

# Re-appraising the potential of naringin for natural, novel orthopedic biotherapies

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**Abstract:** Naringin is a naturally occurring flavonoid found in plants of the *Citrus* genus that has historically been used in traditional Chinese medical regimens for the treatment of osteoporosis. Naringin modulates signaling through numerous molecular pathways critical to musculoskeletal development, cellular differentiation, and inflammation. Administration of naringin increases *in vitro* expression of bone morphogenetic proteins (BMPs) and activation of the Wnt/ $\beta$ -catenin and extracellular signal-related kinase (Erk) pathways, thereby promoting osteoblastic proliferation and differentiation from stem cell precursors for bone formation. Naringin also inhibits osteoclastogenesis by both modifying RANK/RANKL interactions and inducing apoptosis in osteoclasts *in vitro*. In addition, naringin acts on the estrogen receptor in bone to mimic the native bone-preserving effects of estrogen, with few systemic side effects on other estrogen-sensitive tissues. The efficacy of naringin therapy in reducing the osteolysis characteristic of common musculoskeletal pathologies such as osteoporosis, degenerative joint disease, and osteomyelitis, as well as inflammatory conditions affecting bone such as diabetes mellitus, has been extensively demonstrated *in vitro* and in animal models. Naringin thus represents a naturally abundant, cost-efficient agent whose potential for use in novel musculoskeletal biotherapies warrants re-visiting and further exploration through human studies. Here, we review the cellular mechanisms of action that have been elucidated regarding the action of naringin on bone resident cells and the bone microenvironment, *in vivo* evidence of naringin's osteostimulative and chondroprotective properties in the setting of osteolytic bone disease, and current limitations in the development of naringin-containing translational therapies for common musculoskeletal conditions.

**Keywords:** diabetes mellitus, naringin, osteoarthritis, osteoclastogenesis, osteomyelitis, osteoporosis

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## Introduction

The anti-inflammatory, antioxidant, and anti-apoptotic properties of natural flavanones and their glycosides have been established in preclinical *in vitro* and *in vivo* models of atherosclerosis, cardiovascular disease, various cancers, diabetes mellitus, neurodegenerative conditions, osteoporosis, and rheumatic disease. Flavonoids are phenolic compounds widely distributed among vascular plants; there are approximately 4000 known natural products within the flavonoid family.<sup>1</sup> The flavanones and their metabolites are highly lipophilic, with preferential accumulation in the

stomach and reduced accumulation in the brain due to the blood–brain barrier.<sup>2,3</sup> Naringin (naringenin 7-O-neohesperidose), a naturally occurring flavonoid commonly found in tomatoes, grapefruits, and other members of the *Citrus* genus, has been found to protect against retinoid acid-induced osteolytic bone diseases such as osteoporosis.<sup>3–5</sup> Naringin naturally exists as a mixture of chiral isomers of varying proportions associated with the state of fruit maturation and purification.<sup>3</sup> Naringin is the primary active component of *Drynaria fortunei*, a traditional Chinese medicine used for osteoporosis.<sup>5</sup> Plant-based derivatives such as naringin constitute

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**Table 1.** Effects of naringin on the musculoskeletal system.

| Pathology   | Effects  |
|---|--|
| Osteolytic diseases (e.g. osteoporosis secondary to age, estrogen deficiency, diabetes mellitus, or glucocorticoid use) | <ul style="list-style-type: none"> <li>• Directly increases BMP-2 expression</li> <li>• Increases BMP-2 expression through Wnt/<math>\beta</math>-catenin</li> <li>• Increases BMP-4 and RUNX2 expression and MSC differentiation down osteoblastic lineage</li> <li>• Decreases MSC differentiation down adipogenic lineage</li> <li>• Decreases RANK/RANKL interactions</li> <li>• Increases OPG expression</li> <li>• Decreases osteoclastogenic, proinflammatory cytokine expression</li> <li>• Directly acts on estrogen receptor <math>\alpha</math>, and</li> <li>• Upregulates osteoclast death <i>via</i> Fas/FasL</li> </ul> |
| Degenerative joint diseases (e.g. osteoarthritis)   | <ul style="list-style-type: none"> <li>• Reduces the expression of cartilage-degrading cytokines</li> <li>• Increases the expression of reactive oxygen species-neutralizing enzymes</li> <li>• Decreases the production of cartilage-degrading enzymes</li> <li>• Increases the production of cartilage-protective IL-4 and recruits T<sub>reg</sub> cells, and</li> <li>• Decreases pannus formation</li> </ul>  |
| Bone and joint infections (e.g. osteomyelitis)  | <ul style="list-style-type: none"> <li>• Direct antibiotic effects on Gram-positive and Gram-negative bacteria</li> <li>• Direct inhibition of bacterial quorum sensing system</li> </ul>  |

BMP, bone morphogenetic protein; IL, interleukin; MSC, mesenchymal stem cell; OPG, osteoprotegerin; T<sub>reg</sub>, regulatory T-cell.

a major class of widely available, cost-effective, and biologically active materials for the treatment of a number of skeletal pathologies due to their pro-osteogenic potential, effects on multiple signaling pathways, and actions on numerous cellular targets.

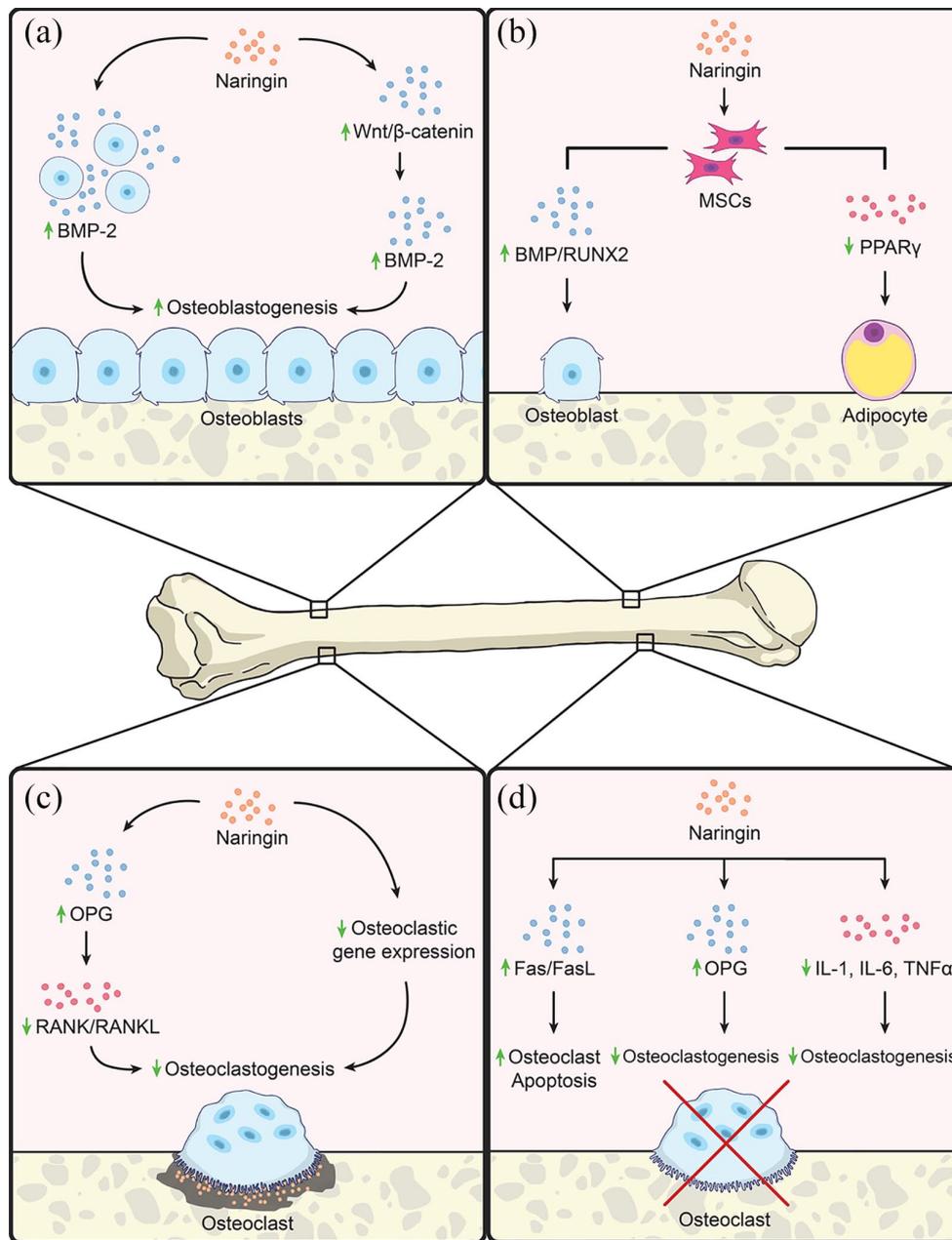
Here, we describe the signaling pathways through which naringin exerts its myriad of effects upon bone and the translational therapeutic potential of naringin for common musculoskeletal pathologies such as osteoporosis, osteoarthritis, avascular necrosis, and osteomyelitis (Table 1). We also briefly describe translational research and pharmacologic challenges that must be overcome to successfully harness the bone-promoting/preserving effects of naringin.

### ***In vitro* characterization of the effects of naringin on bone resident cells**

#### *Bone morphogenetic proteins regulate skeletal formation through osteoblast activation*

The positive effects of naringin upon bone health are many. Naringin enhances the production of cell survival proteins and reduces the expression of genes involved in the inflammatory response.<sup>3</sup> In the musculoskeletal system, bone morphogenetic proteins (BMPs) have been shown to be important in skeletogenesis,<sup>6</sup> limb and digit

formation,<sup>7</sup> and fracture repair.<sup>8</sup> BMP-2 is responsible for the differentiation of multipotent stem cells to an osteoblast-like lineage.<sup>9</sup> This osteoblastic differentiation yields increased bone formation, with clinical potential to restore bone mineral density (BMD) in osteopenia and osteoporosis and enhance fracture healing. Multiple groups have demonstrated that hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors used to treat hypercholesterolemia upregulate BMP-2 expression.<sup>10–13</sup> At concentrations between 0.001 and 0.1  $\mu\text{mol/l}$ , naringin increased osteoblast activity by inhibiting HMG-CoA reductase in a dose-dependent fashion in rat osteoblast-like UMR-106 cells.<sup>14</sup> Given these effects on HMG-CoA, it has been posited that naringin augments BMP-2 expression for osteoblastogenesis and bone formation. Indeed, primary osteoblasts cultured with naringin displayed increased BMP-2 protein and mRNA levels.<sup>5</sup> Yin *et al.* found that naringin upregulated osteoblastogenesis *in vitro* through a BMP-2-mediated mechanism<sup>15</sup> [Figure 1(a)]. Osteoblast activity increased in the presence of naringin in UMR-106 cells, as demonstrated by the colorimetric tetrazolium assay, which evaluates cellular viability, metabolism, and proliferation *via* oxidoreductases, and increased total protein and alkaline phosphatase (ALP) activity.<sup>14</sup> Together, these findings indicate that naringin activates BMP-2 for increased osteoblast and bone production.



**Figure 1.** *In vitro* effects of naringin. (a) Naringin has been shown to increase osteoblastogenesis and thus bone anabolism. Naringin increases osteoblastogenesis through upregulating BMP-2, a protein responsible for the differentiation of MSCs down an osteoblastic lineage, directly and by stimulating the Wnt/ $\beta$ -catenin pathway which converges on further upregulation of BMP-2. (b) MSCs grown in the presence of naringin demonstrate increased osteoblastogenesis and decreased adipogenesis. MSCs in the presence of naringin express increased levels of osteoblastic genes such as BMPs and RUNX2 and decreased gene expression of the adipogenic PPAR $\gamma$ . (c) Naringin has been shown to decrease osteoclastogenesis and thus osteolysis in osteoclastic pathologies, such as osteoporosis in menopause and diabetic osteoporosis. Naringin decreases osteoclastogenesis by inhibiting RANK/RANKL interaction by inducing expression of the receptor decoy OPG as well as directly decreasing expression of osteoclastic genes. (d) Physiological levels of estrogen maintain bone stock in females by inducing osteoclast apoptosis and decreasing inflammatory cytokines that are osteoclastogenic; therefore, the loss of estrogen results in osteoporosis due to increased osteoclastogenesis. Through interactions with estrogen receptor  $\alpha$ , naringin increases interactions between the death receptor Fas and its ligand FasL which increases osteoclast apoptosis, which tips bone homeostasis from a catabolic to an anabolic state, like endogenous estrogen. Naringin also decreases the inflammatory cytokines IL-1, IL-6, and TNF- $\alpha$  that stimulate osteoclastogenesis. BMP-2, bone morphogenic protein 2; IL, interleukin; MSC, mesenchymal stem cell; OPG, osteoprotegerin; TNF, tumor necrosis factor;

#### *Upstream effects of naringin on BMP activation via the Wnt/ $\beta$ -catenin pathway*

Wnt signaling through the canonical Wnt/ $\beta$ -catenin pathway crucially regulates musculoskeletal development.<sup>16,17</sup> Wnt and  $\beta$ -catenin serve as important upstream regulators of BMP signaling, thereby influencing osteoblastogenesis and bone formation.<sup>18</sup> Wang *et al.* showed that naringin upregulated  $\beta$ -catenin mRNA and protein expression in osteoblast-like UMR-106 cells<sup>19</sup> [Figure 1(a)]. Gene expression of the Wnt-related regulators  $\beta$ -catenin and cyclin D1 in human amniotic fluid-derived stem cells (hAFSCs) increased in the presence of naringin.<sup>20</sup> Application of the Wnt-inhibitor DKK-1 reduced ALP activity, which is suggestive of the critical role of Wnt/ $\beta$ -catenin signaling in osteogenesis.<sup>20</sup> Modulation of the Wnt/ $\beta$ -catenin pathway by naringin in this way contributes to bone anabolism.

#### *Differentiation of bone resident cells and osteogenesis*

Naringin increases ALP activity, osteocalcin (OCN) and osteopontin synthesis, and cell proliferation in primary cultured osteoblasts, in addition to promoting human bone mesenchymal stem cell (BMSC) proliferation and osteogenic differentiation.<sup>21–23</sup> hAFSCs exposed to naringin increasingly underwent osteogenic proliferation and differentiation, as evidenced by increased ALP activity, a marker for early osteogenic differentiation, and calcium deposition, a marker for late osteogenic differentiation.<sup>20</sup> Similar effects upon osteogenic proliferation and differentiation have been observed with naringin administration in human BMSCs,<sup>23</sup> periodontal ligament stem cells (PDLSCs),<sup>24</sup> and murine pre-osteoblastic MC3T3-E1 cells.<sup>5,25,26</sup> Moreover, hAFSCs expressed increased levels of BMP-4 and RUNX2 mRNA in the presence of naringin, suggesting that the activation of BMP signaling by naringin promotes osteogenesis<sup>20</sup> [Figure 1(b)]. Inhibition of BMP-4 and RUNX2 using the BMP-inhibitor noggin reduced ALP activity, which further suggests the necessity of BMP signaling pathways for osteogenesis.<sup>20</sup> Moreover, it has been established that the extracellular signal-regulated kinase (ERK) MAPK pathway plays a vital role in osteoblastic differentiation.<sup>27</sup> Naringin has been shown to increase Erk expression and phosphorylation in osteoblasts, promoting osteogenesis. Naringin doses of 10  $\mu$ g/ml demonstrated the greatest pro-osteogenic effects upon rat bone marrow-derived mesenchymal stem cells (BM-MSCs), which persisted for 9 days.<sup>5</sup> The pro-osteogenic potential of naringin may be

harnessed or enhanced through novel formulations and drug delivering biomaterial constructs. Lai *et al.* loaded TiO<sub>2</sub> (titanium) nanotubes with naringin *via* chitosan layers and observed that these naringin-eluting nanotubes enhanced osteoblast spreading and proliferation, ALP activity, and bone mineralization.<sup>28</sup> The application of naringin-loaded polymeric micelles to human adipose-derived stem cells yielded greater osteogenic differentiation, as characterized by increased osteopontin expression and bony matrix mineralization, than was observed in the presence of free naringin.<sup>29</sup> Naringin additionally enhances osteoblast survival by increasing 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> signaling in osteoblasts *in vitro*.<sup>30</sup>

The pro-osteogenic effects of naringin may also be linked to its ability to downregulate peroxisome proliferator-activated receptor (PPAR) $\gamma$  (a key regulator of adipogenesis) expression in BMSCs, as well as its activation of the neurogenic locus Notch homolog protein (Notch) signaling pathway<sup>31,32</sup> [Figure 1(b)]. This downregulation of PPAR $\gamma$  was also associated with increased microRNA (miRNA)-20a expression.<sup>31</sup> Naringin thus concurrently reduces adipogenesis in bone and promotes osteogenic differentiation *via* Notch and miRNA transcriptional regulation, augmenting bone formation. These findings suggest that naringin is a potent natural promoter of osteogenesis with the potential for use in stem cell therapies, which traditionally use dexamethasone for lineage differentiation.<sup>5</sup> Such treatments are often limited by adipogenesis secondary to glucocorticoid exposure, even when conducted in osteogenic media.

#### *Regulation of osteoclastogenesis via RANK activation*

Bone homeostasis is mediated by the delicate balance between osteoblast-mediated anabolism and osteoclast-mediated catabolism.<sup>33</sup> Increased osteoclastogenesis is the hallmark of many bone pathologies, including senile osteoporosis due to increased adipogenesis, post-menopausal osteoporosis secondary to the loss of the osteoblastic and anti-osteoclastic effects of estrogen,<sup>34</sup> and diabetic osteoporosis induced by the proinflammatory state and resultant macroangiopathies and microangiopathies characteristic of diabetes mellitus.<sup>35</sup> Osteoclastogenesis is mediated by interactions between RANK/RANKL and osteoprotegerin (OPG);<sup>36</sup> pathologic states increase the RANK/RANKL ratio present in bone and decrease production of OPG. Moreover, under

proinflammatory conditions, increased expression of cytokines such as interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$  promote osteoclastogenesis.<sup>37</sup>

Naringin has been shown to successfully disrupt osteoclastogenesis *via* the RANK/RANKL pathway [Figure 1(c)]. Incubation of RAW264.7 cells with medium containing naringin for 5 days inhibited osteoclast differentiation and proliferation, bone resorption, and RANK, TRAP, matrix metalloproteinases (MMP)-9 and NFATc1 mRNA expression critical to osteoclast differentiation, while upregulating C-fos expression.<sup>38</sup> Naringin decreases degradation of RANKL-mediated I $\kappa$ B and enhances RANKL-mediated phosphorylation of Erk.<sup>23</sup> Furthermore, naringin enhances OPG release from osteoblasts, reducing interactions between RANKL and its receptor and preventing osteoclast differentiation.<sup>22</sup> The combination of these mechanisms suppresses the expression of genes critical to osteoclast development.

#### *Estrogen and estrogen receptor signaling*

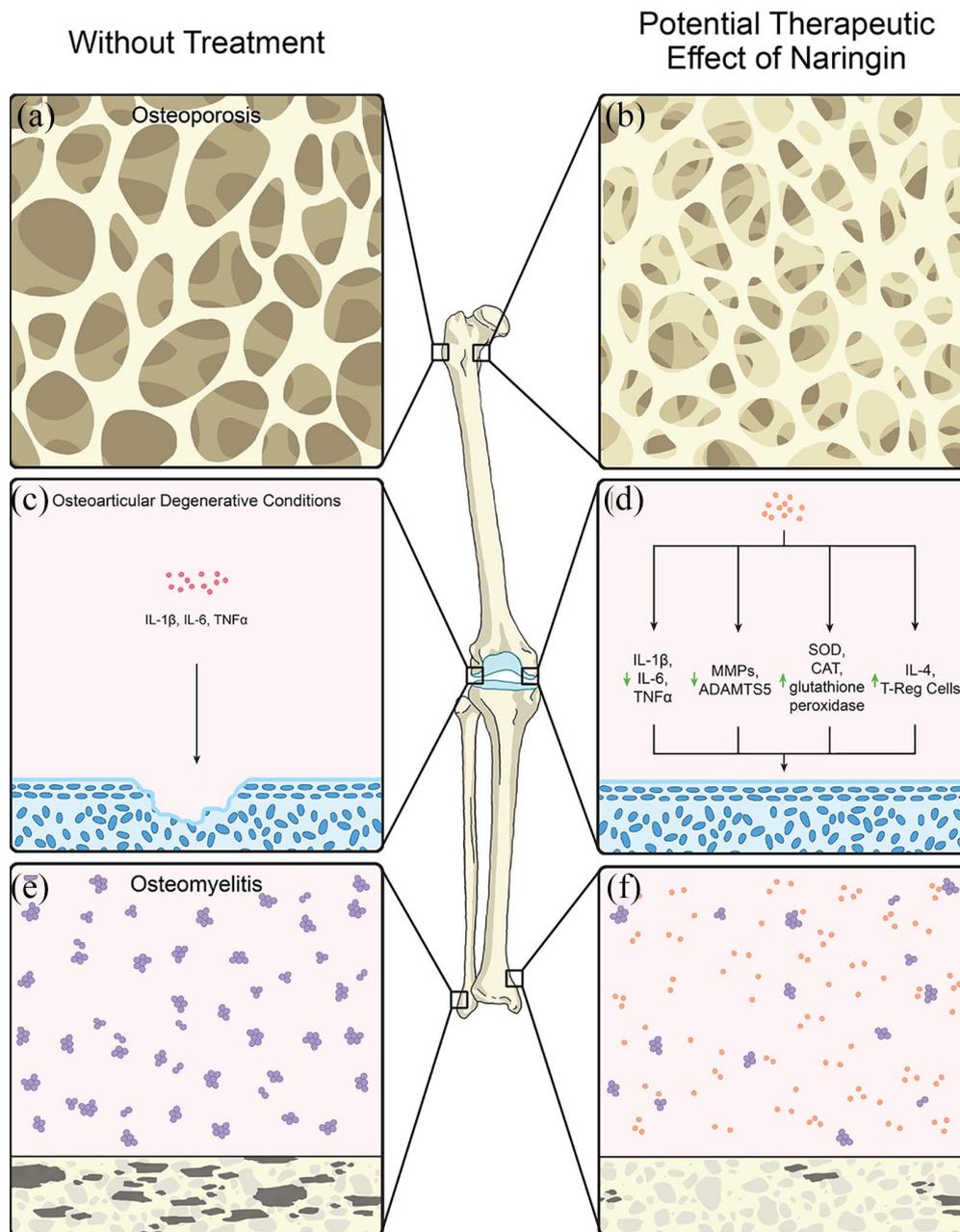
The sex hormone estrogen plays a central role in bone development and metabolism. In childhood and adolescence, estrogen drives progression to skeletal maturity and the maturation and fusion of the epiphyseal growth plates.<sup>39</sup> In adults, estrogen maintains bone stock and influences bone turnover by inhibiting bone resorption. Estrogen mediates its bone-protective effects through estrogen receptor  $\alpha$ .<sup>40</sup> Estrogen also induces apoptosis in osteoclasts by upregulating interactions between the death receptor and its ligand (Fas/FasL).<sup>40</sup> Furthermore, estrogen inhibits osteoblast-mediated osteoclastogenesis by decreasing the production of IL-1, IL-6, and TNF- $\alpha$ , which induce osteoclast differentiation from progenitor cells.<sup>41</sup> Estrogen also modulates the synthesis of OPG, which serves as a decoy receptor for RANKL, by osteoblasts, thus hindering osteoclastogenesis.<sup>42</sup> The loss of estrogen production in menopause is the primary causative factor of diminished BMD and increased predisposition to fractures among post-menopausal women.<sup>43</sup> While hormone replacement is effective against bone loss in menopause,<sup>44–46</sup> estrogen replacement therapy is not without negative sequelae.<sup>47,48</sup> For these reasons, alternative bone-preserving therapies for post-menopausal osteoporosis and other estrogen-depleting conditions without multisystemic side effects are desired.

Naringin has been shown to have estrogen-like effects *in vitro* on UMR-106 cells<sup>49</sup> and is known to bind to the estrogen receptor<sup>50</sup> [Figure 1(d)]. Naringin also downregulates expression of BCL-2 while upregulating expression of BAX, caspase-3, and cytochrome C, suggesting that naringin may induce osteoclast apoptosis through regulation of the mitochondrial apoptosis pathway.<sup>51</sup> The ability of naringin to induce apoptosis in exposed osteoclasts<sup>51</sup> and upregulate OPG mRNA levels in MC3T3-E1 cells hinders osteoclastogenesis in ways that mirror the native effects of estrogen on bone.<sup>30</sup> By targeting pathways vital to the pathogenesis of menopause-induced bone loss, naringin may stem the musculoskeletal consequences of reduced estrogen production for the restoration of bone stock.

#### ***In vivo modeling of the restorative effects of naringin in pathologic conditions of bone***

##### *The dual actions of naringin in the treatment of osteoporotic bone*

Naringin has shown promise as a treatment for osteolytic bone diseases such as osteoporosis, which is characterized by increased osteoclast activity and bone resorption.<sup>3–5</sup> Daily oral naringin administration increased femoral bone mass in healthy mice by enhancing trabecular and cortical bone content and quality<sup>5</sup> [Figure 2(a,b)]. The majority of osteoporosis animal models have employed ovariectomy (OVX) to reduce circulating estrogen levels and induce osteoclast-mediated bone resorption. This model simulates the loss of estrogen production characteristic of menopause, after which the cortical and trabecular BMD and interconnections between bone cells become drastically diminished and the risk of pathologic fracture significantly increases, resulting in osteopenia and osteoporosis [Figure 2(a)]. Orchidectomized,<sup>52</sup> retinoic acid-induced,<sup>4</sup> and disuse-induced animal models of osteoporosis have also been generated to study disease physiology and therapeutic avenues.<sup>53</sup> Li *et al.* demonstrated that naringin decreased bone loss in OVX-induced osteoporosis Sprague–Dawley rats.<sup>51</sup> Naringin has been found to mitigate the loss of bone calcium and phosphorus content,<sup>52</sup> BMD,<sup>19,21,49,51,54–57</sup> bone architecture quality (as measured by bone volume, trabecular thickness, and trabecula count<sup>19,49,51,54,55</sup>) and bone mechanical strength across all murine models of osteoporosis, particularly in OVX mice and rats<sup>19,51,55</sup> [Figure 2(b)]. Treatment of OVX mice with naringin for 6 weeks significantly improved distal femur, proximal tibia, and lumbar spine bone quality, while suppressing the urinary excretion of



**Figure 2.** *In vivo* effects of naringin. (a) Osteoporosis secondary to the loss of estrogen's anabolic effects during menopause, the proinflammatory state of diabetes that promotes osteoclastogenesis, or the loss of p-ERK due to glucocorticoid use leads to decreased trabeculae volume, yielding an increased risk of fracture. (b) Naringin prevents osteoporotic bone fractures by increasing the number and thickness of trabeculae. (c) Osteoarthritic degenerative conditions such as osteoarthritis, ankylosis spondylitis, and rheumatoid arthritis are characterized by increases in the expression of inflammatory cytokines such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , decreases in reactive oxygen species-neutralizing enzymes such as SOD, CAT, and glutathione peroxidase, and increases in cartilage-degrading enzymes, including MMPs and ADAMTS5. (d) Naringin has been shown to be cartilage-protective by decreasing the expression of these inflammatory cytokines, reactive oxygen species, and destructive enzymes. Naringin has also been shown to increase the cartilage-protective effects of IL-4 and T<sub>reg</sub>s. (e) Osteomyelitis is characterized by the proliferation of bacteria that degrade bone both directly *via* bacterial products and indirectly by inducing inflammation productive of osteoclastic cytokines. (f) Naringin demonstrates antibacterial effects against common Gram-positive and Gram-negative organisms commonly implicated in the pathogenesis of osteomyelitis. CAT, catalase; IL, interleukin; MMP, matrix metalloproteinase; SOD, superoxide dismutase; TNF, tumor necrosis factor; T<sub>reg</sub>, regulatory T-cell;

calcium and loss of bone mass and strength characteristic of OVX mice.<sup>49,55</sup> However, the benefits of naringin in bone health are not limited to bone structure and microarchitecture, but also extend to biomechanical strength. A combined 60-day regimen of oral naringin and treadmill exercise in OVX mice more effectively reduced the structural effects of osteoporosis on bone mass and increased bone strength than either monotherapy.<sup>55</sup> Importantly, naringin administration in OVX mice has not been associated with increases in uterus weight, suggesting that the off-target estrogen-modulating effects of estrogen are limited.<sup>49</sup> This represents a significant advantage over traditional post-menopausal hormone replacement therapy, which nonselectively acts upon bone, breast, ovarian, and endometrial tissue.

Perhaps the greatest therapeutic benefit of naringin in the treatment of osteoporosis has been demonstrated in models of disuse-induced osteoporosis. These conditions replicate the loss of bone mechanical loading seen in limited mobility and bed-ridden patients and astronauts in zero-gravity environments.<sup>5</sup> Ma *et al.* created a model of disuse-induced osteoporosis in rats by denervating bone through sciatic neurectomy, which resulted in the loss of BMD, disruption of native trabecular microarchitecture in the distal femurs of experimental animals, and increase in urinary deoxypyridoline excretion.<sup>53</sup> Dose-dependent recovery of trabecular microarchitecture and bone formation rates comparable to the sham group were observed with naringin treatment.

Several theories have been postulated regarding the etiology of naringin's bone-preserving effects in osteoporosis in *in vivo* models. Naringin is believed to limit the upregulation of osteoclast activity and differentiation seen in osteoporosis. Naringin attenuates increases in circulating CTX-1, a biomarker that correlates with osteoclast-mediated resorption in osteoporosis.<sup>51–53,55,57</sup> These reductions in osteoclastogenesis were confirmed by TRAP staining of bone tissue isolated from OVX mice, which revealed reduced osteoclast invasion of and activity within bone.<sup>51,53</sup> This has been attributed to the induction of apoptosis in osteoclasts by naringin<sup>51</sup> and the inhibition of osteoclast differentiation.<sup>53</sup> In addition to mediating osteoclastogenesis, naringin has the potential to induce new bone formation in osteoporotic animals by upregulating the differentiation and activity of osteoblasts, as described previously. Biomarkers thought to correlate with osteoblast activity such as OCN and total procollagen type 1 N-terminal propeptide (P1NP) are

increased in both naringin-treated osteoporotic mice and wild-type mice.<sup>51,53,55</sup> Naringin treatment is histologically marked by increased osteoblast infiltration of bone and increased osteoid formation in *in vivo* models.<sup>4,19,51,53,55</sup> These effects upon osteoblasts are thought to be mediated by the induction of Akt-mediated BMP-2 expression<sup>21,57</sup> and  $\beta$ -catenin signaling through AMPK/Akt and semaphorin-3A (SEM3A) by naringin.<sup>19,53</sup>

Habauzit *et al.* hypothesized that naringin's anti-inflammatory properties may be partly responsible for its bone-protective effects in the setting of osteoporosis, particularly in the setting of age-related bone inflammation.<sup>57</sup> The authors observed that naringin lowered circulating IL-6 and nitric oxide (NO) levels in osteoporotic animal models.<sup>57</sup> Finally, naringin may also prevent microvascular damage to osteopenic and osteoporotic bone. Naringin preserves bone microvascularity in OVX mice and reduces the perturbations of NO and endothelin levels, two key regulators of vasoactivity, seen in osteoporosis.<sup>56</sup> In addition, naringin promotes angiogenesis and neovascularization during fracture callus formation in murine osteoporotic models, likely by way of vascular endothelial growth factor (VEGF) expression changes in osteocytes.<sup>50</sup> The effects of naringin upon the Notch signaling pathway, as described previously, may thus have dual implications for bone health, as endothelial Notch activity simultaneously regulates both bone angiogenesis and osteogenesis, conferring both fracture healing and osteoprotective benefits.<sup>5</sup> These effects resulted in the dose-dependent acceleration of fracture healing in murine models. More work is needed to further elucidate the mechanisms by which naringin exerts its effects upon bone resident cells in osteoporosis.

Pharmacological therapy for osteoporosis targets both osteoclastogenesis and new bone formation with agents such as bisphosphonates and intermittent parathyroid hormone, respectively. However, the reduced responsiveness of protracted disuse osteoporosis to bisphosphonate treatment and transition from bone formation to adipogenesis in bone marrow with age necessitate the adoption of new osteostimulative and osteoprotective therapies.<sup>5</sup>

#### *Modification of metabolic pathways and inflammation in diabetes mellitus*

Diabetes mellitus (DM) is a chronic metabolic disorder associated with impaired new bone formation and fracture healing and increased risk of

pathologic fracture secondary to systemic hyperglycemia, advanced glycation end-product and reactive oxidative species (ROS) formation, and inflammation.<sup>35</sup> On the cellular level, these processes increase osteoclastogenesis and reduce osteoblast levels and activity. Consequently, diabetic rats display reduced trabecular volume and bone remodeling and formation, with increased trabecular spacing in the femur and lumbar spine,<sup>58,59</sup> similar to the findings clinically observed in various forms of osteoporosis [Figure 2(a)]. *In vivo* models of diabetes typically utilize streptozocin injections, which damage the  $\beta$ -cells of the pancreas, to recreate the pathology seen in type 1 DM or prolonged feeding of high fat diets to simulate type 2 DM. Increased levels of superoxide dismutase (SOD) and catalase (CAT) activity in bone marrow are indicative of higher levels of inflammation and oxidative stress,<sup>60</sup> which have been implicated in the pathogenesis of diabetes.<sup>61</sup> Naringin has been found to normalize both SOD and CAT levels in diabetic rats. Naringin administration to diabetic rodents resulted in similar oxidative stress attenuation in the skin,<sup>62</sup> liver,<sup>63–66</sup> pancreas,<sup>64,66</sup> and kidney.<sup>66</sup> As such, naringin has also been shown to slow both structural and compositional trabecular bone quality decline in the long bones and calvaria of streptozocin-induced diabetic rodents<sup>60,67</sup> [Figure 2(b)]. These findings were corroborated by observed increases in serum OCN levels, osteoid production, and osteoblast populations, as well as reduced osteoclastic infiltration of bone, in naringin-treated diabetic rats.<sup>60,67</sup> Furthermore, enhanced bone formation was observed upon subcutaneous naringin administration in a titanium particle-induced diabetic murine calvarial model of osteolysis.<sup>67</sup>

Naringin has demonstrated strong reversing effects of the primary pathologic hallmarks of both type 1 and 2 DM by improving insulin resistance and production, glucose tolerance, and fasting glucose and HbA1c levels *in vivo*.<sup>68</sup> These effects are postulated to stem from the positive regulation of glucose metabolism and GLUT-4 mobilization by naringin.<sup>68</sup> In addition, the effects of naringin upon serum lipid regulation have been extensively investigated *in vivo*. By upregulating PPAR $\gamma$  expression in tissues, particularly the liver and kidney, naringin mollifies the increases in serum low-density lipoprotein, triglyceride, and total cholesterol concentrations and reduced high-density lipoprotein levels characteristic of DM and other

metabolic syndromes.<sup>64,66,68</sup> Naringin may also upregulate heat shock proteins HSP-72 and HSP-27 to influence PPAR $\gamma$  expression and suppress inflammation *via* the nuclear factor (NF)- $\kappa$ B pathway.<sup>66</sup> However, it is possible that the bone-protective effects of naringin in diabetes is independent of these metabolic processes, as naringin has been shown to reduce bone resorption in the absence of changes to these metabolic pathways.<sup>60</sup>

The favorable lipid regulatory profile of naringin may be linked to its positive effects on bone secondary to its promotion of vascular and microvascular health. The relationship between hyperlipidemia and diabetic microangiopathy, resulting in reduced vascular delivery to bone and local hypoxia, inflammation, and oxidative stress responses, is well established.<sup>62</sup> Naringin administration reduces the expression of various serum markers of inflammation in *in vivo* models of DM. These modulatory effects include reductions in serum TNF- $\alpha$ , IL-6, and C-reactive protein, NO,<sup>65,66,69</sup> IL-6, TNF- $\alpha$ , and NF- $\kappa$ B expression in nervous tissue,<sup>70</sup> hepatic expression of NF- $\kappa$ B, IL-6 and cyclooxygenase (COX)-2,<sup>63,66</sup> and TNF- $\alpha$ , IL-6, and IL-1B expression in wound granulation tissue.<sup>62</sup> In the context of diabetic wound healing, naringin treatment improved microvasculature to granulation tissue *via* localized increases in angiogenesis driven by higher local levels of VEGF-c, angiopoietin, and insulin-like growth factor (IGF)-1.<sup>62</sup> The aortae of diabetic mice treated with naringin displayed less endothelial damage, as visualized on transmission electron microscopy, as well as greater vascular functionality, as measured by responsiveness to exogenously administered epinephrine, acetylcholine, and insulin.<sup>71</sup> Moreover, naringin has been observed to induce SEM3A, a local bone micro-environmental factor that both promotes new bone formation and reduces bone resorption, expression *in vivo*.<sup>5</sup> SEM3A deficiency has been directly implicated in the perturbation of bone remodeling and bone loss in diabetic rats.<sup>53,60</sup>

Finally, IGF-1 levels were found to be elevated in nervous tissue in the presence of naringin.<sup>70</sup> Further research clarifying changes in the expression of IGF-1, a potent regulator of anabolism and new bone formation, in bone in the presence of naringin is needed to elucidate the mechanisms by which naringin treatment promotes bone health in osteoporosis, DM, and other inflammatory conditions.

### *Steroid-induced bone loss and its mollification by naringin*

Synthetic glucocorticoids are routinely used in the management of inflammatory and autoimmune disorders such as rheumatoid arthritis, asthma, multiple sclerosis, systemic lupus erythematosus, and inflammatory bowel disease.<sup>72</sup> Although these medications are effective in their ability to suppress the immune system, their deleterious effects are many, particularly upon bone. Chronic exogenous steroid administration contributes to osteopenia, osteoporosis, pathologic fracture, poor fracture healing, and avascular necrosis. *In vivo* models of glucocorticoid-treated mice have demonstrated that glucocorticoids not only reduce trabecular bone volume and whole bone strength, but also alter the osteocyte lacunae microenvironment, thereby decreasing the bone mineral to matrix ratio and increasing bone fragility<sup>73</sup> [Figure 2(a,b)]. In this way, high dose and/or chronic glucocorticoid administration begets osteopenia and osteoporosis. In a rabbit model of steroid-induced avascular necrosis of the femoral head (SANFH), naringin treatment significantly mitigated steroid-associated reductions in serum OCN levels and observed rates of osteonecrosis.<sup>32</sup>

Multiple signaling pathways have been implicated in the pathogenesis of glucocorticoid-induced osteoporosis (GIO). Decreases in phosphorylated ERK (p-ERK) expression have been observed in osteoporotic mice. Chen *et al.* recapitulated these findings in animal models of GIO, as osteoblasts exposed to dexamethasone demonstrated lower levels of p-ERK expression.<sup>74</sup> Jing *et al.* investigated the activation of the Wnt/ $\beta$ -catenin pathway in GIO rats and found that expression of Erk, Lrp-5,  $\beta$ -catenin mRNA levels, as well as p-GSK-3 $\beta$  protein expression, were reduced in GIO.<sup>27</sup> GIO rats demonstrated decreased expression of BMP-2 and RUNX2, thus impairing osteoblastic differentiation.<sup>75</sup> Phosphoinositide 3-kinase (PI3K) and its downstream effector protein kinase B (Akt) are also disrupted in GIO, resulting in perturbed bone growth and formation.<sup>76</sup> Pan *et al.* observed significant reductions in BMD and ALP, OCN, and phosphorylated Akt expression in the bones of GIO animals.<sup>77</sup> Naringin therapy was associated with lower total cholesterol levels and low-density lipoprotein/high-density lipoprotein ratios in SANFH animals.<sup>32</sup> These effects have been attributed to upregulated caspase-3 and ALP activity and RUNX2, transcription factor sp7, PPAR $\gamma$ 2, Notch,  $\beta$ -catenin, and phosphorylated Rac expression with the application of naringin.<sup>32</sup> Together, these findings suggest that naringin is protective

against SANFH through Notch signaling and upregulated PPAR $\gamma$ 2 expression. The modulation of these signaling pathways in bone by naringin, as detailed previously, are indicative of naringin's manifold local and systemic inflammatory effects. Additional *in vivo* research of the effects of naringin in models of GIO, fracture healing, and avascular necrosis are needed to more effectively prevent and combat glucocorticoid-induced conditions of bone.

### *Downregulation of the inflammasome by naringin in degenerative joint disease*

Naringin has demonstrated potential in the treatment of osteoarticular degenerative conditions such as osteoarthritis, ankylosis spondylitis, and rheumatoid arthritis [Figure 2(c,d)]. In a mouse model of ankylosis spondylitis, intraperitoneal flavonoid therapy reduced the expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, signal transducer and activator of transcription (STAT)-3, and Janus kinase (JAK)2 in a dose-dependent fashion.<sup>78</sup> Naringin concomitantly increased the generation of ROS-neutralizing enzymes such as SOD, CAT, and glutathione peroxidase *in vivo*.<sup>78</sup> Moreover, naringin has been shown to reduce the production of prostaglandin E2 (PGE2) and NO in a dose-dependent manner in aqueous humor and RAW 264.6 cells in a lipopolysaccharide-induced model of osteoarthritis.<sup>79,80</sup> Through these mechanisms, oral naringin administration lessens cartilage destruction, intercondylar knee joint damage, pannus formation, and synovial infiltration by leukocytes in surgical and monosodium iodoacetate-induced models of osteoarthritis.<sup>80,81</sup> Naringin therapy also improved weight bearing in osteoarthritic rats by inhibiting the expression of MMP, ADAMTS5, and proinflammatory cytokines, while increasing regulatory T-cell activity and IL-4 expression, thereby preserving cartilage proteoglycan content.<sup>5,82,83</sup> The benefits of naringin in degenerative joint disorders may manifest not only through moderation of the inflammasome, but also the effects of naringin upon estrogen signaling. These effects on estrogen receptors have been described extensively with respect to osteoporosis both *in vitro* and *in vivo* but require further elucidation in the setting of osteoarthritis.

### *The antibacterial and anti-inflammatory potential of naringin in osteomyelitis treatment*

Osteomyelitis is an infection of the bone associated with high morbidity and mortality.<sup>84,85</sup> The mainstay

of osteomyelitis treatment is the administration of an aggressive antibiotic regimen. Osteomyelitis is the product of coexistent infected, nonviable tissue and insufficient host immune responses, resulting in progressive inflammatory bone breakdown.<sup>86,87</sup> The most common response of host cells to infection is necrosis. Established infections are characterized by the formation of fibrous tissue and chronic inflammation around granulation tissue and dead bone, reducing vascular flow to the infection site and the efficacy of the inflammatory response.<sup>86</sup> The spread of infection from bone to surrounding soft tissue reduces vascular supply to bone, allowing for the formation of a sequestrum, or area of dead bone, constraining the medullary and periosteal blood supplies.<sup>86</sup> Given the persistence, recurrence, and severe morbidity and mortality of osteomyelitis despite antibiotic treatment, new and improved therapeutic options are being sought. Though the flavonones promote bone health by reducing inflammatory injury and osteolysis and increasing new bone formation, their effects in osteomyelitis have yet to be thoroughly characterized.

The primary causative agent of osteomyelitis in the United States is *Staphylococcus aureus*, a hardy organism capable of surviving within phagocytes, neutrophils, and osteoblasts, small colony variant and biofilm formation, and antibiotic resistance.<sup>88</sup> Infection of bone by *S. aureus* invokes host immune responses that contribute to progressive and chronic inflammatory bone destruction and the activation of Toll-like receptors and their inflammatory signaling pathways.<sup>87,89,90</sup> *S. aureus*-mediated osteoblast apoptosis is associated with upregulation of TLR2 and the pro-apoptotic JNK pathway and may be a mechanism of inflammation-derived osteomyelitis-associated bone loss.<sup>91</sup> Putnam *et al.* sought to investigate whether *S. aureus* osteomyelitis-induced bone destruction is mediated by proinflammatory cytokine production and signaling.<sup>92</sup> Osteomyelitis is associated with higher levels of circulating proinflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6.<sup>93,94</sup> Johansen *et al.* noted that COX-2 expression was upregulated in osteoblasts within 2 days of *S. aureus* inoculation, as well as significant osteoclastogenesis.<sup>95</sup> *S. aureus* infection of human and murine osteoblasts increases phosphorylation of ERK1 and ERK2, which then promote the phosphorylation of substrates such as the transcription factors ATF-2, Elk-1, and c-Jun. The Erk pathway is a significant target in inflammation-mediated osteolysis.<sup>96</sup> Furthermore, the production of proinflammatory cytokines, chemokines, prostaglandins, and MMPs by macrophages and fibroblasts in the setting of

infection directly degrades bone matrix and activates osteoclasts and indirectly increases RANKL and NF- $\kappa$ B signaling, thus promoting osteolysis.<sup>97</sup> Osteoclast differentiation and bone resorption are upregulated in the setting of *S. aureus* infection and osteomyelitis, with a concomitant decrease in osteoblast viability and differentiation.<sup>97</sup>

*S. aureus* is capable of inducing autophagy in infected cells by escaping from autophagosomes and increasing the expression of tumor necrosis factor-related apoptosis-inducing ligand, which activates caspase-8 to initiate apoptosis in bacterial-challenged osteoblasts.<sup>89</sup> *S. aureus* protein A binding to TNF-1 on pre-osteoblasts initiates the proteolytic apoptotic cascade mediated by caspase-3 and caspase-6.<sup>98,99</sup> *S. aureus* protein A binding to osteoblasts also increases the secretion of RANKL, thus promoting osteoclastogenesis.<sup>98</sup> Furthermore, *S. aureus* reduces the expression of markers of osteoblast growth and replication such as ALP, collagen type I, OCN, and osteopontin, thereby inhibiting *de novo* bone matrix deposition.<sup>98,99</sup> Pyroptosis is the gasdermin-mediated process of programmed cell death that transpires in response to bacterial insult and is characterized by cell swelling, plasma membrane rupture, and the release of proinflammatory contents, including IL-1 $\beta$ , IL-6 and IL-18.<sup>100,101</sup> Mice with *S. aureus* osteomyelitis displayed higher levels of pyroptosis-associated proteins, and inhibition of these proteins reversed bone injury *in vivo*.<sup>102</sup> Through these mechanisms, *S. aureus* infection both exacerbates bone death and inhibits new bone formation.

The flavonones possess varying levels of antibiotic potency against common Gram-positive and Gram-negative organisms [Figure 2(e,f)]. The addition of lipophilic groups to compounds is commonly associated with an increase in antimicrobial activity.<sup>103</sup> The primary site of flavonone action is the cytoplasmic membrane. The cell wall of Gram-positive bacteria permits fatty acid circulation towards the nuclear membrane, imbuing free fatty acids with greater antimicrobial activity against Gram-positive over Gram-negative bacteria.<sup>103</sup> Crude extracts containing flavonoids, triterpenes, and steroids have demonstrated significant activity against *S. aureus*, *Streptococcus faecalis*, and *Escherichia coli*.<sup>1</sup> At a dose of 128 mg/l, the flavonoids rutin, naringin, and baicalin were found to inhibit 25% or less of *Pseudomonas aeruginosa* growth *in vitro*.<sup>104</sup> Naringin has been observed to act upon the bacterial quorum

sensing system of interactions between bacteria and hosts related to bacterial population cell density that mediates gene expression and virulence factor expression.<sup>105</sup> Naringin, naringenin, kaempferol, quercetin, rutin, and neohesperidin, all isolated from *Citrus sinensis*, were found to inhibit quorum sensing signaling pathways *in vitro*.<sup>105</sup> This may allude to the potential of these compounds to disrupt the formation of biofilms and antibiotic resistance.

Baicalin is one of the few flavonones whose effects upon osteomyelitis-induced bone resorption has been investigated. In the setting of *S. aureus*-induced osteomyelitis in a murine model, baicalin reduced TLR2, ALP, OCN, and collagen type 1 expression, serum levels of IL-1 $\beta$ , IL-6, and C-reactive protein, and activity in the MAPK pathway.<sup>106</sup> This altered inflammatory response profile bears semblance to the already elucidated mechanisms of naringin signaling in reducing osteolysis in various models of bone loss. Moreover, the demonstrated ability of naringin to inhibit osteoclastogenesis and bone resident cell apoptosis and promote osteogenic differentiation in other models of osteolytic and inflammatory bone loss is suggestive of its potential to mitigate the osteolytic effects of entrenched bony infection. Further *in vitro* and *in vivo* studies investigating the anti-inflammatory effects of naringin therapy, and with a variety of drug delivery systems, in the setting of musculoskeletal infection are needed.

Given the rise in prevalence of antibiotic infection, particularly that of methicillin-resistant *S. aureus*, there is great need for the development of new treatment regimens and agents to prevent the occurrence, recurrence, and exacerbation of osteomyelitic infection. The demonstrated efficacy of flavonones in preventing osteoclastic bone resorption and promoting osteoblastic differentiation and activity in other osteolytic diseases may carry over to the progressive destruction of bone characteristic of osteomyelitis. The lipophilic nature of the flavonones may make them viable in the treatment of intracellular *S. aureus* infection. In addition, it may be possible to augment their potency through structural and biochemical modifications and alternate methods of compound preparation and administration. Furthermore, the natural occurrence, widespread availability, and low cost of isoflavonoids make them attractive in the treatment of osteomyelitis and in addressing the unequal burden of chronic osteomyelitis in low- and middle-income countries.<sup>107</sup>

#### *Pharmacological profile of naringin and obstacles to translational therapy*

Naringin has yet to obtain approval for clinical therapeutic use as a single and combinatorial agent. The extensive *in vivo* metabolism, low oral bioavailability, and poor water solubility, resulting in poor bodily absorption, of flavonoids significantly limit the therapeutic efficacy of naringin and related flavanones.<sup>5,108,109</sup> Naringin is further degraded in acidic environments such as that of the stomach and is extensively acted upon by intestinal  $\beta$ -glucosidases.<sup>5,109</sup> As a result of these factors, naringin is absorbed slowly and irregularly when administered orally, with significant individual variation due to the heterogeneity of the intestinal microbiome. In order to improve dietary flavonoid bioavailability, solubility, and dissolution, *in vitro* assessment of nanoparticle, microparticle, and water-soluble fiber naringin delivery systems are being increasingly explored.<sup>5,110</sup> Naringin is also significantly degraded when administered intravenously; flavonoids undergo oxidation in circulation and within the liver, are acted upon by hepatic  $\beta$ -glucosidases, and are bound by serum albumin, facilitating excretion and reducing bioavailability.<sup>5,109,111</sup> Nano-carriers for the transport of naringin to skeletal sites have yet to be established, but would confer improved solubility, bioavailability, and pharmacokinetic properties for sustained flavonoid release to bone.

Numerous biomaterial-based platforms have been investigated with the goal of optimizing the therapeutic effects of naringin by reducing its degradation and sustaining its spatiotemporal release within the body. Ji *et al.* embedded naringin within an electrospun nanoscaffold comprised of polycaprolactone (PCL) and poly(ethylene glycol)-*b*-polycaprolactone (PEG-*b*-PCL) nanofibers to study its effects upon bone regeneration.<sup>112</sup> In the presence of these naringin nanoscaffolds, increases in MC3T3-E1 proliferation, osteogenic differentiation, and calcium mineralization by Alizarin Red S staining were observed under culture conditions that lacked additional osteogenic supplementation.<sup>112</sup> This naringin nanoscaffold also suppressed osteoclastogenesis in a critical size defect of mouse calvarial bone, as demonstrated by a significant reduction in TRAP staining compared to blank PCL nanoscaffold controls. Together, these findings are suggestive not only of the successful delivery and persistence of naringin in bone *via* a scaffold construct, but also the potential utility of

naringin in bone tissue engineering.<sup>112</sup> Chen *et al.* developed a porous biodegradable construct from genepin-crosslinked gelatin and  $\beta$ -Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> ceramic microparticles mixed with 10 mg/ml naringin with the goal of facilitating *in vivo* bone repair in a rabbit calvarial defect model.<sup>113</sup> Significant new bone deposition was observed within 8 weeks of implantation of these naringin-loaded gelatin and  $\beta$ -Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> ceramic micro-particle composites. Similar results have been seen upon introduction of porous poly(l-lactide) scaffolds comprised of spray-dried naringin-loaded chitosan microspheres and parthenolide,<sup>114</sup> an 0.85% naringin-loaded pH-responsive hydrogel composed of carboxymethyl-hexanoyl chitosan,  $\beta$ -glycerophosphate and glycerol,<sup>115</sup> and a mineralized collagen coating with embedded naringin-containing metal organic frameworks<sup>116</sup> with respect to new bone formation and bone resident cell differentiation from stem cell precursors. Together, these studies are indicative of the potential of naringin-loaded biomaterial constructs to improve the pharmacokinetic properties of naringin within the body and facilitate its actions upon bone.<sup>5</sup> Further research is needed to characterize the *in vivo* performance of these novel naringin-loaded delivery systems, their effects upon the bioactivity of naringin in various bodily tissues, and their safety and utility in the treatment of diverse musculoskeletal conditions.

In a single ascending-dose randomized controlled trial of 18 adults by Rebello *et al.*, no adverse effects or changes in blood safety biomarkers were observed after oral consumption of 150–900 mg naringenin, the flavonone form of naringin, relative to placebo.<sup>117</sup> Measured serum biomarkers included alanine aminotransferase, creatinine phosphokinase, and potassium, as well as total white blood cell and eosinophil counts as markers of immune system activation. At these doses, naringin metabolites freely circulate and are effectively cleared from the body within 24 h of ingestion. The authors concluded that oral doses of 150–900 mg of naringenin are safe and tolerable in healthy adults, with serum concentrations proportional to the oral doses received.<sup>117</sup> One randomized controlled trial of excessive grapefruit consumption reported constipation and diarrhea as adverse effects, though it is not clear whether this effect can be solely or primarily attributed to naringin.<sup>118</sup> Several other toxicity studies failed to demonstrate the presence of toxic side effects following naringin administration.<sup>119,120</sup>

#### *Clinical evaluation of common “nutraceuticals” and the supplemental potential of naringin*

Natural chondroprotective agents such as glucosamine sulfate, chondroitin sulfate, collagen hydrolysate, hyaluronic acid, antioxidants, and fatty acids are commonly used in the treatment or prevention of degenerative musculoskeletal conditions such as osteoarthritis.<sup>121–124</sup> Within North America, these natural pharmaceutical compounds, “nutraceuticals”, are limited to the designation of “safe dietary supplements.” Supplementation with these natural compounds has been shown to be more clinically and cost-effective than continued nonsteroidal anti-inflammatory drug (NSAID) prescription and use, while increasing patient quality of life.<sup>125–127</sup>

Glucosamine, also known as 2-amino-2-deoxy-D-glucose (C<sub>6</sub>H<sub>13</sub>NO<sub>5</sub>), is an amino acid synthesized from glucose in all bodily tissues that is most prevalent in connective tissue and cartilage.<sup>121</sup> It may also be extracted from the chitinous exoskeletons of crustaceans and cell membranes of mushrooms. Glucosamine is a precursor of glycoprotein and glycosaminoglycan synthesis and is vital for the production of hyaluronic acid, chondroitin sulfate, and keratan sulfate, all components of the extracellular matrices of articular cartilage and synovial fluid.<sup>121</sup> The utility of glucosamine supplementation in the forms of glucosamine sulfate and glucosamine hydrochloride has been explored in multiple randomized clinical trials of osteoarthritis patients.

Exogenous glucosamine undergoes significant first-pass metabolism, such that oral administration is associated with 25% bioavailability.<sup>128,129</sup> The most commonly administered and evaluated dosage of glucosamine sulfate is 1500 mg/day.<sup>121</sup> At this dose, glucosamine sulfate appears to slow the progression of hip osteoarthritis.<sup>130</sup> A systematic review of symptomatic slow-acting drugs in osteoarthritis (SYSADOA) determined that there exists moderate- to high-quality evidence that glucosamine sulfate, among other natural compounds, reduces pain, disease progression, and the risk of future total joint replacement and improves physical function in osteoarthritis patients with few toxic side effects.<sup>131,132</sup> Meta-analyses of all glucosamine studies conducted prior to 2014 determined that glucosamine significantly reduced joint space narrowing, with a modest effect size.<sup>123,133,134</sup> However, the Glucosamine/chondroitin Arthritis Intervention Trial (GAIT) trial determined that glucosamine and chondroitin sulfate administered

both alone and in combination failed to significantly reduce the pain of knee osteoarthritis relative to placebo.<sup>135,136</sup> Several studies have suggested that glucosamine sulfate is superior to placebo and NSAIDs such as ibuprofen,<sup>137</sup> though inconsistent trends between studies and study design effects such as industry funding bias have been reported.<sup>121</sup> Gregory and Fellner critically appraised nutraceuticals such as glucosamine as disease-modifying treatments in osteoarthritis and determined that the strongest evidence of glucosamine's efficacy exists with respect to delaying disease progression.<sup>123</sup> However, the authors classified these data as "inconsistent and unclear" with a likely modest effect size, if any.<sup>123</sup> Due to methodological considerations, the American College of Rheumatology recommended against the use of glucosamine in its 2000 guidelines and has continued to advise against its use in osteoarthritis.<sup>138,139</sup> These considerations included a paucity of standardized case definitions and assessments and insufficient information on study designs. Similarly, the UK's National Institute for Health and Clinical Excellence has also advised against the prescription of glucosamine in osteoarthritis due to a lack of evidence of its clinical utility.<sup>123</sup> According to PJ Gregory, these recommendations were largely based on the results of the GAIT study.<sup>123</sup> Regardless, glucosamine supplements are widely used and anecdotally associated with actual or perceived benefits.<sup>123,140</sup>

Chondroitin sulfate is the most common glycoprotein and glycosaminoglycan in cartilaginous aggregate molecules.<sup>121</sup> Its negative charge facilitates water retention within cartilage for pressure resistance and joint cushioning. Chondroitin sulfate increases synovial cell production of hyaluronan, which maintains the viscosity of synovial fluid.<sup>141</sup> It is also believed to stimulate chondrocyte metabolism, thereby increasing collagen and proteoglycan synthesis, and inhibit the activity of leukocyte esterase and hyaluronidase, which are often upregulated in rheumatic disease.<sup>121,142</sup> Due to the large size of this macromolecule, it is poorly absorbed and has an estimated bioavailability of 10–13%.<sup>143,144</sup> This SYSADOA lessens pain, inflammation, and the rate of disease progression and serves as a structure-modifying drug in osteoarthritis.<sup>121</sup> In a clinical trial of 162 patients given 800 mg of chondroitin sulfate or placebo for 6 months, chondroitin sulfate demonstrated superiority to placebo with respect to global hand pain, hand function, and morning stiffness reduction.<sup>145</sup> Other clinical trials have demonstrated the beneficial effects of chondroitin sulfate

in reducing disease progression and severity in patients with knee osteoarthritis over placebo.<sup>146–152</sup> No significant local or systemic side effects were observed following intramuscular, intra-articular, or oral administration.<sup>153,154</sup> The European League Against Rheumatism bestowed chondroitin sulfate with the highest level of evidence (1A) and recommendation strength (A) in 2003.<sup>155</sup> However, as of 2014, American College of Rheumatology guidelines continued to recommend against the use of chondroitin sulfate.<sup>139</sup>

A randomized, double-blind, placebo-controlled study of a supplement containing glucosamine hydrochloride, chondroitin sulfate, and quercetin glycosides, natural flavonoid compounds similar to naringin, in patients with symptomatic knee osteoarthritis demonstrated improvement in two of four disease-related symptom and function subscales in patients treated with supplements relative to placebo.<sup>156,157</sup> Patients who received the combined supplement also experienced improvement in the ratio of type II collagen synthesis to degradation relative to placebo-treated controls.<sup>156,157</sup> Combined glucosamine and chondroitin sulfate supplementation was also observed to be effective in hindering the progression of spinal disc degeneration in a single patient treated over a 2-year period, though more extensive studies are needed to corroborate this observation.<sup>158</sup>

Four open-label and three double-blind trials have been conducted on the effects of collagen hydrolysate in osteoarthritis.<sup>159</sup> A 24-week multinational double-blind randomized controlled trial of patients with knee osteoarthritis indicated that treatment with 10 g/day of collagen hydrolysate elicited no significant changes in the WOMAC index.<sup>160,161</sup> Though collagen hydrolysate appears to demonstrate equivocal efficacy in reducing osteoarthritis-associated pain, its use is accepted in the treatment of osteoarthritis.<sup>160</sup> Collagen hydrolysates are considered highly safe, with minimal adverse effects such as gastrointestinal fullness and unpleasant taste.<sup>162</sup>

The aforementioned common nutraceutical compounds have been associated with modest improvements in various parameters of degenerative joint disease severity and progression, though inconsistencies exist between clinical trial results and study designs. Moreover, these natural substances are plagued by bioavailability limitations, necessitating the oral consumption of higher doses. Regardless, these nutraceuticals are considered safe and

tolerable due to their improved side-effect profile relative to NSAIDs. Though the described nutraceuticals are commonly used in the prevention and/or treatment of osteoarthritis due to their chondroprotective potential, recommendations regarding their use are conflicting. As previously described, the ability of naringin to improve numerous aspects of musculoskeletal health and protect against degenerative cartilaginous and bone pathology in preclinical studies merits clinical investigation. The favorable chondroprotective effects reported by Kanzaki *et al.* and Tiku *et al.* upon regular oral consumption of a combined supplement containing glucosamine, chondroitin sulfate, and a related flavonoid compound<sup>156,157</sup> hints toward the potential of naringin as a combined or adjunctive supplement, which warrants further evaluation in formal trials. Naringin's mechanisms of action are also distinct from those of the aforementioned nutraceuticals and may contribute to more consistent clinical improvements based on preclinical findings. Despite its inherent pharmacokinetic limitations similar to those of the other natural chondroprotective compounds, the enhanced delivery, release, and action of naringin on bone and connective tissue *via* novel bioformulations may address existent shortcomings in the clinical use and evaluation of nutritional supplements in musculoskeletal pathology. All of these potential therapeutic avenues of naringin have yet to be thoroughly clinically interrogated.

### Conclusion

The multifarious effects of naringin in bone are indicative of its potential in the treatment of many common orthopedic conditions and fracture treatment and prevention. Naringin effectively reduces osteoclastogenesis, inflammation, and adipogenesis and induces osteoblastic differentiation from progenitor cells for the maintenance and preservation of both cartilage and bone. Though naringin demonstrates promise as a therapeutic agent for several forms of osteoporosis, DM, degenerative joint disease, and osteomyelitis, many limitations must be addressed before these early findings can be translated into viable therapies. Additional research is needed into the ways in which the pharmacokinetic properties of naringin can be improved, be it through the use of constructs such as nano-carriers and hydrogels, to optimize naringin delivery, action, and persistence at sites of action. Regardless, naringin represents a ubiquitous, cost-efficient, biologically active, natural compound whose potential can be harnessed to

develop novel treatments for common musculoskeletal conditions.

### Author contributions

Conceptualization: KEY, KDA, and FYL. Data Curation: KEY, KDA, MTM, AMM, and IL. Formal Analysis: KEY and KDA. Investigation: KEY, KDA, MTM, AMM, IL, SVC, H-KK, and JB. Methodology: KEY and KDA. Project Administration: KEY, KDA, JB, and FYL. Resources: FYL. Software: FYL. Supervision: H-KK, JB, and FYL. Validation: KEY, KDA, MTM, AMM, IL, SVC, H-KK, JB, and FYL. Visualization: KEY, SVC, H-KK, and JB. Writing, Original Draft: KEY, KDA, MTM, and AMM. Writing, Review & Editing: KEY, KDA, MTM, AMM, IL, SVC, H-KK, JB, and FYL. All authors approved the final version of this manuscript.

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