Direct Intra-tumour Delivery of Zoledronate is Superior to Systemic Administration for Mitigating Metastasis-induced Bone Destruction

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ABSTRACT

Background: Bone metastases are the most common cause of cancer-related pain and often lead to other complications. Bisphosphonates (BPs) are a class of potent anti-resorptive agents have been found recently to have direct anti-tumour properties. Currently, the use of BPs for cancer therapy is generally restricted to high dose systemic delivery.

Questions/ purpose: (1) whether local delivery of BPs can inhibit tumour-induced osteolysis and tumour growth in an animal model of bone metastases; and (2) whether the efficacy in impairing cancer-induced osteolysis that is achieved by local delivery of BPs is comparable to that of systemic BPs treatment.

Methods: Seven days following intra-tibial inoculation of MDA-MB-231 human breast cancer cells ($N = 10^5$) in athymic nude mice, the experimental group (N = 11) was treated by local administration of 2μg of zoledronate into the tibial lesion (three times/week for three weeks) and compared to vehicle-treated mice (N = 5). The formation of bone metastases and growth of the lesions were followed up by weekly bioluminescence imaging. In a subsequent experiment, a comparison of the effects of local versus systemic delivery of zoledronate on the formation of osteolytic bone metastases was carried in athymic mice (N = 19). The systemic group (N = 5) was treated with zoledronate (0.025mg/kg) once per week for 4 weeks and compared to systemic delivery of vehicle

(N = 4). The local group (N=6) was treated by direct infusion of zoledronate (0.025mg/kg) once per week for 4 weeks and compared to local delivery of vehicle (N = 4). The effect of treatment on bone was determined using microcomputed tomography (μ-CT), and on tumour cells proliferation and apoptosis by immunohistochemistry using antibodies for Ki-67 and caspase-3.

Results: The first experiment showed a statistically significant increase in mean bone volume/tissue volume % (BV/TV) in the treated group (12.30±2.80%) as compared to the control group (7.13±1.22%) ($P<0.001$, 95%CI=3.10-7.30). This corresponded to a net increase of 72.51% in response to zoledronate treatment. Bioluminescence imaging showed insignificant inhibition of the tumour growth in the treated group. Additionally, the treated group had an insignificant decrease in Ki-67-positive cells and this was mirrored with an insignificant increase in the number of caspase-3-positive cells. Comparison between the local and systemic effects of zoledronate also revealed a significant increase in the BV/TV in the locally treated group (10.90±1.25%) when compared to the cohort administered systemic bisphosphonate treatment (7.53±0.75%) ($P<0.001$, 95%CI=2.10-4.81), corresponding to a net increase of 44.8%.

Conclusion: These preliminary results suggest that high dose sustained release of BPs can lead to a significant inhibition of tumour-induced osteolysis. Moreover, comparison between local and systemic delivery revealed that the effect of local BPs administration exceeds the benefits of systemic delivery in terms of osteolysis inhibition. Lastly, the noted effect of BPs local delivery triggers the need for further assessment of its anti-tumour activity.

Clinical relevance: Given the anti-osteolysis effect of BPs, it can be used for palliation and maintaining stability in cases where a complete resection of bone tumour is not possible due to vital structures invasion. Furthermore, Local delivery can provide direct delivery of BPs to the site of bone tumour without passing the systemic circulation, thus, avoiding undesirable side effects and ensuring better bioavailability.

Résumé

Introduction: Les métastases osseuses sont la cause la plus fréquente de douleur causé par le cancer et souvent provoquent d'autres complications comme les fractures pathologiques et la compression de la moelle épinière. Les bisphosphonates (BPs) sont une classe d'agents anti-résorbable puissants qui sont habituellement prescrit pour retarder la progression de l'ostéoporose. Fait intéressant, les BPs pourraient avoir indirectement des propriétés anti-tumorales par leurs effets néfastes sur les macrophages, les ostéoclastes, les cellules endothéliales et par leur habilité à supprimer la route de mevalonate. Présentement, l'utilisation thérapeutique des BPs pour le cancer est généralement restreinte à de doses fortes administrées au niveau systémique. Le but de cette étude était d'investiguer les effets, chez la souris, de l'administration locale et directe de zoledronate au site métastatique de l'os dans un modèle utilisant le cancer métastatique du sein.

Méthodes: Sept jours après l'inoculation intra-tibial des cellules cancéreuses du sein MDA-MB-231 ($N=10^5$) dans les souris athymiques, le groupe expérimental (N=11) a été traité par perfusion direct de 2µg de zoledronate dans la lésion tibiale (trois fois/semaine pendant trois semaines) et a été comparé aux souris contrôles (N=5). À chaque semaine, la formation de métastases osseuses et la croissance de lésions ont été suivies par imagerie bioluminescence. Dans une autre expérience, une comparaison des effets de l'injection local ou systémique de zoledronate sur la formation de métastases osseuses osteolytique a été conduit chez les souris athymiques (N=19). Sept jours suivants l'inoculation de cellules de cancer du sein MDA-MB-231, le groupe avec l'injection

systémique (N=5) a été traité avec zoledronate (0.025mg/kg) une fois par semaine pendant 4 semaines et a été comparé à l'injection systémique reçue par le groupe control (N=4). Le groupe qui a reçu l'injection locale (N=6) a été traité par perfusion directe de zoledronate (0.025mg/kg) une fois par semaine pendant 4 semaines et a été comparé au groupe à injection locale du groupe control (N=4). L'effet du traitement sur l'os a été déterminé par la tomographie aux rayons X assistée par ordinateur (μ-CT), et l'immunohistochimie a été utilisée pour évaluer la prolifération et l'apoptose des cellules cancéreuses à l'aide des anticorps pour Ki-67 et caspase-3.

Résultats: La première expérience a démontré une augmentation statistiquement significative pour la moyenne du pourcentage rationnelle volume/tissue-volume % (BV/TV) pour le groupe qui a reçu le traitement (12.30±2.80%) lorsque comparé au groupe de contrôle (7.13±1.22%) ($P<0.001$). Ceci correspond a une augmentation nette de 72.51% en réponse au traitement ave zoledronate. En plus, le groupe traité a démontré une diminution des cellules Ki-67-positive, ce qui a entrainé la constatation d'une augmentation significative du nombre de cellules caspase-3-positive. En comparant les effets de zoledronate injecté soit localement ou systémiquement, la comparaison révèle une augmentation significative du ratio BV/TV dans le group traité localement (10.90±1.25%) lorsque comparé au groupe qui a reçu le traitement systématique au BP (7.53±0.75%) ($P<0.001$), ce qui correspond à une augmentation nette de 44.8%.

Conclusion: Ces résultats préliminaires suggèrent qu'une libération soutenue de dose forte de zoledronate peut entraîner une inhibition significative de l'ostéolyse d'origine tumorale. De plus, la comparaison entre l'administration locale et systémique révèle que

les effets de l'administration locale de BP excèdent les bénéfices de l'administration systémique en terme de l'inhibition de l'ostéolyse. Finalement, l'effet observé de l'administration locale de zoledronate soulève le besoin de continuer l'évaluation de ses pouvoirs anti-tumoraux.

Relevance clinique: Étant donné les effets anti-ostéolyse du zoledronate, celui-ci pourrait être utilisé à des fins palliatifs et pour la maintenance de la stabilité dans les cas ou la résection complète de tumeur osseuse n'est pas possible dû à l'invasion des structures vitales. De plus, l'injection locale pourrait promouvoir l'administration directe de zoledronate au site de la tumeur osseuse sans passer par la circulation systémique donc éviter ainsi des effets secondaires indésirables et assurer une meilleure biodisponibilité.

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Section 1: Introduction

A) Rational and Objective:

Bone metastases are the most common cause of cancer-related pain and often lead to other complications such as bone fracture and spinal cord compression.[1] All of which can severely erode patient's quality of life. The management of bone metastases consists of a combination of bisphosphonates (BPs), radiotherapy and surgical treatment utilizing a multidisciplinary approach.[2] BPs are potent antiresorptive agent that have been proven to reduce bone metastases pain and the occurrence of SREs.[3,4]. Recently, it has been suggested that high dose of BPs have a direct antitumoural activity.[5] However, the use BPs for cancer therapies is currently limited to intravenous infusion to achieve the doses required for clinical efficacy. High BPs doses can cause severe renal toxicity unless infused slowly while the oral administration has poor bioavailability and poor gastrointestinal tolerability. Side effects of systemic BP treatment can include joint pain, osteonecrosis of the jaw [6] ocular inflammation [7] and compromised bone growth in children.[8] Local delivery of BPs is desirable as it could enhance their bioavailability and possibly efficacy, also reducing necessary doses to achieve anti-tumour effects and reducing costs and systemic side effects. In the present study, our first aim was to examine the effect of localized delivery of BPs in an animal model of bone metastasis. The second aim was to compare the effect of localized delivery of BPs to that of the systemic therapy.

B) *Review of the literature:*

1. Bone Biology

1.1 Bone Structure:

Bone is a living, complex, constantly active tissue composed of highly organized patterns of organic components and minerals including hydroxyapatite and calcium phosphate particles.[9] This organ must be light yet rigid, have a high tensile strength, and not be fragile. To meet these varied criteria, bone is composed of a mixture of two types of bone: cortical (compact) and cancellous (trabecular) bones.[10] The cortical bone represents around 80% of the mature skeletal bone. Cortical bone presents mainly in long bones and is found mainly in the diaphyseal area. In contrast, cancellous bone is primarily located in the metaphyseal-epiphyseal area.[11]

1.2 Bone Cells:

Bone cells adopt particular forms to meet the diverse requirements of bone formation, bone resorption, bone remodeling, and mineral homeostasis. The two primary precursors of bone cells are the mesenchymal stem cell line and the hematopoietic stem cell line. Preosteoblasts, osteoblasts, and osteocytes originate from the mesenchymal stem cell line, whereas monocytes, preosteoclasts, and osteoclasts arise mainly from the hematopoietic stem cell line.

Preosteoblasts:

Preosteoblasts are undifferentiated mesenchymal stem cells that have the potential to proliferate into osteoblasts.[12] Preosteoblasts are present in the bone canals, periosteum, endosteum, marrow cavity, and surrounding tissue.[12] These cells are mononuclear, have minimal cytoplasm, and are irregular in shape. They remain inactive until stimulated by growth factors to proliferate into osteoblasts under certain conditions such as fractures.[13]

Osteoblasts:

Osteoblasts are the bone-forming cells. They line the surfaces of bone and pack tightly against each other.[14] The shape of these cells varies depending on the cells' state of activity. Inactive cells look cuboidal in shape. When these cells become active, however, their shape transforms into a rounded, oval, or polyhedral shape.[15] The primary function of osteoblasts is to generate and secrete the organic matrix of bone, which consists of type I collagen and non-collagenous proteins.[16] Additionally, osteoblasts are rich in the alkaline phosphatase that contributes to the mineralization process.[16] Nevertheless, these are not the only functions of these cells; osteoblasts play several other roles such as maintaining electrolyte balance between the osseous fluid and the extracellular fluid.[16] The life-span of an osteoblast in humans is up to 8 weeks, during which time it builds up around 0.5-1.5 um of osteoid, the unmineralized organic portion of the bone matrix, per day.[17] Furthermore, osteoblast-like cells (bone-lining cells) stimulate osteoclasts by releasing mediators in response to systemic hormones, such as parathyroid hormone, 1,25-dihydroxyvitamin D_3, cytokines, and growth factors.[11] Lastly, some osteoblasts may become confined in their own matrix and change their phenotype and transform into osteocytes.

Osteocytes:

Osteocytes represent more than 90% of bone cells in skeletally mature bone.[18] Osteocytes are derived primarily from osteoblasts. They are smaller than osteoblasts and contain less cytoplasm as well as fewer cell organelles such as ribosomes.[14] Osteocytes have distinctive long dendritic processes or cytoplasmic extensions that interconnect the osteocytes with bone-lining cells through small tunnels in the bone matrix called canaliculi.[19] This complex network of cells is extremely sensitive to stressors.[19] It also helps to control the influx and reflux of ions in and out of the mineralized bone matrix and to organize the ionic change between the extracellular fluid and the blood.

Osteoclasts:

Osteoclasts arise from the hematopoietic stem cell line along with the cells of the monocyte family.[20] The interaction between osteoprotegerin ligand (a transmembranous receptor expressed on osteoblasts) and RANK (a transmembranous receptor expressed on osteoclast precursor cells) initiates a signalling and gene expression cascade resulting in the promotion of osteoclast formation from the hematopoietic stem cell line.[14] When mononuclear osteoclast precursors are stimulated, they proliferate and then merge to form large, multinucleated osteoclasts.[18] These osteoclasts have 3 to 20 nuclei and scores of lysosomes and mitochondria. Typically, these cells are found on the surface of endosteal, haversian, and periosteal bone.[18] They create multiple depressions on cancellous bone or periosteal surfaces known as Howship lacuna, whereas in cortical bone they create resorption cavities.[21] The most distinctive feature of these cells is the brush or ruffled border that forms by a complex folding of the cytoplasmic membrane.[22] These brush

borders play a critical role in the resorption and degradation process of the matrix. Furthermore, when osteoclasts are activated, they attach themselves to the bone surface and release endosomes that enter the cell membrane of bone cells and solubilize the minerals inside by lowering the pH inside the cell membrane and activating digestive enzymes such as tartrate-resistant acid phosphatase.[21,22] Osteoclasts also digest the organic matrix by releasing proteases and phagocytize the remaining fragments within cytoplasmic vacuoles.[21] Once these cells complete their resorptive activities, they transform into mononuclear cells that can be reactivated into new osteoclasts.

1.3 Bone Matrix:

Inorganic Matrix:

The inorganic matrix forms around 60% of the total bone weight. It is mainly an ion reservoir and provides the bone with the bulk of its strength and stiffness.[23] The inorganic matrix contains approximately 99% of total body calcium and 85% of total body phosphorus in addition to many other minerals such as sodium and magnesium in the form of bone mineral crystals.[23] By acting as a reservoir for these minerals, the inorganic matrix plays a critical role in maintaining their extracellular concentration within the ideal ranges needed to meet normal physiological requirements, such as for biochemical reactions, muscle contraction, and nerve conduction.[18] Moreover, rigid calcium-phosphate crystals within the inorganic matrix provide the bone with the mechanical properties needed to withstand the forces across its axis that are imposed by daily activities.[18]

Organic Matrix:

The organic composite of the bone matrix makes up about 20% of the total bone weight. It is similar to the organic composite of fibrous tissue, including tendons and ligaments, and consists primarily of collagen type I along with minimal amounts of type V and type XII.[24] The organic matrix also contains noncollagenous glycoproteins and proteoglycans, but these represent only 10% of the total.[24] The presence of type I collagen in the organic matrix provides the bone with its tensile strength.[18] By contrast, the noncollagenous matrix contains osteocalcin, osteonectin, bone sialoprotein, and bone phosphoproteins, which play an essential role in the mineralization process, in matrix organization, and in the organization of bone cells.[25]

1.4 Bone Modeling and Remodeling:

Bone modeling is the process of new bone formation without necessarily being proceeded by bone resorption. On the other hand, bone remodeling is defined as the renewal process of aged bones resorbed by osteoclasts.[14] Bone modeling typically occurs during the physiological bone growth of the long bones at the metaphyseal and diaphyseal segments.[14] This is followed by bone remodeling, because part of the metaphysis has to be remodeled into a leaner diaphysis.[26] The remodeling process is initiated by a series of resorptive activities at different sites following humoral or local stimulation of osteoclasts.[27] This resorptive stage is followed by an active regenerative phase in which active osteoblasts appear and start to synthesize and deposit unmineralized matrix which later becomes mineralized.[27] Bone remodeling is controlled by the effect of several systemic hormones that are locally produced, and by networking among different bone cells.[28]

2. Bone Metastases

2.1 Epidemiology and Distribution of Bone Metastases:

Bone is the third most common site of metastasis from primary tumours following the lung and the liver.[29] Metastatic bone tumours originate from almost all tumours. The most common primary sites are the breast, prostate, and lung, which together account for more than 80% of bone metastases.[29] In patients with breast cancer, the 10-year incidence of bone metastasis in patients with 3 or more positive lymph nodes at the time of resection reaches up to 30%.[30] In patients with prostate cancer, only 6% of patients have metastatic disease at the time of the diagnosis; however, autopsies show that 90% of patients who die of prostate cancer have metastatic bone disease.[31,32] In lung cancer patients, autopsies show an incidence of metastatic bone disease of approximately 25%.[29] More than 50% of multiple myeloma patients present with symptomatic bone metastases.[33] In patients with carcinoma of the thyroid, kidney, bladder, and melanoma, bone metastases are less frequent.[34]

The variability in metastatic disease distribution is influenced by several factors including biological and molecular characteristics, the primary tumour, and vascular properties. Bone metastases favourably metastasize to the axial skeleton, including the vertebrae, ribs, and hips.[34] The highly vascular metaphyseal bone and its sluggish blood supply provide tumour cells with the opportunity to move in and out of the marrow.[29,35] Additionally, the low-pressure vertebral venous plexus is subjected to arrest and reversal of direction owing to physiological changes in thoracoabdominal pressure, which explains why bone metastases are more often localized in the spine.[36]

2.2 Mechanism of Bone Metastases:

Typically, bone metastases can be classified into lytic, sclerotic, or mixed lesions according to the primary mechanism and the radiographic appearance.[37] As mentioned before, physiological bone remodeling consists of balanced bone resorption by activated osteoclasts and bone formation by activated osteoblasts.[37] Lytic lesions form when bone resorption and focal destruction predominate and new bone formation is unbalanced. Contrariwise, when osteoblastic activity increases, the lesion appears sclerotic.[29] Yet, in many cases both lytic and sclerotic components are present histologically and radiographically in the affected bone. This has been confirmed histologically and biochemically as evidence of increased osteoclastic activity has been found within the sclerotic lesions.[38,39] In breast cancer, the lesion can be lytic, sclerotic, or mixed, whereas it is purely sclerotic in prostate cancer.[36] However, there is evidence of an overall increase in bone resorption in metastatic prostate cancer.[40] In radiographic images, lesions appear to be lytic in patients with lung cancer.[29] Multiple myeloma metastases have a mixed component, whereas patients with small cell lung cancer and some adenocarcinoma appear to be mostly sclerotic.[29]

The development of bone metastases depends on the interaction between tumour cells and the microenvironment of the metastatic site. It has been suggested that bone metastases form in a two-step process: a latent and an active phase.[41] Bone marrow stromal-derived soluble factors, adhesion molecules, and transient cells generate a microenvironment capable of maintaining tumour cells in a latent phase that results in the arrest of proliferation.[42,43] An imbalance between the proliferative and latency signals

leads to a transition from the latent phase to the active phase, which sequentially leads to a clinically demonstrable lesion.[44]

In breast cancer, osteolytic bone metastasis is mediated by the action of activated osteoclasts and increased resorption rather than by the direct destruction of tumour cells in the bone.[37] Several factors have been associated with increased osteoclast activity; however, these factors vary depending on the primary tumour. Tumour-derived parathyroid hormone-related protein (PTHrP) and the bone-derived TGF-β are the primary mediators involved in the osteolytic process.[37] Breast cancer cells produce PTHrP, which has a biological effect similar to that of the parathyroid hormone that is the main regulator of calcium hemostasis. PTHrP stimulates the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) on osteoblasts and stromal cells, which in turn activates osteoclasts and the osteolytic process. The latter releases TGF-β, which interestingly further increases the production of PTHrP. This interaction between the bone environment and breast cancer cells results in an increase in local calcium, which also stimulates the production of PTHrP, leading to a vicious cycle that increases bone destruction.[37] Additionally, breast cancer cells produce prostaglandin E2, which increases the expression of RANKL and enhances further activation of osteoclasts.[44] Moreover, breast cancer cells produce other mediators, such as macrophage colony-stimulating factor, interlukin-1, interlukin-6, and tumour necrosis factor, that may also play a crucial role in the stimulation of osteoclast-induced bone osteolysis.[29,45] In contrast, osteoclast activation is inhibited by osteoprotegerin, which inhibits RANKL binding to the RANK receptor.[46]

On the other hand, the exact mechanism of the osteoblastic process in patients with sclerotic lesions is still unknown. It has been proposed that osteoblast activation and proliferation are stimulated by endothelin-1, which is a vasoconstrictor peptide and an inducer of osteoblast proliferation produced by prostate or breast tumour cells.[47,48] Elevated serum levels of endothelin-1 are associated with the presence of sclerotic lesions in patients with metastatic prostate cancer.[49] Additionally, platelet-derived growth factor-BB has been found to stimulate new bone formation via the induction of osteoblast proliferation.[43]

2.3 Clinical Presentation:

Bone metastases are typically symptomatic and are often the first evidence of disseminated disease.[34] The most common site of bone metastases is the axial skeleton, including the spine, hips, and ribs. Bone metastases are associated with considerable comorbidities and have been found to be the most common cause of pain in patients with malignancy.[50] This pain can have either mechanical or biological etiologies. The mechanical pain results from osteolytic lesions and is related to compression or the mass effect on the tissue and the bone surrounding the tumour. On the other hand, the biological pain is inflammatory in nature and is caused by the local release of chemical mediators and cytokines from tumour cells, which cause irritation of the periosteum, surrounding tissue, and nerves.[34] Skeletal complications in patients with bone metastases are common, and these include bone pain, pathological fracture, hypercalcemia, and spinal cord compression.[34] On average, a breast cancer patient with metastatic bone

disease will experience a skeletal complication every 3 to 6 months depending on the stage, systemic management, and progression of the disease.[50]

Bone Pain:

Bone metastasis pain is often localized and aching or burning nature and can be associated with episodes of discomfort or stabbing pain. Patients might describe a poorly localized or generalized pain, and it is not unusual for more than one distant site to be involved.[34] Additionally, with the ongoing loss of bone strength, pain is usually associated with activities. As bone destruction progresses, pain occurs at rest, becomes severe, is worse at night, and does not respond to analgesics. The pain associated with bone metastases can be due to biological aetiologies including local release of cytokines, chemical mediators, stimulation of the interosseous nerve, and periosteal irritation.[34] On the other hand, spinal instability represents 10% of the causes of back pain in patients with metastatic bone diseases. This pain is mechanical in origin and is relieved when the patient lies down without any movement. Subsequently, as the disease progresses, patients might not be able to stand or sit because of this pain. Because this pain is primarily mechanical, patients will require operative intervention and will not benefit from radiotherapy or systemic treatment.[29] The patterns of bone metastasis pain differ according to the sites involved. Common sites of involvement include vertebral metastases (present with neck and back pain with or without neurological deficits secondary to spinal cord involvement), femoral and pelvic lesions (present with pain in the back and lower limbs and that can be associated with mechanical instability and incident pain), and the base of the skull (present with neuralgias and headache).[50]

Pathological Fracture:

Pathological fracture initially begins as microfractures due to the osteolysis process, which cause pain and subsequently progress to involve both cortices and the trabecular bone. Pathological fracture arises in 10% to 30% of all cancer patients; it occurs in 60% of breast cancer patients and in only 10% of lung cancer patients.[50,51] The most frequent sites of involvement are the vertebral bodies and ribs, and less commonly the long bone, specifically the subtrochanteric region.[29] Fractures in the vertebral bodies result in loss of height and kyphoscoliosis and might lead to restrictive lung disease. Fractures with tumour extension to the epidural space and fractures of the long bones are the most devastating for cancer patients. In fact, a pathological fracture of a long bone in a patient with known metastatic bone disease is a reflection of insufficient clinical management.[29] Mirels et al.[52] proposed a scoring system to predict the risk of pathological fractures which includes a history of localized pain, anatomical site, and radiological features of the lesion.[52] Patients with an impending pathological fracture who have a high risk of fracture are often prophylactically stabilized because it is easier to stabilize the bone while it is intact and shorter rehabilitation will be required.[52] Surgery is often followed by radiotherapy of the lesion to establish local control of the disease and to inhibit further bone destruction.[29] According to the primary tumour, chemotherapy or hormonal therapy might be needed. Patients who are not fit for surgery are often treated with radiotherapy and protective weight bearing.[29]

Spinal Cord Compression:

Spinal cord compression is considered to be one of the most devastating complications of bone metastases and is a medical emergency. Symptomatic spinal metastases are the initial presentation in 10% of patients with cancer.[53] Patients can present with pain due to biological causes, mechanical pain due to instability, radicular pain due to nerve root irritation, or myelopathy findings. As the disease progresses, patients may present with severe pain at the site of the tumour that is exacerbated with activities that increase the intradural pressure, such as coughing and sneezing.[53] Additionally, the pain may become worse at night and may be associated with radiculopathy. At later stages, patients may develop sensory changes, weakness or paralysis, urinary retention, incontinence, or impotence.[29] Patients with a lesion at the level of the conus medullaris can present with cauda equine syndrome.[29] Spinal cord compression should be ruled out by magnetic resonance imaging (MRI) in any patients known to have cancer who present with back pain and radiographic changes.[54]

Hypercalcemia:

Hypercalcemia is a metabolic complication of metastatic bone diseases that occurs in 10% of patients.[29] Hypercalcemia is either due to the extensive bone osteolysis locally as in patients with breast cancer and multiple myeloma or due to paraneoplastic syndromes. Moderate to severe hypercalcemia (serum calcium > 3.0 mmol/L) can lead to significant comorbidity.[29] Patients often present with kidney, gastrointestinal tract, and central nervous system dysfunction. If left untreated, higher calcium levels can lead to renal failure, unconsciousness, arrhythmias, and consequently death.[50]

2.4 Diagnostic Modalities:

The diagnosis of bone metastasis is based on the patient's clinical presentation, laboratory markers, imaging modalities, and biopsy in selected patients. The clinical presentation includes a history of cancer and symptoms and signs of bone metastases. Laboratory markers of bone turnover, such as serum alkaline phosphatase (ALP) and serum calcium, can also aid in the diagnosis.[34] Imaging modalities used to diagnose bone metastasis include plain radiographs, computed tomography (CT) scans, MRI, bone scintigraphy, positron emission tomography (PET) scans, and single photon emission CT (SPECT) scans. [34]

Laboratory Markers:

A routine screening panel includes a complete blood cell count and a platelet count to assess for anemia of chronic disease as well as myelosuppression in the case of hematological malignancies. Bone turnover markers, which include ALP, serum phosphorus, and serum calcium, are useful for identifying hypercalcemia of malignancy. ALP is often elevated in patients with bone metastases; however, normal levels don't exclude bone metastases.[34] A more precise test includes parathyroid hormone levels, which can be low or high depending on the nature of the lesion.[34] Biochemical markers of bone turnover such as N-telopeptide and urine deoxypyridinoline might aid in the diagnosis.[55] Prostate cancer patients with metastatic bone diseases may have elevated prostate-specific antigen and acid phosphatase; however, neither of these markers is pathognomic for bone involvement.

Plain Radiographs:

Plain radiographs are essential to give an overall impression of the bone structure and alignment. Plain radiographs are the first-line imaging modality for the evaluation of bone pain and bone metastases and are highly specific for bone metastases. However, they lack sensitivity for asymptomatic metastases.[56] The appearance of the lesion on radiographs depends on the dominant process of the lesion and whether it is osteolytic or osteoblastic. Osteolytic lesions typically display thinning of the trabeculae, periosteal reactions, cortical breach, and ill-defined margins between the normal and abnormal bone. By contrast, sclerotic lesions appear as rounded, nodular, and well-circumscribed lesions owing to thickened trabeculae.[57] Lesions located in the trabecular bone are harder to detect by plain radiographs than are lesions in the cortical bone because of the limited contrast on the trabecular bone.[58] In addition, lesions less than 1 cm located in the trabecular area might be missed in the initial x-rays. Also, for a lesion to be reliably identified, more than 50% of its trabecular bone should be destroyed.[Choi, 2012 #115,59]Treatment response can also be assessed by using plain films. Nevertheless, plain radiographs are limited to bony changes and do not reflect the status of the soft tissue component. Additionally, 3 to 6 months are required for any identifiable changes to appear on the plain radiographs. These limitations clarify the need for a valid objective measure of treatment response. Overall, sclerotic changes in osteolytic lesions imply bone regeneration, whereas osteolytic changes in sclerotic lesions imply disease progression. Yet it is very challenging to determine whether the tumour is progressing or regressing in the case of a new sclerotic lesion that appears as a primary sclerotic type.[60]

Computed Tomography (CT) Scan:

CT scans provide a more detailed morphology of the bone including the cortical and trabecular components and the joint involved. CT has a sensitivity for the diagnosis of bone metastases of up to 74% and a specificity of 56%.[61] CT has low sensitivity to detect bone metastases within the marrow space before bone destruction occurs compared with MRI.[62] In terms of advantages, CT can be used to guide percutaneous biopsy when tissue diagnosis is required. Furthermore, it can be used to assess the bone treatment response during the staging or restaging of other organs, which decreases the burden of imaging and radiation on patients. A valid objective measure of treatment response can be determined by calculating the change in Hounsfield units within metastatic deposits after BP therapy.[60]

Magnetic Resonance Imaging (MRI):

MRI provides anatomical imaging of the tumour within the bone marrow and the surrounding soft tissues. It is the gold standard imaging modality to evaluate metastatic spread in the bone marrow and the invasion of surrounding structures. It carries a specificity ranging from 89.5% to 99.6% and a sensitivity ranging from 90.1% to 99.7%.[62] Interestingly, it can identify a tumour within the medullary cavity before any cortical destruction or osteolysis appears. MRI shows normal bone marrow as a high-intensity signal on T1-weighted images. The tumour appears as a low-intensity signal owing to infiltration of the tumour cells and replacement of fat cells.[63] Conversely, bone metastases demonstrate high-intensity signal in T2-weighted images owing to high water contents or enhancement with administration of gadolinium owing to increased vascularity.[63] In breast cancer, bone metastases are present in less than 2% of patients at

the time of the diagnosis. Therefore, all radiographic images should be limited to symptomatic cases or to cases in which areas of increased uptake are present on bone scans.[64] MRI is essential as well in the diagnosis of spinal cord compression. An edematous spinal cord will appear as an abnormally high T2 signal. A main advantage of MRI is the absence of ionizing radiation. Thus, it can be used in pregnant women with possible bone metastases. A limitation of MRI is that the cortical bone is poorly visualized owing to the short T2 relaxation time. Hence, bones with high cortical volume and low bone marrow volume are better examined with CT scan.[59] Whole-body MRI is a new modality that allows whole-body screening for bone marrow abnormalities via fast pulse sequences over different anatomic locations. Interestingly, several studies reported sensitivity and specificity of whole-body MRI superior to that of bone scintigraphy.[59,65] MRI is a helpful tool in assessing tumour response to treatment because of its high sensitivity and specificity. Recently, several studies suggested the use of quantitative diffusion-weighted imaging to assess tumour response to treatment.[66] However, further studies are needed to determine precise evaluation guidelines.

Bone Scintigraphy:

Radionuclide bone scintigraphy is a functional imaging modality that identifies metastatic lesions on the basis of osteoblastic activity in response to bone destruction and the associated increase in blood flow.[67] Bone scintigraphy is the most commonly used diagnostic modality to screen for bone metastases because of its wide availability and high sensitivity, which reaches up to 98%. Additionally, it provides a complete skeletal visualization within a short period at reasonable cost.[64] Interestingly, it has been reported

that bone scintigraphy can detect sclerotic lesions up to 18 months earlier than plain radiography.[59] Despite its high sensitivity in detecting widespread lesions, bone scintigraphy has high false-positive rates in cases of increased bone turnover, such as fractures, degenerative processes, and benign lesions including enchondroma.[68] False-negative findings have also been seen in rapidly growing, pure osteolytic lesions, when bone regeneration is slow, or when the lesion site is avascular.[69] Therefore, suspicious lesions on bone scintigraphy should be further correlated with clinical presentation and evaluated with the use of more accurate modalities such as plain x-ray, CT scan, or MRI to accurately determine the nature of the lesion, the involvement of soft tissues, and the risk of fractures.

Certain features in bone scintigraphy can differentiate between benign and malignant lesions. For instance, a vertebral body fracture appears as a transverse linear pattern of increased tracer accumulation. In contrast, benign lesions appear as multiple linear abnormalities of varying intensity of tracer accumulation. Importantly, lesions that extend from the vertebral body to involve the posterior element are suspicious of metastases.[70]

Another limitation of bone scintigraphy is its poor resolution, which can lead to inaccurate measurement of the lesion as well as difficulty localizing it.[58] Bone metastases that respond to treatment will display a reduction or an absence in tracer uptake compared with pre-treatment scans. In the early stages of treatment, some lesions might demonstrate an increase in uptake (flare phenomenon) due to the stimulation of bone-forming cells during the regenerative process, which can be misinterpreted as disease

progression or treatment failure.[62] Importantly, disease progression will demonstrate new lesions in different sites or an interval increase in the size of the early lesions.[60]

Positron Emission Tomography (PET) Scan:

PET scan is a functional nuclear modality that produces high-resolution images based on the uptake of positron-emitting radioisotopes (^{18}F NaF or ^{18}F FDG) by neoplastic cells.[59] The absorption of these radioisotopes varies based on the metabolic activity of these cells, specifically glucose metabolism.[59] Several studies have shown that the PET scan is superior to bone scintigraphy regarding the sensitivity and specificity of detecting osteolytic lesions, with specificity and sensitivity reaching up to 100%. However, bone scintigraphy is favorable for sclerotic lesions, because PET scans have high false-positive and false-negative rates for sclerotic lesions owing to their reduced metabolic activity.[2,64,71,72]

PET scans provide the ability to assess tumour response to treatment by objectively measuring uptake between a series of scans. However, the flare phenomenon can be encountered in the early stages of treatment, which makes the assessment of tumour response challenging.[73] PET scans can also detect metastatic lesions in organs other than bone, such as lung or lymph node metastases.[74] Nevertheless, the high cost of the PET scan, the lack of availability, and the additional examination time limit extensive use of this modality.

Single Photon Emission Computed Tomography (SPECT):

SPECT imaging is a functional nuclear imaging method that utilizes the principles of conventional bone scintigraphy. However, the images are acquired in a cross-sectional

fashion rather than a planar fashion.[65] SPECT scans are beneficial in the evaluation of tumour area that is surrounded by extensive soft tissue, such as the pelvis and the thoracolumbar spine.[64] The advantage of SPECT over the conventional technique is the better localization of the metastatic lesion owing to the cross-sectional imaging. The sensitivity and specificity of SPECT reach up to 87% and 91%, respectively.[73] Compared with PET, SPECT cannot provide a quantitative measurement of isotope uptake by tumour cells.[66]

Biopsy:

In patients with bone metastases of unknown origin, histological diagnosis is essential for therapeutic and prognostic purposes.[75] Additionally, biopsy is mandatory in an attempt to diagnose the primary tumour.[76] Biopsy should be performed in the most accessible bone or visceral lesions and the samples should be sent for histochemistry, electron microscopy, and immunohistochemistry. In addition, the samples should be sent for special stains for certain neoplasms, such as staining for estrogen and progesterone receptors and tumour and hormonal markers.[76] The importance of histological diagnosis lies in confirming the primary tumour and ruling out other tumours such as sarcoma or myeloma. Nevertheless, Rougraff et al.[77] concluded that bone diagnosis alone was not sufficient to identify the primary site in 60% of cases. A biopsy can be performed by use of a core needle biopsy or as an open procedure. If surgical fixation is already planned, then an open biopsy with a frozen section is needed before surgery.

2.5 Current Therapies:

The objectives of managing skeletal metastases are pain control, improved quality of life, bone stabilization, and local tumour control. Asymptomatic bone metastases that do not have a risk of spinal instability or pathological fractures are often observed.[2] Thus, the choice of therapeutic intervention depends on the patient's overall condition, expected survival, and quality of life. The currently available treatment options for bone metastasis are systemic therapy, radiotherapy, and surgical management. Treatment of bone metastases requires a multidisciplinary approach by medical, surgical, and radiation oncologists working as a team with pathologists, interventional radiologists, and rehabilitative specialists. Early diagnosis and appropriate therapy can improve functional dependence and quality of life in these patients.

Systemic Therapy:

Analgesics:

Bone metastases are the most common cause of cancer-related pain. Analgesics are the first-line treatment in patients with bone metastases. In the case of mild pain, the initial treatment is nonsteroidal anti-inflammatory drugs. In the case of mild to moderate pain, nonopioid analgesics are recommended. In patients with uncontrolled pain, opioids might be of benefit. Importantly, optimal pain control in patients with severe bone pain can be achieved by using a regular, fixed dose of analgesics. For breakthrough pain, short-acting opioids such as hydromorphone are proven to be sufficient.[78,79] Other medications such as corticosteroids, nerve block, and tricyclic antidepressants can be used as adjuncts to improve pain control.[80] It is important to combine different analgesic treatments to target

the different pathways of pain to achieve optimal pain control. Symptom monitoring and evaluation also play a critical role in controlling pain, as modification of the drug regimen might be needed to optimize pain control and to strike a balance between adverse effects of the drugs and pain relief.[81]

Bisphosphonates:

BPs are potent antiresorptive agents that bind to the hydroxyapatite mineral bone matrix to inhibit osteoclast-mediated osteolysis. BPs acts primarily by inhibiting the maturation and proliferation of osteoclasts and by inducing osteoclast apoptosis through inhibition of the mevalonate pathway.[82,83] Also, it has been proposed that BPs have direct anti-tumour activities by inhibiting the proliferation and migration of tumour cells as well as by inducing apoptosis.[84,85] Regarding their role in bone metastases, BPs have been shown in randomized clinical trials to delay and decrease the incidence of SREs in patients with bone metastases.[86-88] Several classes of BPs have been used to treat and delay SREs; intravenous zoledronic acid demonstrated the broadest clinical efficacy.[89-91] Rosen et al.[92] compared 4 mg of intravenous zoledronic acid to 9 mg of intravenous pamidronate in a large randomized clinical trial of 1684 patients. Their results showed that zoledronate reduced the risk of SREs in breast cancer patients over pamidronate by 20%. Additionally, zoledronate was superior to pamidronate in terms of time to first SRE and the reduction in risk of skeletal morbidity and complications. Furthermore, BPs proved to be effective in significantly reducing bone cancer pain.[93,94] BPs are also used in addition to intravenous fluid hydration to treat hypercalcemia of malignancy.[95] The optimal duration of BP treatment is controversial, however. Amadori et al.[96] demonstrated that

there is no significant difference in reducing SREs between administrating zoledronate once every 12 weeks for 12 weeks or administrating 4 mg of zoledronate every 4 weeks, after 12 to 15 months of monthly treatment. Overall, systemic BPs are well tolerated. Nevertheless, there are several reports of adverse events with the prolonged use of BPs, including acute renal failure, osteonecrosis of the jaw, bone turnover suppression, increased risk of infection, and gastrointestinal symptoms.[97] Nowadays, studies are examining the possibility of using a localized delivery system for BPs to allow greater efficacy and to avoid such adverse events. It has been revealed that locally delivered BPs remain mainly localized with minimal systemic distribution, thus it may reduce systemic side effects.[98] Furthermore, local delivery of BPs demonstrated less maxillary bone loss in animal models of osteonecrosis of the jaw, compared to the systemic group.[99]

Anti-RANKL Therapy:

Denosumab is a human immunoglobulin monoclonal antibody against RANKL. It acts by binding and neutralizing RANKL and preventing its interaction with its receptor RANK and reversibly inhibits osteoclast-induced osteolysis.[100] Regarding its efficacy concerning the delay and reduction of SREs, Stopeck et al.[101] assigned 2046 patients to receive either subcutaneous denosumab and intravenous placebo or intravenous zoledronate and intravenous placebo. Their results showed that denosumab was superior to zoledronate regarding time to first SREs, pain control, and pain relief. In terms of adverse events, patients in the denosumab group experienced hypocalcemia and osteonecrosis of the jaw at a rate of 2%. Renal complications occurred more in the zoledronate group and the rate of osteonecrosis was 1.4%. In a similar randomized clinical trial, denosumab proved

superior to zoledronate in terms of delaying time to first SREs. [102] Regarding adverse events, the denosumab group experienced higher rates of hypocalcemia, whereas the rate of osteonecrosis of the jaw was similar in both groups.[102] Given that hypocalcemia was reported in these studies in addition to other randomized clinical trials, it is recommended that patients be maintained on supplemental calcium and vitamin D throughout the treatment period.

Radiotherapy:

Radiotherapy is most commonly used as a palliative treatment for bone metastasis pain and is associated with responses ranging from 50% to 90%.[103,104] Additionally, radiotherapy has been used to reduce the risk of SREs and the risk of neurological deficits due to spinal cord compression.[105] The exact underlying mechanism of action of radiotherapy is unknown; however, it has been suggested that radiotherapy acts by permanently destroying the DNA of tumour cells, thus preventing tumour cell repair, and promoting the ossification of lytic lesions.[2] Furthermore, radiotherapy relieves bone metastasis pain by reducing the mechanical compression of the tumour and decreases neoplastic inflammatory cytokines.[29] Several randomized clinical trials have compared the use of different dosing regimens including 30 Gy in 10 fractions, 24 Gy in 6 fractions, 20 Gy in 5 fractions, and a single 8-Gy fraction. The results were comparable in terms of pain relief efficacy. Although rates of pain recurrence and the need for irradiation were higher in the single-fraction regimen, there is no evidence that a single 8-Gy fraction is inferior to multiple-fraction regimens.[106] Additionally, the duration of treatment did not correlate with the treatment response.[107] Overall, studies have shown that patients with

good prognosis and estimated longer survivorship, experienced lower re-treatment rates with multiple fractions radiotherapy, compared to patients who received single fraction. Apart from this, single fraction radiotherapy is recommended in patients with poor prognosis owing to the cost of the treatment and considering the inconvenience to the patient.[2]

Radionuclide therapy delivers beta radiation to the site of bone metastases through binding to hydroxyapatite in areas of active calcification and rapid bone turnover.[108] Studies have shown that radionuclide therapy is superior to external beam radiation in the case of diffuse multiple sclerotic bone metastases.[1] Currently available radiopharmaceuticals include samarium-153 and strontium-89, which are beta-emitting agents.[1] Radionuclides act by inhibiting lymphocyte-associated cytokines, coupled with the alteration of osteoblastic or osteoclastic activity.[109] Radionuclide therapy was shown to be effective in the palliation of sclerotic lesions, with a decrease in analgesic intake ranging from 50% to 90%.[110] The average time to response (pain relief) is 3 to 6 months following treatment. [1] There are few disadvantages of radiopharmaceuticals, which include bone marrow suppression, which can lead to leukopenia and thrombocytopenia in some cases. Additionally, chemotherapy is only allowed at 6 months after radionuclide treatment in patients with visceral metastasis because of the myelosuppressive effect.[1,111,112] A combination of external beam radiation therapy and radionuclide therapy has been shown to be promising and safe and to efficiently provide palliation of both diffuse and localized bone metastases.[113]

Surgical Management:

Surgical management of bone metastases has been shown to improve pain, improve quality of life, and maintain or improve range of motion and ambulatory status.[29,114] Nevertheless, orthopaedic surgeons should weigh these benefits with the potential comorbidities and risks of complications in this population to ensure that surgery is the most appropriate treatment. Surgical management of bone metastases focuses mainly on relieving symptoms, preventing fractures, or promoting healing following pathological fracture.[115-117] Another category is patients who present with a solitary metastatic lesion of a curable cancer such as thyroid or renal cancer. Owing to the expected prolonged survival in these patients, the goal of the treatment is curative and the metastasis should be treated as for sarcomas with wide resection and reconstruction.[118,119]

Spinal Metastases:

The spine is the most common site of skeletal metastases. The objectives of management in patients with spinal metastases include pain relief, mechanical stabilization, and neurological preservation.[120] In an attempt to define patients who will benefit from surgical intervention for spinal metastases, Tokuhashi et al.[121] proposed a scoring system based on the type of primary tumour, the number of vertebral metastases, the presence of visceral or extraspinal metastases, the general condition of the patient, and the neurological status. Patients with a sum score greater than 9 out of 12 are candidates for surgical removal. Patients with a sum score of less than 5 are not candidates for surgery and are planned for palliative therapy with limited decompression and stabilization.[121] Up to 10% to 20% of spinal metastases have unknown primary origin[122]; thus, repeated oncological staging including CT scan of the chest, abdomen, and pelvis and a whole-

body scan is required. Additionally, percutaneous or open biopsy is essential for a definitive diagnosis, because the choice of surgical treatment depends on the histological findings.[54] The type of surgical approach depends mainly on the location of the tumour within the vertebra, the spine segments involved, the histological findings, and the type of surgery.[54] The main indications for surgery in spine metastases are symptomatic metastases not responding to medical treatment, spinal instability, spinal cord compression, and radioresistant tumours.[54] Generally, tumours in the cervical and thoracic spine are approached anteriorly, whereas tumours in the lumbar spine are approached posteriorly.[54] Surgical treatment varies depending on the location of the tumour and the segment involved. Generally, patients may undergo posterior laminectomy, intra-lesional excision, or en bloc resection and reconstruction with autologous bone graft, titanium mesh, and/or polymethylmethacrylate.[54,120,123,124] Patients who complain of pain due to spinal metastases without spinal cord compression or instability are perfect candidates for percutaneous vertebroplasty or kyphoplasty, which can result in dramatic pain relief.[125] Additionally, patients who are poor surgical candidates and have a life expectancy less than 3 months can be treated with vertebroplasty or kyphoplasty. Regarding spinal instability, the factors that have been shown to cause spinal instability include significant vertebral body involvement, segmental junctional involvement, and facet joint involvement. Patients with solitary metastases who are expected to have a long survivorship are perfect candidates for en bloc resection because it eliminates the need for radiotherapy and results in disease-free

status. However, this procedure is technically demanding and has a high rate of morbidities.[126]

Long Bone Metastases:

Long bone metastases can present as symptomatic metastases that do not respond to medical treatment, as impending fractures, and as pathological fractures. The decision on the surgical procedure should depend on surgeon preference, the region involved, facilities, cost, and the patient's estimated survival.[115,127] Impending fractures carry a risk of fractures and may require prophylactic fixation to avoid unnecessary complications.[128] Mirels proposed criteria to predict the risk of fracture in patients with long bone metastases.[52] Mirels criteria consist of four parts that include the location of the tumour, size, presence of pain, and the matrix. Each part is assigned a score from 1 to 3. Patients with a sum score equal to or less than 7 have less than a 15% chance of fracture and do not need prophylactic fixation. On the other hand, patients with a sum score equal to 9 or more have a more than 30% chance of fracture and prophylactic fixation is needed.[52] The goal of fixing impending fractures prophylactically before they fracture is to minimize blood loss, decrease hospital stay, promote faster recovery, improve patient's function, and improve survival.[128] Bone can be fixed by using intramedullary (IM) nails, plating, or joint replacement. In patients with a peri-articular lesion, lesions are often resected and reconstructed with arthroplasty procedures. When the lesion is invading a large part of the bone, the lesion can be resected and replaced by a cemented endoprosthesis.[115] IM

nail fixation is reliable and safe for treating long bone pathological or impending fractures with or without cementation.[129] Lesions at the hip can be treated with femoral nails (antegrade or retrograde). The use of proximal interlocking screws with antegrade nailing is recommended to obtain purchase into the femoral neck and the head.[129,130] Humeral lesions with no articular extension are often treated with intramedullary nailing as well.[131] By contrast, management of pathological fractures with plate fixation allows for curettage of the lesion and reconstruction with bone cement or polymethylmethacrylate to provide stability. The choice of plate, whether locking or nonlocking, depends on the quality of bone and the amount of bone stock left following curettage. Several authors have successfully reported the use of locking plates in pathological fracture because these plates improve bone fixation in the setting of poor bone quality and decrease the incidence of the screws pulling out.[132] Furthermore, a diaphyseal lesion can be reconstructed as well with a diaphyseal prosthesis.[133] Several papers recommend curettage of the tumour before fixation as it offers considerable advantages, including debulking the tumour allowing for better radiotherapy response, the ability to use bone cement, and faster recovery. Disadvantages include longer operation times, extensive dissection, and increased blood loss.[134] An important aspect of this population is patient functionality. It is important to restore function immediately in this population because of the possibly short life-span. Some authors favour the use of a cemented implant over the use of a construct that relies on bone healing or ingrowth into stem and cups, which requires more time to restore function.[115,134] Additionally, bone

grafts and allografts are not recommended because postoperative radiation will reduce bone to graft incorporation.[134]

2.6 Conclusion:

Metastatic bone disease is the most common bone tumour and the most common cause of cancer-related pain. The consequences of this disease are devastating to patients and the health care system. The management and the intervention are often tailored to each patient according to many factors, including the patient's general condition, the primary tumour, the site of the tumour, and expected survival. Nevertheless, in the majority of patients, treatment of bone metastases is palliative rather than curative. A multidisciplinary approach combining systemic treatment, radiotherapy, and surgical treatment provides the opportunity to deliver optimal treatment to each patient.

3. Bisphosphonates

3.1 Background:

Bisphosphonates (BPs) are structural analogues of pyrophosphate that consist of two phosphate groups attached to a central carbon on either side (P-C-P).[135] BPs were introduced in 1865 by Menschutkin.[136] They were first used in industrial processes as a corrosion inhibitor, in textile production, in the fertilizer industry, and as a water softener.[137] Medically, BPs were first used by Fleisch et al.[138,139] in 1968, who described the inhibitory effect of two types of BPs, etidronate and clodronate, on crystal formation, vascular calcification, and bone resorption. In 1969, the first clinical application of BPs in humans was published in which etidronate was administered to a child with fibrodysplasia ossificans progressiva.[140] Since this initial clinical use, many studies have

shown in both in vitro and in vivo models that BPs inhibit osteoclast-mediated bone resorption. First, Russell et al.[141] showed that BPs inhibit the dissolution of hydroxylapatite crystals in vitro. In addition, BPs were shown to inhibit the rise in serum calcium in thyroparathyroidectomized rats. In another study, BPs retarded the removal of bone and cartilage in healthy rats.[142] Clinically, BPs have been used to prevent heterotopic ossifications following arthroplasty[143] as well as calcifications following spinal cord injury. However, the most impressive clinical application is in various bone diseases with excessive osteoclastic activity, such as Paget's disease of bone, osteoporosis, metastatic bone disease, and hypercalcemia of malignancy, in which BPs are becoming the treatment of choice.[140]

3.2 Mechanism of Action:

BPs are structural analogues of pyrophosphate that consist of two phosphate groups attached to a central carbon (P-C-P).[135] The (P-C-P) component is responsible for the high affinity of BPs for hydroxylapatite crystals.[144] The structure of BPs varies according to substitution in the R1 and R2 position attached to the carbon atom. The presence of a hydroxyl group at the R1 position was thought to be the primary reason for the increased affinity of BPs for calcium crystals.[144] Later it was shown that the presence of a nitrogen atom in the R2 side chains increases the potency of BPs 10- to 100-fold; these nitrogen-containing BPs (N-BPs) include pamidronate, risedronate, and zoledronate.[145] Although the R2 side chain is an important element of anti-resorptive effectiveness, it is important to mention that both phosphate groups should be present for the drug to be pharmacologically active.

BPs acts on both cellular and molecular levels. At the cellular level, BPs are internalized into the intracellular vacuoles of osteoclasts by endocytosis during the process of bone resorption.[146] Several studies have shown that BPs inhibit osteoclasts by inhibiting their adhesion, proliferation, and resorptive activity as well as by inducing their apoptosis.[82,83] In addition, morphological changes have been observed in BP-treated osteoclasts such as the absence of ruffled borders, disruption of the cytoskeleton, and the lack of an invaginated plasma membrane.[83] These morphological changes have been explained by an interruption of the intracellular signalling within osteoclasts.[140]

At the molecular level, there are two major molecular mechanisms by which BPs act on osteoclasts, and BPs can be divided into two groups according to these mechanisms.[147] The first group is the non-nitrogen BPs, which lead to the intracellular accumulation of non-hydrolysable analogues of ATP (AppCp) nucleotides within osteoclasts following several chemical reactions.[148] The accumulation of these metabolites has been shown to inhibit mitochondrial ADP/ATP translocase and thus will inhibit osteoclast function and may lead to osteoclast death.[149] By contrast, N-BPs interfere with osteoclast function through several mechanisms. The first is by interacting with farnesyl pyrophosphate synthase (FPPS), which is a crucial enzyme in the mevalonate biosynthetic pathway that catalyzes the conversion of isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate (DMAPP) to form farnesyl pyrophosphate (FPP).[150] Moreover, N-BPs interfere with geranylgeranyl pyrophosphate synthase (GGPPS), which is downstream of FPPS in the mevalonate pathway.[151] These two enzymes are essential for the phenylation of GTPases, which are important signaling proteins that regulate the structure and

function of osteoclasts.[152,153] In addition, disruption of the mevalonate pathway leads to the accumulation of IPP, which later transforms into a cytotoxic ATP analogue called ApppI.[154]

3.3 Clinical Application:

BPs have been used clinically as an inhibitor of bone resorption in diseases with excess osteoclastic activity. However, the first clinical use of BPs was as an inhibitor of calcification. Specifically, etidronate was used in patients diagnosed with fibrodysplasia ossificans progressiva.[155] Etidronate also showed promise in preventing heterotopic ossification after hip arthroplasties and in preventing ectopic calcification after spinal cord injury.[155] BPs have also been used as a diagnostic modality to detect bone metastases by utilizing the high affinity of BPs at sites of increased bone turnover as well as their ability to link to a gamma-emitting technetium isotope.[156] Regarding diseases with increased osteoclastic activity, Paget's disease was the first medical condition in which BPs were administered to inhibit bone resorption.[157] Currently, BPs are considered the treatment of choice for Paget's disease because they inhibit bone resorption and are efficient in relieving pain.[158] Studies have shown that zoledronate is more effective than other BPs for reducing pain and improving quality of life.[159] Moreover, BPs have proven beneficial in terms of reducing the risk of fractures in patients with osteoporosis. BPs were first introduced in the management of osteoporosis in 1990 by Watts et al.[160] Their results showed that etidronate significantly increased spinal bone mass and reduced the incidence of vertebral fractures in women with postmenopausal osteoporosis. Following this study, several studies reported the use of different BPs, such as alendronate,

risedronate, and zoledronic acid, for osteoporosis and showed a significant reduction in fracture risk in both vertebral and nonvertebral fractures.[160-162]

Additionally, multiple studies have shown that BPs might be of benefit in adult patients with hip osteonecrosis. Agarwala et al.[163] showed that BPs could delay surgical intervention in patients with hip osteonecrosis. In another study by Lai et al.,[164] alendronate was used in the treatment of nontraumatic osteonecrosis of the femoral head. Only 2 of 29 femoral heads collapsed in the alendronate group compared with 19 of 25 in the control group.

BPs have been used as well to improve implant fixation and joint arthroplasty in patients with periprosthetic bone loss. In cementless joint arthroplasty, the stability of the implant depends on the growth of new bone into the prosthesis at the prosthesis-bone surface. Early excessive implant migration due to osteolysis and bone resorption can lead to early implant failure.[165] Therefore, several studies have investigated the use of BPs early in the postoperative period to help to maintain the position of the implant by inhibiting osteolysis and allowing bone formation at the bone-prosthesis interface. Friedl et al. [166] demonstrated a reduction in the migration of cementless acetabular prosthesis over 2 years after a single dose of zoledronate (4 mg) in patients undergoing total hip arthroplasty for osteonecrosis of the femoral head. Also, in animal models, implants coated with BPs were shown to have increased mechanical strength at the implant-bone interface as well as increased peri-implant bone mineral density.[167,168]Yet, the effects of BPs on implant fixation and stability are controversial. Some studies have not demonstrated a significant treatment effect.[169,170] Moreover, some reports have

investigated a possible inhibitory effect of BPs on allograft resorption. Aspenberg et al.[171] showed that soaking a morselized allograft in ibandronate before femoral impaction resulted in increased bone mineral density in the region distal to the femoral implant tip. It was difficult to differentiate whether this was from the BPs or the allograft itself.

3.4 Role of Bisphosphonates in Bone Tumours:

BPs have played a significant role in treating oncological diseases as a palliative or adjuvant therapy. This role can be traced to the 1980s when multiple authors reported the use of BPs for the treatment of hypercalcaemia of malignancy due to multiple myeloma and bone metastases.[172,173] In the 1990s, a large randomized trial demonstrated that BPs are the standard of care for the treatment and prevention of skeletal-related events (SREs) associated with bone metastases from breast cancer and myeloma.[4] Moreover, BPs were demonstrated to be beneficial in bone metastases from prostate and lung cancers.[174,175] Additionally, BPs have been used as an effective palliative therapy for bone pain due to bone metastases and as an adjunctive to radiotherapy and analgesics.[3] Besides their analgesic effect and the reduction in SREs, BPs have been shown to decrease the time to first and consequent SREs by 30-50%, which has been proven to maintain the patient's function and quality of life.[176] The potential adjuvant and palliative effects of BPs have led investigators to conduct a variety of preclinical studies to examine the anti-cancer properties of BPs.[177,178]

Effects of BPs In Vitro:

At the cellular level, BPs have been shown to have anti-tumour properties in vitro, including inhibiting tumour cell adhesion, invasion, and proliferation and inducing

tumour cell apoptosis.[84] It has been demonstrated that N-BPs induce tumour cell apoptosis in vitro by interfering with the mevalonate pathway, which mimics the anti-resorptive effect of BPs on osteoclasts in vivo.[84] Räikkönen et al.[179] confirmed this by showing that mevalonate pathway intermediates inhibited BP-induced apoptosis of breast cancer cells. Additionally, it has been shown that N-BPs inhibit the farnesylation of oncogene Ras (an important regulator of tumour cell proliferation), which thus inhibits its interaction with the tumour cell membrane and induces tumour cell apoptosis.[84] Furthermore, another mechanism by which N-BPs prompt apoptosis was described by Ory et al.[180] They showed that zoledronate induces cell death in osteosarcoma cells by nuclear alteration and activation of a mitochondrial pathway via translocation of apoptosis-inducing factor and endonuclease-G. They noticed as well a decrease in Bcl-2 expression and an increase in Bax expression. Moreover, Mönkkönen et al.[154] established that N-BPs inhibit mitochondrial adenine nucleotide translocase (ANT), which has been demonstrated to play a part in inducing cell apoptosis. The inhibition of the mevalonate pathway leads to the accumulation of APPPI, which inhibits mitochondrial ANT.[154] Apart from the mevalonate pathway, several studies demonstrated that the BP analogue apomine induces cell apoptosis in breast cancer cells through activation of caspase and p38 MAPK independently of the mevalonate pathway.[181] Regarding the anti-proliferative activity of N-BPs, zoledronate has been shown to inhibit tumour cell growth by inducing cell cycle arrest in the S-phase. This effect can be coupled as well with the apoptotic effect of zoledronate.[85]

BPs also inhibit tumour cell adhesion and invasion processes that are required for the formation of metastases. BPs have been shown to inhibit the adhesion of prostate and breast neoplastic cells to the extracellular matrix in vitro.[182] BPs may interfere with cell adhesion molecules and cell surface receptors.[84] Zoledronate has been shown to interfere with the cell surface receptor integrin by inhibiting G-protein prenylation, specifically geranylgeranylation.[183] Furthermore, BPs have been shown to inhibit cancer cell invasion in vitro.[184] The underlying mechanism is thought to be due to RhoA-inhibition.[185] BPs have also been shown to inhibit metalloproteinases (MMPs) that are essential for the degradation of the extracellular matrix by cancer cells in the invasion process.[186] In a prostate cancer cell line, zoledronate was found to inhibit the transcription of cysteine-rich angiogenic inducer 61 (CYR61), which promotes the migration and invasion of cancer cells.[187]

Indirectly, BPs inhibit tumour cells in vitro through an antiangiogenic effect. Numerous studies have reported that BPs inhibit vascular endothelial cell function in vitro by inhibiting the migration and proliferation of endothelial cells.[188] The underlying mechanisms are suggested to be due to inhibition of focal adhesion assembly,[189] Rho geranylgeranylation,[189] and suppression of sustained activation of protein kinase B/Akt.[188] These mechanisms suggest that the antiangiogenic activity of BPs is somewhat related to their ability to inhibit FPPS activity.

Effects of BPs In Vivo:

BPs exhibit an anti-tumour effect in vivo, reducing the skeletal tumour burden and the occurrence of metastatic bone disease in animal models of prostate, breast, and lung

cancers as well as in models of multiple myeloma and osteosarcoma.[84] This anti-tumour activity in pre-clinical models has been achieved by directly inhibiting osteoclast-mediated bone resorption. One such mechanism is by reducing the amount of bone-derived growth factors such as TGF-β released from the resorbed bone, which are essential for tumour cell growth.[84] Additionally, Korpal et al.[190] demonstrated that pamidronate (another N-BP) inhibits metastatic lesion formation by interfering with the TGF-β-SMAD signaling pathway in breast cancer cells inoculated into bone. On the other hand, Hirbe et al.[191] reported that zoledronate inhibited tumour growth in mice with defective osteoclasts, which suggests that BPs have direct anti-tumour properties besides their anti-osteoclastic effect. Interestingly, Fournier et al.[192] suggested that BPs with low mineral affinity inhibit tumour growth in vivo but do not inhibit osteolysis. Indirectly, BPs inhibit tumour cells via an antiangiogenic effect in vivo. This has been demonstrated by evidence of an antiangiogenic effect of zoledronate in the chick chorioallantoic membrane assay.[84] Moreover, zoledronate was shown to inhibit the vascularization of subcutaneous tissue coupled with growth factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor.[188] However, the underlying mechanism of the inhibitory effect of BPs in vivo and their impact on growth factors is not yet entirely understood. It has been suggested that zoledronate may inhibit MMP-9-mediated mobilization of stromal VEGF.[193] Yet, some reports demonstrated tumour inhibition in a mouse model of ovarian tumour via inhibition of vascularization without inhibition of tumour-derived VEGF expression.[189] Interestingly, zoledronate did not inhibit vascularization in animal models of bone healing.[*194*]

Effects of BPs on Vγ9Vδ2 T Cell Cytotoxicity:

Another proposed mechanism of the anti-tumour action of BPs is through induction of the anti-tumour activity of human Vγ9Vδ2 T cells. The effect of BPs on human Vγ9Vδ2 T cells was first noticed when patients who received their first dose of pamidronate developed flu-like acute phase reactions.[194] BPs have been suggested to induce human Vγ9Vδ2 T cells indirectly by inhibiting the mevalonate pathway, leading to intracellular accumulation of IPP.[195] Human Vγ9Vδ2 T cell receptors recognize the IPP as tumour antigen, causing activation of the Vγ9Vδ2 T cells and killing of the tumour cells.[84] In vitro, human tumour cell lines treated with BPs activate human Vγ9Vδ2 T cells to target and lyse tumour cells.[195] This finding is supported by the fact that many tumour cell lines can accumulate IPP intracellularly.[179,196] Additionally, in vivo studies have shown that zoledronate-treated human Vγ9Vδ2 T cells inoculated in mouse models with UMUC-3 bladder cancer cell line,[197] SBC-5 small lung cancer cell line,[198] or melanoma cells[195] were associated with significantly prolonged survival of these animals. Moreover, Vγ9Vδ2 T cells can be expanded from peripheral blood mononuclear cells extracted from patients diagnosed with breast and prostate cancer treated with zoledronate and IL-2.[199] The potential effect of these cells in vitro and their ability to expand from peripheral blood mononuclear cells of cancer patients treated with BPs may make them beneficial for future autologous cancer therapy.

3.5 Conclusion:

BPs are a potent anti-resorptive medication prescribed for diseases with excess osteoclastic activity and have both direct and indirect anti-tumour effects in preclinical

studies and clinical trials, in particular an established role in preventing SREs in patients with breast cancer. The direct actions of BPs are to induce cancer cell apoptosis and inhibit cancer cell proliferation, migration, and invasion in vitro. In vivo studies have suggested that BPs inhibit tumour cells mainly by inhibiting osteoclast-mediated bone resorption. On the other hand, an indirect effect was proposed via the inhibition of tumour angiogenesis and the stimulation of Vγ9Vδ2 T cells. However, the exact mechanism of action in vivo is still unclear and further clinical research is required. In addition, further clinical studies should be conducted to optimize the clinical dose and to investigate the synergistic effect of BPs with other therapeutic agents. Last, the mode of drug delivery should be examined to maximize efficacy and minimize adverse events.

Section 2: Manuscript

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Intra-tumour Delivery of Zoledronate is Superior to Systemic Administration for Mitigating Metastasis- induced Bone Destruction.

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Intra-tumour Delivery of Zoledronate is Superior to Systemic Administration for Mitigating Metastasis-induced Osteolysis

Abstract:

Background/Aim: Bisphosphonates (BPs) have been found recently to have direct anti-tumour properties. In the present study, we examined whether local delivery of BPs inhibits tumour-induced osteolysis and tumour growth in an animal model of bone metastasis

Materials and Methods: Following the establishment of an intra-tibial model of bone metastases in athymic mice (N=15), the experimental group (N=11) was treated by local administration of zoledronate into the tibial lesion. In a subsequent experiment, a comparison of the effect of local versus systemic delivery of zoledronate on the formation of tumour-induced osteolysis was carried in athymic mice (N=19).

Results: The first experiment showed a statistically significant increase in mean bone volume/tissue volume % (BV/TV) of the treated group (12.30±2.80%) compared to the control group (7.13±1.22%) (P<0.001). Additionally, the comparison between the local and systemic effect of zoledronate revealed a significant increase in the BV/TV in the locally treated group (10.90±1.25%) when compared to the systemically treated group (7.53±0.75%) (P<0.001).

Conclusion: These preliminary results suggest that local delivery of BPs can lead to a significant inhibition of tumour-induced osteolysis. Moreover, local BPs administration is superior to systemic delivery in terms of osteolysis inhibition.

Introduction:

Bone metastases, the most common cancer affecting the bone, are mostly derived from solid tumours such as breast, prostate, lung and bladder cancers.[1] They are also the most common cause of cancer-related bone pain and often lead to additional complications such as pathological fracture and spine compressions, all of which can severely affect patients' quality of life.[2] Treatment of bone metastases imposes a huge burden on the healthcare system, and with the advancement in healthcare and increase in cancer life expectancy, metastatic bone disease are projected to increase dramatically.[3] The currently available treatment options for bone metastasis are surgical therapy, radiotherapy, Anti receptor activator of nuclear factor-kappaB ligand (RNKL) antibody, and systemic bisphosphonates (BPs).

BPs are potent anti-resorptive agents that inhibit bone resorption by inhibiting osteoclastic cell activity, which is responsible for bone removal during turnover.[4] Since most studies showed that there is an increase in osteoclast numbers and activity in metastatic bone disease, the anti-resorptive activity of BPs is used to reduce bone cancer pain, bone destruction, and bone tumour growth.[5] Interestingly, BPs have also been suggested to have anti-tumour properties by negatively regulating macrophages, endothelial cells and tumour cells. [4,6] Furthermore, BPs have been shown to elicit combinatorial effects with chemotherapeutic agents and are often administered to breast or prostate cancer patients with a metastatic bone disease as a single intravenous dose or course of treatment as part of the standard care regimen.[7,8] It has also been shown that local delivery of BPs by elution from porous materials can be used to enhance bone formation.[9]

Clinically, proximity to vital structures often hinders complete surgical resection of the tumour. In addition, there are complications that render systemic BPs administration unsuitable for some patients [10]. Therefore, we sought to examine whether local delivery of BPs can inhibit tumour-induced osteolysis and tumour growth in an animal model of bone metastasis; and whether the efficacy in impairing cancer-induced osteolysis that is achieved by local delivery of BPs is comparable to that of systemic BPs treatment.

Material and Methods

Study subjects and participants:

MDA-MB-231 human breast cancer cell line (provided by Cedarland, USA) were cultured in a DMEM cell culture medium (Gibco, Invitrogen, Cat#11965092) supplemented with 10% fetal bovine serum and 1% antibiotics (HyClone brand from Thermo Scientific, Cat# SV30010) at 37°C in a humidified atmosphere of 5% carbon dioxide (CO2). Zoledronic acid was purchased from Sigma, USA (Cat# SML0223), and D-luciferin was purchased from PerkinElmer, USA (Cat# 770504). The experimental design used 35 female athymic nude mice (490, Homozygous), aged 9-12 weeks, were purchased from Charles River, USA. The average weight was 25g (range, 22.7-27.6 g). The mice were maintained in pathogen-free conditions. The Mcgill Animal Care and Use Committee approved all the experimental procedures.

Establishment of an intra-tibial mice model of bone metastasis

MDA-MB-231 human breast cancer cell line (N= 10^5) were resuspended in 20 µl of Phosphate buffered saline (PBS) and injected into the marrow space of the right tibia using a 27½ gauge needle coupled to a Hamilton syringe under imaging guidance [11]. Five days following inoculation, the presence of tumour cells was confirmed using in vivo bioluminescence imaging (IVIS spectrum, PerkinElmer, USA). Mice were randomly assigned to different groups according to the design of each experiment. At the end of each experiment, the mice were sacrificed using the American Association for Laboratory Animal Science (IACUC) approved method (CO2 asphyxiation).

Zoledronate treatment

Local administration: Following successful implantation of MDA-MB-231 human breast cancer cells, mice were divided randomly into two groups, the treatment group (2µg/mice zoledronate, delivered intra-tibially three times/week for three weeks, N = 11), and the control group (30µl/mice PBS, intra-tibially three times/week for three weeks, N= 5). Zoledronate treatment was started one week after the successful implantation of tumour cells. The tumour growth was monitored weekly using in vivo bioluminescence imaging and clinically for any signs of tumour development. After three weeks of treatment, the mice were

sacrificed and tibias were removed and dissected for micro-computed tomography (Skyscan1172, Skyscan, Belgium) and histological analysis.

Local versus systemic administration

Following successful injection of MDA-MB-231 human breast cancer cells, nineteen athymic nude mice were divided randomly into four groups, the local treatment group (0.025mg/kg zoledronate, delivered intra-tibially once/week for four weeks, N = 6), the local control group (30µl/mice PBS, delivered intra-tibially once/week for four weeks, N = 4), the systemic treatment group (0.025mg/kg zoledronate, delivered sub-cutaneously once/week for four weeks, N = 5), and the systemic control group (100µl/mice PBS, injected sub-cutaneously once/week for four weeks, N = 4). Doses were calculated based on an average weight of 25g.[12] The treatment was started one week following successful inoculation of the breast cancer cells. After four weeks of treatment, the mice were sacrificed, and tibias were removed and dissected for micro-computed tomography and histological analysis.

In vivo bioluminescence imaging

The growth of MDA-MB-231 derived tibial lesions was assessed by longitudinal bioluminescence imaging. The mice were imaged using IVIS spectrum following an intra-peritoneal injection of D-luciferin solution (PerkinElmer, USA) (150 mg/kg body weight) under gas anesthetic. Bioluminescence images were taken 20 minutes after D-luciferin injection and acquired until the peak signal was reached. Photon emission was quantified using Living Image software and graphed according to the average radiance (photons/s/cm^2/sr).

Micro-computed tomography (µ -CT) analysis

Tibiae were dissected from mice at necropsy and excised tibia scanned using a high-resolution micro-tomographic system. Each of the three-dimensional images was constructed from approximately 550 individual micro-CT images (8.9 μm/image) starting from the growth plate of the tibia and moving distally. Image reconstruction was performed using NRecon (Version 1.6.2.0; SkyScan). The CT analyzer (1.11.8.0; SkyScan) was used to measure static histomorphometric parameters of the region of interest. The bone density was expressed as a percentage of bone volume/ tissue volume % (BV/TV).

Immunohistochemistry:

Tissue fixation and immunohistochemical (IHC) staining were carried out as previously described.[13] The proliferative index in the bone metastatic lesions was assessed by staining with a Ki67 antibody (1 μg/ml; Cat. #: ab15580; Abcam, Toronto, ON, Canada). Anti- Cleaved-Caspase 3 staining (0.2 μg/ml dilution; Cat. #: 9661; Cell Signaling, Whitby, ON, Canada) was performed to quantify apoptosis within the bone lesions. Following incubation with the primary antibody, secondary biotin-conjugated antibody (Jackson Laboratories) was applied for 30 minutes. After washing with distilled water, slides were developed with diaminobenzidine (Dako) as the chromogen. All slides were counterstained using Harris haematoxylin before being scanned using a Scanscope XT digital slide scanner (Aperio). Quantification was performed by analyzing bone metastases with Imagescope software (Aperio) using positive pixel count algorithm for Ki67 and Cleaved Caspase-3 staining. For quantification of Ki67 and Cleaved Caspase-3 staining, positively stained nuclei were represented as a percentage of total nuclei per field.

Statistical Analysis

All statistical analyses were conducted using SPSS Version 21 (Armonk, NY: IBM Corp). The Student t-test was used to test for significance. All data were expressed as Mean ± SEM. A p-value <0.05 A p-value of less than 0.05 was considered statistically significant.

Results

Local administration of zoledronate suppresses breast cancer-induced osteolysis:

Following 3 weeks of zoledronate treatment, the treated group demonstrated a statistically significant increase in the BV/TV (12.30±2.80%) compared to the control group (7.13±1.22%) (P<0.001, 95%CI=3.10 to 7.30); net increase is 72.51% (Fig.1A). Three-dimensional (3D) reconstruction of μ-CT images, established using the coronal (midline) and axial cut (5mm from the growth plate), demonstrated complete absence of the osteolytic lesions and an increase in trabecular bone formation in the zoledronate-treated tibiae compared to the control group (Fig.1B). To quantify tumour growth, mice were imaged weekly by longitudinal bioluminescence imaging. The control group showed an increase in photon emission (expressed as average radiance) from day five onwards, unlike treatment group that showed a slowdown of

tumour growth. However, a comparison between the mean photon emissions at the end of the experiment demonstrated no significant difference between the two groups. (*P*=0.24) (Fig.2). Additionally, an examination of the effect of zoledronate on tumour cells proliferation and apoptosis using Ki-67 antibody (marker of cell proliferation) and antibody active against CC3 (marker of cell apoptosis), revealed a decrease in Ki-67-positive cells (29.4±14.4%) in the treatment group as compared to the control group (44.9±7.7%). However, this decrease was not statistically significant (P=0.07, 95% CI=-2.30 to 33.21). The decrease in Ki-67 in the treated group was mirrored with an increase in the number of caspase-3-positive cells (0.03±0.02%) in the treatment group when compared to the control group (0.001±0.001%) (P=0.05, 95%CI= 0.00 to 0.10) (Fig.3).

Local versus systemic administration of zoledronate is superior for reducing cancer-induced osteolysis:

A significant statistical difference was found in BV/TV between the treated systemic group (7.53±0.75%) and the control systemic group (5.70±0.94%) (*P*= 0.01, 95% CI=0.61 to 3.20) (Fig.4A). In agreement with our previous results, a significant statistical difference was found in the BV/TV between the treated local group (10.90±1.25%) and the control local group (6.01±0.50%) (P<0.001, 95%CI=2.10 to 4.81) (Figure.4A). Comparing the BV/TV between the treated local group (10.90±1.25%) and the treated systemic group (7.53±0.75%) showed a significant statistical difference as well (*P*<0.001, 95%CI=2.01 to 4.81) with a net increase of 44.8% (Fig.4A). Three-dimensional (3D) reconstruction of μ-CT images, established using the coronal (midline) and axial cut (5mm from the growth plate), demonstrated an increase in trabecular bone volume in the locally-treated tibiae when compared to the systemically-treated group (Fig. 4B)

Discussion:

Local delivery recently gained wide popularity in the field of orthopaedic oncology. Multiple studies have investigated the potential of local delivery of therapeutic agents in at the site of bone tumour mainly for palliative measures [14] [15-18]. Gangi et al. reported the use of percutaneous alcohol injection directly into bone metastases in 25 patients with painful vertebral metastases leading to effective pain reduction and quality of

life improvement [14]. On the other hand, several studies reported local delivery of BPs for treating multiple bone pathologies [19,20]. Local elution of zoledronate from titanium implants in animal studies showed to increase in the net bone formation with clear signs of improved vascularity, maturity, and remodelling [19]. Locally-delivered BPs was also used to improve implants mechanical stability [21], and to enhance bone formation in an osteoporotic model [22]. Nevertheless, this is the first study to examine the effect of direct delivery of BPs into the site of bone metastasis.

In this study, we have used an intra-tibial model of bone metastasis that represents a model for late events in the bone metastatic process and allow us to examine the effect of localized delivery of BPs on the progression of established metastatic bone disease [23,24]. Our results demonstrated that local delivery of BPs significantly inhibited tumour-induced osteolysis in the treatment group; in fact, it preserved the bone completely. However, despite the substantial effect of BPs on tumour induced osteolysis, it failed to inhibit tumour growth significantly. On the other hand, a comparison between the local and the systemic delivery of BPs demonstrated that the anti-osteolytic effect of local delivery of BPs in bone metastasis model exceeds that of the systemic effect. Several studies have investigated the effect of systemic zoledronate on tumour-induced osteolysis [23,25-27]. Buijs et al. [23] examined the effect of systemic zoledronate in an intra-tibial model of bone metastasis and demonstrated a significant inhibition of tumour-induced osteolysis in an intra- tibial model of bone metastasis. Similarly, Peyruchaud et al.[26] showed that 3μg of zoledronate given daily, inhibited the formation of new lytic lesions and the progression of the established lesions [26]. The underlying mechanism of the inhibition of osteolysis has been mainly found to be due to the inhibition of osteoclast-mediated bone resorption [28].

Despite using a relatively high dose of zoledronate (2μg) directly at the site of bone tumour one week after cancer cell implantation, our results showed that tumour growth was not significantly different between treatment and control groups. These results were consistent with what is reported in the literature [29,30]. Buijs et al. [23] demonstrated that zoledronate significantly inhibited tumour-induced bone resorption. However, it did not inhibit local tumour growth. In addition, Pluijm et al. [31] examined the effect of pamidronate and olpadronate in an intra-tibial model of bone metastases. Treatment regimen started at day three after cancer

cells implantation. Their results showed that BPs inhibited osteolysis but it did not affect tumour growth as well. From the review of the literature, it seems that BPs treatment inhibits tumour growth in preclinical animal models only when treatment starts before the establishment of bone metastases. However, once the tumour reaches a certain size it is difficult to control the growth of this tumour with BPs administration only [31]. Moreover, it has been suggested that the BPs' high affinity for bone minerals bound their direct anti-tumour effect on the tumour cells in vivo [32].

To further justify the use of local delivery, we compared the local delivery of zoledronate to the systemic delivery with equal dose (0.025mg/kg), which represents the clinical dosage that has been used in human for treatment of bone metastases [12]. To minimize bias, we divided the mice into two control groups, one for systemic and one for local drug delivery. The rationale behind this randomization is a hypothetical effect of the increase in bone formation in the local group due to the repeated micro-fractures caused by our injection. However, the comparison between the two controls revealed no significant statistical difference (P=0.6). On the other hand, a significant statistical difference (P<0.001, 95%CI=2.10-4.81) in the BV/TV between the local group (10.90±1.25%) and the systemic group (7.53±0.75%), a net increase of 44.8%. These results showed that the effect of local delivery of zoledronate in bone metastases model exceeds that of the systemic effect. This has been explained by the fact that half the dose of systemic zoledronate reaches the bone with a short half-life of ten days [33]. Our results suggest that a large percentage of the dose reaches the bone; however, to our knowledge, there are no studies on the pharmacokinetic of direct injection of zoledronate.

Two major factors favor the use of local over systemic delivery with BPs. Clinically; local drug delivery of zoledronate can be utilized in cases where a complete resection of bone tumour is not possible due to vital structures invasions such as a spinal cord or major blood vessel. Given the anti-osteolysis effect of zoledronate, it can be used for palliation and maintaining stability. In addition, our results showed that other modalities used for local elution, including bone cement and different implants, can also be utilized in bone tumours. However, the effect on tumour growth is still questionable and needs further investigation. On the other hand, high doses of systemic BP is required to achieve clinical efficacious concentration for

neoplastic bone metastasis. However, high systemic BP route can cause severe renal toxicity, while oral administration is complicated by poor bioavailability (< 1 % in humans) and poor gastrointestinal tolerability. In addition, other side effects of systemic BPs treatment have been reported in the literature include joint pain, jaw osteonecrosis[10], ocular inflammation[34], and compromised bone growth in children [35]. Local delivery can provide direct delivery of zoledronate to the site of bone tumour without passing the systemic circulation, thus, avoiding undesirable side effects and ensuring better bioavailability.

In conclusion, lntra-tumour delivery of zoledronate demonstrated a substantial inhibitory effect of tumour-induced osteolysis. Additionally, the comparison between local and systemic delivery unexpectedly revealed that local delivery was more effective in osteolysis inhibition. Although tumour growth was not significantly inhibited, the noted effect of intra-tumour delivery of zoledronate triggers the need for further assessment of its anti-tumour properties. Also, further work needs to be performed to compare systemic effects of zoledronate in the non-affected skeleton as well as a dose reduction study to determine the minimum effective treatment for lysis inhibition.

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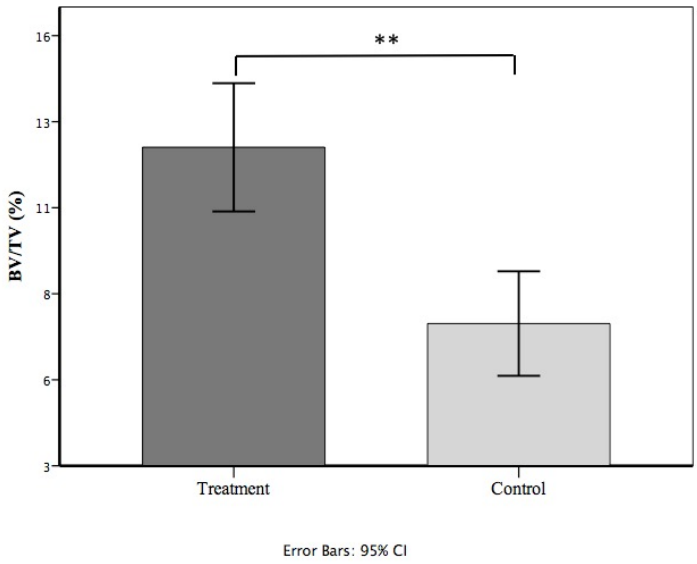
Figures

A)



B)

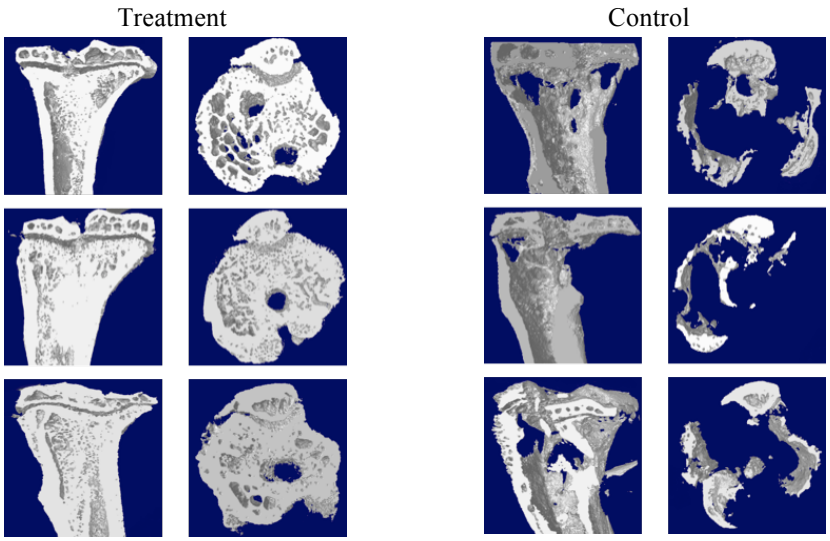
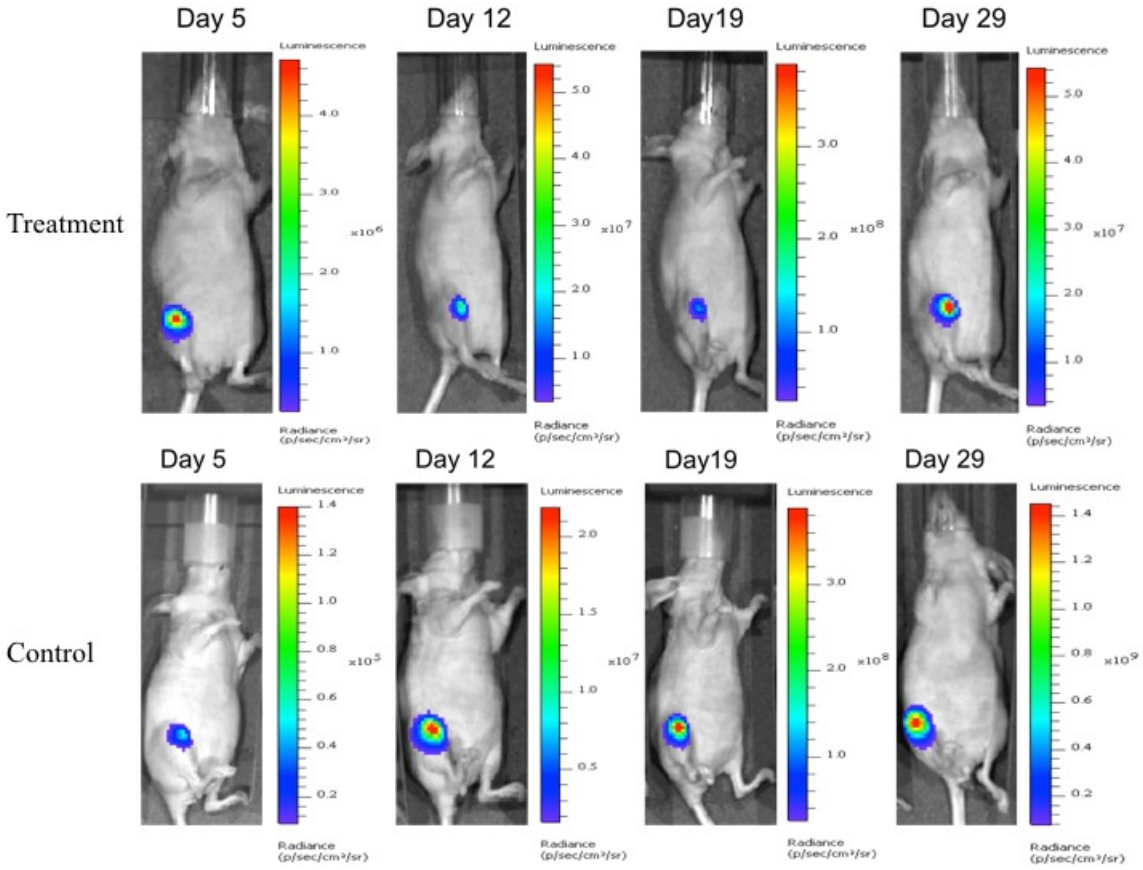


Figure 1. Quantitative and qualitative assessment of BV/TV using μ-CT. A) The treated group showed a significant increase in the BV/TV as compared to the control group **P<0.001. B) 3D constructed coronal and axial μ-CT images demonstrated a qualitative increase in the trabecular bone volume in the treated tibia when compared to the control.

A)



B)

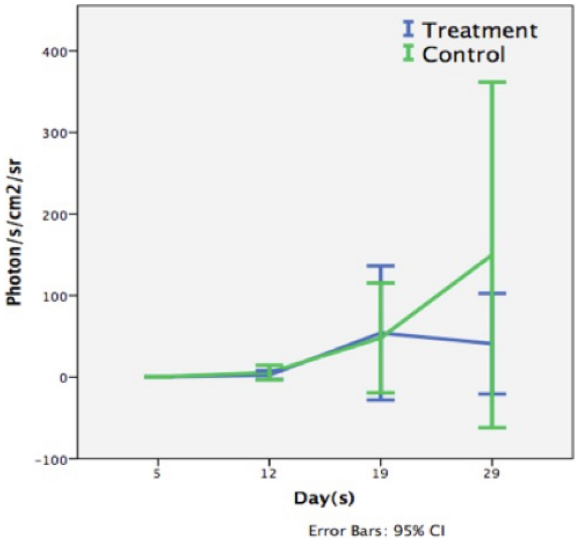
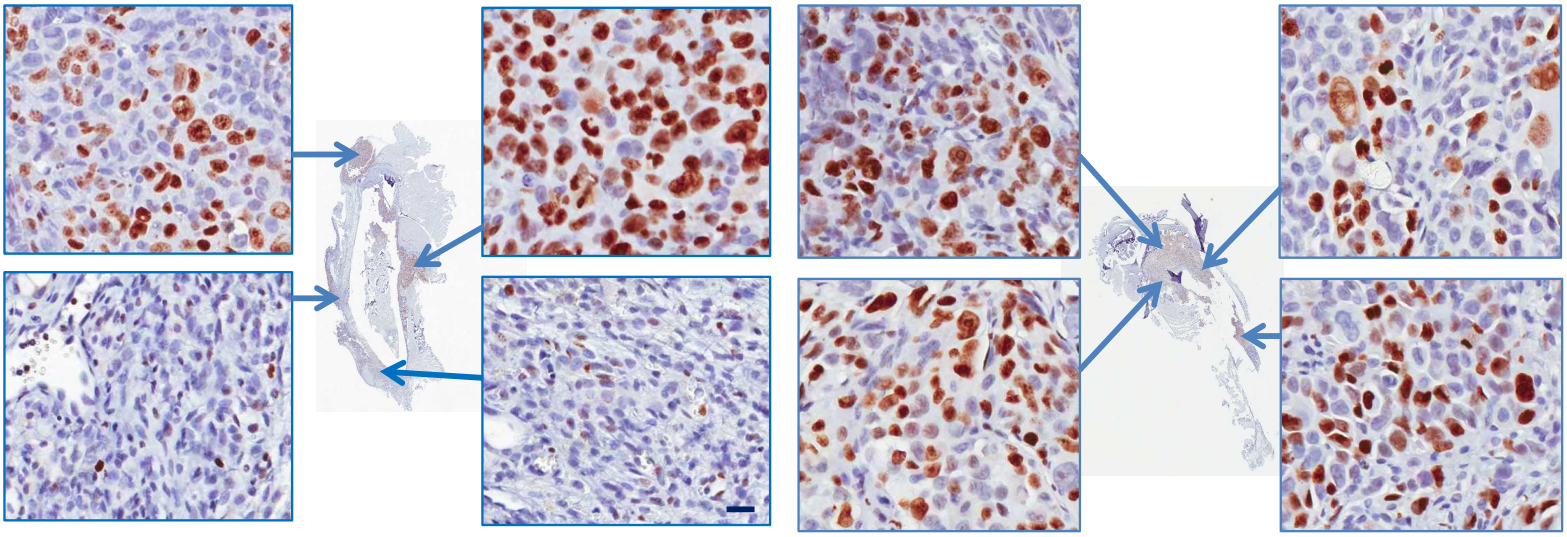


Figure 2. Real Tumours growth during local zoledronate treatment in intra-tibal breast cancer model. A) Representative images of tumours growth obtained from each group at different time points. B) Graph

showed the BIL measurements according to the average radiance. Treatment with zoledronate showed an insignificant statistical difference at day 29. (P=0.2)

A)

Treatment Control



B)

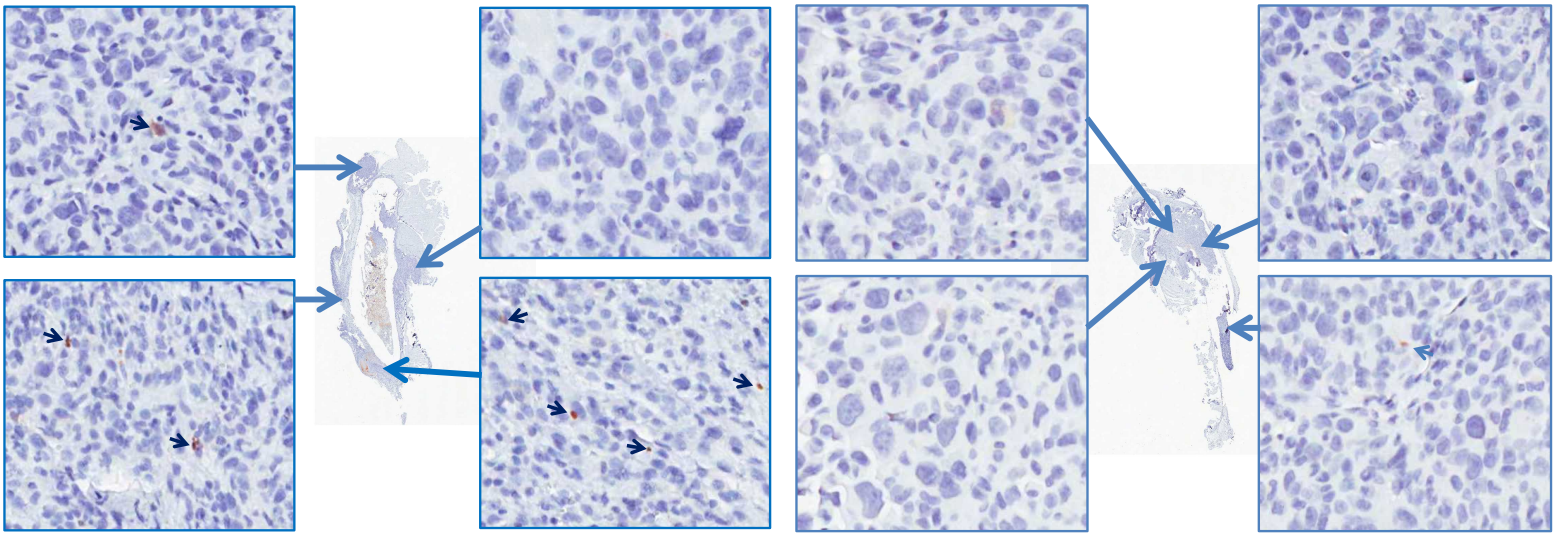
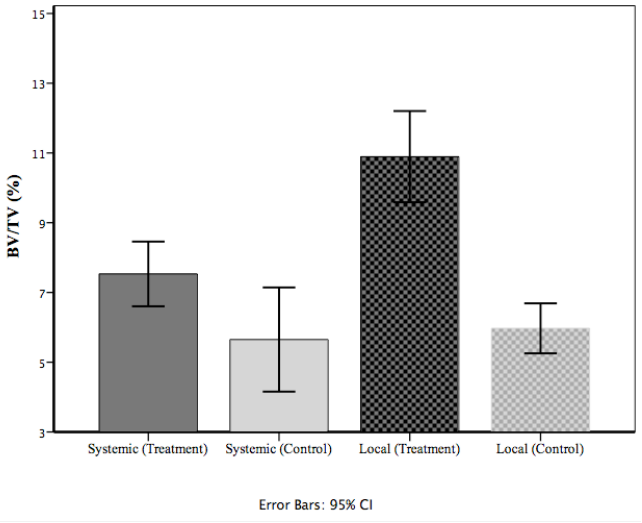


Figure 3. The effect of zoledronate (2μg, three times/week for three weeks) on tumour cells proliferation and apoptosis using immunohistochemistry. (A) Ki-67 antibody (marker of cell proliferation) revealed a statistically insignificant decrease in Ki-67-positive cells (29.4±14.4%) in the treatment group when compared to the control group (44.9±7.7%) (P=0.07). (B) Anti CC-3 antibody (marker of cell apoptosis) demonstrated a statistically insignificant significant increase in the number of caspase-3-positive cells (0.03±0.02%) in the treatment group when compared to the control group (0.001±0.001%)(P=0.05). Scale bar, 20μm

A)



B)

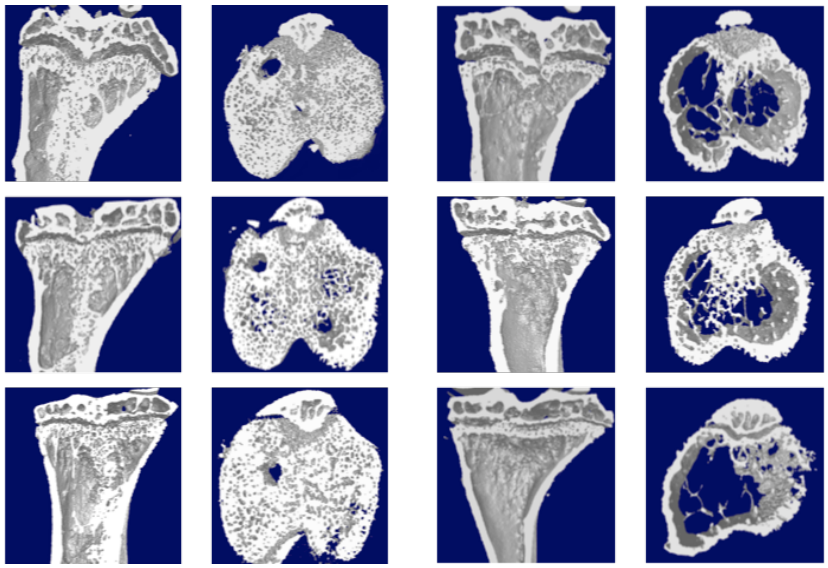


Figure 4. Quantitative and qualitative assessment of BV/TV using μ-CT. A) Our results showed a significant statistical difference in the BV/TV between the local group (10.90±1.25%) and the systemic group (7.53±0.75%)**P<0.001. B) 3D constructed coronal and axial μ-CT images demonstrate an increase in the trabecular bone volume in the locally treated tibia when compared to the systemically treated tibia.

Section 3: Conclusion

This study has shown that local delivery of BPs substantially inhibited tumour-induced osteolysis in an animal model of bone metastasis. The underlying mechanism of the inhibition of osteolysis has been mainly found to be due to the inhibition of osteoclast-mediated bone resorption.

A comparison between local and systemic delivery unexpectedly revealed that local delivery was more effective in osteolysis inhibition. Given the significant anti-osteolytic effect of locally delivered BPs, it can be utilized for palliation and to maintain stability at the site of tumour resection. Furthermore, it has been shown that that locally delivered BPs remains mainly localized with minimal systemic distribution thus, avoiding undesirable systemic side effects and ensuring better bioavailability.

Although tumour growth was not significantly inhibited, the noted effect of intra-tumour delivery of BPs triggers the need for further assessment of its anti-tumour properties. Additionally, further work needs to be performed to examine the effect of locally delivered BPs in the non-affected skeleton as well as a dose reduction study to determine the minimum effective treatment for lysis inhibition.

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