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Review

Targeting bone metastatic cancer: Role of the mTOR pathway

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ABSTRACT

One of the great challenges of cancer medicine is to develop effective treatments for bone metastatic cancer. Most patients with advanced solid tumors will develop bone metastasis and will suffer from skeletal related events associated with this disease. Although some therapies are available to manage symptoms derived from bone metastases, an effective treatment has not been developed yet.
The mammalian target of rapamycin (mTOR) pathway regulates cell growth and survival. Alterations in mTOR signaling have been associated with pathological malignancies, including bone metastatic cancer. Inhibition of mTOR signaling might therefore be a promising alternative for bone metastatic cancer management. This review summarizes the current knowledge on mTOR pathway signaling in bone tissue and provides an overview on the known effects of mTOR inhibition in bone cancer, both in in vitro and in vivo models.

Contents

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1. Introduction

Bone is the preferred site of metastasis: it is estimated that up to 70% of advanced breast and prostate tumors will form bone metastases and that 15–30% of lung, colon, stomach, bladder, uterus, rectum, thyroid or kidney tumors will also spread to the bone [1]. In recent years, cancer research has allowed the development of more efficient anti-cancer therapies that has resulted in a higher patient survival. However, if these patients suffer from disease recurrence and bone metastasis formation, no specific therapies are available to treat them.

Bone metastatic cancer is associated with skeletal-related events (SREs), such as pathological fractures, spinal cord compression, palliative radiotherapy for bone pain, and orthopedic surgery, that negatively influence the quality of life of cancer patients and represent an economic burden for healthcare services worldwide [2]. Although some therapies are available to ameliorate and alleviate symptoms associated with bone metastases and SREs, clinicians do not have the necessary tools to cure these patients. New molecular targets are thus required to fight bone metastases and associated SREs.

mTOR inhibition has already been shown to be a suitable strategy for the control of tumor growth in different types of cancer [3,4]. Emerging evidence suggests that the mTOR pathway is also involved in bone turnover and may play a role in the development and progression of bone cancer. Of note, mTOR inhibitors have shown clinical activity in trials involving primary bone sarcoma patients [5–8].

Our goal in this review will be to provide an overview of the relevant literature concerning mTOR signaling in the bone. We will describe preclinical data from in vitro and in vivo studies on the effects of mTOR inhibition in osteoclasts and osteoblasts from normal and metastatic bone, in different solid tumors.

2. Emerging molecular targets in bone metastases treatment

Maintenance of bone homeostasis is a tightly controlled process involving not only key cells like osteoblasts – responsible for bone formation – and osteoclasts – involved in bone resorption – but also many factors that regulate the differentiation and activation of these cells [9]. Tumor cells that arrive and adhere to the bone matrix break this equilibrium and initiate a vicious cycle that feeds tumor development and accelerates bone loss [2]. In the bone, cancer cells secrete several cytokines, including parathyroid hormone-related peptide (PTHrP), that induce changes in the bone microenvironment. For example, PTHrP can induce the expression of Receptor Activator of Nuclear Factor-kB Ligand (RANKL) on marrow stromal cells and osteoblasts. RANKL binds its receptor RANK in osteoclast precursors and activates the NF-kB and JNK signaling cascades that induce differentiation and activation of osteoclasts leading to increased bone resorption [10,11]. In response, matrix and bone cells release growth factors, such as TGF-β, that stimulate tumor cells to produce PTHrP and inhibit bone formation by osteoblasts, thereby closing a cycle that accentuates the unbalance between bone formation and resorption [12,13].

The complex sequence of events that lead to the onset of bone metastases not only involve processes common to any other metastasis (establishing of a pre-metastatic niche in the host tissue, chemotaxis of tumor cells into the host tissue or extravasation of tumor cells from blood vessels) but also processes that are more specific to the bone tissue (tumor cell invasion in the bone environment, implantation of tumor cells in bone marrow, osteomimicry, deregulation of osteoblast/osteoclast activity) [14].

One way to break this cycle is to target molecular players involved in the unbalancing of bone destruction/production that characterizes bone metastases. In recent years, several inhibitors have been developed that can act on molecular pathways involved in bone metastases establishment and progression, including denosumab that blocks the RANKL pathway [15], abiraterone for targeting the androgenic pathway in prostate [16]; dasatinib and saracatinib able to inhibit src [17], and anti-dkk1 antibodies targeting wnt/dkk pathway [18]. None of these treatments is totally efficient in stopping the natural progression of bone metastases thus, effective therapies against bone secondary tumors are a medical unmet need in the management of bone malignancies.

3. Role of mTOR in osteoclast and osteoblast function and differentiation

The mTOR pathway is an important regulator of cell signaling with known roles in physiological processes, including cell growth, survival and autophagy [19]. It is also recognized that abnormal mTOR signaling has profound effects in cell homeostasis and can lead to the development of pathological states, such as cancer [20]. Over the past decade, some studies have provided data that argues for a role of mTOR also in bone diseases. Indeed, several groups have shown that mTOR is implicated in osteoclastogenesis, the process of differentiation and activation of osteoclasts. Glantschnig and colleagues demonstrated for the first time in their 2003 study that M-CSF, RANKL and TNF-α – factors known to be necessary for osteoclast survival – exert their anti-apoptotic effects on osteoclasts through the mTOR/S6K pathway, summarized in Fig. 1 [21]. The authors treated osteoclasts with rapacymin, an inhibitor of mTOR, and observed a decrease in survival of osteoclasts in vitro. Later, these results were confirmed by a study showing that macrophage colony-stimulating factor (M-CSF)-dependent mTOR activity is required for the survival of osteoclast precursors [22]. Downregulation of mTOR, through a siRNA approach, was able to induce apoptosis in osteoclast precursors, a result that is consistent with a previous result showing that rapamycin inhibited osteoclastogenesis [23]. More recently, in a study aimed at understanding the metabolic regulation of osteoclast activation, the authors used genetic and pharmacological inhibitors to further confirm that mTOR activity is required for osteoclast formation [24]. In particular, the constrasting effects of anabolic mTOR and catabolic AMPK on differentiation and activation of osteoclast suggest that a balanced equilibrium between these two molecular pathways plays a central role in bone turnover [24].

Hyperactivation of mTOR has also been shown to be responsible for abnormal osteoclastogenesis using primary osteoclast-like cells (OCLs) derived from a neurofibromatosis Type 1 mouse model (Nf1 heterozygous; Nf1 +/− mice) [25]. NF1 disease is characterized by bone deficiencies associated with enhanced osteoclastogenesis. The authors observed that mTOR is hyperactive in NF1 +/− OCLs and that this enhanced activation induces osteoclast hyperproliferation. Nf1 +/− animals have more and bigger OCLs but treatment with rapacymin in vitro was able to decrease cell size and number to levels observed in control OCLs, showing that inhibition of mTOR is able to block abnormal osteoclast formation. Evidence for a role of mTOR in bone disease came also from a study using animal models of experimental arthritis [26]. In the presence of mTOR inhibitors, reduced osteoclast formation in vivo and osteoclast survival in vitro was observed. In addition, mTOR inhibition reduced bone erosion and structural damage of the joints of arthritic animals, which confirms a pathological role for mTOR in bone disease.

mTOR is able to regulate osteoclastogenesis through the mTOR-c/EBPbeta-MaFB axis [27]. In osteoclasts, two isoforms of c/EBPbeta protein can be produced: one short, named LIP and one long, known as LAP. Smink and colleagues showed that overactivation of mTOR induces the production of short c/EBPbeta isoforms which leads to the repression of MafB. MafB is a known inhibitor of osteoclast differentiation since it can impair RANK-induced osteoclast differentiation by acting on transcription factors necessary for osteoclast maturation, such as c-Fos, NFATc1 and Mitf [28]. Thus, repression of MafB via mTOR enhances osteoclast differentiation.

On the other hand, mTOR may also be involved in osteoblast differentiation. Osteoblasts produce osteoprotegerin (OPG), which by inhibiting RANKL binding to RANK – located on the surface of

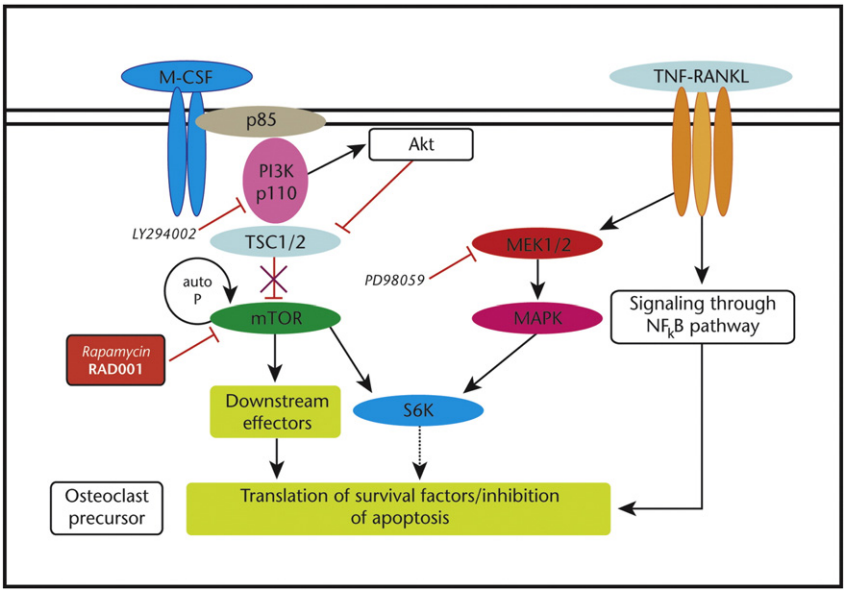

Fig. 1. The potential role of mTOR signaling in osteoclast survival: M-CSF, TNF-alpha and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. Abbreviations: MAPK, MAP kinase; mTOR, mammalian target of rapamycin; NFkB, nuclear factor kappa-B; PI3K, phosphoinositol 3 kinase; S6K, ribosomal S6 kinase; TNF/RANKL, tumor necrosis factor/receptor activator of nuclear factor kappa-B ligand.
Adapted from Glantschnig H et al. [21].

osteoclasts – impairs osteoclastogenesis [1]. OPG can also inhibit the differentiation of osteoclasts by interfering with the signaling interaction between stromal ST2 cells and osteoclast progenitors [29]. The relationship between mTOR pathway and OPG levels has been investigated in stromal mouse bone marrow-derived stromal ST2 cells [30]. Rapamycin treatment induced an increase in OPG levels in these cells; however, in the presence of a rapamycin concentration sufficient to induce OPG production, no effects on cell cycle and proliferation were observed.

To test the effects of mTOR on osteoblast differentiation, Yeh and colleagues [31], treated fetal rat calvarial cells with rapamycin and observed the inhibition of basal osteogenic and lipogenic marker expression and bone nodule mineralization. A previous study had already shown that prolonged rapamycin treatment induces the expression of osteoblast markers osterix, osteocalcin, osteoprotegerin, osteonectin, and bone sialoprotein in human embryonic cells while downregulating stem cell markers [32].

While mTOR signaling may function to affect osteoblast proliferation and differentiation, conflicting results have been reported about whether inhibition of mTOR signaling by rapamycin decreases or increases bone formation.

Together these data demonstrate the involvement of mTOR in the differentiation and activation of both osteoclasts and osteoblasts and suggest that inhibition of its downstream effects might alter bone turnover.

4. Inhibition of the mTOR pathway and the effects on bone turnover

As previously discussed, inhibition of mTOR due to rapamycin treatment can induce osteoclast apoptosis [21]; another consequence of inhibiting the mTOR signaling cascade has also been shown to be a decrease in bone resorption as assessed by the release of collagen-I degradation product in vitro [21].

In a subsequent study by Kneissel and colleagues [33], everolimus (RAD-001), an analog of rapamycin, was shown not only to inhibit osteoclastogenesis, but also to reduce the expression of cathepsin K. The protease cathepsin K is the main collagen-degrading enzyme in osteoclasts. A reduction of this protease in osteoclasts after everolimus treatment suggests that mTOR inhibition can directly decrease bone resorption both in healthy bone and in bone metastases.

The results obtained in these studies therefore argue for the possibility to block osteolysis (bone degradation) through the downregulation of mTOR signaling. The mechanisms underlying the observed effects of mTOR inhibition still remain unknown. We envisage three possible scenarios: i) mTOR inhibition might occur directly in bone microenvironment cells and in osteoclasts; ii) mTOR inhibition occurs in tumor cells of bone metastases or iii) mTOR inhibition occurs in primary tumor cells (Fig. 2) [34].

5. Effects of mTOR inhibition on the vicious cycle

Several studies have addressed the effects of mTOR inhibition in bone tumors (see Table 1 for a summary of in vivo models). Hussein and colleagues [35] have shown that rapamycin is able to decrease bone degradation (osteolysis) associated with experimental bone metastasis. The mouse mammary carcinoma model was developed by injecting breast cancer cells 4T1 into the mice tibia. Rapamycin treated mice show less lytic lesions and survived longer than non treated mice

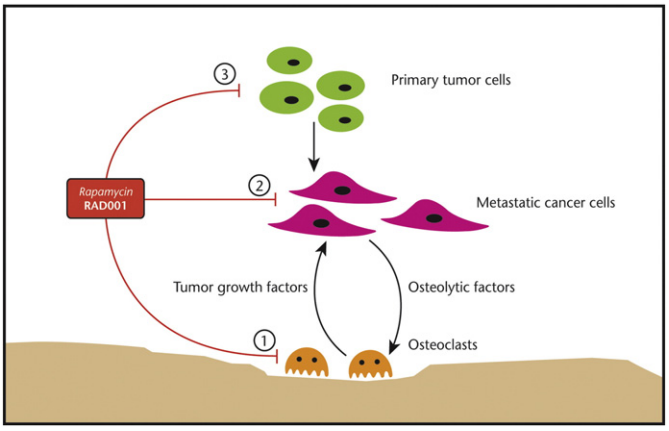

Fig. 2. Potential targets for mTOR inhibition within the vicious circle scheme.
Adapted from Smink et al. [34].

Table 1
In vivo models where the inhibition of mTOR pathway has demonstrated some effects on bone metastases.

Model	Species (strain)	Inoculated cells	Treatment	Outcome	Reference
Mammary carcinoma	Mouse (female BALB/c)	4T1 cells	Rapamycin	– Reduced number of lytic lesions – Increased survival (21 versus 16 days)	Hussein et al. [35]
Neuroblastoma	Mouse (male CB-17 SCID)	CHLA-20 and NB1691 cells	Rapamycin	– Higher OPG serum levels – Improved bone thickness	Hartwich et al. [37]
Oral squamous cell carcinoma	Mouse (female BALB/c)	HSC-2 cells	Temsirolimus	– Impaired tumor formation – Inhibition of osteolytic lesions	Okui et al. [38]
Osteoblastic osteosarcoma	Mouse (male C57BL/6J)	MOS-J cells	Everolimus + ZOL	– Slower tumor progression – Enhanced bone mass	Moriceau et al. [44]
Osteolytic osteosarcoma	Mouse (male C3H/He)	POS-1 cells	Everolimus + ZOL	– Slower tumor progression – Enhanced bone mass	Moriceau et al. [44]
Prostate cancer	Mouse (male SCID)	C4-2 cells	Everolimus/docetaxel/ZOL	– Decreased tumor volume	Morgan et al. [45]

Abbreviations: ZOL — Zoledronic acid, OPG — Osteoprotegerin.

(21 versus 16 days). Even if rapamycin did not reduce the size of metastatic lesions nor did it inhibit cell proliferation, treatment with this inhibitor was able to block abnormal production of osteoclasts by late osteoclast precursors stimulated with 4T1 conditioned medium. These results suggest the potential role of breast cancer derived factors in the mTOR pathway of osteoclast precursors.

Smink and colleagues [36] showed that rapamycin inhibits osteoclast formation and resorption in giant cell tumors in vitro. The giant cell tumor disease is characterized by the development of osteolytic lesions due to the high activity of giant, multinucleated osteoclasts. Due to the role of mTOR in osteoclast activity, the authors hypothesized that giant cell enhanced activity could be blocked by rapamycin. The authors showed that multinucleated osteoclasts derived from patients with giant cell tumors expressed the short c/EBPbeta isoform. Treatment with rapacymin was able to induce the expression of the long isoform LAP in giant cells which induced the upregulation of MafB. Expression of the MafB inhibitor reduced osteoclast formation and impaired bone resorption in giant cell tumors.

Following previous studies by Mogi et al. [30], demonstrating that mTOR inhibition by rapamycin enhances OPG production, Hartwich and colleagues [37] studied the effect of rapamycin treatment in neuroblastoma bone metastases. The authors showed that rapamycin treatment induces the production of OPG and inhibits osteoclast formation in vitro. In vivo, they observed higher OPG serum levels and improved bone thickness in the neuroblastoma xenograft model after inhibiting mTOR with rapamycin. Of note, the time necessary to develop pathologic fractures was delayed in xenografted treated animals in comparison with control animals. These results demonstrate that rapamycin can inhibit osteolysis in neuroblastoma bone metastases.

Finally, Okui et al. [38] tested mTOR inhibition in a murine model of bone metastases from oral squamous cancer. They treated HSC-2 oral squamous cell carcinoma cells with the inhibitor temsirolimus and observed a reduction in cellular proliferation and migration in comparison with control cells. In addition, temsirolimus was able to inhibit osteoclast formation and activity. In vivo, temsirolimus impaired tumor formation in animals injected with HSC-2 cells and inhibited osteolytic lesions.

Taken together, these data clearly indicate the potential benefit of mTOR inhibition in treating metastases-associated osteolytic disease and give a mechanistic basis for trials involving the drug or its analogs in a clinical setting.

6. mTOR inhibition synergizes with other molecular pathways

Inhibition of the mevalonate pathway through the use of nitrogen-containing bisphosphonates (N-BP), namely zoledronate (ZOL), is currently a standard therapy used to target osteoclasts in bone diseases [39–41]. These compounds are able to inhibit enzymes of the mevalonate pathway and induce osteoclast apoptosis. Interestingly, mevalonate and mTOR pathways are related through upstream/downstream targets.

After being synthesized in the cells from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), mevalonate can be metabolized into numerous end products. One of those, farnesyl pyrophosphate (FPP) is made up of a farnesyl moiety necessary for the post-translational modification of Ras- and Ras-related proteins [42]. Ras proteins can induce malignancy in normal cells but can also stimulate the mTOR pathway [43].

Moriceau and colleagues [44] tested whether combination therapy with ZOL and everolimus could impair the growth of human and mouse osteosarcoma cells.

Everolimus treatment reduced the number of osteosarcoma cells in vitro, as did ZOL; however, when used together these compounds showed an additive inhibitory effect. Importantly, ZOL and everolimus combined treatment exerted an antiproliferative effect on an osteosarcoma cell line resistant to everolimus. To test these effects in vivo, two osteosarcoma animal models were used: one osteoblastic, induced by injection of MOS-J cells that in vitro are resistant to ZOL and everolimus used alone, and one osteolytic, where tumors with osteolytic lesions develop from POS-1 cells. In this latter model, treatment with everolimus did not have an effect on the growth of POS-1 tumors while ZOL induced a small, although non-significant, reduction in tumor volume. In both models, combination treatment with ZOL/everolimus slowed down tumor progression and enhanced bone mass. However, no effect was seen on bone resorption. The mechanism of action of ZOL and everolimus on osteosarcoma cells and the crosstalk between mTOR and mevalonate pathways is summarized in Fig. 3.

Another study provided evidence for the effectiveness of targeting both mevalonate and mTOR pathways as an anti-bone metastasis therapeutic approach [45]. The authors showed that the inhibitory effect of everolimus in bone tumors derived from prostate cancer is enhanced by combination treatment with docetaxel and zoledronic acid. Indeed, in vivo, the highest reduction in tumor volume was observed in response to treatment with all three drugs (everolimus/docetaxel/zoledronic acid).

Other compounds are thought to be able to interact with the mTOR pathway and to enhance its antiproliferative effect in bone tumors. The biologically active form of vitamin D, 1,25-dihydroxyvitamin D (1,25OH2D) is involved in a wide range of physiological processes: besides being critical for calcium absorption and bone mineralization, the active form of vitamin D is also known to have antiproliferative and differentiative properties [46]. Of note, 1,25OH2D also exerts a regulatory effect on the mTOR pathway. Vitamin D targets several cellular molecules in cancer, including EGFR and downstream members of its intracelular signaling pathways that lead to tumor growth and metastasis in colon and breast cancer and MAPK signaling in breast and blood cancer [47]. Lisse and colleagues have shown that in cancer cells vitamin D can induce the expression of DNA damage-inducible transcript 4 (DDIT4), DDIT4 (DNa-damage-inducible transcript 4)

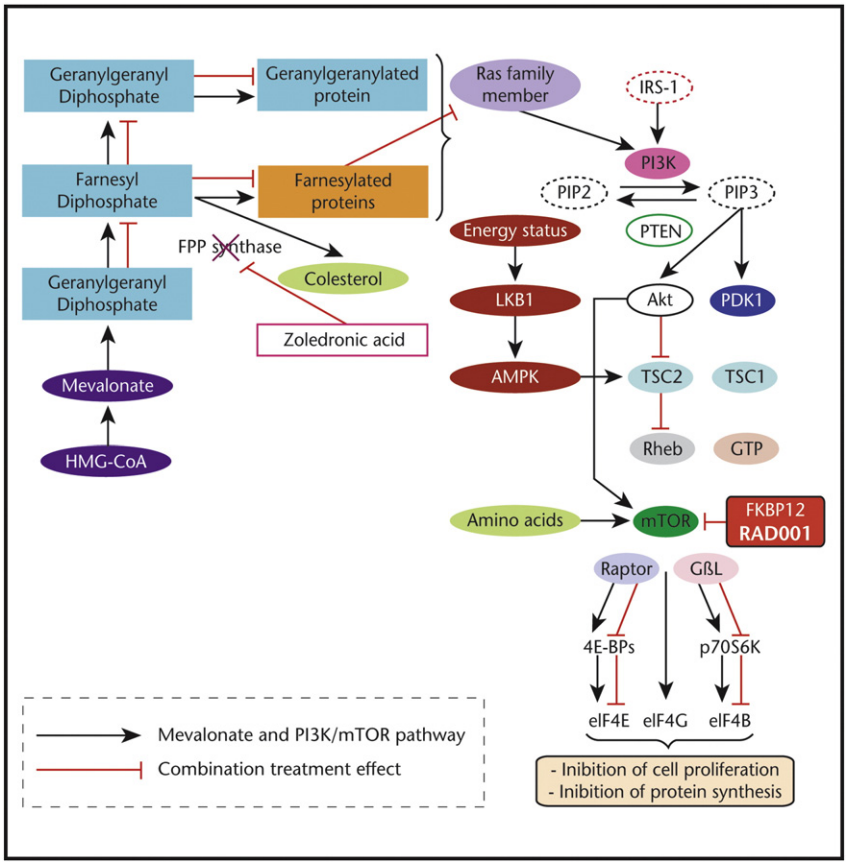

Fig. 3. Summary of the mechanism of action of Zoledronic acid and RAD01 (Everolimus) on osteosarcoma cells and the crosstalk between mTOR and mevalonate pathways. Adapted from Moriceau G et al. [44].

facilitates the assembly and activation of the tuberous sclerosis complex (tSC)1/2 complex for eventual suppression of downstream mTOR activity through actions on ras homolog enriched in the brain (rheb) [48,49]. This result supports the idea of a synergetic interaction between mTOR inhibitors and vitamin D [50]. Indeed, the finding that everolimus can enhance the antiproliferative action of 1,25OH2D in acute myelogenous leukemia cells argues for their use as combination therapy in cancer [51].

Controlling autophagy may also be considered as an alternative strategy to enhancing the anti-tumorigenic effects of mTOR inhibition. Indeed, a recent study has shown that the combination of everolimus and cloroquine (an inhibitor of autophagy) can potentiate the antiproliferative action of everolimus on endothelial progenitor cells [52]. Everolimus and cloroquine in combination were found to have a synergistic effect and reduced the number of endothelial progenitor cells. This anti-proliferative effect was shown to be mediated by increased apoptosis and reduced everolimus-induced autophagy. These findings demonstrate that the combined inhibition of both mTOR and autophagy – the latter occurring in response to mTOR inhibition – may represent an effective strategy to block angiogenesis and tumor progression also at bone level.

7. The effects of everolimus on bone metastases: clinical evidence in solid tumors

It is recognized that a high percentage of patients with advanced breast cancer will go on to develop bone metastases [1]. Adding to this, endocrine therapies (such as aromatase inhibitors) used in breast cancer treatment can produce negative effects on bone health by enhancing bone turnover [53]. The maintenance of bone health in advanced breast cancer patients is, thus, an unmet clinical need. In the clinical trial BOLERO-2 involving 724 advanced breast cancer patients, the combined effect of everolimus and of the aromatase inhibitor exemestane was assessed on progression-free survival (PFS) and bone turnover marker levels [54,55]. Patients treated with exemestane were randomized to everolimus (combination treatment) or placebo (exemestane only) and the results obtained were promising. Indeed, after 18 months, the addition of everolimus to exemestane therapy reduced the incidence of bone progression (i.e. appearance of new bone metastases or progression of pre-existing bone metastases) in the overall population. The PFS in bone also was significantly higher in the subset of patients with bone metastases at baseline treated with the combination therapy compared to controls. Interestingly, bone turnover (expressed as bone-specific alkaline phosphatase, amino-terminal propeptide of type 1 collagen and C-terminal cross-linking telopeptide of type 1 collagen) was inhibited at 6 and 12 weeks in patients treated with everolimus and exemestane to a significantly greater extent than in the control group. This suppression in the combination arm was irrespective of the presence of bone metastases and bishosphonate use at baseline. High bone turnover markers reported in placebo plus exemestane group express not only osteoclast and osteoblast increased activity at bone metastasis level but also at health bone level, a well known effect of aromatase inhibitors on bone metabolism. Furthermore, high bone turnover in prostate, lung and breast cancer patients with bone metastases, has been shown to be related to a high risk of developing skeletal related events, bone progression and death [56].

Since it was shown from this study that everolimus inhibits bone turnover in addition to anticancer activity, we can conclude that mTOR inhibition could play an independent effect on bone metastasis and on bone mass/quality that both in turn contribute to the inhibition of bone progression and preservation of bone health, therefore improving survival in breast cancer patients treated with aromatase inhibitors.

It is worth noting that other studies have also examined the efficacy of mTOR inhibition in treating metastatic renal cell carcinoma. The clinical management of this disease is complicated and currently relies on targeting multiple pathways, including mTOR [57]. Indeed, everolimus has been shown to provide significant survival benefit, compared to sorafenib, as 3rd line therapy in patients with bone metastases from renal carcinoma (authors unpublished data).

8. Future perspectives

Collectively, data available to date on mTOR inhibition in bone metastases indicates that this pathway might represent a novel target for single or combination therapies aimed at improving bone health in patients with metastatic tumors. However, additional evidence regarding the action of mTOR inhibitors in bone tumor environment is still needed to define the best therapeutic option to develop. Further studies assessing: i) the effect of everolimus/bisphosphonate combination and everolimus in combination with other molecules either on osteoclasts, on tumor cell lines or on osteoclasts stimulated with tumor cells conditioned medium; ii) bone turnover in patients treated with everolimus, other than in breast cancer; iii) incidence and delay of SRE in bone metastatic patients treated with everolimus; and iv) the effect of new generation compounds able to inhibit mTOR signaling on differentiation and activation of osteoblasts/osteoclasts, will be necessary to gain a better understanding of how to impair bone tumor progression through the inhibition of mTOR.

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