PHARMACOLOGICAL EVALUATION OF AN ADVANCED FORMULATION OF CURCUMIN TO PREVENT BREAST CANCER BONE METASTASES

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A thesis submitted in partial fulfilment of the requirements for the degree

of

Master of Philosophy

Faculty of Pharmacy

The University of Sydney

2018

STATEMENT OF ORIGINALITY

I hereby declare that I am submitting this thesis to The University of Sydney for the degree of Master of Philosophy in fulfilment of the requirements of the University. I also certify that this is my own work, carried out in the Faculty of Pharmacy under the supervision of **Dr. Pegah Varamini** and **Prof. David Hibbs and Prof. Ramin Rohanizadeh** and it does not have any material published by any other person.

I also certify that I acknowledged all the persons who helped to prepare this thesis. I also declare that the intellectual content of this thesis is the product of my own work and any work done by all co-authors in publication have been declared and appendix have been signed by the corresponding authors.

I also certify that this thesis has not been submitted in this University or any other institute for any degree either in full or partial fulfilment.

……..

September 2018

CONTENTS

Acknowledgement.....v

Glossary…….…..vi

Thesis chapter abstracts

Chapter 1. ……..... 7

Chapter 2…….……..7

Chapter 3……...…7

Chapter 4…….……..……8

Chapter 5……...…8

Chapter 1: Introduction

1. Thesis organization……..……..…….9

2. Reference……..……10

3. Aims & objective……..……11

Chapter 2: Different targeting strategies to prevent breast cancer bone metastases.

Abstract……..……..…12

1. Introduction ……..……13
2. Normal bone functions……..…..…..14
3. Cancer bone metastases……...…..…15
4. Bone pain……..……16
5. Treatment strategies……...…16
6. Radiation therapy……..……17
7. Bone targeted agents……..…...18
8. Novel bone targeted agents……..….21
9. Some FDA approved drugs for cancer treatment available in market……..……26
10. Conclusion…….……...28
11. Consent for publication…….……...28
12. Conflict of interest……..…..28

13. Acknowledgments……..……..28
14. References……..……..…29

Chapter 3: Pharmacological evaluation of an advanced formulation of curcumin to prevent breast cancer bone metastases.

Abstract……..36

1. Introduction…….…..…37
2. Materials and methods…….…....40
3. Results……..……42
4. Discussion…….....….49
5. Conclusions……..…52
6. Conflict of interest……...….52
7. Acknowledgments…….……...52
8. References…….....….53

Chapter 4: Conclusion, future directions and final remarks

1. Conclusion…….……...59
2. Future directions…….……..59
3. Final remarks…….……...59

Chapter 5: Appendices

1. Appendix 1…….……60
2. Appendix 2…….……72
3. Appendix 3…….……75
4. Appendix 4…….……76

ACKNOWLEDGEMENT

I would like to extend my thanks to all of those who help me to make this thesis a reality with their kind support and help

Foremost, I want to pay thanks to my **God Almighty** for bestowing upon me wisdom, strength and health to finish this project.

In preparing my thesis I would like to thank the following people:

I would be very appreciative and grateful to my Supervisor & advisor **Dr Pegah Varamini** for imparting her knowledge and apprehension to this study. Her timely help, guidance and supervision enable me to complete this goal.

I would like to convey my thanks to my **Family** for motivating me in completion of this thesis. I want to pay my gratitude to my "**Parents"** whose prayers always sheltered me in all troubles of life. I am greatly indebted to my supportive and beloved husband **Aftab Ahmed**, who was always by my side in times of need. I am also very grateful to my kids **Areesha & Inaya** who always cooperated with mummy's busy schedule.

I would greatly appreciate my friend **Annim Mohammad** who helped me in editing introduction part of second chapter of my work. She is PhD candidate at The University of Sydney.

I would like to thank my sweet friend "**Priyanka Thakur"** whose timely suggestions helped me out in achieving this goal.

I would like to say thanks my colleagues **Sepideh Khazeni** for helping me out in performing and analyzing uptake studies **& Ghada Aboueid** for conducting out IncuCyte live cell analysis with me.

I am very thankful to **Dipesh Khanal**, **Kamini, Nadeem Ahmed** and **Amjaad** for their suggestions and guidance.

Without all of these people help and support I would never be able to achieve my target.

Thank you very much everyone!!!

GLOSSARY

Following abbreviations are used in this thesis

NPs ……. Nanoparticles

Aln-Cur-NPs…...Alendronate conjugated curcumin nanoparticles

Cur-NPs……...Curcumin nanoparticles

CLSM……. Confocal laser scanning microscopy

OPG……..... Osteoprotegrin

M-CSF…… Macrophage colony stimulating factor

TGF-β……. Transforming growth factor-β

RANKL……...Receptor activator of NF- κB ligand

PTHrP……. Parathyroid hormone related protein

PDGF……...Platelet derived growth factor

IGF……...Insulin like growth factor

HPLC……...High performance liquid chromatography

LC……..... Loading capacity

IL……. Interleukin

SRE……. Skeletal related events

CTSK……Cathepsin K

BMP-2……. Bone morphogenetic protein

PEG…….....Poly ethylene glycol

MTTT……...3-(4,5-Dimethylthiazole-2-Yl)-2,5-Diphenyltetrazolium Bromide

THESIS CHAPTERS ABSTRACTS

CHAPTER 1

It is about thesis introduction and thesis chapters. It also includes historical perspective of breast cancer bone metastasis and aims and objectives of my work.

CHAPTER 2

Abstract: *Background:* Breast cancer is the most frequently diagnosed malignancy in women worldwide. Breast cancer tends to metastasize to bone. Around 70% of the breast cancer patients eventually develop bone metastasis. After the bone invasion, metastatic cells alter the balance between osteoblastic and osteoclastic activities, leading to skeletal complications, characterized by pain and pathological fractures and hence worsening the patient's quality of life. Once tumor invades the bone, it is hard to treat it with the so-far available treatments options (e.g. bisphosphonates and denosumab). Bone metastasis should be essentially controlled, in cancer treatment and there is a strong need to explore new, more efficient therapeutic targets. This review discusses the bone physiological processes and the recent advances in exploring different pathways involved in bone metastasis. Furthermore, some novel treatment options, which are under preclinical and clinical investigations, are highlighted. *Conclusion:* A deeper understanding of these metastatic pathways can provide oncology researchers with novel avenues for treating bone metastasis, one of the main challenges to cure breast cancer. The restoration of healthy bone environment will not only improve the patient's quality of life but also reduces the tumor burden.
Keywords: Bone Metastasis, Targeted strategies, Osteoblasts, Osteoclasts, Bone resorption, Novel targets, RANKL/RANK,

CHAPTER 3

Abstract

The most common cancer among women is breast cancer. According to an estimation by breast cancer network Australia, 18,087 women will be diagnosed with breast cancer in 2018. About 70% of the breast cancer patients develop bone metastasis. In pre-clinical investigations, curcumin reported to be non-toxic even at doses of 12 g per day. However, with this high dose of curcumin, only 50 nM plasma concentration is achieved. The reason for this low plasma concentration of curcumin is low water solubility and instability. We have previously developed a new nanoformulation of curcumin (Cur-NP) with enhanced physicochemical properties as well as improved antitumor activity in breast cancer cell lines. Furthermore, we have formulated alendronate-conjugated curcumin nanoparticles (Aln-Cur-NPs) for the targeted delivery of the drug payload (curcumin in this project) to the bone. This project aims to investigate the *in vitro* biological effects of Aln-Cur-NPs that are developed to prevent breast cancer bone metastasis. The loading capacity and particle size of the new batch fabricated for this study was determined and was shown to be consistent with previous batches of Aln-Cur-NPs and Cur-NPs. The loading capacity was found to be 4% and 5.7%, and the size was 28 nm and 23 nm for Aln-Cur-NP and Cur-NP, respectively. *In vitro* anti-tumor activity of the curcumin nanoparticles with and without alendronate conjugation, was evaluated in three different breast cancer cell lines and reported as IC_{50} values equivalent to the concentration of curcumin. A significantly higher antitumor activity was observed for Aln-Cur-NP compared to Cur-NP with IC_{50} values of 13.9, 22.2 and 7.7 µg/mL for MCF-7, MDA-MB-231 and SK-BR-3, respectively. This study showed the enhanced anticancer activity of

curcumin nanoparticles conjugated with alendronate compared to Cur-NPs, which strongly supports the synergistic effect of curcumin/bisphosphonates combination considering the similar amount of uptaken curcumin by the cancer cells for both nanoparticle formulations. The impact of nanoparticles on the viability of MDA-MB-231 cells was also investigated using recording time lapse image technology by IncuCyte® Zoom over two days. It was demonstrated that the uptake of raw curcumin was much less, and it precipitated outside the cells while, curcumin encapsulated in nanoparticles was effectively uptaken by the cancer cells. In the same experiment, we observed that Aln-Cur-NPs reduced the viability of the cells more effectively than Cur-NPs and raw curcumin.

The uptake of Aln-Cur-NPs and Cur-NPs in nucleus and cytoplasm in MDA-MB-231after 24 hours of treatment was revealed by Confocal Scanning Laser Microscopy. The qualitative analysis of confocal images showed higher uptake for Aln-Cur-NPs compared to raw curcumin ($p < 0.0001$) and no uptake for the untreated (PBS) control. Parathyroid Hormone Related Protein (PTHrP) release is increased by cancer cells in bone microenvironment and promotes osteoclastic activity and contribute to osteolytic bone metastases. The effect of our Nanoparticles on the release of PTHrP was determined by PTHrP ELISA assay for quantitative measurement of human PTHrP concentration released by MDA-MB-231 cells. MDA-MB-231 cells were treated with alendronate-modified and non-modified curcumin nanoparticles. Results showed a reduction in the release of PTHrP by MDA-MB-231 cell lines by both curcumin nanoparticles compared to the negative control (PBS-treated). Cur-NP and Aln-Cur-NPs showed twice higher activity in reducing the release of PTHrP compared to raw curcumin. These results suggested the possibility of reducing osteolytic activity of the cancer cells in bone metastasis. These preliminary data suggest Aln-Cur-NPs can offer promises in preventing and treating breast cancer bone metastases.

CHAPTER 4

It includes the conclusion and future directions..

CHAPTER 5

It includes appendices related to my publications and conference presentations.

CHAPTER 1

INTRODUCTION

1.1. Thesis Organization

This thesis is comprised of four chapters. **Chapter 1** encompasses the introduction of thesis. It also includes aims and objective of my work. **Chapter 2** includes a review article which has already been published in the journal "Current Pharmaceutical design". The publication's title is: "Different targeting strategies to prevent breast cancer bone metastases". This chapter includes introduction to breast cancer bone metastases, normal bone functioning, mechanism of breast cancer bone metastases, treatment strategies for breast cancer bone metastases, novel treatment strategies for preventing breast cancer bone metastases.

Chapter 3 is comprised of the manuscript of the paper which we are intended to publish soon on the topic "The pharmacological evaluation of an advanced formulation of curcumin to prevent breast cancer bone metastases'. This chapter includes the *In-vitro* evaluation of Alendronate –conjugated curcumin nanoparticles (Aln-Cur-NPs). *In-vitro* experiments include drug loading capacity measurement, anticancer activity of Aln-Cur-NPs, uptake studies of nanoparticles using confocal laser scanning microscopy (CLSM) and live cell analysis using IncuCyte. **Chapter 4** is about conclusion, future directions and final remarks. **Chapter 5** includes different appendices attached to thesis.

1.2. Historical Perspective

Cancer that spreads beyond breast to other organs is called breast cancer. According to a survey conducted by Australian Breast Cancer Network, 15,600 women and 145 men were diagnosed with breast cancer in 2015. It's almost 42 women each day were diagnosed having breast cancer. For women of 85 years old age group, 1 in 8 women was diagnosed to have breast cancer. In 2020, this number is expected to increase up to 17,210 women with breast cancer (1). About 90-95% of breast cancer patients are diagnosed in early stages. Breast cancer tends to metastasize to distant organs. Almost 20-30% of the breast cancer patients develop metastatic disease (2).

Bone is the most common metastatic organ (1). About 70% of metastatic patients develop bone metastases. Bone metastases is associated with skeletal morbidity (2). Furthermore, Bone is the storage area for different growth factors like transforming growth factor (TGF-β), insulin-like growth factor (IGF)-I and II, fibroblasts growth factor (FGF)-I and II and platelet derived growth factors. Once these growth factors get stimulated by cancer cells they further support the tumor growth in bone. About half of the patients suffer skeletal related events (SREs). SREs include spinal cord compression, pathological fractures and pain requiring radiation therapy or surgery (3, 4). High blood flow to the bone marrow and bone environment favors the residency and growth of cancer cells in the bone (5).

Currently available treatment options for bone metastases are bisphosphonates and denosumab. Other than these, radiopharmaceuticals, radiotherapy and surgery are of clinical value in managing bone metastases. Currently available treatment options focus on improving patient's quality of life by improving functional independence, preventing further SREs, managing pain and reducing pain. Unfortunately no preventive treatment is available (6).

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AIMS & OBJECTIVE

Once breast cancer spreads to the bones it is incurable, thus there is an absolute need for a preventative treatment against bone metastasis. Curcumin, a non-toxic plant extract has recently attracted much attention in medicine due to its remarkable therapeutic actions. We have demonstrated that formulating curcumin in nanoparticles significantly promotes its anti-cancer activities. In this project, we will extend this research by investigating the possible combination therapy to prevent breast cancer cells from spreading to the bones using curcumin and bisphosphonates, which are well-known anti-bone-resorptive agents currently used in palliative treatment in patients with bone metastatic cancer. The project will test the hypothesis that curcumin nanoparticles coated with bisphosphonates will reduce the risk of breast cancer bone metastasis.

The overall objectives of this research are:

- Preparation of nanoparticles following our previous work.
- Determination of loading capacity of nanoparticles.
- Determination of anti-proliferative effects of nanoparticles.
- Investigation of uptake of nanoparticles by MDA-MB-231 cells.
- Determining the inhibitory effect of curcumin nanoparticles on the release of PTHrP peptide.

Nanoparticles are safe and effective in cancer treatment. Nanoparticles also provide targeted therapy to cancer cells with direct killing of cancer cells without damaging the healthy cells. That is why they are most widely researched as a treatment option for cancer.

Firstly, curcumin nanoparticles were made according to our previous work. Our first target was to determine the loading capacity of nanoparticles and loading of curcumin in each nanoparticle should be determined.

Secondly, anti-proliferative effect of curcumin nanoparticles should be evaluated to determine IC_{50} values of curcumin nanoparticles.

Thirdly, to assure cellular internalization of nanoparticles, up-take studies were done.

Fourthly, effect of our nanoparticles was determined on the release of PTHrP from cancer cells.

CHAPTER 2

DIFFERENT TARGETING STRATEGIES FOR TREATING BREAST CANCER BONE METASTASES.

This chapter has been published in current pharmaceutical design as

Irshad I, Varamini P. *Different Targeting Strategies for Treating Breast Cancer Bone Metastases.* Current pharmaceutical design. 2018 Jun 19.

Curr Pharm Des. 2018 Jun 19. doi: 10.2174/1381612824666180619165728. [Epub ahead of print]

Different Targeting Strategies For Treating Breast Cancer Bone Metastases.

Irshad I[1], Varamini P[1].

Author information

Abstract

BACKGROUND: Breast cancer is the most frequently diagnosed malignancy in women worldwide. Breast cancer tends to metastasize to bone. Around 70% of the breast cancer patients eventually develop bone metastasis. After the bone invasion, metastatic cells disrupt the balance between osteoblastic and osteoclastic activities, leading to skeletal complications, characterized by pain and pathological fractures and hence worsening the patient's quality of life. Once tumor invades the bone, it is hard to treat it with, the so-far available treatments options (e.g. bisphosphonates and denosumab). Bone metastasis should be essentially controlled, in cancer treatment and there is a strong need to explore new, more efficient therapeutic targets. This review discusses the bone physiological processes and the recent advances in exploring different pathways involved in bone metastasis. Furthermore, some novel treatment options, which are under preclinical and clinical investigations, are highlighted.

CONCLUSION: A deeper understanding of these metastatic pathways can provide oncology researchers with novel avenues for treating bone metastasis, one of the main challenges to cure breast cancer. The restoration of healthy bone environment will not only improve the patient's quality of life but also reduces the tumor burden.

KEYWORDS: ; Bone Metastasis; Bone resorption; Novel targets; Osteoblasts; Osteoclasts; RANKL/RANK; Targeted strategies

PMID: 29921200 DOI: 10.2174/1381612824666180619165728

DIFFERENT TARGETING STRATEGIES FOR TREATING BREAST CANCER BONE METASTASES.

Breast Cancer Bone Metastasis: Different targeting strategies.

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Abstract

Abstract: *Background:* Breast cancer is the most frequently diagnosed malignancy in women worldwide. Breast cancer tends to metastasize to bone. Around 70% of the breast cancer patients eventually develop bone metastasis. After the bone invasion, metastatic cells alter the balance between osteoblastic and osteoclastic activities, leading to skeletal complications, characterized by pain and pathological fractures and hence worsening the patient's quality of life. Once tumor invades the bone, it is hard to treat it with, the so-far available treatments options (e.g. bisphosphonates and denosumab). Bone metastasis should be essentially controlled, in cancer treatment and there is a strong need to explore new, more efficient therapeutic targets. This review discusses the bone physiological processes and the recent advances in exploring different pathways involved in bone metastasis. Furthermore, some novel treatment options, which are under preclinical and clinical investigations, are highlighted. *Conclusion:* A deeper understanding of these metastatic pathways can provide oncology researchers with novel avenues for treating bone metastasis, one of the main challenges to cure breast cancer. The restoration of healthy bone environment will not only improve the patient's quality of life but also reduces the tumor burden.

Keywords: Bone Metastasis, Targeted strategies, Osteoblasts, Osteoclasts, Bone resorption, Novel targets, RANKL/RANK,

1. INTRODUCTION

According to the Australian Institute of health and welfare 2017 report, 17730 Australians (17,586 of women and 144 of men) are diagnosed with breast cancer and this number will increase to 18,235 Australians by 2018. Around 3,000 patients died of breast cancer in 2017 (1), mainly due to advanced breast cancer. Patients with advanced breast cancer disease undergo aggressive therapy and most of them experience severe side effects. Roughly, 70% of metastatic breast cancer patients develop bone metastasis, which may be complicated or uncomplicated bone metastasis. Uncomplicated bone metastasis can be characterized as the metastasis involving painful bone but not associated with existing pathologic fracture, spinal cord compression or cauda equina compression, while complicated bone metastasis is characterized by pathological fractures and spinal cord and cauda equina compression (2) (Fig. **1.1**). Sometimes those associated with soft tissue components or those within weight bearing bones at high risk of fracture are also considered complicated. Bone metastases result in skeletal-related events (SREs) that can be described as spinal cord compression hypercalcemia, pathological fractures (excluding significant traumas), necessity for surgery to bone or bone radiation therapy (3). The microenvironment, where bone linked with bone marrow is ideal for tumor growth (4). Transcriptional analysis showed the involvement of gene for chemokines (CXCR4) involved in homing, matrix metalloproteinases (MMPs) involved in invasion, fibroblast growth factor (FGF) involved in angiogenesis, Interleukin-11(IL-11) and osteopontin (OPN) involved in osteolysis. It was shown that tumor cells that cause bone metastasis are characterized to invade healthy bone

tissue, increasing their multiplication and causing skeletal destruction (5, 6). This ongoing process eventually leads to an increase in bone pain, immobilization and progressively worsening the quality of life (7).
This review will first provide an insight into the healthy bone physiological processes. Subsequently, mechanisms involved in breast cancer metastasis to bone in addition to some novel targets and treatment options that are under investigation will also be discussed.

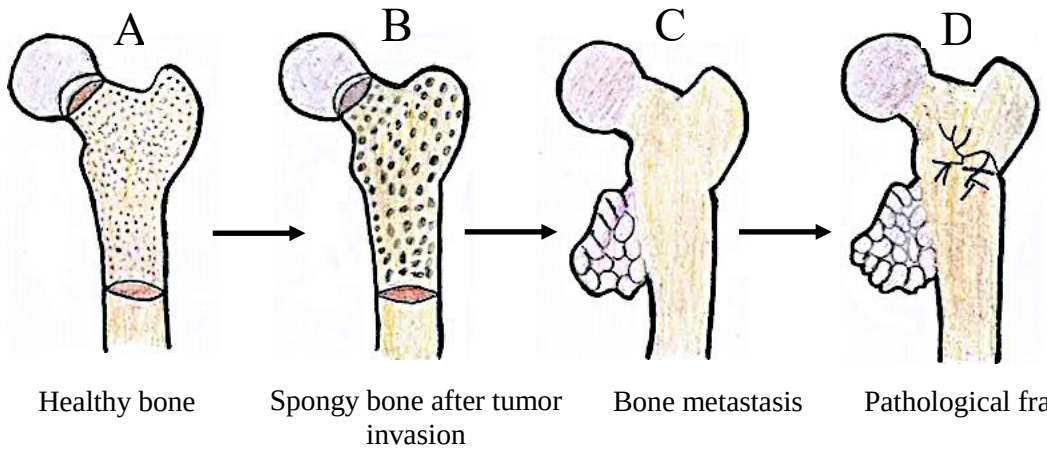


Fig. (1.1). Changes in the bone structure from healthy bone tissue (A) to pathological fractures due to the cancer cell invasion and bone metastasis (D).

2. NORMAL BONE FUNCTIONS

Bone constitutes human skeleton. Human skeleton has structural and locomotor functions as well as being a calcium reservoir. During growth, the bone size is increased. Bone mineralization occurs during childhood and adolescence period (8).

2.1. Modelling

Through modelling the bones are shaped and adapt to load bearing and other influences. Modeling leads to bone mass, size and geometrical changes.

2.2. Remodelling

Microfracture repair happens regularly in normal individuals throughout their lives. This involves existing bone resorption, new bone deposition and mineralization. The whole process is called remodeling. An adult's skeleton undergoes complete remodeling every decade. Bone remodeling regulation is crucial to explain bone metastasis as malignant tumor exploit these pathways to boost cancer growth and bone destruction. Bone remodeling involves the contribution of 2 types of cells including 1) osteoblasts liable for bone matrix production, mineralization, and remodeling process initiation and 2) osteoclasts accountable for bone resorption.

2.3. Bone Formation and Resorption

Bone growth, modelling and remodeling are based on bone formation and bone growth. Osteoblasts are responsible for bone formation.

2.3.1. Osteoblasts

Osteoblasts contribute to the synthesis and mineralization of osteoid. Osteoid is a material responsible for bone shape, hardiness, and resilience. Some parameters that can be used to measure bone formation include 1) osteoid components like osteonectin, osteopontin and osteocalcin and 2) bone-specific alkaline phosphatase (BSAP).

2.3.2. Osteocytes

Osteoblasts which get captured into the new bone matrix (9, 10) are named as osteocytes. Osteocytes constitute 90% of bone cells and are developed from osteoblasts who have completed their role in bone formation.

Osteocytes develop into the osteocyte-osteoclast-osteoblast network system. Osteocytes are responsible for healing microfractures and harmonize remodeling (11). When microfracture occurs, osteocytes undergo apoptosis and sends signals to osteoclasts to begin bone resorption and remodeling. Osteocytes have regulatory roles on osteoclasts (8).

2.3.3. Osteoclasts

Osteoclasts execute bone resorption through the fusion to the bone, constituting a ring of firm junctions that are regulated by α5β3 integrins (12). After binding, osteoclasts secrete acids and proteases (e.g., lysosomal cathepsins, MMPs phosphatases). Acids dissolve hydroxyapatite from bone and cathepsin, in contact with MMPs, degrades the collagen matrix. Osteoclasts endocytose debris from bone degradation. Later, osteoclasts discharge their content (high levels of calcium, magnesium, phosphates and products of collagen) into the blood stream and thus can be used to determine the value of overall bone resorptive activity of serum or urine. Osteoclasts differentiation is critically effected by receptor activator of nuclear factor-κB (RANK) ligand and macrophage colony-stimulating factor (M-CSF) (8).

2.3.4. Mechanism of Normal Bone Remodeling

In normal bone remodeling, osteoblasts express RANKL (NF- κB ligand) that binds to RANK on the surface of osteoclasts and their precursors. This binding regulates the osteoclasts differentiation from their precursors. Osteoclasts activation and survival lead to increased bone resorption. However, osteoblasts secrete osteoprotegrin (OPG) that inhibit excessive bone resorption by binding to RANKL and prevent binding with RANK. Hence, the balance between RANKL/Osteoprotegrin expression determines the bone mass in both normal and disease state (13). Osteoblasts and osteoclasts are the basic units of normal bone remodeling (Fig. **1.2A**). Osteoblasts are derived from mesenchymal stem cells under control of osteoblastic transcription factor called Runx2. Mononuclear myeloid precursors are fused to form pre-osteoclast. Pre-osteoclasts are differentiated into activated, multinuclear osteoclasts. This differentiation is controlled by colony-stimulating factor (M-CSF) and RANKL (receptor activator for NF- κB ligand). After activation, osteoclasts get adhered to bone and cause degradation of bone. Osteoblasts also produce a decoy receptor to RANKL called osteoprotegerin (OPG). The RANKL to OPG ratio is determinant of osteoclast activity. Bone lining cells and osteocytes also constitute osteoblastic lineage.

3. CANCER BONE METASTASES

3.1. Mechanism of Bone Metastasis

After invading the bone marrow microenvironment (Fig. **1.2B**) tumor cells disrupt the RANKL/osteoprotegrin (OPG) expression balance that leads to the over-production of osteoclasts. Additionally, tumor cells induce angiogenesis that enhances bone resorption and makes the bone tissue irregular and weak, causes abnormal bone formation via osteoblasts (14, 15), structural malformation, fracture and bone pain (16). Bone resorption is responsible for the release of various factors such as transforming growth factor (TGF-β and IGF1) and calcium. These further aggravate tumor growth and deregulation of RANKL/OPG expression. This is a vicious tumor growth cycle where increased bone resorption reinforces more tumor growth and *vice versa* (17). Relocation of cancer cells to the bone disturbs the normal cycle of the bone turnover, forms lytic, sclerotic tissue or mixed metastasis, which leads to substantial pain and reduced prognosis (18, 19). Once cancer cell crosses the intrinsic barriers, it will take over the control of additional homeostatic factors (20). Different environmental barriers that cancer cells have to cross include physical barrier (basement membrane), chemical barriers (hypoxia, reactive oxygen species and low pH), and biological barriers (immune surveillance, regulatory extracellular matrix, inhibitory cytokines) (21, 22). Breast cancer cells establish strong interaction with the microenvironment once released from primary tumor site and reside in the bone marrow (23). After that, breast cancer cells secrete factors that activate NF- κB ligand (RANKL)-dependent and -independent stimulation of osteoclast bone resorption (24). Fig. **1.2A** shows some pathways in normal bone environment and Fig. **1.2B** shows mechanism of metastatic bone environment. Breast cancer cells in malignant bone microenvironment secrete growth factors, cytokines and parathyroid hormone-related protein (PTHrP) which have negative impact on osteoblast function. In malignant bone environment, RANKL is increased and OPG is reduced which leads to more osteoclast formation and bone

degradation. A significant reduction occurs in osteoblasts differentiation and no more osteoid is available to compensate osteoclastic bone resorption.

Current therapeutic targets include RANKL, PTHrP and bone hydroxyapatite. Matrix metalloproteinases (MMPs), cathepsin K and transforming growth factors (TGF)-β Insulin-like growth factor (IGF), monocyte chemotactic protein-1 (MCP-1), Platelet-derived growth factor (PDGF), Vascular endothelial growth factor (VEGF) are also under investigation to target bone metastases (25) (Fig. **1.2B**).

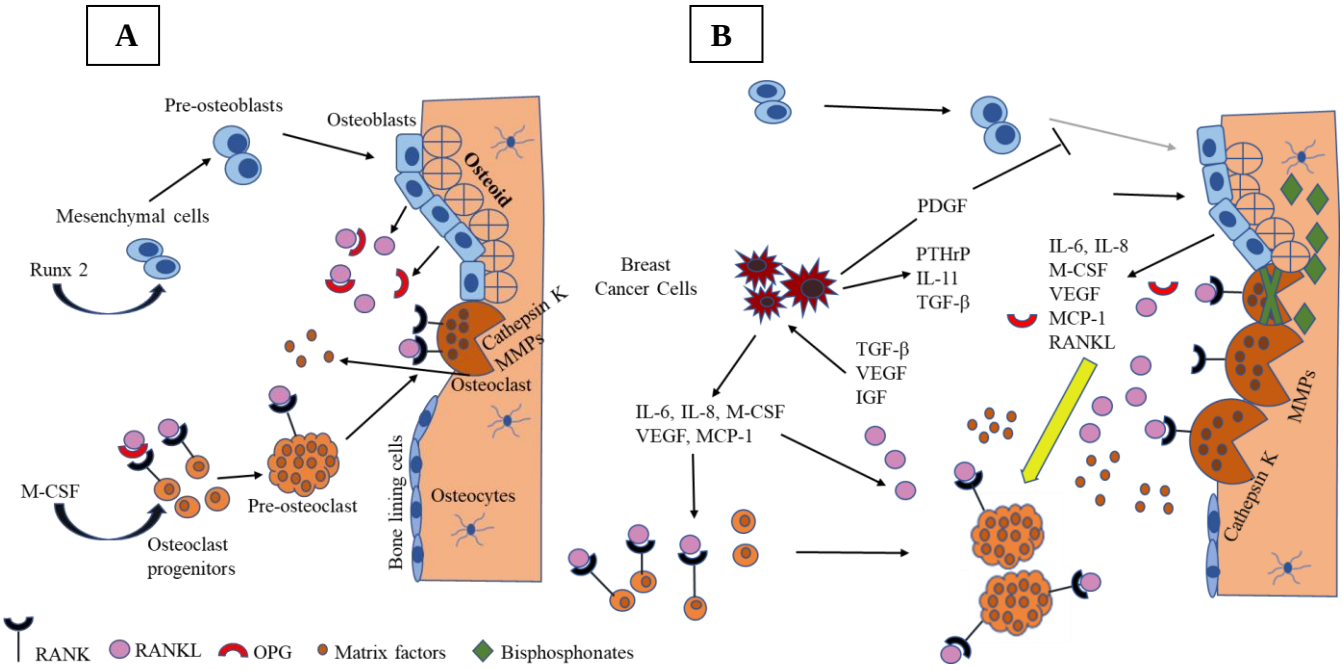


Fig. (1.2). The Bone Microenvironment (25). A) Normal bone remodeling processes, B) osteolytic bone metastases.

3.2. Bone Pain

Cancer pain is caused mostly due to metastatic bone disease (26-28). However, it is notable that not all bone metastasis lead to pain and pain intensity is not always proportional to size and degree of metastatic lesions in the bone. Metastatic bone pain is mostly a neuropathic pain, transmitted by primary efferent nociceptor peripheral nerves. These peripheral nerves have many types of receptors for noxious stimuli detection, including acidity, lipid metabolites, heat and inflammatory molecules. Persistent acidic and inflammatory environment of metastatic lesions cause sustained stimulation, allodynia (central pain sensitization) and hyperalgesia (hypersensitivity to pain). Thus, any agent that has potential to antagonize inflammatory mediators can be a potential therapeutic agent for managing cancer pain. A single or multiple radiotherapy sessions delivering 8Gy or 20Gy was helpful in managing this type of pain (29). Radioisotopes can be administered as a drink, capsule or injection into a vein (30). Radioisotopes are easy to administer, less toxic and effective in subclinical metastatic sites but cannot be delivered in precise doses (31). Analgesics are recommended for managing metastatic bone pain. Standards of care should be accompanied with bone-modifying agents to manage cancer bone pain as they could exert a synergistic effect (3).

4. TREATMENT STRATEGIES

4.1. Treatment for Uncomplicated Bone Metastases

Treatment options available for treating uncomplicated bone metastasis include bone-targeted agents along with radiation therapy. It has been proved that both single and multi-fractionated radiotherapy is equally effective for treating uncomplicated bone metastasis (32).

4.2. Treatment for Complicated Bone Metastases

Treatment options for complicated bone metastasis mainly include bone surgery and radiotherapy. Zoledronic acid, pamidronate or denosumab are recommended to be administered because they have been shown to delay the use of analgesics. In a phase III randomized clinical trial, a single dose of 8 Gy radiation was found to be effective for palliating spinal cord compression. For the patients who were suffering from bone metastases neuropathic pain however, multifractionated treatment (20Gy in 5 fractions) was shown to be more effective than a single fractionated treatment (8 Gy in 1 fraction).

4.3. Radiation Therapy

Mechanism of pain relief following radiation therapy is poorly understood. Many clinical trials with different scoring and reporting methods are available but guidelines for irradiation are still unclear because of great variation in beneficial results. Three different types of radiation therapy are used including local-field, wide-field and radionuclide therapy are shown in Table **1.1** (33).

Table 1.1: Radiation therapy for bone metastases (33).

Radiation Therapy	**Delivering method**	**Pain relief rate**	**Examples**	**Indications**
Local-field radiation therapy Conventional treatment	Delivered using photons	80-90%	40-46 Gy/ 20-23 fractions 30-36 Gy/10-12 fractions Gy	Used for patients with localized pain: less than four metastatic sites without visceral sites (lung, liver, central nervous system).
Wide-field radiation therapy Systemic radiation therapy	MeV units (from Co 60 to 15 MeV linear acceleration)	64-100% 50-66% of patients maintain pain relief for remaining life	Upper wide field treatment (from skull to L2-3) is 6 Gy Lower-wide field (from L3-4 to above the knees) or mid-body wide field treatment (from L1 to upper third of the femurs) is 8 Gy	Used for widespread symptomatic bone metastases or as an adjuvant to local-field irradiation to reduce frequency of re-treatment.
Radionuclide therapy Systemic radiation therapy	Radioisotopes (high linear energy transfer)	37-91% from 89St* 58% from 186Re* 72% of 153Sm*	89St 186Re 153Sm 223Ra	It is used in combination with bisphosphonates and radiation therapy in the treatment of bone metastatic disease.

*St: Strontium, Re: Rhenium, Sm: Samarium, Ra: Radium

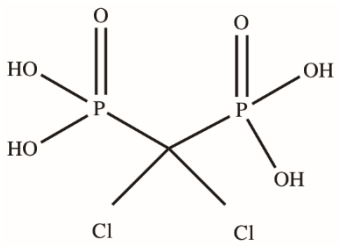
5. BONE-TARGETED AGENTS

5.1. Inorganic Pyrophosphates

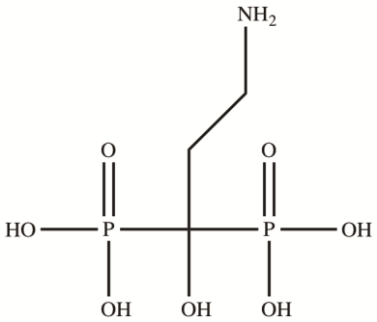
Inorganic pyrophosphate analogs, also called bisphosphonates, can exert their role *via* two mechanisms (34):

1) Interfering with and hampering the osteoclast survival process.
2) Stimulating the apoptosis of osteoclasts

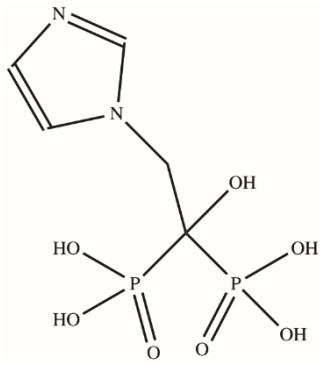
Through these mechanisms, bisphosphonates can regulate bone turnover and reduce tumor-related bone resorption (34). Bisphosphonates can be classified into amino-bisphosphonates and non-amino bisphosphonates. Among these, amino-bisphosphonates are predominantly utilized in clinical interventions (34, 35). Ibandronate, pamidronate and zoledronic acid (amino-containing) and clondronate (non-amino containing), are bisphosphonates (Fig. **1.3)** available in clinic to treat bone metastasis from breast cancer (34), prostate cancer (36), lung cancer (37) and multiple myeloma (38, 39). Some examples of derivatives in clinical trials are shown in Table **1.2 (40)**.



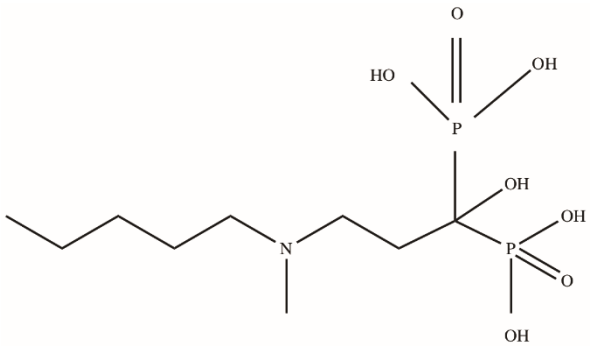
Clondronic acid



Pamidronic acid



Zoledroni acid



Ibandronic acid

Fig. (1.3). Chemical structures of some bisphosphonates

Table 1.2: Clinical Trials for Bisphosphonates (40).

Goal	Study Type	Country	Clinical trial Gov. Identifier	Status
Oral bisphosphonates(Alendronate sodium) to prevent bone loss in postmenopausal women with early breast cancer, receiving anastrozole therapy and determine how long treatment is needed.	Phase III	Australia	NCT00122356	Completed 13/03/2013
Identification of Risk Factors` for skeletal related events in breast cancer patients receiving bisphosphonates for bone metastasis.	Cohort	Canada	NCT01144481	Completed 07/01/2015
Studying long term bone quality in women with breast cancer receiving bisphosphonates (Clondronate sodium, demeclocycline hydrochloride, ibandronate sodium, tetracycline hydrochloride, zoledronic acid)	Observational	United States	NCT00873808	Withdrawn 10/04/2013
Safety and efficacy of zoledronic acid when added to standard therapies in patients with breast cancer and metastatic bone lesions.	Phase III	Germany	NCT00372710	Terminated 23/11/2009

5.2. Denosumab

Denosumab is a human monoclonal anti-RANKL antibody used for the treatment of osteoporosis, bone metastasis, treatment-induced bone loss, and giant cell tumor of bone (41). It inhibits the activation of RANK receptors by directly binding to these receptors on the surface of osteoclasts (42). Inhibition of RANKL-RANK receptor interaction by denosumab causes reduction in tumor-induced bone demolition (41, 42). Some clinical trials involving denosumab are shown in Table **1.3 (40)**.

Table 1.3. Clinical Trials for Denosumab (40).

Name	Study Phase	Country	Clinical trial Gov. Identifier	Status
Can denosumab prevent recurrence in the bone when given in early stage breast cancer?	Phase III	Argentina and 40 other countries	NCT01077154	Ongoing
Does denosumab reduces the rate of first clinical fracture in women with non-metastatic breast cancer receiving aromatase inhibitor?	Phase III	Austria, Sweden	NCT00556374	Ongoing
Study of denosumab with zoledronic acid in treatment of bone metastasis in subjects with Breast cancer.	Phase III	Argentina and 35 other countries	NCT00321464	Completed 08/03/2017
Study of denosumab in breast cancer subjects with bone metastasis who have not previously been treated with bisphosphonates therapy.	Phase II	United States	NCT00091832	Completed 28/01/2017
A study to evaluate denosumab in young patients with primary breast cancer.	Phase III	Australia, Belgium	NCT01864798	Terminated 05/09/2017
Study of denosumab as adjuvant treatment for women with high risk early breast cancer receiving neoadjuvant or adjuvant therapy.	Phase III	Argentina and 40 other countries	NCT01077154	Ongoing

5.3. Effectiveness of bisphosphonates in Clinical Trials.

Bisphosphonates including clodronate, pamidronate, ibandronate and zoledronic acids were widely studied in women with breast cancer bone metastasis. These Placebo-controlled trials indicated the effectiveness of bisphosphonates for reducing SREs and are summarized in Table **1.4** (43-52).

Table 1.4. Placebo controlled trials of bisphosphonates (53).

Name	**Hypercalcemia**	**Skeletal Morbidity**	**SREs**	**Pain**
Clodronate 1600 mg orally daily *vs*. Placebo (43-45)	Total hypercalcemic events reduced	Reduced Fractures	Increased time to first SREs	Reduced Pain intensity
Pamidronate 45 mg i.v. every 3 weeks *vs*. placebo (46)				Increased Pain relief
Pamidronate 90 mg i.v. every 3-4 weeks *vs*. placebo for 2 years (47)			Reduced Proportion of patients With SREs complications	
Pamidronate 60 mg i.v. every 4 weeks *vs*. placebo (48).	Increased time to hypercalcemic events		Reduced SREs	Increased time to progression of pain
Ibandronate 2 mg or 6 mg i.v. every 3-4 weeks *vs.* placebo for 2 years (49)		Reduced mean no. of bone events	Increased time to first SREs	
Ibandronate 50 mg orally daily *vs*. placebo for 96 weeks (50)		Reduced skeletal morbidity	Decreased risk of skeletal related events	
Ibandronate 6 mg i.v. every 4 weeks *vs.* placebo for 24 months (51)			Reduced Proportion of patients With SREs complications Increased time to first SREs	
Zoledronic Acid 4 mg i.v. every 4 weeks *vs*. placebo for 1 year (52)			Reduced rate of SREs Increased time to first SREs	

5.4. Adverse Effects and Management of Bone-Targeted Therapies

5.4.1. Acute Phase Reaction

Acute phase reaction (APR) is a systemic host defense response by which the innate immune mechanisms are activated due to inflammation, injury or infection. About 10 to 30 % of patients who have been treated with zoledronic acid and denosumab were shown to experience APR side effects. This can be observed during first three days after treatment. During this process, there is an increase in the number of acute phase proteins (APRs) that are involved in homeostasis, causing influenza-like symptoms, chills, fever, lethargy, increased protein catabolism, reduced appetite, flushing, bone pain, hypotension, myalgia, and arthralgia (54-57). Laboratory analysis shows increased tumor necrosis factor-alpha (TNFα) and interleukin-6, neutrophilia, and leukocytosis.

Most of the reactions are spontaneously reversed after 72 hours of first dose application or can also be managed with non-steroidal anti-inflammatory drugs and antipyretics (54-56, 58).

5.4.2. Nephrotoxicity

Bisphosphonates have a renal route of excretion. About 40-70% of administered dose of bisphosphonates is excreted through kidneys (57, 59, 60). Unmetabolized bisphosphonates accumulate in tubular cells and can cause apoptosis which leads to acute kidney injury. This type of renal injury could be reversible (e.g. zoledronic acid) or irreversible (e.g. pamidronate). Denosumab is least likely to cause renal injury and can be used as a choice of medication for patients with kidney failure and dependent on dialysis (56, 61, 62). Preventive measures include monitoring of phosphate, serum creatinine, calcium levels and avoiding the administration of multiple nephrotoxic drugs. Patients with renal impairment should get a reduced dose of zoledronic acid (63).

5.4.3. Hypocalcemia

Chances of hypocalcemia with bisphosphonates therapy are 3.4-6% and with denosumab treatment is 5.5-13%. Clinical manifestation could be, general weakness, lethargy, and fatigue (64-66). Calcium and vitamin D supplements are vital, especially for patients having pre-existing vitamin D or renal insufficiency, hypomagnesaemia, impaired thyroid and parathyroid activity, geriatric patients or patients having gastric surgery.

5.4.4. Jaw Osteonecrosis

Jaw osteonecrosis is caused by vascularization defects in the maxilla or the mandibular bone. This may occur followed by head and neck radiotherapy, use of bisphosphonates or denosumab (67, 68). During the last decade, 2% of cases of jaw osteonecrosis have been linked with denosumab therapy and 1.4% with zoledronate therapy. Oral health evaluation during bisphosphonates and denosumab therapy is critical to consider. It is essential to avoid invasive dental procedures (69, 70).

5.4.5. Rare Side Effects

Denosumab and bisphosphonates can cause conjunctivitis, scleritis, uveitis (70-75), dermatitis, eczema, rashes (76, 77) or rare atypical femur bone fracture (78, 79).

6. NOVEL BONE-TARGETED AGENTS

Although denosumab and bisphosphonates are potential agents in improving the quality of life of patients with breast cancer bone metastasis, they have not been proved to provide progression-free and overall survival improvement from the disease. So, research to explore new potential therapeutic agents is going on. Bone destruction due to breast cancer is a complicated process and mediators that can serve as the basis for developing novel targeted agents are under investigation (80-82).

6.1. Novel targets for osteoclast-mediated bone resorption inhibition

6.1.1. RANKL/RANK

RANKL/RANK pathway plays a key role in the regulation of bone resorption (41). Osteoblasts have RANKL which is a transmembrane surface protein and can be cleaved by proteases into soluble form (83). RANKL (both Soluble and membrane-bound forms) can bind to RANK receptors present on the surface of osteoclast precursor. After binding with the receptor, they will cause osteoclastogenesis. OPG is a cytokine receptor and a RANKL antagonist which is produced by osteoblasts and has the ability to inhibit RANKL/RANK interaction (41). Deregulation of RANKL and OPG balance is observed in breast cancer (84). Thus, OPG has potential to reduce bone destruction and reduce SREs in breast cancer bone metastasis. This activity is exhibited by enhanced osteoclast activity and is confirmed in OPG knockout mice (85-87).

6.1.2. c-Src Kinase Inhibitors

Cellular Src Kinase (c-Src) is a member of Src family (non-receptor tyrosine kinases), also known as proto-oncogene c-Src. C-Src phosphorylates specific tyrosine residues in other proteins. Elevated c-Src levels are associated with cancer progression (88, 89). c-Src is engaged in performing multiple functions including adhesion,

invasion, migration, metastasis, and angiogenesis via chemokine receptor signaling (CXCL12/CXCR4/Akt) pathway or by inhibiting the functions of apoptosis - inducing ligand pathway (90). Enhanced expression and increased activity of c-Src has been investigated in a variety of cancers. Inhibitors of c-Src kinases have been proven to play a pivotal role in tumor cell invasion and proliferation. Selective tyrosine kinase inhibitors (TKIs) cause inhibition of c-Src kinases by blocking osteoclast differentiation (91, 92). Some preclinical investigations reported that dasatinib, bosutinib, and saracatinib have inhibited osteoclast differentiation (90, 93, 94). Dasatinib monotherapy has proven efficacious in advanced breast cancer bone metastasis patients (95, 96). Related clinical trials are shown (Table **1.5)**.

Table 1.5. Investigated targets for the treatment of bone metastasis (40).

Target	Compound	Phase	Country	Clinical trial Gov. Identifier	Status
Cathepsin K	odanacatib	Phase III	United States	NCT00691899	Withdrawn 12/08/2016
C-Src	dasatinb	Phase II	United States	NCT00410813	Completed 02/072017
Avβ3 integrin	etaracizumab	None			
TGFβ	fresolimumab	None			
	trabedersen	None			
	galunisertib	None			
CXCL12/CXCR4	plerixafor	None			
	LY2510924	None			

6.1.3. Cathepsin K (CTSK)

Cysteine cathepsins are among hydrolytic enzymes and members of the family of papain-like cysteine proteases in lysosome. A cysteine lysosomal protease, called cathepsin K or CTSK is primarily present in osteoclasts. It induces degradation of bone collagen and ultimately causes bone resorption (84, 97). A preclinical investigation done in animal models of breast cancer bone metastasis showed cathepsin K inhibitors are effective in preventing bone destruction. Furthermore, cathepsin K antagonist can play their role not only in bone resorption inhibition but also in stimulation of bone formation (98). Cathepsin K may directly act on cancer cells. Odanactinib (a cathepsin K inhibitor) has been proved to successfully reduce the level of bone resorption marker called urinary N-telopeptide of type-I collagen. However, there may be some disadvantages associated with cathepsin K inhibitors. For example, balicatib (AAE-581, Novartis) is a nitrogen-containing cathepsin K has the ability to accumulate in lysosomes. Due to this accumulation, activities of other lysosomal cysteine cathepsins are inhibited which may lead to severe adverse effects like stroke and skin reactions. For example, morphea-like skin reactions are noticed in a phase II clinical trial in which patients received balicatib therapy for 12 months. As a result, balicatib was withdrawn from clinical trials (97, 99). This adverse effect is not shown by odanacatib (MK-0822, Merck), which is under clinical trial investigation for osteoporosis treatment. Odanacatib has successfully reduced bone resorption markers in a phase-II trial in women having breast cancer bone metastasis after four weeks of

therapy (100). But odanacatib was withdrawn from the regulatory approval process due to increased risk of stroke (101).

6.1.4. Integrins

Integrins belong to a heterodimeric transmembrane glycoprotein family that mediates adhesion to extracellular matrix proteins and immunoglobulins. So far, 24 heterodimers have been developed by incorporating 18α and 8β subunits. Many types of integrins have an association with bone metastasis but αvβ3 performs a more crucial role in osteoclast function and bone metastasis (102). According to a preclinical study, some peptidic (e.g. S247, cilengitide, ATN-161) and non-peptidic (e.g. PSK1404) compounds that target αvβ3 could inhibit osteolysis and tumor growth in bone metastasis animal models (103, 104). These αvβ3 inhibitors, not only antagonized the osteoclast-mediated bone resorption but PSK1404 also prevented bone colonization by cancer cells expressing αvβ3 integrins at the dosage regimen that does not block bone resorption (103). GLPG0187, ATN-161, IMGN388, cilengitide are different αvβ3 antagonists which are in clinical trials for breast cancer bone metastasis (105).Clinical trial investigations revealed that L-000845704 (a non-peptide antagonist developed by Merck) could, inhibit bone resorption in osteoporosis. Investigations are underway to study its applications in oncology as well (105).

6.1.5. Proteasome

Proteasome is an extra-lysosomal proteolytic enzyme complex. The ubiquitin-proteasome system is involved in degrading intracellular proteins. This system involves the tagging of many intracellular proteins with ubiquitin (which is a small regulatory protein) and then these intracellular proteins are recognized by 26S proteasome complex, resulting in the degradation of these proteins into small peptides. Many proteasome inhibitors (PIs) are under clinical investigation. Preclinical data suggested that PIs exert their effect on three kinds of cells. First, by inhibition of osteoclast differentiation and their function (106). Second, they enhance bone formation through stimulating osteoblasts differentiation, up-regulating bone morphogenetic protein 2 (BMP-2) and inhibiting runt-related transcription factor (RUNX2) (107). Finally, PIs block cell proliferation and activate apoptosis in many cancer cells (108, 109) and induce osteolysis in breast and prostate cancer bone metastasis in animal models(110, 111). However, clinical trials did not show the expected results.

6.1.6. Hedgehog

Cancer progression involves the activation of Hedgehog (Hh) signaling pathway (112) which is also important in the regulation of cancer stem cells. Hh ligand (Desert, Indian and Sonic Hh) bind to transmembrane protein receptors (Patched receptors). Hh Inhibitors exert direct cytotoxic effects on cancer cells. In preclinical animal models, Hh inhibitors blocked osteoclastogenesis and bone metastasis. A phase II clinical trial was designed to investigate the effect of selective SMO (it is a smoothened protein encoded by *SMO* gene) antagonist (sonidegib) in early stage breast cancer (NCT01757327), but it was withdrawn before enrolment.

6.2. Novel targets for restoration of osteoblast functions

6.2.1. Dickkopf-1 (DKK-1)

Wnt signaling pathways are protein signal transduction pathways that pass signals into the cell through cell surface receptors. DKK-1 is a glycoprotein with a significant role in amphibian's head formation via antagonizing the Wnt signaling pathway. Osteoblastogenesis process involves Wnt signaling pathway. Wnt proteins in association with low density lipoprotein receptor-related proteins 5 and 6 (LRP5/6), bind Frizzled receptors (G Protein-coupled receptors) and initiates signaling via β-catenin. This process activates different genes involved in osteoblastogenesis (113). DKK-1 binds to LRP5/6 and blocks its binding with Wnt-1, causing breakdown of β-catenin and inhibit osteoblast differentiation. DKK-1 was shown to be elevated in serum and bone marrow of patients with multiple myeloma (114). Neutralizing antibodies that block DKK-1 cause reduction in osteolysis, skeletal tumor growth in addition to an increase in the osteoblast number and osteocalcin level in the serum (115, 116). There are some preclinical and clinical evidence that breast cancer cells that metastasize to bone secrete DKK-1 (117). A Phase I clinical trial investigates a combination of DKK1-neutralizing antibody, BHQ880 and zoledronate in myeloma patients(117).

6.2.2. Sclerostin

Sclerostin is a secreted glycoprotein that is encoded by *SOST* gene. It is produced by osteocytes. Sclerostin promotes migration, invasion of cancer cells and osteolysis and has anti-anabolic effect on bone formation. Sclerostin binding to LRP5 receptors can be blocked by antibodies that neutralize sclerostin (118). Sclerostin neutralizing antibody is used to treat osteoporosis e.g. romosozumab (119). However, no clinical trial is available to study the effects of sclerostin-blockers on metastatic bone disease.

6.2.3. Activin A

Activin A is widely distributed in all human tissues and belongs to TGF-β family of growth factors. Serine and threonine kinase transmembrane receptors mediate the effects of activin A. Activin A activates ActR1B or ALK-4 type 1 receptors that leads to the phosphorylation of receptor-regulated Smad proteins (RSmad4), Smad2, Smad3 and Smad4. Activin A gets entered in the nucleus that results in gene transcription regulation in bone cells. Activin A activates bone degradation, triggers osteoclast differentiation and inhibits osteoblast differentiation (120). Higher serum levels of Activin-A are found in breast cancer patients with bone metastasis as compared to the patients without bone metastasis (121). Therefore, this cytokine can be regarded as a potential target for more specific treatment measures for skeletal metastasis. In an *in vivo* humanized multiple myeloma induced bone disease model, Activin A targeting by a soluble decoy receptor, reversed osteoblast inhibition and inhibited tumor growth (122). RAP-011 is an activin type IIA receptor fused to a murine IgG-Fc fragment can restore bone mass (123). Recently, different groups have shown the combined effect of RAP-011 with Act RIIA receptors to serve as potential therapeutic targets in treatment of skeletal metastasis. RAP-011 can be measured as biochemical marker of bone metastatic disease (121). Sotatercept (ACE-011), a recombinant activin receptor type IIA and human globulin G (IgG), binds to activin A receptors. Sotatercept is potentially important for preventing bone loss and deposition of new bone in myeloma patients with osteolytic lesions (124). Sotatercept treatment demonstrated clinically significant decrease in bone pain, increase in the bone formation biomarkers, antitumor activity and increase in hemoglobin levels (125, 126).

6.2.4. Endothelin-1

Endothelins are peptides that constrict blood vessels. They produce their effect by binding to their receptors, ET_A and $ET_{B1,}$ ET_{B2} and ET_C receptors. Breast cancer cells produce endothelin-1 (ET-1) that activates mitogenesis in osteoblasts, resulting a reduction in osteoclast activity (127). ET_A antagonist ABT-627 (atrasentan) could inhibit osteoblastic breast cancer bone metastasis (128). Bosentan is a dual endothelin receptor antagonist (ET_A and ET_B receptor) approved to be used in treatment of pulmonary artery hypertension. This mixed inhibitor was shown to block breast cancer bone metastasis *in vivo* (129).

6.3. Novel Targets for Bone-Derived Growth Factors

6.3.1. Transforming growth factor-Beta (TGF-β) Signaling

TGF-β is a multifunctional cytokine of transforming growth factor superfamily, having four different isoforms (TGF-β1, TGF-β2, TGF-β3 and TGF-β4).

TGF-β binds to TGF-β type I receptor (ALK5) and TGF-β type II receptors (TβRII) which are serine/threonine heterodimeric kinases. It phosphorylates Smad2 and Smad3 which are TGF-β specific mediators. This Phosphorylated complex then binds to Smad4 and translocates to the nucleus and regulates TGF-β genes. TGF-β in turn regulates the growth of many factors like IL-6, IL-8, IL-11, integrin αvβ3, MMP-1 and CXCR-4 which play a key role in bone metastasis (130).

Hence inhibition of the TGF-β signaling can be considered as a potential target to reduce bone metastasis. Many strategies have been developed to block TGF-β signaling including TβRI inhibitors, dominant negative TβRII, neutralizing TGF-β antibodies and antisense oligonucleotides. These have been investigated to inhibit bone metastasis to breast cancer in preclinical trials. Although the effects of these TGF-β inhibitors have been investigated in different types of cancers, no clinical trials have been performed to explore their effect in breast cancer bone metastasis (130, 131).

Epithelial-to-mesenchymal transition (EMT) process has been found to play a role in cancer and metastasis progression. In this process, epithelial cells gain migratory and invasive properties and become mesenchymal

stem cells and initiate metastasis. TGF-β signaling through Smad pathway serves as an effector of this process (132). Exogenous Bone morphogenetic protein-7 (BMP-7) inhibits TGF-β signaling which antagonizes EMT signaling in prostate and breast cancer bone metastasis model in animals (133, 134). Another animal study revealed the role of TGF-β signaling in the regulation of the Jagged1-Notch pathway. Jagged1 is a cell surface protein that regulates Notch signaling pathway. Up-regulation of JAG 1 has been found to be associated with poor breast cancer survival rates. MRK-003, a γ-secretory inhibitor, has shown to inhibit Jagged1-Notch signaling pathway and hence cause a reduction in bone metastasis to breast cancer (135). These findings revealed that a strategy against breast cancer bone metastasis can be developed based on TGF- β-dependent EMT signaling, γ-secretase or BMP-7 inhibitors.

6.3.2. Insulin-like Growth Factors (IGFs)

Insulin-like growth factors (IGF-I and IGF-II) exist abundantly in bone and have been involved in spreading, development and aggressiveness of many different cancers. IGFs exert their action by binding to IGF type I receptors (IGF-IR). IGFs activate IGF-IR/Akt/NF-kB pathway, stimulates proliferation and increases bone tumor burden (136). An IGF-IR inhibitor e.g, PQIP (Chemical formula C3OH31N7) reduced the osteolytic lesion size in breast cancer bone metastases (137).

6.4. Novel Agents Targeting Bone Environment

6.4.1. Chemokine Receptor Signaling (CXCL-12/CXCR-4)

Almost all types of cells secrete chemokines. Most of the chemokines are involved in adaptive and innate immune systems, while a few of chemokines such as CXCL-12 that are produced by the osteoblasts, play a pivotal role in the regulation of cellular trafficking. It is proved that chemokines play a vital role in cancer metastasis (138). Chemokine receptors like, CXCR3, CCR4, CXCR4, CCR5 and CCR7 and especially CXCR, are found to be involved in the metastasis regulation process. CXCR4 is found to play a fundamental role in organ-specific breast cancer metastasis, including liver, lung and bone metastasis. In these organs CXCL-12 (CXCR4 ligand) is produced in high quantity (138).

The proposed mechanism is that after CXCL-12 binds to CXCR4 and activates the non-receptor Src, tyrosine kinase, AKT pathway is activated in bone marrow breast cancer cells (139). Consequently, the CXCL-12/CXCR-4 pathway can serve as a targeted therapy to treat bone metastasis. Synthetic peptide antagonist like CTCE-9908 and antibodies could block this CXCL-12/CXCR-4 pathway and reduce bone and lung metastases caused by breast cancer cells in preclinical experiments (140, 141).

6.4.2. Cadherin-11

Osteoblasts and bone marrow stromal cells express cadherin-11, which is a member of type 2 cadherin family. In one animal study it was demonstrated that the overexpression of cadherin-11 in breast cancer cells was associated with metastasis to bone but not to the lungs. This finding suggested that cadherin-11 can be used as a specific and novel target for treating bone metastasis. Yet, no agent has reached clinical trial (136).

6.4.3. Targeting Runx2

The bone transcription factor Runx2 that is a member of Runt-Related Transcription factor (Runx) family has crucial role in bone development by controlling osteoblasts and osteoclast processes (142, 143). It has been proved that Runx2 facilitate the interaction between cancer cells and the microenvironment of bone. Runx2 suppresses the ubiquitination of oculo-dento-digital dysplasia-hypoxia inducing factor (ODDD) HIF-1α by directly binding to ODDD-HIF-1α. Vascular angiogenesis during endrochondral bone formation is regulated by HIF-1α and vascular endothelial growth factor (VEGF). Runx2 has been identified to be involved in tumor invasion by regulating matrix metallopeptidase 9 (MMP9) (144). It has also been proved to play a crucial role in osteoclasts activation by gene regulation for OPN, M-CSF and PTHrP. Runx2 indirectly blocks Wnt signaling pathway and promotes activation of osteoclasts (144).

6.4.4. Targeting microRNAs (miRNAs)

MicroRNAs (miRNAs) belong to 21-23nucleotide- noncoding, long RNAs which are transcribed by RNA polymerase types II and III. Generally, miRNA cause either mRNA degradation or translational silencing by binding to their complementary site at the 3-untranslated region (145). It is evident that normal and cancer cells have different expressions for miRNAs. They can either enhance or inhibit the development and progression of the tumor. Many types of miRNAs have been found to be involved in regulation of bone metastasis (146). Thus, miRNAs involved in bone metastasis development can serve as a target for treating breast cancer bone metastasis. Very few miRNAs, *e.g.* miR-141 and miR-219, are found to inhibit osteoclast activity and osteolytic activity in breast cancer bone metastasis. miR-203 and miR-219 also have reducing effects on breast cancer bone metastasis (147). Several miRNAs associated with cancer have been discovered in humans, including miR-10b, miR-16-2, miR26a1, miR26-a2, miR-126, miR-17-92, miR-15b (148).

Several miRNAs, either directly or indirectly, regulate Runx2 in breast cancer progression. miRNAs are associate with bone metastasis initiation (let-7g, miR-146a, miR-335, osteolytic activity (miR-133a, miR-190). Further investigation is required to explore the regulatory role of Runx2 via miRNA and its potentials as a novel target for bone metastases (144).

6.5. Targeting Cancer Stem Cells

Stem-like cells (CSCs) are tumorigenic cells and may generate tumors through the stem cell renewal and differentiation (149, 150). The bone marrow biopsy sample from cancer patients showed that majority of early metastatic cells have CSC markers (150, 151). In a recent pre-clinical study, CD44-positive CSC-like cells were shown to have an increased capacity to metastasize to bone (152). Cancer stem cells markers include CD44 (breast, prostate and liver cancers), E-Cadherin (prostate, breast and brain cancers), CD166 (cellular proliferation), CD13 (liver cancer), CD90 (liver, breast and lungs cancers), CD105 (renal, breast and liver cancers (153, 154))The CSC biology is yet to be fully understood. CSCs and their niches could be considered as targets for preventing and treating breast cancer bone metastasis (154).

6.6. FDA approved Drugs for Cancer Treatment available on the market.

Some of the FDA approved breast cancer drugs are given in Table **1.6.**

Table 1.6: Some FDA approved drugs for breast cancer available on the market (19)

Brand Name	Generic Name	Manufacturer	Drug Type	Indication	Approval Date
Perjeta	Pertuzumab	Genentech	Monoclonal Antibody	First line treatment of $HER2^+$ metastatic breast cancer	June 2012
Halaven	Eribulin mesylate	Eisai	Macrocyclic Ketone Analogue	Metastatic breast cancer	November 2010
Xgeva	Denosumab	Amgen	Human Monoclonal Antibody	Preventing skeletal-related events in patients with bone metastasis from solid tumors	November 2010
Evista	Raloxifene hydrochloride	Eli Lilly	Estrogen receptor modulator	Prevention/Treatment of osteoporosis and reduction of breast cancer risk in postmenopausal women	September 2007
Ixempra	ixabepilone	Bristol-Myers Squibb	Epothilone B Analog	Breast Cancer	October 2007
Tykerb	lapatinib	GlaxosmithKline	Dual Tyrosine Kinase Inhibitor	Breast cancer	March 2007
Herceptin	Trastuzumab	Genentech	Monoclonal Antibody	Metastatic breast cancer	October 1998
Nolvadex	Tamoxifen citrate	AstraZeneca	Selective estrogen receptor modulator	Breast Cancer	October 1998
Xeloda	Capecitabine	Roche	Antimetabolite	Advanced breast cancer tumors	April 1998
Quadramet	Samarium Sm 153 Lexidronam Injection	Dupont Merck Pharmaceutical Company	Chelated complex	Pain associated with bone cancer	March 1997
Aredia	Pamidronate disodium for injection	Chiron	Nitrogen containing Bisphosphonates	Osteolytic bone metastasis of breast cancer	August 1996
Arimidex	Anastrozole	Astrazeneca	Aromatase Inhibitor	Advanced breast cancer in postmenopausal women	January 1996
Taxotere	Docetaxel	Rhone Poulenc Rorer	Microtubule Inhibitor	Locally advanced or metastatic breast cancer	May 1996

7. CONCLUSION

Bone metastasis significantly affects the quality of life of patients with breast cancer and new targeted strategies are in urgent demand to prevent and palliate skeletal events. Currently available clinical treatments can often shrink or slow the growth of bone metastases. However, these treatments are not able to eradicate bone metastatic foci. Bone metastasis progresses over time and leads to SREs, substantial morbidity and mortality and there is insufficient evidence available to demonstrate which bone modifying agent is the preferred choice. Advances in the discovery of different novel targets described in this review, not only provides insights into making a better use of the currently available agents but also the development of new targeted therapeutic interventions. These novel targets can also be used in combination with the treatment options available in clinic to effectively inhibit the development of bone metastasis in women with breast cancer. More in-depth preclinical and clinical investigations are required to optimize the current treatment strategies by elucidating the interactions between tumor cells and bone microenvironment to reach maximum effectiveness. Further investigations are warranted to discover new agents that can prevent bone metastasis in breast cancer patients to avoid the associated morbidity and mortality due to the bone metastasis.

CONSENT FOR PUBLICATION

Not applicable

CONFLICT OF INTERST

The authors declare no conflict of interest

ACKNOWLEDGEMENTS

We acknowledge National Breast Cancer Foundation Research Fellowship to Pegah Varamini (PF-16-007).

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CHAPTER 3

PHARMACOLOGICAL EVALUATION OF AN ADVANCED FORMULATION OF CURCUMIN TO PREVENT BREAST CANCER BONE METASTASES

We are planning to submit the paper to Nanomedicine (IF: 6.692)

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Abstract

The most common cancer among women is breast cancer. According to an estimation by breast cancer network Australia, 18,087 women will be diagnosed with breast cancer in 2018. About 70% of the metastatic breast cancer patients develop bone metastasis. In pre-clinical investigations, curcumin was reported to be non-toxic even at doses of 12 g per day. However, with this high dose of curcumin, plasma concentration of curcumin is only 50 nM. The reason for this low plasma concentration of curcumin is low water solubility and instability. We have previously developed a new nanoformulation of curcumin (Cur-NP) with enhanced physicochemical properties as well as improved antitumor activity in breast cancer cell lines. Furthermore, we have formulated alendronate-conjugated curcumin nanoparticles (Aln-Cur-NPs) for the targeted delivery of the drug payload (curcumin in this project) to the bone. This project aims to investigate the *in vitro* biological effects of Aln-Cur-NPs that are developed to prevent breast cancer bone metastasis. The loading capacity and particle size of the new batch fabricated for this study was determined and was shown to be consistent with previous batches of Aln-Cur-NPs and Cur-NPs. The loading capacity was found to be 4% and 5.7%, and the size was 28 nm and 23 nm for Aln-Cur-NP and Cur-NP, respectively. *In vitro* anti-tumor activity of the curcumin nanoparticles with and without alendronate conjugation, was evaluated in three different breast cancer cell lines and reported as IC_{50} values equivalent to the concentration of curcumin. A significantly higher antitumor activity was observed for Aln-Cur-NP compared to Cur-NP with IC_{50} values of 13.9, 22.2 and 7.7 µg/mL for MCF-7, MDA-MB-231 and SK-BR-3, respectively. This study showed the enhanced anticancer activity of curcumin nanoparticles conjugated with alendronate compared to Cur-NPs, which strongly supports the synergistic effect of curcumin/bisphosphonates combination considering the similar amount of uptaken curcumin by the cancer cells for both nanoparticle formulations. The impact of nanoparticles on the viability of MDA-MB-231 cells was also investigated using recording time lapse image technology by IncuCyte® Zoom over two days. It was demonstrated that the uptake of raw curcumin was much less, and it precipitated outside the cells while curcumin encapsulated in nanoparticles was effectively uptaken by the cancer cells. In the same experiment, we observed that Aln-Cur-NPs reduced the viability of the cells more effectively than Cur-NPs and raw curcumin.

The uptake of Aln-Cur-NPs and Cur-NPs in nucleus and cytoplasm in MDA-MB-231after 24 hours of treatment was revealed by Confocal Scanning Laser Microscopy. The qualitative analysis of confocal

images showed higher uptake for Aln-Cur-NPs compared to raw curcumin ($p < 0.0001$) and no uptake for the untreated (PBS) control. Parathyroid Hormone Related Protein (PTHrP) release is increased by cancer cells in bone microenvironment and promotes osteoclastic activity and contribute to osteolytic bone metastases. The effect of our nanoparticles on the release of PTHrP was determined by PTHrP ELISA assay for quantitative measurement of human PTHrP concentration released by MDA-MB-231 cells. MDA-MB-231 cells were treated with alendronate-modified and non-modified curcumin nanoparticles. Results showed a reduction in the release of PTHrP by MDA-MB-231 cell lines by both curcumin nanoparticles compared to the negative control (PBS-treated). Cur-NP and Aln-Cur-NPs twice higher activity on the reduction in the release of PTHrP compared to raw curcumin. These results suggested the possibility of reducing osteolytic activity of the cancer cells in bone metastasis. These preliminary data suggest Aln-Cur-NPs can offer promises in preventing and treating breast cancer bone metastases.

1. Introduction

The most commonly diagnosed cancer in women is breast cancer (1). About 70% of the metastatic breast cancer patients develop bone metastasis (2). Median survival for patients with breast cancer bone metastasis is 19-25 months (3). Bone metastasis is a major cause of morbidity as it leads to impaired mobility, pathologic fractures, severe pain, bone marrow aplasia, spinal cord compression and hypercalcaemia (2). The biggest problem encountered in treating cancer is the inability to deliver effective drug to the cancer cells without affecting the normal cells (4). The new treatment strategy for treating cancer requires targeted delivery of drug to only cancer cells with more advantages and less side effects (5).

Tumor cells interact with the microenvironment of specific organs to produce metastatic lesions (6). According to Stephen Paget's 'seed and soil' hypothesis, tumor cells act as 'seeds' and have affinity for particular 'soil', that is, the 'organ' (7, 8). Once cancer cells target a specific organ, they take control of the whole environment (Fig. **2.1**). Cancer cells during epithelial-to-mesenchymal transition (EMT), loose epithelial polarization and cell surface intercellular adhesion proteins in order to exhibit mesenchymal properties (9). Subsequently, tumor cells release proteolytic enzymes to dissolve extracellular matrix of tumor stroma (10).Then, cancer cells can invade local tissue, migrate to the surrounding cells (11, 12) and enter the systemic circulation, known as circulating-tumor cells (CTC) (13-15). Furthermore, tumor cells develop certain mechanisms to escape from immune cells through mechanisms that involve up-regulation of CD47 proteins (16, 17). Cancer cells develop different signaling pathways to promote CTCs to develop metastatic lesions. One of these signaling pathways is the development of chemokine receptor (CXCL12-CXCR4) signaling for cancer cell adhesion and survival (18-23). Different studies demonstrated the expression of non-receptor cytoplasmic tyrosine kinase (Src) in the bone marrow through stimulation of CXCL12-CXCR4 receptors and by increased resistance to tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) in bone marrow microenvironment (24).

After invasion to bone, cancer cells become either osteolytic (promote bone break down) or osteoblastic (promote bone formation) (25). Breast cancer normally cause osteolytic lesions and have the highest rates of fracture (26).

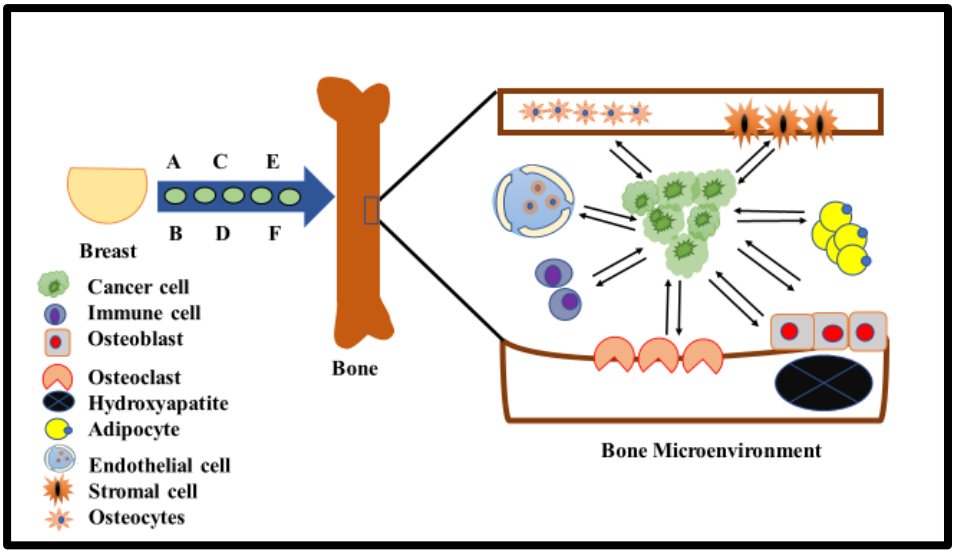


Fig. (2.1): Effect of cancer cells on bone microenvironment

Recently, it is demonstrated that T cells and B cells immune cells can also produce receptor activator of nuclear factor kappa-B ligand (RANKL binds RANK on osteoclasts), affect osteoclastogenesis and proliferate bone metastatic environment (27). Adipocytes support cancer cells to survive as an energy source (28, 29). Tumor cells secrete osteolytic factors such as vascular endothelial growth factor receptor (VEGF), PTHrP, Interleukin-6 (IL-6), IL-8 and IL-11. These factors stimulate osteoclastic bone resorption either directly stimulating osteoclast or indirectly by increasing the RANKL/OPG ratio. Osteoprotegerin (OPG) is a decoy receptor to RANKL produced by osteoblasts. Tumor cells secrete various growth factors like platelet-derived growth factors (PDGFs), bone morphogenetic proteins (BMPs), transforming growth factor (TGF-β) and fibroblasts growth factors (FGFs) help in osteoblasts differentiation (30, 31). Osteoblasts form osteocytes and get captured in the bone. Osteocytes regulate osteoclast development through macrophage colony stimulating factor (M-CSF), receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG). They also inhibit osteoblasts differentiation (Fig. **2.2**) (27, 32).

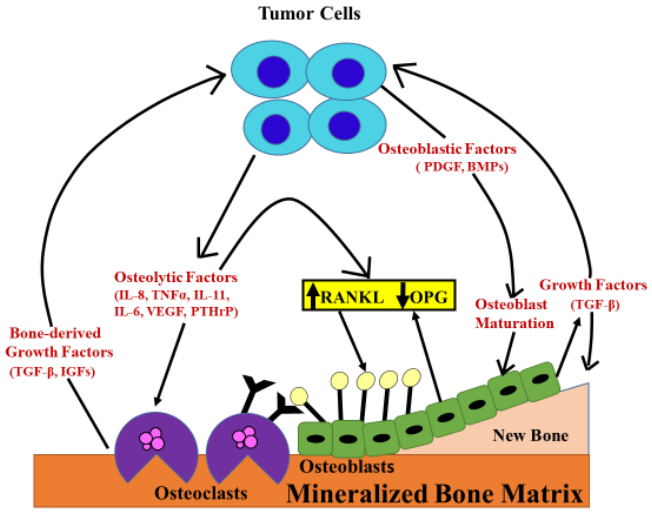


Fig. (2. 2): Different mediators released during bone metastases

Osteoblasts also secrete TGF-β and IGFs into mineralized bone matrix. Hydroxyapatite (bone mineral structure) liberates BMPs, TGF-β, IGFs and FGFs. These factors further worsen the metastatic lesions (33). PTHrP predominantly increase osteolytic lesions (34).

Treatment strategy for bone metastases revolves around three main principles [33]. These principles include; 1 treatment of cancer cells to prevent their invasion to the bone; 2) targeting bone microenvironment to inhibit the vicious cycle phenomenon caused by bone resorption as a consequence of bone metastatic cancer and; 3) use of palliative therapies to improve quality of life of cancer patients (35). Bone metastases are incurable and associated with significant morbidity due to so-called skeletal-related events (SREs) defined as pathological fractures, pain, spinal cord compression, etc. and reduced quality of life in women with advanced breast cancer (36, 37). Despite the use of these increasingly potent bone-targeted agents, progress in terms of absolute reductions in the occurrence of SREs is modest, more effective therapies are clearly needed.

Curcumin, the active ingredient of turmeric (*Curcuma longa*) possesses anti-oxidant and anti-metastatic properties (38-42). It is non-toxic even at high doses (8–12 g/day) (42-46). However, several properties limit its therapeutic potential such as its low metabolic stability and poor water solubility (i.e, 0.001 mg/mL) (43-46). Different strategies can be used to improve the solubility, stability and accumulation of drug molecules in cancerous cells. Use of nanodrug delivery systems has been shown to be a promising strategy to address these issues (47-51). Moreover, surfactant used in such micellar preparations (the commonly used ones being polyethylene glycol (PEG), pluronic F-127 (52) and chitosan (50)) prevent protein adsorption, reducing the chances of reticuloendothelial system (RES) clearance and improving the enhanced permeability and retention (EPR) effect in tumors (49).

We have previously developed a nanoparticle drug delivery system which could improve the solubility issue associated with the use of curcumin (53). In the current project, we used the targeting and anti-bone-resorptive potential of bisphosphonates (54) together with anticancer and anti-bone-resorptive effects of curcumin (55, 56) to prevent and treat breast cancer bone metastasis.

To target curcumin to the bone, we conjugated the nanoparticles with alendronate. Alendronate will increase the accumulation of the nanoparticles to the bone. Alendronate is a bisphosphonates drug used for treating osteoporosis and other bone diseases and it inhibits bone demineralization (57). Alendronate is one of the most extensively studied bisphosphonates in treating osteoporosis. Bisphosphonates exert their effect after binding to the bone mineral due to their high affinity to bone calcium, appearing at a high concentration in resorption lacunae (cavities formed by osteoclasts for bone resorption) (Fig. **2.3**). After binding, bisphosphonates are internalized by the osteoclasts, leading to a disruption in bone resorption processes (3, 58). Several studies suggest that bisphosphonates cause apoptosis of osteoclasts and thus may have direct apoptotic effect on tumor cells (3, 58). In cancer treatment, bisphosphonates are considered as standard treatment for tumor-induced hypercalcemia and bone metastasis (59). They are also clinically effective in osteoporosis, osteogenesis imperfecta (brittle bone disease) and Paget's disease (abnormal enlargement and weakening of bone disease) (60-62).

In our current study, we have studied the biological characteristics of alendronate-modified curcumin nanomicelles in breast cancer cells. In this current study, we have performed various *in-vitro* biological evaluations.

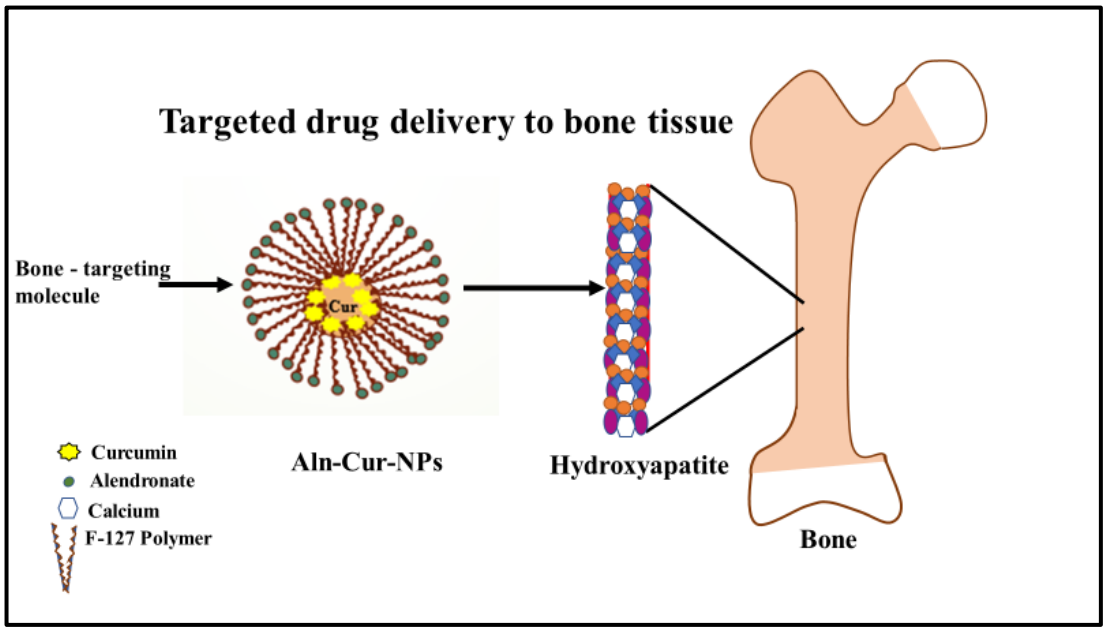


Fig. (2.3): Targeted drug delivery system to the bone.

2. Materials and Methods:

2.1. General

Compounds: Curcumin (purity ≥ 80%) and pluronic acid® F-127 were purchased from Sigma-Aldrich Australia. Alendronate sodium trihydrate was obtained from Alcon-Biosciences PVT.LTD. India.

Cancer Cells: Human breast cancer cells MDA-MB-231, MCF-7 and SK-BR-3 were gifts from Professor Robert Baxter's laboratory which were purchased from ATCC. MDA-MB-231cells were cultured in 5% Fetal Bovine Serum (FBS) Roswell Park Memorial Institute (RPMI) medium. MCF-7 and SK-BR-3 cells were cultured in 10% Fetal Bovine Serum (FBS) Roswell Park Memorial Institute (RPMI) medium. Breast cancer cells were maintained at 37º C humidified 5 % CO_2 and 95 % O_2 atmosphere.

Instruments: High performance liquid chromatography (HPLC), Leica Spe-ll Confocal Laser Scanning Microscopy (CLSM), IncuCyte Zoom, IncuCyte S3, Human PTHLH® ELISA Kit was kindly supplied by Wuhan Fine Biological Technology Co., LTD. Flat-bottomed well plates and pipettes were supplied by Corning. Australia.

Solvents: HPLC grade dichloromethane (DCM), HPLC grade acetonitrile (MeCN), HPLC grade methanol (MeOH), DMSO were purchased from sigma. ProLong Gold Antifade mounting media, Hoechst labelling solution were purchased from Solarbio. Australia., Triton-X 100, 4% formaldehyde, Phosphate Buffere saline (PBS), FBS, MTT reagent were obtained from sigma. TGF-β was supplied by Prospec-Tany Technogene.LTD. Purified deionized water was prepared using the Milli-Q system.

2.2. Preparation of Nanoparticles

Curcumin nanoparticles and alendronate nanoparticles were prepared using anti-solvent method according to our previous studies which has been submitted for publication. Poloxomer F-127 (10 g) was converted to F-127-COOH by dissolving in DCM (45 mL) by succinic anhydride reaction. F-127-COOH wasbe purified by precipitation with ice cold water. Sodium alendronate (500 mg) was dissolved in water. It was added to the mixture of F-127-COOH (2 g) and Milli-Q water (70mL). Amino terminal of alendronate was bind to the carboxyl group of modified F-127. After dialysis for 24 hours, Aln-Cur-NP was freeze-dried and characterization was done by NMR (52).

Characterization of Nanoparticles:

2.2.1. Determination of Loading Capacity (LC%) & Drug Encapsulation Efficiency

High performance liquid chromatography (HPLC) was used to determine drug loading (LC%) of the new prepared batches of nanoparticles. About 5 mg of curcumin-loaded nanoparticles were dissolved in water. Unloaded curcumin was removed by centrifugation at 10,000 RPM for 10 min. Supernatant was collected, lyophilized and dissolved in 20 mL of dichloromethane (DCM) to disrupt micelles. Extra DCM was removed by evaporation, and dry mass (entrapped curcumin) was collected. This dry mass was dissolved in HPLC solvent (5 ml) to achieve a 1 mg/mL solution (As we have used curcumin loaded nanoparticles).. Briefly, 50 µL of sample was injected into the HPLC system using Solvent A (40% methanol + 10% water) and Solvent B (50% acetonitrile) as the mobile phase at a flow rate of 1 mL/min with an isocratic pump at 25 ^{0}C and C18 column (Nova-Pak, 150 x 4.6 mm, 4µm). The following equation was used to calculate drug loading and a standard curve was plotted for raw curcumin (Figure 4).

(%) LC = [Entrapped Drug / Nanoparticles weight] x 100

Drug encapsulation efficiency (DEE) was determined using following equation

DEE = (Experimental drug loading / Theoretical drug loading (TDL)) x 100

2.3. *In vitro* biological evaluations

2.3.1. *In vitro* anticancer activities of the nanoparticles (MTT Viability Studies)

The *in vitro* anti-cancer properties of nanoparticles were investigated against three breast cancer cell lines with different receptor expression characteristics including MDA-MB-231, MCF-7 and SK-BR-3 using MTT cell viability assay. The cells were passaged and plated (at 90 µL/well) in flat-bottomed 96-well plates at 2×10^5 cells/mL. Drug solutions were prepared by dissolving Aln-Cur-NPs and Cur-NPs and void nanoparticles in PBS while raw curcumin solution was prepared by dissolving it in 0.5% DMSO at curcumin equivalent concentrations of 0.1, 1, 10, 25, 50 or 100 μM (n = 3 in triplicate). Treated cells were incubated for 48 h followed by MTT assay (63). Void nanoparticles made from F-127 were used as control. IC_{50} values were interpolated and normalized based on the loading capacity data.

2.4. Cellular Uptake Studies

Sub-confluent MDA-MB-231 cells were passaged and seeded at 80,000/200 µL cells per well in 24 well plate and allowed to adhere for 2 days. After 2 days, medium was renewed with 5%FBS RPMI for 30 minutes. Cells were treated with Aln-Cur-NPs, Cur-NPs and raw curcumin at 10 µM concentration equivalent to curcumin and PBS (negative control) for 24 h. Next day, all wells were washed three times

with 100 µL of PBS. Cells were fixed using 4% formaldehyde. Triton-X 100 was used to permeabilize the cells. Hoechst labelling solution was used for staining the nuclei. Washing was repeated to remove Hoechst solution. The slides were mounted with mounting media and viewed under Leica spe-ll confocal laser scanning microscope at Bosch Institute, The University of Sydney. Curcumin is naturally fluorescent in the green spectrum.

2.5. Live Cell Imaging:

IncuCyte® Zoom & S3 Live-Cell Analysis System (Essen Bioscience, USA) were used to examine the cytotoxic effect and uptake of NPs by MDA-MB 231 cell lines in two independent experiments. The uptake of NPs was determined depending on the natural fluorescence of curcumin which can be detected in the green channel of IncuCyte® S3. MDA-MB 231 cells were seeded at 9000 cells/well in a flat-bottomed 96 well plate and allowed to adhere for 24 h. Cells were treated with Cur-NP, Aln-Cur-NP and raw curcumin in triplicates and at concentrations of 12.5, 25, 50 µM based on the amount of equivalent curcumin. PBS was used as a negative control. Time lapse images were taken using 20x magnification power at 2 h intervals for 48 h.

In another experiment, the effects of our NPs on cell viability was determined against MDA-MB-231 cell lines using IncuCyte® Zoom. MDA-MB-231 cells were seeded at 2000 cells/well and were allowed to adhere overnight. Cur-NP, Aln-Cur-NP, Raw curcumin at their IC_{50} concentrations (obtained from MTT studies) were added to the wells. Cytotoxic effect was determined over the period of 3 days.

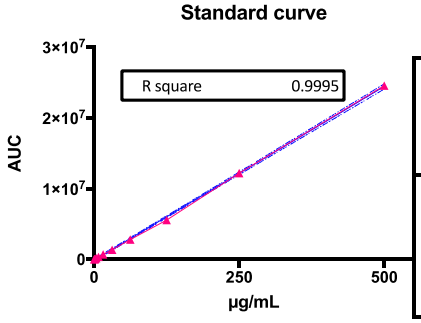
2.6. PTHrP ELISA Assay

PTHrP ELISA assay was performed using Human PTHLH® (Human Parathyroid Hormone-related Protein) ELISA kit, 96 tests (Fine Test). Sub-confluent human breast cancer MDA-MB-231 cells were plated at $5x10^4$ cells per well (n=2) in 96 well plate and allowed to adhere for 24 h. Next day, cells were preincubated in 80 µL of medium and with 20 µL of Cur, Cur NP and Aln-Cur-Np at IC_{25} and IC_{50} concentrations (based on MTT assay results) for 4 h. Medium was then refreshed with the addition of 100 µL of recombinant human TGF-β1 (5 ng/mL) for 24 h. Samples were diluted by addition of 50 µL of supernatant to 75 µL of sample dilution buffer in Eppendorf tubes. Cell culture supernatant was centrifuged for 20 minutes to remove insoluble impurities and cell debris at 1000×g at 2 - 8°C. Clear supernatant was collected and used in the assay immediately. ELISA plate wells were washed 2 times before adding standard, sample and control wells. After that all ELISA assay steps were done according to manufacturer's instructions. At the end ELISA plate was read using microplate reader at 450nm immediately.

3. Results

3.1. Determination of loading capacity

Loading capacity for new batches of Aln-Cur-NPs (batch #3) and Aln-Cur-NPs (batch #4) was found to be 4% and 3.7% respectively. Loading capacity for Cur-NPs was found to be 5.7%. Loading capacity of Cur-NPs was higher than Aln-Cur-NPs (Fig. **2.4**).



Nano-particles	Interpolated Values	Loading Capacity	Encapsulation Efficiency
Aln-Cur-NPs#3	39.5 µg/ml	4%	80%
Aln-Cur-NPs#4	36.7 µg/ml	3.7%	74%
Cur-NPs	57 µg/ml	5.7%	57%

Fig. (2. 4): Loading Capacity (%) of two batches of alendronate-conjugated curcumin-loaded nanoparticles.

3.2. Schematic Representation of Aln-Cur-NP

Figure **2.5** represents scheme for formation of Aln-Cur-Nanoparticles.

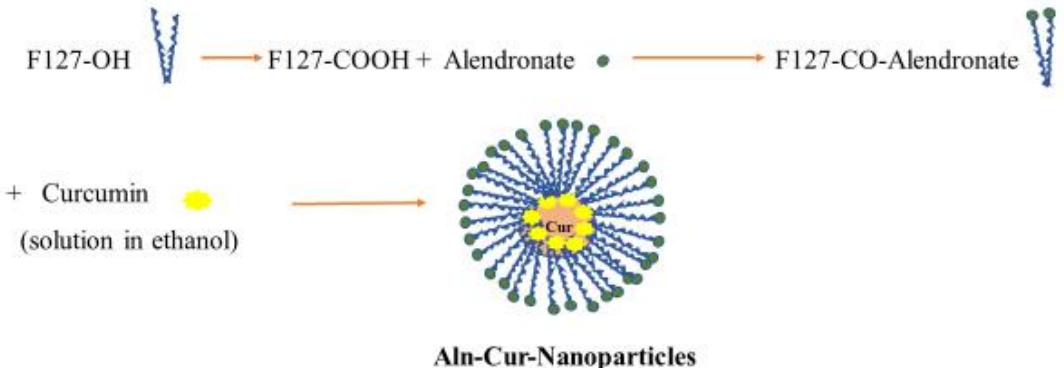


Fig. (2.5): Schematic representation of formation of Aln-Cur-Nanoparticles.

3.3. *In vitro* anticancer activities of curcumin nanoparticles

Aln-Cur-NPs and Cur-NPs were studied for their direct antiproliferative properties on three breast cancer cells, MCF-7, MDA-MB-231 and SK-BR-3 (Table **2.1).**

Table 2.1. IC_{50} values obtained against breast cancer cells

	NP or Control	**IC_{50} (µg/mL)**
MCF-7	NP or control	IC_{50} (µg/mL)
	Aln-Cur NP[a]	13.9 ± 1.6
	Cur-NP[b]	31.0 ± 4.0
	Void NP[c]	>1000
MDA-MB-231	NP or control	IC_{50} (µg/mL)
	Aln-Cur NP	22.2 ± 4.8
	Cur-NP	51.6 ± 21.7
	Void NP	>1000
SK-BR-3	NP or control	IC_{50} (µg/mL)
	Aln-Cur NP	7.7 ± 2.5
	Cur-NP	61.6 ± 10.9
	Void NP	>1000

[a]alendronate-conjugated curcumin nanoparticles, [b]curcumin nanoparticles, [c] void nanoparticles.

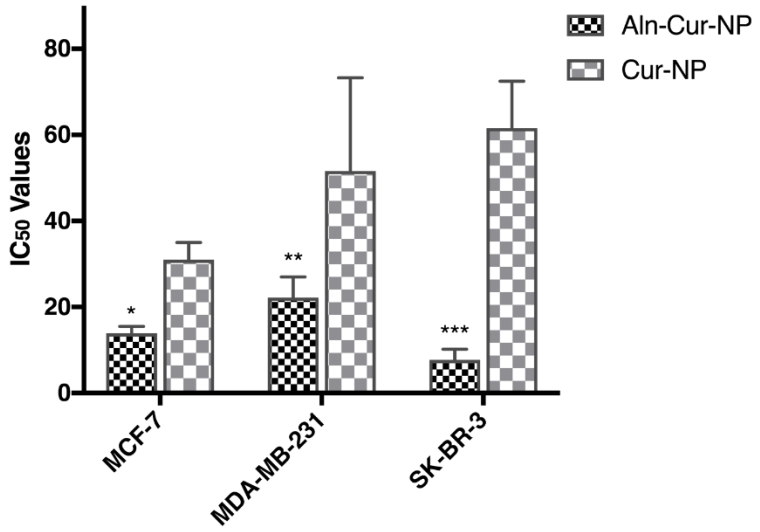


Fig. (2.6). Comparison between the *in vitro* antitumor activity of alendronate-conjugated (Aln-Cur-NPs) vs. unconjugated NPs (Cur-NPs). Two Way ANOVA, Dunnett's Post Hoc multiple comparisons test. * P<0.05, ** P<0.01 and *** P<0.001 when compared with Cur-NP (mean + SD).

Antitumor activity of the raw curcumin and curcumin nanoparticles with/without alendronate conjugation is shown as IC_{50} values in Table **2.1**. A higher antitumor activity was observed for Aln-Cur-NP as compared to Cur-NP with IC_{50} values obtained at 13.9, 22.2 and 7.7 µg/mL for MCF-7, MDA-MB-231 and SK-BR-3, respectively. IC_{50} values greater than 1000 µg/mL for void nanoparticles indicate that there was no adverse effect of the polymer alone against tested cancer cell lines. Figure 5 demonstrates a comparison between Aln-Cur-NPs and Cur-NPs. This suggests that the addition of alendronate to the formulation enhanced the anti-cancer properties of the NPs, which indicates the hypothesis of curcumin/bisphosphonates combination synergistic effect.

3.4. Cellular internalization by confocal laser scanning microscopy (CLSM)

In vitro nanoparticles uptake and drug internalization were evaluated using CLSM in MDA-MB-231 breast cancer cells after 24 h of drug treatment. Preliminary studies were done to optimize nanoparticles concentration and incubation times (data not shown). Curcumin is auto-fluorescent in nature. Cellular internalization is shown in the form of green fluorescence intensity. Cellular internalization of Aln-Cur-NPs and Cur-NPs to MDA-MB-231 cell lines was found to be more than raw curcumin. MDA-MB-231 cancer cells treated with raw curcumin were shown to achieve weaker fluorescence intensity, i.e. less drug uptake was noticed. In the negative control group (PBS), no green fluorescence was detected (Fig. **2.7**). We also performed quantitative analysis which confirmed statistically significant increase in the uptake of curcumin in both NPs forms as compared to raw curcumin (Fig. **2.8**). However, there was no statistically significant difference between the two different NPs, Aln-Cur-NPs and Cur-NPs.

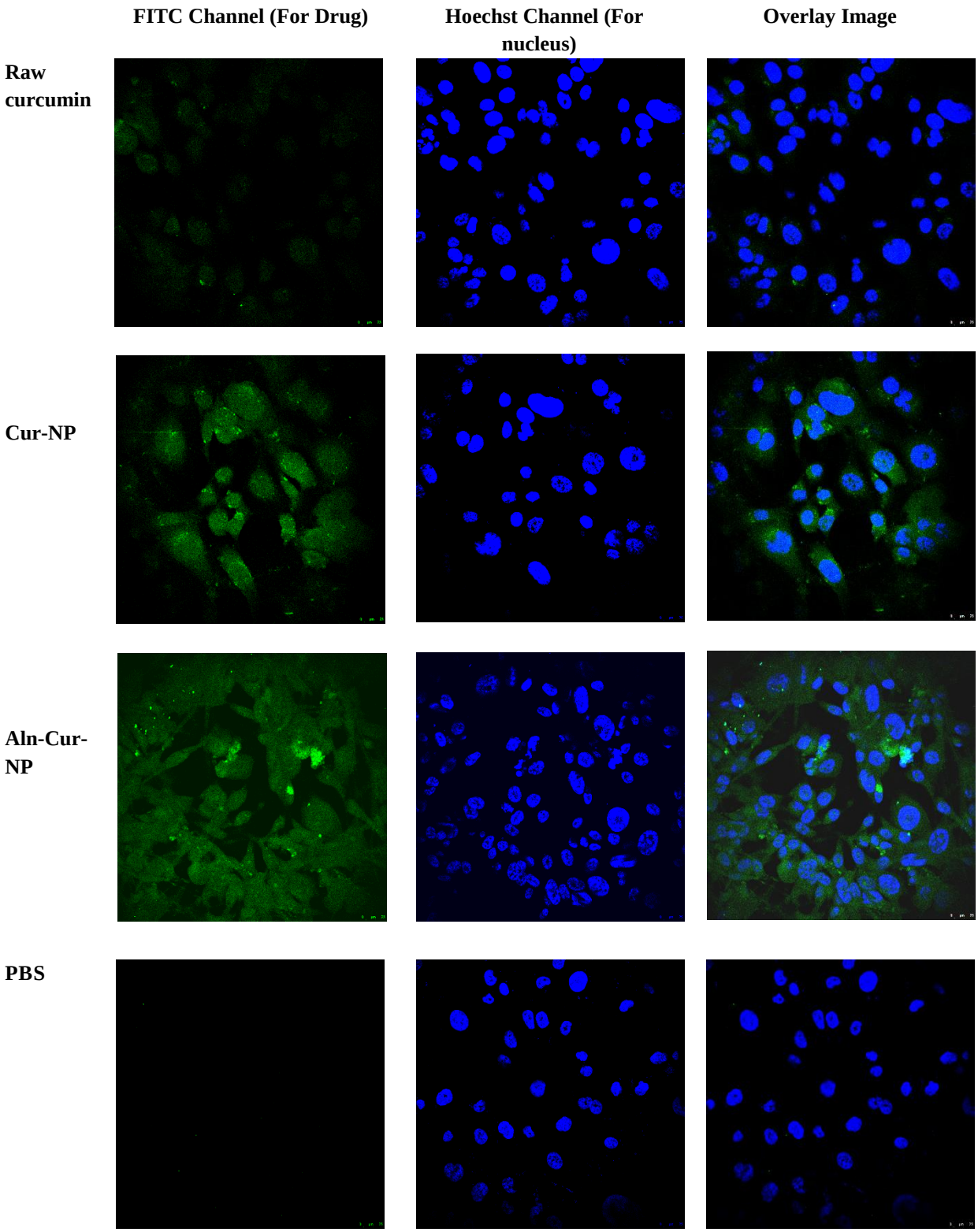


Fig. (2. 7). Cellular internalization of curcumin in MDA-MB-231 cancer cells after treating the cells at 10 µM concentration. Confocal Laser Scanning microscopy images. Cells were incubated with nanoparticles for 24 hours at 37°C in MDA-MB-231 cell lines. Curcumin appears in green fluorescence, nuclei appear in blue fluorescence and overlay appear as combination of green and blue image.

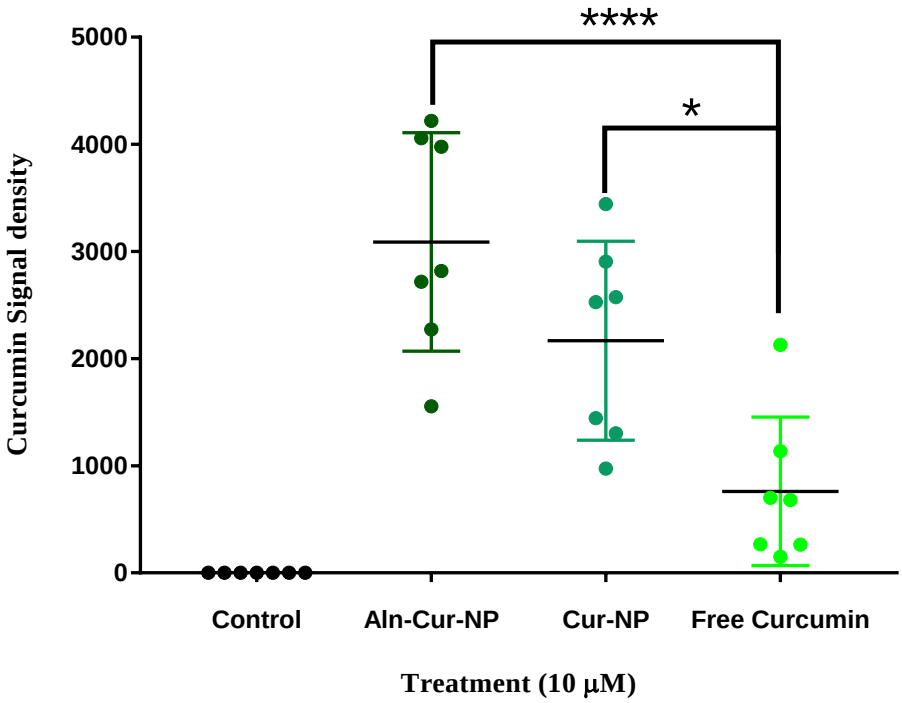


Fig. (2. 8): Statistical analysis of uptake of Aln-Cur-NP and Cur-NPs by MDA-MB-231 cancer cells as compared to raw curcumin showed there is significant difference between uptake of NPs and raw curcumin, while no significant difference was observed in the uptake of Aln-Cur-NPs and Cur-NPs. * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001.

6.3 Live cell imaging

The results of IncuCyte® S3 and IncuCyte® Zoom live cell imaging clearly showed a significant uptake of nanoparticles by MDA-MB-231 cells. The uptake of raw curcumin was much less than the two NP forms, and it could be mostly seen outside the cells (green dots) whereas the nanoparticles were detected inside the cells (Fig. **2. 9A and** Fig. **2.9B**). Furthermore, a lower viability was observed by the Aln-Cur-NP-treated cells as compared to Cur-NP-treated cells and raw curcumin. One important finding was that curcumin inside nanoparticles converted to curcumin crystals after they had killed the cells. More crystals were observed with Aln-Cur-NPs as compared to Cur-NPs.

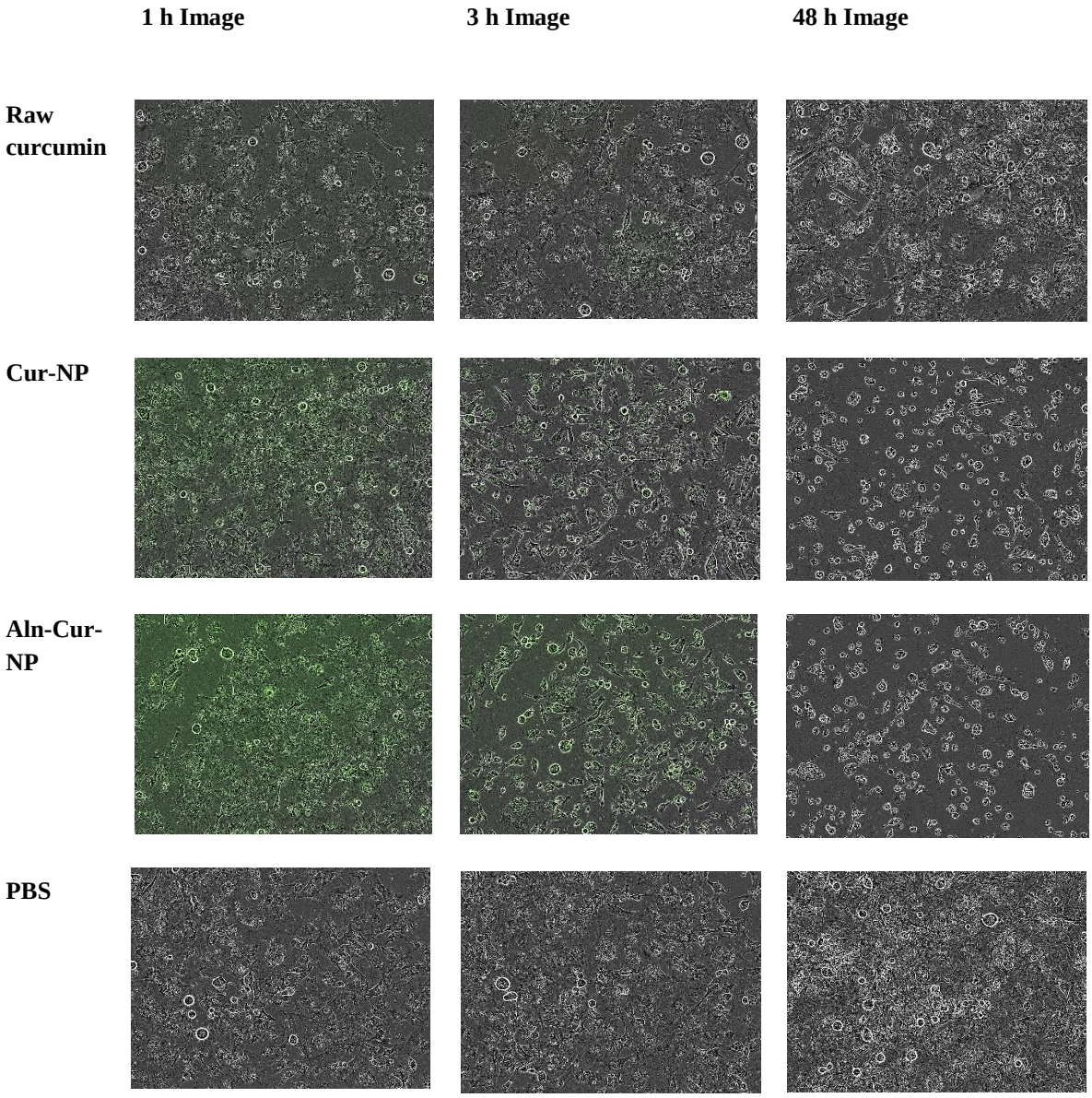


Fig. (2. 9A). IncuCyte®S3 images comparing the uptake of Cur-NP, Aln-Cur-NP and raw curcumin at 1h, 3h, and 48 h after adding the drug at 12.5 µM concentration based on the amount of equivalent curcumin.

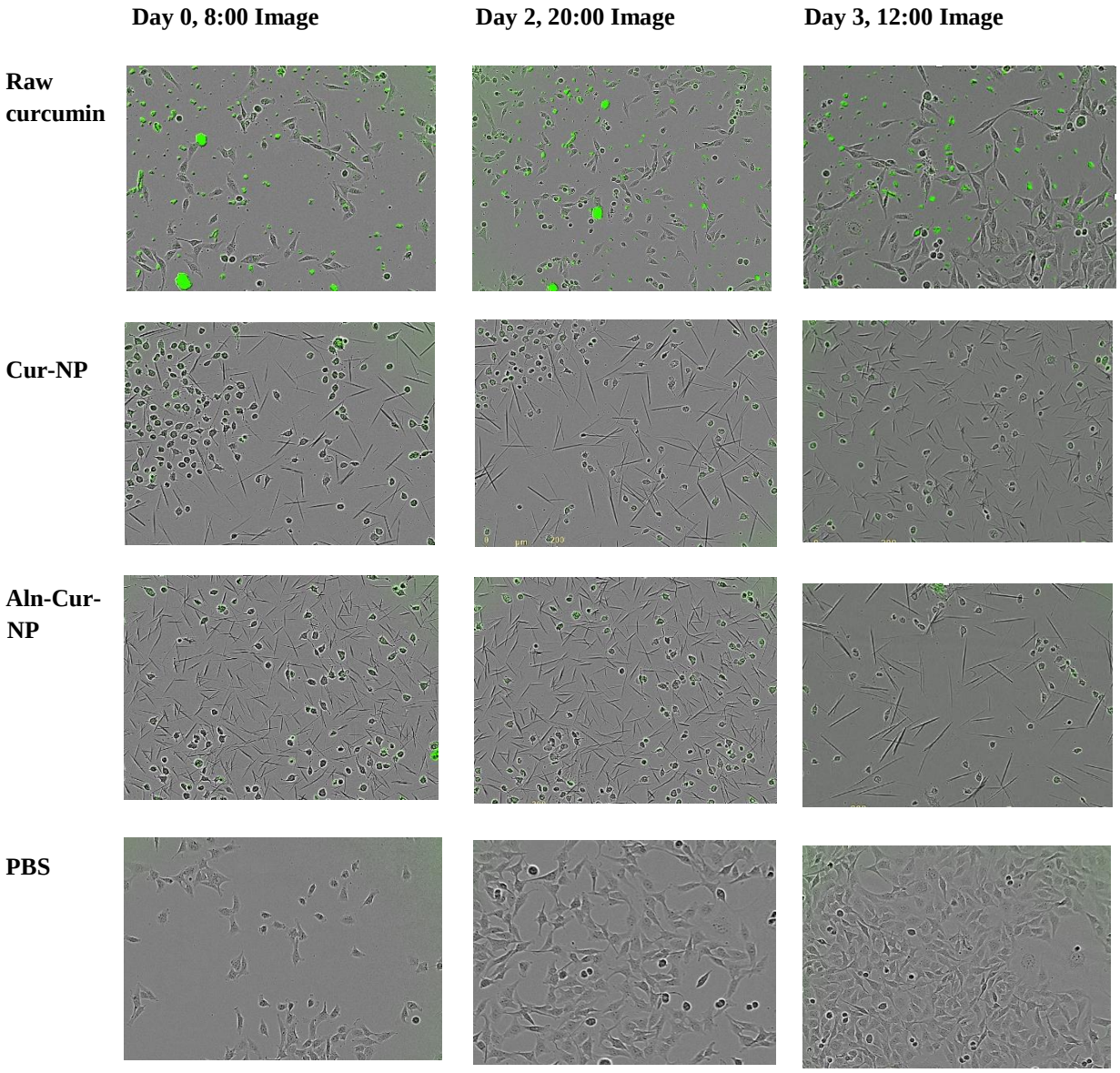


Fig. (2. 9B). IncuCyte®Zoom images comparing the cytotoxicity of raw curcumin, Cur-NP, Aln-Cur-NP over the period of 3 days after adding the drug at IC_{50} concentration.

6.3. PTHrP ELISA Assay

Effect of nanoparticles on the release of PTHrP by MDA-MB-231 cells was determined after 24 h of treatment. TGF-β was also added to stimulate the release of PTHrP by MDA-MB-231 cancer cells. Aln-Cur-NPs, Cur-NPs, raw curcumin were used at IC_{25} and IC_{50} concentrations. At the end of experiment absorbance was measured using microplate reader at 450 nm. The amount of PTHrP released by MDA-MB-231 cells treated with Aln-Cur-NP at IC_{50} and IC_{25} concentrations equivalent of curcumin was reduced to 152.6 pg/mL and 137.4 pg/mL, respectively, compared to the negative control (PBS) at above 1669.2 pg/mL. Cancer cells treated with Cur-NPs at IC_{50} and IC_{25} concentrations, reduced the release of PTHrP to 217.4 pg/mL and 141.1 pg/mL, respectively. Raw curcumin also showed some inhibitory effect on release of PTHrP by MDA-MB-231 cancer cell lines with values equal to 189.7 pg/mL and 330.3 pg/mL IC_{50} and IC_{25} concentration (Fig. **2.10**).

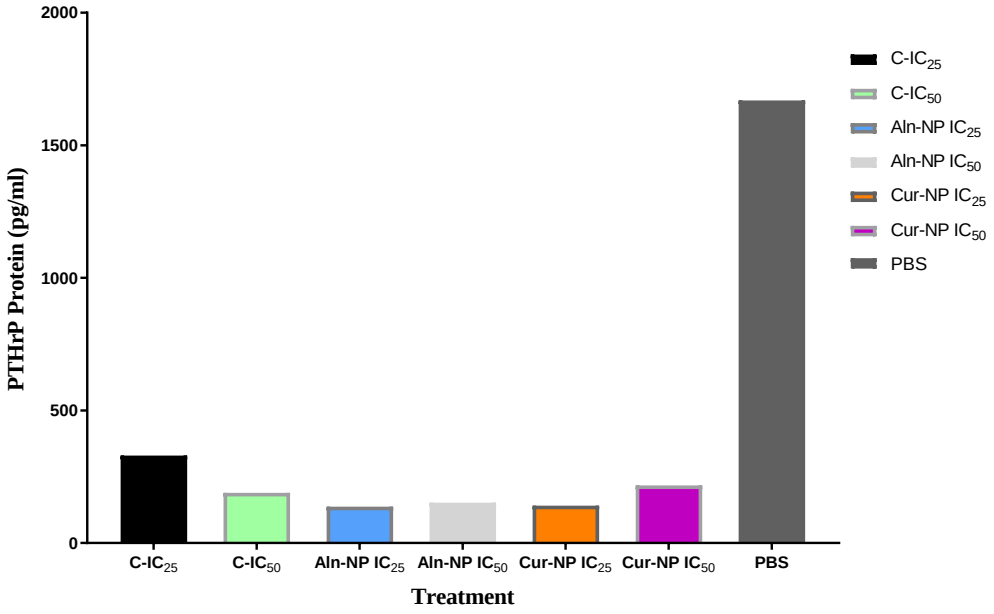


Fig. (2.10). Effect of curcumin (C-IC_{50}, C-IC_{25}), curcumin nanoparticles (Cur-NP IC_{50}, Cur-NP IC_{25}), alendronate nanoparticles (Aln-Cur-NP IC_{50} and IC_{25}) and PBS on the release of PTHrP in MDA-MB-231 cells was determined using Human PTHLH (Parathyroid hormone-related protein) ELISA kit (Fine Test).

Results clearly showed nanoparticles have reducing effect on release of PTHrP from MDA-MB-231 cells as compared to the negative control (PBS). Aln-Cur-NP showed maximum reducing effect on PTHrP release from MDA-MB-231 cells.

Discussion

In this study, we investigated the biological behavior of curcumin nanoformulations with and without alendronate conjugation for the prevention and treatment of breast cancer bone metastasis. Curcumin is extracted from rhizome of *Curcuma longa* and is a widely studied molecule (64). It has cytotoxic, anti-oxidant, anti-proliferative potential. However, the challenge with its application as a medicine is the poor bioavailability due the very low solubility in water. Curcumin has poor absorption, biodistribution and bioavailability. Most of the curcumin get metabolized in the intestine and liver which result in a rapid degradation and elimination from the body. Two major pathways identified in curcumin metabolism in the intestine are O-conjugation (form to form curcumin glucuronide and curcumin sulfate) and reduction (to form tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol) (65, 66). Curcumin may also undergo intensive second metabolism in the liver. The major metabolites are glucuronides of tetrahydrocurcumin and hexahydrocurcumin, with dihydroferulic acid and traces of ferulic acid as further metabolites (65, 66). Most of elimination occurs through feces and negligible amount of curcumin is excreted in the urine.

We used a nanoformulation strategy to improve the solubility and poor stability of curcumin.

Nanoformulation has been shown to be a strategy to improve the antitumor properties of curcumin in against breast, prostate, cervical and pancreatic cancer cells (67-69). Bisphosphonates are a class of drugs that are used for osteoporosis to prevent bone loss. Bisphosphonates are characterized as

compound having double C-P bonds. Bisphosphonates have high affinity for the hydroxyapatite of the bone. Bone will selectively uptake bisphosphonates in two steps 1) first due to the bone resorption effect of osteoclasts, hydroxyapatite crystals are exposed (physicochemical action) and 2) bisphosphonates get attracted towards hydroxyapatite of the bone and are uptaken by osteoclasts and inhibit bone resorption (cellular effect) (70). All bisphosphonates possess common P-C-P bond. The only difference is in their side chain which produces different chemical structures for bisphosphonates (71).

Preclinical data suggested that bisphosphonates may also reduce cell viability and proliferation by increasing apoptosis in tumor cells. They may also possess anti-angiogenesis, anti-neoplastic and immunomodulatory effects. Bisphosphonates can also decrease tumor cell adhesion and invasion (72, 73). Due to anti-osteoclastic properties of bisphosphonates, they are potent inhibitors of bone resorption. Other mechanism for their direct inhibition of cancer cells growth might be their anti-angiogenic potential (74). In many studies, alendronate has been proved to exert anti-proliferative effects against different cancer cells such as breast, myeloma, neuroblastoma and melanoma (75).

Recently, polymeric micelles have been widely investigated as promising anticancer drug delivery carriers (76, 77). Drugs encapsulated in micelles have been shown to have a more accumulation in solid tumors in comparison with free drugs. This increased accumulation of drug might be because of enhanced permeability and retention effect (EPR). Pluronic acid (Fig. **2.11**) is the most widely used polymeric vehicle for micelle formation. It is highly compatible with biological fluids. Pluronic acid is a triple block structure comprised of poly(ethylene oxide (PEO)) and poly(propylene oxide)(PPO)) chains. PEO segment of F-127 is hydrophilic in nature while PPO segment is hydrophobic. PPO segment incorporates hydrophobic drugs. Due to the self-assembly nature of F-127, it forms spherical core at critical micelles concentration (CMC) (78). CMC for F-127 is about 0.26-0.8wt% (79) and we have previously optimized the ratios of curcumin to F127 within this CMC range to achieve the best particle size and stability (data under publication).

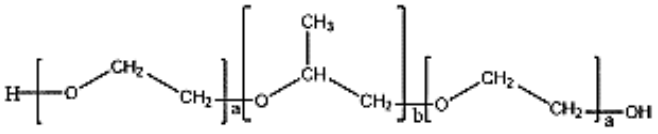


Fig. (2.11). Chemical structure of F-127

In our study, we have conjugated curcumin nanoparticles with alendronate aiming to achieve synergistic anticancer in addition to anti-bone resorption effect. It was proposed that alendronate not only target curcumin nanoparticles to the bone but also will exert some direct anti-bone resorption and cytotoxic effect in combination and synergistic effect with curcumin.

We have formulated two new batches of Aln-Cur-NPs. Loading capacity for Aln-Cur-NPs is 4 % and 3.7 % while for Cur-NPs it is 5.7 %. Nanomicelles proved to increase water solubility.

The effect of Aln-Cur-NPs and Cur-NPs was also determined on the proliferation of MDA-MB-231, MCF-7 and SK-BR-3 cells through cell viability MTT assay. Mitochondrial reduction of MTT in three breast cancer cells by Aln-Cur-NPs and Cur-NPs was found to occur in a dose dependent manner. We compared the antiproliferative activity and IC_{50} values of Aln-Cur-NPs and Cur-NPs with raw curcumin and void-NPs. Increased antiproliferative effect of Aln-Cur-NPs relative to Cur-NPs and raw curcumin, indicated the direct inhibitory effect of alendronate when it is in combination with curcumin in the micellar formations.

Curcumin was released from nanomicelles in the form of crystals after being used by the cells. In previous studies, it was shown that following cell death due to curcumin's effect, curcumin was first precipitated out as amorphous nanospheres. Amorphous nanospheres then aggregated to form needle shaped crystals (80). In our experiments we showed that the amorphous form of curcumin which was loaded in the nanoparticles was the effective form.

Cellular internalization of nanomicelles was determined using CLSM. CLSM provides exciting opportunities for imaging nanomicelles internalization into breast cancer cells. CLSM has capacity to reject out-of-focus light and provides sharp and high-contrast images of cells (81). Curcumin is auto-fluorescent in nature. The internalization of curcumin by MDA-MB-231 cells at concentration of 10μM equivalent to curcumin after 24 h of treatment was observed for Aln-Cur-NPs and Cur-NPs and raw curcumin. Curcumin showed green fluorescence in the images obtained with CLSM, suggesting the curcumin is released from nanomicelles. The cell's nucleus was counterstained with Hoechst labelling solution and was appeared as blue in CLSM images. While curcumin appeared as bright green signals in CLSM images. Brighter green signals were noticed in MDA-MB-231 cells treated with Aln-Cur-NPs compared to cells treated with Cur-NPs while much less green signals were observed for MDA-MB-231 cells treated with raw curcumin and no green signals were observed in the negative control. This indicated that in both nanomicelle-treated cells there is a higher amount of curcumin.

In two different experiments the effect of nanoparticles on live cells was examined using IncuCyte. The uptake of Cur-NPs and Aln-Cur-NP was more than raw curcumin. While no significant difference was observed in uptake of Cur-NP and Aln-Cur-NP. These results from IncuCyte were in line with our quantitative analysis performed using confocal images. The nonsignificant difference in the uptake of Cur-NPs and Aln-Cur-NP indicates that the higher cytotoxic effect of Aln-Cur-NPs on MDA-MB-231 cells confirmed by both MTT and IncuCyte is due to the synergistic effect of alendronate and curcumin.

PTHrP exerts its action by acting via PTHrP receptors (PTHrP-R). It is a protein that mediates autocrine (secreted and acted on the same cell through autocrine receptors) and paracrine (secreted by cell and acts on neighboring cells) functions (82). On the bone, PTHrP exerts its effect through endocrine action (83, 84). PTHrP has role in development of normal breast growth and physiology. PTHrP released by epithelial cells contributes to the development of breast in embryos (85). In adult's breast, myoepithelial cells release PTHrP and it acts on periductal stroma and inhibits ductal extension. During lactation, PTHrP secreted by alveolar epithelial into maternal circulation and stimulates milk production (86). PTHrP is produced by tumor cells and promotes osteoclastic activity and osteolytic bone metastasis (87). The role of PTHrP in the development of primary tumor is not clear. About 60% of primary breast cancer patients and 70% of patients with bone metastases have increased PTHrP levels. A higher PTHrP and mRNA 1-139 expression is correlated with the development of invasive bone metastasis (83). Tumor-derived PTHrP stimulates vicious cycle for bone metastasis. It also stimulates tumor cell adhesion, proliferation and survival (88). During osteolytic resorption, TGF-β is released by bone matrix. TGF-β stimulates the release of PTHrP from tumor cells (89). Then PTHrP causes bone resorption and stimulates vicious cycle for bone metastasis (83). Results of a study revealed that knocking down of PTHrP in MDA-MB-231 cells could be a potential treatment option for breast cancer and skeletal metastasis. Knocking down of PTHrP stimulates different mechanisms like, decrease in A1 proteins & cyclins D1 levels, increase in levels of LC3-II & Beclin 1, autophagosomes formation, cleavage of caspase 8 and induced tumor cells apoptosis. All these processes can inhibit tumor growth

and hence skeletal metastasis (90). Curcuminoids are proved to have inhibitory effect on PTHrP secretion by MDA-MB-231 cells (91) and also prevent TGf-β induction of PTHrP (92). In our study, the effect of our nanoparticles (Aln-cur-NPs and Cur-NPs) at IC_{50} and IC_{25} concentrations showed a decrease in the release of PTHrP by MDA-MB-231 cells. Alendronate has proved to have cytotoxic effect (93). Aln-Cur-NPs and Cur-NPs showed the highest reducing effects on release of PTHrP by MDA-MB-231 cells. While with cells treated with PBS, a high concentration of PTHrP was released by MDA-MB-231 cells. These results are in line with previous reports on the preventative effects of curcumin on the secretion of PRHrP in breast cancer cells, which results in the inhibition of bone resorption activation (55).

Conclusion

Curcumin was encapsulated in pluronic F-127 nanoparticles. Alendronate not only made our formulation targeted to the bone, but it also synergized the anticancer activity. The results of MTT assay confirmed the anticancer effect of Aln-Cur-NPs on different cancer cells. CLSM images confirmed the uptake of nanoparticles by MDA-MB-231 cells. The uptake and cytotoxicity of NPs was confirmed by IncuCyte®. The results of PTHrP ELISA confirmed the inhibitory effect of Aln-Cur-NPs on release of PTHrP by MDA-MB-231 cells and can be evaluated by in-vivo studies.

In conclusion, our results showed that alendronate-conjugated curcumin nanoparticles are promising candidate for *in-vivo* studies for enhanced anticancer effect of curcumin in breast cancer bone metastases.

We are hoping to develop a promising targeted drug to prevent breast cancer bone metastases with less side effect profile as compared to conventional therapy.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise

Acknowledgement

We acknowledge National Breast Cancer Foundation Research Fellowship to Pegah Varamini (PF-16-007). I also acknowledge the efforts of Annim Mohammad, who put her efforts to edit my writing.

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CHAPTER 4

CONCLUSIONS, FUTURE DIRECTIONS & FINAL REMARKS

The advent of nanotechnology opened a new horizon in direct targeting of cancer cells without harming the normal cells. This thesis research tried to fill a gap of lack of preventive therapy for breast cancer bone metastases. Different experiment performed with Alendronate conjugated nanoparticles shown promising results in preventive and treating breast cancer bone metastases. Loading capacity determination showed the efficient loading of curcumin into nanoparticles. Antiproliferative activities of Aln-Cur-NPs was found to be better than Cur-NPs in MDA-MB-231, MCF-7 and SK-BR-3 cancer cells. CLSM verified the uptake of NPs by MDA-MB-231 cancer cells. PTHrP assay showed the inhibitory effect of Aln-Cur-NPs on release of PTHrP peptide and hence inhibition of bone metastases. We conclude that this combination of Aln and curcumin has shown synergistic effect on killing cancer cells. This synergistic effect is because of alendronate itself effect on cancer cells and bone and cytotoxic effect of curcumin.

FUTURE DIRECTIONS

The results obtained validate the efficacy of Aln-Cur-NPs as compared to raw curcumin and Cur-NPs.

In future we may investigate the affinity of Aln-Cur-NPs for bone and evaluate its effect on bone-resorption.

Our promising results may also point towards the in vivo biological evaluation of alendronate conjugated curcumin nanoparticles to prevent breast cancer bone metastases.

FINAL REMARKS

We anticipate the alendronate conjugated curcumin nanoparticles developed in our project has great potential to prevent breast cancer bone metastases. There is a strong need to evaluate this research more in *in vivo* studies.

CHAPTER 5

APPENDIX 1

Publications during my MPhil

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Current Pharmaceutical Design, 2018, *24*, 1-12 1

REVIEW ARTICLE

Different Targeting Strategies for Treating Breast Cancer Bone Metastases

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ARTICLE HISTORY

Received: April 10, 2018
Accepted: June 15, 2018

DOI:
10.2174/1381612824666180619165728

Abstract: ***Background:*** Breast cancer is the most frequently diagnosed malignancy in women worldwide. Breast cancer tends to metastasize to bone. Around 70% of the breast cancer patients eventually develop bone metastasis. After the bone invasion, metastatic cells disrupt the balance between osteoblastic and osteoclastic activities, leading to skeletal complications, characterized by pain and pathological fractures and hence worsening the patient's quality of life. Once tumor invades the bone, it is hard to treat it with, the so-far available treatments options (e.g. bisphosphonates and denosumab). Bone metastasis should be essentially controlled, in cancer treatment and there is a strong need to explore new, more efficient therapeutic targets. This review discusses the bone physiological processes and the recent advances in exploring different pathways involved in bone metastasis. Furthermore, some novel treatment options, which are under preclinical and clinical investigations, are highlighted.

Conclusion: A deeper understanding of these metastatic pathways can provide oncology researchers with novel avenues for treating bone metastasis, one of the main challenges to cure breast cancer. The restoration of healthy bone environment will not only improve the patient's quality of life but also reduces the tumor burden.

Keywords: Bone Metastasis, Targeted strategies, Osteoblasts, Osteoclasts, Bone resorption, Novel targets, RANKL/RANK.

1. INTRODUCTION

According to the Australian Institute of health and welfare 2017 report, 17730 Australians (17,586 of women and 144 of men) are diagnosed with breast cancer and this number will increase to 18,235 Australians by 2018. Around 3,000 patients died of breast cancer in 2017 [1], mainly due to advanced breast cancer. Patients with advanced breast cancer disease undergo aggressive therapy and most of them experience, severe side effects. Roughly, 70% of advanced disease patients suffer from bone metastasis, which may be complicated or uncomplicated bone metastasis. Uncomplicated bone metastasis can be characterized as the metastasis involving painful bone but not associated with existing pathologic fracture, spinal cord compression or cauda equina compression, while complicated bone metastasis is characterized by pathological fractures and spinal cord and cauda equina compression [2] as shown in Figure 1. Sometimes those associated with soft tissue components or those within weight bearing bones at high risk of fracture are also considered complicated. Bone metastases result in skeletal-related events (SREs) that can be described as the spinal cord compression hypercalcemia, pathological fractures (excluding significant traumas), necessity for surgery to bone or bone radiation therapy [3]. The microenvironment, where bone linked with bone marrow is ideal for tumor growth [4]. Transcriptional analysis showed the involvement of gene for Chemokines (CXCR4 involved in homing), matrix metalloproteinases (MMPs involved in invasion), fibroblast growth factor (FGF involved in angiogenesis), Interleukin-11(IL-11) and osteopontin (OPN involved in osteolysis). It was shown that tumor cells that cause bone metastasis are characterised to invade healthy bone tissue, increasing their multiplication and causing skeletal destruction [5, 6]. This ongoing process eventually leads to pain elevation, immobilization and progressively worsening the quality of life [7].

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This review will first provide an insight into the healthy bone physiological processes. Subsequently, mechanisms involved in breast cancer metastasis to bone in addition to some novel targets and treatment options that are under investigation will also be discussed.

2. NORMAL BONE FUNCTIONS

Bone constitutes human skeleton. Human skeleton has structural and locomotor functions as well as being a calcium reservoir. During growth, the bone size is increased. Bone mineralization occurs during childhood and adolescence period [8].

2.1. Modeling

Through modeling the bones are shaped and adapt to load bearing and other influences. Modeling leads to bone mass, size and geometrical changes.

2.2. Remodeling

Microfracture repair process occurs regularly in normal individuals throughout their lives. This involves, existing bone resorption, new bone deposition, and mineralization. The whole process is called remodeling. An adult's skeleton undergoes complete remodeling every decagon. Bone remodeling regulation is crucial to explain bone metastasis as malignant tumor exploits these pathways to boost cancer growth and bone destruction. Bone remodeling involves the contribution of 2 types of cells including1) osteoblasts liable for the bone matrix production, mineralization, and remodeling process initiation and 2) osteoclasts accountable for bone resorption.

2.3. Bone Formation and Resorption

Bone growth, modeling and remodeling are based on bone formation and bone growth. Osteoblasts are responsible for bone formation.

2.3.1. Osteoblasts

Osteoblasts contribute to the synthesis and mineralization of osteoid. Osteoid is a material responsible for bone shape, hardiness, and resilience. Some parameters that can be used to measure bone

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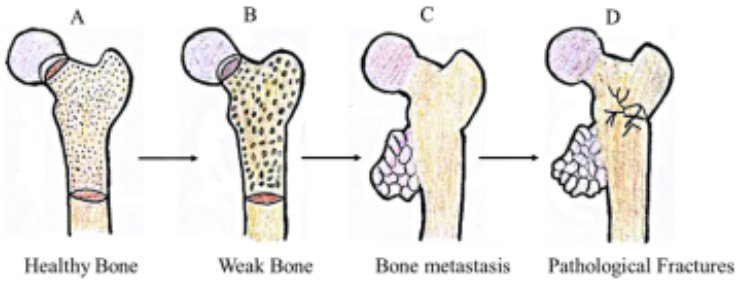

Fig. (1). Changes in the bone structure from healthy bone tissue (A) to pathological fractures due to the cancer cell invasion and bone metastasis (D).

formation include 1) osteoid components like osteonectin, osteopontin and osteocalcin and 2) bone-specific alkaline phosphatase (BSAP).

2.3.2. Osteocytes

Osteoblasts which get captured into the new bone matrix [9, 10] are named as osteocytes. Osteocytes constitute 90% of bone cells and are developed from osteoblasts who have completed their role in bone formation. Osteocytes develop into the osteocyte-osteoclast-osteoblast network system. Osteocytes are responsible for healing microfractures and harmonize remodeling [11]. When microfracture occurs, osteocytes undergo apoptosis and sends signals to osteoclasts to begin bone resorption and remodeling. Osteocytes have regulatory role on osteoclasts [8].

2.3.3. Osteoclasts

Osteoclasts execute bone resorption through the fusion to the bone, constituting a ring of firm junctions that are regulated by α5β3 integrins [12]. After binding, osteoclasts secrete acids and proteases (e.g., Cathepsin K). Acid dissolves hydroxyapatite from bone and cathepsin K degrades the collagen matrix. Osteoclasts endocytose debris from bone degradation. Later, osteoclasts discharge their content (High levels of calcium, magnesium, phosphates and products of collagen) into the blood stream and thus can be used to determine the value of overall bone resorptive activity of serum or urine. Osteoclasts differentiation is critically affected by receptor activator of nuclear factor-κB (RANK) ligand and macrophage colony-stimulating factor (M-CSF) [8].

2.3.4. Mechanism of Normal Bone Remodeling

In normal bone remodeling, osteoblasts express RANKL (NF-κB ligand) that binds to RANK on the surface of osteoclasts and their precursors. This binding regulates the osteoclasts differentiation from their precursors. Osteoclasts activation and survival lead to increased bone resorption. However, osteoblasts secrete osteoprotegrin (OPG) that inhibit excessive bone resorption by binding to RANKL and prevent binding with RANK. Hence, the balance between RANKL/Osteoprotegrin expression determines the bone mass in both normal and disease state [13]. Figure **1** is showing the mechanism of normal bone microenvironment. (A) Osteoblasts and osteoclasts are the basic units of normal bone remodeling. Osteoblasts produce osteoid, bone matrix and osteoclasts which are responsible for degradation of mineralized bone. Osteoblasts are derived from mesenchymal stem cells under control of osteoblastic transcription factor called Runx2. Mononuclear myeloid precursors are fused to form pre-osteoclast. Pre-osteoclasts are differentiated into activated, multinuclear osteoclasts. This differentiation is controlled by colony-stimulating factor (M-CSF) and RANKL (receptor activator for NF-κB). After activation, osteoclasts get adhered to bone and cause degradation of bone. Osteoblasts also produce a decoy receptor to RANKL called osteoprotegerin (OPG). The RANKL to OPG ratio is determinant of osteoclast activity. Bone lining cells and osteocytes also constitute osteoblastic lineage.

3. CANCER BONE METASTASES

3.1. Mechanism of Bone Metastasis

After invading the bone marrow microenvironment (Fig. **2**) tumor cells disrupt this RANKL/osteoprotegrin (OPG) expression balance that leads to the over-production of osteoclasts. Additionally, tumor cells induce angiogenesis that enhances bone resorption and makes the bone tissue irregular and woven, causes abnormal bone formation via osteoblasts [14, 15], structural malformation, fracture and bone pain [16].

Bone resorption is responsible for the release of various factors, such as transforming growth factor (TGF-β and IGF1) and calcium. These further aggravate tumor growth and deregulation of RANKL/OPG expression. This is a vicious tumor growth cycle where increased bone resorption reinforces more tumor growth and *vice versa* [17]. Relocation of cancer cells to the bone disturbs the normal cycle of the bone turnover, forms lytic, sclerotic tissue or mixed metastasis, which leads to substantial pain and reduced prognosis [18, 19].

Once cancer cell crosses the intrinsic barriers, it will take over the control of additional homeostatic factors [20]. Different environmental barriers that cancer cells have to cross include physical barrier (basement membrane), chemical barriers (hypoxia, reactive oxygen species and low pH), and biological barriers (immune surveillance, regulatory extracellular matrix, inhibitory cytokines) [21, 22].

Breast cancer cells establish strong interaction with the microenvironment once released from primary tumor site and reside in the bone marrow [23]. After that, breast cancer cells secrete factors that activate NF- κB ligand (RANKL)-dependent and -independent stimulation of osteoclast bone resorption [24].

Figure **1A** demonstrates some pathways in normal bone environment and Figure **1B** shows mechanism of metastatic bone environment. Breast cancer cells in malignant bone microenvironment secrete growth factors, cytokines and parathyroid hormone-related protein (PTHrP) which have negative impact on osteoblast function. In malignant bone environment, RANKL is increased and OPG is reduced which leads to more osteoclast formation and bone degradation. A significant reduction occurs in osteoblasts differentiation and no more osteoid is available to compensate osteoclastic bone resorption.

Current therapeutic targets include RANKL, PTHrP and bone hydroxyapatite. Matrix metalloproteinases (MMPs), cathepsin K and transforming growth factors (TGF)-β Insulin-like growth factor (IGF), monocyte chemotactic protein-1 (MCP-1), Platelet-derived

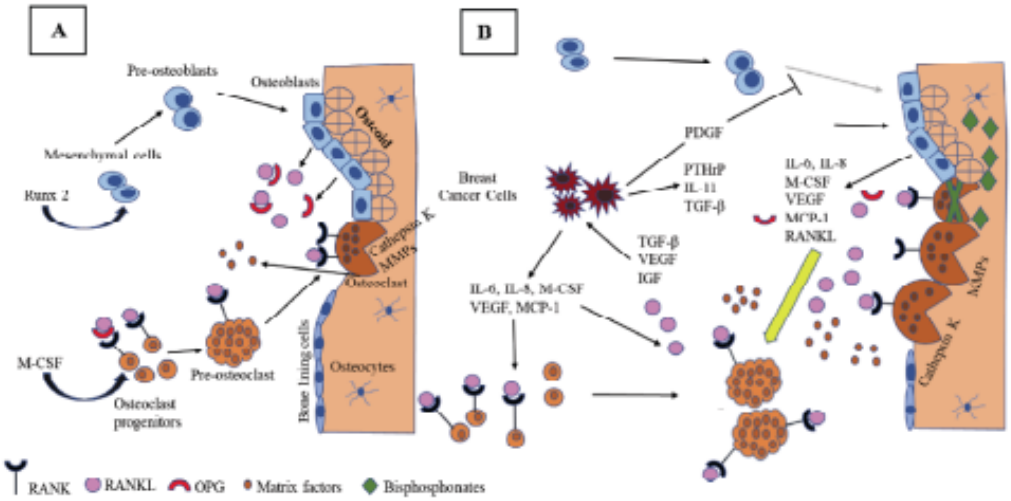

Fig. (2). The Bone Microenvironment [25]. A) Normal bone remodeling processes, B) osteolytic bone metastases.

growth factor (PDGF), Vascular endothelial growth factor (VEGF) are also under investigation to target bone metastases [25] (Figure **1B**, green text).

3.2. Bone Pain

Cancer pain is caused mostly due to the metastatic bone disease [26-28]. However, it is notable that not all bone metastasis lead to pain and pain intensity is not always proportional to size and degree of metastatic lesions in the bone. Metastatic bone pain is mostly a neuropathic pain, transmitted by primary efferent nociceptor peripheral nerves. These peripheral nerves have many types of receptors for noxious stimuli detection, including acidity, lipid metabolites, heat and inflammatory molecules. Persistent acidic and inflammatory environment of metastatic lesions cause sustained stimulation, allodynia (central pain sensitization) and hyperalgesia (hypersensitivity to pain). Thus, any agent that has potential to antagonize inflammatory mediators can be a potential therapeutic agent for managing cancer pain. A single or multiple radiotherapy sessions delivering 8Gy or 20Gy was helpful in managing this type of pain. Radioisotopes are easy to administer, less toxic and effective in subclinical metastatic sites but cannot be delivered in precise doses. Analgesics are recommended for managing metastatic bone pain. Standards of care should be accompanied with bone-modifying agents to manage cancer bone pain as they could exert a synergistic effect [3].

4. TREATMENT STRATEGIES

4.1. Treatment for Uncomplicated Bone Metastases

Treatment options available for treating uncomplicated bone metastasis include bone-targeted agents along with radiation therapy. It is demonstrated that both single dosing and multi-fractionated dosing of radiotherapy has been proved to be equally effective for curing uncomplicated metastasis of bone [29].

4.2. Treatment for Complicated Bone Metastases

Treatment options for complicated bone metastasis mainly include bone surgery and radiotherapy. Zoledronic acid, pamidronate or denosumab are recommended to be administered because they have shown to delay the use of analgesics.

In a phase III randomized clinical trial, a single dose of 8 Gy radiation was found to be effective for palliating spinal cord compression. For the patients who were suffering from bone metastases neuropathic pain however, multifractionated treatment (20Gy in 5 fractions) was proved to be more effective than a single fractionated treatment (8 Gy in 1 fraction).

4.3. Radiation Therapy

Mechanism of pain relief following radiation therapy is poorly understood. Many clinical trials with different scoring and reporting methods are available but guidelines for irradiation are still unclear because of great variation in beneficial results. Three different types of radiation therapy are used including local-field, wide-field and radionuclide therapy are shown in Table **1** [30].

5. BONE-TARGETED AGENTS

5.1. Inorganic Pyrophosphates

Inorganic pyrophosphate analogs, also called bisphosphonates, can exert their role *via* two mechanisms [31]:

1) Interfering with and hampering the osteoclast survival process.
2) Stimulating the apoptosis of osteoclasts

Through these mechanisms, bisphosphonates can regulate bone turnover and reduce tumor-related bone resorption [31]. Bisphosphonates can be classified into amino-bisphosphonates and non-amino bisphosphonates. Among these, amino-bisphosphonates are predominantly utilized in clinical interventions [31, 32]. Ibandronate, pamidronate and zoledronic acid (amino-containing) and clondronate (non-amino containing), are bisphosphonates (Fig. 3) available in clinic to treat bone metastasis to breast cancer [31]. Some examples of derivatives in clinical trials are shown in Table 2 [33].

5.2. Denosumab

Surfaces of osteoclasts and their precursors have RANK receptors. Denosumab is a human monoclonal anti-RANKL antibody

Table 1. Radiation therapy for bone metastases [30].

Radiation Therapy	Delivering method	Pain relief rate	Examples	Indications
Local-field radiation therapy Conventional treatment	Delivered using photons	80-90%	40-46 Gy/ 20-23 fractions 30-36 Gy/10-12 fractions Gy	Used for patients with localized pain: less than four metastatic sites without visceral sites (lung, liver, central nervous system).
Wide-field radiation therapy Systemic radiation therapy	MeV units (from Co 60 to 15 MeV linear acceleration)	64-100% 50-66% of patients maintain pain relief for remaining life	Upper wide field treatment (from skull to L2-3) is 6 Gy Lower-wide field (from L3-4 to above the knees) or mid-body wide field treatment (from L1 to upper third of the femurs) is 8 Gy	Used for widespread symptomatic bone metastases or as an adjuvant to local-field irradiation to reduce frequency of re-treatment.
Radionuclide therapy Systemic radiation therapy	Radioisotopes (high linear energy transfer)	37-91% from 89St* 58% from 186Re* 72% of 153Sm*	89St 186Re 153Sm 223Ra	It is used in combination with bisphosphonates and radiation therapy in the treatment of bone metastatic disease.

*St: Strontium, Re: Rhenium, Sm: Samarium, Ra: Radium

Table 2. Clinical Trials for Bisphosphonates [33].

Goal	Study Type	Clinical trial Gov. Identifier	Status
Oral bisphosphonates to prevent bone loss in postmenopausal women with early breast cancer, receiving anastrozole therapy and determine how long treatment is needed.	Phase III	NCT00122356	Completed 13/03/2013
Identification of Risk Factors' for skeletal related events in breast cancer patients receiving bisphosphonates for bone metastasis.	Cohort	NCT01144481	Completed 07/01/2015
Studying long term bone quality in women with breast cancer receiving bisphosphonates	Observational	NCT00873808	Withdrawn 10/04/2013
Safety and efficacy of zoledronic acid when added to standard therapies in patients with breast cancer and metastatic bone lesions.	Phase III	NCT00372710	Terminated 23/11/2009

used for the treatment of osteoporosis, bone metastasis, treatment-induced bone loss, and giant cell tumor of bone [34]. It inhibits the activation of RANK receptors by directly binding to these receptors on the surface of osteoclasts [35]. Inhibition of RANKL-RANK receptor interaction by denosumab causes reduction in tumor-induced bone demolition [34, 35]. Some clinical trials involving denosumab are shown in Table 3 [33].

5.3. Effectiveness of Bisphosphonates in Clinical Trials

Bisphosphonates including clodronate, pamidronate, ibandronate and zoledronic acids were widely studied in women with breast cancer bone metastasis. These Placebo-controlled trials indicated the effectiveness of bisphosphonates for reducing SREs and are summarized in Table 4 [36-45].

5.4. Adverse Effects and Management of Bone-Targeted Therapies

5.4.1. Acute Phase Reaction

Acute phase reaction (APR) is a systemic host defense response by which the innate immune mechanisms are activated due to inflammation, injury or infection. About 10 to 30 % of patients who have been treated with zoledronic acid and denosumab were shown to experience APR side effects. This can be observed during first three days after treatment. During this process, there is an increase in the number of acute phase proteins (APRs) that are involved in homeostasis, causing influenza-like symptoms, chills, fever, lethargy, increased protein catabolism, reduced appetite, flushing, bone pain, hypotension, myalgia, and arthralgia [47-50]. Laboratory analysis shows increased tumor necrosis factor-alpha (TNFα) and interleukin-6, neutrophilia, and leukocytosis. Most of the reactions are spontaneously reversed after 72 hours of first dose application or can also be managed with non-steroidal anti-inflammatory drugs and antipyretics [47-49, 51].

5.4.2. Nephrotoxicity

Bisphosphonates have a renal route of excretion. About 40-70% of administered dose of bisphosphonates is excreted through kidneys [50, 52, 53]. Unmetabolized bisphosphonates accumulate in tubular cells and can cause apoptosis which leads to acute kidney injury. This type of renal injury could be reversible (e.g. zoledronic acid) or irreversible (e.g. pamidronate). Denosumab is least likely to cause renal injury and can be used as a choice of medication for patients with kidney failure and dependent on dialysis [49, 54, 55]. Preventive measures include monitoring of phosphate, serum creatinine, calcium levels and avoiding the administration of multi-

Table 3. Clinical Trials for Denosumab [33].

Name	Study Phase	Clinical trial Gov. Identifier	Status
Can denosumab prevent recurrence in the bone when given in early stage breast cancer?	Phase III	NCT01077154	Ongoing
Does denosumab reduces the rate of first clinical fracture in women with non-metastatic breast cancer receiving aromatase inhibitor?	Phase III	NCT00556374	Ongoing
Study of denosumab with zoledronic acid in treatment of bone metastasis in subjects with Breast cancer.	Phase III	NCT00321464	Completed 08/03/2017
Study of denosumab in breast cancer subjects with bone metastasis who have not previously been treated with bisphosphonates therapy.	Phase II	NCT00091832	Completed 28/01/2017
A study to evaluate denosumab in young patients with primary breast cancer.	Phase III	NCT01864798	Terminated 05/09/2017
Study of denosumab as Adjuvant treatment for women with high risk early breast cancer receiving Neoadjuvant or Adjuvant therapy.	Phase III	NCT01077154	Ongoing

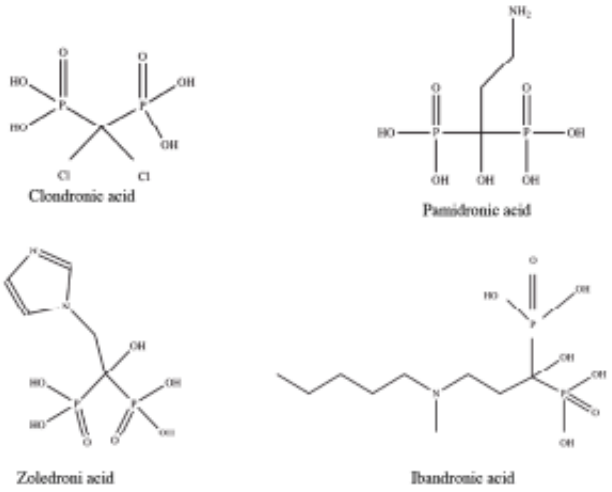

Fig. (3). Chemical structures of some bisphosphonates.

ple nephrotoxic drugs. Patients with renal impairment should get a reduced dose of zoledronic acid [56].

5.4.3. Hypocalcemia

Chances of hypocalcemia with bisphosphonates therapy are 3.4-6% and with denosumab treatment is 5.5-13%. Clinical manifestation could be, general weakness, lethargy, and fatigue [57-59]. Calcium and vitamin D supplements are vital, especially for patients having pre-existing vitamin D or renal insufficiency, hypomagnesaemia, impaired thyroid and parathyroid activity, geriatric patients or patients having gastric surgery.

5.4.4. Jaw Osteonecrosis

Jaw osteonecrosis is caused by vascularization defects in the maxilla or the mandibular bone. This may occur followed by head and neck radiotherapy, use of bisphosphonates or denosumab [60, 61]. During the last decade, 2% of cases of jaw osteonecrosis have been linked with denosumab therapy and 1.4% with zoledronate therapy. Oral health evaluation during bisphosphonates and denosumab therapy is critical to consider. It is essential to avoid invasive dental procedures [62, 63].

5.4.5. Rare Side Effects

Denosumab and bisphosphonates can cause conjunctivitis, scleritis, uveitis [63-68], dermatitis, eczema, rashes [69, 70] or rare atypical femur bone fracture [71, 72].

6. NOVEL BONE-TARGETED AGENTS

Although, denosumab and bisphosphonates are potential agents in improving quality life of patients with breast cancer bone metastasis, they have not been proved to provide progression-free and overall survival improvement from the disease. So, research to explore new potential therapeutic agents is going on. Bone destruction due to breast cancer is a complicated process and mediators that can serve as the basis for developing novel targeted agents are under investigation [73-75].

Table 4. Placebo controlled trials of bisphosphonates [46].

Name	Hypercalcemia	Skeletal Morbidity	SREs	Pain
Clodronate 1600 mg orally daily *vs.* Placebo [36-38]	Total hypercalcemic events reduced	Reduced Fractures	Increased time to first SREs	Reduced Pain intensity
Pamidronate 45 mg i.v. every 3 weeks *vs.* placebo [39]				Increased Pain relief
Pamidronate 90 mg i.v. every 3-4 weeks *vs.* placebo for 2 years [40]			Reduced Proportion of patients With SREs complications	
Pamidronate 60 mg i.v. every 4 weeks *vs.* placebo [41].	Increased time to hypercalcemic events		Reduced SREs	Increased time to progression of pain
Ibandronate 2 mg or 6 mg i.v. every 3-4 weeks *vs.* placebo for 2 years [42]		Reduced mean no. of bone events	Increased time to first SREs	
Ibandronate 50 mg orally daily *vs.* placebo for 96 weeks [43]		Reduced skeletal morbidity	Decreased risk of skeletal related events	
Ibandronate 6 mg i.v. every 4 weeks *vs.* placebo for 24 months [44]			Reduced Proportion of patients With SREs complications Increased time to first SREs	
Zoledronic Acid 4 mg i.v. every 4 weeks *vs.* placebo for 1 year [45]			Reduced rate of SREs Increased time to first SREs	

6.1. Novel Targets for Osteoclast-Mediated Bone Resorption Inhibition

6.1.1. RANKL/RANK

RANKL/RANK pathway plays a key role in the regulation of bone resorption [34]. Osteoblasts have RANKL which is a transmembrane surface protein and can be cleaved by proteases into soluble form [76]. RANKL (both Soluble and membrane-bound forms) can bind to RANK receptors present on the surface of osteoclast precursor. After binding with the receptor, they will cause osteoclastogenesis. OPG is a cytokine receptor and a RANKL antagonist which is produced by osteoblasts and has the ability to inhibit RANKL/RANK interaction [34]. Ion of RANKL and OPG balance is observed in breast cancer [77]. Thus, OPG has the potential to reduce bone destruction and reduce SREs in breast cancer bone metastasis. This activity is exhibited by enhanced osteoclast activity and is confirmed in OPG knockout mice [78-80].

6.1.2. c-Src Kinase Inhibitors

Cellular Src Kinase (c-Src) is a member of Src family (non-receptor tyrosine kinases), also known as proto-oncogene c-Src. C-Src phosphorylates specific tyrosine residues in other proteins. Elevated c-Src levels are associated with cancer progression [81, 82]. c-Src is engaged in performing multiple functions including adhesion, invasion, migration, metastasis, and angiogenesis via chemokine receptor signaling (CXCL12/CXCR4/Akt) pathway or by inhibiting the functions of apoptosis - inducing ligand pathway [83]. Enhanced expression and increased activity of c-Src has been investigated in a variety of cancers. So, inhibitors of c-Src kinases have been proven to play a pivotal role in tumor cell invasion and proliferation. Selective Tyrosine kinase inhibitors (TKIs) cause inhibition of c-Src kinases by blocking osteoclast differentiation [84, 85]. Some preclinical investigations reported that dasatinib, bosutinib, and saracatinib have inhibited osteoclast differentiation [83, 86, 87]. Dasatinib monotherapy has proven efficacious in advanced breast cancer bone metastasis patients [88, 89]. Related clinical trials are shown (Table 5).

6.1.3. Cathepsin K (CTSK)

Cysteine cathepsins are among hydrolytic enzymes and members of the family of papain-like cysteine proteases in lysosome. A cysteine lysosomal protease, called cathepsin K or CTSK is primarily present in osteoclasts. It induces degradation of bone collagen and ultimately causes bone resorption [77, 90]. A preclinical investigation done in animal models of breast cancer bone metastasis showed cathepsin K inhibitors are effective in preventing bone destruction. Furthermore, cathepsin K antagonist can play their role not only in bone resorption inhibition but also in stimulation of bone formation [91]. Cathepsin k may directly act on cancer cells. Odanactinib (a cathepsin K inhibitor) has been proved to successfully reduce the level of bone resorption marker called urinary N-telopeptide of type-I collagen.

Some disadvantages are associated with cathepsin K inhibitors. For example, balicatib (AAE-581, Novartis) is a nitrogen-containing cathepsin K has the ability to accumulate in lysosomes. Due to this accumulation, activities of other lysosomal cysteine cathepsins are inhibited which may lead to severe adverse effects like stroke and skin reactions. For example, morphea-like skin reactions are noticed in 12-month phase III trial patients receiving balicatib therapy. As a result, balicatib was withdrawn from clinical trials [90, 92]. This adverse effect is not shown by odanacatib (MK-0822, Merck), which is under clinical trial investigation for osteoporosis treatment. Odanacatib, has successfully reduced bone resorption markers in a phase-II trial in women having breast cancer bone metastasis after four weeks of therapy [93].

Table 5. Investigated targets for the treatment of bone metastasis [33].

Target	Compound	Phase	Clinical trial Gov. Identifier	Status
Cathepsin K	odanacatib	Phase III	NCT00691899	Withdrawn 12/08/2016
C-Src	dasatinb	Phase III	NCT00692458	Completed 12/08/2016
Avβ3 integrin	etaracizumab	Phase II	NCT00072930	Completed 5/01/2008
TGFβ	fresolimumab	None		
	trabedersen	None		
	galunisertib	None		
CXCL12/CXCR4	plerixafor	None		
	LY2510924	None		

6.1.4. Integrins

Integrins belong to a heterodimeric transmembrane glycoprotein family that mediates adhesion to extracellular matrix proteins and immunoglobulins. So far, 24 heterodimers have been developed by incorporating 18α and 8β subunits. Many types of integrins have an association with bone metastasis but αvβ3 performs a more crucial role in osteoclast function and bone metastasis [94]. According to a preclinical study, some peptidic (e.g. S247, cilengitide, ATN-161) and non-peptidic (e.g. PSK1404) compounds that target αvβ3 could inhibit osteolysis and tumor growth in bone metastasis animal models [95, 96]. These αvβ3 inhibitors, not only antagonized the osteoclast-mediated bone resorption but PSK1404 also prevented bone colonization by cancer cells expressing αvβ3 integrins at the dosage regimen that does not block bone resorption [95]. GLPG0187, ATN-161, IMGN388, cilengitide are different αvβ3 antagonists which are in clinical trials for breast cancer bone metastasis [97].Clinical trial investigations revealed that L-000845704 (a non-peptide antagonist developed by Merck) could, inhibit bone resorption in osteoporosis. Investigations are underway to study its applications in oncology as well [97].

6.1.5. Proteasome

Proteasome is an extra-lysosomal proteolytic enzyme complex. The ubiquitin-proteasome system is involved in degrading intracellular proteins. This system involves the tagging of many intracellular proteins with ubiquitin (which is a small regulatory protein) and then these intracellular proteins are recognized by 26S proteasome complex resulting degradation of these proteins into small peptides. Many proteasome inhibitors (PIs) are under clinical investigation. Preclinical data suggested that PIs exert their effect on three kinds of cell. First, by inhibition of osteoclast differentiation and their function [98]. Second, they enhance bone formation through stimulating osteoblasts differentiation, up-regulating bone morphogenetic protein 2 (BMP-2) and inhibiting runt-related transcription factor (RUNX2) [99]. Finally, PIs block cell proliferation and activate apoptosis in many cancer cells [100, 101] and induce osteolysis in breast and prostate cancer bone metastasis in animal models [102, 103]. However, clinical trials did not show the expected results.

6.1.6. Hedgehog

Cancer progression involves the activation of Hedgehog (Hh) signaling pathway [104] which is also important in the regulation of cancer stem cells. Hh ligand (Desert, Indian and Sonic Hh) bind to transmembrane protein receptors (Patched receptors). Hh Inhibitors exert direct cytotoxic effects on cancer cells. In preclinical animal models, Hh inhibitors blocked osteoclastogenesis and bone metastasis. A phase II clinical trial was designed to investigate the effect of selective SMO (it is a smoothened protein encoded by *SMO* gene) antagonist (sonidegib) in early stage breast cancer (NCT01757327), but it was withdrawn before enrolment.

6.2. Novel Targets for Restoration of Osteoblast Functions

6.2.1. Dickkopf-1 (DKK-1)

Wnt signaling pathways are protein signal transduction pathways that pass signals into the cell through cell surface receptors. DKK-1 is a glycoprotein with a significant role in amphibian's head formation via antagonizing the Wnt signaling pathway. Osteoblastogenesis process involves Wnt signaling pathway. Wnt proteins in association with low density lipoprotein receptor-related proteins 5 and 6 (LRP5/6), bind Frizzled receptors (G Protein-coupled receptors) and initiates signaling via β-catenin. This process activates different genes involved in osteoblastogenesis [105]. DKK-1 binds to LRP5/6 and blocks it's binding with Wnt-1, causing breakdown of β-catenin and inhibit osteoblast differentiation. DKK-1 was shown to be elevated in serum and bone marrow of patients with multiple myeloma [106]. Neutralizing antibodies that block DKK-1 cause reduction in osteolysis, skeletal tumor growth in addition to an increase in the osteoblast number and osteocalcin level in the serum [107, 108]. There are some preclinical and clinical evidence that breast cancer cells that metastasize to bone secrete DKK-1 [109]. A Phase I clinical trial investigates a combination of DKK1-neutralizing antibody, BHQ880 and zoledronate in myeloma patients [109].

6.2.2. Sclerostin

Sclerostin is a secreted glycoprotein that is encoded by *SOST* gene. It is produced by osteocytes. Sclerostin promotes migration, invasion of cancer cells and osteolysis and has anti-anabolic effect on bone formation. Sclerostin binding to LRP5 receptors can be blocked by antibodies that neutralize sclerostin [110]. Sclerostin neutralizing antibody is used to treat osteoporosis e.g. romosozumab [111]. However, no clinical trial is available to study the effects of sclerostin-blockers on metastatic bone disease.

6.2.3. Activin A

Activin A is widely distributed in all human tissues and belongs to TGF-β family of growth factors. Serine and threonine kinase transmembrane receptors mediate the effects of activin A. Activin A activates ActR1B or ALK-4 type 1 receptors that leads to the phosphorylation of receptor-regulated Smad proteins (RSmad4), Smad2, Smad3 and Smad4. Activin A gets entered in the nucleus that results in gene transcription regulation in bone cells. Activin A activates bone degradation, inhibits osteoblast differentiation and stimulates osteoclast differentiation [112]. Higher serum levels of

Activin-A are found in breast cancer patients with bone metastasis as compared to the patients without bone metastasis [113]. Therefore, this cytokine can be regarded as a potential target for more specific treatment measures for skeletal metastasis.

RAP-011 is an activin A receptor ligand. Recently, different groups have shown the combined effect of RAP-011 with Act RIIA receptors to serve as potential therapeutic targets in treatment of skeletal metastasis. RAP-011 can be measured as biochemical marker of bone metastatic disease [113].

6.2.4. Endothelin-1

Endothelins are peptides that constrict blood vessels. They produce their effect by binding to their receptors, ET_A and ET_{B1}, ET_{B2} and ET_C receptors. Breast cancer cells produce endothelin-1 (ET-1) that activates mitogenesis in osteoblasts, resulting a reduction in osteoclast activity [114]. ET_A antagonist ABT-627 (atrasentan) could inhibit osteoblastic breast cancer bone metastasis [115]. Bosentan is a dual endothelin receptor antagonist (ET_A and ET_B receptor) approved to be used in treatment of pulmonary artery hypertension. This mixed inhibitor was shown to block breast cancer bone metastasis *in vivo* [116].

6.3. Novel Targets for Bone-Derived Growth Factors

6.3.1. Transforming Growth Factor-Beta (TGF-β) Signaling

TGF-β is a multifunctional cytokine of transforming growth factor superfamily, having four different isoforms (TGF-β1, TGF-β2, TGF-β3 and TGF-β4).

TGF-β binds to TGF-β type I receptor (ALK5) and TGF-β type II receptors (TβRII) which are serine/threonine heterodimeric kinases. It phosphorylates Smad2 and Smad3 which are TGF-β specific mediators. This Phosphorylated complex then binds to Smad4 and translocates to the nucleus and regulates TGF-β genes. TGF-β in turn regulates the growth of many factors like IL-6, IL-8, IL-11, integrin αvβ3, MMP-1 and CXCR-4 which play a key role in bone metastasis [117].

Hence inhibition of the TGF-β signaling can be considered as a potential target to reduce bone metastasis. Many strategies have been developed to block TGF-β signaling including TβRI inhibitors, dominant negative TβRII, neutralizing TGF-β antibodies and antisense oligonucleotides. These have been investigated to inhibit bone metastasis to breast cancer in preclinical trials. Although the effects of these TGF-β inhibitors have been investigated in different types of cancers, no clinical trials have been performed to explore their effect in breast cancer bone metastasis [117, 118].

Epithelial-to-mesenchymal transition (EMT) process has been found to play a role in cancer and metastasis progression. In this process, epithelial cells gain properties to migrate and invade and become mesenchymal stem cells and initiate metastasis. TGF-β signaling through Smad pathway serves as an effector of this process [119]. Exogenous Bone morphogenetic protein-7 (BMP-7) inhibits TGF-β signaling which antagonizes EMT signaling in prostate and breast cancer bone metastasis model in animals [120, 121]. Another animal study revealed the role of TGF-β signaling in the regulation of the Jagged1-Notch pathway. Jagged1 is a cell surface protein that regulates Notch signaling pathway. Up-regulation of JAG 1 has been found to be associated with poor breast cancer survival rates. MRK-003, a γ-secretory inhibitor, has shown to inhibit Jagged1-Notch signaling pathway and hence cause a reduction in bone metastasis to breast cancer [122]. These findings revealed that a strategy against breast cancer bone metastasis can be developed based on TGF- β-dependent EMT signaling, γ-secretase or BMP-7 inhibitors.

6.3.2. Insulin-like Growth Factors (IGFs)

Insulin-like growth factors (IGF-I and IGF-II) exist abundantly in bone and have been involved in spreading, development and aggressiveness of many different cancers. IGFs exert their action by binding to IGF type I receptors (IGF-IR). IGFs activate IGF-IR/Akt/NF-kB pathway, stimulates proliferation and increases bone tumor burden [123]. An IGF-IR inhibitor e.g, PQIP (Chemical formula C3OH31N7) reduced the osteolytic lesion size in breast cancer bone metastases [124].

6.4. Novel Agents Targeting Bone Environment

6.4.1. Chemokine Receptor Signaling (CXCL-12/CXCR-4)

Almost all types of cells secrete chemokines. Most of the chemokines are involved in adaptive and innate immune systems, while a few of chemokines such as CXCL-12 that are produced by the osteoblasts, play a pivotal role in the regulation of cellular trafficking. It is proved that chemokines play a vital role in cancer metastasis [125]. Chemokine receptors like, CXCR3, CCR4, CXCR4, CCR5 and CCR7 and especially CXCR, are found to be involved in the metastasis regulation process. CXCR4 is found to play a fundamental role in organ-specific breast cancer metastasis, including liver, lung and bone metastasis. In these organ CXCL-12 (CXCR4 ligand) is produced in high quantity [125].

The proposed mechanism is that after CXCL-12 binds to CXCR4 and activates the non-receptor Src, tyrosine kinase, AKT pathway is activated in bone marrow breast cancer cells [126]. Consequently, the CXCL-12/CXCR-4 pathway can serve as a targeted therapy to treat bone metastasis. Synthetic peptide antagonist like CTCE-9908 and antibodies could block this CXCL-12/CXCR-4 pathway and reduce bone and lung metastases caused by breast cancer cells in preclinical experiments [127, 128].

6.4.2. Cadherin-11

Osteoblasts and bone marrow stromal cells express cadherin-11, which is a member of type 2 cadherin family. In one animal study, it was demonstrated that the overexpression of cadherin-11 in breast cancer cells was associated with metastasis to bone but not to the lungs. This finding suggested that cadherin-11 can be used as a specific and novel target for treating bone metastasis. Yet, no agent has reached clinical trial [123].

6.4.3. Targeting Runx2

The bone transcription factor Runx2 that is a member of Runt-Related Transcription factor (Runx) family has crucial role in bone development by controlling osteoblasts and osteoclast processes [129, 130]. It has been proved that Runx2 facilitate the interaction between cancer cells and the microenvironment of bone. Runx2 suppresses the ubiquitination of oculo-dento-digital dysplasia-hyoxia inducing factor (ODDD) HIF-1α by directly binding to ODDD-HIF-1α. Vascular angiogenesis during endrochondral bone formation is regulated by HIF-1α and vascular endothelial growth factor (VEGF). Runx2 has been identified to be involved in tumor invasion by regulating matrix metallopeptidase 9 (MMP9) [131]. It has also been proved to play a crucial role in osteoclasts activation by gene regulation for OPN, M-CSF and PTHrP. Runx2 indirectly blocks Wnt signaling pathway and promotes activation of osteoclasts [131].

6.4.4. Targeting microRNAs (miRNAs)

MicroRNAs (miRNAs) belong to 21-23nucleotide- noncoding, long RNAs which are transcribed by RNA polymerase type II & III. Generally, miRNA cause either mRNA degradation or translational silencing by binding to their complementary site at the 3-untranslated region [132]. It is evident that normal and cancer cells have different expressions for miRNAs. They can either enhance or inhibit the development and progression of the tumor. Many types of miRNAs have been found to be involved in regulation of bone metastasis [133]. Thus, miRNAs involved in bone metastasis development can serve as a target for treating breast cancer bone metastasis. Very few miRNAs, e.g. miR-141 and miR-219, are found to inhibit osteoclast activity and osteolytic activity in breast cancer bone metastasis. miR-203 and miR-219 also have reducing effects

Table 6. Some FDA approved drugs for breast cancer available in market [137].

Brand Name	Generic Name	Manufacturer	Drug Nature	Indication	Approval Date
Perjeta	Pertuzumab	Genentech	Monoclonal Antibody	First line treatment of HER2$^+$ metastatic breast cancer	June 2012
Halaven	Eribulin mesylate	Eisai	Macrocyclic Ketone Analogue	Metastatic breast cancer	November 2010
Xgeva	Denosumab	Amgen	Human Monoclonal Antibody	Preventing skeletal-related events in patients with bone metastasis from solid tumors	November 2010
Evista	Raloxifene hydrochloride	Eli Lilly	Estrogen receptor modulator	Prevention/Treatment of osteoporosis and reduction of breast cancer risk in postmenopausal women	September 2007
Ixempra	ixabepilone	Bristol-Myers Squibb	Epothilone B Analog	Breast Cancer	October 2007
Tykerb	lapatinib	GlaxosmithKline	Dual Tyrosine Kinase Inhibitor	Breast cancer	March 2007
Herceptin	Trastuzumab	Genentech	Monoclonal Antibody	Metastatic breast cancer	October 1998
Nolvadex	Tamoxifen citrate	AstraZeneca	Selective estrogen receptor modulator	Breast Cancer	October 1998
Xeloda	Capecitabine	Roche	Antimetabolite	Advanced breast cancer tumors	April 1998
Quadramet	Samarium Sm 153 Lexidronam Injection	Dupont Merck Pharmaceutical Company	Chelated complex	Pain associated with bone cancer	March 1997
Aredia	Pamidronate disodium for injection	Chiron	Nitrogen containing Bisphosphonates	Osteolytic bone metastasis of breast cancer	August 1996
Arimidex	Anastrozole	Astrazeneca	Aromatase Inhibitor	Advanced breast cancer in postmenopausal women	January 1996
Taxotere	Docetaxel	Rhone Poulenc Rorer	Microtubule Inhibitor	Locally advanced or metastatic breast cancer	May 1996

on breast cancer bone metastasis [134]. Several intronic miRNAs associated with cancer have been discovered in humans, including miR-10b, miR-16-2, miR26a1, miR26-a2, miR-126, miR-17-92, miR-15b [135].

Several miRNAs, either directly or indirectly, regulate Runx2 in breast cancer progression. miRNAs are associate with bone metastasis initiation (let-7g, miR-146a, miR-335, osteolytic activity (miR-133a, miR-190). Further investigation is required to explore the regulatory role of Runx2 via miRNA and its potentials as a novel target for bone metastases [131].

6.5. Targeting Cancer Stem Cells

Stem-like cells (CSCs) are tumorigenic cells and may generate tumors through the stem cell renewal and differentiation. The bone marrow aspirates from cancer patients showed that majority of early metastatic cells have CSC markers. In a recent pre-clinical study, CD44-positive CSC-like cells were shown to have an increased capacity to metastasize to bone [136]. The CSC biology is yet to be fully understood. CSCs and their niches could be considered as targets for preventing and treating breast cancer bone metastasis.

6.6. FDA Approved Drugs for Cancer Treatment Available in Market

Some of the FDA approved breast cancer drugs are given in Table 6.

CONCLUSION

Bone metastasis significantly affects the quality of life of patients with breast cancer and new targeted strategies are in urgent demand to prevent and palliate skeletal events. Currently available clinical treatments can often shrink or slow the growth of bone metastases. However, these treatments are not able to eradicate bone metastatic foci. Bone metastasis progresses over time and leads to SREs, substantial morbidity and mortality and there are not sufficient evidences available to demonstrate which bone modifying agent is the preferred choice. Advances in the discovery of different novel targets described in this review, not only provides insights into making a better use of the currently available agents but also the development of new targeted therapeutic interventions. These novel targets can be used with the currently available treatment options available in clinic to effectively inhibit the development of

bone metastasis in women with breast cancer. More in-depth preclinical and clinical investigations are required to optimize the current treatment strategies by elucidating the interactions between tumor cells and bone microenvironment to reach maximum effectiveness. Further investigations are warranted to discover new agents that can prevent bone metastasis in breast cancer patients to avoid the associated morbidity and mortality due to the bone metastasis

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

We acknowledge National Breast Cancer Foundation Research Fellowship to Pegah Varamini (PF-16-007).

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APPENDIX 2

Presentations……

- Presented in “Postgraduate Conference” at The University of Sydney in 2017.
- Presented in “3 MT Presentation” at The University of Sydney in July 2018.

- Presented in a conference on “Joint Event on Global Pharmacovigilance and Advanced Pharmacy” held during July 16-17, 2018 in Sydney, Australia

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Prof/Dr. **Iram Irshad**

University of Sydney, Australia

for his phenomenal and worthy oral presentation on

“Pharmacological evaluation of an advanced formulation of curcumin to prevent breast cancer bone metastasis”

at the “Joint Event on Global Pharmacovigilance and Advanced Pharmacy”

held during July 16-17, 2018 in Sydney, Australia

Oweikumo Eradiri
Colchester Hospital University NHS Foundation Trust, UK

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- Presented couple of times at “Sydney Pharmacy Joint Cancer Journal Club” during 2017-2018.
- Presented couple of time in joint group presentation with “Project Consumer” during 2017-2018.

APPENDIX 3

Thesis Authorship attribution statement

Chapter 2 is published as:

Irshad I, Varamini P. Different Targeting Strategies for Treating Breast Cancer Bone Metastases. Current Pharmaceutical Design. 2018 Apr.

Dr. Pegah Varamini codesigned and supervised the review article and provided intellectual input into it. Iram Irshad wrote the manuscript and provided intellectual output.

Chapter 3 is being prepared for submission to Nanomedicine (IF: 6.692) as:

Iram Irshad[1], Sepideh Khazeni, Ghada Aboueid, Ramin Rohanizadeh[2], Pegah Varamini[1*]. Pharmacological Evaluation of an Advanced Formulation of Curcumin to Prevent Breast Cancer Bone Metastasis.

Dr. Pegah Varamini initiated, co-designed and supervised the project. She also provided her intellectual input into the project. Iram Irshad co-designed and carried out the core experiments. Dr. Pegah Varamini has performed cell viability analysis. Sepideh Khazeni performed "Uptake Studies" with Iram Irshad. Sepideh Khazeni performed statistical analysis for uptake studies. Ghada Aboueid has performed "IncuCyte Live Cell Analysis" with Iram Irshad. Iram Irshad has written the manuscript for publication and Dr. Pegah Varamini has provided scientific input into the manuscript. Annim Mohammad has edited the Introduction part of manuscript.

Iram Irshad

Dated 13/09/18

As a supervisor of the candidature upon which thesis is based, I can confirm that the authorship attribution statements above are correct.

Dr. Pegah Varamini

Dated: 13/09/18

APPENDIX 4

Other Contributions

- Other than above research work, I have also contributed equally with my colleague in writing a manuscript which is ready for submitting to a journal.

"**LHRH-Receptor Mediated Targeting of Cancer Cells Using Curcumin Nano-micelle Formulation".**

Nadeem Ahmed[1], Iram Irshad[1], Sepideh Khazeni, Ghada Aboueid, Ramin Rohanizadeh[2], Pegah Varamini[1*]

- I have also collected mice tissues for my colleagues Sepideh Khazeni & Ghada Aboueid working on breast and prostate cancer projects.