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## Anabolic Agents and the Bone Morphogenetic Protein Pathway

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A major unmet need in the medical field today is the availability of suitable treatments for the ever-increasing incidence of osteoporosis and the treatment of bone deficit conditions. Although therapies exist which prevent bone loss, the options are extremely limited for patients once a substantial loss of skeletal bone mass has occurred. Patients who have reduced bone mass are predisposed to fractures and further morbidity. The FDA recently approved PTH (1–34) (Teriparatide<sup>®</sup>) for the treatment of postmenopausal osteoporosis after both preclinical animal and clinical human studies indicated it induces bone formation. This is the only approved bone anabolic agent available but unfortunately it has limited use, it is relatively expensive and difficult to administer. Consequently, the discovery of low cost orally available bone anabolic agents is critical for the future treatment of bone loss conditions.

The intricate process of bone formation is co-ordinated by the action of many different bone growth factors, some stored in bone matrix and others released into the bone microenvironment from surrounding cells. Although all these factors play important roles, the bone morphogenetic proteins (BMPs) clearly play a central role in both bone cartilage formation and repair. Recent research

into the regulation of the BMP pathway has led to the discovery of a number of small molecular weight compounds as candidate bone anabolic agents. These agents may usher in a new wave of more innovative and versatile treatments for osteoporosis as well as orthopedic and dental indications. © 2007, Elsevier Inc.

## I. Introduction

Skeletal architecture plays a crucial role in bone by providing both shape and structure to withstanding repetitive loading. The function of bone tissue is to afford both a load-bearing material and an available repository for mineral. One of the major medical issues today is the increasing incidence of osteoporosis with subsequent morbidity. This is a disabling disease characterized by compromised bone strength, predisposing patients to increased risks of fracture. It affects at least one-quarter of all postmenopausal white women in the United States and the proportion rises to 70% in women older than 80 years (Conference-Report, 1993; Melton, 1997; WHO, 2003). One in three women older than 50 years will have an osteoporotic fracture that causes a considerable social and financial burden on society (Melton *et al.*, 1992). One person in the European Union sustains an osteoporotic fracture every 30 s, and the annual first-year direct cost of treating all osteoporotic fractures is estimated at 25 billion (Compston *et al.*, 1998). The disease is not limited to women; older men can also be affected (Akin *et al.*, 2004). By 2050, the worldwide incidence of hip fracture is projected to increase by 310% and 240% in men and women, respectively (Gullberg *et al.*, 1997). The combined lifetime risk for hip, forearm, and vertebral fractures presenting clinically is around 40%, equivalent to the risk for cardiovascular disease (Kanis, 2002). Osteoporotic fractures, therefore, cause substantial mortality, morbidity, and economic cost.

Bone loss occurs in both adult women and men, probably due to a decline in the volume of bone formed as a natural part of aging. The rate of loss is slow in young adulthood because the remodeling rate is low; however, it accelerates in women at menopause due to increased bone turnover and results in trabecular thinning, disappearance and loss of connectivity, cortical thinning, and increased intracortical porosity. These changes compromise the material and structural properties of bone and lead to increased propensity for fracture. Most of the treatments in use today are antiresorptive medications including estrogens, selective estrogen receptor modulators (SERMs) such as raloxifene, bisphosphonates (alendronate, risedronate, and ibandronate), and calcitonins all aimed at inhibiting osteoclastic resorption reducing the progression of trabecular thinning, loss of connectivity, cortical thinning, and porosity.

One of the main issues with these antiresorptive therapies is although they are successful at reducing loss of bone and its eventual devastating consequences, they lack the ability to replace lost bone. The ultimate effectiveness of these agents is dependent on early diagnosis of the onset of this osteoporotic condition. If substantial bone has been lost at important sites in the skeleton, then these agents do little to augment skeletal structural integrity. Because of the mechanism of action of these antiresorptive agents, they are ineffective treatments for one of the most serious consequences of osteoporosis, which are fractures. In fact, they appear to delay remodeling of fracture callus woven bone into lamellar bone resulting in delayed healing of the fracture itself and possibly weaker bone (Komatsubara and Mori, 2005).

Therefore, a critical need exists for inexpensive skeletal anabolic agents to treat bone deficit conditions such as osteoporosis as well as fractures and other orthopedic and dental indications. Recent clarification of the complex pathways involved in bone formation and repair indicates that growth factors play essential roles in the intricate cascade leading to mature bone formation. This has raised the possibility of using growth factors to help increase bone mass or repair bone tissues. However, while BMP-2 and platelet-derived growth factor (PDGF) are approved for local bone repair, in general, growth factors have obvious limitations in their ability to act or to be used systemically. In addition, together with their inherent high cost of manufacturing, they are restricted in their use for local indications only.

For a successful anabolic agent, they must fit important therapeutic criteria such as ease of administration, strong therapeutic efficacy, limited side effects, and low toxicity. They should increase bone mass and reverse deficit conditions such as osteoporosis, while having the ability to enhance healing of fractures and replacement of bone tissue. To date, no acceptable drug exists according to these criteria. While most of the approved agents for bone act as antiresorptive agents, parathyroid hormone (PTH) is the only systemically active anabolic agent available. However, its use is extremely limited due to its difficulty to administer, side effects, and cost. While BMP-2 and PDGF are approved for local use, they are also limited in their use mainly due to their cost (Table I).

Other factors and compounds, with anabolic activity, are being investigated as potential candidates as anabolic agents (Table II). However, in general, the larger the molecule, the higher the cost and the more difficult it is to administer. With these important restrictions in mind, this chapter focuses attention on current anabolic therapies and possibility of small innovative and more versatile agents as future potential therapies.

The use of clinically available small molecular weight systemic agents for local repair of fractures has met with a number of difficulties. For instance, although local application of bisphosphonates, in animal studies, increases

**Table I** Clinical Use of Bone Anabolic Growth Factors

Drug	Oral Availability	Use	Stage of Development*				
			Pre	PI	PII	PIII	NDA
BMP-2 (Infuse <sup>®</sup> )	No	Local					
PDGF (GEM 215 <sup>®</sup> )	No	Local					
PTH (1–34) (Teriparatide <sup>®</sup> )	No	Systemic					
PTH (Preos)	No	Local					
FGF	No	Local					
TGF- $\beta$	No	Local					
VEGF	No	Local					
IGF-1,2	No	Local					
Sclerostin antagonists	No	Systemic					

\*Pre—Preclinical; PI—Phase clinical studies; PII—Phase II clinical studies; PIII—Phase III clinical studies; NDA—New drug application.

**Table II** Clinical Use of Small Molecular Weight Bone Anabolic Agents

Drug	Oral Availability	Use	Stage of Development*				
			Pre	PI	PII	PIII	NDA
Strontium ranelate (PROTELOS <sup>®</sup> )	Yes	Systemic					
Chrysalin	No	Local					
Calcilytics	Yes	Systemic					
Statins	Yes	Local/Systemic					
Proteasome inhibitors	Yes	Local/Systemic					
Prostaglandin agonists	Yes	Local/Systemic					
Flavonoids	Yes	Local/Systemic					
$\beta$ -Blockers	Yes	Systemic					
AC100	No	Local					

\*Pre—Preclinical; PI—Phase clinical studies; PII—Phase II clinical studies; PIII—Phase III clinical studies; NDA—New drug application.

bone area next to dental implants (Miller and Marks, 1993a) and reduces bone resorption following surgical trauma (Binderman *et al.*, 2000; Kaynak *et al.*, 2000), it appears this approach leads to delayed healing and weaker bone (Komatsubara and Mori, 2005). These currently used clinical drugs for the treatment of osteoporosis (bisphosphonates, calcitonin, estrogen, and vitamin D analogs) inhibit bone resorption instead of primarily stimulating

new bone formation and clearly would be of limited if any benefit for the treatment of bone conditions requiring the establishment of new bone growth.

A key approach to finding new potential bone anabolic agents is to focus on those pathways critically important for bone formation. However, the action of these agents must be proximal enough, in these pathways, to activate the multitude of complex downstream signaling cascades responsible for initiating and sustaining the intricate process of bone formation.

## II. Bone Metabolism

Bone is a metabolically active organ in which the organizational pattern of the mineral and organic components determine the successful mechanical function of the skeleton. This is achieved by a combination of dense, compact, and cancellous (trabecular) bone, reinforced at points of stress (Glimcher, 1968, 1987). Defined agents and mechanisms regulate bone formation and bone resorption, the two major processes of bone remodeling.

Bone formation *in vivo* is a complex phenomenon whereby recruitment and replication of mesenchymal precursors of osteoblasts, differentiation into preosteoblasts, osteoblasts, and mature osteoblasts ultimately result in the accumulation and mineralization of the extracellular matrix. Since the formation of new bone is primarily a function of the osteoblast, agents regulating bone formation act by either increasing or decreasing the replication of cells of the osteoblastic lineage or modifying the differentiated function of the osteoblast.

Both systemic and local factors control bone formation. These local regulators of bone formation are growth factors that act directly on cells of the osteoblastic lineage. Growth factors are polypeptides with important effects on cell function. Some are also present in the circulation and may function as systemic agents, but for the most part, work locally in specific tissues as regulators of cell metabolism. Production of new bone during embryogenesis occurs through a complex series of cellular interactions controlled by growth factors that communicate the information needed for correct pattern formation and the signals required for differentiation of cells into cartilage and bone. Most bones in the body start as cartilage models, then are ultimately replaced by bone through the process of endochondral bone formation. In contrast, bones of the craniofacial skeleton form directly by the conversion of mesenchymal progenitors into osteoblasts through intramembranous bone formation. The result of either developmental path, controlled by growth factors, is a bone surrounded by a periosteal layer rich in progenitor cells and containing a mature marrow cavity and vascular

supply. It is the expression of these bone growth factors and the appropriate signaling of their pathways that plays a central role in the regulation of the anabolic response in bone.

### A. Bone Anabolic Growth Factors

Systemic factors do influence skeletal integrity including PTH, 1, 25 dihydroxy vitamin D3, and more recently the possible role of the sympathetic nervous system. A wide variety of locally derived growth factors positively impact bone formation including insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), hedgehogs (Shh and Ihh), transforming growth factor  $\beta$  (TGF- $\beta$ ), PDGF, vascular endothelial growth factor (VEGF), Wnts, and BMPs probably being the most important of these local bone growth factors.

Therefore, due to the importance of the BMPs in bone formation, factors that limit the effect of BMP activity play very important roles in the regulation of both the temporal and spatial aspects of bone formation suggesting that they might be potential therapeutic targets. These include proteins such as noggin, dan, chordin, follistatin, and cerberus. The *SOST* gene product, sclerostin (a BMP antagonist) has been shown to exhibit a pattern of expression in trabecular bone only suggesting it as a potential therapeutic target (Kusu *et al.*, 2003; Poole *et al.*, 2005; van Bezooijen *et al.*, 2005b).

Although all these growth factors and hormones alone affect bone formation, it is the interaction of all of these growth factors and their inhibitors together that results in the complex process of bone formation.

### B. Platelet-Derived Growth Factor

hPDGF is the major growth factor of human blood serum. The hPDGF heterodimer and its two isoforms, the PDGF-1(A) and PDGF-2(B) homodimers, are potent mitogens and chemoattractants for target cells such as diploid fibroblasts, osteoblasts, arterial smooth muscle cells, and brain glial cells. PDGF, an osteoblast mitogen, accelerates fracture healing and periodontal bone repair when applied locally *in vivo* (Giannobile *et al.*, 1994), while systemic administration of PDGF increases bone density and strength throughout the skeleton (Mitslak *et al.*, 1996). PDGF was recently shown to improve periodontal regeneration in humans (Camelo *et al.*, 2003; Nevins *et al.*, 2003, 2005) and has been approved by the FDA for the treatment of bone loss associated with advanced periodontal disease when combined with the synthetic bone matrix,  $\beta$ -tricalcium phosphate ( $\beta$ -TCP).

### C. Transforming Growth Factor $\beta$

The first local bone growth factor purified to homogeneity was TGF- $\beta$ . Early on, TGF- $\beta$  promised to be one of the key factors involved in coupling bone formation to previous bone resorption (Mundy, 1991). This potent osteotropic polypeptide is abundant in the bone matrix, and produced in response to factors that stimulate osteoclastic bone resorption. It is a very potent stimulator of osteoblastic bone formation, causing chemotaxis, proliferation, and differentiation in committed osteoblasts. TGF- $\beta$  has complex effects on bone resorption, it inhibits osteoclast formation and osteoclast activity (Dieudonne *et al.*, 1991). TGF- $\beta$  is released from bone in a biologically inert state due to the presence of at least two proteins that appear to regulate its activity. Release of active TGF- $\beta$  from these latent complexes occurs during bone resorption and is mediated by osteoclasts (Bonewald *et al.*, 1991). Knowledge of the mechanisms responsible for these activation processes may be vital to understanding the role of TGF- $\beta$  in bone remodeling. A single application of human recombinant TGF- $\beta$ 1 to skull defects induced a dose-dependent increase in intramembranous bone formation in rabbits (Beck *et al.*, 1991, 1993) and defects in sheep (Moxham *et al.*, 1996). Alternatively, evidence suggested that high dose TGF- $\beta$  delayed or inhibited mineralization of newly formed osteoid (Broderick *et al.*, 2005). TGF- $\beta$  is also known to regulate chondrocyte proliferation and hypertrophic differentiation and has marked effects on cartilage growth (Tuli *et al.*, 2002). It is clear TGF- $\beta$  is a pivotal growth factor during osteogenesis and systemic bone disease.

### D. Fibroblast Growth Factors

Fibroblast growth factors and FGF receptors (FGFRs) comprise a signaling system conserved throughout evolution. Twenty-two FGFs and four FGFRs have been identified in humans and mice (Itoh and Ornitz, 2004). FGFs are key regulators of several developmental processes in which cell fate and differentiation to various tissue lineages are determined. The importance of the proper spatial and temporal regulation of FGF signals is evident from human and mouse genetic studies which show that mutations leading to the dysregulation of FGF signals cause a variety of developmental disorders including dominant skeletal diseases and cancer (Dailey *et al.*, 2005). A number of years ago, the overexpression of FGF-2 caused a variety of skeletal malformations including shortening and flattening of long bones and moderate macrocephaly (Coffin *et al.*, 1995), while continuous slow administration of a small amount of FGF-2 accelerates bone-derived osteogenic cytokine-induced new bone formation (Kimoto *et al.*, 1998). It was

also shown that both local and systemic FGF-1 increases new bone formation and bone density, and can restore bone microarchitecture and prevent bone loss associated with estrogen-withdrawal (Dunstan *et al.*, 1999). rhFGF-4 was shown to stimulate bone formation around titanium implants in bone (Franke Stenport *et al.*, 2003). Other uses in bone have been the acceleration of surgical angiogenesis in necrotic bone with a single injection of FGF-2 (Nakamae *et al.*, 2004). As with these other growth factors, FGF is an extremely important growth factor in the bone metabolism.

### **E. Vascular Endothelial Growth Factors**

The family of vascular endothelial growth factors (VEGFs) currently includes VEGF-A, -B, -C, -D, -E, and placenta growth factor (PlGF). Several of these factors, notably VEGF-A, exist as different isoforms, which appear to have unique biological functions. The VEGF family proteins bind in a distinct pattern to three structurally related receptor tyrosine kinases, denoted as VEGF receptors-1, -2, and -3. One of the main influences of VEGFs is to stimulate angiogenesis (Baumgartner and Isner, 1998) which appears to play an important role in cancer (Carmeliet, 2005). This has led to the discovery of VEGF inhibitors as potential therapies in cancer treatment (Cardones and Banez, 2006; Ferrara, 2005). VEGF has been shown to promote bone growth (Peng *et al.*, 2002; Young *et al.*, 2002) probably by its effects on angiogenesis (Filvaroff, 2003; Kent Leach *et al.*, 2006; Kleinheinz *et al.*, 2005; Peng *et al.*, 2005).

### **F. Growth Hormone/Insulin-Like Growth Factors**

The GH/IGF axis is important for long bone development, homeostasis, and disease (Fisher *et al.*, 2005; Kasukawa *et al.*, 2004; McCarthy and Centrella, 2001), and IGF 1 and IGF 2 have both systemic and local effects on bone growth and fracture repair (Aspenberg *et al.*, 1989; Isgaard *et al.*, 1986; Kawata *et al.*, 2002; Linkhart *et al.*, 1996; Marie, 1997; McCarthy *et al.*, 1989; Miyakoshi *et al.*, 2001; Stabnov *et al.*, 2002; Wildemann *et al.*, 2004) through a complicated series of growth factor-binding proteins which regulate their effects on bone (Yamaguchi *et al.*, 2006). There seems to be a role for IGF in the downstream signaling of PTH on bone where in mouse osteoblast cultures PTH treatment increased IGF-I mRNA and protein levels, and alkaline phosphatase activity, which were accompanied by phosphorylations of IGF-I receptor, insulin receptor substrate 1 (IRS-1), and IRS-2, essential adaptor molecules for the IGF-I signaling (Miyakoshi *et al.*, 2001). Further results indicate that the PTH bone anabolic action is

mediated by the activation of IRS-1 as a downstream signaling of IGF-I that acts locally as an autocrine/paracrine factor (Yamaguchi *et al.*, 2005).

### G. Bone Morphogenetic Proteins

The BMPs are probably the most important bone growth factors and account for the major proportion of the osteoinductive potential of bone extracts (Hoffmann and Gross, 2001). Over 20 BMPs family members have been identified and characterized (Cao and Chen, 2005; Wan and Cao, 2005; Wozney, 1992; Wozney *et al.*, 1988). All these BMPs are members of the TGF- $\beta$  superfamily. The BMPs are critically important for the regulation of bone formation, and it is these factors which is the focus of this chapter.

## III. The BMP Pathway and Bone Anabolic Therapies

Bone deficit conditions such as osteoporosis, osteopenia, nonunion fractures, and bone loss from traumatic injury are extremely difficult to treat with currently available agents that only prevent the loss of bone while lacking substantial bone anabolic activity.

The stimulation of local bone formation by either local or systemic application of bone anabolic agents would vastly improve the clinical treatment and repair of bone fractures, help integrating and stabilizing orthopedic implants, and markedly improve the repair of isolated bony defects. Growth factors, including recombinant BMP-2, FGF, and PDGF have the ability to stimulate bone formation and increase repair rates in animal models. Because of the lack of systemic availability, the use of these factors is restricted to their local application. Further, the use of these recombinant human growth factors for local application, to stimulate bone formation in humans, has been unfortunately variable and concerns have been raised about the expense and drug stability in such therapies (Schilephake, 2002). While understanding that there are many growth factors and hormones involved in bone formation, the discovery of small molecular weight compounds that can elicit bone anabolic activity will come from a closer understanding of the signaling pathway that are critical for bone formation.

There are multiple signaling cascades and processes required to be either activated or inhibited to elicit bone formation. One of the main signaling cascade events that stimulates bone formation is the BMP/SMAD pathway. Although other pathways are involved in the anabolic response in bone, it is the BMP/SMAD pathway that has received the most attention to date and a number of compounds have been discovered which can affect this pathway and lead to bone formation.

## A. BMP/SMAD Signaling Pathway

Bone morphogenetic proteins are very powerful inducers of both bone and cartilage and are implicated in a variety of non-osteogenic developmental processes. The BMPs are multifunctional growth factors that belong to the TGF- $\beta$  superfamily family. Type I and II BMP receptors and the downstream molecules, SMAD1, 5, and 8, mediate BMP signals. Phosphorylated SMAD1, 5, and 8 form complexes with SMAD4 and translocate to the nucleus where they interact with numerous other important transcription factors such as Runx2/cbfa1 to elicit bone formation. BMPs were first discovered by Marshall Urist when he found that the osteoinductive activity present in bone could not be accounted for by any known single growth factor or combination of growth factors, suggesting the existence of a novel bone-inductive protein (Urist *et al.*, 1977, 1979, 1982). To date, over 20 BMPs family members have been identified and characterized (Cao and Chen, 2005; Wan and Cao, 2005; Wozney, 1992; Wozney *et al.*, 1988).

Subsequent investigation led to the realization that the BMP pathway plays an integral role in bone formation not only in embryogenesis but also in the adult bone metabolism and plays a very important role in maintaining adult skeletal integrity. BMP-2 and other BMPs heal bone defects when applied locally. However, there are limitations to its use. Its efficacy appears limited to local use as no convincing evidence exists of its systemic activity. However, when used locally with an appropriate matrix such as collagen, BMPs show strong anabolic effects.

Preclinical models and clinical trials have shown the ability of BMP-2 to stimulate bone formation. In these animal models, bone defects are large enough so they will not heal without a therapeutic intervention allowing the ability of BMP-2 to induce bone and heal nonunion defects. Healing of long bone critical-sized defects by BMP-2 has been shown in a number of species including rats, rabbits, dogs, sheep, and non-human primates (Murakami *et al.*, 2002). Gene therapy studies indicate that bone defects can be healed by the local implantation of a bioresorbable polymer mixed with bone marrow mesenchymal stem cells to which adenovirus BMP-2 is transferred (Chang *et al.*, 2003). Studies show that rhBMP-2 delivered in an injectable formula with a calcium phosphate carrier or with a liposome carrier accelerates bone healing in a rabbit ulna osteotomy model and a rat femoral bone defect model (Li *et al.*, 2003; Matsuo *et al.*, 2003). Clinical studies show rhBMP-2 can be utilized as complete bone graft substitutes in spinal fusion surgery. In some circumstances, the efficacy of BMP-2 for inducing successful fusion is superior to that of autogenous bone graft, while BMIP-2 has been shown to be efficacious in several fusion applications, including intervertebral and lumbar posterolateral fusion (Boden *et al.*, 2002; Sandhu, 2004). BMP-2 has also been shown to induce new dentine formation and has a potential

application as a substitute for root canal surgery and BMP-2 is an effective bone inducer around dental implants for periodontal reconstruction (Cochran and Wozney, 1999).

One promising use of the BMPs is their application for local repair of bone or stimulation of new bone formation where loss has occurred (Asahina *et al.*, 1997; Hong *et al.*, 1998; Southwood *et al.*, 2004; Springer *et al.*, 2005). BMP-2 is FDA approved for a spinal fusion indication, although its cost and lower than expected efficacy have limited its usage in its current form (Boden, 2005; Khan and Lane, 2004). Unfortunately, BMP-2 appears to be ineffective when administered systemically where it is unable to increase bone formation or prevent trabecular bone loss induced by unloading in rats (Zerath *et al.*, 1998), probably because of its limited pharmacokinetics. One approach would be to stimulate the production of BMPs at local sites in bone by small compounds, resulting in bone formation where needed. This approach has led to the discovery of some small molecular weight anabolic agents that act by increasing BMP-2 protein at local sites.

## B. Agents That Act on the BMP Pathway

There is growing interest in the discovery of new and more versatile small molecular weight anabolic agents. Although the time has not quite come for these agents to make their impact on the market, a lot of preclinical and developmental studies suggest the possible use of small molecular weight agents as anabolic agents. These include agents, which not only enhance the expression of anabolic growth factors such as BMPs in the bone microenvironment, but also agents that can directly enhance the signaling pathways critically involved in the process of bone formation. Interestingly, a great proportion of the agents that have been reported to have bone anabolic potential, have the ability to affect the BMP pathway either directly or indirectly. Below is a table of the reported anabolic agents that affect the BMP pathway (Table III).

### 1. Statins

Initial identification of statins as small molecular weight anabolic agents was by the use of a cell-based screening assay that enhances BMP-2 transcription. This assay employed the 2T3 osteoblast cell line transfected with the murine BMP-2 promoter operatively linked to the firefly luciferase reporter. Screening of a natural products collection led to the identification of an extract that specifically stimulated the BMP-2 promoter in these cells. Purification of this extract identified lovastatin as the active constituent. Statins including lovastatin, simvastatin, pravastatin, atorvastatin, fluvastatin, rosuvastatin,

**Table III** Anabolic Agents That Affect or Enhance the BMP Pathway

Drug	BMP	
	Expression	Signaling
BMP-2	Yes	Yes
Statins	Yes	Yes
Proteasome inhibitors	Yes	Yes
Flavonoids	Yes	?
Sclerostin antagonists	?	Yes
Prostaglandin agonists	Yes	?
PTH (1–34) (Teriparatide <sup>®</sup> )	?	Probably
PTH (Preos)	?	Probably

**Table IV** Increase of New Bone Formation *In Vitro* by Lovastatin

Dose ( $\mu\text{g/ml}$ )	New Bone Formation ( $\text{mm} \times 10^{-3}$ )
0	$3.7 \pm 0.3$
0.075	$4.5 \pm 0.3$
0.15	$7.1 \pm 0.4^*$
0.3	$10.6 \pm 0.5^*$
0.6	$11.8 \pm 0.6^*$
1.2	$11.3 \pm 0.4^*$

\*Significantly greater than vehicle treated group. ANOVA  $p < 0.05$ .

pitavastatin, and cerivastatin are widely used agents for lowering cholesterol and reducing heart attacks. They provide an important and effective approach to the treatment of hyperlipidemia and arteriosclerosis (Hunninghake, 1998; Spin and Vagelos, 2003) and all stimulated BMP-2 promoter activity except pravastatin.

Subsequently, statins have been shown to increase the expression of BMP-2 in human and rodent bone cells (Mundy *et al.*, 1999) confirmed by many others (Emmanuele *et al.*, 2003; Maeda *et al.*, 2001, 2004; Mundy *et al.*, 1999; Sugiyama *et al.*, 2000). *In vitro* models of bone formation indicate simvastatin elicits marked increases in osteoblast accumulation and new bone formation over 4–7 days of culture (Mundy *et al.*, 1999) (Table IV), while pravastatin could not, consistent with its inability to stimulate the BMP-2 expression (Maeda *et al.*, 2001; Mundy *et al.*, 1999; Sugiyama *et al.*, 2000). Pravastatin cannot enter cells other than hepatocytes (Nakai *et al.*, 2001; Yamazaki *et al.*, 1996; Ziegler *et al.*, 1994), resulting in its reduced pleiotropic effects correlating with its inability to stimulate new bone formation.

It has been reported that compactin (mevastatin) at doses of 1–100  $\mu\text{M}$  suppresses osteoclastic bone resorption *in vitro* by inducing apoptosis of osteoclasts (Luckman *et al.*, 1998). Studies confirm the mechanism is through inhibition of the fusion of preosteoclastic cells and the disruption of actin ring in osteoclasts (Woo *et al.*, 2000). This effect is because of the inhibition of prenylation of target proteins by prenyl protein transferases, similar to that seen with bisphosphonates. These findings suggest statins, while able to stimulate osteoblasts are also capable of inhibiting resorbing osteoclasts. However, these *in vitro* effects occur at markedly different doses where inhibition of osteoclastic activity is between 1–100  $\mu\text{M}$ , while the bone anabolic effects of these agents occur at doses as low as 0.06  $\mu\text{M}$ . Given the hepatoselective nature of these statin drugs and the high doses required, it is unlikely they would be able to inhibit bone resorption *in vivo* as suggested previously (Luckman *et al.*, 1998). Alternatively, it is clear statins, unlike bisphosphonates, can affect osteoblastic activity at low doses, show anabolic effects *in vitro*, and there is increasing evidence this translates to effects *in vivo*.

Statins stimulate local formation in mice resulting in a 30–50% increase in calvarial bone thickness after 21 days (Mundy *et al.*, 1999). Lovastatin incorporated in biodegradable polymer discs, releasing lovastatin at a constant rate, resulted in a 50–70% increase in cranial bone thickness over a 21-day period in mice (Whang *et al.*, 2005). High dose of simvastatin formulated in a gel stimulates cranial bone apposition and the bone formed remained for up to 22 days after dosing (Thylin *et al.*, 2002). These findings pose the possibility that statins can be utilized for local bone repair to enhance new bone formation in orthopedic indications such as device stabilization, in surgical repair of bone defects as well as in conditions such as periodontal bone disease. There are numerous advantages for the local use of statins to stimulate bone formation. First, it is an inexpensive drug to manufacture compared with recombinant proteins such as BMPs or FGFs, second, it has a long history of clinical systemic usage with very acceptable good toxicity profiles, and finally, it is relatively easy to incorporate into biodegradable matrices that regulate its release to enhance its effectiveness.

Systemically, when lovastatin is given to ovariectomized rats, there is a marked increase in bone density (Mundy *et al.*, 1999). Long bones from rats treated with cerivastatin showed a 43% increase in tibial trabecular volumes and a 38% increase in tibial trabecular volumes in rats treated orally with simvastatin (Garrett *et al.*, 2001b). Both cerivastatin and simvastatin increase trabecular bone in rats in a similar manner to that previously seen by acidic FGF (Dunstan *et al.*, 1999). There was a 46% increase in both BFR and a 32% increase in MAR in the tibiae of rats treated with cerivastatin at 0.1 mg/kg/day (Garrett *et al.*, 2001b). Cerivastatin has also been shown to improve cortical bone strength in ovariectomized rats when used in doses as low as

0.1 mg/kg/day, and in addition significantly increased bone mineral density (BMD), bone formation rate, osteocalcin mRNA levels, as well as resistance to fracture (Wilkie *et al.*, 2000). Lovastatin, applied transdermally in a gel preparation to intact rats, increased bone volume up to 33% and the BFR was still increased by 166% after 30 days following the last dose (Gutierrez *et al.*, 2000). Further studies show simvastatin given orally to rats significantly increases cancellous bone compressive strength in the vertebral bodies of these rats (Oxlund and Andreassen, 2004), while others show an increase in cortical bone in young male rats using a single local administration (Crawford *et al.*, 2001). In mice, given a diet prepared with simvastatin, there was a marked improvement in fracture healing (Skoglund *et al.*, 2002). Work has also shown that simvastatin, when administered locally to a fracture site, demonstrated a dramatic positive effect on the strength of a healing fracture and indicated that the local delivery of statins could be used to promote fracture healing (Skoglund and Aspenberg, 2006).

These findings indicate statins have an anabolic effect by increasing the rate at which bone is forming and show statins have the potential to stimulate bone formation both *in vitro* and *in vivo* in rats. As statins appear to be bone anabolic agents in rats, with low relative toxicity in man, they could provide an important treatment when administered in the correct fashion for osteoporosis and fracture healing.

**a. Mechanism of Action.** Statins inhibit the rate-limiting step in the mevalonate pathway and the addition of downstream metabolites mevalonate, farnesyl pyrophosphate, or geranylgeranyl pyrophosphate inhibited statin-stimulated bone formation. As geranylgeranyl pyrophosphate reverses these effects, inhibition of prenylation appears to play a major role in the stimulation of bone formation by this drug. Prenylation is important for the activity of important intracellular molecules including GTPases, such as Rho, Rac, Rab, and Rap. These proteins play important roles in cellular proliferation and differentiation and therefore any perturbation of their activity influences cellular activity. Initial studies focused on nitric oxide synthase (eNOS) activity and NO which has been shown to be needed for bone formation in mice and in cultured mouse calvaria (Armour *et al.*, 2001; Feron *et al.*, 2001; Garrett *et al.*, 2001a), and prenylation of Rho GTPase regulates eNOS (Ming *et al.*, 2002). The inhibition of Rho GTPase prenylation upregulates eNOS activity providing the beneficial effects in the endothelium (Laufs *et al.*, 1997). Statin stimulation of bone resulted in upregulation of eNOS mRNA in murine osteoblasts and increased expression of mRNA for BMP-2. Interestingly, the expression of both eNOS and BMP-2 mRNAs peaked at 6 hours after exposure to the drug and disappeared by 24 hours. Furthermore, eNOS protein levels as well as NO production increased by 24 hours in human osteoblastic MG63 cells, which is at the same time

BMP-2 protein levels have been found to be increased in cells treated with statins (Garrett *et al.*, 2001b). Another possible impact of prenylation inhibition is on the small GTPase Rab23, a negative regulator of hedgehog signaling. Downstream signaling of hedgehog is facilitated by the members of the Gli family of transcription factors (Cohen, 2003; Eggenschwiler *et al.*, 2001, 2006; Evans *et al.*, 2003, 2005). As regulation of the BMP-2 promoter is known to be under the control of these Glis transcription factors (Garrett *et al.*, 2003), reduction in prenylation by statins would cause a decrease in Rab23 prenylation and a subsequent increase in Gli signaling and BMP expression. These findings link the prenylated Rab23 and eNOS expression and activity with BMP-2 mRNA in time, although the mechanism whereby the activation of eNOS activity and Rab23 would lead to BMP-2 protein increases is still under investigation.

**b. Clinical Findings.** Large databases were examined and indicated there a possible relationship between statin use, BMD, and subsequent fractures (Bauer, 2003). Since then, published studies indicate a significant increase of BMD associated with taking statins in postmenopausal women (Edwards *et al.*, 2000), and a protective effect against nonpathological fracture among older women (Chan *et al.*, 2000). Another association between statin use by elderly patients and reduction in the risk of hip fracture was seen (Wang *et al.*, 2000), while others suggested current exposure to statins is associated with a decreased risk of bone fractures in individuals aged 50 years and older (Meier *et al.*, 2000a,b). Further studies indicated a 60% reduction in fracture risk in women is associated with statin use (Pasco *et al.*, 2002) with similar findings in men (Funkhouser *et al.*, 2002). In male patients with type 2 diabetes mellitus, it was shown HMG-CoA reductase inhibitors increased BMD of the femur (Chung *et al.*, 2000). A recent prospective 1-year study found simvastatin treatment resulted in a significant increase in bone alkaline phosphatase with no significant decrease in the bone resorption marker C-terminal fragment of type I collagen. Simvastatin increased BMD at 6 and 12 months in women, and the authors concluded simvastatin had a positive effect on bone formation and BMD. Another study indicated simvastatin increased serum osteocalcin levels in patients (Chan *et al.*, 2001). One prospective study of 91,052 patients indicated statin use was associated with a 36% (odds ratio, 0.64; 95% confidence interval, 0.58–0.72) reduction in fracture risk when compared with no lipid-lowering therapy and a 32% (odds ratio, 0.67; 95% confidence interval, 0.50–0.91) reduction when compared with non-statin lipid-lowering therapy (Scranton *et al.*, 2005).

Other preliminary reports have indicated little or no effect of orally administered statins on bone (Cauley *et al.*, 2000; LaCroix *et al.*, 2003; Rejnmark *et al.*, 2002, 2004; van Staa *et al.*, 2001). The major drawback of all these studies is they are retrospective. The compliance of patients taking

statins is unknown and statins ineffective on bone, such as pravastatin, are included, while the dose of statin used varies considerably.

The *in vitro* findings suggest statins increase BMP-2 expression and stimulate osteoblast differentiation leading to new bone formation *in vivo*. However, all of the statins currently on the market target the liver and decrease cholesterol biosynthesis. Consequently, the biodistribution of active statin or metabolites to bone and other peripheral tissues is small, making it uncertain if current statins administered orally for lipid lowering will have beneficial effects on bone in humans. There are several possibilities for improving biodistribution to bone. The more recent potent statins such as cerivastatin or atorvastatin may get past the liver in sufficient amounts to cause beneficial effects on bone, and animal studies suggest this may be the case. Alternative modes of administration of the statins such as transdermal application through a skin patch may also solve the problem of poor biodistribution to bone (Gutierrez *et al.*, 2006). Another possibility would be to administer statins at local sites, bypassing the peripheral distribution problem, to stimulate bone formation (Skoglund and Aspenberg, 2006). This turns out to be a feasible mode of administration to stimulate new bone. A fourth possibility is that there may be other drugs of this class that were not selected for development as cholesterol-lowering agents because of their relatively greater biodistribution to peripheral tissues. These may be ideal drugs for use as bone-active agents.

Perhaps the most important consequence of these findings is that not only would statins themselves be effective drugs for bone loss conditions, but these findings focus attention on the mevalonate pathway and its relationship to BMP-2 expression and bone formation. This could lead to the identification of other potential molecular targets for drug discovery as well as other therapeutic approaches to enhance bone formation and produce the ideal anabolic agent for osteoporosis.

## 2. Proteasome Inhibitors

The proteasome is an abundant multicatalytic enzyme complex present in the cytoplasm and nucleus of all eukaryotic cells. The primary function of the proteasome is to degrade proteins. While it can act primarily as a cellular “garbage disposal” that removed damaged or misfolded proteins from cells, the proteasome also removes various short-lived proteins that regulate the cell cycle, cell growth, and differentiation. By regulating the turnover of these proteins via timely degradation and recycling, the proteasome plays a critical role in the maintenance of cellular homeostasis. Substrates of the proteasome include cell-cycle regulators, signaling molecules, tumor suppressors, transcription factors, and anti-apoptotic proteins; over 80% of all cellular proteins are recycled through the proteasome.

The ubiquitin/proteasome system plays an important role in the regulation of activity of bone cells. Osteoblastic function is suppressed by the cAMP pathway through proteolytic degradation of Cbfa1/Runx2 involving a ubiquitin/proteasome-dependent mechanism (Tintut *et al.*, 1999) and that the anti-apoptotic effect of PTH is prolonged by inhibition of proteasomal activity resulting in elevated levels of cbfa1/Runx2 (Bellido *et al.*, 2003). Treatment of cells with the proteasome inhibitors also induced ATF4 accumulation, an important regulator of bone cell activity, resulted in activation of an osteocalcin promoter (Yang and Karsenty, 2004). Other evidence supporting the role of the proteasome in bone cells shows that Smurf1, an E3 ligase responsible for targeting Cbfa1 and other important transcription factors to the proteasome, appears to be an important regulatory factor in osteoblast differentiation and a potential molecular target for identification of bone anabolic agents (Zhao *et al.*, 2003) and confirmed where Smurf1 induces Runx2 degradation in a SMAD6-dependent manner (Shen *et al.*, 2006). Smurf1 also physically interacts with MEKK2, an important mediator of BMP signaling, and promotes the ubiquitination and turnover of MEKK2 which negatively regulates osteoblast activity and response to BMP through controlling MEKK2 degradation (Yamashita *et al.*, 2005). Proteasomal activity in osteoblasts plays a pivotal role in regulating the intracellular levels of molecules important for many of the critical signaling pathways. These include the intricate hedgehog signaling mechanism of the Gli family of transcription factors where the activity of Gli2 and Gli3 are regulated by the proteasome (Cohen, 2003; Kalderon, 2002, 2005; van den Heuvel, 2003; Yu and Miller, 2004). Regulation of Gli transcription factors by proteasome inhibitors leads to the enhanced expression of BMP-2 and results in bone formation (Garrett *et al.*, 2003). Bone morphogenetic protein is a powerful stimulator of bone formation acting through its receptors BMPR-I and -II and through the downstream effector molecules known as SMADs. The cytoplasmic levels of these SMADs signaling molecules are also regulated by the proteasome (Heldin and ten Dijke, 1999; Johnsen *et al.*, 2002; Nishimori *et al.*, 2001; Shen *et al.*, 2006; Zhang *et al.*, 2001) indicating a probable enhancement of BMP signaling by proteasome inhibitors.

Another pathway which plays an important role in the anabolic activity of osteoblasts is the Wnt/ $\beta$ -catenin pathway (Holmen *et al.*, 2005; Iwao *et al.*, 1999; Mbalaviele *et al.*, 2005; Urano, 2006). Upregulation of this pathway has been shown to play a role in bone metabolism and appears to play a cooperative role with the BMP pathway in stimulating bone formation (Mbalaviele *et al.*, 2005). Although the Wnt/ $\beta$ -catenin pathway is extremely complicated, it has been shown that endogenous regulators such as sFRP (soluble Frizzled-related peptide) play a critical role in its effect on bone (Bodine *et al.*, 2005).  $\beta$ -catenin levels inside the cell are maintained by action

**Table V** Increase of New Bone Formation *In Vitro* by Proteasome Inhibitor 1

Dose ( $\mu\text{g/ml}$ )	New Bone Formation ( $\text{mm} \times 10^{-3}$ )
0	$4.1 \pm 0.2$
0.005	$4.2 \pm 0.4$
0.0125	$6.1 \pm 0.4^*$
0.025	$8.6 \pm 0.6^*$
0.05	$9.9 \pm 0.5^*$
0.1	$8.3 \pm 0.5^*$

\*Significantly greater than vehicle treated group. ANOVA  $p < 0.05$ .

of the proteasome (Aberle *et al.*, 1997; Bonvini *et al.*, 1999; Easwaran *et al.*, 1999; Salomon *et al.*, 1997). This indicates another potential pathway in which proteasomes and their inhibitors may influence bone metabolic activity.

Therefore, it is not surprising that inhibition of proteasomal activity in osteoblasts by proteasome inhibitors activity would lead to pronounced effects on bone tissues. In fact proteasome inhibitors are very potent anabolic agents *in vitro* (Table V) in the low nanomolar range as well as *in vivo* (Garrett *et al.*, 2003). In the clinic Bortezomib<sup>®</sup> or Velcade<sup>®</sup> (PS-341), the first proteasome inhibitor evaluated in human clinical trials, has been approved by the US Food and Drug Administration for use in patients with refractory or relapsed multiple myeloma with minimal side effects and has been shown to significantly elevate alkaline phosphatase from baseline (Zangari *et al.*, 2005). The rise in alkaline phosphatase together with a parallel increase in PTH after Bortezomib<sup>®</sup> suggested that response to Bortezomib<sup>®</sup> in myeloma is closely associated with osteoblastic activation (Shimazaki *et al.*, 2005; Zangari *et al.*, 2005) indicating a possible therapeutic activity not only treat myeloma itself but also reverse the bone loss common in the associated myeloma bone disease.

Proteasome inhibitors, such as Bortezomib<sup>®</sup>, therefore have the potential to be potent anabolic agents, systemically to treat bone deficit conditions locally to effectively enhance fracture healing, where bone regeneration is required.

### 3. Flavonoids

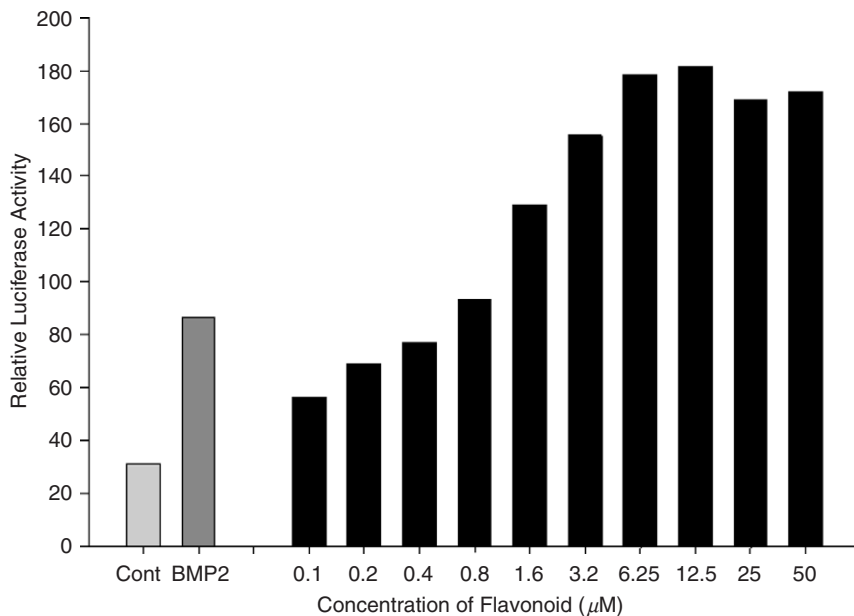
Flavonoids are found ubiquitously in higher plants and constitute an important component of the majority of people's daily diets. The biological activities of flavonoids cover a very broad spectrum, from anticancer and antibacterial activities to effects on bone. Although flavonoids may not be developed as

pharmaceutical agents for the treatment of bone deficit conditions, mostly because of the multitude of effects ascribed to them, numerous papers have suggested these agents act on bone in many ways. The effects of flavonoids on bone tissues were first noted sometime ago when the flavonoid Catergen (Zyma) showed an improvement in bone quality (Jones *et al.*, 1984). Since then, many flavonoids have been shown to have effects on bone. A synthetic flavonoid, Ipriflavone, has been shown to have effects on bone cells *in vitro* (Benvenuti *et al.*, 1991; Bonucci *et al.*, 1992; Hagiwara *et al.*, 1999; Kakai *et al.*, 1992; Ribari and Sziklai, 1987). It also has effects on bone metabolism in rats (Cecchini *et al.*, 1997; Foldes *et al.*, 1988; Ozawa *et al.*, 1992; Shino *et al.*, 1988; Yamazaki, 1987) and in patients (Agnusdei *et al.*, 1989; de Aloysio *et al.*, 1997; Gambacciani *et al.*, 1993; Kovacs, 1994; Mazzuoli *et al.*, 1992; Melis *et al.*, 1992; Nakamura *et al.*, 1992; Passeri *et al.*, 1992; Reginster, 1993) and is now used in the clinic in many countries. However, there have been a number of papers showing no effect of this agent either *in vitro* or in the clinic (Alexandersen *et al.*, 2001; Deyhim *et al.*, 2005; Ghezzeo *et al.*, 1996).

$\alpha$ -glucosylhesperidin significantly prevented this bone loss in rats (Chiba *et al.*, 2003) while kaempferol positively affected osteoblasts (Miyake *et al.*, 2003). Quercetin has pronounced effects on bone cells preventing bone loss as well as having marked bone-building properties (Horcajada-Molteni *et al.*, 2000; Prouillet *et al.*, 2004; Rassi *et al.*, 2005; Ross, 2005; Singh *et al.*, 2001; Son *et al.*, 2006; Sziklai and Ribari, 1995; Wattel *et al.*, 2003, 2004; Woo *et al.*, 2004; Wood, 2004; Yamaguchi and Jie, 2001; Zhang *et al.*, 1996). The flavonoids eupalitin 3-*O*- $\beta$ -D-galactopyranosyl-(1-- > 2)- $\beta$ -D-glucopyranoside, eupalitin3-*O*- $\beta$ -D-galactopyranoside, and 6-methoxy-kaempferol 3-*O*- $\beta$ -D-(1-- > 6)-robinoside were shown to have effects on bone (Li *et al.*, 1996). Another flavonoid naringin, which can be found in citrus fruit, apparently can act to inhibit HMG-Co reductase and can increase local new bone formation possibly being used as a bone graft material (Wong and Rabie, 2006).

A great portion of these flavonoid molecules seem to have their effects on bone through an estrogen-like activity and have been therefore termed phytoestrogens (Vaya and Tamir, 2004). Although the majority of the effects of flavonoids are on preventing resorption, recently it has been shown they are capable of stimulating new bone formation and therefore can be considered anabolic agents.

An example of the effects of these flavonoids on the BMP-2 promoter activity is shown in Fig. 1 where Robustone from the *Derris robusta* plant (Dakora, 1995; Dakora and Ndakidemi, 2003) caused a dose-dependent increase in BMP-2 promoter activity in osteoblastic cells. These cells were stably transfected with a BMP-2-promoter—Luciferase construct and exposed to this flavonoid for 24 hours. There was a marked increase



**Figure 1** Increase in BMP-2 promoter stimulated by the flavonoid Robustone.

**Table VI** Increase of New Bone Formation *In Vitro* by Robustone

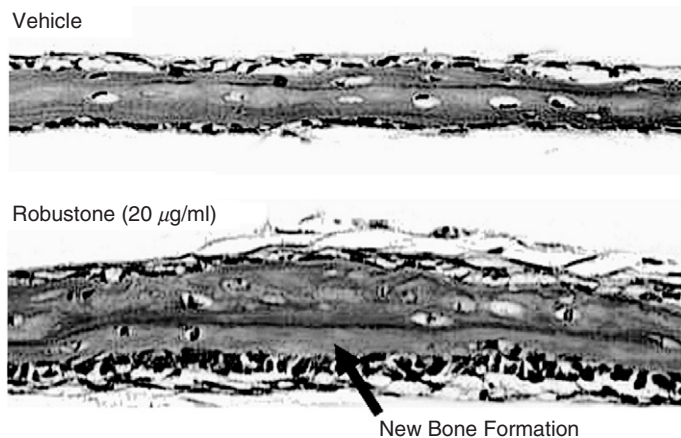
Dose (µg/ml)	New Bone Formation (mm × 10 <sup>-3</sup> )
0	4.2 ± 0.2
0.02	4.1 ± 0.3
0.2	5.3 ± 0.3*
2	6.4 ± 0.2*
20	8.3 ± 0.2*

\*Significantly greater than vehicle treated group. ANOVA  $p < 0.05$ .

in BMP-2 promoter activity around 1 µM and a maximum stimulation at 5 µM.

This flavonoid also dose dependently stimulated new bone formation when assessed in an *in vitro* murine calvarial assay (Table VI, Fig. 2), where over 7 days, there is a significant increase in new bone formation in cultured neonatal murine calvaria.

Many flavonoids affect bone metabolism and most inhibit osteoclastic resorption and reduce bone loss. However, there is a subset of these compounds that affects BMP expression and the BMP signaling pathway stimulating and



**Figure 2** Histology of neonatal calvaria treated with Robustone.

enhancing bone formation. Therefore, some flavonoids do affect the BMP pathway and are potential anabolic agents.

Whether these agents can clinically increase bone mass and reduce fractures is still to be determined. They do have the potential to be clinically relevant bone anabolic agents. Although, it is unclear if these agents, mostly from dietary intake, would reach sufficient serum concentrations to elicit a systemic bone anabolic response this may not preclude their use for local administration for fracture and bone defect repair.

#### 4. Antagonists of Sclerostin

Unlike the other growth factors, sclerostin is an osteocyte-expressed negative regulator of bone formation with amino acid sequence similarity with the DAN family of secreted glycoproteins that share the capacity to antagonize BMP activity. While it binds BMPs and antagonizes their bone forming capacity, it cannot antagonize all BMP responses. Sclerostin's mechanism of action is, therefore, distinct from that described for classical BMP antagonists. Because of its unique expression pattern and role in reducing bone formation (Kusu *et al.*, 2003; Ohyama *et al.*, 2004; Sutherland *et al.*, 2004; van Bezooijen *et al.*, 2004; Winkler *et al.*, 2003, 2004, 2005), it has been suggested that blocking the activity of this factor would lead to anabolic activity leading to enhanced bone formation (Ott, 2005; Poole *et al.*, 2005; van Bezooijen *et al.*, 2005a,b). It has been shown that antibodies developed to sclerostin cause marked increases in bone mass and bone formation (Paszty, 2006). This alternative approach to the treatment of bone deficit

conditions is particularly interesting and provides us with another way to treat bone diseases. The effectiveness of this treatment would be limited to sites where sclerostin is expressed and regulating bone formation. It is therefore unclear if this would be true at fracture sites or other sites of bone formation.

## 5. Prostaglandin Agonists

Prostaglandins play a significant role in bone metabolism (Tashjian *et al.*, 1972). Strong evidence indicates that prostaglandins play a role in stimulating bone resorption (Caniggia *et al.*, 1978; Dietrich and Raisz, 1975; Dowsett *et al.*, 1976; Harris *et al.*, 1973a,b; Kato, 1980; Katz *et al.*, 1981; Raisz and Koolemans-Beynen, 1974; Raisz *et al.*, 1974, 1979; Robinson *et al.*, 1975; Sakamoto *et al.*, 1979; Tashjian and Levine, 1978; Tashjian *et al.*, 1977; Yamasaki *et al.*, 1980; Yu *et al.*, 1979). Early evidence indicated prostaglandin E2 could not only cause bone resorption but it could also stimulate bone formation (Chyun and Raisz, 1984). Subsequent work has confirmed this where prostaglandins either enhance bone formation or prevent bone loss (Akamine *et al.*, 1992; Ito *et al.*, 1993; Jee *et al.*, 1990, 1992; Jorgensen *et al.*, 1988; Ke *et al.*, 1993; Li *et al.*, 1993, 1995; Lorenzo and Sousa, 1988; Ma *et al.*, 1994; Marks and Miller, 1988; McCarthy *et al.*, 1991; Miller and Marks, 1993a,b; Mori *et al.*, 1990, 1992; Raisz and Fall, 1990; Raisz *et al.*, 1993; Schmid *et al.*, 1992; Yang *et al.*, 1993). This early work led to the discovery that PGE2 mediates its tissue-specific pharmacological activity via four different G-protein-coupled receptor subtypes, EP1–4 (Paralkar *et al.*, 2003; Raisz and Woodiel, 2003). Bone effects have been reported with many of the prostanoid receptors, with most interest focused on the anabolic effects of EP2, EP4, and FP receptors. Current data suggests activity of the EP2 receptor stimulates formation and activity of the EP4 receptor stimulates resorption (and possibly formation), while activity of the FP receptor produces new trabeculae (Hartke and Lundy, 2001).

These studies have led to the synthesis of a number of EP4 receptor selective prostaglandin E2 agonists which enhance bone formation (Hagino *et al.*, 2005; Ito *et al.*, 2006; Ke *et al.*, 2006; Shamir *et al.*, 2004) and can augment BMP-induced bone formation (Toyoda *et al.*, 2005). These agents have been further investigated in preclinical studies for their ability to enhance fracture healing (Paralkar *et al.*, 2003; Tanaka *et al.*, 2004). Although no evidence exists which suggests these agents utilizes the BMP signaling pathway to elicit its activity, these agents do appear to enhance the effects of BMP on bone tissues (Arikawa *et al.*, 2004; Toyoda *et al.*, 2005).

**6. Parathyroid Hormone**

Full-length human parathyroid hormone (PTH), like PTH-peptide (Teriparatide<sup>®</sup>), is an effective anabolic agent in preclinical studies and is being developed under the name of Preos for treatment in osteoporosis although unlike PTH-peptide it has not yet been approved. Preos demonstrated significant fracture risk reductions in postmenopausal women with osteoporosis, but noted the higher incidence of hypercalcemia with Preos compared to placebo (NPS, 2006). PTH is expensive to manufacture and can only be given by injection which like the PTH-peptide (Teriparatide<sup>®</sup>) limits its use for the treatment of osteoporosis and bone diseases. Of the agents available on the market today to treat bone deficit conditions, PTH-peptide remains the only agent with significant bone anabolic activity. While PTH has been shown to interact with BMP to increase osteoblastogenesis and decrease adipogenesis (Chang *et al.*, 2003), other studies show that PTHrP and PTH inhibited BMP induced osteogenesis (van der Horst *et al.*, 2005). PTH has been shown to reduce sclerostin, a very potent inhibitor of the BMP signaling pathway, and it was suggested that this is one of the mechanisms of action of PTH (Keller and Kneissel, 2005).

**C. Agents That Do Not Act on the BMP Pathway**

A few reported anabolic agents exist which elicit their bone anabolic activity apparently through BMP independent pathways. Unlike Strontium, AC100, and Chrysalin, the Calcilytics agents increase PTH production. And from studies PTH affects the BMP pathway by interacting with BMP (van der Horst *et al.*, 2005) and possibly by inhibiting sclerostin expression (Keller and Kneissel, 2005). So it is entirely possible that the calcilytics do affect the BMP pathway although to date this has not been shown (Table VII).

**Table VII** Bone Agents That Do Not Affect or Enhance the BMP Pathway

Drug	BMP	
	Protein Expression	Signaling
Strontium ranelate	No	No
Chrysalin	No	No
Calcilytics	No	No
$\beta$ -blockers	No	No
AC100	No	No

### 1. Strontium Ranelate

Strontium ranelate (PROTELOS) is a newer anti-osteoporotic agent that appears to reduce bone resorption by decreasing osteoclast differentiation and activity (Ammann, 2005). The di-strontium salt strontium ranelate, a novel orally active agent consisting of two atoms of stable strontium and the organic moiety ranelic acid, has been developed for the treatment of osteoporosis (Reginster, 2002; Reginster *et al.*, 2003). It has been reported to enhance osteoblastic cell replication and increase collagen synthesis while decreasing pre-osteoclast differentiation and bone-resorbing activity of mature osteoclasts *in vitro* (Marie, 2005). The anti-fracture efficacy of strontium ranelate was assessed in two large, randomized, controlled trials conducted in postmenopausal women. Over the 3-year treatment period significantly fewer patients had height loss and fewer patients reported new or worsening back pain in the treated group than in the control group (Delmas, 2005; Rizzoli, 2005). These results demonstrate strontium ranelate is a new therapeutic option in the prevention of osteoporotic vertebral fractures in postmenopausal women.

Pharmacological studies in animals have shown that strontium ranelate decreases bone resorption and increases bone formation, resulting in increased bone mass. In ovariectomized rats, strontium ranelate prevented the reduction in bone mineral content and the decrease in trabecular bone volume induced by estrogen deficiency suggesting a possible anabolic activity. In this model, strontium ranelate decreased bone resorption, whereas bone formation was maintained at a high level as documented by plasma biochemical markers and histomorphometric indices of bone formation (Marie, 2005). The mechanism of action of strontium ranelate is unclear, however, no evidence exists indicating this agent elicits any of its effects through modulation of the BMP pathway.

### 2. Chrysalin

The  $\alpha$ -thrombin peptide, TP508 or Chrysalin, accelerates the healing of full-thickness wounds in both normal and ischemic skin. In wounds treated with TP508, a pattern of increased vascularization is consistently observed both grossly and microscopically when compared to wounds treated with saline (Norfleet *et al.*, 2000). TP508, binds to high-affinity thrombin receptors and mimics cellular effects of thrombin at sites of tissue injury (Stiernberg *et al.*, 2000). This thrombin peptide was shown to be active in repairing segmental bone defects in rabbits (Sheller *et al.*, 2004), and its mode of action was thought to be through thrombin-induced angiogenesis (Tsopanoglou *et al.*, 2004). This thrombin-related peptide was then shown to enhance bone formation during distraction osteogenesis (Li *et al.*, 2005). TP508 was shown

to promote fracture repair through a mechanism that involves an increased induction of a number of growth factors, enhanced expression of inflammatory mediators, and angiogenesis-related genes (Wang *et al.*, 2005). This peptide has also been shown to exert maturation specific effects on chondrocytes in the endochondral lineage, promoting cartilage extracellular matrix synthesis over endochondral differentiation in resting zone cells and proliferation over differentiation of growth zone cells (Schwartz *et al.*, 2005). Phase 3 clinical trials with this synthetic peptide Chrysalin (R) (TP508) in unstable, displaced distal radius (wrist) fractures showed that the treatment with 10  $\mu\text{g}$  Chrysalin did not demonstrate a statistically significant benefit compared to placebo in the primary efficacy endpoint of time to removal of immobilization. A secondary endpoint, radiographic evidence of time to radial cortical bridging, showed a statistically significant benefit for Chrysalin-treated subjects ( $p = 0.049$ ) (Othologic, 2006). The mechanism of action of Chrysalin does not appear to be due to effects on BMP expression or signaling and so the agent probably works through an alternative independent pathway.

### 3. Calcilytics

A number of years ago, the receptor sensing calcium (CaR) was discovered on the surface of parathyroid cells. Although  $\text{Ca}^{2+}$  receptors are expressed throughout the body and in many tissues that are not intimately involved in systemic  $\text{Ca}^{2+}$  homeostasis, their physiological and/or pathological significance remains speculative and their value as therapeutic targets is unknown (Nemeth, 2004a,b). Stimulation of CaR induced by an increase of extracellular ionized calcium concentration resulted in an increase of intracellular calcium and subsequent decrease of PTH secretion from parathyroid cells (Hoppe and Rybczynska, 2000). Agents were then synthesized that block this receptor activity and act as antagonist at the  $\text{Ca}^{2+}$  receptor. When infused intravenously in normal rats, they caused a rapid and large increase in plasma levels of PTH (Nemeth *et al.*, 2001). These are known as calcilytic agents and they increase endogenous levels of circulating PTH to an extent that stimulates new bone formation (Nemeth, 2004a,b). These agents could replace the use of exogenous PTH or its peptide fragments in treating osteoporosis (Nemeth, 2002a,b). There have been designs and new synthesis, of new calcium receptor antagonist which are novel 3H-pyrimidin-4-ones (Shcherbakova *et al.*, 2005a,b). These newer calcilytic agents are promising new therapeutic tools allowing for tight control of plasma PTH and restoration of circadian PTH rhythmicity (Schmitt *et al.*, 2005). Other effects are now being seen with these agents where calcilytic drugs prevent NO-induced damage and death of human neurons (Dal Pra *et al.*, 2005). Although calcilytics show no direct effects on the BMP pathway, it is interesting to

postulate that due to their capacity to increase PTH expression they may still affect this pathway.

#### 4. $\beta$ -Blockers

A number of years ago,  $\beta$ -adrenergic blocking agent propranolol was shown to increase bone formation in rats (Minkowitz *et al.*, 1991), and the direct effects of physiologically relevant propranolol concentrations on osteoblastic cells can be attributed principally to  $\beta$ -adrenergic blockade (Majeska *et al.*, 1992).

It has been shown that  $\beta$ -adrenergic stimulation enhances osteoclastogenesis and bone resorption (Arai *et al.*, 2003; Takeuchi *et al.*, 2001). Because of these findings,  $\beta$ -adrenergic antagonists have been investigated in the clinic and have been shown to be associated with a reduction in fracture risk and higher BMD (Pasco *et al.*, 2004; Schlienger *et al.*, 2004). While studies in rats using  $\beta$ -agonists confirm the deleterious effect of  $\beta_2$  agonists on bone mass (Bonnet *et al.*, 2005), more recent studies indicate that  $\beta$ -blockers might suppress bone resorption with relative preservation of bone formation (Pasco *et al.*, 2005). However, others report no evidence of  $\beta$ -blocker drugs stimulating bone formation and if anything, propranolol reduces osteoblast activity (Reid *et al.*, 2005). Although there does appear to be a relationship between BMP and responses in adrenergic cells (Varley and Maxwell, 1996; Varley *et al.*, 1998; Xu *et al.*, 2003; Zhang *et al.*, 2004), there appears to be little if any evidence that  $\beta$ -blockers elicit their bone activity by acting on BMP or enhancing its activity.

#### 5. AC-100 (MEPE Peptide)

Matrix extracellular phosphoglycoprotein (MEPE) is a 56.6-kDa protein and is expressed exclusively in osteoblasts, osteocytes, and odontoblasts with markedly elevated expression found in X-linked hypophosphatemic rickets (Hyp) osteoblasts and in oncogenic hypophosphatemic osteomalacia (OHO) tumors (Rowe *et al.*, 2004). AC-100, a central 23-amino acid fragment of MEPE, contains motifs that are important in regulating cellular activities in the bone microenvironment although it is unclear just how this happens. This fragment AC-100 apparently requires inducible cyclooxygenase-2 to exert potent anabolic effects on normal human marrow osteoblast precursors (Nagel *et al.*, 2004). Work has focused on the ability of the synthetic peptide fragment of human MEPE stimulates new bone formation *in vitro* and *in vivo* (Hayashibara *et al.*, 2004). It is therefore possible that this fragment of MEPE, AC-100, represents a potential treatment for bone repair in periodontal and orthopedic applications and a novel biological approach to dentistry. A phase I clinical study demonstrated equivalent

safety seen in preclinical studies. Two phase II clinical studies of AC-100 were initiated in 2005, one for periodontal regeneration, the other for dental pulp protection. Results for these studies will be available in early 2006. In addition, this product is being developed for an orthopedic application ([Acologix, 2006](#)). No evidence exists which suggests this peptide utilizes the BMP signaling pathway to elicit its activity.

#### IV. Conclusions and Future Directions

There are numerous factors, signaling pathways, and transcription factors critically involved in the stimulation of bone growth. These include BMP/SMAD, Wnt/ $\beta$ -catenin, Hedgehog/Gli, IGF, and FGF pathways. Lately, there has been the suggestion that the sympathetic nervous system is involved in bone metabolism, and it is intriguing to suggest that it might be possible to modulate bone metabolism by manipulating the important molecules that influence this pathway. While many of these pathways play an essential role in growth responses during patterning and embryogenesis of bone, there is also evidence these pathways play important roles in adult bone metabolism. It would be naive to think that any single pathway can stimulate bone formation without a major influence on these other pathways. Mounting evidence indicates that the BMP, Wnt, and hedgehog pathways interact as regulators and/or modulators of each other. This would be especially true when considering the intricate control and regulation of the cascade of events responsible for bone formation ([Burstyn-Cohen \*et al.\*, 2004](#); [Fisher \*et al.\*, 2005](#); [Garrett \*et al.\*, 2003](#); [Grotewold and Ruther, 2002](#); [Huang and Klein, 2004](#); [Kawai and Sugiura, 2001](#); [Litsiou \*et al.\*, 2005](#); [Mbalaviele \*et al.\*, 2005](#); [Raible and Ragland, 2005](#); [Rawadi \*et al.\*, 2003](#); [Theil \*et al.\*, 2002](#); [Tian \*et al.\*, 2005](#); [Tzahor \*et al.\*, 2003](#); [Zhang and Stott, 2004](#)). Although these different pathways control anabolic activity, it is vital to understand that the negative regulation of bone growth by inhibitors of these pathways may play a more important role in controlling where and when bone growth occurs. It is clear, however, that the BMP pathway is one of the major pathways responsible for initiating the complex process of bone formation and small molecular weight agents that enhance this pathway by either upregulating the expression of BMPs, increasing downstream signaling or reducing the negative regulators of this pathway would be good potential candidates for bone anabolic agents. A major need exists for low cost effective anabolic agents that could be used orally or delivered locally for the treatment of bone deficit conditions. In addition, agents that influence the BMP pathway such as the statins or agents that affect the proteasome clearly show promise as anabolic agents for the treatment of bone deficit conditions. These new and powerful small molecular

weight bone anabolic agents may offer new innovative and versatile treatments for bone disease in the future.

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