CLINICS

REVIEW

Pharmacological management of osteogenesis

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Osteogenesis and bone remodeling are complex biological processes that are essential for the formation of new bone tissue and its correct functioning. When the balance between bone resorption and formation is disrupted, bone diseases and disorders such as Paget's disease, fibrous dysplasia, osteoporosis and fragility fractures may result. Recent advances in bone cell biology have revealed new specific targets for the treatment of bone loss that are based on the inhibition of bone resorption by osteoclasts or the stimulation of bone formation by osteoblasts. Bisphosphonates, antiresorptive agents that reduce bone resorption, are usually recommended as first-line therapy in women with postmenopausal osteoporosis. Numerous studies have shown that bisphosphonates are able to significantly reduce the risk of femoral and vertebral fractures. Other antiresorptive agents indicated for the treatment of osteoporosis include selective estrogen receptor modulators, such as raloxifene. Denosumab, a human monoclonal antibody, is another antiresorptive agent that has been approved in Europe and the USA. This agent blocks the RANK/RANKL/OPG system, which is responsible for osteoclastic activation, thus reducing bone resorption. Other approved agents include bone anabolic agents, such as teriparatide, a recombinant parathyroid hormone that improves bone microarchitecture and strength, and strontium ranelate, considered to be a dual-action drug that acts by both osteoclastic inhibition and osteoblastic stimulation. Currently, anti-catabolic drugs that act through the Wnt-β catenin signaling pathway, serving as Dickkopf-related protein 1 inhibitors and sclerostin antagonists, are also in development. This concise review provides an overview of the drugs most commonly used for the control of osteogenesis in bone diseases.

KEYWORDS: Antiresorptive Drugs; Bone Formation; Osteoblasts; Osteogenesis; RANKL Inhibitors.

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INTRODUCTION

Osteoblasts play a crucial role both in the promotion of bone formation and, indirectly, in the modulation of osteoclast differentiation through the expression of the receptor activator of nuclear factor NFκB ligand (RANKL) and of osteoprotegerin (OPG), which are known, together with RANK, to regulate osteoclast formation and activity (1,2). RANKL, a transmembrane protein that is highly expressed by pre-osteoblasts and osteoblasts (3), periosteal cells (4), and osteocytes (5), binds and activates its receptor RANK, which is mainly expressed by osteoclasts and their precursors (6). After binding to RANK, RANKL stimulates the formation, activity and survival of osteoclasts (7,8), resulting in increased bone resorption (9). OPG, a member of the tumor necrosis factor (TNF) superfamily of proteins that is secreted by osteoblasts, is another key molecule in this process because it inhibits RANKL-induced osteoclastogenesis (13). In fact, OPG binds to RANKL with high affinity and competes with RANK for binding to RANKL on the surface of osteoclasts and their precursors (10,11). This RANK/RANKL/OPG system is regulated by various cytokines (interleukin (IL)-1, 4, 6, 11 and 17 and TNF-α), hormones (glucocorticoids, vitamin D and estrogen), and mesenchymal transcription factors (cbfa-1 and peroxisome proliferator-activated receptor gamma) (9) and determines osteoclast activity (Figure 1).

Bone is continually reabsorbed and formed. This process is called bone remodeling, in which bone cells have an extremely important role in ensuring a balance between the processes of bone formation and resorption. When this balance is disrupted, various diseases and conditions, such as osteogenesis imperfecta, tumors, osteoarthritis and osteoporosis may arise. In particular, osteoporosis is characterized by a progressive loss of bone mass and microarchitecture, which leads to increased fracture risk.

Currently, the available drugs used in the treatment of bone diseases can be divided into two categories: antiresorptive agents, such as bisphosphonates (BPs), estrogen, selective estrogen receptor modulators (SERMs) and RANKL inhibitors that inhibit osteoclastogenesis and bone-forming agents that increase bone strength by increasing

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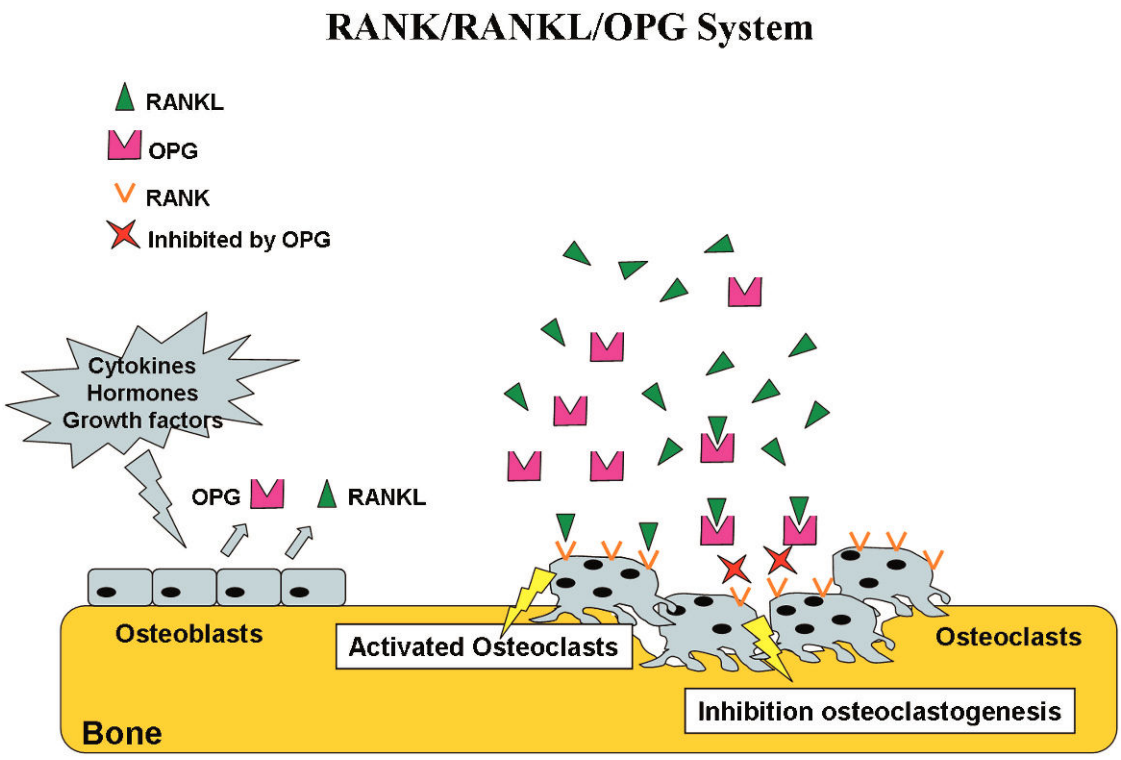

Figure 1 - RANK/RANKL/OPG system. Osteoblasts produce RANKL and OPG under the control of various cytokines, hormones, and growth factors. OPG binds and inactivates RANKL, resulting in the inhibition of osteoclastogenesis. In the absence of OPG, RANKL activates its receptor, RANK, expressed on osteoclasts and preosteoclast precursors. The RANK-RANKL interaction leads to preosteoclast recruitment and fusion into multinucleated osteoclasts and to osteoclast activation and survival.

bone mass, such as parathyroid hormone (PTH) peptides, strontium ranelate (SR) and anti-Dickkopf-related protein 1 (DKK1) and anti-sclerostin (SOST) antibodies (Figure 2 and Table 1).

The purpose of this review is to provide an overview of the drugs commonly used for the control of osteogenesis in bone diseases.

Bisphosphonates

BPs are a class of drugs generally used in the treatment of bone disorders that are characterized by excessive osteoclastic bone resorption, such as osteoporosis, Paget's disease, fibrous dysplasia, hypercalcemia of malignancy, and inflammation-related bone loss (12-15).

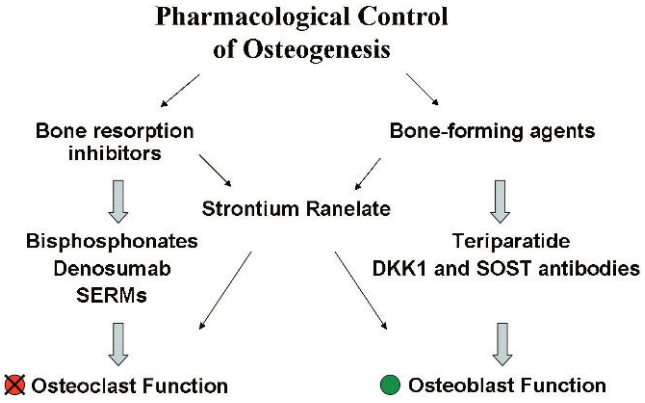

Figure 2 - Summary of the main drugs used in the control of osteogenesis.

The clinical efficacy of BPs primarily stems from two key properties: their ability to bind strongly to bone mineral and their inhibitory effects on mature osteoclasts (16).

In fact, these drugs are able to bind with high affinity to hydroxyapatite crystals, where they remain for prolonged periods. The drugs then act selectively on osteoblasts, particularly in areas of high bone turnover, resulting in an antiresorptive effect (17,18). The drugs are subsequently released from the bone matrix upon exposure to acid and enzymes secreted by active osteoclasts (19,20).

Studies to date suggest that the mechanisms by which BPs are internalized by osteoclasts are similar for different BPs, which can be divided into two categories: nitrogen-containing BPs and non- nitrogen-containing BPs.

Nitrogen-containing BPs, such as alendronate, ibandronate, pamidronate, risedronate, and zoledronate, have a side chain that contains a nitrogen atom, in contrast to the non-nitrogen-containing BPs, such as clodronate and etidronate.

Nitrogen-containing BPs principally act by inhibiting farnesyl pyrophosphate (FPP) synthase, an enzyme in the cholesterol synthesis pathway and preventing the prenylation of small guanosine triphosphate (GTP)-binding proteins, which are indispensable for cytoskeletal organization and vesicular traffic in the osteoclast, causing osteoclast inactivation (21,22).

In contrast, in osteoclasts' cytosol, non-nitrogen-containing BPs are metabolized into adenosine triphosphate (ATP) analogs that block osteoclast function and induce osteoclast apoptosis (23).

Table 1 - Comparisons of the principal drugs used in bone diseases.

Drugs	Bone resorption inhibitors	Bone-forming agents	*In vitro* effects	*In vivo* effects
Bisphosphonates	√		- Inhibition of osteoclast activity and differentiation - Induction of osteoclast apoptosis	- Reduction in the risk of new vertebral, non-vertebral, and hip fractures
Denosumab	√		- Inhibition of osteoclast differentiation, activation and survival	- Increase in bone mass and decrease in the risk of fractures
Selective Estrogen Receptor Modulators (SERMs)	√		- Reduction in the number of preosteoclasts and mature osteoclasts	- Reduction in the risk of new vertebral fractures
Intermittent PTH_{1-34} Therapy		√	- Increase in the number and activity of osteoblasts - Increase in osteogenesis and chondrogenesis during skeletal repair	- Increase in bone mineral density - Enhancement of the cortical thickness and trabecular bone volume and improved bone microarchitecture
Strontium Ranelate	√	√	- Induction of the osteoblastic differentiation of human MSCs - Increase in osteoblast proliferation, survival and differentiation - Reduction in osteoblast apoptosis - Decrease in osteoclast differentiation - Increase in osteoclast apoptosis	- Increase in bone mineral density - Reduction in the risk of new vertebral, non-vertebral, and hip fractures
Anti-DKK1 and Anti-SOST Antibodies		√	- Increase in osteoblastogenesis and proliferation	- Increased bone formation, trabecular thickness, and bone mass and strength

In vitro, several BPs inhibit osteoclast differentiation in human bone marrow cultures (24) and promote the apoptosis of murine osteoclasts, which was also confirmed by *in vivo* studies in mice. More specifically, *in vitro* studies have shown that BPs are not always selective for osteoclasts and can inhibit cell growth and induce apoptosis in a wide range of cell types (16,19,25-28) and in many cancer cell types (20) at high doses.

In the 1990s, *in vitro* studies demonstrated that osteoblasts treated with BPs did not exhibit osteoclastogenesis (29,30). Additionally, numerous studies performed to evaluate the effects of BPs on osteoblasts have demonstrated the non-selectivity of these drugs for osteoclastic cells.

In addition, BPs are able to inhibit the apoptosis of osteocyte cell lines and primary murine osteoblasts (31), as well as human osteoblasts (32).

Nitrogen-containing BPs appear to induce collagen type I (COLIA1) gene expression (28). Moreover, alendronate and etidronate enhance IL-6 production in osteoblasts (33).

Clodronate stimulates osteoblast differentiation in ST2 and MC3T3-E1 cells, whereas etidronate promotes osteoinduction only in MC3T3-E1 cells (34). In addition, it has been shown that BPs decrease the expression of RANKL and increase the expression of OPG in human osteoblastic cells (35,36). Finally, trabecular cultures of MG-63 cells and primary human bone have shown that risedronate and alendronate each increase osteoblast and osteoblast progenitor numbers and also enhance the gene expression of bone morphogenetic protein 2 (BMP-2), COLIA1, and osteocalcin (OCN) (37,38).

It has been demonstrated that these drugs increase the proliferation and formation of mineralized nodules in murine and human bone marrow cultures *in vitro* (25,39-42) and promote early osteoblastogenesis in both young and aged mice *in vivo* (39). In contrast, other studies have demonstrated that BPs decrease proliferation and inhibit osteoblast differentiation and mineralization (27,28,43,44). In particular, an *in vitro* study has demonstrated that pamidronate and zoledronate decrease osteoblast proliferation in a dose-dependent manner and increase differentiation and bone-forming activities among immortalized human fetal osteoblasts (28). However, another *in vitro* study on mouse calvarial osteoblasts has shown that pamidronate and alendronate inhibit osteoblast growth and bone nodule formation (43).

These conflicting results are explained by the fact that low concentrations of BPs, from 10^{-9} M to 10^{-6} M, were shown to increase growth and have induction effects, whereas concentrations higher than 10^{-5} M had inhibitory effects (45). Finally, BPs such as alendronate, risedronate, and zoledronate have been shown to reduce the risk of new vertebral, non-vertebral, and hip fractures (46-49). Interestingly, the long-term use (up to 10 years) of BPs in the treatment of osteoporosis has been associated with a good safety profile (50), although several studies have associated BP therapy with a potential risk of osteonecrosis of the jaw and atypical subtrochanteric femoral fractures (51-53).

Denosumab

The RANK/RANKL/OPG pathway is key to maintaining the balance between the activities of osteoblasts and

osteoclasts to prevent bone loss and ensure normal bone turnover. Thus, manipulation of the RANKL system has been a target of pharmaceutical development. In particular, human OPG constructs, such as OPG fusion proteins (OPG-Fc) (54), have been valuable research tools because they strongly inhibit bone resorption in a variety of species, including rats (55,56), pigs (57), monkeys (58), and humans (54,59). However, the clinical development of OPG-Fc was abandoned in favor of denosumab due to several limitations concerning half-life and specificity. Denosumab (AMG 162) is currently the only RANKL-targeted therapy available, offering a new approach in the treatment of osteoporosis (60,61). This human monoclonal IgG2 antibody was developed using transgenic mouse technology. Denosumab binds RANKL with high affinity and specificity, thereby inhibiting osteoclastogenesis, as demonstrated by numerous studies (61-65) and also increasing bone mass and reducing the risk of fractures (66).

Finally, several studies have demonstrated that denosumab is able to reduce the expression of specific markers of bone resorption in postmenopausal women (67) and in subjects with bone metastases or multiple myeloma (68).

Selective Estrogen Receptor Modulators

SERMs, such as estrogen, are potent inhibitors of bone resorption and are currently Food and Drug Administration (FDA) approved for the prevention and treatment of osteoporosis in postmenopausal women (69). In particular, estrogen is a systemic hormone with direct effects on bone that plays an important role in osteoporosis. In postmenopausal women, the deficiency of estrogen leads to an upregulation of RANKL on bone marrow cells, resulting in an increase in bone resorption (70).

In contrast, estrogen itself stimulates OPG production in osteoblasts and thus exerts antiresorptive effects on bone (71). The extraskeletal effects of estrogen deficiency are mainly based on increased renal calcium excretion and decreased intestinal calcium absorption (72,73). Tamoxifen was the first SERM to be widely used in clinical practice, based on its now well-recognized estrogen antagonist activity in the breast.

The prolonged use of tamoxifen was associated with an increase in uterine cancer (74), leading to the search for other SERMs with different pharmacological profiles. Thus, raloxifene, a new SERM, was developed for the treatment and prevention of postmenopausal osteoporosis, with the goal of improving the drug safety profile. Raloxifene has a spectrum of tissue-specific agonist-antagonist effects on estrogen target tissues but acts on bone as an estrogen agonist (75). This drug has been extensively studied and data support its estrogen agonist profile in the skeletal system. The drug specifically acts on estrogenic receptor-α and estrogenic receptor-β, binding to the receptors in the same ligand-binding pocket as does estradiol, and causes the C-terminal α-helix of the receptor to change its conformation to block access to the activation function-2 region of the receptor. This event in turn likely blocks access to the transcriptional coactivators necessary to facilitate the activation of estrogen-responsive genes (76). In the ovariectomized (OVX) rat model, raloxifene acts as an antiresorptive, with preservation of both bone mineral density (BMD) and bone strength (76). It has been demonstrated that raloxifene modulates the homeostasis of bone cells *in vitro* by inhibiting osteoclastogenesis and bone resorption, reducing the number of preosteoclasts and mature osteoclasts in OVX rats (77) by suppressing osteoblast apoptosis and increasing osteoblast proliferation and differentiation in MC3T3-E1 cultures (78-80). Other studies in OVX rats have shown that raloxifene was able to decrease RANKL and increase OPG expression (77,81,82). Finally, an *in vitro* study on human fetal osteoblast cell lines treated with raloxifene, which expressed a G-protein-coupled receptor (GPR30) but lacked estrogen receptor, has shown that this drug was able to induce cell proliferation, although the function of GPR30 in bone remains unclear (83).

Parathyroid Hormone Therapy

The first molecule to be approved by the FDA as the only anabolic therapy for osteoporosis was a PTH analog (84). This analog is available in the form of human recombinant PTH peptide 1-34 (teriparatide, or PTH_{1-34}), a fragment of PTH that has similar affinity for PTH receptor-1.

PTH is released from the parathyroid gland, and its secretion is chiefly controlled by serum [Ca^{2+}] through negative feedback (85).

Pharmacologically, when PTH is administered intermittently (once daily) at low doses, it has an anabolic effect on osteoblasts (85), stimulating bone formation both *in vitro* and *in vivo* and increasing in BMD (84).

Many studies have demonstrated the efficacy of PTH_{1-34} therapy in a variety of skeletal repair models, suggesting that PTH_{1-34} enhanced and accelerated not only bone remodeling but also osteogenesis and chondrogenesis during skeletal repair (87). In 1999, Andreassen et al. were the first to report the efficacy of intermittent PTH_{1-34} therapy on rat tibial fracture healing (88). In particular, it has been shown that intermittent PTH administration promotes bone formation by increasing the number and activity of osteoblasts, enhances the mean cortical thickness and trabecular bone volume and improves bone microarchitecture (89). At the molecular level, PTH enhances Wnt signaling through inhibition of the Wnt antagonist SOST and induces the local production of bone anabolic growth factors such as insulin-like growth factor 1 (IGF1) (86). Furthermore, PTH_{1-34} enhances the differentiation of mesenchymal stem cells (MSCs) into osteoblasts via the induction of osterix (OSX) and Runt-related transcription factor 2 (RUNX-2) expression *in vitro*, increasing both OSX expression at the fracture site *in vivo* and the expression of osteoblastic marker genes, including COLIA1 and OCN (90). Several studies have shown that PTH_{1-34} can promote the proliferation and differentiation of MSCs in the early phase of bone healing (91) and to induce the proliferation of chondroprogenitors at a fracture site, contributing to increased bone formation during fracture healing (92) and accelerating articular cartilage repair (87,93,94), respectively. These data were supported by clinical studies that have demonstrated positive effects of intermittent PTH therapy, including increasing bone mass and reducing the bone fragility associated with osteoporosis due to age, sex hormone deficiency and glucocorticoid therapy (95).

Conversely, in certain studies, toxicity has been reported for the use of PTH therapy. In particular, the toxic effect of treatment with teriparatide or parathyroid hormone 1-84, which appears to be unique to animals and not applicable to human subjects, is osteosarcoma (96). In fact, it has been reported that rats treated with high doses of either

teriparatide or parathyroid hormone 1-84 for prolonged periods of time developed osteosarcoma (97-99).

Treatment with teriparatide is approved by the FDA for a limited duration, from 18-24 months and in many European countries, approval is limited to 18 months. However, in several studies, the period of treatment with teriparatide was prolonged to 24-30 months (100,101).

Although it has been reported that the teriparatide-related risk of osteosarcoma development is low (102), there are still no clear scientific data. The general recommendation for this treatment is to closely follow patients who have risk factors, *i.e.*, subjects with Paget's disease, prior skeletal irradiation, or unexplained increases in serum bone-specific alkaline phosphatase (ALP) and adolescents in whom the epiphyses have not yet closed (96).

Strontium Ranelate

SR is a drug commonly used for the treatment of osteoporosis and fragility fractures (103,104). SR consists of two cations of strontium, representing the active component, and one anion of ranelate, which acts as a carrier (105). In contrast to other drugs, SR has a dual effect on bone remodeling, both stimulating bone formation and decreasing bone resorption. *In vitro* experiments have shown that SR increased osteoblastic activity, enhancing preosteoblastic cell proliferation and differentiation (7,11,106,107) and stimulating osteoblastic differentiation markers, such as ALP, hydroxyapatite (HA) deposit formation, bone sialoprotein (BSP), and OCN, in primary murine osteoblasts (108).

In addition, in *in vitro* animal models, SR was observed to reduce osteoblast apoptosis (9,107) and to decrease osteoclast differentiation marker expression, with an enhancement of osteoclast apoptosis (109-111). Furthermore, *in vitro* data on primary human osteoblasts indicate that this drug promotes the ultimate differentiation of osteoblasts into osteocytes, as indicated by the increased expression of SOST, a marker of osteoblast differentiation (106). *In vitro* studies on rodent (112,113) and human (106) primary osteoblast cultures have shown that SR, similar to calcium, acts as an agonist of the calcium-sensing receptor (CaSR), promotes cell proliferation (106,112) via activation of the CaSR, and increases bone cell differentiation (106,113) and bone cell survival (106).

SR induces osteoblastic differentiation of human MSCs, stimulating the expression of genes of the bone extracellular matrix: COLIA1, BSP, OCN and RUNX-2. These genes are essential for osteoinduction (114). Furthermore, numerous studies using various cellular models have been performed to evaluate the effects of strontium in combination with different biomaterials on osteogenesis (115-119). In particular, it has been demonstrated that strontium released into the culture medium by a previously loaded amidated carboxymethylcellulose (CMCA) hydrogel was able to promote osteoinduction as detected based on the production of ALP and the formation of HA deposits in a clonal cell line derived from human adipose tissue-derived MSCs (120).

Wnt/β-catenin Pathway Antagonists

The Wnt/β-catenin pathway plays an important role in the main processes controlling osteogenesis (121). This pathway regulates the gene transcription of proteins important for osteoblast function (63).

In vitro and *in vivo* experiments have shown that activation of the canonical Wnt/β-catenin pathway induces the cellular replication and differentiation of osteoblasts, reducing adipogenic differentiation in MSCs (122,123).

The Wnt pathway is composed of Wnt proteins, frizzled transmembrane receptors and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). Wnt signaling is activated by the presence of the Wnt ligand, which interacts with its receptor, thereby inhibiting the receptor. This interaction leads to cytoplasmic accumulation of β-catenin, which translocates to the nucleus, activating a fundamental transcription factor, RUNX-2, involved in osteogenic differentiation. However, in the absence of the Wnt ligand, β-catenin is phosphorylated by glycogen synthase kinase 3 beta (GSK3B), leading to its degradation, and gene transcription is halted (124). Various studies have demonstrated that modifications in Wnt signaling contributed to age-related bone loss in mouse models (125). In aged or OVX osteopenic mice, with the use of GSK3B, the Wnt signaling cascade enhanced bone formation and increased trabecular and cortical bone density and bone strength (126,127).

Studies of the Wnt/β-catenin pathway have led to the further discovery of inhibitors of Wnt signaling that are secreted by osteocytes. These inhibitors include SOST and DKK1 protein, which are Wnt antagonists specific to bone. Both block the binding of Wnt to LRP5, thereby inhibiting osteoblast stimulation (64,128). In fact, it has been observed that a loss-of-function mutation of SOST leads to an increase in bone formation and bone mass (129). Many forms of cancer are associated with such mutations within the Wnt signaling pathway (130,131).

Currently, based on promising results in animal models, monoclonal antibodies designed to block the inhibitory action of both SOST and DKK1 have been introduced for use in clinical trials (65,66,132-134).

The development of pharmacological SOST and DKK1 antagonists that increase bone formation and bone mass is a new strategy in the treatment of bone disorders. *In vivo* studies on monkeys and OVX rats have shown that systemic administration of an anti-SOST MAB increased bone formation, bone mass, and strength (135). Furthermore, the anti-SOST antibody was able to enhance bone formation markers in postmenopausal women (136). Finally, an increase in bone formation on trabecular, periosteal, endocortical, and intracortical surfaces, without increased bone resorption and with enhanced trabecular thickness, BMD and bone strength, was shown in preclinical studies with the administration of SOST-neutralizing monoclonal antibodies (137,138).

Today, the prevention and treatment of several bone disorders are possible. This progress is due to the development of a variety of drugs that act to halt excessive bone resorption by inhibiting osteoclasts or by promoting bone formation.

BPs such as alendronate and zoledronic acid have been demonstrated to significantly reduce the risk of vertebral, non-vertebral and femoral fractures by decreasing bone remodeling via the inhibition of osteoclasts with increased bone mass, although their long-term use has been correlated with the occurrence of atypical femoral fractures (53). However, a new approach targeting the inhibition of osteoclast activity inhibits RANKL, which is involved in the survival and differentiation of mature osteoclasts.

Denosumab is among the RANKL inhibitors that have been most studied and used, and numerous studies have demonstrated that denosumab exerts an inhibitory action on osteoclastogenesis (61-65).

In animal models, it has been demonstrated that teriparatide accelerates bone fracture healing, thereby enhancing bone remodeling (139). In studies of bone histomorphometry, PTH_{1-34} was able to increase the trabecular bone mass in postmenopausal women (140). Raloxifene also modulates the homeostasis of bone cells *in vitro* by inhibiting osteoclastogenesis and bone resorption, with a reduction in the numbers of preosteoclasts and mature osteoclasts in OVX rats (77). Additionally, it has been shown that raloxifene suppressed osteoblast apoptosis in MC3T3-E1 cells (78) and increased osteoblast proliferation and differentiation in murine cell cultures (79,80). Finally, *in vitro* studies have demonstrated that SR promotes the survival, proliferation and differentiation of osteoblasts and inhibits osteoclastic activity and clinical studies have shown that SR improves bone strength, increasing BMD. Furthermore, no change in the porosity of bone was evident in patients treated with SR (141).

In conclusion, an understanding of the molecular and cellular mechanisms of bone fragility is essential for the development of successful cell therapies that support new pharmacological approaches.

AUTHOR CONTRIBUTIONS

Nardone V conceived and designed the study and was responsible for manuscript writing. D'Asta F was responsible for bibliographic control. Brandi ML approved the final version of the manuscript.

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