drugs and the real impact on natural history of a variety of solid tumors. In the future, the results from clinical trials and the integration with pharmacogenetic data will offer the possibility to better targeting bortezomib therapy, optimizing the combination with cytotoxic agents, radiotherapy and other novel anticancer therapies.

Declaration of interest

The authors have nothing of significance to declare.

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