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Targeting the Proteasome in Melanoma

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1. Introduction

Malignant melanoma, once disseminated, is a malignant neoplasm extremely resistant to conventional anticancer treatment, such as chemo or radiation therapies. Therefore, new therapeutic strategies are under investigation as, for instance, immunotherapy, gene therapy or so called targeted therapy. Proteasome appears as one of these new possible targets.

The ubiquitin proteasome pathway is a complex multicatalytic system specialized in the degradation of proteins of intracellular origin, unlike lysosomes that are specialized in the degradation of proteins of extracellular origin.

Many of the proteins degraded by the proteasome are molecules involved in cell proliferation and apoptosis, such as cyclins and cyclin dependent kinases, the proapoptotic protein p53 or the nuclear transcription factor NFkappaB. It has been demonstrated that inhibition of proteasome induces cell death, more strongly in neoplastic cells than in normal cells, and, even more, that proteasome inhibition sensitizes neoplastic cells to other proapoptotic stimulus such as chemo o radiation therapy, probably by the NFkappaB pathway. Therefore, the proteasome could be a good target for cells so resistant to apoptosis as melanoma cells are.

We and others have demonstrated that melanoma cells are sensitive *in vitro* to Bortezomib and other proteasome inhibitors, that are able to decrease melanoma cell viability, to induce a reduction in cell proliferation rate and a cell cycle arrest and to trigger apoptotic cell death through both caspase dependent and independent pathways.

Bortezomib is a commercially available proteasome inhibitor, mainly used for the treatment of multiple myeloma and other malignant hematological disorders. Although the only published phase II clinical trial using single agent Bortezomib in patients with advanced melanoma yielded disappointing results, the potential use of proteasome inhibitors for the treatment of metastatic melanoma patients is still under assessment. Based on the knowledge of the physiological role of the proteasome system and on preclinical studies, employment of proteasome inhibitors in combined therapies seems the best way to afford the use of these compounds for advanced melanoma treatment.

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One of the first drugs employed to this aim was Temozolamide, a chemotherapeutic agent that has shown to exert an antitumour effect in a synergic manner when administrated with Bortezomib in melanoma animal models. A phase I clinical trial combining Bortezomib and Temozolamide that enrolled 19 patients with advanced malignant melanoma has been recently published. Although the study has been designed to define phase II dose schedule, some limited results (partial response, disease stabilization) have been observed.

Other multiple combinations containing proteasome inhibitors have been tested in both *in vitro* and *in vivo* pre-clinical studies. Some of the therapies that have been shown to synergize with proteasome inhibitors against melanoma cells are several chemotherapeutic agents, calpain inhibitors, interferon, tyrosin-kinase inhibitors, different types of cell mediated immunotherapy, etc. As proteasome inhibitors are drugs with pleiotropic effects on proliferation and suppression of apoptosis, cell invasion and angiogenesis, multiple pathways have been proposed to explain how proteasome inhibition can reduce or avoid tumor growth, but ultimate mechanisms remain unclear. In that sense, a great research effort is necessary to elucidate the molecular basis of the proteasome inhibitors action on melanoma cells in order to better design combined therapeutic strategies. We hope that in the future one or more of these or other possible combinations will reach successfully the clinical setting.

Finally, a new promise on the employment of proteasome inhibitors for the treatment of metastatic melanoma, are the second generation proteasome inhibitors. They include the peptide boronic acid analogs MLN9708 and CEP-18770, the peptide epoxyketones carfilzomib and PR-047, and the beta-lactone compound NPI-0052, all of which show a potent *in vitro* proteasome inhibitory activity. They differ in enzyme binding kinetics, which might affect their pharmacological properties, efficacy and toxicity. All these features will define their future clinical use.

In the present chapter we will review the structure and function of the proteasome, the role of proteasome inhibitors as anti-cancer agents and the current status of preclinical and clinical knowledge about the potential use of proteasome inhibitors for metastatic melanoma treatment.

2. The proteasome

The ubiquitin proteasome pathway is a complex multicatalytic system specialized in the degradation of proteins of intracellular origin (Voorhees 2006). Protein synthesis and protein degradation are important processes in cellular homeostasis that ensure maintenance of protein regulation (Gallastegui 2010). Nevertheless, the concept of protein turnover is quite new. At the beginning of the last century, body proteins were viewed as essentially stable constituents, and diet proteins were believed to function primarily as energy-providing products, which were independent from the structural and functional proteins of the organism. The discovery of the lysosome, as one of the compartments for cell protein processing, along with other experiments that were carried out at the same time, have strengthened the notion that cellular proteins are indeed in a constant state of synthesis and degradation (Ciechanover 2010). However, proteolysis in lysosomes is a nonspecific process. In higher eukaryotes, only membrane-associated proteins and alien proteins captured during endocytosis (viral, bacterial, etc.) are destroyed in lysosomes. Degradation of the vast majority (80-90%) of intracellular proteins is realized by the proteasome (Sorokin 2009).

The proteasome is a large cylindrical particle consisting of at least 33 subunits, with a total molecular weight of approximately 2.5 MDa. There are several variants of the proteasome

that perform slightly different functions. For example, cells of the immune system contain a particular form of the proteasome, the immunoproteasome, that produces peptides for display at the cell surface (Schrader 2009). The peptides generated by the immunoproteasome are not subjected to further degradation by cell peptidases and are used for antigen presentation (Sorokin 2009). The version of the proteasome that is found in all cells and is responsible for the specific degradation of regulatory proteins and the removal of damaged proteins is called the 26S proteasome. The 26S proteasome is composed of a 20S core particle capped by two 19S regulatory particles at both ends (Fig 1). The 20S core particle is composed by four heptameric rings, which are assembled to form a cylindrical structure. The outer two rings are made of α subunits, and the inner two rings are composed of β subunits, which contain the proteolytic active sites in a central cavity. The degradation chamber can be reached through a channel that runs along the long axis of the core particle. The entrance to the channel is narrow, such that folded proteins must be at least partially unfolded before they can be translocated into the 20S core particle and degraded. The 19S regulatory particle is composed of at least 19 subunits arranged into two subcomplexes: the lid and the base. (Schrader 2009).

Ubiquitin is a 76-amino-acid-residue protein. It is highly conservative in eukaryotes, but is absent in bacteria and archaea. In eukaryotes, several genes encode ubiquitin. Activation of ubiquitin requires processing by so called deubiquitinating enzymes (Sorokin 2009). Proteins targeted for degradation by the 26S proteasome are first "labeled" by covalent linkage of a polyubiquitin chain, via a three-step cascade mechanism utilizing the following three enzymes: E1, the ubiquitin-activating enzyme, E2, the ubiquitin-carrier protein, and E3, the ubiquitin-protein ligase. Successive conjugation of ubiquitin moieties generates a polyubiquitin chain. A polyubiquitin chain consists at minimum of four molecules. These polyubiquitinated substrates are recognized by the 26S proteasome and rapidly broken down into short peptides. One important function of the 19S regulatory particle is to recognize ubiquitinated proteins and other potential substrates of the proteasome. A second function of the 19S regulatory particle is to open an orifice in the α-ring that will allow entry of the substrate into the proteolytic chamber. Also, since a folded protein would not be able to fit through the narrow proteasomal channel, it is assumed that the 19S particle unfolds substrates and inserts them into the 20S core particle. Both the channel opening function and the unfolding of the substrate require metabolic energy, and indeed, the base of the 19S regulatory particle contains six different ATPase subunits. Following degradation of the substrate, short peptides derived from the substrate are released, as well as reusable ubiquitin (Fig. 1) (Ciechanover 2010, Kim 2011). This process has been named the "ubiquitin-dependent degradation of protein" (Sorokin 2009).

Substrates for this non-lysosomal protein degradation pathway include misfolded and defective proteins, as well as others that are selectively polyubiquitin-tagged and targeted for degradation by the ubiquitin-proteasome system (Potts 2010). Proteins differ greatly from each other in lifetime, and the lifetime of protein molecules in an organism depends on their role. So, some structural proteins can remain unchanged for many years, whereas regulatory proteins are frequently required only for a few minutes to trigger a certain process and after completing their function they should be destroyed. Moreover, in the course of time, cells accumulate a large amount of aberrantly folded and oxidized protein that should be also eliminated. The proteasome is the basis of a complex multicomponent cellular machine for getting rid of these unwanted cell proteins (Sorokin 2009).

Proteasomes in eukaryotic cells are localized both in the cytoplasm and in the nucleus. Nuclear/cytoplasmic distribution of proteasomes seems to be tissue-specific. The

distribution of proteasomes between the cytoplasm and the nucleus also changes remarkably during embryogenesis. In spermatozoids and ovules, proteasomes are concentrated in the cytoplasm, at early stages of subdivision they translocate to the nucleus, and by the blastocyst stage the intracellular localization of proteasomes is close to their distribution in adult somatic cells. Besides, intracellular distribution of proteasomes changes dynamically in accord with the cell cycle phase. Proteasomal degradation of cyclins in the nucleus is a necessary condition for the normal course of the cell cycle (Sorokin 2009).

During the last years, experimental data have pointed about the existence of deviations from the classical protein degradation pathway. The vast majority of examples of non-classical proteasomal degradation are associated with the ubiquitin-independent degradation of proteasome substrates. Some of the proteins that can undergo ubiquitin-independent degradation are ornithine decarboxylase, p21, p53 and oncosupressive Rb proteins (Sorokin 2009).

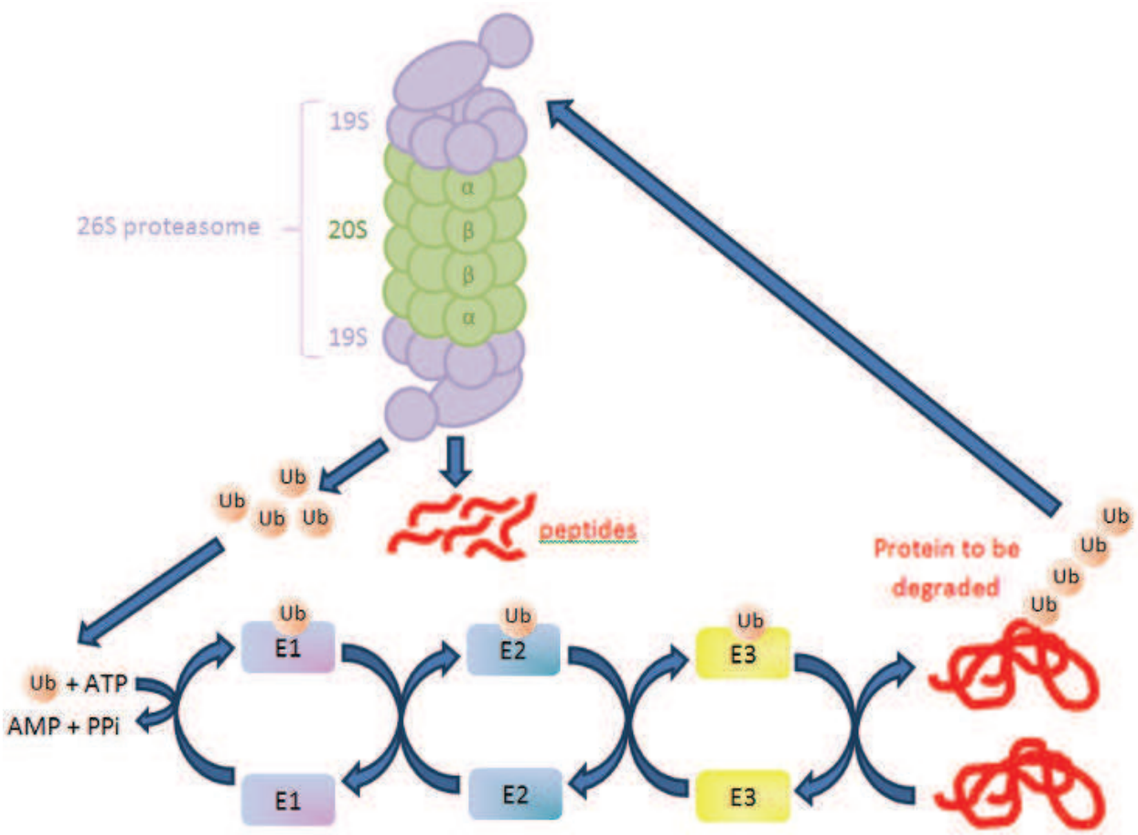


Fig. 1. The ubiquitin-proteasome pathway: The scheme shows the main steps of the ubiquitin-proteasome pathway in eukaryotic cells. First, the ATP consumption is needed for the binding of ubiquitin (Ub) with the ubiquitin activating enzyme (E1). Second, the ubiquitin-carrier protein (E2) takes the ubiquitin molecule from E1. Third, ubiquitin molecule is transferred from the E2 to the ubiquitin-protein ligase (E3). Fourth, the target proteins bind up to four molecules of ubiquitin. Fifth, the proteasome recognizes the protein and finally the protein is degraded by the catalytic core 20S, releasing free molecules of ubiquitin and digested peptides

In addition, recent emerging evidences suggest the existence of many other non-proteolytic functions of both the proteasome and ubiquitin. Non-proteolytic functions of proteasome include DNA repair, transcription initiation and transcription elongation. Ubiquitin has even more diverse non-proteolytic functions, such as membrane trafficking, protein kinase activation, DNA repair, and chromatin remodelling. The relationship of non-proteolytic functions between proteasome and ubiquitin is not clear. (Kim 2011, Kwak 2011, Livnat-Levanon 2011).

The discovery that ubiquitin modification plays a role in routing proteins to the lysosome/vacuole and that modification by specific and unique ubiquitin-like proteins controls autophagy demonstrated that the two distinct proteolytic systems, the lysosomes and the proteasome, communicate with one another (Ciechanover 2010).

With the many processes and substrates targeted by the ubiquitin pathway, it is not surprising to find that aberrations in the system underlie, directly or indirectly, the pathogenesis of many diseases (Ciechanover 2010).

3. Proteasome inhibitors as anticancer agents

In addition to providing a mechanism for cellular protein quality control, the ubiquitin-proteasome pathway facilitates essential cell functions ranging from antigen processing to signal transduction, cell cycle control, proliferation, differentiation, angiogenesis and apoptosis. Thus, besides involving in normal cellular functions and homeostasis, the alteration of proteasomal activity contributes to the pathological states of several clinical disorders including inflammation, neurodegeneration and cancer. These critical roles, together with the ubiquitious nature of the proteolytic 20S core particle, suggest multiple potential applications for proteasome inhibition for several pathological conditions such as inflammation / autoimmune diseases and cancer therapy (Potts 2010, Chen Current Protein 2010).

Many of the proteins degraded by the proteasome are molecules involved in cell proliferation and apoptosis, such as cyclins and cyclin dependent kinases, the proapoptotic protein p53, members of the Bcl2 family, the nuclear transcription factor NFkappaB, etc. Thus, the inhibition of proteasome activity could have important downstream consequences that can be used to advantage in tumor cells. It has been demonstrated that inhibition of the proteasome induces cell death in both normal and neoplastic cells, that cancer cells possess elevated levels of proteasome activity and are more sensitive to proteasome inhibitors than normal cells and, even more, that proteasome inhibition sensitizes neoplastic cells to other proapoptotic stimulus such as chemo o radiation therapy, probably by the NFkappaB pathway. These and other findings provided strong rationale for targeting the proteasome for the treatment of cancer (Voorhees 2006, Potts 2010, Chen 2010b).

One of the principal actions of proteasome inhibitors are the regulation of cell cycle control molecules. Ubiquitin-dependent proteolysis mediates the normal turnover of p53, the guardian of human genome. Consequently, proteasome inhibition leads to p53 accumulation and subsequently induces the transcription of cyclin-dependent kinase inhibitor p21. Proteasome inhibition also induces p53 phosphorylation. Some studies reveal that the ubiquitin-proteasome system is responsible for the degradation of p21 and p27, both of which are important cell cycle regulators whose expression are down-regulated in various malignancies. So, an important biologic effect of proteasome inhibitors is the accumulation of p27 (Voorhees 2006, Wu 2010, Chen 2010b).

Another main action of proteasome inhibitors is the regulation of pro- and anti-apoptotic proteins. The execution of apoptosis is largely governed by opposing activities of pro-(Bax, Bak, Bik,Bim, Bad, Bid, HRK, NOXA, PUMA, and BNIP3) and antiapoptotic (Bcl-2, Bcl-xL, Bcl-w, A1, and Mcl-1) members of the Bcl-2 family. Altogether, such group of proteins regulates mitochondrial membrane permeability, cytochrome C release, generation of reactive oxygen species, and caspase activation. Proteasome inhibition has been shown to favor balance to pro-apoptotic signaling. Therefore, proteasome inhibitors block the degradation and upregulate the expression of Bax, Bik, Bim, and NOXA. Proteasome inhibition also activates transcription of PUMA and promotes Mcl-1 cleavage. Moreover, proteasome inhibitors downregulate the expression of a class of proteins, known as inhibitors of apoptosis (IAPs), that suppresses the effector caspases. To this end, proteasome inhibitors reduce the expression of several IAPs, including cIAP-1, XIAP and survivins (Voorhees 2006, Wu 2010, Chen 2010b)

As above mentioned, proteasome inhibitors have been shown to synergize with several chemotherapeutic drugs inducing apoptosis of malignant cells. This action has been mainly explained by their inhibitory effect on NFκB activation. NFκB is a heterodimeric transcription factor, mostly composed of 65 and 50 kDa subunits, which is prevented from translocation into the nucleus through association with a family of inhibitory proteins called IκB. Upon stimulation through several factors (basically chemokines and cytokines, such as TNFα, free radicals, ultraviolet radiation or bacterial components) the IκB is phosphorylated, ubiquitinated and subsequently degraded in the 26S proteasome. Then, free NFκB translocates into the nucleus, where it activates transcription of genes whose products can inhibit apoptosis by mediating cellular survival responses. This signal pathway has been called "canonical" NFκB activation, in front of "non-canonical" or different mechanisms of activation of NFκB. As NFκB also regulates angiogenesis, cell cycle control, adhesion and migration, strategies employed by malignant tumors to evade antitumoral therapies include upregulation of NFkB. Thus, on the one hand, many tumor cells, in contrast to their normal counterparts, show constitutive activation of NFkB. On the other hand, treatment of malignancies with radiation therapy or some cytostatic compounds, such as anthracycline drugs, leads to induced activation of NFkB. The latter mechanism is considered a major cause for the development of inducible chemoresistance. Thus, strategies to inhibit NFkB in malignant tumors are considered a worthwhile addition to the current therapeutic options. One way to indirectly inhibit the NFκB pathway is via the inhibition of the 26S proteasome. Lack of degradation of IκB induces increased levels of the IκB inhibitory subunit that prevents NFκB from translocation into the nucleus, and subsequently activation of anti-apoptotic and survival signals. In addition, the proteasome has also shown to have a role in the "non-canonical" NFκB activation. Consequently, proteasome inhibitors are strong potential substances for NFκB blockade of and chemosenzitation of cancer cells (Voorhees 2006, Yang 2006, Testa 2009, Amschler 2010).

Thus, proteasome inhibitors induce tumoral cell-cycle arrest and apoptosis, inhibit cell-adhesion, migration and release of angiogenic factors and sensitize malignant cells to pro-apoptotic stimulus such as chemotherapeutic agents or radiation therapy (Amschler 2010, Testa 2009, Wu 2010, Chen 2010b)

Other important actions of proteasome inhibitors are the accumulation of misfolded proteins and endoplasmic reticulum stress, the induction of oxidative stress, the down

regulation of the PI3K/AKT pathway, the activation of bone morphogenetic protein signaling, the repression of global protein translation, the immunosensitization of cancer cells to the cytotoxicity of lymphocytes, etc. In addition, proteasome inhibitors can induce cytoprotective responses that attenuate their antitumor efficacy, such as upregulation of heat-shock proteins, induction of macroautophagy, or activation of some other prosurvival signaling pathways, even NFκB. In summary, the specific effects and precise mechanism of action of proteasome inhibition in malignancy remain unclear and are subject to further investigation (Rajkumar 2005, Voorhees 2006, Wu 2010).

There are five main types of proteasome inhibitors that bind either reversibly or irreversibly to the active enzyme sites in the 20S proteasome, primarily the chymotrypsin-like site, thus inhibiting their proteolytic function. These types include peptide vinyl sulfones, peptide boronates, peptide epoxyketones (Epoxomycin and Eponomycin) and -lactones (Lactacystin and derivatives). Only a few compounds have progressed to clinical development, however, with others deemed unsuitable owing to metabolic instability, potency issues or lack of specificity (Dick 2010).

Bortezomib (PS-341) is the first-in-class proteasome inhibitor that reached human clinical use. Other first-in-class proteasome inhibitors, not available for clinical use in humans (such as MG-132, ALLN, Lactacystin, Epoxomicin, etc), have been extensively employed in the experimental setting in order to better understand the ubiquitin-proteasome pathway and the potential clinical use of the whole group. Bortezomib is a dipeptidyl boronic acid analog that reversibly inhibits the 26S proteasome by binding to N terminal threonine residues in the active site of the chymotrypsin-like catalytic región. It has been approved for the treatment of multiple myeloma and relapsed mantle cell lymphoma. However, it has generally been ineffective as monotherapy for the treatment of a wide variety of solid tumors. Bortezomib can overcome or reverse chemoresistance and increase sensitivity to chemotherapeutic agents, including Melphalan, Doxorubicin, Mitoxantrone, and to Dexamethasone. Such combinations have been approved for relapsed or newly diagnosed multiple myeloma. Moreover, combinations of Bortezomib and novel targeted therapies may act synergistically to increase antitumor activity and overcome specific cellular resistance and/or antiapoptotic mechanisms. Those targeted therapies include protein deacetylase inhibitors, kinase inhibitors, farnesyltransferase inhibitors, heat-shock protein 90 inhibitors, pan-Bcl-2 family inhibitors, and other classes of targeted inhibitors. Based in the results of preclinical studies, some early-phase clinical trials combining Bortezomib and other targeted therapies are ongoing, basically for the treatment of multiple myeloma patients (Voorhees 2006, Testa 2009, Orlowski 2008, Wright 2010 Eisenle 2010).

In clinical trials of multiple myeloma patients, Bortezomib adverse events have been reported in at least 10% of cases. They include anemia, anorexia, constipation, dehydration, diarrhea, dizziness, fatigue, headache, limb pain, nausea, neutropenia, peripheral neuropathy, pyrexia, rash, thrombocytopenia, vomiting, and weakness. Thrombocytopenia and neuropathy are probably the most limiting in the clinic (Orlowski 2008).

With the validation of the proteasome as a target for cancer therapy, interest has focused on the possibility that proteasome inhibitors other than Bortezomib could offer some advantages. Various second-generation agents are now in development. Among peptide boronic acid analogs, two new molecules have to be mentioned. MLN9708, which hydrolyses immediately in plasma to MLN2238, is a reversible inhibitor of the chymotrypsin-like subunit of the 20S proteasome that is distinct from Bortezomib in having a substantially shorter dissociation half-life. CEP-18770 (Cephalon) is a P2 threonine boronic

acid that is another reversible inhibitor, primarily of the chymotrypsin-like activity of the proteasome. Two compounds in the peptide epoxyketone class are being developed. Carfilzomib (formerly PR-171) is an irreversible inhibitor of the chymotrypsin-like activity of the proteasome, and PR-047 is an orally bioavailable analog of Carfilzomib, again being an irreversible inhibitor of the β5 subunit. Finally, several natural compounds have been identified as inhibitors of the proteasome. Marizomib (NPI-0052, or salinosporamide A), is a β-lactone compound derived from the marine bacterium Salinospora tropica; like Carfilzomib and PR-047, it is also an irreversible inhibitor of the β5 subunit. Given their low nanomolar IC50 values for the β5 subunit, Bortezomib and the second-generation inhibitors, all represent very effective inhibitors of proteasome activity. NPI-0052 also has a low nanomolar IC50 for the trypsin-like (β2) subunit. Two second-generation agents have entered phase I trials: NPI-0052 and Carfilzomib. Both, unlike Bortezomib, bind irreversibly the proteasome, abrogating one mechanism of recovery from proteasome inhibition, such as release of the target by the drug. Preclinical studies have shown that both at least partially overcome Bortezomib resistance in vitro. Moreover, in a number of models, including multiple myeloma and chronic lymphocytic leukemia, these inhibitors have shown enhanced potency compared with Bortezomib, suggesting they may have a broader spectrum of activity. Early results from phase I studies of Carfilzomib indicate that it is well tolerated, even on a dose intense schedule, and may have less neurotoxicity than Bortezomib. Evidence of antitumor activity is being seen in multiple myeloma and Waldenstron's macroglobulinemia, including in myeloma patients with previously Bortezomib-refractory disease. In phase I clinical trials employing Marizomib for patients with leukemia, lymphoma and other solid tumors, Marizomib did not appear to induce the limiting toxicities associated with Bortezomib, such as peripheral neuropathy, neutropenia and thrombocytopenia, in spite of eliciting levels of proteasome inhibition that equal or exceed those produced by Bortezomib. Anti-tumor activity was also seen in multiple myeloma patients previously treated with Bortezomib. Marizomib phase I clinical trials combining second generation proteasome inhibitors with other targeted therapies, as for instance Marizomib plus Vorinostat (a hystone deacetilating agent), have been initiated. Second-generation proteasome inhibitors might address some of the key issues associated with Bortezomib, such as improving the efficacy of proteasome inhibition in solid tumors, and limiting therapy-associated peripheral neuropathy. Combinatory therapy employing two different proteasome inhibitors, such as Bortezomib and Marizomib has also been proposed. Extensive clinical investigation of the second-generation inhibitors will be required, however, to determine whether the pharmacologic differences between these agents and Bortezomib will result in differences in efficacy and safety in patients. (Orlowski 2008, Testa 2009, Einsele 2010, Dick 2010, Potts 2010, Bettignies 2010, Berkers 2010, Potts 2011).

Finally, another interesting concept about the use of proteasome inhibitors as anticancer agents is the role of the feedback regulation of proteasome gene expression as a possible mechanism of proteasome inhibitors resistance in solid tumors. The proteasome can be regulated at different levels. The 26S proteasome is composed of 33 distinct subunits each encoded by a different gene. Regulation of proteasome gene expression is another important mechanism that controls proteasome homeostasis. The discovery of feedback regulation of proteasome gene expression has several important implications in cancer therapy that targets the proteasome. First, it provides a clue to understand the cause of proteasome overexpression often-detected in cancers. Second, the feedback mechanism may contribute

to drug resistance in cancer therapy. The feedback induction, which normally occurs only when the proteasome activity is suppressed, may become constitutively active in cancer cells. As already mentioned, Bortezomib is the only proteasome inhibitor in clinical use. Although this drug has shown promising results in the treatment of multiple myeloma and mantle cell lymphoma it has limited efficacy in other cancer types. Whereas the compromised efficacy of Bortezomib by the feedback mechanisms may still be sufficient to kill myeloma tumor cells, it may not be strong enough to be effective in other cancers, especially solid tumors. Thus, the feedback pathway presents a potential target for cancer therapy. To date, proteasome inhibitors attacking the catalytic sites of the proteasome are the only tool to reduce the proteasome activity. However, knockdown of individual proteasome genes combined with proteasome inhibitors may present a promising alternative in cancer therapy. Further investigation of these mechanisms will provide more choices for proteasome-targeting cancer therapy (Xie 2010).

4. Melanoma and proteasome inhibitors

Cutaneous melanoma is the most aggressive form of skin cancer. Its treatment is based on early detection and surgical excision. Once in an advanced stage, metastatic melanoma presents a very poor prognosis as it becomes resistant to conventional anticancer treatments, such as chemo or radiation therapies. At the moment, the methylating agent Dacarbazine is still the standar therapy for metastatic melanoma allowing clinical objective responses in only 10-20% of patients, with a complete response rate of less than 5%, and a short median response duration of 4-6 months. Therefore, new therapeutic strategies are under investigation as, for instance, immunotherapy or so called targeted therapy (Ibrahim 2009, Lutzky 2010).

Some of the mechanisms related to the aggressive behavior of melanoma cells are 1) constitutive activation of growth factor receptors (c-Kit, PDGFR-α, EGFR), 2) constitutive activation of the MAP-kinase pathway (RAS/RAF/MEK/ERK), 3) constitutive activation of the PI3K/AKT pathway (partially due to loss, mutation, or epigenetic silencing of PTEN tumor suppressor gene), 4) constitutive activation of transcription factor NFκB, 5) disregulation (deletions, silencing, mutations) of proteins involved in cell cycle control (CDKN2A/CDK4/CCND1), 6) impairment of transcriptional activities of proapoptotic protein p53 and 7) overexpression of antiapoptotic Bcl-2 protein family (Fecher 2007, Rother 2009, Nathanson 2010). Besides these mechanisms, directly involved in cell proliferation and survival, melanoma cells also employ other strategies that allow them to invade and migrate (p.e. aberrant expression of adhesion molecules) (Kuphal 2005, Abdullah 2010, Braeuer 2011), to grow (p.e.secretion of angiogenic factors) (Basu 2009, Ria 2010), and to escape the immune system control (Gajewski 2007). Theoretically, any molecule involved in hot points of this altered cell machinery could be a good target for melanoma treatment.

Up to now, targeted therapies that have reached the best results on the clinical setting are specific inhibitors of BRAF (PLX4032) in melanomas with the V600E BRAF mutation, c-Kit inhibitors in melanomas harboring c-Kit mutations (Davies 2010) and anti-CTLA-4 antibodies, such as Ipilimumab or Tremelimumab, that overcome the mechanisms of immunotolerance (Boasberg 2010). Although the introduction of these drugs has been a great advance for the treatment of patients with metastatic melanoma, this only represents the end of the very beginning. First, despite impressive clinical responses to PLX4032 treatment, most responsive patients ultimately relapse because of acquired resistance.

Second, melanomas with c-Kit mutations constitute only a very low percentage of the whole group of metastatic melanomas and, from what we know about other neoplasms with c-Kit mutations, relapse because of acquired resistance will also occur. Third, we continue without a good alternative for melanoma patients with tumors presenting a molecular profile different to BRAF/V600E or c-Kit mutations. And, finally, we lack biomarkers to identify subgroups of patients that will respond to anti-CTLA-4 therapy (Flaherty 2010, Shepherd 2010, Robert 2009).

For most authors, a possible therapeutic approach to avoid drug resistance developed in patients treated with single agent therapy is the use of combinatory treatments that simultaneously target different cellular pathways. In this context, proteasome inhibitors, that have pleiotropic effects on proliferation, survival, migration, invasion and angiogenesis, and that can synergize with several other drugs or therapies, appear as a good tool for metastatic melanoma treatment (Tawbi 2009).

4.1 Preclinical evidences of usefulness of proteasome inhibitors in melanoma

4.1.1 Effect of single-agent proteasome inhibitors on melanoma cells

First observations about the *in vitro* effect of proteasome inhibitors on proliferation and survival of melanoma cells were published by our group and others during 2004-08. These studies demonstrated that several different proteasome inhibitors (Bortezomib, that was the only one used in clinical practice, and ALLN, MG-132 and Epoxomicin, that were exclusively used in experimental studies) were able to decrease the viability of melanoma cell lines by inhibiting their proliferation, causing cell cycle arrest and inducing apoptotic cell death by caspase-dependent and -independent mechanisms, AIF related (Fernandez 2005, Qin 2005, Nikiforov 2007, Sorolla 2008).

Investigation of underlying molecular mechanisms to the effect of proteasome inhibitors in melanoma cells indicated that Bortezomib-mediated release of mitochondrial death inducers is not preceded by a significant cleavage of Bid nor down-regulation of apoptotic factors frequently related with the NFκB pathway (Bcl-2, Bcl-xL, XIAP, FLIP, TRAF-2), all previously associated with melanoma chemoresistance (Fernandez 2005). Comparing benign melanocytes with melanoma cells and other neoplastic cells, these studies also showed that proteasome inhibitors have the feature of promoting a dramatic induction of the proapoptotic protein NOXA in a tumor cell-restricted manner (Fernandez 2005, Qin 2005). The induction of NOXA by proteasome inhibitors was P53 (Qin 2005), HIF-1 and E2F-1 independent but directly dependent on the oncogene c-MYC. Thus, c-MYC appeared as a direct modulator of NOXA, essential for the regulation of NOXA by the proteasome in neoplastic cells, providing a molecular explanation for the preferential selectivity of proteasome inhibitors toward tumor cells (Yerlicava 2008, Fuchs 2008). The role of NOXA in the response of melanoma cells to Bortezomib was validated in xenograft murine model systems (Fernandez 2005, Qin 2005).

A recent work presented a genome-wide siRNA screen for modulators of cell death induced by Bortezomib on colon cancer, cervical cancer and malignant melanoma cells. The authors found that a common set of 39 genes was responsible for conferring sensitivity to Bortezomib in the different tumor cell types. They causally linked Bortezomib-induced apoptosis to the accumulation of ASF1B, MYC, ODC1, NOXA, BNIP3, Gadd45α, p-SMC1A, SREBF1, and p53. These results suggested that proteasome inhibition promotes cell death primarily by dysregulating MYC and polyamines, interfering with protein translation, and disrupting essential DNA damage repair pathways; in summary, leading to the inhibition of

multiple homeostatic responses that finally drive tumor cells to engage programmed cell death. They considered that such information could be useful to the design of pharmacodynamic biomarkers and the application of combination chemotherapy regimens containing Bortezomib (Chen 2010a).

Additional studies of our group and others also showed that antioxidant agents can block apoptosis triggered by proteasome inhibition in melanoma and other tumor types (Fernandez 2006, Llobet 2008). In detail, the antioxidant compound Tiron completely inhibits proteasome-induced apoptosis caused by the boronic acid-based proteasome inhibitor Bortezomib, but has no effects on the aldehyde proteasome inhibitors MG-132 and ALLN. Conversely, the antioxidant molecule, called Edaravone, blocks the MG-132 and ALLN-induced apoptosis, but does not have significant effects on apoptosis induced by Bortezomib. Vitamin C has been also shown to abrogate the ability of Bortezomib to induce apoptosis in several other cancer cell lines. (Llobet 2008, Zou 2006). These results indicated that different antioxidants are able to block different proteasome inhibitors in a specific way and may have important implications for the design of drug mixtures containing proteasome inhibitors.

Finally, Bortezomib-induced endoplasmic reticulum stress and autophagy in melanoma cells are other antineoplastic functions of proteasome inhibitors that have become a point of great interest in the last few years (Hill 2009, Armstrong 2011).

4.1.2 *In vitro* and *in vivo* melanoma preclinical models about proteasome inhibitor-based combinatory therapy

As melanoma is almost universally resistant to chemotherapy and shows a constitutive activation of transcription factor NFκB, inhibition of the proteasome seemed a good option to overcome melanoma cell chemoresistance.

One of the experimental studies, that most encouraged the clinical use of combinatory therapy containing proteasome inhibitors and chemotherapeutic agents in melanoma, was published in 2004. The authors showed that Bortezomib enhanced Temozolamide induced growth inhibition of melanoma cells *in vitro* and *in vivo* in a xenograft tumor model. Tumor growth decrease was related to inhibition of nuclear translocation of NFκB, stabilization of p53 and p21 levels and, ultimately, induction of apoptosis. The combination also significantly inhibited tumoral angiogenesis when compared with the use of Temozolamide alone (Amiri 2004). Significantly enhanced killing of melanoma cells was also achieved by simultaneously triggering production of NOXA (using Bortezomib) as well as reducing Mcl-1 levels (using Fludarabine) (Qin 2006).

More recently, another study demonstrated that the proteasome inhibitor Bortezomib led to a significant synergistic enhancement of the antitumoral activity of the chemotherapeutic agent Camptothecin on melanoma cells. This effect consisted in broader induction of cell apoptosis, suppression of cell invasion, as well as inhibition of *in vivo* metastasis in a murine model. A reduced degradation of IkB and, consecutively, a reduced activity of NFκB were observed, as expected. The *in vivo* model allowed to assess the inhibition of nuclear NFkB translocation and the induction of melanoma cell apoptosis in pulmonary melanoma metastases. In addition Bortezomib exerted pleiotropic and/or off-target effects. These effects were, at least in part, independent of proteasome inhibition or independent to NFκB inhibition and differed from those induced by the selective IKKb inhibitor, KINK-1. For instance, it was found that Camptothecin-induced upregulation of the Bcl-2 family protein NOXA was markedly augmented by Bortezomib, whereas KINK-1 achieved a less

pronounced increase. Bortezomib also led to a marked increase of the Camptothecin induced release of cytochrome C (a critical step in the mitochondrial pathway of apoptosis) that was not seen after melanoma cells incubation with KINK-1 (Amschler 2010).

The second generation proteasome inhibitor Marizonib (NPI-0052 or salinosporamide A) seems also to sensitize prostate cancer and malignant melanoma cells to Cisplatinum (Potts 2011).

Other agents that showed a synergistic effect on melanoma cells when combined with proteasome inhibitors are radiation therapy (Munshi 2004), Geldanamycin and Geldanamycin analogues (that target the Hsp 90 protein chaperone) (Bonvini 2001, Mimnaugh 2004, Banerji 2009), the Hsp70 inhibitors KNK-437 and Schisandrin-B (Yerlikaya 2010), Mistletoe Lectin-I and the PPAR-A agonist Rosiglitazone (Freudlsperger 2007), Gossypol (an inhibitor of the anti-apoptotic proteins Mcl-1/Bcl-2/Bcl-xL) (Wolter 2007) and the BH3 mimetic ABT-737 (inhibitor of Bcl-2/Bcl-X(L)/Bcl-w) (Miller 2009), the cytokines Interferon-alpha (Lesinski 2008, Lesinski 2009) and IL-29 (Guenterberg 2010), Bacitracin (a protein disulfide isomerase inhibitor) (Lovat 2008), Decitabine (a demethylating agent) (Halaban 2009), Fenretinide (a synthetic retinoid inducing endoplasmic reticulum stress) (Hill 2009), Evodiamine (Wang 2010), GRP78-specific subtilase toxin (that inhibits GRP78, a vital unfolded protein response mediator) (Martin 2010) and newly developed SMAC-mimetics (Lecis 2010). A combination of the new generation proteasome inhibitor Marizomib with histone deacetylase inhibitors was also tested in preclinical melanoma models, with not yet published results (Potts 2011).

Proteasome inhibition also enhanced the effect of cell-mediated immunotherapies in melanoma animal models, such as dendritic cell-based immunization/activation (Schumacher 2006) and adoptive transfer of tumor-specific T lymphocytes (Seeger 2010, Jazirehi 2011). Nevertheless, some paradoxical responses to this type of approach have been observed (Lundqvist 2010).

Recently, our group showed that a combined exposure to the multikinase inhibitor Sunitinib and Bortezomib resulted in a synergistic decrease of cell viability and an increase in caspase activation and apoptosis in two Sunitinib sensitive melanoma cell lines (M16 and M17) (Yeramian 2011). We demonstrated that constitutive activated PDGFRα and VEGFR2, respectively, were the targets of the observed Sunitinib effect. Proteasome inhibition did not show any additive or synergistic effect on Sunitinib resistant cell lines. In Sunitinib sensitive cell lines, Sunitinib inhibited Akt phosphorylation in its two residues (Thr 308. Ser 473), suppressed the phosphorylation of ribosomal protein p70S6KSer240/244 and downregulated the levels of cyclin D1. In addition, Sunitinib partially inactivated ERK in M17 but not in M16 cell line because M16 harbored the BRAF/V600E mutation that maintains the ERK pathway active despite the complete inactivation of PDGFRα by Sunitinib. Moreover, LY294002, a PI3K inhibitor, sensitized melanoma cells to Bortezomib treatment, suggesting that down-regulation of phospho-Akt by Sunitinib mediates the synergy obtained by Bortezomib plus Sunitinib co-treatment. Altogether, our results suggest that melanoma cells harbouring an activated tyrosin-kinase receptor may be clinically responsive to pharmacologic receptor tyrosin-kinase inhibition by Sunitinib, and a strategy combining Sunitinib and Bortezomib, may provide therapeutic benefit. Moreover, our results also highlighted that subgrouping of melanomas by their molecular profile will be important in the design of personalized combined therapies containing proteasome inhibitors. Different responses to Bortezomib therapy in BRAF wild type or BRAF mutated melanoma cells have also lately described by other authors (Armstrong 2011).

4.2 Clinical studies employing proteasome inhibitors in patients with metastatic melanoma

Bortezomib is the proteasome inhibitor that has been preferentially clinically evaluated for the treatment of metastatic melanoma, as single agent or in combination with conventional chemotherapeutic drugs.

The first phase II clinical trial has employed Bortezomib as single agent administered twice weekly for 2 weeks, every 3 weeks at a intravenous dose of 1.5 mg/m^2. The study was intended to treat 45 patients, but it was closed after an interim analysis due to early evidence of insufficient clinical efficacy. Twenty-seven patients with a median age of 56 years (range, 32–77 years) were included. Objective responses were not observed. Only 6 patients (22%) achieved stable disease. Of these 6 patients, 4 were still stable after 4 cycles of treatment, but were removed from the study due to toxicity. The median time to disease progression was 1.5 months (95% confidence interval, 1.4 –1.6) and the median overall survival was 14.5 months (95% confidence interval, 9–22). No Grade 4/5 treatment-related toxicities were reported. Eleven patients (42%) had Grade 3 toxicities including sensory neuropathy, thrombocytopenia, constipation, fatigue, ileus, abdominal pain, and infection without neutropenia. After these results, the authors concluded that single-agent Bortezomib was not found to be effective in the treatment of patients with metastatic melanoma, and that exploration of combination regimens may be warranted (Markovic 2005).

Based in previous *in vitro* and *in vivo* experimental evidence of the existence of a synergic effect between Bortezomib and the chemotherapeutic agent Temozolamide on melanoma cells (Amiri 2004), a second phase I trial was designed. Objectives included defining a maximum tolerated dose for the combination, characterizing biomarker changes reflecting inhibition of both proteasome and NFκB activity in blood and tumor samples, and characterizing antitumor activity. Nineteen melanoma patients with poor prognostic factors (including 17 patients with M1c type metastasis and 10 with raised serum LDH) were enrolled onto four escalating dose levels of Temozolomide and Bortezomib. Bortezomib, 1.3 mg/m^2, and Temozolomide, 75 mg/m^2, proved to be the maximum tolerated dose. Dose-limiting toxicities were neurotoxicity, fatigue, diarrhea, and rash. Objective responses included only a partial response of 8 months of duration. Three more patients achieved stabilization. A significant reduction in proteasome-specific activity in peripheral blood mononuclear cells was observed 1 hour after infusion at Bortezomib. Nevertheless, consistent effects on NFκB activation could not be detected. Authors gave different explanations to this fact including the possibility that Bortezomib acts on melanoma cells through other mechanisms such as a c-MYC–dependent increase in NOXA. A phase II trial is already in progress (Su 2010).

Finally, a recent phase II clinical trial has been published about the effect on metastatic melanoma patients of the combination of Paclitaxel, Carboplatin, and Bortezomib. This trial was based in preclinical studies demonstrating that Bortezomib has anticancer additive/synergistic effects when combined with several chemotherapeutic agents, including Paclitaxel and Platinum, and the results of a previous phase I trial of this 3-drug combination that included patients with metastatic melanoma and other tumor types (Ma 2007). Bortezomib was administered at a dose of 1.3 mg/m^2 intravenously, Paclitaxel at a dose of 175 mg/m^2, and Carboplatin at an area under the concentration. Seventeen patients were enrolled. A median of 4 cycles were administered. Three patients discontinued treatment due to persistent grade 4 neutropenia with grade 3 leukopenia or grade 4 pulmonary embolism. Grade 3 toxicities included neutropenia, leukopenia, thrombocytopenia,

and arthralgia. Two partial responses and four stabilization of disease were observed. The median progression-free survival was 3.2 months, and the median overall survival was 7.0 months. The authors concluded that the combination of Paclitaxel, Carboplatin and Bortezomib in patients with metastatic melanoma lacks sufficient clinical activity and was associated with significant toxicity to warrant further investigation (Croghan 2010).
Clinical trials of malignant melanoma patients based on combinatory treatments employing proteasome inhibitors and therapeutic strategies other than conventional chemotherapy have been not published so far.
Additional phase I clinical trials combining Bortezomib with interferon-alfa (Kendra 2008) or with Dacarbazine (Roberts 2006) have also been reported at ASCO meetings. Although the primary objective of the trials was to determine the safety, tolerability and dose limiting toxicities, the antitumoral activity of the combinations was quite limited and, to our knowledge, phase II clinical trials employing these combinatory therapies are not currently ongoing. Other two phase I clinical trials employing de second-generation proteasome inhibitor, Marizomib, have also been reported at the 2008 and 09 ASCO meetings. 23 and 30 patients with different tumor types were enrolled, including patients with myeloma, lymphomas, leukemias, and solid tumors. Stable disease was induced in one and two patients with melanoma, respectively. The toxicity profile was tolerable and dissimilar to Bortezomib in spite of reaching higher levels of proteasome inhibition (Aghajanian 2008, Townsend 2009).

5. Conclusion

In conclusion, biologic properties of proteasome inhibitors and preclinical studies suggested that this type of pharmacological agents could be a good therapeutic approach for the treatment of many cancer types. However, first human clinical assays employing the first commercially available proteasome inhibitor, Bortezomib, demonstrated that this drug is quite effective in some hematologic malignancies but not in solid tumors, such as malignant melanoma, nor as single agent nor in combination with conventional chemotherapeutic products. Nevertheless, multiple preclinical studies, carried out on *in vitro* or *in vivo* melanoma models, support that proteasome inhibitors could be useful in combinations with several targeted therapies or different immunotherapeutic strategies. As we currently know that melanoma is a molecular heterogeneous disease, studies designed to increase our knowledge about underlying mechanisms to the combined action of proteasome inhibitors and other treatments on the different melanoma subtypes are warranted. In this way, we could have a rational basis to select those groups of melanoma patients in which proteasome inhibitor-based therapy could be a good choice. Finally, second generation proteasome inhibitors appear as a chance for the treatment of solid tumors. Probably in the coming years we will see to what extent this can be a reality in melanoma.

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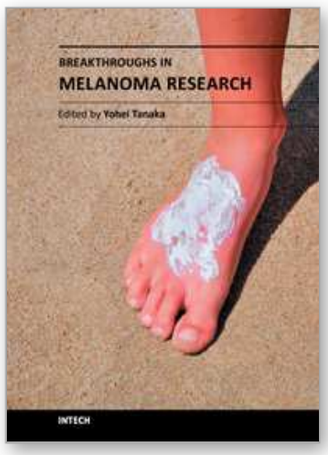
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Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

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