

Review

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The phytochemical plumbagin reciprocally modulates osteoblasts and osteoclasts

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Abstract: Bone metabolism is essential for maintaining bone mineral density and bone strength through a balance between bone formation and bone resorption. Bone formation is associated with osteoblast activity whereas bone resorption is linked to osteoclast differentiation. Osteoblast progenitors give rise to the formation of mature osteoblasts whereas monocytes are the precursors for multi-nucleated osteoclasts. Chronic inflammation, auto-inflammation, hormonal changes or adiposity have the potential to disturb the balance between bone formation and bone loss. Several plant-derived components are described to modulate bone metabolism and alleviate osteoporosis by enhancing bone formation and inhibiting bone resorption. The plant-derived naphthoquinone plumbagin is a bioactive compound that can be isolated from the roots of the *Plumbago* genus. It has been used as traditional medicine for treating infectious diseases, rheumatoid arthritis and dermatological diseases. Reportedly, plumbagin exerts its biological activities primarily through induction of reactive oxygen species and triggers osteoblast-mediated bone formation. It is plausible that plumbagin's reciprocal actions – inhibiting or inducing death in osteoclasts but

promoting survival or growth of osteoblasts – are a function of the synergy with bone-metabolizing hormones calcitonin, Parathormone and vitamin D. Herein, we develop a framework for plausible molecular *modus operandi* of plumbagin in bone metabolism.

Keywords: bone remodeling; ligand–receptor interaction; molecular docking; osteoblast; osteoclast; plumbagin.

Introduction

Bone provides an essential endoskeletal structure and functions for support, movement, and protection. It stores minerals, mainly calcium and phosphate, and of course harbors the bone marrow (Datta et al. 2008; Robling et al. 2008). It is therefore part of this essential primary lymphoid organ wherein hematopoietic stem cells (HSCs) reside and further differentiate into immune cells. Hematopoiesis occurs in the perivascular site at the side of the sinusoids and osteogenesis occurs in the endosteal niche. However, both compartments interact under physiological as well as pathological conditions (Chen et al. 2021).

Bone is constituted of four types of cells, viz. osteoblasts, bone lining cells, osteocytes, and osteoclasts (Mohamed 2008). These bone cells have distinct roles in the process of “bone remodeling.” Osteoblasts are cuboidal cells located along the bone surface comprising 4–6% of the total resident bone cells. They are known mainly for their bone-forming function (Capulli et al. 2014). The bone lining cells are the quiescent flat-shaped osteoblasts and have the capacity to redifferentiate into osteoblasts and produce bone matrix whenever needed and additionally are involved in transport processes (Allen and Burr 2014; Florencio-Silva et al. 2015). Osteocytes being abundant in bone tissue comprise 90–95% of the total bone cells with a lifespan of up to 25 years. They are terminally differentiated osteoblasts embedded into the bone matrix (Franz-Oendaaal et al. 2006; Qin et al. 2020; Sarutipaiboon et al. 2020). Osteoclasts originate from hematopoietic precursor cells that differentiate to mature multi-nucleated osteoclasts

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under the influence of the cytokines macrophage colony-stimulating factor (M-CSF) and receptor of nuclear factor kappa-B ligand (RANKL) secreted by bone marrow stromal cells and osteoblasts (Bar-Shavit 2007). Among these four types of bone cells, osteocytes are the primary cells responsible for bone calcification. Bone calcification and resorption are processes that are tightly coupled with each other in "Bone remodeling". Different factors including diet, hormones, injury, friction during movements, calcium/phosphate homeostasis, anti-calcifying proteins, matrix composition, calciprotein particles (i.e. colloidal calcium phosphate nanoparticles), etc. play an essential role in bone remodeling (Weilbaecher et al. 2011). This physiological process continues throughout life to maintain mineral homeostasis and in response to mechanical loading. It can be disturbed by chronic inflammation, cancer, diabetes or loss of steroid hormones resulting in disbalance and loss of bone material and increased fracture risk.

Types of bone cells

Osteoblasts

Osteoblasts have a basophilic cytoplasm that can be stained with toluidine blue. Osteoblasts are generally activated when there is damage or fracture in a bone, or quality of bone is compromised, which triggers the cells to synthesize new bone matrix. They show the highest activity during embryonic skeletal formation and growth. Osteoblasts synthesize bone matrix proteins such as collagen type 1 alpha 1, alkaline phosphatase (ALP) and osteocalcin (Jensen et al. 2010). Osteoblasts are late responders to a given signal, as it takes four months to complete new bone matrix synthesis by the mature osteoblast. In adults, osteoblasts are active only to rebuild depleted bone matrix or to reform the defect (Canalis 2008; Rutkovskiy et al. 2016). Osteoblasts express Parathyroid hormone (PTH) receptors which exert their bone formation effect through osteocytes. Mechanical loading on bone and PTH signaling simultaneously inhibit the expression of sclerostin from osteocytes which removes the inhibition of Wnt signaling (Allen and Burr 2014). Osteoblasts can be further distinguished as pre-osteoblast cells, differentiated osteoblasts, and mature osteoblasts. Osteoblasts have an important role in osteoclast differentiation, as they can produce RANKL, the cytokine necessary for osteoclast differentiation (Han et al. 2018; Xing et al. 2018). RANKL-deficient mice develop osteopetrosis with blockage of bone marrow space in the endosteal space (Kong et al. 1999) due to the absence of

mature multinucleated osteoclasts despite the presence of osteoclast progenitors. The precursors retain the ability to differentiate into mature osteoclasts when co-cultured with wildtype osteoblasts (Dougall et al. 1999). While the action of RANKL is non-redundant and cannot be replaced by any other cytokine, RANKL is still a pleiotropic cytokine that is additionally involved in the function and survival of dendritic cells (DC), the control of self-tolerance through the interaction of DCs with regulatory T cells and the formation of lymphoid organs as well as the differentiation of intestinal M cells (epithelial cells of the mucosa involved in antigen transport) and epithelial cells in the medulla of the thymus (Akiyama et al. 2012).

Osteoblast differentiation

Osteoblasts are the bone building cells derived either from mesenchymal progenitors or via an osteochondral progenitor (Rutkovskiy et al. 2016). During skeletogenesis, formation of skull and clavicles occurs through intramembranous ossification where osteoblast formation takes place directly from the mesenchymal progenitors. However, formation of axial skeleton and limbs occurs by endochondral ossification where skeleton is built by an intermediate class of perichondral cells which are derived from osteo-chondro progenitors (Nishimura et al. 2012). These osteo-chondro progenitors are capable of producing both osteoblasts and hypertrophic chondrocytes, which help regulate the conversion of perichondral cells to pre-osteoblasts (Rutkovskiy et al. 2016). Osteoblasts can be found on the surface of the newly formed osteoid. They are post-mitotic but not terminally differentiated cells and their main function is the formation of extracellular bone matrix (osteogenesis) that subsequently mineralizes. The osteoblasts localized towards the periosteum surface of bone become bone lining cells. The activity of osteoblasts is highest during embryonic growth and development of skeleton. However, in adults, the osteoblasts are active only to rebuild depleted bone matrix or to reform the defect (Rutkovskiy et al. 2016). Bone formation by osteoblasts is regulated by important transcription factors such as Runx2 and other growth factors such as bone morphogenic proteins (BMPs) which belong to the transforming growth factor beta superfamily (Russell et al. 2006) (Figure 1A). BMP, Wnt, Notch are the crucial signaling pathways for osteoblastogenesis driving the expression of the runt-related transcription factor (*RUNX2*), distalless homeobox 5 (*DLX5*), and osterix (*OSX*) that are essential for osteoblast differentiation (Lin et al. 2011; Liu and Lee 2013). BMP signaling pathways occur through the canonical SMAD-dependent pathway comprising BMP

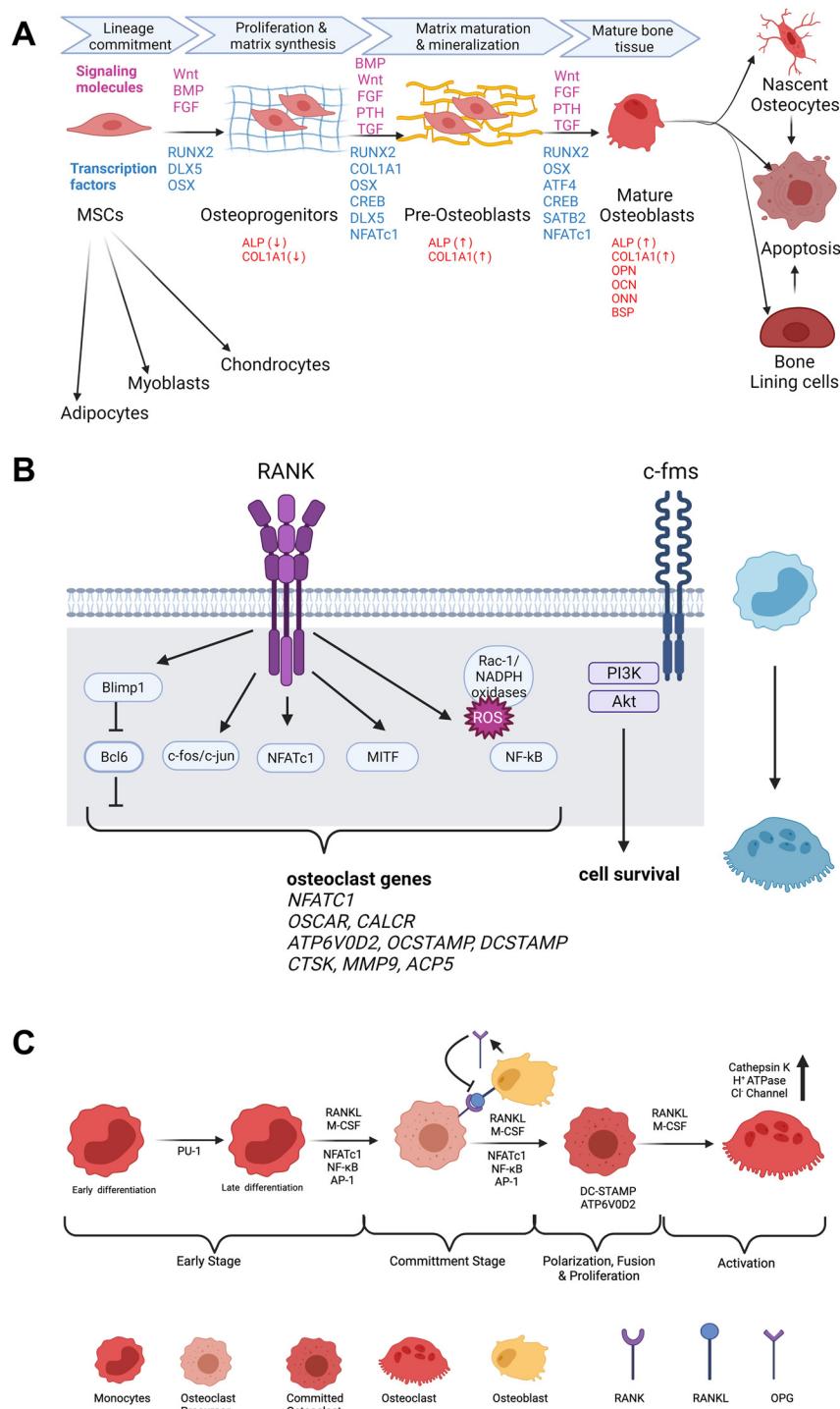


Figure 1: Osteoblast and osteoclast differentiation.

(A) Different stages of osteoblast differentiation from mesenchymal stem cells (MSCs) showing the role of various signaling molecules (in pink) and transcription factors (blue). (B) RANKL and M-CSF-mediated signal transduction pathways trigger differentiation of monocyte precursors into mature osteoclasts. (C) Early stage: early monocyte to late monocyte differentiation is mediated through PU.1. 2. Late stage: In the late monocytic stage M-CSF binds to the c-fms on the late monocytes and differentiates them into the osteoclast precursor. 3. Commitment stage: Osteoblasts produce RANKL which promotes the transition of precursor osteoclasts to committed osteoclasts with the help of transcription factors like NFATc1, AP-1 and NF-κB in the commitment step; 4. Polarization and fusion: Committed osteoclasts express DC-STAMP and ATP6v0D2 which causes the fusion and polarization of committed osteoclasts. 5. Activation: In this step multinucleated osteoclasts are formed which show high expression of the chloride channel, H⁺ ATPases, carbonic anhydrase II. Figures were created using BioRender.com.

receptors, BMP ligands and SMAD. BMP signaling also occurs through the non-canonical SMAD-independent pathway comprising p38 mitogen-activated protein kinases. Both signaling pathways converge to activate RUNX2 for driving osteoblast differentiation from its progenitors (Chen et al. 2012). Studies in mice suggested that RUNX2 is the master gene in osteoblast differentiation (Jensen et al. 2010). The second major signaling cascade for osteoblastogenesis is the Wnt pathway. Canonical Wnt signaling pathway requires GSK-3 β inhibition and subsequent accumulation of β -catenin and its entry into the nucleus which stimulates transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF) for RUNX2 transcription. Wnt signaling activities that are independent of β -catenin constitute different non-canonical Wnt signaling pathways. Notch signaling is the third important pathway for osteoblast differentiation from mesenchymal stem cells (MSC). This pathway involves Notch, Jagged or membrane bound ligands Delta on the surface of neighboring cells, Notch intracellular domain (NCID), co-activator mastermind-like (MAML) and Notch target genes such as hairy and enhancer of split-1 (*HES*) and Hairy-Related Transcription Factor 1 (*HEY*) (Bolós et al. 2007; Watt et al. 2008).

Osteoblast differentiation is further influenced by other factors like Fibroblast growth factors (FGF), miR-29 and connexin-43 (Kapinas et al. 2010). Connexin-43 is an important gap junction protein of bone cells which regulates cell survival, mechanotransduction, and cell signaling pathways in bone cells (Buo and Stains 2014). Studies show that a mutation in connexin-43 causes defective osteoblast differentiation and malformation of the skeleton (Flenniken et al. 2005). FGF2-null mice show reduced trabecular bone mass, decreased bone mineralization coupled with increased adipocyte production, indicating an important role for FGF2 in regulating bone mass and bone mineralization (Montero et al. 2000). Additionally, recombinant human (rh)FGF18 treatment stimulates osteoblast differentiation in MSCs by increasing RUNX2 expression along with other osteoblast-specific markers *in vitro* (Hamidouche et al. 2010).

The synthesis of bone matrix takes place in two steps: synthesis of the organic matrix, followed by bone matrix mineralization. During the first stage, osteoblasts synthesize type-I collagen and non-collagen proteins like Osteocalcin, Osteopontin, Osteonectin, Bone Sialoproteins type-I and II, along with proteoglycans like decorin and biglycan that form the organic matrix. After this, bone mineralization occurs in a vesicular and a fibrillar phases (Yoshiko et al. 2007). In the vesicular phase, vesicles of a size of 30–200 nm that contain calcium inside are released from the osteoblast apical membrane. These vesicles are attached to the proteoglycan on the newly synthesized bone matrix. Due to the negative

Table 1: Signaling pathways involved in osteoclast and osteoblast during bone remodeling.

Signaling pathways in osteoblasts	Signaling pathways in osteoclasts
TGF- β -Smad signaling	RANKL signaling
FGF signaling	NF- κ B signaling
Wnt signaling pathway	Macrophage-colony stimulating factor
Ephrin signaling	Src signaling
Hedgehog signaling	ITAM signaling
PTH signaling	TGF- β -Smad signaling

charge, sulphated proteoglycans can immobilize calcium ions inside the matrix vesicles (Arana-Chavez et al. 1995; Yoshiko et al. 2007). The proteoglycan is then degraded and the immobilized calcium passes through the calcium channel, which is composed of annexin (Anderson 2003). Subsequently, ALP activity releases phosphate by degrading the phosphate-containing compounds. This phosphate moves into the vesicle, where it nucleates with calcium and forms hydroxyapatite crystals (Glimcher 1998). In the fibrillar stage, excess accumulation of hydroxyapatite causes the vesicle's rupture, and hydroxyapatite is released into the nearby bone matrix (Boivin et al. 2008; Boivin and Meunier 2002). Osteoblasts show cytoplasmic structures that move to the osteocytes through the bone matrix (Aguirre et al. 2006; Manolagas 2000). TUNEL positive and ovoid structures containing dense bodies were observed inside the osteoblast vacuoles. This study suggested that in addition to professional phagocytes, osteoblasts also show phagocytic activity by engulfing and degrading apoptotic bodies during alveolar bone formation (Cerri 2005). The central signaling pathways involved in bone remodeling (Pandey et al. 2018) are listed in Table 1.

Osteocytes

The osteocytogenesis and osteocyte maturation require a membrane-associated protein called E11 and MMP14 metalloprotease for dendrites and canaliculi formation (Zhang et al. 2006). The long dendrites extend and interconnect through a tunnel structure called "canalliculi" within the bone matrix after mineralization. Dendrites also interact with the osteoblast on the bone surface (Kamioka et al. 2001). Genes involved in the dendritic formation, such as E11/GP38, CD44, and fimbrin, decide the osteocytes' morphology. Dendritic projections can also reach to periosteal and endocortical surfaces of the bone and establish

communication through the cell-to-cell interaction between osteocytes and other bone cells (osteoblasts, lining cells, and osteoclasts). With the help of capillaries derived from the Haversian Canal, osteocytes can establish direct contact with blood vessels within the marrow (Bonewald 2011). Tracer experiments had indicated that osteocytes secrete transport proteins of a molecular weight up to 70 kD and 70 nm in diameter in the fluid, which could run through canaliculi to reach its target cell (Kramer et al. 2010; Wang et al. 2004). A study by Paic et al. in 2009 identified differential expression of genes between osteoblasts and osteocytes. For example, unlike osteoblasts, osteocytes express a decreased level of alkaline phosphatase and type-I collagen but a high level of osteocalcin and genes for mineralization and phosphate metabolism (Paic et al. 2009).

Additionally, osteocytes also express phosphate regulating neutral endopeptidase dentin matrix protein 1 (DMP-1), extracellular matrix phospho-glycoprotein, and fibroblast growth factor (Bonewald 2011; Paic et al. 2009). Moreover, BMP and Wnt genes involved in osteoblast differentiation are repressed by osteocyte Dkk1 and sclerostin (product of *SOST* expressed predominantly by osteocytes), by binding to LRP5 and LRP6, thus repressing bone formation. So, the production of osteocytes can directly control the formation and activity of osteoblasts.

Osteocytes sense and respond to mechanical signals and occurring bone fracture or damage, which will initiate the bone remodeling process (Verborgt et al. 2002). The sclerostin level is altered in response to mechanical stimuli. Bones under continuous mechanical stress show decreased levels of sclerostin, which ultimately maintains high bone mass and strength by activating the Wnt signaling pathway, whereas bone with low mechanical stress shows high expression of *SOST* (Robling et al. 2008). This has also been experimentally proven as deletion of *SOST* results in progressive high bone mass (Lin et al. 2009). On the other hand, transgenic mice overexpressing *SOST* show a low bone mass (Rhee et al. 2011). In humans, an increased bone mass disorder such as van Buchem disease is caused by a noncoding deletion that removes a *SOST*-specific regulatory element in bone (Sebastian and Loots 2018), suggesting the possible treatment of post-menopausal osteoporosis by targeting osteocytes to increase bone mass and strengthen the bone (Paszty et al. 2010).

Bone lining cells

Osteoblasts that have not died by induced cell death and did not become osteocytes can remain on the bone surface as bone lining cells. These cells have a quiescent phenotype

and can be reactivated if appropriately stimulated, e.g., by PTH treatment or Sclerostin inhibition. They have therefore an interesting therapeutic potential in the therapy of osteoporosis (Kim et al. 2017).

Osteoclasts

In the bone marrow, HSCs give rise to the various cells of the innate and adaptive immunity. The common myeloid progenitors can differentiate into common monocyte progenitors which serve as the progenitor cells for osteoclast differentiation (Xing et al. 2005). Osteoblasts produce RANKL, which serves as the ligand for RANK receptor present on pre-osteoclast cells. This signal is essential for the differentiation of osteoclasts and the coordination between these two cell types is vital to maintain bone homeostasis. Progenitor cells then fuse to form polycaryons that further mature into extremely large (often $\geq 100 \mu\text{m}$), multinuclear (≥ 3 nuclei) osteoclasts that express the tartrate resistant acid phosphatase (TRAP) as well as several proteases that play a role in bone resorption. As the progenitor cells are macrophage-like cells with phagocytic activity, osteoclasts are able to phagocytose osteocytes as part of the bone degradation process (Soysa et al. 2012). Osteoclasts also retain the ability to act as antigen presenting immune cells towards T cells via MHC I/II, and osteoclast-produced inflammatory cytokines enhance T cell activation (Roper et al. 2020). However, osteoclasts seem also to be able to act as potential immune suppressors, comparable to myeloid-derived suppressor cells, as both cell types originate from the same heterogenous population of precursor cells (Gabrilovich and Nagaraj 2009). This phenotype is to be especially relevant in the setting of bacterial infection, where bacterial products aim to down-regulate the response of the immune system (Seebach and Kubatzky 2019).

Osteoclast differentiation

Haematopoietic cells give rise to myeloid and lymphoid progenitors, which through several differentiation stages form multinucleated and resorbing osteoclasts. The differentiation occurs under the influence of M-CSF, secreted by osteoblasts and osteoprogenitor mesenchymal cells. In addition to osteoblasts, osteocytes, and stromal cells express RANKL (Francis et al. 2009). M-CSF binds on the precursor osteoclast to its cognate receptor c-fms, stimulating osteoclast proliferation and inhibiting apoptosis (Yavropoulou and Yovos 2008; Yoshida et al. 1990)

(Figure 1B). RANKL binds to RANK receptor present on the osteoclast precursor and leads to osteoclasts formation (Sodek and McKee 2000). On the contrary, stromal cells, gingival cells, and periodontal fibroblasts secrete osteoprotegerin (OPG) that antagonizes RANKL and acts as a decoy receptor (Boyce and Xing 2008). This serves as a mechanism to inhibit the interaction between RANKL and RANK in order to moderate osteoclastogenesis (Phan et al. 2004). Downstream of RANK, the transcription factor NFATc1 gets activated resulting in the induction of several osteoclastic genes but also *Nfatc1* itself to amplify the RANKL-mediated signaling. The action of NFATc1 is further supported by the activation of AP-1 (c-fos/c-jun), MITF and canonical and non-canonical NF- κ B signaling as well as the repression of the transcriptional inhibitor Bcl6 through Blimp1 (Kurotaki et al. 2020). Osteoclastic genes can be grouped into genes with a function in bone resorption such as the gene for TRAP (*ACP5*), matrix metallopeptidase (*MMP9*) or Cathepsin K (*CTSK*), genes involved in the osteoclast fusion processes (*DCSTAMP*, *OCSTAMP*, *ATP6VOD2*) and co-receptors, for examples OSCAR or Calcitonin receptor (*CALCR*) (Longhini et al. 2014).

Osteoclasts show four types of membrane domains, viz., sealed zone, ruffled border, basolateral domain, and functional secretory domains (Mulari et al. 2003). Osteoclasts bind extracellular mineralized matrix via $\alpha_5\beta_3$ integrin and CD44 through an osteoclastic structure known as “podosome” (Chabadel et al. 2007; Lakkakorpi et al. 1991). The ruffled border consists of the microvilli structures of osteoclasts which form a clear zone separated from the surrounding area known as the “sealing zone”. This sealed zone is created by formation of an actin ring and proteins like Actin, Paxillin, Talin, Vinculin, Tensin, and Actin-associated proteins such as Fimbrin, Dynamin, Gelsolin, α -Actinin (Luxenburg et al. 2007) and Rho GTPases are critically involved in the process (Touaitahuata et al. 2014). Osteoclast activity requires the maintenance of the ruffled border, formed by intense trafficking of lysosomal and endosomal components. In this region, Cathepsin K, TRAP, and MMP9 are transferred into “Howship’s lacunae,” which causes bone degradation (Faloni et al. 2012; Graves et al. 2008). The degraded products transcytose to the plasma membrane by endocytosing through the ruffled border (Arana-Chavez and Bradaschia-Correa 2009; Teitelbaum 2007). Vesicular transport towards ruffled borders increases their surface area and various proteases as well as protons are secreted lowering the pH to 4.5 in the area of resorption lacuna formed between the ruffled border and resorbing bone area (Väänänen et al. 2000). The neighboring cells are protected due to the sealing mechanism. This process helps to regulate the

mineral homeostasis and maintains the calcium balance in the blood.

Abnormal activation of osteoclasts is found in pathological conditions like bone metastasis and inflammatory arthritis in which periarticular erosion and painful osteolytic lesions occur (Feng and McDonald 2011; Seeman and Delmas 2006; Takayanagi 2007). Under these conditions, pro-inflammatory cytokines like TNF- α , IL-6 or IL-1 β can support and aggravate osteoclast-induced bone resorption (Adamopoulos and Mellins 2015). Osteoclasts produce “clastokines” (such as TRAcP, S1P, BMP6, Wnt10b, SOST, HGF and CTHRC1) that control osteoblasts during the bone remodeling cycle (Charles and Aliprantis 2014; Teti 2013). Osteoclasts can also determine the formation of the HSC niche in the bone marrow (Mansour et al. 2012). Earlier studies have revealed that loss in osteoclast activity in the HSC niche results in defective endochondral ossification in a mouse model. Moreover, the loss of osteoclast activity leads to decreased osteoblast differentiation with a corresponding increase in the number of mesenchymal progenitors (Mansour et al. 2012).

Bone remodeling

Bone remodeling is a dynamic process involving synergistic and sequential stages of bone resorption and formation. Bone remodeling takes place through five steps, namely activation, resorption, reversal, formation and termination. During this process, various bone cells act sequentially, e.g., the osteoclasts are placed in the forward direction at the resorption site, whereas the osteoblasts are active at the rear of the resorption site. For the remodeling process, osteoclasts and osteoblasts form a temporary structure called Basic Multi-cellular Unit (BMU). Along with the osteoclasts and osteoblasts, bone lining cells form a canopy above the lacunae. Collectively, these cells form the anatomical unit, which is known as the bone remodeling compartment (BRC), which carries out the whole bone remodeling process. The spatial and temporal arrangement of cells in the BMU is critical for the bone remodeling process and assures coordination between them to carry out the sequential operations as shown in Figure 2A (Hauge et al. 2001).

Activation: In this phase, osteocytes sense signals like mechanical structure damage or hormonal stimulation and transfer them into a chemical or biological signal (Stern et al. 2012). In the presence of any such signal, osteocytes produce transforming growth factors (TGF- β) that inhibit osteogenesis so that the osteocytes undergo apoptosis (Heino et al. 2002), which is the ultimate signal for

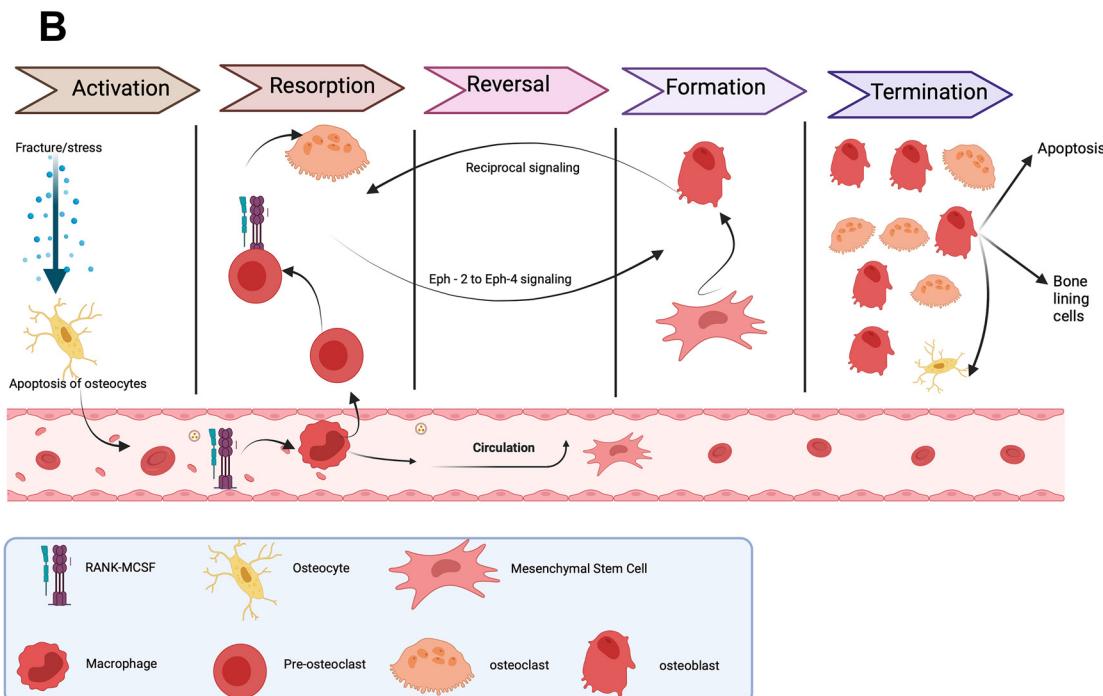
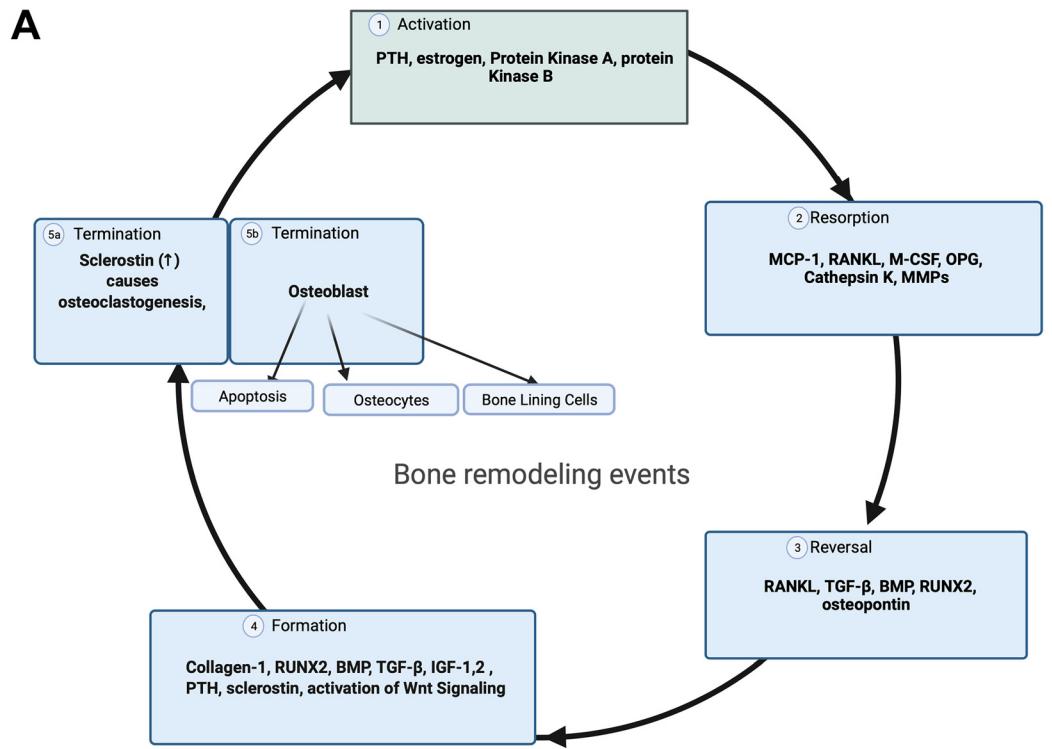


Figure 2: Process of bone remodeling.

(A) Schematic diagram of bone remodeling events depicting sequential processes 1) Activation: In this step the microfracture or microdamage signal causes apoptosis of osteocytes. Osteocyte apoptosis promotes RANKL and M-CSF production in osteoblasts. 2) Resorption: In this phase RANKL and M-CSF activate osteoclast differentiation and resorption of the damaged site. 3) Reversal: When the damaged bone proteins are removed by osteoclasts, the signal is sent to the osteoblasts via ephrin2. Additionally, osteoblasts inhibit the activity of osteoclasts through ephrine4 to ephrine2 reciprocal signaling. 4) Formation: Osteoblasts deposit mineralized organic matrix. 5) Termination: Osteoblasts may differentiate into bone lining cells, or osteocytes (which remains immersed into the matrix) or may undergo apoptosis. Rise in sclerostin

osteoclastogenesis to start (Aguirre et al. 2006). Parathyroid hormone or parathormone (PTH) is a calcitropic signal and has a function in calcium homeostasis. It is secreted in response to reduced serum calcium levels and has targets like kidneys, bone, and intestine. PTH activates seven-transmembrane G protein-coupled receptors on the osteoblasts (Jüppner et al. 1991). The PTH binds the osteoblast receptor and activates protein kinase C, protein kinase A, and intracellular calcium signaling pathways, which induces transcriptional signals to differentiate precursor osteoclasts into mature osteoclasts, and establishes bone resorption (Swarthout et al. 2002).

Resorption: Osteoblasts sense the PTH signals from the osteocytes. In response to these signals, they produce macrophage chemoattractant protein 1 (MCP1) and RANKL. MCP1 acts on osteoclasts precursors, leading to the recruitment of pre-osteoclasts at the resorption site, where RANKL triggers differentiation (Li et al. 2007; Siddiqui and Partridge 2017). Osteoblasts produce matrix metalloprotease (MMP13) in response to the mechanical (Yang et al. 2004) and endocrine (Partridge et al. 1987) signals which degrade unmineralized osteoid and expose RGD (Arginine-glycine-aspartic acid) adhesion motif having a role in cell recognition and cell adhesion site. Osteoclasts anchor to the RGD site via the vitronectin receptor $\alpha_v\beta_3$ integrin (McHugh et al. 2000). By anchoring on the RGD site, it forms a sealed zone between the matrix and osteoclast. In this sealed zone, H^+ is pumped, which causes the acidification of the surrounding matrix, and creates "Howship's lacunae" (Teitelbaum 2000). Followed by Howship's lacunae formation Cathepsin K is released into the lacunae, which acts at low pH and degrades the remaining organic matrix by collagenolytic action (Saftig et al. 1998).

Reversal: There are specialized cells called the reversal cells, which reverse the resorption phase to the formation phase and coupling of the mechanism within the BMU. After resorption, the osteoclasts leave patches of demineralized collagen in the Howship's lacunae (Everts et al. 2002). Mononuclear cells remove the undigested collagen patches and prepare the bone surface for addition of bone matrix. Mesenchymal bone lining cells and osteal macrophages work in synergy in the reversal phase. Macrophages produce MMPs to degrade the undigested matrix and the osteopontin is incorporated in mineralized tissue

(Newby 2008; Takahashi et al. 2004). On the contrary, mesenchymal bone lining cells can deposit osteopontin-rich cement lines within Howship's lacunae (Everts et al. 2002). The reversal cells' possible role is to receive or produce the coupling signal causing a transition from bone resorption to bone formation within the BMU.

Formation: Insulin-like growth factor (IGF) I, II, and TGF β are coupling factors which start the formation step. Studies demonstrated that coupling factors which act as signals are stored in the bone matrix and therefore get released during the resorption process. TGF- β acts as a recruitment signal of the mesenchymal cells at the resorption site (Crane and Cao 2014). Under natural conditions, the osteocytes release Sclerostin, which ceases the osteoblast differentiation by binding with low-density lipoprotein receptor-related protein 1 (LRP5/6), which prevents the Wnt/ β -catenin signaling pathway (Li et al. 2005). Mechanical and PTH signals via PTH receptors on the osteocytes inhibits sclerostin causing LRP5/6 activation and Wnt- β -catenin signaling for osteoblast differentiation and bone formation (Robling et al. 2008). The recruitment of mesenchymal stem cells follows the release of the bone-forming Type-I collagen molecules from osteocytes at the bone lacunae. Non-collagenous proteins like proteoglycan and glycosylated protein such as non-specific alkaline phosphatases, small integrin-binding ligand-protein, bone sialoprotein, Gla protein (like bone matrix protein and osteocalcin) compose organic material (Robey et al. 2006). The inorganic phosphate allows mineralization by nucleotide pyrophosphatase. Phosphodiesterase and ANK (progressive ankylosis), combined with calcium, form hydroxyapatite that gets incorporated into the bone matrix (Harmey et al. 2004).

Termination: When the equal amount of resorbed bone is replaced by newly formed bone, the termination process is initiated. Ultimately, osteoblasts undergo apoptosis or become osteocytes or bone lining cells, respectively. In some studies, round or oblong structures containing dense bodies and TUNEL positive structures have been observed in osteoblasts vacuoles. This suggests that along with professional phagocytes, osteoblasts can also engulf and degrade apoptotic bodies during bone formation (Cerri 2005). During termination, the sclerostin concentration gradually starts to rise, resulting in osteoclastogenesis (Figure 2B).

concentrations promotes the osteoclastogenesis. (B) Diagram visualizing the bone remodeling process involving different events with cells 1) When there is fracture or stress occurs in the bone, it causes the apoptosis of osteocytes. This apoptotic signal is passed to the pre-osteoclast cell through the bone lining cells. 2) The precursor osteoclast cells then differentiated into the mature osteoclast through the RANKL and M-CSF secreted by osteoblasts. Then the osteoclasts are recruited at bone resorption site which cause the resorption of the bone. 3) The mononucleated osteoclasts acts as reversal cells which cause coupling mechanism leading to start of the bone formation and end of the resorption. 4) After reversal phase the reciprocal signaling (e.g. Eph-2 to Eph-4 signaling) starts due to which bone formation takes place by the osteoblasts. 5) When equal amount of bone formation takes place the termination process is initiated where osteoblasts are converted into osteocytes, bone lining cells or undergoes apoptosis. Figures were created using BioRender.com.

Phytotherapy

From ancient times, plant-derived natural compounds, explicitly secondary metabolites, have been used for medicinal purposes. Secondary metabolites are generally derived from various parts of the plant body and possess a broad spectrum of bioactivities such as plant defense against herbivory and also towards ecological or predator stress, but are rarely needed for plants growth and development (Seca and Pinto 2018). Secondary plant metabolites can be broadly divided into three major classes, viz., Terpenoids, Alkaloids, and Phenolics, which have plenty of natural products with practical pharmacological activities (Savithramma et al. 2011). Phytochemicals serve as backbone for the synthesis of novel therapeutic agents. Many seem to be specific for the tumor environment, but features like anti-oxidant activity of phytochemicals can also be useful in cancer prevention (Zubair et al. 2017). Several phytochemicals have made a successful transition into clinical use. Clinically relevant examples of phytochemicals are the two cancer therapeutics Taxol/Paclitaxel from *Taxus brevifolia* or vincristine from *Cantharantus roseus* that was approved as the first plant-derived cancer treatment by the FDA (Seca and Pinto 2018; Weaver 2014). Up to 50% of anti-cancer drugs are natural products or derived from such compounds, which highlights the importance of these biologicals (Efferth and Koch 2011). Given the wealth of plant derivatives that are used in traditional medicine, it could be expected that an even higher number of plant compounds can be turned into effective and safe treatment. However, the positive effects seen in many *in vitro* studies does not translate into clinical use and the poor bioavailability as well as the fast metabolic turnover of the substances hamper their use (Zubair et al. 2017).

Plumbagin is a secondary metabolite obtained from plants belonging to the family *Plumbaginaceae*. The *Plumbaginaceae* family mainly grows in central Asia, but can also be found in certain Mediterranean regions (Tripathi et al. 2019). Recent research addresses a potential role of Plumbagin in bone-related diseases such as osteoporosis.

Properties of plumbagin

Plumbagin is an important compound found in an Ayurvedic plant – *Plumbago zeylanica*, also known as Chitrak. Chitrak is a perennial subscandent shrub from the family *Plumbaginaceae*. *P. zeylanica* Linn. is found throughout

India. Chitrak-derived products are claimed to provide strength, intelligence, and longevity, to cure leprosy, piles, cough, and inflammation and to enhance digestive capacity.

Chemically, plumbagin is a 2-methyl-5-hydroxy-1,4-naphthoquinone ($C_{11}H_8O_3$), that is structurally similar to the vitamin K structure. Like vitamin K (1–2 h), its half-life is rather short (4 h). It is poorly soluble in water and can be easily dissolved in organic solvents. Therefore, it has a poor bioavailability of app. 39%, as determined in rats (Hsieh et al. 2006). *In vivo*, plumbagin can compete with vitamin K, resulting in decreased platelet adhesion and prolonged bleeding time (Vijayakumar et al. 2006). In an attempt to create Plumbagin derivatives with less adverse side effects and enhanced tumor activity *in vivo*, chemical analogues were synthesized that made it possible to assign distinct functions to the chemical groups attached to the naphthalene ring structure (Rajalakshmi et al. 2018). Most important for its biological functions is the hydroxy group at the C5 position which increases the molecule's electrophilicity. The ability to act as an electron acceptor is crucial for Plumbagin's ability to generate ROS, a feature that seems central to plumbagin bioactivity and its described anti-tumorigenic effect (Klotz et al. 2014; Rajalakshmi et al. 2018).

Plumbagin is described to possess antioxidant, anti-inflammatory, anticancer, antimicrobial and neuro-protective properties (Padhye et al. 2012). Plumbagin was found to possess anti-oxidant capacity in hydroxyl radical and superoxide radical scavenging assays, but a low scavenging activity for the nitrogen radical in a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Kumar et al. 2013). When the authors compared the activity to the chemical analogues 5-hydroxyl-1,4-naphthoquinone juglone (isolated from black walnuts *Juglans nigra*) and synthetical menadione (2-methyl-1,4-naphthoquinone), they found that the existence of the methyl group was central for the anti-oxidant function, as it was absent in juglone. The underlying mechanism of plumbagin's anti-inflammatory activity is less defined on the biochemical level. It has been reported that plumbagin inhibits LPS-mediated inflammation by causing down-regulation of NF- κ B and MAPK pathways in RAW264.7 cells and suppressed TNF- α , IL-1 β , IL-6 and iNOS expression (Wang et al. 2014). In various cancer cell lines plumbagin was found to provoke apoptosis through the downregulation of NF- κ B, induction of Caspase-3 activity and cell cycle arrest (Tripathi et al. 2019). All of these changes in signaling processes could however also be a consequence of plumbagin-mediated ROS production and a resulting decrease in cell viability. Antimicrobial functions of plumbagin were found against the malaria parasite *Plasmodium falciparum*, the bacterial strains *Bacillus*

subtilis, *Mycobacteria* spp. and *Staphylococcus aureus* and *Candida albicans* fungi (Bhattacharya et al. 2013; Nair et al. 2016; Sumsakul et al. 2014). Other strains such as *Escherichia coli* and *Salmonella typhimurium* were found to be unaffected by the presence of plumbagin (de Paiva et al. 2003).

Plumbagin and cancer

The best characterized function of plumbagin is its ability to interfere with signal transduction processes in cancer cell lines, ultimately leading to cell death. A modulation of the central pathways of MAPK, Akt, STAT3 and NF- κ B was described in many cases, but the exact mechanism how plumbagin targets these pathways is still not fully understood (Tripathi et al. 2019) (Figure 3A). Jamal and

colleagues performed a computational analysis to understand the inhibitory effects of plumbagin on these molecules. The data showed the interaction of Plumbagin with central residues within the structure of the respective protein resulting in the loss of function due to the inhibition of DNA binding abilities for STAT3 and the p50/p65 NF- κ B heterodimer (Jamal et al. 2014). For Akt, plumbagin may act as an allosteric inhibitor for ATP binding and thus could prevent Akt activation. *In vitro*, cytotoxic, ROS-dependent effects of plumbagin were found for prostate, lung, breast, cervical, colorectal, esophageal, gastric, ovarian, pancreatic and renal cancers, osteosarcoma, leukemia, hepato-cellular carcinoma, melanoma and glioma. The main findings have been summarized in other reviews (Tripathi et al. 2019; Yin et al. 2020). Plumbagin's cytotoxic effects could be corroborated in various *in vivo* tumor models as well, but less data are available here.

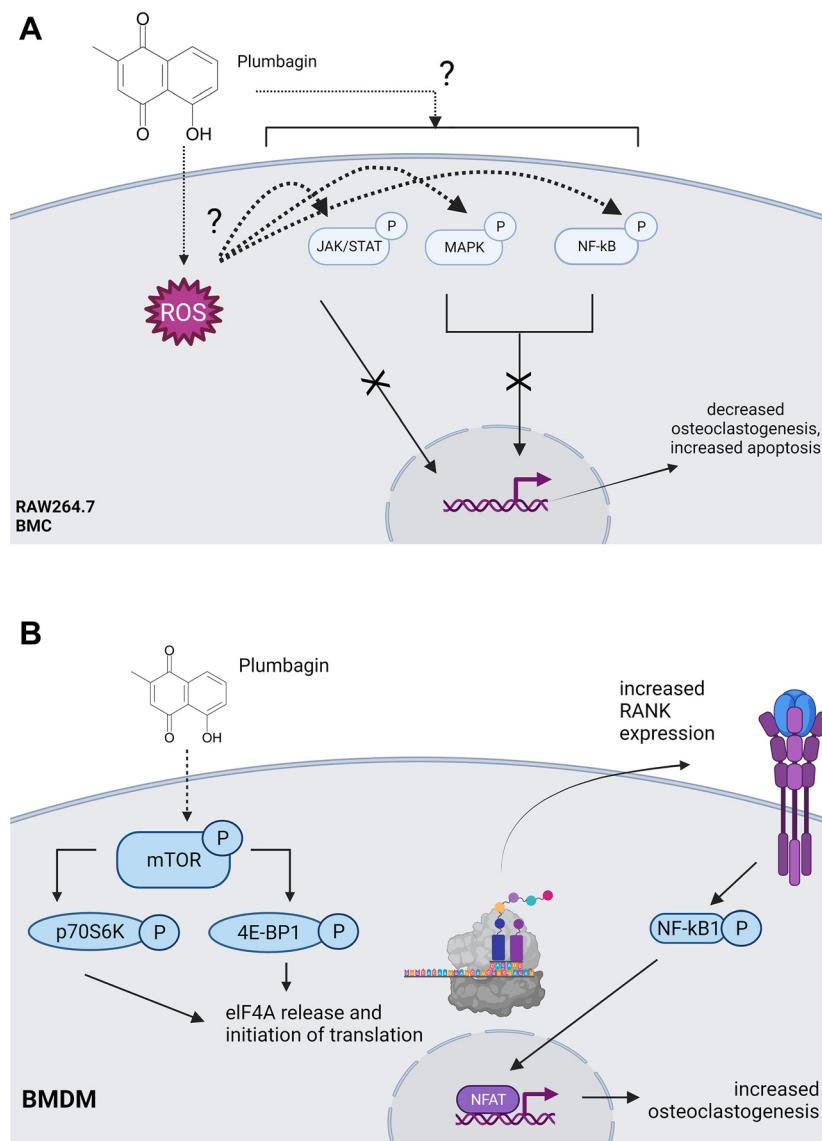


Figure 3: Plumbagin-mediated effects on osteoclasts.
 (A) Effect of plumbagin on NF- κ B and MAPK signaling pathways by inhibition of phosphorylation of different proteins and the production of cytotoxic reactive oxygen species (ROS). Overall, this results in decreased osteoclastogenesis. (B) Plumbagin enhances osteoclast differentiation in bone marrow-derived macrophages through upregulation of the mTOR pathway and an increase in protein translation, which results in enhanced RANK-mediated signal transduction and the upregulation of osteoclast specific genes. Figures were created using BioRender.com.

The integrity of bone can also be affected by cancer, since multiple myeloma, breast cancer, prostate cancer, lung cancer, renal carcinoma, melanoma, gastrointestinal tumors and cancers of head and neck frequently metastasize to the bone (Danieau et al. 2019). Osteolytic lesions are more prevalent with metastasis from breast cancer, multiple myeloma, melanoma and non-small cell lung cancer whereas osteoblastic lesions are more frequently found in prostate and small cell lung cancer (Chen et al. 2021). Osteosarcoma and Ewing sarcoma are primary bone cell tumors (Danieau et al. 2019). In both cases, the tumor microenvironment is characterized by the presence of proinflammatory cytokines that causes increased osteoclast differentiation and enhanced bone resorption. In tumor models for breast cancer and osteosarcoma, plumbagin was found to reduce osteolytic lesions and exert a beneficial impact on bone structure. This was shown by two *in vivo* studies using MDA-MB-231 breast cancer tumor bearing nude mice, where plumbagin injection for 4 weeks was found to decrease osteolytic lesions (Sung et al. 2011). Using a similar model system, Li and coworkers showed that plumbagin treatment lowered the production of proinflammatory cytokines IL-1 β and IL-6, which would also positively influence the bone environment (Li et al. 2012). In the same studies, the breast cancer cell lines themselves were found to express lower amounts of proinflammatory cytokines and other osteoclast-activating factors. In addition, the expression of the osteoclast differentiation inhibitory factor OPG at the cell surface of MDA-MB-231 cells was investigated, as OPG counteracts RANKL signaling and is often decreased in cancer settings. Indeed, OPG levels were increased by plumbagin treatment, which resulted in reduced RANKL signaling on osteoclast progenitors. It was further found that cancer cell migration and invasiveness were reduced by plumbagin via diminished NF- κ B and STAT3 signaling *in vitro* and *in vivo* (Yan et al. 2013, 2014). In the human osteosarcoma cell lines MG-63, U2OS and HOS plumbagin was found to trigger cell death through ROS induction, caspase activity and cell cycle arrest (Chao et al. 2017; Tian et al. 2012).

Effects of plumbagin on osteoclasts

From the data described above it was hypothesized that plumbagin might also target osteoclastogenesis directly. Indeed, data obtained from the murine macrophage cell line RAW264.7 and bone marrow cells from mice seemed to suggest that this is the case, since increasing amounts of plumbagin inhibited the differentiation into TRAP positive, multinucleated osteoclasts (Li et al. 2012; Sung et al. 2011).

This was attributed to a decreased induction of RANKL-mediated downstream signaling events such as TRAF6 binding to RANK, I κ B α degradation and NF- κ B activation (Li et al. 2012; Sung et al. 2011). However, osteoclastogenesis is not inhibited by plumbagin in primary bone marrow-derived macrophages (BMDM), while the same experimental set-up inhibits osteoclastogenesis in control RAW264.7 macrophages (Sultanli et al. 2021) (Figure 3A, B). Quite surprisingly, 24 h of pre-stimulation with plumbagin even enhanced osteoclastogenesis. The difference between the two model systems is not completely unexpected, as RAW264.7 is an immortalized macrophage-like cell line, established from a tumor induced by the Abelson murine leukemia virus and thus displays typical features of tumor cells. Primary macrophages have the ability to produce ROS as part of their immune response, so they can be expected to have a higher tolerance towards ROS. ROS can act as a secondary messenger for osteoclast progenitor cells and inhibition of ROS prevents osteoclast formation (Kubatzky et al. 2018). Cancer cells on the other hand produce increased amounts of ROS due to their hyperactivated metabolism, which should render them more sensitive towards a further increase in ROS (Perillo et al. 2020). This is corroborated by results from Abimannan and colleagues, where plumbagin-mediated Erk activation prevented the reactivation of Th1 and Th17 subsets by increasing the expression of the Th2 lead cytokine IL-4, also using ROS as a secondary messenger (Abimannan et al. 2016). The severe negative effect of plumbagin on RANKL-treated RAW264.7 cells observed by us and other investigators (Li et al. 2012; Sultanli et al. 2021; Sung et al. 2011) remains to be elucidated. According to Binding DB (Liu et al. 2007), which compares the structural similarity between the compounds, we found that plumbagin potentially targets almost 40 proteins. Of these, eight are essential in osteoclast differentiation and apoptosis (Table 2). The above data show that Plumbagin affects osteoclastogenesis by modulating NF- κ B, MAPK, and RANK expression. In primary cells, plumbagin targeted similar proteins, but caused a transient upregulation of the mTOR pathway resulting in an upregulation of translational activity via the translational initiation factor eIF4E and an increased expression of RANK which supposedly is the reason for the increased responsiveness towards RANKL (Sultanli 2021). We hypothesize that cells with a higher ROS sensitivity such as tumor cells, immortalized cell lines, non-phagocytic bone marrow cells, or periodontal ligament stem cells have an increased susceptibility towards the ROS producing effect of plumbagin. This consistently results in the inhibition of NF- κ B, MAPK, and JAK/STAT pathways (Figure 3)

Table 2: List of target proteins obtained from the Binding database^a for *Homo sapiens*.

Target name	Uploaded compounds generating hits	Max similarity	Hits (All compounds)	K_i data	IC_{50} data	K_d data	EC_{50} data	Gene name	UniProt ID
Histone acetyltransferase p300	1	1	1	0	2	0	0	EP300	Q09472
Histone acetyltransferase PCAF	1	1	1	0	1	0	0	KAT2B	Q92831
NF-kappa-beta-inducing kinase (NF- κ B)	1	1	1	0	1	0	0	MAP3K14	Q99558
Bcl-2-related protein A1	1	0.97	2	0	0	0	2	BCL2A1	Q16548
Eukaryotic translation initiation factor 4 gamma, 1 isoform 4	1	0.97	1	0	1	0	0	EIF4G1	Q04637
Glutathione S-transferase	1	0.97	1	0	0	0	1	GSTM1	P09488
Apoptotic peptidase activating factor 1	1	0.89	1	0	2	0	0	APAF1	Q14727
Caspase-9	1	0.89	1	0	1	0	0	CASP9	P55211
Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1	1	0.81	1	0	1	0	0	PIN1	Q13526
Beta-secretase 1	1	0.76	5	0	5	0	0	BACE1	P56817
Aldehyde oxidase	1	0.75	1	2	5	0	0	AOX1	Q06278
Aldehyde oxidase (AO)	1	0.75	1	1	1	0	0	AOX1	H9TB17
Amine oxidase [flavin-containing] A	1	0.75	6	2	5	0	0	MAOA	P21397
Cdc25C	1	0.75	1	0	2	0	0	CDC25C	P30307
Dual specificity phosphatase	1	0.75	3	0	4	0	0	CDC25B	P30305
Cdc25B									
Glutathione reductase (GR)	1	0.75	1	2	0	0	0	GSR	P00390
Indoleamine 2,3-dioxygenase 1	1	0.75	2	0	3	0	0	IDO1	P14902
Perilipin-1	1	0.75	1	0	1	0	0	PLIN1	O60240
Protein-glutamine gamma-glutamyltransferase 2 (TG2)	1	0.75	1	0	1	0	0	TGM2	P21980
Ubiquitin C-terminal hydrolase L3 (UCH-L3)	1	0.75	1	0	1	0	0	UCHL3	P15374
Ubiquitin carboxyl-terminal hydrolase isozyme L1	1	0.75	1	0	1	0	0	UCHL1	P09936
Quinone reductase 1)	1	0.72	2	0	2	0	0	PIM1	P11309
Amine oxidase [flavin-containing] B	1	0.7	2	2	0	0	0	MAOB	P27338
Aurora kinase A	1	0.7	1	0	1	0	0	AURKA	Q14965
Dual-specificity tyrosine-phosphorylation regulated kinase 1A	1	0.7	1	0	1	0	0	DYRK1A	Q13627

^aBinding database measures binding affinity of a ligand to the predicted active site in the target protein. The data of binding Database are linked to PDB and PubMED. Currently, the database has approximately 20,000 experimentally determined binding affinities data for protein-ligand complexes of 110 proteins and their isoforms with 11,000 ligands. The output file of the interaction queries (protein-ligand) is obtained as SD files for further downstream analyses. In our study, listed target proteins for Plumbagin were found to form a complex. The initial eight target proteins are essential for osteoclast differentiation and apoptosis e.g., APAF1, CAS9, EP300 and KAT2B promotes apoptosis; BCL2A1, prevents apoptosis; EIF4G1, promotes cell survival and prevents apoptosis; GSTM1, promotes differentiation and suppresses apoptosis; AP3K14, promote differentiation and apoptosis in cellular stress.

(Zheng et al. 2017a,b). All of the above studies show that Plumbagin is a potent phytochemical directly affecting osteoclastogenesis by inducing apoptosis in susceptible osteoclast precursors and anti-cancerous properties in osteosarcoma and bone metastases. Model-dependent differences need to be taken into account when evaluating the inhibitory effects of plumbagin on osteoclasts.

Effect of plumbagin on osteoblasts

Pathologies such as osteoporosis, periprosthetic osteolysis, rheumatoid arthritis and bone cancers show increased bone resorption that especially in osteoporosis and bone cancers is accompanied by impaired bone formation (Bi et al. 2017). As the response of osteoblasts and osteoclasts is varied

towards different pharmacological intervention, identifying phytochemicals having protective properties for osteoblasts will be useful for treating bone related diseases. The effect of plumbagin on osteoblasts is not widely studied. However, ROS is an important modulator of osteoblast differentiation, as it regulates several pathways that are involved when the stimulus of mechanotransduction is received by osteoblasts (Schröder 2019; Tao et al. 2020). It has been demonstrated that acute ROS generation by Nox2 contributes to osteoblast formation (Schröder 2019). Moreover, normal ROS generation also supports adhesion of osteoblast by generating energy into the mitochondria but excess ROS generation leads to its apoptosis (Tao et al. 2020). Work using the osteoblast cell line MC3T3 shows that plumbagin protects against glucocorticoid-induced osteoporosis through the Nrf-2 pathway (Zhang et al. 2015). Plumbagin treatment reduces the levels of ROS and lipid peroxide and increases Nrf-2 levels, a mechanism also described for other electrophilic drugs (Satoh et al. 2014). It also shows anti-apoptotic effects in MC3T3 cells. Plumbagin increases osteocalcin, osteopontin, and RUNX2 expression and overcomes the stress induced by dexamethasone (Yan et al. 2015).

To further understand how plumbagin might be acting on osteoblasts, we collected data from the STRING database (Padhye et al. 2012; Szklarczyk et al. 2018) along with metadata from research articles. According to the Binding database, plumbagin binds to different target proteins which modulates signaling pathways, and regulates RUNX2 expression (Table 2). RUNX2 is the first and most significant marker for osteoblast differentiation (Komori 2005; Soltanoff et al. 2009), thus an interactome study on RUNX2 was conducted. The proteins essential for RUNX2 expression (Table 3) include its regulators, co-activators, repressors and receptors. The data show that plumbagin

Table 3: List of proteins essential for RUNX2 expression which is a key marker in osteoblast differentiation.

RUNX2 regulators	Proteins
Upstream regulators	MSX2, NKX3-1, DLX5, HOXA10, MEF2C
Co-activators	CBFB, KAT6A, JUN, MEN1, CEBPB, CEBPD, ETS1, HES1, SMAD1, SMAD5, TLE5, RB1, TAZ, SATB2
Inhibitory regulators	HOXA2, TP53, TWIST1, TWIST2, SMURF1, SMAD6, MSX2, MSX1
Co-repressors	HDAC6, YAP1, TLE5, DLX3, LEF1, SMAD3, HEY1, STAT1, PPARG
Receptors involved	TGFBR1, ADRB2, PTCH1, FGFR2, FGFR3, FGFR1, PTH2R
Signaling molecules	BMP2, BMP4, TGFB1, SMAD6, SMURF1, epinephrine, Hedgehog, FGF2, FGF18, PTH

interacts with downstream targets related to three receptors, i.e., TGF- β receptor, ADRB2, PTCH1 receptor (Figure 4). Plumbagin may thus effect osteoblast differentiation via regulation of these regulators and repressors. As TGF- β 1 and PTCH1 receptors are involved, plumbagin may act through these two pathways.

Results of molecular docking experiment

Our binding DB and interactome studies showed that plumbagin interacts with TGFB1, PTCH1 and ADRB2 receptors (Figure 4). Thus, molecular docking analysis of the ligand–receptor complex structure was carried out using AutoDockTools-1.5.6 software comprising plumbagin (National Center for Biotechnology Information 2021) as a ligand against TGFB1 (Sawyer et al. 2004), PTCH1 (Qi et al. 2018) and ADRB2 (Hanson et al. 2008) receptors. After completion of the molecular docking, a total of 10 conformations of the ligand–receptor complex were obtained which were ranked according to the binding energy and one having the most negative binding energy was chosen (Table 4). Docking studies demonstrate that plumbagin had favorable interactions with all these receptors specific for osteoblast differentiation (Figure 5A–C). The present computational work needs to put forth experimental validation before considering plumbagin as a potential lead molecule for osteoblast differentiation.

Outlook

Plumbagin is a naphthoquinone with the ability to induce anti-oxidant and ROS-inducing signaling. While this was proven in many *in vitro* model systems or even mouse *in vivo* models, developing plumbagin into a safe drug is impeded by its hydrophobicity, low bioavailability and short half-life (Rajalakshmi et al. 2018). Consequently, the development of novel drug delivery systems led to the first small clinical trial in humans (Chrastina et al. 2018; Kyriakopoulos et al. 2019). The results show that a synthetic plumbagin-loaded nanoemulsion (PCUR-101) was safe and might have beneficial anti-inflammatory effects in a combination therapy for a subset of prostate cancer patients (mCRPC) (Kyriakopoulos et al. 2019). Plumbagin may also have beneficial effects on bone metabolism. Among the phytochemicals implicated in bone metabolism, plumbagin stands out due to a dual effect on osteoclasts and osteoblasts. In osteoporosis, the rate of bone resorption is higher than the rate of bone formation, leading to high morbidity as well as increased health care costs. Most

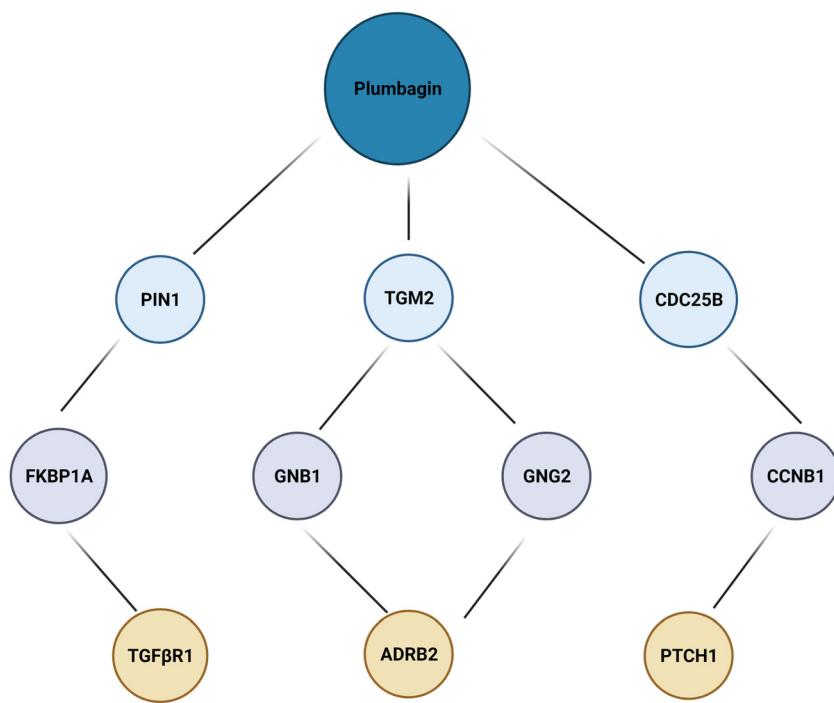


Figure 4: Interactome of plumbagin with receptors TGFBR1, ADRB2 and PTCH1. Plumbagin interacts with PIN1, TGM2 and CDC25 according to BindingDB. These proteins interact with FKBP1A, GNB1 & GNG2, CCNB1, which further interact with respective receptors. There are three receptors TGFBR1, ADRB2 and PTCH1 which are involved in RUNX2 expression pathway. Figure was created using BioRender.com.

Table 4: List of probable receptors and their parameters for plumbagin binding based on molecular docking.

Receptor	Binding energy (kcal/mol)	Inhibition constant (K_i , μM)	Intermolecular energy (kcal/mol)	Total internal energy (kcal/mol)	Number of hydrogen bonds formed
TGFB1	-7.66	2.42	-7.96	-0.61	1
PTCH1	-6.38	21.22	-6.67	-0.64	2
ADRB2	-6.47	18.15	-6.77	-0.64	0

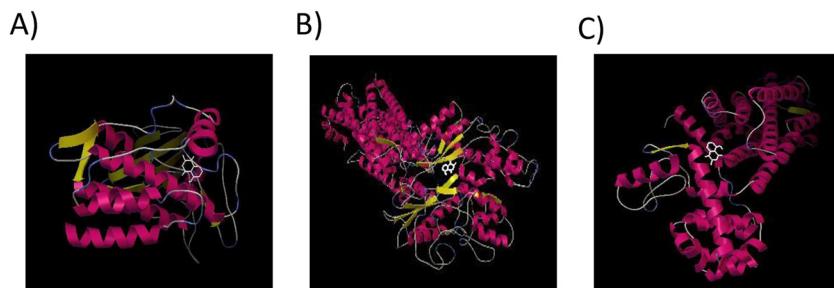


Figure 5: 3D-Structural representative of receptor-ligand interaction. (A) PB-TGF β ; (B) PB-PTCH1; (C) PB-ADRB2. Molecular docking analysis of the ligand-receptor complex structure was carried out using AutoDockTools-1.5.6 software. During the docking process, inhibitor and water molecules were removed for every receptor. SDF file for plumbagin was taken from PubChem database which was converted to PDB file in PyMol software. For this, the genetic algorithm and docking default algorithm was applied and output was saved in Lamarckian GA (4.2) format.

conventional drugs block osteoclast differentiation or activity, but PTH is the only treatment that can actively support osteogenesis. Plumbagin has been shown to inhibit osteoclast formation in certain scenarios, but its

positive effect on osteoblast formation remains less documented. Although different transcription factors, regulatory microRNAs and intracellular factors that have been implicated in bone metabolism, also seem to play a role in

plumbagin-mediated bone metabolism, it remains to be worked out how plumbagin can modulate these factors. Unbiased and thorough data from these future studies will help formulate a strategy to use plumbagin as a potential pharmaceutical for the treatment of osteoporosis and other bone-related diseases along with the conventional treatment regime to reduce the dosage and toxicity.

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