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Review

The Role of Proteasome Inhibitors in Multiple Myeloma Bone Disease and Bone Metastasis: Effects on Osteoblasts and Osteocytes

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Abstract: The alterations of bone remodeling are typical of multiple myeloma (MM) patients where the uncoupled and unbalanced bone remodeling caused the onset of osteolytic lesions. Moreover, bone metastasis occurs in the majority of patients with breast and prostate cancer. Skeletal-related events negatively impact on quality of life by increasing the vulnerability to fractures. Several bone-targeting treatments have been developed to control bone pain and pathological fractures, including bisphosphonates and Denosumab. Nevertheless, these agents act by inhibiting osteoclast activity but do not improve bone formation. Proteasome inhibitors (PIs) have shown bone anabolic effects and encouraging results in stimulating osteoblast differentiation and bone healing. Among these, the first-in-class bortezomib and the second-generation PIs, carfilzomib, and ixazomib regulate the bone remodeling process by controlling the degradation of several bone proteins. PIs have been recently proven to also be efficacious in blocking MM-induced osteocyte death providing new possible therapeutic use in the management of bone loss. PIs have significant side effects that limit their use as bone anabolic strategy. Multiple alternative approaches have been made. The conjugation of PIs with bisphosphonates, which can target them to bone, showed good results in terms of bone anabolic activity. However, the clinical implications of these effects require further investigations.

Keywords: bone disease; bone remodeling; osteoblasts; osteocytes; proteasome inhibitors

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1. Introduction

Bone remodeling is a tightly controlled process responsible for the repair of skeletal damages, thereby maintaining skeletal health. Different cellular types are involved in the regulation of all phases of bone remodeling such as osteoblasts (OBs), osteoclasts (OCLs), osteocytes, and bone lining cells. Bone resorption and formation, operated by OCLs and OBs, respectively, are coupled and strictly balanced ensuring the preservation of bone mass [1]. Disruption of bone remodeling is a typical feature of metastatic bone cancers, as well as multiple myeloma (MM) and non-malignant diseases such as osteoporosis. In all these pathological conditions, patients experience bone fragility and increased vulnerability to fractures with a negative impact on quality of life [2,3]. Bisphosphonates are the cornerstone of the therapeutic armamentarium for the management of bone lesions in cancers and in osteoporotic patients. In addition, Denosumab, the inhibitor of receptor activator of nuclear factor κ-B ligand (RANKL), has been approved for the treatment of patients with bone metastasis and for the treatment of osteoporosis in postmenopausal women [4]. Denosumab has been recently approved by the FDA for the prevention of skeletal-related events in newly diagnosed MM patients [5]. Nevertheless, these strategies only target OCLs with no effects on bone formation. For this reason, increasing efforts have been made to develop strategies that prevent the inhibition of bone formation. Since the discovery of the involvement of proteasome in controlling the activity of bone-related

proteins, several studies have focused their attention on the potential use of proteasome inhibitors (PIs) to improve bone anabolism [6]. Early in vitro studies clearly showed that the inhibition of proteasome positively affects OB differentiation in MM bone disease and bone cancers of solid tumors. In particular, the first-in-class PI bortezomib showed encouraging results in controlling MM-induced bone disease by targeting both OBs and osteocytes [7]. Afterwards, the next-generation PIs carfilzomib and ixazomib had been developed and tested for their potential bone anabolic activity. In a clinical perspective, PIs showed a great ability to normalize the levels of bone turnover markers in MM patients and improve bone formation.

This review focuses on the main mechanisms underlying the proteasome-dependent control of bone remodeling and the anabolic effects of PIs. We summarize preclinical and clinical findings obtained with PIs in bone disease focusing on the impact on OBs and osteocytes.

2. Methods and Results

2.1. Literature Search

PubMed and Web of Science databases were searched for eligible published articles until March 2021, using specific free words. Different combination of the following terms was used: osteoblast, osteocytes, proteasome, proteasome inhibitors, bone, bone regeneration, bone remodeling. Of the results obtained, only articles published in English were included. Other potential articles were identified from references within the selected articles or reviews related to the topic.

Inclusion criteria are listed as: (a) human MSC; (b) human osteoblast; (c) human osteocytes; (d) proteasome; (e) proteasome inhibitors; (f) studies focused on the role of proteasome on bone remodeling; (g) studies focused on the role of proteasome on bone remodeling in MM and metastatic bone cancers; (h) in vitro studies; (i) in vivo studies; (l) case reports; (m) review; (n) conference abstracts. Exclusion criteria are listed as (a) article does not meet inclusion criteria, (b) studies focused on the role of PIs on osteoclast formation.

The titles and abstracts were screened to assess the suitability of the results. Then, the full text of the selected studies were analyzed to assess whether or not they satisfied the inclusion criteria. Only articles published in English were included, which may leave out other eligible publications that were reported in other languages. Therefore, the results should be interpreted cautiously due to the limited data.

2.2. Results

By using the search terms “osteoblasts”, “proteasome inhibitors”, “osteocytes”, and “bone”, we identified 98 papers including reviews and research articles. Preliminary exclusion during the screening stage was made by the titles’ and abstracts’ relevancy. During the eligibility stage, articles that did not meet the inclusion and exclusion criteria were excluded. Additional relevant articles were added after manual selection from references according to eligibility.

3. Bone Remodeling

Bone remodeling is a lifelong process that allows the repair of micro damage and the substitution of old or damaged bone with new bone. During physiological bone remodeling, the activity of bone-forming OBs and bone-resorbing OCLs are coupled and strictly controlled in order to maintain bone homeostasis [1]. Indeed, the amount of resorbed bone is totally replaced by new bone in terms of location and amount [1]. Generally, it is customary to distinguish between targeted and non-targeted remodeling. Non-targeted bone remodeling refers to the regulation by hormones (e.g., parathyroid hormone (PTH), estrogens, growth factors), while targeted bone remodeling is responsible for the removal of damaged bone by targeted resorption [8]. As widely described by others, bone remodeling takes place within the basic multicellular unit (BMU), an organized structured consisting of OBs, OCLs, and osteocytes, which allows the formation and

resorption activities to occur in a balanced and coupled manner [9–11]. OBs derive from mesenchymal stromal cells (MSC) and are responsible for bone matrix synthesis and mineralization. OCLs derive from mononuclear progenitors of the monocyte lineage that fuse to form giant multinucleated cells. Osteocytes are terminally differentiated cells of the OB lineage, embedded in the bone matrix within the lacuno-canalicular system. Osteocytes are mechano-sensors and capable of sensing mechanical stimuli and convert these signals into a cellular response to control bone resorption and formation [12,13]. Lastly, bone lining cells belong to the OB lineage that migrate over the remodeling area, in particular near OCLs, preventing the inappropriate interactions of OCL precursors with the bone surface.

The remodeling phase consists of four overlapping steps named activation, resorption, reversal, and formation. The resorption step comprises the early migration of mononuclear OCL precursors from marrow capillary to the bone surface. When differentiated OCLs complete bone resorption, specialized cells remove the undigested product of bone resorption, preparing the bone surface for the subsequent bone formation and providing signals for OB migration (reversal step). In the formation step, OBs begin to deposit unmineralized bone matrix until the resorbed bone is completely rebuilt. When this step is complete, the bone surface is covered by lining cells [1,14]. In humans, the length of bone remodeling is approximately 4–6 months.

The resorption step is preceded by the activation step in which different signals, such as mechanical and nutritional stress as well as hormones (e.g., PTH, estrogen), provide the key message to ensure a proper OCL recruitment [1]. Osteocytes play a pivotal role in this phase. In particular, during targeted bone remodeling, dying or dead osteocytes (e.g., induced by structural damage of bone matrix) induce the secretion of RANKL by OBs. In addition, osteocytes can themselves produce RANKL, thus contributing to the recruitment of OCL precursors [13].

The beginning of the resorption step is mediated by cells of OB lineage, osteocytes, lining cells, and preOBs, which respond to signals described above. For example, in response to PTH stimulation, OBs produce monocyte chemoattractant protein-1 (MCP-1), a chemoattractant chemokine for OCLs [15]. Moreover, OBs secrete RANKL and macrophage colony-stimulating factor (M-CSF). The interaction of RANKL with the receptor RANK expressed by OCL precursors results in the activation and differentiation of OCL precursors. M-CSF interacts with its receptor c-Fms expressed by OCL precursors promoting their proliferation and survival as well as motility and cytoskeletal organization in mature OCLs [16]. Osteoprotegerin (OPG) is a glycoprotein belonging to the tumor necrosis factor (TNF) receptor family secreted by cells of the OB lineage that acts as a soluble decoy receptor for RANKL [17]. OPG inhibits the activation and differentiation of OCLs and induces their apoptosis. These effects are reversible, since OPG is not incorporated into bone matrix. Other factors, such as matrix metalloproteinases (MMPs), are produced by OBs and degrade the unmineralized bone matrix by exposing the adhesion sites for OCLs' attachment via $\alpha v\beta 3$ integrin [1]. In this way, OCLs form the "sealed zone" below the cell, in which OCLs pump hydrogen ions to the dissolute bone matrix and produce the Howship's resorption lacunae [18]. Collagenolytic enzymes, such as cathepsin K, degrade the remaining organic bone matrix [18]. Once the resorption is completed, OCLs die by apoptosis or move along the bone to start resorption in another site.

In the subsequent reversal step, reversal cells from the OB lineage (probably lining cells and other specialized cells [19]) cover the eroded surface, clean the resorption lacunae by enwrapping and digesting non-mineralized collagenous proteins, and integrate signal and coupling factors derived from OCLs. Different OCL-derived factors are responsible for the stimulation of bone formation during this step: (i) factors released during resorption, such as transforming growth factor-beta (TGF-β), bone morphogenetic protein-2 (BMP-2), platelet-derived growth factor, (PDGF), and insulin-like growth factors; (ii) factors secreted by OCLs, such as cardiotrophin-1, sphingosine-1-phosphate, collagen triple helix repeat containing 1, and complement factor 3a; (iii) EphB4·ephrin-B2 bidirectional signaling complex expressed on OBs and OCLs, respectively.

In the formation step, OBs synthetize a new unmineralized bone matrix called osteoid, composed of collagen type I and non-collagenous proteins such as osteopontin, osteocalcin, bone sialoprotein, proteoglycans, and alkaline phosphatase. During mineralization, calcium hydroxylapatite is then incorporated into the new osteoid [1]. Once mineralization is completed, mature OBs can become quiescent lining cells, die by apoptosis, or differentiate into osteocytes and remain entombed in the bone matrix. This phase is tightly regulated by osteocytes, which secrete antagonists of osteoblastogenesis such as sclerostin and Dickkopf-1 (Dkk-1) [13].

4. Overview of Abnormal Bone Remodeling in MM and Bone Cancers

Cancer in bone disrupts the balance between OBs and OCLs activities resulting in perturbation of bone remodeling, bone pain, fractures, and increased tumor growth. According with the seed-and-soil hypothesis proposed by Paget in 1889, multiple interactions between tumor cells and the bone microenvironment are essential for the development of bone metastasis [20]. In particular, tumor cells possess adhesive molecules for the binding to stromal cells and bone matrix. This interplay induces the production by cancer cells of angiogenic and bone-resorbing factors that, in turn, increase cancer growth in bone. Moreover, numerous growth factors released during bone resorption (e.g., TGF-β, BMP, FGF, calcium) make up the fertile soil needed for cancer cells growth [20].

In MM, the accumulation of malignant plasma cells (PCs) into the bone marrow (BM) disrupts the physiological bone remodeling process, ultimately leading to bone destruction [3]. The stimulation of bone-resorbing OCLs and the suppression of bone-forming OBs by MM occur either directly and through multiple cell-to-cell contact with bone microenvironment cells. At the same time, these alterations support the growth and survival of MM cells within the BM niche contributing to the vicious cycle of tumor growth and bone destruction. Cytokines and chemokines released either by MM cells and produced by stromal cells after physical interactions with MM cells are responsible for the abnormal bone remodeling in MM. Specifically, MM cells upregulate the expression of RANKL in BM stromal cells and decrease the release of OPG, thus supporting OCL recruitment [3,21]. Moreover, MM patients show an increased RANKL/OPG ratio that correlates with the extent of bone disease [22,23]. In addition to RANKL, other OCL-activating factors, such as chemokine (C-C motif) ligand (CCL)-3, interleukin (IL)-1, IL-3, IL-6, activin A, and TNFα, are upregulated by MM cells in BM microenvironment [24–28].

The reciprocal interplay between MM cells and stromal cells also hinders OB differentiation. MM cells reduce the activity of Runt-related transcription factor (Runx)-2 in human OB progenitors by cell-to-cell contact leading to an impaired OB differentiation [29]. Other factors, such as IL-7 and hepatocyte growth factor (HGF), were found to decrease Runx2 activity and characterize BM MM patients compared with controls. [30,31].

Moreover, MM cells produce Wnt inhibitors Dkk-1, soluble frizzled-related protein (sFRP), and sclerostin, thus contributing to the inhibition of OB differentiation. In this regard, MM patients have a high level of Dkk-1, sFRP-2, and sFRP-3 in BM [32–36].

In bone metastasis of solid tumors, the BM microenvironment plays a pivotal role in controlling the survival of cancer cells. In prostate carcinoma, bone provides a series of adhesion and growth factors responsible for the growth and proliferation of cancer cells in the skeleton [37]. In particular, prostate carcinoma skeletal metastases are mainly characterized as osteoblastic lesions. In this case, cancer cells induce a series of bone remodeling alterations that increase OBs' activation and bone production. The increased OBs-mediated mineralization outbalances the induction of bone resorption resulting in the formation of osteoblastic lesions [37]. Many factors responsible for the formation of osteoblastic lesions have been identified. Among these, BMPs and endothelin directly stimulate the differentiation of OBs' precursors as well the production of OBs' specific proteins. Parathyroid hormone-related protein (PTHrP) reduces OBs' apoptosis [37]. In contrast, metastatic lesions characterizing breast cancer are predominantly osteolytic. Brest cancer cells produce PTHrP, which stimulate the production of RANKL by OBs and the

subsequent increase in OCL activity and bone resorption [38]. The release of growth factors stored in bone such as insulin growth factor-1 (IGF-1) and TGF-β, allows the proliferation of breast cancer cells within the bone. In addition to MM, the dysregulation of the axis RANKL/OPG has been extensively studied in the abnormal bone remodeling process of solid tumors. Breast and lung cancer showed an upregulation of the RANKL/OPG signaling, whereas prostate cells increase the level of OPG, thus leading to increased OBs' activity. Interestingly, breast cancer bone metastases are characterized by increased levels of Dkk-1, whereas it declines during progression from primary tumor to metastasis in prostate cancers [38].

5. The 26S Proteasome: Structure and Function

The proteasome–ubiquitin system (UPS) is the main process responsible for the degradation of damaged or no longer necessary proteins involved in several cellular functions such as cell cycle, apoptosis, and DNA repair [39]. It comprises two steps: the attachment of multiple ubiquitin molecules to the target protein and the degradation of the polyubiquitinated protein by the 26S proteasome complex. The 26S proteasome is an ATP-dependent complex of different subunits composed of a core catalytic complex called 20S proteasome and two 19S regulatory complexes, which recognize the ubiquitinated proteins and are responsible for their translocation into the core complex [40,41]. Specifically, each 19S regulatory complex has a lid and a base attached to the ends of the 20S core complex. While the base is involved in the deubiquitination process, the lid is responsible for the recruitment, unfolding, and translocation of target proteins into the 20S core complex [42].

The 20S region is a cylinder-shaped protease composed of 28 subunits organized in four rings in a $\alpha 7\beta 7\beta 7\alpha 7$ configuration. The two outer α-rings act as a link for the 19S complexes, whereas the two inner β-rings are responsible for proteolysis. The first step of the proteasome-mediated proteolysis involves three different enzymes belonging to the ubiquitin system: ubiquitin-activating enzyme or UBA (E1), ubiquitin-conjugating enzyme or UBC (E2), and ubiquitin ligase (E3) enzyme. Briefly, E1 is responsible for the ATP-dependent activation of ubiquitin, which moves to E2 enzyme and then to a target protein. The specificity is conferred by E3 enzyme, which catalyzes the binding between ubiquitin and the target protein. Once the proteasome recognizes the polyubiquitinated protein, deubiquitinating enzymes (DUBs) remove the ubiquitin molecules and the target protein moves into the 20S proteolytic core. Three different catalytic activities, chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L), each located in three different β-subunits (β5, β2, and β1, respectively), are responsible for the degradation of proteins into small fragments [40].

6. The UPS and Bone Metabolism

Over the last decade, numerous efforts have been made to understand the role of UPS in bone remodeling. The early evidence arose from the observations that several bone proteins, such as those belonging to BMP and Wnt pathway, as well as the transcription factors Runx2 and Osterix were modulated by ubiquitin-mediated proteasome degradation [43–45]. A preliminary study from Murray et al. showed that the proteolytic activity is essential for OB proliferation and that OBs have high levels of the main three catalytic activities of proteasome [46]. Moreover, Runx2 accumulation in OBs is regulated by ubiquitination, and different E3 ubiquitin ligases have been involved in the proteasome-dependent Runx2 degradation [46]. Specifically, Zhao and colleagues showed that the E3 ubiquitin ligase Smad ubiquitin regulatory factor 1 (Smurf1) interacts with Runx2 favoring its degradation by proteasome [47]. Consistently, overexpression of Smurf1 in OBs inhibits bone formation without inducing OB apoptosis, whereas deletion of Smurf1 in mice markedly increases OB activity and bone mass [47,48]. In OBs, Smurf1 also regulates the degradation of Smad1, the downstream molecule of the BMP pathway, thus impairing OB formation. Importantly, the interplay between Runx2 and Smurf1 explains the mechanisms by which PTH acts on OBs. It is widely known that PTH induces bone resorption when

administered continuously, whereas intermittently, it stimulates bone formation [49,50]. The anabolic effect of PTH is mainly due to the reduced OB apoptosis through the transcription of Runx2-dependent survival genes. At the same time, PTH decreases the level of Runx2 by inducing its Smurf1-dependent proteasomal degradation, thus explaining why repeated exposure of PTH is needed to exert its anabolic effect [51]. PTH also stimulates all proteolytic activities in OBs as well as the accumulation of polyubiquitinated proteins [46].

Many other E3 ubiquitin ligases are involved in Runx2 degradation as evidenced by reports that Schnurri-3 (Shn3), a zinc finger adaptor protein, recruiting the E3 ubiquitin ligase WWP1, inhibits Runx2 activity through polyubiquitination and proteasome degradation [52]. Wnt signaling, one of the main pathways involved in OB formation, is tightly regulated at proteasome level. Indeed, Wnt inhibitors, by blocking the interplay between Wnts and their own receptors, lead to β-catenin ubiquitination by β-TrCP1 and proteasomal destruction, thus preventing its translocation into the nucleus [53]. Importantly, the transcription factor Osterix has been proven to be tightly regulated by ubiquitination and degradation. Specifically, the E3 ubiquitin ligases Cbl-b and c-Cbl inhibit OB differentiation of MSCs through the proteasome-dependent degradation of Osterix [44]. More recently, other mechanisms potentially involved in the regulation of proteasome-dependent bone metabolism have been investigated. In particular, the ubiquitin-specific peptidase 53 (USP53), a member of the DUBs family, has been identified as a positive regulator of OB differentiation both in vitro and in vivo [54]. The authors showed that USP53 interacts with the ubiquitin ligase FBXO31 during OB differentiation and regulates β-catenin degradation. Interestingly, USP53 expression decreases in osteoporotic patients and in a mouse model of osteoporosis [54]. These preliminary findings provide novel insights into the involvement of DUBs in bone disease and potential therapeutic strategies for bone regeneration.

7. Proteasome Inhibitors

The inhibition of proteasome as an approach to induce cell apoptosis is especially effective in hematological cancer cells, such as MM cells, because of their dependence on proper proteasome function to ensure the degradation of aberrantly folded proteins [55,56]. Indeed, the vast number of immunoglobulins produced by malignant PCs makes these cells more sensitive to the proteasome inhibition than the normal ones [56]. One of the early mechanism of action imputed to PIs was the abrogation of the degradation of inhibitory proteins of the kappa-beta (kB) family, IkB, leading to cytoplasmic recruitment and inhibition of nuclear factor kappa-light-chain enhancer of activated B cells (NFkB) [57]. This aspect has special relevance for MM, where the adhesion of malignant PCs to BM microenvironment cells leads to the NF-κB-dependent production of antiapoptotic proteins and IL-6 [58]. Additionally, proteasome inhibition alters the equilibrium between protein synthesis and removal of misfolded proteins, which leads to endoplasmic reticulum (ER) stress and unfolded protein response (UPR) and, ultimately, to apoptosis [55,59]. Based on this evidence, several PIs have been developed. According to their chemical structures, they have been divided in different classes: boronic acid derivatives, epoxyketones, and salinosporamides. A brief description of these classes of PIs will be provided in the next paragraph. A schematic diagram of the 26S proteasome and the subunit specificities of PIs are reported in Figure 1.

7.1. Boronates

Bortezomib, the first in-class dipeptide boronate PI, was approved in 2003 by the FDA for the treatment of MM [60]. It is a slowly reversible inhibitor that binds to β5 subunit of proteasome and, at a higher concentration and in a much less potent manner, to the β1 and β2 subunits [61,62]. Beside the abovementioned effects of proteasome inhibition, bortezomib increases the pro-apoptotic protein Noxa [63], which induces apoptosis by multiple mechanisms such as the release of proapoptotic protein from the mitochondria, the degradation of the pro-survival protein myeloid leukemia cell differentiation protein

(Mcl1), and the phosphorylation of the anti-apoptotic protein B-cell lymphoma-extra-large (Bcl-xL) [64].

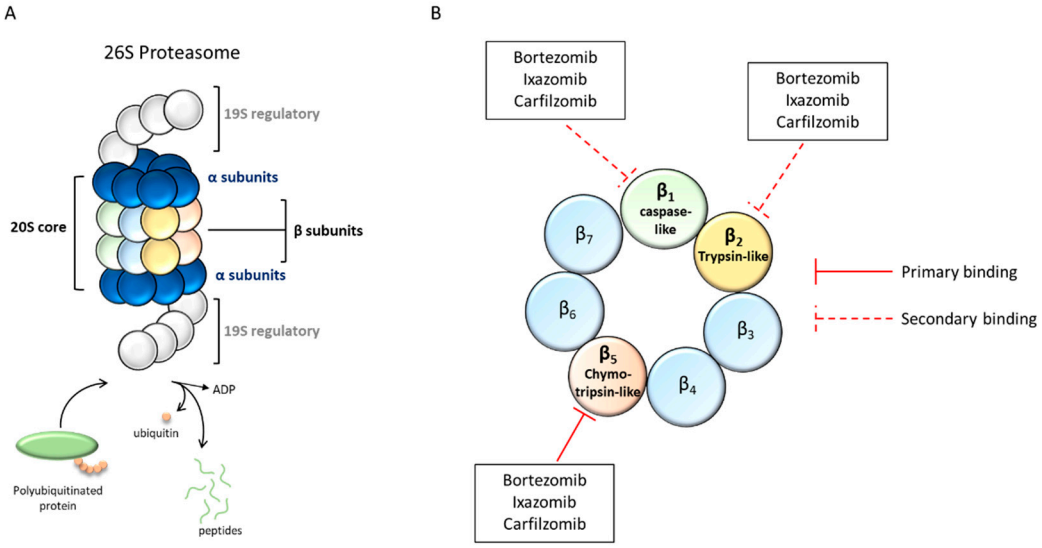

Figure 1. Structure of 26S proteasome and subunit specificities of PIs. (**A**) The 26S proteasome is composed of a core catalytic complex called 20S proteasome and two 19S regulatory complexes, which recognize the ubiquitinated proteins. The 20S region is a cylinder-shaped protease composed of different subunits organized in four rings in a α7β7β7α7 configuration. The two outer α-rings act as a link for the 19S complexes, whereas the two inner β-rings are responsible for proteolysis. (**B**) Cross-section of β-subunits. Three different catalytic activities, chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L), are located in β5, β2, and β1 subunit, respectively. Bortezomib slowly reversibly inhibits the chymotrypsin-like activity (β5 subunit) and, at a higher concentration and in a much less potent manner, also targets the trypsin-like and caspase-like activities (β2 and β1 subunits, respectively). Carfilzomib irreversibly binds mainly the chymotrypsin-like activity, whereas ixazomib reversibly inhibits the chymotrypsin-like activity. Both drugs at very high concentrations interact with the β2 and β1 subunits (**B**).

Although the introduction of bortezomib improved the progression-free survival and overall survival of MM patients [65], the presence of drug resistance and the dose-limiting side effects led to the development and approval of second-generation PIs. Ixazomib is one such molecule, an orally administered dipeptidyl leucine boronic acid, approved by the FDA in 2015 for the treatment of MM [66]. The biologically active form, named MLN2238, is released after hydroxylation upon exposure to aqueous solution or plasma displaying a shorter dissociation half-life than bortezomib [67,68]. Similar to bortezomib, it exerts a time-dependent reversible proteasome inhibition by blocking the β5 subunit of proteasome and, at higher concentrations, the β1 and β2 proteolytic sites. Apoptosis induced by MLN2238 is caspase-dependent, since it activates both caspase 9 and caspase 8 through the involvement of other signaling axes, such as p53-p21, p53-NOXA-PUMA, and endoplasmic reticulum stress [67,68]. Additionally, it is responsible for the inhibition of cell cycle in MM cells by reducing the levels of cyclin D1 and CDK6 [69].

7.2. Epoxyketones

Carfilzomib is an epoxyketone-based PI approved for the treatment of patients with relapsed and refractory MM [70,71]. It is an analog of the natural compound epoxomicin and irreversibly blocks the β5 subunit of proteasome, resulting in the accumulation of ubiquitin–protein conjugates as well as the induction of programmed cell death. Past preclinical studies revealed that carfilzomib had less neurotoxicity than bortezomib and greater selectively for the CT-L subunit. At very high concentrations, it interacts with the β1 and β2 subunits [72]. Carfilzomib induces both external and internal apoptotic pathways as well as the activation of JNK and cytochrome c release.

8. Proteasome Inhibitors and Osteoblast Functions

Increased understanding of the mechanisms underlying bone disease and the involvement of UPS in regulating bone proteins prompted the scientists to investigate the potential effects of PIs on the bone remodeling process. The majority of observations arose from in vitro and in vivo studies conducted in MM. Although the ability of bortezomib to regulate bone remodeling relies on both the inhibition of OCLs and the stimulation of OBs' activity, this review will focus on the potential beneficial effects of proteasome inhibition on OBs' compartment. The main mechanisms underlying the bone anabolic activity of PIs have been summarized in Figure 2.

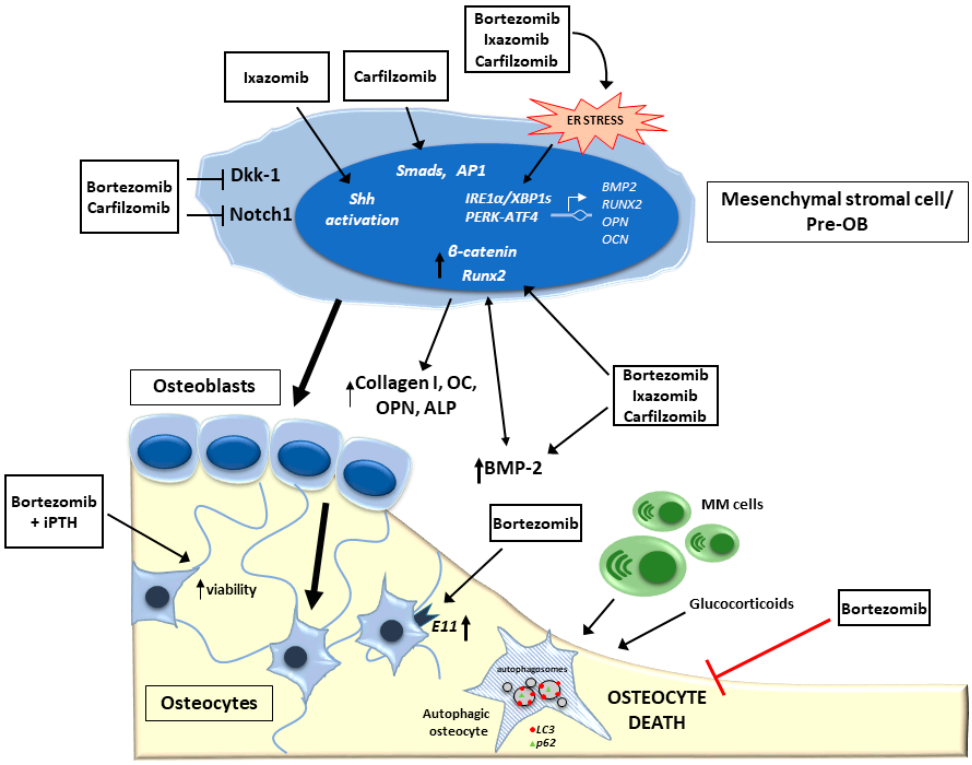

Figure 2. Biological consequences of proteasome inhibition in MM bone microenvironment. PIs increase the activity of Runx2 in pre-OBs with a consequent increased expression of collagen I, OC, OPN, and ALP through a Wnt-independent stabilization of β-catenin. Bortezomib also increases BMP-2 expression in osteoblastic cells that, in turn, increase the levels of the transcription factor Runx2, enhancing bone formation. The treatment with carfilzomib enhances the activity of Smads and AP1 transcription factors, promoting early OB progenitors' commitment. The bone anabolic effect of PI treatment, in particular bortezomib and carfilzomib, has been correlated with Notch1 and Dkk-1 inhibition with subsequent enhancement of OB formation. Bortezomib and ixazomib promote the ER stress, mainly through the activation of IRE1α/XBP1s and PERK-ATF4 signaling pathways leading to increased expression of OB markers. The anabolic effect of ixazomib is also associated with the activation of SHH pathway by inducing the nuclear translocation of Gli1 in MSCs and OB differentiation. MM cells and high doses of glucocorticoids induce autophagic death in osteocytes by modulating the levels of two autophagic markers LC3 and p62. Bortezomib blunts osteocyte death by reducing LC3 and increasing p62 protein levels. Bortezomib also potentiates the effect of intermittent PTH on osteocyte viability. Additionally, bortezomib stabilize the transmembrane glycoprotein E11, leading to increased osteoblast–osteocyte transition.

8.1. Bortezomib

Early preclinical studies showed the ability of PIs to induce OB differentiation via multiple mechanisms in different models. Garrett et al. firstly showed that PIs, such as epoxomicin, proteasome inhibitors-1, and lactacystin, promoted bone formation in mice

in a dose-dependent manner [73]. The same study attributed this effect to the increased expression of BMP-2 in OBs following PIs administration. Afterwards, by using a neonatal mouse calvarial organ culture, Oyajobi et al. confirmed the ability of bortezomib to induce new bone formation via BMP2 stimulation [74]. Additionally, they reported that bortezomib is a potent inhibitor of Dkk1, a known antagonist of Wnt signaling, at both mRNA and protein levels, thus pointing out the capability of bortezomib to regulate Wnt signaling in bone [74]. In line with these results, Giuliani and coworkers demonstrated that bortezomib induced the OB phenotype in primary human MSCs with no effects on the number or viability of mature OBs by increasing the activity of Runx2 and the expression of osteocalcin and type I collagen [75]. Noteworthy, MM patients who responded to bortezomib treatment had a significantly higher number of Runx2-positive OBs compared with the non-responder ones [75]. The authors supposed that either bortezomib has a direct effect on OB differentiation or the removal of malignant cells from the BM may recover the ability of precursors cells to differentiate into OBs. Interestingly, by using SCID-rab mice engrafted with primary MM cells, it has been demonstrated that the antimyeloma response of bortezomib was associated with increased bone mineral density (BMD), OB numbers, and reduced number of OCLs in both myelomatous and non-myelomatous implanted bones [76]. This anabolic effect was not seen in mice treated with melphalan, suggesting that the effect of bortezomib on skeletal homeostasis is not due to a reduced tumor burden [76]. These results have been further validated in a 5T2MM mouse model [77] and C57BL/6 mice [78]. Despite the increasing evidence supporting the bone-protection effect of PIs, the underlying molecular mechanism remains poorly understood. Only recently, a possible mechanism that connects proteasome inhibition and osteogenic differentiation has been proposed. Zhang et al. showed that while increasing osteogenesis, bortezomib induces G_0/G_1 phase cell cycle arrest in murine BM MSCs by inducing $p21^{Cip1}$ and $p27^{Kip1}$ expression [79]. Notably, this upregulation is transcriptionally dependent on the activation of ER stress signaling pathways IRE1α/XBP1s induced by bortezomib. The same group demonstrated that both IRE1α/XBP1s and PERK-ATF4 stress signaling are activated during PI-induced OB differentiation. Moreover, XBP1s overexpression triggered the expression of OB differentiation-related genes as well as the induction of ATF4, whereas its inhibition abolished new bone formation in mice after bortezomib administration [80]. These findings strongly support the notion that the signaling cascade of IRE1α/XBP1s is a key mediator of the PIs-induced bone formation. In addition to this, it has been demonstrated that bortezomib, and its analog ixazomib, promote OB differentiation of osteogenic cells by enhancing Osterix protein levels [81]. An interesting study proposed by Chandra et al. examined the potential bone-protective effects of bortezomib against radiation damage in a mouse focal radiation model [82]. Intriguingly, bortezomib preserves bone architecture and strength after radiation, as well as OB number and mineralizing surface compared with non-irradiated controls. To further understand the underlying cellular mechanism, the authors established a fluorescent reporter mouse model to visualize mesenchymal lineage cells within BM. Of note, bortezomib administration reduces the percentage of apoptotic OB and increases DNA repair in OB in irradiated bones. The same protective effect was seen on osteocytes [82]. As reviewed by others, clinical studies have demonstrated that bortezomib and carfilzomib administration is associated with increased levels of markers of bone formation, such as bone ALP, OC and serum PTH, and reduced levels of bone resorption markers, such as Dkk-1, in MM patients [7,36,83–87]. Histomorphometric and imaging studies supported this biomarker evidence, demonstrating that PIs have beneficial effects on bone health in MM patients [7].

In breast cancers, bortezomib reduced the growth of osteolytic lesions in mice intratibially inoculated with breast cancer cell lines [88]. Moreover, bortezomib administration increased trabecular bone formation in both tumor-containing tibias and in distal femurs of non-tumor bearing mice. In order to assess the ability of bortezomib to protect bone from subsequent osteolysis, the authors evaluated bortezomib treatment for a period of time before tumor cell inoculation. They found that bortezomib administration before tumor

inoculation provides protection from osteolysis, suggesting that bortezomib may protect the skeleton in advanced breast cancer [88]. The analysis of gene expression of breast cancer cell lines treated with bortezomib revealed that bortezomib decreases the expression of genes promoting tumor growth such as *RUNX2*, *MMP-9*, and *VEGF* [88]. It has been also demonstrated that bortezomib prevents bone metastasis of prostate cancer by inhibiting the E3 ubiquitin protein ligases, WWP1, Smurf1, and Smurf2, which are known to be upregulated in metastatic patients compared with those without bone metastases [89]. Moreover, by using a murine intratibial injection model, Whang et al. showed that bortezomib is able to inhibit the growth of osteolytic lesions but not their initial formation [90].

8.2. Ixazomib

Early preclinical studies showed that ixazomib promoted OB formation and activity of osteoprogenitors cells from MM patients through the activation of TCF/β-catenin signaling, UPR response via IRE1α–XBP1 pathway, and upregulation of BMP2 in MSC precursors [45,91]. Moreover, in a mouse model of MM, ixazomib showed equal effectiveness of bortezomib in reducing tumor burden and preventing tumor-associated bone loss. In keeping with these data, Tibullo et al. have recently showed that ixazomib activates the Sonic Hedgehog (SHH) pathway by inducing the nuclear translocation of Gli1 in human MSCs and OB differentiation [45]. An interesting study from Yang et al. showed how ixazomib is able to enhance the PTH-induced beta catenin signaling. By using CRISPR/Cas9 genome-editing technology, the authors demonstrated that ixazomib enhances PTH-induced cAMP generation in OBs by facilitating the dissociation of β-catenin from the PTH receptor and its nuclear translocation [92]. These findings suggest the possible use of ixazomib to improve the therapeutic efficacy of PTH in bone disease.

8.3. Carfilzomib

Carfilzomib, an epoxyketone-based PI, was recognized to influence bone remodeling process. In terms of bone anabolic activity, carfilzomib stimulates osteogenic differentiation and mineralization of primary MSCs in vitro under both transient and continuous conditions. Interestingly, it reduces the mRNA levels of DKK-1 in differentiated MSCs and stimulates the activity of Smad2/3/4 and AP1 transcription factors, promoting early progenitor commitment and OB differentiation [93]. As with bortezomib and ixazomib, carfilzomib upregulation of the IRE1α component of the UPR results in enhanced OB differentiation. In both the non-tumor bearing model and in a model of disseminated MM, carfilzomib increased bone volume and prevented bone loss [93]. Two independent studies showed that the bone anabolic effect of carfilzomib is associated with the Wnt-independent activation of β-catenin/TCF signaling [94] as well as the downregulation of Notch signaling [95].

8.4. Proteasome Inhibitors and Osteocyte Functions

As far as osteocytes are concerned, little is known about the involvement of UPS during osteocytogenesis. In this regard, Staines et al. found that the protein E11/Podoplanin, critically implicated in the early OB-osteocyte transitions, is regulated post-translationally by proteasome degradation [96]. The inhibition of proteasome by bortezomib and other PIs promotes E11–Podoplanin stabilization and the acquisition of osteocyte phenotype in pre-osteocyte cells [96]. As described above, osteocytes play a key role in regulating bone remodeling process in MM. Toscani et al. recently examined the effect of bortezomib-based therapy on osteocyte viability on MM BM biopsies [97]. In this study, MM patients who underwent bortezomib therapy showed a significant improvement in the number of viable osteocytes compared with patients treated without bortezomib. Moreover, bortezomib counteracted the negative effect of dexamethasone on osteocyte viability. As described above, MM cells induce autophagic death in cocultured osteocytes. The ex vivo analysis performed on BM bone biopsies showed that patients treated with bortezomib display a reduction in autophagic osteocytes compared with those treated without bortezomib,

thus providing a novel potential mechanism underlying the bone protective activity of PIs. The same study showed that bortezomib is able to counteract the reduction in osteocytes viability induced by MM cells in vitro [97]. Interestingly, this effect is linked to the ability of bortezomib to downregulate the autophagic death induced by MM cells and high doses dexamethasone by modulating the levels of the autophagic markers LC3 and p62. Additionally, bortezomib potentiates the anabolic effects of intermittent PTH in murine osteocytes [97].

9. Side Effects of PIs and Future Perspectives

PIs have shown significant side effects in patients with MM, including hematologic toxicity (thrombocytopenia, neutropenia, anemia) resulting from the systemic distribution of the drug. Peripheral neuropathy is the main extra-hematological side effect of bortezomib. On the other hand, carfilzomib has been associated with cardiovascular events, in particular hypertension [98,99]. All these aspects limit the use of this class of drugs as bone anabolic therapy. For these reasons, there is an urgent need for a new strategy to selectively deliver PIs into the bone and prevent the toxic effects.

The conjugation of bortezomib to bisphosphonates and the design of bone-specific nanoparticles have been recently proposed [100]. Wang et al. showed that bisphosphonate–bortezomib complex prevents bone loss in a mouse model of MM more effectively than Btz with less systemic side effects [101]. Moreover, this approach showed high bone anabolic activity and enhanced fracture repair in aged mice. Since bortezomib inhibits the proteasomal degradation of proteins non-specifically, this approach may cause side effects in bone that are not acceptable for non-malignant patients, such as osteoporotic patients, who need long-term therapy. To overcome these limitations, several attempts have been made to target specific proteins known to increase bone mass, such as BMPs, PTH, or OPG [102–104]. An interesting study performed in rats showed that local application of PIs enhances fracture healing [105]. Immunohistochemical analysis revealed that PIs increase BMP-2 expression within the fracture callus during early phases of healing. Lastly, as recently reviewed by Rothe et al., the development of biocompatible polymers such as implant coatings, scaffolds, or particle-based materials provide novel strategies to enhance bone healing [106]. In this regard, the use of a novel model to study bone healing and formation has been proposed. Falacho et al. performed a histomorphometric evaluation of bone healing in a model of rabbit bone defects after implanting them with different forms of heterologous porcine bone [107]. This model may be considered as a new alternative approach to assess bone healing.

10. Conclusions

Alteration of the bone remodeling process is a characteristic feature of MM and metastatic bone tumors. Affected patients experienced bone pain, risk of fractures, and reduced quality of life. For these reasons, developing new therapies to increase bone mass is a critical clinical need. Over the last years, growing observations suggested that UPS regulates the activities of bone proteins involved in bone resorption and formation.

The findings reviewed herein suggest the PIs affect bone remodeling and the activities of bone proteins. In particular, bortezomib has been proven to influence bone formation and resorption both in preclinical and in clinical models of MM. Preliminary evidence suggests that the next-generation PIs, i.e., ixazomib and carfilzomib, have beneficial effects on bone remodeling. Although PIs target bone resorption activity of OCLs, a more profound effect has been seen on OB compartment with a stimulatory effect on MSC, mature OBs, and osteocytes. For these reasons, targeting proteasome may be an efficient strategy to improve bone formation in patients with bone disease.

The preclinical evidence of the bone anabolic activity of PIs is confirmed in MM patients treated with bortezomib and carfilzomib, who showed an improvement in bone remodeling markers. Histomorphometric studies showed that MM patients treated with bortezomib display an increased bone formation process and bone healing.

Overall, these data strongly sustain the use of PIs to improve bone integrity in patients with metastatic bone cancers and MM.

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