4332 J. Med. Chem. 2010, 53, 4332–4353
DOI: 10.1021/jm9018756

Journal of Medicinal Chemistry Perspective

Emerging Targets in Osteoporosis Disease Modification

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Received December 20, 2009

Introduction

Bone health in adult life depends on the coordinated activities of bone forming osteoblasts and bone resorbing osteoclasts that function together in a basic multicellular unit (BMU[a]) on the bone surface.[1] Bone turnover reflects a balance between these anabolic and catabolic cellular functions and ensures that the mature skeleton can repair itself when damaged and sustain its endocrine function by release of minerals such as calcium and phosphorus into the circulation. In addition, the skeleton can adapt to changes in physical activity and alter its size in response to cellular signaling in osteocytes (specialized osteoblasts surrounded by mineralized bone) which likely act as mechanosensors in bone that sense changes in mechanical load.[2] Normal bone turnover can be altered, however, in many disease states resulting in increases or decreases in bone mass or changes in bone quality linked to mineralization defects that can compromise strength and increase the risk of fracture.[3] Gradual loss of bone mineral density (BMD) referred to as osteopenia and severe loss of bone known as osteoporosis may represent the early and late manifestations of alterations in bone turnover that result in bone disease.

The peak bone mass attained by the skeleton during adolescent growth may predict the risk for development of osteoporosis in later life.[4] In fact the accumulation of bone during growth is strongly heritable with genetic differences accounting for as much as 50−85% of the variation in BMD.[5] With advancing age, however, the BMU creates an environment in bone where the amount of bone resorbed typically exceeds its replacement capacity leading to a net decrease in BMD. Although normal age-dependent loss of bone mass occurs in both men and women, the rapid loss of bone associated with estrogen deficiency following menopause in women accounts for the majority of clinical cases of osteoporosis. Other major disease-related causes of osteoporosis in addition to hypogonadism include but are not limited to endocrine disorders such as Cushing's syndrome (and the related secondary loss of bone associated with the use of steroids for other medical conditions) and renal insufficiency.[3,6]

The general principles involved in bone remodeling and the role of the BMU are shown in Figure 1. The normally quiescent bone surface lined by inactive osteoblasts is thought to receive activating signals, possibly including those from underlying osteocytes, which trigger initiation of bone resorption via osteoclasts. A cascade of events leads to the formation of mature multinucleated osteoclasts from osteoclast precursor cells in the bone marrow that attach to the bone surface. Significant progress has been made over the past decade or so that establishes a fundamental role for OPG/RANKL interactions in regulating the level of osteoclast bone resorbing activity.[7,8] Binding of osteoprotegerin (OPG) to RANKL prevents RANKL-dependent activation of the receptor activator of nuclear factor κB (RANK) receptor on osteoclasts, thus limiting the potential for resorptive activity. The etching effect of osteoclast action and degradation of bone matrix result in the formation of resorption pits, which is then followed by apoptosis of the invading osteoclasts. In normal bone, the demineralized space becomes occupied by bone forming osteoblasts that synthesize new bone matrix to repair the pitted surfaces. Signals from osteoclasts themselves could be involved in the osteoblast recruitment phase. The new

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[a]Abbreviations: BMU, basic multicellular unit; BMD, bone mineral density; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor κB; SERM, selective estrogen receptor modulator; SARM, selective androgen receptor modulator; PTH, parathyroid hormone; RGD, arginine−glycine−aspartate; DXA, dual energy X-ray absorptiometry; CTX-I, C-terminal telopeptides of type I collagen; NTX-I, N-terminal telopeptides of type I collagen; FPPS, farnesyl diphosphate synthase; MSC, mesenchymal stem cell; CAT, cathepsin; OVX, ovariectomized; mAb, monoclonal antibody; HSA, human serum albumin; BMP, bone morphogenic protein; TGF, transforming growth factor; IGF, insulin-like growth factor; FGF, fibroblast growth factor; WNT, wingless-type MMTV integration site; LRP5/6, lipoprotein receptor-related protein 5/6; RUNX2, runt-related transcription factor 2; ATF4, activating transcripton factor 4; OC, osteocalcin; ALP, alkaline phosphatase; PINP, N-terminal cross-linking propeptide of type I procollagen; PICP, C-terminal cross-linking propeptide of type I procollagen; PTH1R, parathyroid hormone receptor; MMTV, mouse mammary tumor virus; DSH, disheveled; GSK, glycogen synthase kinase; APC, adenomatous polyposis coli; TCF, T-cell factor; LEF, lymphoid enhancer-binding factor; SOST, sclerostin; DKK-1, dickkopf-1; sFRP-1, secreted frizzled related protein-1; WIF, wingless-type MMTV integration site inhibitory factor-1; FZD, frizzled receptor; CREB, cyclic adenosine monophosphate response element binding protein; COL1, type-1 collagen; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; GPCR, G-protein-coupled receptor; EP, prostaglandin receptor; PG, prostaglandin; cAMP, cyclic adenosine monophosphate; BMC, bone mineral content; CB, cannabinoid; SRC, sarcoma proto-oncogene; PBMC, primary bone marrow cells; CDK, cyclin dependent kinase; LCK, leukocyte-specific protein tyrosine kinase; YES, Yamaguchi sarcoma viral oncogene homologue; ABL, Abelson murine leukemia viral oncogene homologue 2; KIT, Hardy−Zuckerman 4 feline sarcoma viral oncogene homologue; CSK, carboxy-terminal SRC kinase; PYK, proline-rich tyrosine kinase; FAK, focal adhesion kinase; BMM, bone marrow macrophage; EGCG, (−)-epigallocatechin gallate; NFkB, nuclear factor κ-light-chain-enhancer of activated B cells; m-CSF, macrophage colony-stimulating factor; SPA, scintillation proximity assay; TPH, tryptophan hydroxylase; HTR, hydroxytryptamine receptor; ADR2β, β2 adrenergic receptor; SSRI, selective serotonin reuptake inhibitor; SHN3, schnurri3; WWP1, WW domain−containing protein 1; SMURF1, small mothers against decapentaplegic ubiquitin regulatory factor 1; SMAD, small mothers against decapentaplegic.

 Published on Web 03/10/2010

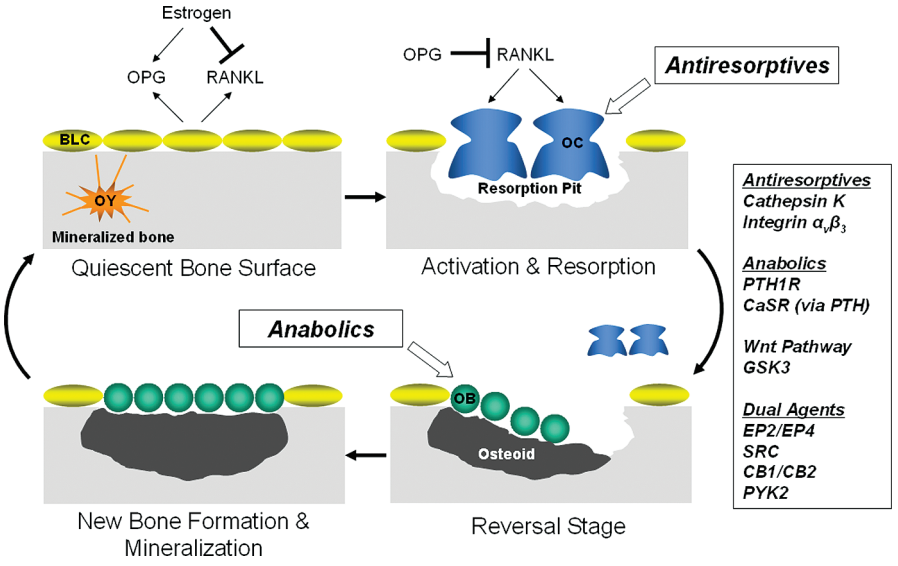

Figure 1. Role of OPG/RANKL and emerging new targets in bone remodeling. OPG and RANKL are expressed in many tissues and cell types including osteoblasts. Cellular locations of emerging antiresorptive, anabolic, and potential dual-acting agents are shown and described in the text. Abbreviations are as follows: BLC, bone lining cell; OY, osteocyte; OC, osteoclast; OB, osteoblast.

matrix (osteoid) deposited by osteoblasts is then mineralized, and the loss of old bone is effectively balanced by replacement of new bone. In postmenopausal osteoporosis, however, the loss of estrogen that can normally inhibit RANKL expression tips the OPG/RANKL ratio in favor of RANKL-stimulated osteoclast activity and accelerated bone resorption. Although bone formation also increases, it does so at a relatively slower rate where the bone forming capacity of osteoblasts fails to totally replace the lost bone. Thus, a net loss of bone occurs over time that can be associated with increased fracture risk. An understanding of the central role of RANKL in driving bone resorption led to the development of the fully human anti-RANKL antibody denosumab (Prolia) by Amgen, which is currently under review in the U.S. and Europe for osteoporosis and other bone-related indications.[9,10]

Among current marketed osteoporosis drugs, the bisphosphonate class of oral small molecule antiresorptives celebrated 40 years of clinical use in 2009 for the treatment and prevention of osteoporosis. Bisphosphonates arguably represent the current standard of care for osteoporosis, and several classes of bisphosphonates are available[11,12] including, for example, Novartis' recently approved Reclast (zoledronic acid) as a once a year infusion. Calcium and vitamin D supplementation represents an additional mainstay of osteoporosis treatment. Other therapies are available that address the hypogonadal function associated with low bone mass in women including use of estrogen and selective estrogen receptor modulators (SERMs).[13] Calcitonin is another approved antiresorptive for the treatment of osteoporosis. Whereas bisphosphonates represent the first line treatment for osteoporosis, approved therapies associated with osteoblast anabolic function are also available. These include Eli Lilly's PTH-related anabolic agent Forteo (teriparatide) which was approved as a biological agent in 2002 and Nycomed's PTH molecule Preotact which was approved in Europe in 2006. However, while indicated for treatment of osteoporosis via enhanced bone-forming activity, the Forteo label limits its use to 2 years because of the risk of osteosarcoma. Other compound classes with potential anabolic activity that have received particular attention include the selective androgen receptor modulators (SARMs)[14] and Servier's Protelos (strontium ranelate) which, although approved in Europe for osteoporosis, has a less well understood mechanism of action.

The emerging understanding of osteoclast and osteoblast biology has led to the identification of several new targets with potential antiresorptive, anabolic, and dual action properties. The focus of the current Perspective is to describe progress in the advancement of bone antiresorptive or anabolic small molecule programs described in the last five years (or since recent reviews).[15−17] We have placed an emphasis on targets with a known biochemical mechanism in bone metabolism, and that have been validated by testing with selective small molecule agents in cell culture or in vivo. However, compounds discovered in cell based and natural products screening with unknown biochemical mechanism are also included.

Assessment of Bone Mass and Use of Serum Biomarkers

Bone is constructed of both outer cortical material that is dense and compact with superior strength and inner trabecular material that forms a porous network with a large surface area that is in contact with the marrow. Bone tissue can vary in the proportion of cortical and trabecular material present and regional changes in bone mass, and the effects of bone modulating agents can be monitored in a number of ways. Direct assessment of BMD is typically made noninvasively by dual energy X-ray absorptiometry (DXA), whereas measurement of serum biomarkers is used to indirectly evaluate changes in the rates of bone formation and resorption. In preclinical studies and in patients taking antiresorptive medications, serum or urine C-terminal and N-terminal cross-linking telopeptides of type I collagen (CTX-I and NTX-I) represent examples of several biomarkers used to assess bone resorption. Enhanced osteogenesis is associated with increased differentiation and activity of osteoblast cells, as characterized by the secretion of proteins associated with mineralization and the production of new collagen. These changes can be monitored in preclinical studies and in patients on medication using serum biomarkers including osteocalcin (OC), bone-specific alkaline phosphatase (ALP), and N-terminal

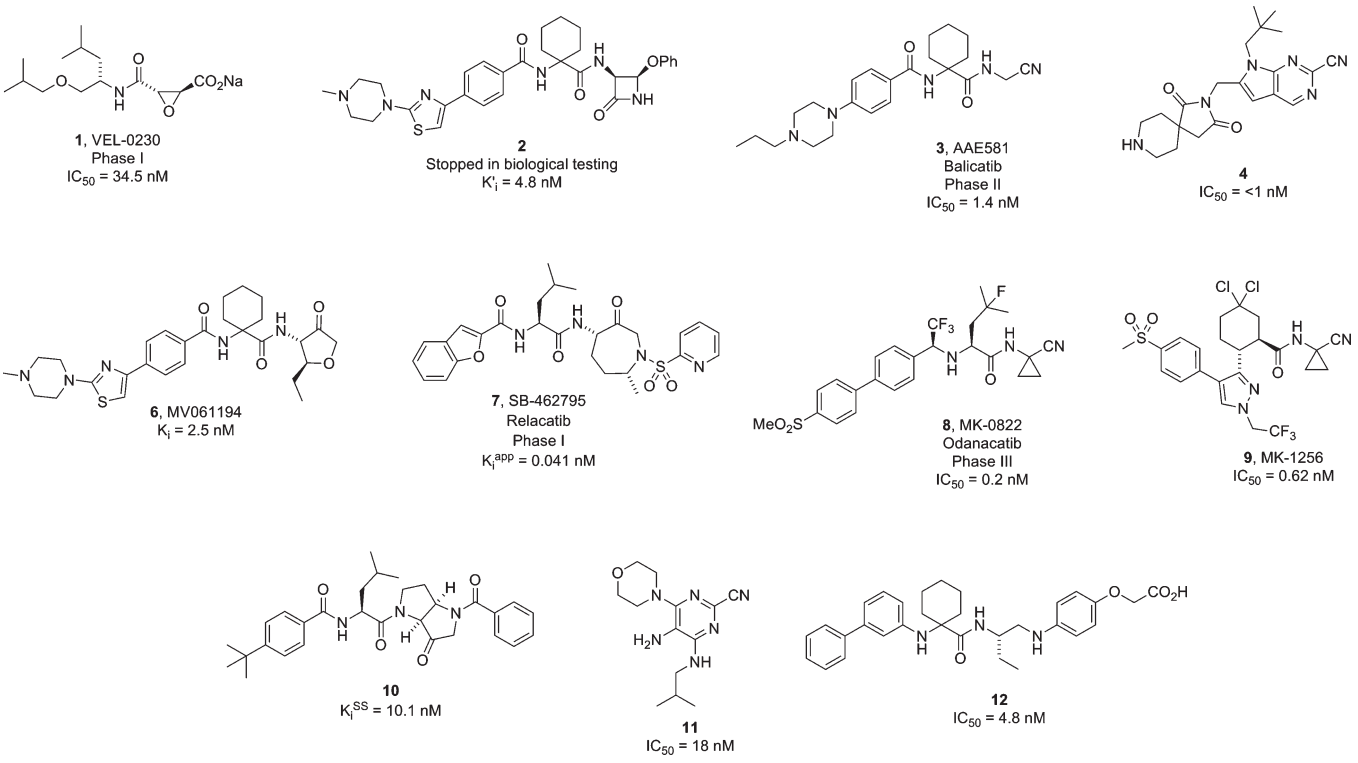

Figure 2. Cathepsin K inhibitors in various stages of development.

and C-terminal cross-linking propeptides of type I procollagen (PINP and PICP respectively).

Bone Antiresorptive Targets

Bone remodeling is initiated by the activity of osteoclasts as they migrate to the bone surface.[7] Bisphosphonates inhibit osteoclast activity via stimulation of apoptosis and inhibition of farnesyl diphosphate synthase (FPPS), which is required for prenylation of small GTPases and osteoclast survival.[2] Osteoclasts attach to proteins containing an arginine−glycine−aspartate (RGD) sequence in the extracellular matrix of mineralized bone largely via $\alpha_v\beta_3$ integrin receptor interactions. The activated osteoclasts establish a sealing zone next to bone that allows for acidification by hydrochloric acid secretion and breakdown of the mineral component. This localized production of hydrochloric acid depends on the activities of an H^+ATPase, Cl^- channel, and HCO_3^- exchanger. The type I collagen exposed during demineralization becomes a substrate for the protease cathepsin K, and the loss of collagen results in the formation of a resorption pit leading to erosion of the bone surface. Recent progress is described below on the development of small molecule inhibitors of cathepsin K and $\alpha_v\beta_3$ integrin as novel antiresorptive agents.

Cathepsin K

Cathepsin K is one of 11 human cysteine proteases in the papain family. Expressed primarily and at high levels in osteoclasts, cathepsin K plays an important role in the breakdown of type 1 collagen in bone matrix. Loss of the cathepsin K gene results in pcynodysostosis in humans[18] and osteopetrosis in mouse models,[19] conditions characterized by short dense bones, providing genetic evidence for the role of cathepsin K in osteoclast function. Recent developments have been reviewed,[20−22] and advanced compounds from several groups have been published including four clinical candidates that have provided valuable data on cathepsin K inhibition in humans (Figure 2). Early tool compounds used in preclinical studies of cathepsin K pharmacology were irreversible covalent inhibitors that resulted in antigenic and immunologic complications in vivo. Reversible covalent binders were next developed, followed by noncovalent inhibitors. Decreased affinity of the noncovalent binders for cathepsin K was compensated for by incorporating basic functionality in the S3 pocket. Subsequently it was recognized that inhibitors containing basic functionality may be lysosomotropic and could reach concentrations in acidic subcellular organelles sufficient to cause inhibition of other cysteine proteases or cause other off-target effects despite excellent biochemical selectivity. By optimization of other interactions in neutral scaffolds, potency was improved and lysosomal accumulation was avoided. Data on each class of cathepsin K inhibitor have been recently published and are summarized below (Table 1).

The cathepsin K active site (Figure 3) is characterized by the Cys25-His159-Asn175 triad containing the catalytic cysteine Cys25 that forms a reversible or irreversible covalent bond with many reported inhibitors via an epoxide, hydrazide, semicarbazone, vinylsulfone, aldehyde, amide, ketone, or nitrile. Flanking Cys25 are binding sites S and S′, the former of which has been divided into the S1, S2, and S3 pockets and is occupied by the corresponding P1, P2, and P3 inhibitor substituents.[23] For example, corresponding to S2, the P2 substituent makes van der Waals contacts with Leu157, Leu205, and Tyr67. 1-Amino-1-cyclohexanecarboxylic acid and leucine derivatives are two P2 groups that have provided high levels of cathepsin K selectivity while maintaining potency. The carbonyl and amine of the P2 group satisfy critical hydrogen bonds with Gly66. The S1 and S′ pockets may or may not be occupied, and the S3 pocket is defined by

Table 1. Inhibitor Enzymatic Potency on CAT K and Isoform Selectivity

compd	IC_{50} (nM) CAT K	*n*-fold CAT B	CAT L	CAT S	CAT V	CAT H	CAT F
1	34.5	8	17	5			
2	4.8[a]	71	500	3542			
3	1.4	3428	359	46429			
4	< 1.0		> 1000	> 10000			
5	1.7[d]	> 882	> 882	> 882	> 882	> 882	397
6	2.5[d]	> 4000	> 40000	> 40000		> 4000	
7	0.041[b]	317	1.65	39	1.54		
8	0.2	5170	14975	300	3810	> 50000	3975
9	0.62[c]	> 16000	4700	4300	1100	> 140000	110
10	10.1[e]	> 990	> 347	> 446			
11	18	> 556	111	31			
12	4.8	> 4167	> 4167	2083			

[a] K_i'. [b] K_i^{app}. [c] Biochemical data determined using humanized rabbit enzyme. [d] K_i. [e] K_i^{ss}.

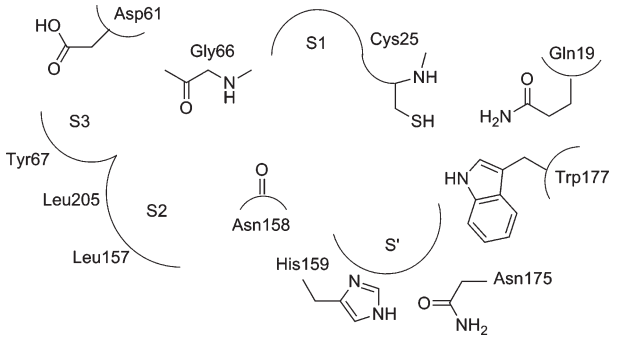

Figure 3. Schematic representation of the cathepsin K active site.[23]

Tyr67 and Asp61 and appears to tolerate π-stacking aromatic or basic substituents that form ionic interactions.

An example of an irreversible covalent inhibitor of cathepsin K is VEL-0230 (**1**), developed by Velcura and Nippon Chemiphar (Figure 2). The carboxylate epoxysuccinyl group binds in the region of the Cys25 and His159 and reacts irreversibly with the catalytic Cys25, providing a moderately potent and selective cathepsin K inhibitor (CAT K IC_{50} (nM), 34.5; selectivity (fold), CAT B, 8; CAT L, 17; CAT S, 5). Compound **1** significantly inhibited osteoclast-dependent resorption pit formation on dentin slices and CTX-I release by mature osteoclasts at 1 μM.[24] In addition to the antiresorptive behavior known for other cathepsin K inhibitors, **1** has also been reported to show osteogenic properties,[25] possibly via stimulation of differentiation of mesenchymal stem cells (MSC) into osteoblasts.[26] Velcura has reported the completion of phase Ia clinical trials of **1** at 150−450 mg in which a significant reduction of CTX-I compared to placebo was observed within 15 min of dosing and persisted over 12 h. Approximately 80% inhibition of CTX-I was observed at 6 h.[27] Velcura reported in May 2008[28] initiation of phase I clinical trials for the treatment of rheumatoid arthritis, metastatic bone diseases, myeloma, and osteoporosis.

Basic substituents in the S3 pocket have been positioned in order to form a highly favorable ionic bonding interaction with Asp61. 3,4-Disubstituted azetidinones, exemplified by compound **2**, recently published by Celera Genomics, form a covalent thioester bond on reaction with the cathepsin K active site, in a mechanism analogous to β-lactam reaction with serine proteases.[29,30] A similar thioester bond was shown to hydrolyze in vitro with $t_{1/2}$ = 26 min for another analogue. Compound **2** was potent and selective (CAT K K_i' (nM), 4.8; selectivity (fold), CAT B, 71; CAT S, 3542; CAT L, 500), but rat pharmacokinetics were poor, and further optimization was discontinued. Also containing a piperazine group, AAE581 (**3**) from Novartis was potent and selective[31] (CAT K IC_{50} (nM), 1.4; selectivity (fold), CAT B, 3428; CAT L, 359; CAT S, 46429). In phase II clinical trials, a 50 mg dose of compound **3** decreased bone resorption markers serum CTX-I (61%) and urinary NTX-I (55%) as normalized to creatine at 1 month.[32] Furthermore, 50 mg of **3** provided 4.46% and 2.25% increases in BMD at lumbar spine and hip, respectively, compared to 0.25% and 0.25% for placebo at 1 year.[32] Novartis has reportedly stopped development of this compound,[21] since skin adverse events (mainly pruritus) were observed as well as a small number of patients experiencing scleredema or morphea-like skin changes.[32] Noting the basic piperazine group in the molecule, these adverse findings may have been linked to inhibition of other cathepsins or CAT K itself following lysosomal accumulation of **3**.[31,33] For example, cathepsins B, K, L, and G are all expressed in skin and skin-derived cells, and their inhibition could lead to excessive deposition of extracellular matrix proteins in the dermis and thereby account for the adverse events.[34]

A second series of inhibitors based on a novel pyrrolopyrimidine-2-carbonitrile scaffold has been published more recently by Novarits.[35−37] Compound **4**[38] was potent and selective (CAT K IC_{50} (nM), < 1.0; selectivity (fold) CAT S, > 10000; CAT L, > 1000). In rats, **4** showed high clearance and high volume of distribution (*F*, 98%; CL, 14.3 (L/h)/kg; Vd_{ss} 97.2 L/kg). It was noted that compounds in this series accumulated in bone marrow, the site of action (compound **4** had bone marrow/serum concentration ratio of 34.9 after 11 days of dosing 100 mg/kg po in rats). This bone marrow partitioning may lead to improved efficacy. Following administration of 2 mg/kg po q.d. for 14 days to cynomolgus monkeys, **4** significantly reduced CTX-I levels over the entire 24 h cycle and throughout the 14-day study.

Another compound to enter clinical trials was MV-701 (**5**, structure not disclosed), developed by Medivir AB. Compound **5** is a selective and potent inhibitor of cathepsin K (CAT K K_i (nM), 1.7; selectivity (fold), CAT L, S, B, V, and H > 882; CAT F, 397), and oral administration to male cynomolgus monkeys (16 mg/kg) resulted in a rapid reduction in CTX-I levels within 2 h to a maximum of 80% after 4−8 h. Similar inhibition of CTX-I was seen in multiple day dosing po q.d., and inhibition was reversible, returning to pretreatment levels within 48 h. In a phase I clinical study, CTX-1 was

also reduced by >50% at trough concentration between doses following 300 mg po q.d. for 14 days.[39] Although the structure of **5** has not been released, a compound recently published by this group is shown in Figure 2, MV061194 (**6**). This compound bears some similarity to **2** and features a reversible ketone electrophile and a piperazine S3 substituent. Compound **6** is potent and selective (CAT K K_i (nM), 2.5; selectivity (fold) CAT L and S, >40000; CAT B and H, >4000).[40]

Another example of a ketone electrophile was the clinical compound developed by GSK, SB-462795 (**7**). This compound placed a large 7-methylazepanone group in the S1 pocket and occupied the S′ pocket with a pyridin-2-ylsulfonamide group. The azepanone[41] was able to lock in the inhibitor's bioactive conformation, and with the addition of the C7 *S*-methyl group, the azepane chair conformation required for binding was favored, thereby improving potency.[42] In addition, the stereogenic center adjacent to the reactive ketone was more configurationally stable in the azepane than in smaller ring or acyclic[43] analogues, and the azepane was also observed to improve rat pharmacokinetic properties. X-ray cocrystallography showed that the benzofuran carboxamide formed an edge-to-face $\pi-\pi$ stacking interaction with Tyr67 in the S3 pocket. The pyridine-2-ylsulfonamide group occupied the S′ binding pocket with one of the sulfonamide oxygens forming a hydrogen bond with the indole N−H of Trp184.[42] Compound **7** was a very potent and reasonably selective cathepsin K inhibitor (CAT K $K_{i,app}$ (nM), 0.041; selectivity (fold) CAT L, 1.65; CAT V, 1.54; CAT S, 39; CAT B, 317) that showed cellular inhibition of osteoclast resorption ($IC_{50} \approx 70$ nM). Pharmacokinetics in rat (CL = 19.5 (mL/min)/kg, Vd_{ss} = 1.79 L/kg, F = 89%) and cynomolgus monkey (CL = 11.7 (mL/min)/kg, Vd_{ss} = 0.95 L/kg, F = 28%)[42] were acceptable. A single dose of **7** at 10 mg/kg or five daily doses at 3 mg/kg po in cynomolgus monkeys showed a significant decrease in urinary and serum NTX-I of up to 57%. A year of daily dosing in male cynomolgus monkeys at 10, 30, and 1000 mg/kg was well tolerated, although exposure in the 1000 mg/kg group was only 4 times that in the 30 mg/kg cohort. Compared to control, at 30 and 1000 mg/kg doses BMD was significantly increased at lumbar spine, femoral neck, and distal radius (predominantly trabecular bone), and in midfemur and proximal radius (predominantly cortical bone). In addition, bone strength was significantly increased (42% and 49% at 30 and 1000 mg/kg, respectively) in lumbar vertebrae.[44] Although clinical trials were reported[21] to have started in 2007, no human data have been published. GSK has also recently published a less potent series of semicarbazone-based cathepsin K inhibitors.[45]

In a series of publications, Merck in collaboration with Celera Genomics discussed their discovery and optimization of odanacatib (MK-0822, **8**),[46] which was reported to be in phase III clinical trials.[47] This compound was designed from a known dipeptide using structural data with the strategy of improving contacts with cathepsin K and replacing peptidic bonds with bioisosteres.[48] Early lead compounds containing P2 leucine[49] or 1-amino-1-cyclohexanecarboxylic acid[50] were potent (CAT K IC_{50} = 0.2 nM), but much of the potency was driven by an ionic interaction with Asp61 via an S3 pocket piperidine. Basic functionality was soon recognized to lead to lysosomotropic compounds.[31] The S3 piperazine was replaced with methylsulfone, resulting in an approximately 40-fold loss of potency.[51] However, replacing the amide link between P3 and P2 with a trifluoroethylamine resulted in a 10- to 20-fold increase in potency on cathepsin K and also improved metabolic stability.[52] The trifluoroethylamine was itself nonbasic and was able to maintain and enhance the crucial P2−Gly66 hydrogen bonding interaction. A fluorine atom was introduced into the P2 leucine group in order to block oxidative metabolism. P1−P2 amide hydrolysis was reduced by incorporation of a cyclopropyl group into P1 as 1-amino-1-cyanocyclopropane.

Compound **8** showed excellent biochemical potency and selectivity (CAT K IC_{50} (nM), 0.2; selectivity (fold), CAT B, 5170; CAT L, 14975; CAT S, 300; CAT F, 3975; CAT V, 3810; CAT C, H, Z >50 000) and was reported to show excellent pharmacokinetics in the dog (F up to 100%) and rat (F up to 38%) with long half-lives.[53] In humans weekly 50 mg doses po over 24 months significantly increased BMD at lumbar spine (5.5%) and total hip (3.2%) compared to almost no change for placebo. Treatment at the same dose decreased the bone resorption marker NTX-I in urine by 52%.[53]

Primary amides[54] and ketones,[55,56] which allowed exploration of the S′ pocket, as replacements of the nitrile electrophile of **8** have resulted in selective but somewhat less potent inhibitors. Continued studies[57] on bioisosteric replacement of the P2−P3 amide resulted in a second series that yielded another nonbasic lead compound, MK-1256 (**9**). The pyrazole core[58] served as an isosteric replacement in the region of the P2−P3 amide and was selected to avoid a steric clash with Asn158 in S2 and to provide additional polar functionality. Increased molecular polarity had a beneficial effect on cell shift in moving from biochemical to cell based measures of potency. The addition of the *N*-(2,2,2-trifluoroethyl) group blocked metabolic dealkylation, and the *gem*-dichloro group on the cyclohexyl ring blocked oxidative metabolism while improving potency. As was seen in the optimization of **8**, the S3 methylsulfone and S1 cyclopropyl groups improved metabolic stability. Compound **9** was a potent and selective cathepsin K inhibitor (humanized rabbit CAT K IC_{50} (nM), 0.62; selectivity (fold), CAT B, >16000; L, 4700; S, 4300; C, 6000; H, >140000; V, 1100; Z, 1700; F, 110), with excellent pharmacokinetics in rat (CL, 7.8 (mL/min)/kg; V_d, 2.8 L/kg; F, 105%) and rhesus monkey (F, 104%. CL, 5 (mL/min)/kg; $t_{1/2}$, 13 h; V_d, 3.4 L/kg).[59] When dosed at 1 mg/kg daily for 1 week in ovariectomized (OVX) rhesus monkeys, a mean 72% reduction in urinary NTX-I normalized to creatine was achieved.[59]

Amura Pharmaceuticals published a novel nonbasic cis-fused 5,5-bicyclic ketone cathepsin K inhibitor, **10**, which was made configurationally stable by virtue of its cis-fusion and was able to access both S and S′ binding sites (CAT K K_i^{ss} (nM), 10.1; selectivity (fold), CAT L, >347; CAT S, >446; CAT B, >990). Compound **10** inhibited human osteoclast bone resorption in vitro (CTX-I 75% I at 100 nM).[60]

A nitrile-based inhibitor was recently published by AstraZeneca (**11**).[61] This compound was optimized for reduced electrophilicity toward glutathione on the premise that less reactive electrophiles may be more selective for cathepsin K. The isobutyl group occupied S1, the morpholino group occupied S2, and although the 5-amino group was directed toward S3, this position was not further substituted in order to reduce nitrile electrophilicity. Thus, there were no contacts in the S3 pocket. Nevertheless, with minimal molecular recognition elements, **11** achieved good potency and selectivity (CAT K IC_{50} (nM), 18; selectivity (fold), CAT S, 31; CAT L, 111; CAT B >556), and its low molecular weight resulted in favorable rat pharmacokinetic properties.

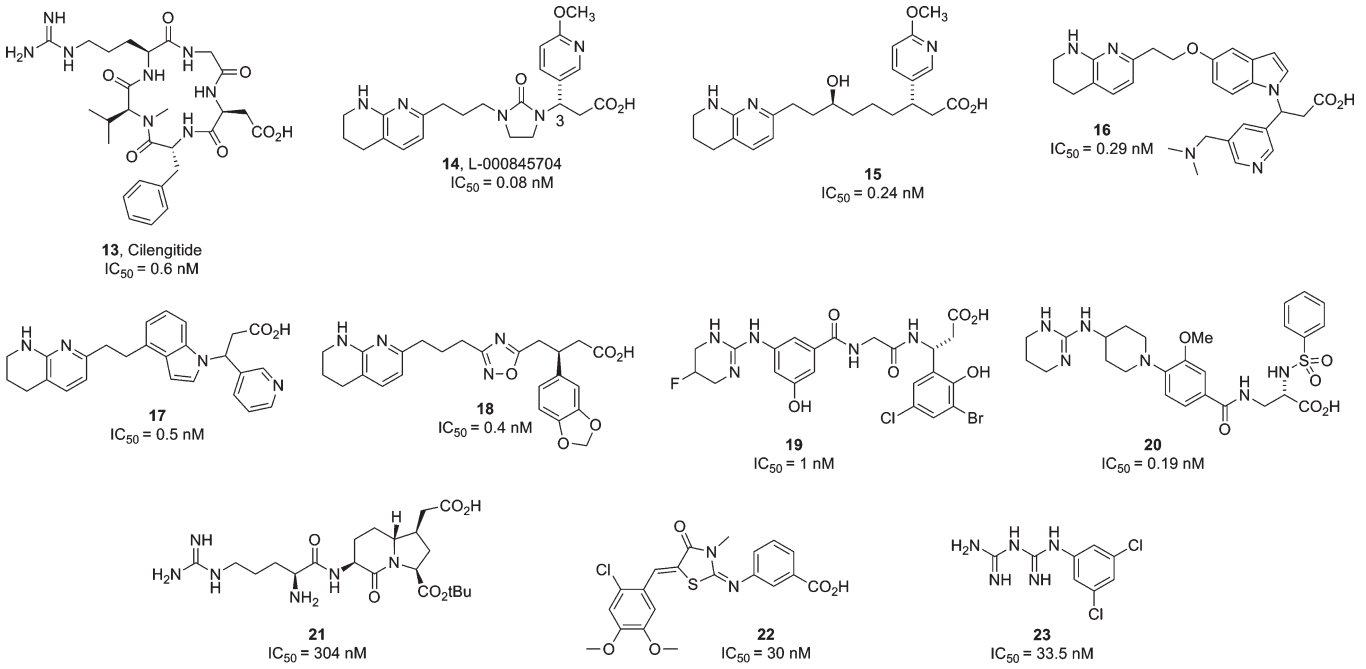

Figure 4. Examples of integrin $\alpha_v\beta_3$ inhibitors.

A series of noncovalent reversible inhibitors related to an earlier series from Novartis has been published recently by Sankyo (**12**).[62] The 3-biphenylamino, cyclohexyl, ethyl, and acetic acid groups of **12** occupied the S3, S2, S1, and S′ pockets, respectively. Perhaps because of the lack of a covalent interaction with the catalytic cysteine, good selectivity was achieved (selectivity (fold): CAT S, 2083; CAT B, L, >4167), and potency was maintained on both human and rat (CAT K IC_{50} (nM), 4.8 and 58, respectively). With biochemical potency on the rat protein and reasonable PK following 50 mg/kg oral dosing (C_{max} = 9.2 μg/mL; AUC = 103.9 μgh/mL), **12** showed a trend toward increased BMD in OVX rat femur following b.i.d. dosing for 42 days, although the improvement was not statistically significant. In addition to the clinical programs listed above, Ono Pharmaceuticals has an ongoing phase II clinical trial to investigate a cathepsin K inhibitor in osteoporosis,[63] but no structure, biological, or clinical data have been published. Additional clinical data describing the efficacy of cathepsin K inhibitors in the treatment of osteoporosis are expected in due course.

Integrin $\alpha_v\beta_3$

Integrin $\alpha_v\beta_3$ interacts with many RGD-containing proteins in different cell types but is relatively highly expressed in osteoclasts. Binding of integrin $\alpha_v\beta_3$ to bone matrix proteins such as osteopontin and bone sialoprotein in a noncovalent manner is a key step in the initiation of bone resorption.[64] Soluble integrin $\alpha_v\beta_3$ binders such as vitronectin, echistatin, and the synthetic cyclic pentapeptide cilengitide (**13**)[65] inhibit osteoclast activity in culture and prevent bone loss in OVX mice and rats.[66] Up-regulation of the integrin $\alpha_v\beta_3$ receptor has been associated with osteoporosis. There has been much interest in this target over the years, and the literature has been recently reviewed from a chemistry perspective.[67] A humanized mAb to integrin $\alpha_v\beta_3$, Vitaxin (MEDI-522), is in phase II clinical trials most recently in combination with bevacizumab for the antiangiogenic treatment of metastatic kidney cancer. Related to its inhibition of osteoclast activity in vitro,[68] Vitaxin was also reported for possible use in rheumatoid arthritis and related diseases.[69]

Small molecule drug-discovery efforts toward integrin $\alpha_v\beta_3$ inhibitors have focused on nonpeptidic RGD mimetics and were accelerated by structural data available from recent crystal structures of the native and cilengitide-bound $\alpha_v\beta_3$ receptor extracellular domain.[70,71] In the cilengitide cocrystal structure, binding interactions were mostly electrostatic, with minor hydrophobic interactions observed. Cilengitide was shown to span a pocket near the extracellular N-termini of the α_V and β_3 subunits. The guanidine group formed a bidentate salt bridge with α_V-Asp218 and α_V-Asp150, and the aspartic acid group bound a Ca^{2+} or Mn^{2+} metal ion closely associated with the β_3 subunit and formed hydrogen bonds to β_3-Arg214 and the backbone amide of β_3-Asn215 and β_3-Tyr122. The aspartic acid C-β and arginine C-ζ were separated by ~13.8 Å, an important element in the pharmacophore of $\alpha_v\beta_3$ peptidomimetics.

Ligand complementarity to the highly charged $\alpha_v\beta_3$ binding pocket has resulted in analogues with challenging issues of poor oral bioavailability and high plasma protein binding. A common strategy has been to discover lipophilic isosteres of RGD arginine and aspartate functionality. For example, as shown in Figure 4, research at Merck resulted in the advanced compound L-000845704 (**14**) that replaced the guanidine group with less basic, more lipophilic tetrahydronaphthyridine ($\alpha_v\beta_3$ IC_{50} = 0.08 nM).[72] Tetrahydronaphthyridine also improved selectivity for the integrin $\alpha_v\beta_3$ isoform ($\alpha_v\beta_5$, 125-fold selective). The peptide backbone could be replaced with alkylimidazolidinone,[73] increasing lipophilicity and increasing conformational constraint.[74] The polarity and basicity of the 3-substituent (Figure 4) were modified in order to reduce protein binding (human plasma protein binding, 88%) and improve oral bioavailability, clearance, and half-life. Pharmacokinetic properties of **14** were determined in rat (F, 26%; CL, 47 (mL/min)/kg; $t_{1/2}$, 3 h), dog (F, 64%; CL, 6.4 (mL/min)/kg; $t_{1/2}$, 3.5 h), and monkey, and increased BMD was observed at the distal femoral metaphysis relative

to cortex in OVX rats at 10 mg/kg (8.9% over vehicle) and 30 mg/kg (12.8% over vehicle) b.i.d. for 28 days.[74] Urinary NTX-I in OVX rhesus monkeys was reduced by 39% following 15 mg/kg q.d. for 14 days.[74] Dosing in postmenopausal osteoporotic women for 12 months at 200 mg b.i.d. was generally well tolerated and increased BMD at the lumbar spine (3.5%), hip (1.7%), and femoral neck (2.4%).[75]

An alternative approach to reducing plasma protein binding in the same series was to hydroxylate the aliphatic tether between the guanidine mimetic and carboxylic acid groups. On binding $\alpha_v\beta_3$, the alcohol group was predicted to lie in a solvent exposed region. Compound **15** showed good potency ($\alpha_v\beta_3$ IC_{50} = 0.24 nM), pharmacokinetic properties (dog PK: *F*, 60%; CL, 2.5 (mL/min)/kg; $t_{1/2}$, 6.1 h; human plasma protein binding, 93%), and good selectivity versus the closely related target $\alpha_{IIb}\beta_3$.[76]

A group at Johnson and Johnson took a similar approach in order to improve free fraction and thereby in vivo efficacy. Polar functionality was investigated in a series of alkyltetrahydronaphthyridine substituted indole-based $\alpha_v\beta_3/\alpha_v\beta_5$ dual inhibitors that were directed at oncology and osteoporosis applications.[77] Introduction of *N*,*N*-dimethylaminomethyl to the unsubstituted pyridine ring of an earlier lead resulted in **16** with good potency (IC_{50} $\alpha_v\beta_3$, 0.29 nM; $\alpha_v\beta_5$, 0.16 nM; $\alpha_v\beta_5$, 5.3 nM; $\alpha_{IIb}\beta_{IIIa}$, > 1 μM) and reduced human serum albumin (HSA) binding (lead compound HSA bound fraction, 96.5%; **16**, 40%). Reduced lipophilicity also improved microsomal stability. Interestingly, the indole isomer **17** was designed on the basis of docking studies using the published $\alpha_v\beta_3$ cilengitide cocrystal structure[71] and was found to have similar potency as **16** (ELISA IC_{50} $\alpha_v\beta_3$, 0.5 nM; $\alpha_v\beta_5$, 0.96 nM; $\alpha_5\beta_1$, 250 nM; $\alpha_{IIb}\beta_{IIIa}$, > 1 μM) and good CACO-2 permeability.[77]

A Pfizer approach took the strategy of replacing the amide backbone of peptidomimetic $\alpha_v\beta_3$ antagonists with the known amide isostere 1,2,4-oxadiazole.[78] Compound **18** contained a tetrahydronaphthyridine guanidine isostere to reduce the basicity of the molecule and was a potent inhibitor of $\alpha_v\beta_3$ (IC_{50} = 0.4 nM) and selective over $\alpha_{IIb}\beta_3$ (67-fold). Adhesion of $\alpha_v\beta_3$-expressing 293 cells was blocked at 0.65 nM **18**, but adhesion of $\alpha_v\beta_1$- and $\alpha_v\beta_5$-expressing HEK-293 cells was blocked at 2.6 and 1.7 nM, respectively, indicating modest selectivity. Adhesion of $\alpha_v\beta_6$-expressing HT29 cells was blocked at 2.7 μM. Good rat bioavailability was observed (*F*, 95%), but clearance was high and $t_{1/2}$ was reduced (CL, 10.9 (mL/min)/kg; $t_{1/2}$, 1.4 h). A second effort from Pfizer directed toward oncology applications identified **19** that inhibited adhesion of $\alpha_v\beta_3$-expressing HEK-293 cells at 1 nM and of $\alpha_v\beta_6$-expressing HT29 cells at 366 nM, indicating good selectivity. Mouse pharmacokinetic properties, however, were poor (*F*, 5.9%; $t_{1/2}$, 2 h).[79]

In a series of papers from Meiji Seika Kaisha, Ltd., studies of amide **20**, a dual $\alpha_v\beta_3/\alpha_{IIb}\beta_3$ inhibitor for the treatment of ischemic diseases, were described. The scaffold was reportedly designed on the basis of structure and literature data and optimized for dual inhibition.[80] Aqueous solubility and $\alpha_v\beta_3$ potency were improved by methoxy substitution on the central phenyl ring because of improved contacts in the binding site and torsion around the phenylpiperidine linkage: IC_{50} $\alpha_v\beta_3$, 0.19 nM; $\alpha_{IIb}\beta_3$, 0.44 nM; inhibition of aortic smooth muscle cells binding to vitronectin, 110 nM. Rat pharmacokinetic and solubility properties were amenable to intravenous infusion: rat $t_{1/2}$, 46 min; CL, 47 (mL/min)/kg; aqueous solubility, 1.3 mg/mL.[81] This compound was reported to be under license for preclinical development as MN-447.[82]

Other recent developments in $\alpha_v\beta_3$ integrin inhibitors include a hexahydroindolizin-5(1*H*)-one scaffold with moderate potency for $\alpha_v\beta_3$ and selectivity over $\alpha_v\beta_5$ (**21**, competitive binding IC_{50} 304 nM, $\alpha_v\beta_5$, 3.5-fold).[83] The discovery was reported of a potent 3-methylthiazolidin-4-one-based $\alpha_v\beta_3$ inhibitor through pharmacophore virtual screening followed by similarity searching among commercial compounds. The most potent $\alpha_v\beta_3$ binder was **22** (competitive binding IC_{50} of 0.03 nM). Compound **22** showed inhibition of cell growth at 20 μM in several cancer cell lines, but biochemical selectivity data were not provided.[84] A series of phenybiguanides was reported that was also discovered by virtual screening followed by SAR analysis of a screening hit. The most potent analogue **23** inhibited $\alpha_v\beta_3$-mediated cell adhesion (IC_{50} 33.5 nM), but selectivity data was not provided.[85]

Bone Anabolic Targets

Whereas antiresorptive agents inhibit osteoclast function, a complementary approach that targets enhancement of osteoblast function underlies the basis for development of bone anabolic agents. Following activation and resorption of bone by invading osteoclasts, the eroded surfaces become occupied by osteoblasts within the BMU that subsequently engage in the deposition of new matrix in a "coupling" process designed to complete the cycle of new bone formation.[1] Although the development of pharmacological agents that stimulate bone formation is less advanced compared to antiresorptive therapies, several pathways are known to facilitate osteoblast function including activity via bone morphogenic proteins (BMPs), transforming growth factor β (TGFβ), parathyroid hormone (PTH), insulin-like growth factor (IGF), fibroblast growth factor (FGF), and wingless-type MMTV integration site (WNT) signaling.[2,6] The transcription factor runt-related transcription factor 2 (RUNX2) acts a master regulator that orchestrates many of the downstream effects of these pathways and in its absence the skeleton will not mineralize. Other transcription factors such as osterix and activating transcripton factor 4 (ATF4) also contribute to the expression of osteoblast-specific genes.

Parathyroid Hormone Receptor (PTH1R)

Analogues of parathyroid hormone (PTH) peptide are the only class of bone anabolic agents currently approved for the treatment of osteoporosis. PTH has been clinically shown to increase bone formation rates, increase bone mineral density, and reduce the incidence of bone fractures.[86] In response to low levels of serum calcium, the parathyroid gland normally secretes PTH, which acts on the parathryoid hormone receptor (PTH1R) in bone to release calcium. Prolonged exposure to PTH can cause excessive bone resorption,[87,88] but when dosed intermittently, PTH has anabolic effects on bone in part by preventing osteoblast apoptosis.[89,90] Therefore, approaches have been taken to develop orally active compounds based on identification of small molecule PTH mimetics and compounds that act directly on the parathyroid gland to stimulate secretion of PTH.

Given the size of PTH (~9.5 KDa), it was originally thought unlikely that small molecule mimetics could elicit similar pharmacological responses compared to the natural peptide ligand. Nevertheless, scientists at GlaxoSmithKline were able to identify a small molecule mimic of PTH

(Figure 5) that contained the pyrimidine 2,4,6-trione core.[91] Compound AH3960 (**24**), identified through high-throughput screening, had EC_{50} = 1.5 μM for cAMP production and EC_{50} = 3.2 μM for intracellular mobilization in HEK293 cells overexpressing PTH1R. In the same assays, hPTH(1−34) had EC_{50} values of 0.3 and 1 nM, respectively. In human MSC, both compounds inhibited adipogenesis after intermittent dosing and they also increased ALP, providing evidence for osteoblastic differentiation. The mechanism through which **24** acts on the PTH1R was not reported, and no further work on this compound has been disclosed. However, two patents (published in 2005)[92,93] from GlaxoSmithKline cover small molecule PTH agonists and disclose structures related to **24** (see Figure 5, **25** and **26**).

Calcium Sensing Receptor (CaSR)

PTH secretion from the parathyroid gland is regulated by the calcium sensing receptor (CaSR). Binding of calcium to CaSR normally inhibits secretion of PTH. However, compounds that antagonize the effect of Ca^{2+} (calcilytics) disrupt CaSR signaling, which results in a release of PTH into the circulation. The first calcilytic reported was NPS 2143 (**27**, Figure 6), a compound containing a central amino alcohol core discovered at NPS Pharmaceuticals and GlaxoSmithKline.[94,95] Compound **27** had IC_{50} = 43 nM in HEK 293 cells expressing human CaSR. In cultured bovine parathyroid cells, **27** was shown to have the desired effect of increasing PTH secretion with EC_{50} = 41 nM. This effect was mimicked in rats, where **27** raised plasma PTH 4-fold during a 2 h infusion. Compound **27** was also tested in OVX rats.[96] After being dosed for 8 weeks, **27** raised PTH levels but did not produce a significant improvement in BMD in the proximal tibia. In contrast, OVX rats treated with PTH for 8 weeks showed an increase in BMD in the proximal tibia that matched the sham/vehicle treated rats. One explanation for the difference in BMD between **27** and PTH was thought to be related to the duration of PTH elevation. Compound **27** elevated PTH levels for at least 4 h, whereas PTH treated animals had elevated hormone levels for only 1 h. The authors suggested that the longer elevated PTH observed with **27** may result in activation of bone resorptive mechanisms. These data suggested that a shorter acting calcilytic may be beneficial for treating osteoporosis.

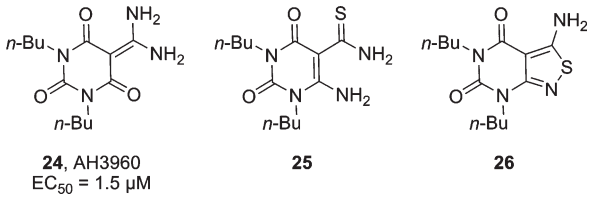

Figure 5. Parathyroid hormone mimetics.

In an effort to transiently raise PTH levels and thus mitigate the potential for bone resorptive effects, efforts have been made to identify calcilytics with a shorter half-life compared to **27**. Figure 6 summarizes some compounds where in vivo effects on PTH have been reported. NPS Pharmaceuticals published on a series of calcilytics that contained either a quinazolinone (**31**)[97] or pyrimidinone (**32**)[98] core, functionality that is not present on **27**. Representative examples from this new series of compounds were shown to elevate PTH levels in normal rats for only 5−10 min (**31**, 10 μmol/kg iv, PTH $C_{max} \approx 400$ pg/mL; **32**, 3 μmol/kg iv, PTH $C_{max} \approx 600$ pg/mL). In addition to this work, Bristol-Myers Squibb identified a series of calcilytics that contained an aminopyridine core (**33**).[99] One example from this series was shown to elevate PTH levels with a maximal effect at 2 min (7 μmol/kg iv, 1100 pg/mL), returning to baseline after 10 min in normal rats. While these groups have identified compounds that raise PTH transiently, efficacy in models of osteoporosis has yet to be reported.

The strongest evidence for a calcilytic achieving a PTH profile beneficial for bone formation comes from data reported on ronacaleret (**28**), a drug co-developed by GlaxoSmithKline and NPS Pharmaceuticals, which had entered the clinic for osteoporosis. Ronacaleret was shown to increase PTH when given orally at 30, 60, or 120 mg/kg to OVX rats (PTH C_{max} of 33, 66, and 68 pM, respectively).[100] Bone formation rates and increases in osteoid perimeter at the lumbar spine were observed in the 120 mg/kg treated animals, but the PTH profile associated with these results has not yet been published. In addition results from a phase I study in healthy postmenopausal women were reported showing that after 28 days of dosing at 475 mg, **28** significantly increased biomarkers for bone formation (OC, 63%; PINP, 79%; ALP, 35% relative to placebo).[101] According to GlaxoSmithKline's Web site, **28** is currently in phase II clinical studies.[102] Japan Tobacco and Novartis[103] have also reported having calcilytics

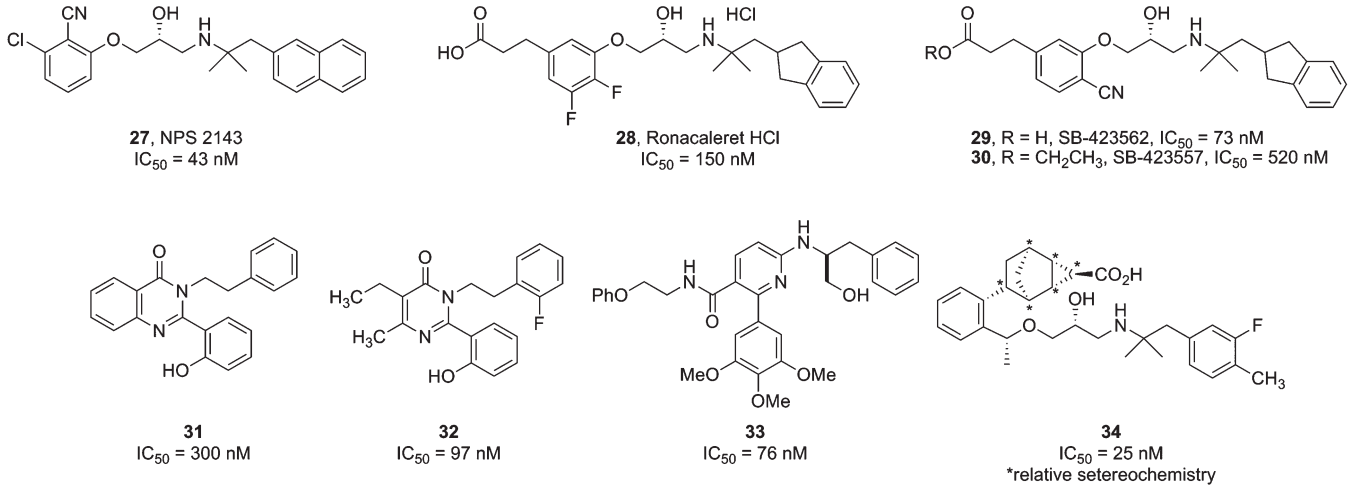

Figure 6. Calcium sensing receptor antagonists.

in the clinic (JTT-305 and ATF-936, respectively); however, the structures of these compounds have not yet been disclosed.

Analogues of **28** have also recently been described by GSK and NPS. The carboxylic acid SB-423562 (**29**) was a potent CaSR antagonist (IC_{50} = 73 nM) but had high clearance (rat CL = 89 (mL/min)/kg), was poorly absorbed, and suffered high first pass hepatic extraction.[104] The corresponding ethyl ester SB-423557 (**30**) was well absorbed and readily cleaved to the acid in vivo. Thus, **30**, when dosed in vivo, resulted in dose-dependent and transient systemic levels of the acid **29**, while the ester was not detected. Accordingly, PTH stimulation with **30** was dose dependent and short-lived, returning to baseline levels within 2 h following 5, 15, or 50 mg/kg po doses in rats.[104] In OVX rats, 50 mg/kg dosed daily po increased BMD and strength of the lumbar spine to near sham surgery control levels but not as well as 1 μg/kg sc PTH(1−34). Pharmacokinetic properties were similar in humans, with short half-life (1.4−3.7 h). Furthermore, the t_{max} (1−2 h) and duration of PTH elevation (0.5−6.5 h) after oral administration of **30** in humans closely matched the profile observed after intermittent sc dosing of PTH(1−34).[104] This suggested that oral adminstration of **30** might improve BMD in humans. The risk of hypercalcemia associated with calcilytics does not appear to be a concern with **30**, since after a dose of 400 mg in healthy volunteers, the increase in serum ionized calcium was considered to be within the normal range.[104]

In a publication by Pfizer scientists, use of GSK's **28** was shown to have modest activity against the human CaSR (IC_{50} = 150 nM), with an oral bioavailability of 12% in rats.[105] In an effort to improve on **28**, this group prepared analogues that contained more hydrophobic substituents adjacent to the carboxylic acid moiety and replaced the phenoxy ring with a benzyloxy group.[105] Their work led to compound **34**, which had CaSR IC_{50} = 25 nM and rat oral bioavailability of 60%. The PTH response with compound **34** in Sprague−Dawley rats was considered robust, but a time course profile was not reported.

Wingless-Type MMTV Integration Site (WNT) Pathway

The wingless-type MMTV integration site (WNT) family of ligands is required in a variety of developmental and regenerative processes.[106] In bone, activation of the canonical WNT signaling pathway is associated with differentiation, proliferation, and survival of osteoblasts.[107−109] The role of the WNT co-receptor lipoprotein receptor-related protein 5/6 (LRP5/6) in the regulation of bone mass has received particular attention over the past few years. In humans, loss or gain of function mutations in the LRP5 gene is associated with low and high bone mass phenotypes, respectively. Binding of WNTs to LRP5/6 and frizzled receptor (FZD) results in LRP5/6 phosphorylation and the formation of a complex containing disheveled (DSH), glycogen synthase kinase-3β (GSK-3β), axin, and adenomatous polyposis coli (APC).[110] As part of this complex, GSK-3β is unable to phosphorylate β-catenin which can subsequently translocate to the nucleus, bind to T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) transcription factors, and activate transcription of bone related genes. Importantly, however, genetic studies of β-catenin mice with gain and loss of function mutations in osteoblasts suggest that, unlike LRP5 mutations that can affect bone formation parameters, β-catenin impacts bone mass via effects on resorption mediated by changes in OPG expression.[111] These contrasting genetic findings suggest that LRP5 and β-catenin may not be linked directly via WNT signaling to the control of bone mass. Nevertheless, canonical WNT signaling is regulated by several types of WNT inhibitors such as sclerostin (SOST), dickkopf-1 (DKK1), secreted frizzled related protein-1 (sFRP1), and WNT inhibitory factor-1 (WIF-1), which are known to have profound inhibitory effects on bone mass.[108] As such, this pathway has opened up new opportunities for therapeutics to treat bone diseases.

Targeted approaches for activating the canonical WNT signaling pathway include (1) blocking the antagonists that bind to the LRP5/6−FZD complex, (2) inhibiting the proteins that sequester WNTs and neutralize WNT activity, and (3) increasing intracellular β-catenin levels by inhibiting the kinase GSK-3β (see below). Monoclonal antibodies against sclerostin, developed by Amgen and UCB, had marked bone anabolic effects in preclinical rodent models of postmenopausal osteoporosis[112] and inflammation-induced bone loss.[113] In a follow-on human study, administration of a sclerostin-neutralizing monoclonal antibody in healthy postmenopausal women resulted in dose-dependent increases in biochemical markers of bone formation.[114] Amgen also reported on a monoclonal antibody to DKK1 which showed efficacy in rodents and also in a mouse model of rheumatoid arthritis.[115,116] A human anti-DKK1 neutralizing antibody (BHQ880) was recently shown to stimulate bone formation in mouse myeloma models,[117,118] and Novartis has initiated a clinical trial for osteolytic bone loss associated with multiple myeloma.

Recently, Wyeth reported a small molecule with a 2-aminopyrimidine structure that showed evidence of WNT signaling agonist activity in osteosarcoma cells. WAY-262611 (**35**) was reported to have micromolar cellular activity and furthermore showed increased trabecular bone formation rate in the tibia in OVX rats (10 mg/kg po q.d., 28 days, 1.5 ($\mu m^2/\mu m$)/day).[119] Importantly, **35** did not appear to inhibit GSK-3β, suggesting that the compound may act by enhancing WNT/LRP5/FZD interactions at the cell membrane. More recently naphthylpyrimidine **36** was developed from a high-throughput screening campaign. Compound **36** had EC_{50} = 6.8 μM (4.9-fold induction at 20 μM) in a Wnt3a and DKK1-dependent TCF-luciferase cellular assay. Furthermore, C57BL/6 mice given a local subcutaneous injection (0.1 mg/kg **36**, q.d., 7 days) over the right side of the calvaria showed a dose-dependent increase in mineral apposition rate (~150%) used as a measure of in vivo bone anabolic activity.[120] Since compounds showed minimal effects on GSK-3β, it was proposed that agonistic activity was mediated via WNT3a/DKK1 interaction.

In a different approach, Wyeth identified two series of sulfonamides that disrupted the binding of WNT to sFRP-1.[121,122] By use of human U2OS osteosarcoma cells overexpressing sFRP-1 and WNT-3a, compound **37** containing a bis-phenylsulfone core was obtained from a high throughput screening campaign (Figure 7).[121] Compound **37** showed EC_{50} = 3.9 μM in a TCF-luciferase assay, a measure of WNT signaling pathway activation, and a 2.5-fold induction of TCF-luciferase gene expression at 15 μM. Through SAR studies, compound **38** was obtained which had a higher EC_{50} of 7 μM and showed a 3-fold increase in TCF-luciferase gene expression at 15 μM. At 5 μM, **38** also increased the total bone area (~150%) and the number of osteoblasts (~130%) in an ex vivo mouse calvarial study used as a secondary assay to assess effects on bone formation.

In a second related sulfonamide series, Wyeth compound WAY-316606 (**39**) demonstrated an EC_{50} = 0.65 μM in the TCF-luciferase assay,[122] and at 10 μM, **39** caused an 8.7-fold

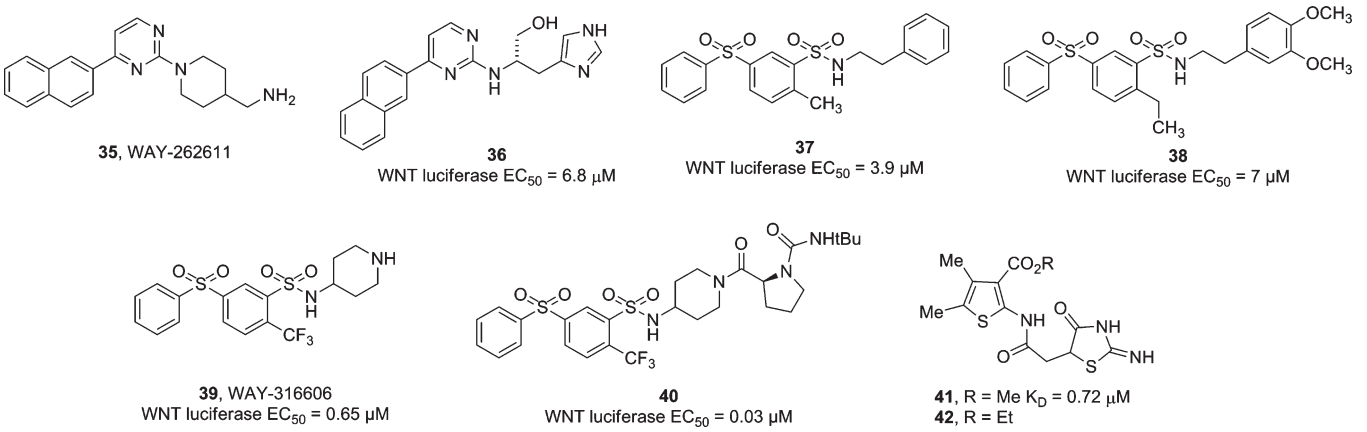

Figure 7. WNT pathway inhibitors.

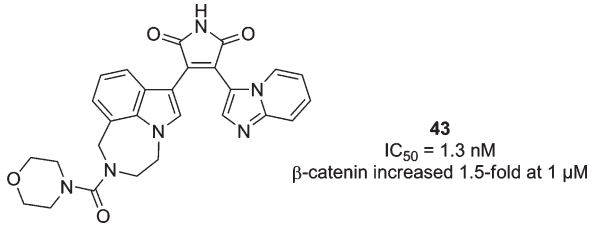

Figure 8. Eli Lilly GSK inhibitor **43**.

increase in gene expression.[123] Compound **39** also increased total bone area 60% in the ex vivo mouse calvarial assay at 10 nM.[123] Through extensive SAR studies, compound **40** was identified which had an EC_{50} = 30 nM and was able to cause a 4-fold increase in WNT-Luc gene expression at this concentration.[122] In the ex vivo mouse calvarial assay, **40** increased total bone area 75% at 1 nM. While compounds like **39** and **40** increased bone formation in an ex vivo model, the compounds lacked sufficient exposure in vivo and data were not reported from preclinical models of osteoporosis. Nonetheless, this set of sFRP-1 inhibitors illustrates how relatively low-molecular-weight compounds can be identified with the potential for disrupting protein/protein interactions in the WNT-signaling pathway.

Other series disclosed by Wyeth include a series of iminooxothiazolidines that also inhibited sFRP-1. Methyl ester **41** was discovered in a cell-based screen of Wnt-pathway activation and was shown to selectively bind sFRP-1 with K_D = 0.72 μM based on changes in protein tryptophan fluorescence. The corresponding ethyl ester (**42**) showed a 2.3-fold increase in TCF-luciferase gene expression at 15 μM in transfected U2OS cells and increased total bone area (~120%) and the number of osteoblasts (~140%) in an ex vivo calvarial bone formation assay at the same concentration.[124]

Glycogen Synthase Kinase 3 (GSK3)

GSK3 has been implicated in a number of diseases including neurological disorders,[125] cancer,[126] and diabetes.[127] The GSK-3α and related GSK-3β genes are expressed ubiquitously in tissues but are not functionally redundant. The β isoform of GSK3 and more recently the α isoform[128] have been shown to interrupt canonical WNT signaling by lowering the intracellular levels of β-catenin via phosphorylation, which leads to removal of β-catenin by proteasomal degradation. Since canonical WNT signaling appears to be important for regulation of bone mass,[107−110] inhibitors of GSK3 in principle have the potential to act as bone anabolic agents.

While many groups have published on potent GSK3 inhibitors, there are relatively few examples where GSK3 inhibitors have been used in preclinical models of osteoporosis. Lithium chloride is known to weakly inhibit GSK-3β (K_i = 2 mM).[129] Nevertheless, when normal C57BL/6, osteopenic SAMP6, and *Lrp5*$^{-/-}$ mice were dosed with lithium chloride (200 mg/kg po q.d., for 1 month), bone volume and bone formation rates were increased in all strains. The anabolic responses to lithium were greatest in the *Lrp5*$^{-/-}$ and SAMP6 mice where bone formation rates increased ~130% compared to ~30% in C57BL/6 mice.[130] These interesting results therefore suggested that the bone anabolic effects of lithium were occurring via canonical WNT signaling but downstream of the LRP5 WNT co-receptor. It was proposed that prospective studies of patients receiving lithium therapy might demonstrate a link with increased bone mass.

A group from Eli Lilly indentified a dual GSK-3α and GSK-3β inhibitor, compound **43** (Figure 8), through SAR work on a series of bis-arylmaleimides, a compound class present in other GSK3 inhibitors.[131] Compound **43** had an IC_{50} = 1.3 nM on GSK-3β and was 500-fold selective over a panel of 11 other kinases.[132] In mouse mesenchymal C3H10T1/2 cells, **43** was shown to dose-dependently increase the levels of β-catenin (1.5-fold at 1 μM). In the same cell line, WNT signaling measured by TCF-luciferase gene expression increased by 7-fold at 0.1 μM but decreased to about 2-fold at 10 μM. Increased expression of genes associated with osteoblast differentiation and activity such as ALP and OC was also observed in C3H10T1/2 cells treated with **43**. In a 2-month study with OVX rats, compound **43** dosed 3 mg/kg q.d., po, was also shown to restore both mineral apposition rate in tibia and mechanical properties of L5 vertebrae to levels similar to those of sham control.[131]

While the preclinical observations with GSK3 inhibitors on bone formation are promising, there is concern that inhibition of GSK3 may be tumor promoting.[126] GSK3 suppresses not only WNT signaling but also hedgehog and notch pathways which play fundamental roles in cell growth, differentiation, and proliferation. Inhibition of GSK3 may activate these pathways, causing an increase in cell proliferation.[133] However, GSK3 inhibitors have been shown to inhibit the growth and proliferation of pancreatic[134] and colorectal tumors.[135]

Whole Cell Screening

In addition to targeting specific mechanisms of bone anabolism, research groups have also screened compounds in

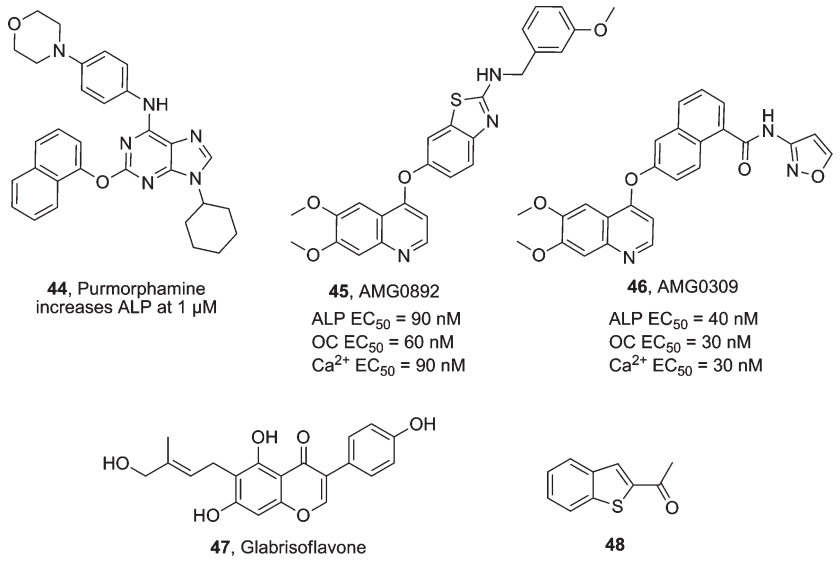

Figure 9. Osteogenic compounds identified through whole cell screening.

whole cell assays that provide functional assessments of osteogenesis. In a proof of concept study from the Schultz group,[136] ~100 000 compounds were screened in mouse C3H10T1/2 cells using ALP as a marker of osteogenesis. This work led to the identification of a purine analogue, purmorphamine (**44**, Figure 9), that increased ALP activity at concentrations as low as 1 μM. Osteoblast differentiation was confirmed in **44** treated cells by increased expression of the bone-specific transcription factor RUNX2. Compound **44** was also shown to increase ALP activity synergistically with bone morphorgenetic protein-4 (BMP-4), a growth factor that induces C3H10T1/2 differentiation. Additional work[137] demonstrated that **44** activated the hedgehog signaling pathway and that smoothened was the specific protein target in the hedgehog pathway.[138]

At Amgen,[139] ~45 000 compounds were screened in a mouse MC3T3-E1 osteoblast cell line, using ALP and OC as markers of osteogenesis and calcium as a marker of mineralization. Two of the most potent compounds identified from this screen, AMG0892 (**45**) and AMG0309 (**46**), increased ALP (15- and 25-fold, respectively), OC (6- and 5-fold, respectively), and calcium (2.5-fold) with EC_{50} values between 30 and 100 nM. These two compounds also increased the expression of RUNX2, confirming osteoblast differentiation. The underlying mechanism responsible for the osteogenic activity of **45** and **46** was also examined. Both compounds inhibited MC3T3-E1 cell growth and had no effect on apoptosis, suggesting a link between growth inhibition and increased osteogenesis. In cell-based studies using luciferase reporter gene assays driven by BMP, WNT, and CREB response elements, **45** and **46** were shown to activate the CREB pathway but not the BMP or WNT signaling pathways.[139] These results suggested that the osteogenic effects seen with **45** and **46** were due to CREB pathway activation, a pathway that has been implicated in osteoblast biology.[140,141]

Compounds affecting osteogenesis were also identified in a high-throughput screen in MCT3-E1 cells using a green fluorescence protein reporter gene assay, driven by the type I collagen promoter (Col1a1GFP).[142] After the screening of ~2500 compounds, a natural product obtained from the licorice plant *Glycyrrhiza glabra* was identified, named glabrisoflavone (**47**), which increased expression of GFP and transcription of OC (~58% over control) and ALP (~58% over control) mRNA at 30 μM. The osteogenic activity of glabrisoflavone was also confirmed in mouse primary osteoblasts. Mechanistic studies were conducted in luciferase assays containing BMP, WNT, or RUNX2 response elements. Glabrisoflavone had no significant effect in these settings, which suggested that the osteogenic effects of glabrisoflavone operated independently of the BMP, WNT, or RUNX2 pathway.

Finally, a high-throughput screen in MCT3-E1 osteoblast cells was performed for compounds that increased activity of the BMP-2 promoter.[143] Benzthiophene **48** was shown to induce BMP-2 expression by 35.6% at 4 μM. HMG-CoA reductase inhibitors are also known to increase BMP-2 expression,[144] and in this report 0.4 μM lovastatin was shown to increase BMP-2 expression 20.9%. When compound **48** was dosed orally at 15 and 30 mg/kg for 90 days in OVX rats, it significantly increased trabecular bone volume (19.4% and 21.0%, respectively) compared to vehicle treatment and it returned bone resorption surface to levels close to those seen in sham operated animals.

Dual Antiresorptive and Anabolic Targets

Some targets appear to be expressed in both osteoblasts and osteoclasts and may hold promise for a synergistic effect in treating osteoporosis. Prostaglandin and cannabinoid receptors and SRC and PYK2 kinase pathways in osteoclasts and osteoblasts have been targeted for potential dual antiresorptive and anabolic treatments of osteoporosis.

Prostaglandin E2 Receptors EP2 and EP4

Prostaglandin E2 (PGE2, **49**) signals through at least four known receptors (EP1−4). Local administration of **49** in animals stimulates new bone formation and increases bone mass and strength, although therapeutic use is limited by side effects that include diarrhea, lethargy, and hypotension. These side effects may be due to **49** activity[145] at EP1 and EP3 receptors;[146] thus, isoform selective agonists of EP2 and/or EP4 receptors could be valuable as anabolic treatments of osteoporosis.[147−149] The relative importance of EP2 and EP4 receptors in regulating bone mass was recently reviewed,[150]

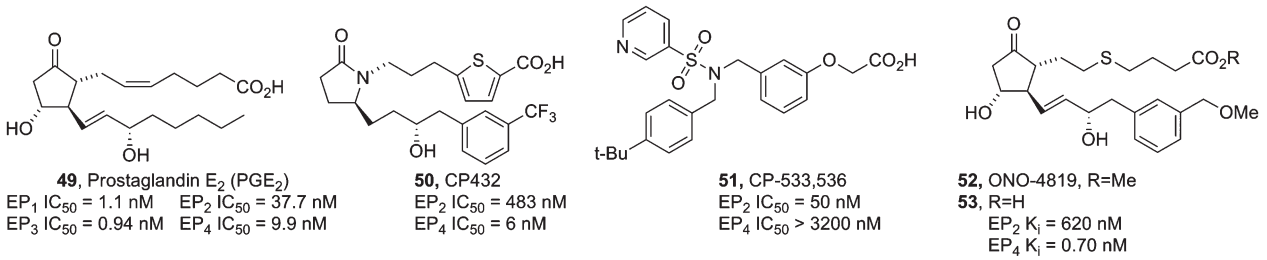

Figure 10. Prostaglandin E2 receptor EP2 and EP4 agonists.

suggesting that the evidence for a role of both receptors in bone formation was more consistent than a direct role for either on osteoclastogenesis. Both EP2 and EP4 receptors appeared to be important for bone formation in animal models, but a role for the human EP2 receptor remains to be confirmed.[150]

Pfizer previously initiated programs on both EP2 and EP4 receptor agonists for bone disease. An EP4 selective agonist based on a scaffold related to the natural ligand **49** was developed from a screening effort. Introduction of a thiophene ring into the N-linked alkanoic acid side chain blocked a β-oxidation clearance pathway, improving metabolic stability. The aryl ring in the C-linked side chain was optimized for selectivity and potency, resulting in CP432 (**50**, rat IC_{50} values EP4, 6 nM; EP2, 483 nM; EC_{50} cAMP activation in HEK-293 expressing rat EP4, 0.5 nM, 102% of **49** response, Figure 10).[151] Rat oral pharmacokinetics were poor (CL, 70 (mL/min)/kg; *F*, 1%; $t_{1/2}$, 0.2 h), but 1 mg/kg dosed daily sc to 12-month-old ovariectomized rats for 6 weeks increased total bone mineral content (20.6%) and total BMD (22.9%) over vehicle and was well tolerated. Increased mineralizing surface and mineral apposition rate indicated increased recruitment and activity of osteoblasts.[152]

In a parallel program, Pfizer designed CP-533,536 (**51**) based on an earlier published sulfonamide that was known to be EP2 selective. Phenoxyacetic acid was introduced as an isostere of **49** heptenoate and provided an attractive balance of EP2 potency and EP4 selectivity. Arylsulfonamide and 3-pyridyl in particular provided good functional potency while maintaining selectivity. The *tert*-butylphenyl group, being highly metabolized, was added to prevent systemic exposure of the compound following local administration by injection into the bone marrow cavity. These studies resulted in **51** (rat IC_{50} values EP2, 50 nM; EP4, >3200; EC_{50} cAMP activation in HEK-293 expressing rat EP2, 0.3 nM, full agonist compared to **49**)[153] that was selective among seven other prostanoid receptors[146] and did indeed show a short plasma half-life (1 mg/kg iv rat CL, 56 (mL/min)/kg; $t_{1/2}$, 0.33 h). In a model of bone growth,[146] **51** (0.3 mg/kg) was injected directly into the rat periosteum in an extended release formulation. After 2 weeks volumetric bone mineral content (BMC) and bone area were found to be increased 18% and 25%, respectively, over vehicle. In a model of fracture healing, the rat femur was fractured and **51** (0.5 mg) injected directly into the fracture site. After 3 weeks, total callus area was found to be increased 19% over vehicle and mean force to failure by torsional testing was increased by 34%.[146] Recently, this compound entered a phase II clinical trial for tibial shaft fracture healing.[154]

Ono Pharmaceuticals has also developed an EP4 agonist, ONO-4819 (**52**). Introduction of sulfur in the α-chain was found to improve potency, and its position in the chain was important for selectivity and chemical stability.[155] Installation of phenyl in the ω-chain improved selectivity but reduced EP4 potency. Potency was recovered by meta-substitution with methoxymethyl. Compound **52** was brought forward as the methyl ester of **53** (data for **52**: mouse competitive binding K_i values, EP4, 0.70 nM; EP2, 620 nM; EP3, 56 nM; five other receptors >10 μM; c-AMP production in CHO cells expressing mouse EP4 EC_{50}, 1.6 nM)[156] because of increased chemical stability of the ester. Compound **52** has been shown to accelerate BMP-induced osteoblast differentiation[157] and to augment the BMP-dependent bone mineralization in mice. Polymer discs containing BMP (5 μg) and **52** (300 μg) implanted in the dorsal muscle pouch in mice were mineralized to a greater extent (BMC ~3-fold higher) than discs containing BMP alone after 3 weeks.[158] Furthermore, in OVX rats, **52** at 3 μg/kg sc b.i.d. for 11 weeks restored trabecular BMD from 220 mg/cm^3 (OVX vehicle) to 378 mg/cm^3.[159]

Cannabinoid Type 1 and Type 2 Receptors (CB1 and CB2)

Differentiated osteoblasts and osteoclasts express cannabinoid type 2 receptors (CB2) on their surfaces[160] and produce the endocannabinoids 2-arachidonoylglycerol and anandamide.[161] Mouse gene knockout data and in vitro cellular studies using selective agonists and antagonists indicated that CB2 signaling was important in osteoblastic colony formation, ALP activity, and matrix mineralization. CB2 signaling may also suppress osteoclast differentiation.[160] In vivo, *Cb2* knockout animals showed high bone turnover with overall bone loss at 1 year and increased bone loss compared to wild-type following ovariectomy.[162] In humans polymorphisms in the *CNR2* gene that encodes CB2 have been associated with osteoporosis. On the other hand, CB1 is highly expressed in bone sympathetic nerve terminals and may function by suppressing norepinephrine release in the vicinity of osteoblasts, possibly leading to increased bone formation.[163] However, *Cb1* knockout studies in mice[164] have demonstrated low bone mass, normal bone mass, or high bone mass phenotypes depending on gender and mouse background strain.[161]

Selective and nonselective CB1 and CB2 small molecule agonists and antagonists have been made available through their development for the treatment of central and peripheral neurological disorders and have been important tools in the elucidation of cannabinoid receptor pharmacology in bone metabolism (Figure 11). Bone anabolic effects were associated with CB1 and CB2 dual agonists, and antiresoptive effects were demonstrated for CB2-selective antagonists. CP55,940 (**54**),[165] a nonselective CB agonist, stimulated bone nodule formation in MSC cultures at 100 nM. In contrast, bone nodule formation was inhibited by AM251 (**55**),[166] a selective CB1 antagonist, at 100 nM.[164] AM630 (**56**),[167] a selective CB2 antagonist, inhibited osteoclast differentiation (IC_{50} = 0.33 μM)

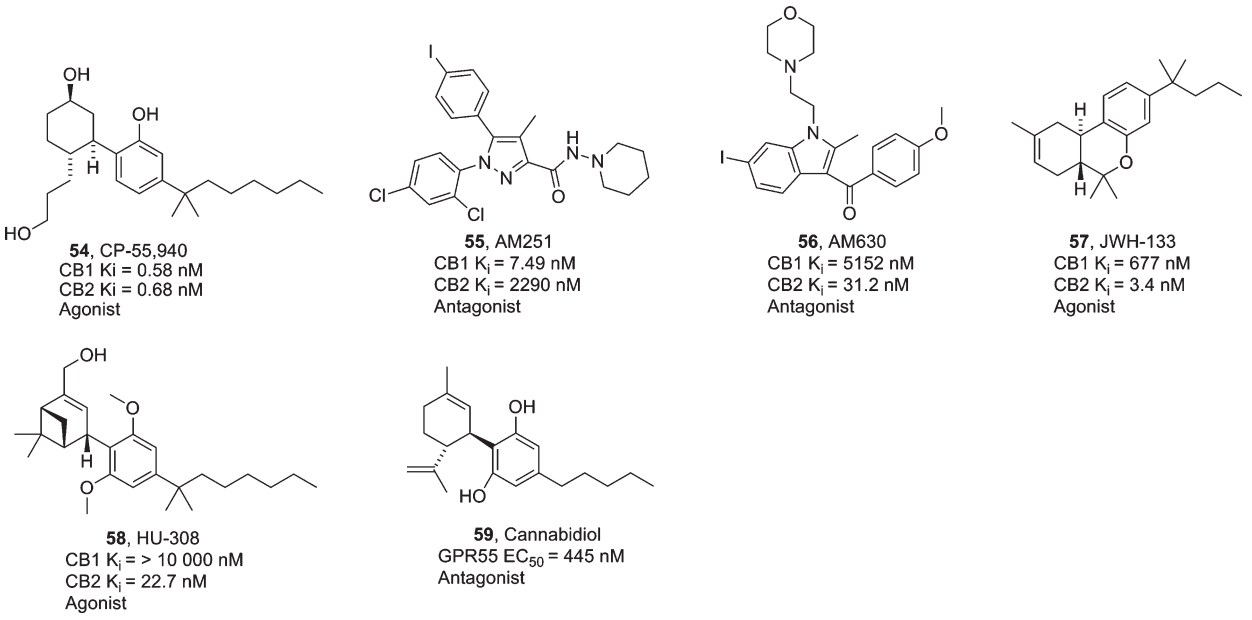

Figure 11. Cannabinoid CB1 and CB2 binders and GPR55 antagonist **59**.

and activity (IC_{50} = 7.2 μM) in vitro. Furthermore, **56** at 1 mg/kg restored bone volume in OVX wild-type mice compared to sham/vehicle treated animals.[162,168] CB2 selective agonists JWH-133 (**57**)[169] and HU308 (**58**)[170] promoted osteoclast differentiation at concentrations from 10 to 300 nM.

Recent reports of other small molecule cannabinoid receptor ligands have appeared but have not included data associated with bone metabolism. Benzimidazole derivatives as CB2 agonists have been recently disclosed by Astra-Zeneca.[171] Diverse CB2 partial agonists, antagonists, and inverse agonists of moderate affinity were discovered by virtual screening.[172] A series of 1-alkyl-2-aryl-4-(1-naphthoyl)pyrroles as CB1 and CB2 dual binders have also been described.[173]

A new report suggested a potential role for the CB receptor-related molecule GPR55 in regulating osteoclast activity and bone mass.[174] GPR55 was found to be expressed in both osteoclasts and osteoblasts, and in Gpr55 knockout mice bone volume was increased in the tibia and femur (136% and 149%, respectively) of 12-week-old male (but not female) mice. The increased bone mass was associated with impaired osteoclast function, despite increased osteoclast numbers and no change in bone formation parameters. Cannabidiol (**59**) has a low affinity for CB1 and CB2 receptors (K_i from 2.4 to 10 μM)[175] but is an antagonist of GPR55 (EC_{50} = 445 nM). When tested on human osteoclasts cultured on dentin discs for 5 days, **59** inhibited osteoclast activity induced by the GPR55 agonist O-1602. Moreover, treatment of male mice for 8 weeks with 10 mg/kg cannabidiol (3 times per week) decreased bone resorption (serum CTX-I, −18%) and was associated with a nonsignificant increase (10%) in bone volume.

Sarcoma Proto-Oncogene (SRC) Tyrosine Kinase

Sarcoma proto-oncogene (SRC) kinase is a well recognized target in oncology with several inhibitors or dual SRC/Abelson murine leukemia viral oncogene (ABL) inhibitors in advanced clinical trials or marketed (KX2-391, phase I; AZD-0530 (**60**), phase II; dasatinib, launched). Previously $Src^{-/-}$ mice[176] were demonstrated to present an unusual osteoclast morphology with an inability to resorb bone[177]

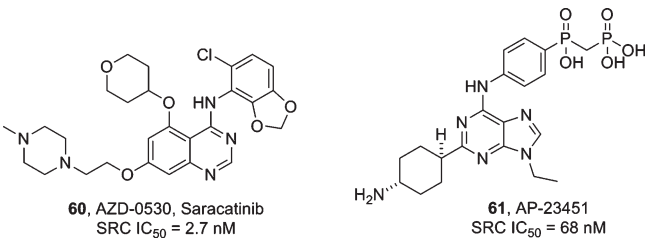

Figure 12. SRC tyrosine kinase inhbitors.

and an osteopetrotic phenotype. SRC also negatively regulates osteoblast differentiation, and inhibitors may have a complementary effect in both cell types.[178]

Although under development for oncology applications, the effect of **60** (Figure 12) on osteoclasts in vitro and in vivo has been studied. Compound **60** binds the SRC kinase ATP binding site where the tetrahydropyran-4-yloxy group occupies the ribose pocket improving selectivity, and the chlorobenzodioxolyl group makes important hydrophobic contacts in the SRC selectivity pocket (IC_{50} values: SRC, 2.7 nM; LCK, < 4 nM; YES, 4 nM; ABLl, 30 nM; KIT, 200 nM; CSK, 840 nM; 18 other kinases reported were > 2 μM).[179] Compound **60** inhibited formation and activity of human osteoclasts in vitro. Bone resorption on bovine cortical bone in PBMC/osteoclast culture was completely blocked by 1 μM **60** over 3 weeks.[180] In men, markers of bone resorption reported from a multiple ascending dose study of **60** were reduced following 10−14 days dosing at 250 mg (serum CTX-I, −88%; urinary NTX-I, −67%).[181]

One series of SRC inhibitors has been specifically developed for applications in osteoporosis. AP23451 (**61**) was developed from a known series of purine-based cyclin-dependent kinase (CDK) inhibitors.[182] The diphosphonic acid group was included in order to target the inhibitor to active sites of bone remodeling, was predicted by modeling to be projected into solvent on binding to SRC, and was shown not to interfere with binding. The carbon linked *trans*-4-aminocyclohexyl group particularly improved SRC kinase potency.[183] Compound **61** was equipotent on SRC and ABL (IC_{50}, 68 nM) but

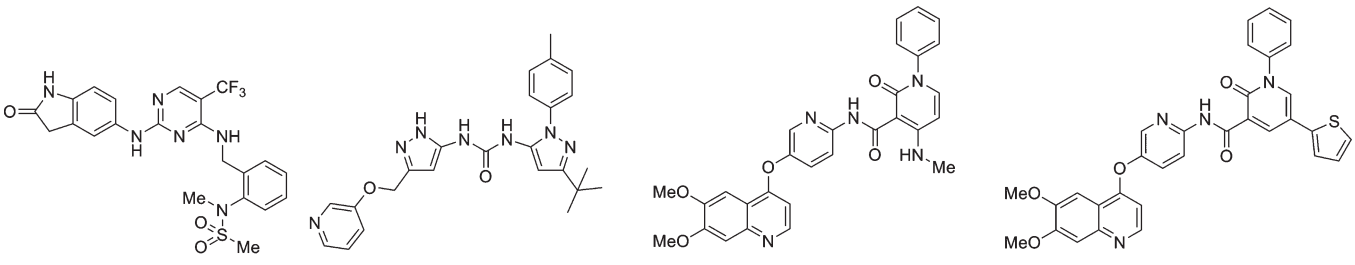

Figure 13. PYK2 inhibitors.

at least 30-fold selective over CDK2 and a panel of 35 other kinases. In a mouse model of osteoclast activation and bone resorption, **61** (3 or 10 mg/kg sc b.i.d. for 5 days) reduced serum calcium levels in mice treated with PTH (20 μg/kg q.i.d. for 3 days) to levels at or below those of untreated animals. [^{14}C]**61** was effectively localized to bone and retained there for several weeks.[184] However, it is difficult to differentiate effects due to specific SRC inhibition from those due to bisphosphonate-type osteoclast inhibition.

Proline-Rich Tyrosine Kinase 2 (PYK2)

Proline-rich tyrosine kinase 2 (PYK2) is another enzyme implicated in the regulation of both osteoblast and osteoclast activity.[185] Several studies point toward a role for PYK2 in the osteoclast sealing zone and its ability to alter bone resorption. In fact osteopetrosis and impaired osteoclast function are phenotypic features of *Pyk2*$^{-/-}$ mice.[186] Moreover, an independent study of *Pyk2*$^{-/-}$ mice also showed that increases in osteoblast number and activity contributed to increased bone mass, raising the possibility of a dual role for PYK2 in bone biology.[187] In this latter study, PF-431396 (**62**, Figure 13) was developed as a combined PYK2/FAK inhibitor (IC_{50} values: PYK2, 11−32 nM; FAK, 1.5 nM) and over 28 days of dosing (30 mg/kg, po) was able to maintain total bone content and total bone density in OVX rats. Increased mineralizing surface (30.4% OVX plus vehicle versus 40.6% OVX plus **62**) and mineral apposition rate (1.43 μm/day OVX plus vehicle versus 1.96 μm/day OVX plus **62**) in proximal tibia cancellous bone were consistent with increased osteoblast activity. Because of the lack of kinase selectivity, it is difficult to know the contribution of PYK2 or FAK inhibition to in vivo efficacy. Another compound from this series PF-562271 (IC_{50} values: PYK2, 14 nM; FAK, 1.5 nM) has completed a phase I study in oncology applications.[188] More recently PF-4618433 (**63**) was published as part of a structural analysis and although less potent on PYK2 (IC_{50} 637 nM) may provide a more selective starting point for optimization.[189]

Screening at Amgen identified a pyridinone series of compounds (**64** and **65**) as PYK2 inhibitors (IC_{50} values 35 and 53 nM, respectively) that did not inhibit FAK (IC_{50} values of 17 and > 125 μM, respectively).[190] Modeling studies indicated that selectivity may have been achieved by an unusual binding conformation wherein **64** simultaneously occupied both the DFG-in ATP pocket and the protein substrate binding cleft. These compounds increased ALP, OC, and calcium in MC3T3 cells cultured over 3 weeks ($EC_{50} \approx 10$ nM).[190] Selectivity was demonstrated over a limited number of other high homology kinases (IC_{50} values of > 1 μM), but nonselective effects via inhibition of other kinases could not be ruled out.

Natural Products

While most of the compounds for osteoporosis have been generated using a targeted based approach, a number of researchers have identified natural products that inhibit osteoclasts or activate osteoblasts in vitro (Figure 14). These natural products have frequently been isolated from traditional remedies and folk medicines where anecdotal evidence suggested that they may improve BMD. For example, green tea, used as a curative for many ailments,[191] contains polyphenol natural products that may help in treating osteoporosis. One compound extracted from green tea, D-catechin (**66**), is known to inhibit bone resorption in embryonic mouse calvaria.[192] A related analogue, (−)-epigallocatechin gallate (EGCG, **67**), was shown to induce apoptosis in osteoclasts. More recently, **67** was shown to inhibit RANKL-induced differentiation of mouse osteoclasts in culture (17% at 10 μM, 32% at 20 μM).[193] The authors suggest that **67** may block RANKL mediated NF-κB transcriptional activation that would result in decreased osteoclastogenesis.

Flavonoids, a class of compounds related to the catechins, have also been isolated from plants used in folk medicines. For example, the compound naringin (**68**), which was isolated from *Drynaria fortunei*, a plant used in traditional Chinese medicine, was found to stimulate proliferative activity (~150% at 1 nM) in the ROS 17/2.8 osteosarcoma cell line.[194] In a more recent publication,[195] researchers reported the isolation of another flavonoid (**69**) from *D. fortunei* that showed increased proliferation in the rat osteosarcoma cell line UMR106 (10 nM, 35.9%). More complex flavonoids, like the biflavonoid sciadopitysin, were also shown to have beneficial effects on osteoblasts. Isolated from the leaves and twigs of the traditional Korean medicinal plant *Cephalotaxus koreana*, sciadopitysin (**70**) was shown to increase ALP activity (121% at 1 μM) in primary mouse osteoblasts.[196]

The anthracene glycosides **71** and **72** were isolated from the traditional Vietnamese medicinal plant *Rhodomyrtus tomentosa* and were shown to increase ALP activity (**71**, 135% at 0.01 μM), collagen synthesis (**72**, 111% at 0.01 μM), and mineralization (**71**, ~115%; **72**, ~125% at 1 μM) of the murine osteoblast cell line, MC3T3.[197] Another plant used in traditional Vietnamese medicine is *Manglietia phuthoensis*, which has been extracted to yield a phenyl glycoside and a lignan glycoside that stimulate osteoblasts.[198] These two compounds, named mangliesides B (**73**) and E (**74**), respectively, also increased ALP activity (**73**, ~114%; **74**, ~112% at 0.03 μM), collagen synthesis (**73**, ~123%; **74**, ~118% at 0.03 μM), and mineralization (**73**, ~118%; **74**, ~109% at 0.03 μM) in MC3T3-E1 cells.

Besides polyphenols and polyhydroxylated compounds, a few terpenes have been isolated from folk medicines and have

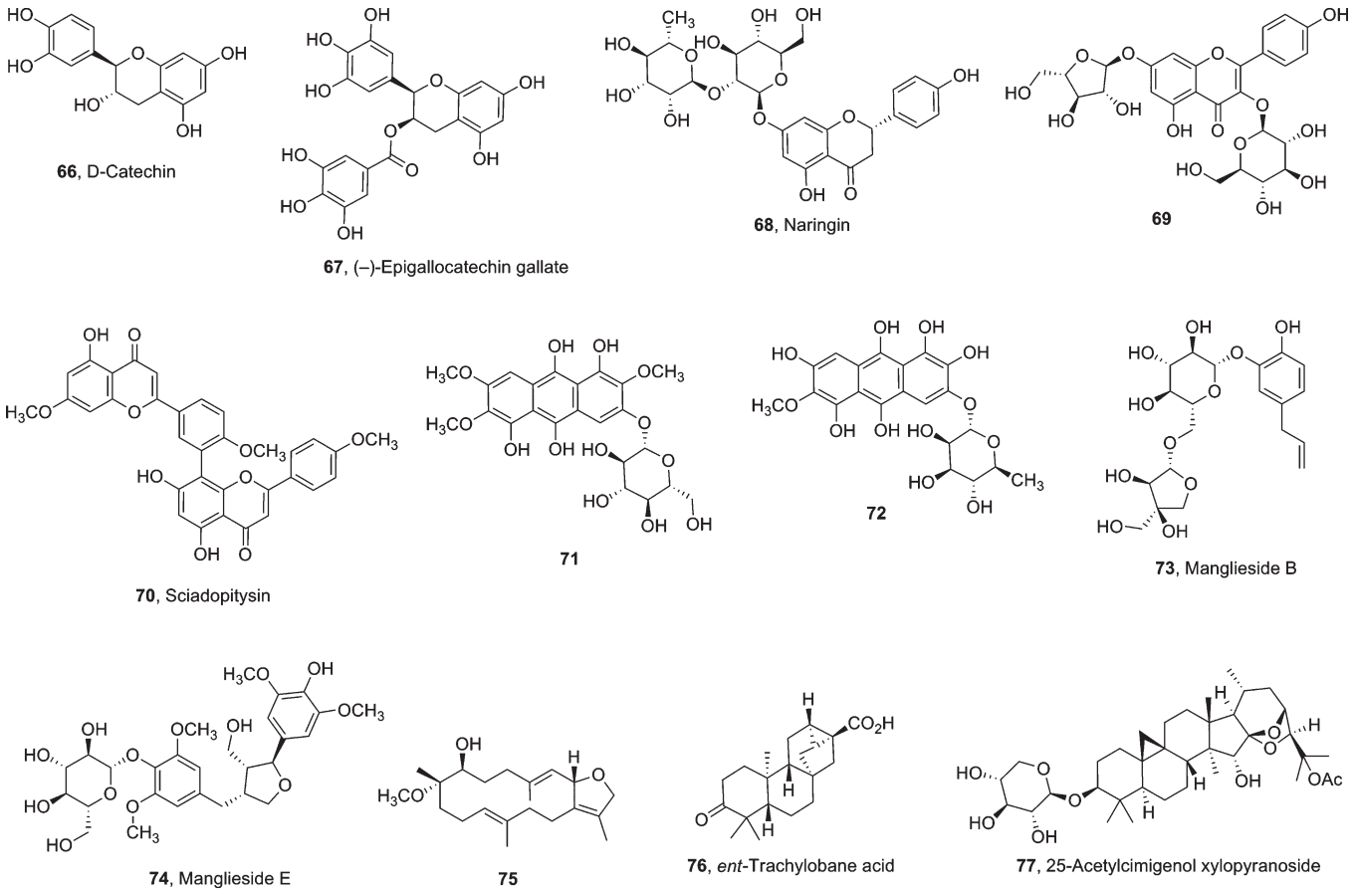

Figure 14. Natural products that modify bone metabolism.

been shown to either stimulate osteoblasts or inhibit osteoclastogensis. A compound from the class of diterpenes known as the cembranoids, compound **75**, was isolated from the soft coral *Sarcophyton mililatensis* and was shown to increase ALP activity (127.8% at 0.3 μM), collagen content (108.2% at 0.3 μM), and mineralization (110.6% at 0.3 μM) in MC3T3-E1 cells.[199] A diterpenoid, ent-trachylobane acid (**76**), isolated from a plant used in Tibetan folk medicine, *Euphorbia wallichii*, inhibited osteoclastogenesis induced by M-CSF and RANKL (IC_{50} of 4 μM).[200] Finally, the triterpene 25-acetylcimigenol xylopyranoside (**77**), isolated from the roots and rhizome of black cohosh, a traditional Native American medicine, was shown to fully inhibit RANKL induced osteoclastogenesis at 10 μM.[201]

Perspective

Antiresorptive therapy represents the current standard of care for treatment of osteoporosis.[12] Branded bisphosphonates have been used with great success to treat patients with primary and secondary forms of osteoporosis, and the recent availability of generic bisphosphonates will likely continue this trend. In addition to advances in the understanding of the mechanism of action of bisphosphonates, other recent key findings in osteoclast biology have been described that have created new therapeutic opportunities to treat bone disease. As mentioned earlier, over the past several years the critical role of the OPG/RANKL system in regulating osteoclast activity and bone mass has been elucidated.[7,8] The discovery of this essential pathway has led to advances in the development of fully human antibodies against RANKL (denosumab) as a potential new option for the treatment of osteoporosis and other bone-related diseases.[8−10]

Nevertheless, it has been proposed that availability of additional anabolic agents would also make a contribution to osteoporosis treatment.[202,203] Thus, therapeutic agents capable of stimulating bone formation would arguably enhance the options for treating osteoporosis. New anabolic therapies may allow combination with antiresorptive therapies in ways that could prolong efficacy and provide sustained protection against the risk of fracture. Indeed, dual acting single agent therapeutics may have improved convenience and safety in use and improved efficacy.

Recently the peripheral and central effects of serotonin signaling on bone mass regulation were described suggesting potential opportunities for the design of new therapeutics for the treatment of osteoporosis. The production of serotonin by tryptophan hydroxylase 1 (TPH1) in gut enterochromaffin cells was proposed as a new mechanism whereby its release into the circulation as a hormone was important for regulation of bone mass.[204] Several lines of mouse genetic evidence pointed to serotonin's peripheral inhibition of bone formation via the hydroxytryptamine receptor 1b (HTR1b) serotonin receptor expressed on osteoblasts. Thus, inhibition of TPH1 may be an attractive new target for enhancing bone formation. Although most serotonin is produced peripherally, additional new work suggested that the ~5% synthesized in the brain appeared to play a dominant and opposite role in the control of bone mass. Remarkably, the serotonin produced by TPH2 in brainstem neurons was shown to signal to HTR2c receptors in the ventromedial hypothalamus, leading to a decreased peripheral sympathetic tone.[205] It was proposed that reduction in

sympathetic activity mediated by $\beta 2$ adrenergic receptors (ADR2β) receptors expressed on osteoblasts was responsible for stimulation of bone mass. Patients taking selective serotonin reuptake inhibitors (SSRIs) show evidence of osteoporosis, suggesting a link between sertotonin signaling and bone mass.[206]

Other potential bone anabolic targets reported in the literature are supported by mouse genetic or biochemical data but currently lack pharmacological validation. For example, a remarkable high bone phenotype driven by increases in bone formation was observed in Schnurri3 (Shn3) knockout mice.[207] In this study it was proposed that Shn3 participated in the ubiquitination and degradation of Runx2 by the E3 ubiquitin ligase WW domain-containing protein 1 (WWP1). Thus, inhibition of SHN3/WWP1 activity could potentially provide an opportunity to develop bone anabolic treatments. Similarly, small mothers against decapentaplegic (SMAD) ubiquitin regulatory factor 1 (SMURF1) E3 ubiquitin ligase[208,209] was also shown to be involved in the regulation of osteoblast activity, suggesting that E3 ubiquitin ligases may present a new target class for further investigation. However, current work in the field of ligase inhibitors has highlighted the difficulties associated with small molecule tractability of this target class. SMURF1 and WWP1 are involved in ubiquitination of several proteins associated with BMP and TGFβ signaling including a number of SMAD regulatory proteins. The central role of SMAD signaling in the regulation of bone mass was recently reported from a new angle where bone formation was shown to be induced in mice following treatment with a soluble activin type IIA receptor.[210] These results suggested that inhibition of activin signaling via SMAD2/3 led to enhanced osteoblast activity. Acceleron's fully human ActRIIA:Fc fusion protein (ACE-011) is currently in phase I clinical trials for osteoporosis.[211]

While the search for new bone anabolic targets continues, there is a growing interest in the potential for small molecules to be used as therapeutic inducers of cell fate during tissue repair and cell regeneration.[212] As such, new compounds could see applications in, for example, the differentiation of osteoblast cells to enhance bone formation for indications such as osteoporosis and fracture healing. In a recent example, the compound reversine was shown to stimulate the differentiation of committed C2C12 myoblasts into osteoblasts.[213] Similarly, when the proteasome inhibitor bortezomib was administered to mice, it induced MSCs to differentiate into osteoblasts and also increased bone formation in a mouse OVX model of osteoporosis.[214] It is therefore theoretically possible that MSCs located in the periosteum and bone marrow, for example, could be coaxed into an osteoblast phenotype with selective small molecules. Other recent work showed that PTH stimulation of osteoblasts was associated with an increase in hematopoietic cells within the hematopoietic stem cell niche located on bone marrow endosteal surfaces.[215,216] These results raise the possibility of using small molecule approaches for altering stem cell activity that may benefit treatment of bone diseases and other related diseases.

Acknowledgment. The authors thank Dr. Andrew Tasker for his critical review.

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Philip Babij obtained his Ph.D. from Kings College London. After postdoctoral fellowships at UTHSC Houston and the University of Vermont he joined the faculty at University College London as Assistant Professor. He moved to Wyeth-Ayerst Research before joining Amgen where he is currently Principal Scientist involved in drug research and development in bone and metabolic disorders.

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