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Osteogenic effects of resveratrol *in vitro*: potential for the prevention and treatment of osteoporosis

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There are a number of pharmacological agents for the treatment of bone mineral loss and osteoporosis. Hormone replacement therapy (HRT) with estrogen is an established treatment, but it has several adverse side effects and can increase the risk of cancer, heart disease, and stroke. There is increasing interest in nutritional factors and naturally occurring phytochemical compounds with the potential for preventing age-related and postmenopausal bone loss. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a polyphenolic phytoestrogen with osteogenic and osteoinductive properties. It can modify the metabolism of bone cells and has the capacity to modulate bone turnover. This paper provides an overview of current research on resveratrol and its effects on bone cells *in vitro*, highlighting the challenges and opportunities facing this area of research, especially in the context of providing nutritional support for postmenopausal women who may not benefit from HRT and older patients with various forms of arthritis, metabolic bone disease, and osteoporosis.

Keywords: resveratrol; phytoestrogen; osteogenesis; osteoporosis; menopause

Introduction

Despite being calcified, bone is a living and dynamic tissue that constantly turns over and renews itself. The osteon in cortical bone is the micromechanical unit that responds to the biomechanical, endocrine, and nutritional stimuli that are responsible for the physiological maintenance of bone extracellular matrix (ECM).[1] Old bone is degraded by bone-resorbing osteoclasts and is replaced with new bone produced by bone-synthesizing osteoblasts.[2] However, the delicate balance between bone synthesis and bone degradation is lost with aging, resulting in low bone density and osteoporosis. Osteoporosis is a metabolic disease of bones that causes them to become more porous, gradually making them weaker, more brittle, more fragile, and more likely to fracture. The increased porosity is the result of an imbalance in the bone remodeling process, whereby bone resorption, mediated by osteoclasts, outpaces bone formation, mediated by osteoblasts. Consequently, osteoporosis reduces bone mineral density and mass, making long bones significantly more prone to fractures. Osteoporosis-induced bone fractures commonly occur in the spine, wrist, and hips, but can affect other bones such as the arm or the pelvis. Reduced bone mineral density can be a feature of bone remodeling in arthritis; subchondral bone sclerosis is also associated with age-related joint degeneration.[4]

Nonhormonal treatments for osteoporosis: bisphosphonates

Significant progress has been made over the past five decades in the nonhormonal treatment of osteoporosis.[5] Bisphosphonates (BPs) are a class of drugs that are used to treat osteoporosis and related bone diseases by preventing the loss of bone mass.

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These drugs were developed in the early 1960s as potential treatments for bone diseases. Five decades later, BPs are the most frequently prescribed drugs for the treatment of osteoporosis and other diseases characterized by increased bone resorption. In patients with postmenopausal osteoporosis, BPs reduce osteoclast activity back to healthy, premenopausal levels, thereby decreasing the rate of bone loss. BP drugs increase bone mass, strengthen bones, and reduce the incidence of fractures, including severe fractures of the hip and spine. BPs approved for the treatment and/or prevention of osteoporosis include alendronate (Fosamax, Fosamax Plus D; Merck, Whitehouse Station, New Jersey, USA), ibandronate (Boniva; Genentech, South San Francisco, California, USA), zoledronic acid (Reclast; Novartis, Basel, Switzerland), and risedronate (Actonel, Actonel with Calcium, and Atelvia; Warner Chilcott, Dublin, Ireland). Other BPs include etidronate, raloxifene, and teriparatide, for the secondary prevention of osteoporotic fragility fractures in postmenopausal women. In addition to osteoporosis, BPs are also used to lower calcium levels in the blood and to treat Paget's disease of bone (which causes bones to become weak and deformed) and bone-related cancers, alleviating pain and weakness. In addition, BPs are also used following other forms of cancer treatment, such as chemotherapy and hormone therapies, both of which can weaken bones. They can also prevent some cancers from spreading to bone. Therefore, BPs are an enormously important class of drugs in modern medicine, and in addition to their therapeutic potential for osteoporosis and related bone diseases, they are potential therapeutic agents for disease modification in osteoarthritis.[6] Discussion of the side effects of BPs is beyond the scope of this review. We refer the readers to the NIH website for further information (http://nihseniorhealth.gov/osteoporosis/treatmentandresearch/01.html). However, bone is also highly responsive to sex hormones, particularly estrogen, and its turnover can be regulated by estrogen-like compounds.

Hormonal treatments for osteoporosis: estrogen

Estrogen plays a fundamental role in skeletal growth and bone homeostasis in both men and women.[7] Although the term *estrogen* actually refers to a large number of steroidal and nonsteroidal molecules capable of inducing estrus, in the context of this article, *estrogen* refers to a pharmacological agent in hormone replacement therapy (HRT) to prevent postmenopausal bone loss in older women.[8] Taking estrogen orally in various formulations or as an adhesive skin patch increases circulating levels of this hormone and compensates for its loss after menopause, thus relieving the symptoms of menopause. It is especially recommended after menopause to prevent osteoporosis in women. It slows down the loss of bone minerals and increases bone thickness. Estrogen has been shown to prevent bone loss and lower the risk of hip fractures in postmenopausal women. Estrogen deficiency can also affect young women, and oral estrogen is prescribed to young women with hormonal imbalances.[9]

Estrogen deficiency is also linked to disturbances in the redox state. The acute loss of estrogens has been shown to increase the levels of reactive oxygen species (ROS), activate nuclear factor κB (NF-κB), and stimulate the production of proinflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α).[10] The production of proinflammatory cytokines is a common feature of many different types of inflammatory bone and joint disorders. Interestingly, proinflammatory cytokine production and release is attenuated by HRT.[10] Therefore, for long-term preservation of bone mineral density, women are advised to take estrogen for at least seven years after menopause.[8] This strategy is thought to reduce the risk of developing osteoarthritis and osteoporosis. However, this duration of therapy may have little effect on bone density in women over 75, who have the highest risk of developing fractures.[8] Studies also suggest that age-related bone loss may be the result of estrogen deficiency in men as well as postmenopausal women.[11] These studies highlight the fact that estrogen is important for the maintenance of bone mineral density in both men and women.

However, estrogen use is associated with a number of undesired side effects that include headache, fluid retention, weight gain, and swollen breasts. In addition to these minor side effects, there are reports that suggest an increased risk of breast or uterine cancer, heart failure, or stroke in some women. Therefore, it may not be recommended for women who have a family or personal history of heart disease, stroke, blood clots, or breast cancer. The Women's Health Initiative (WHI) study linked the

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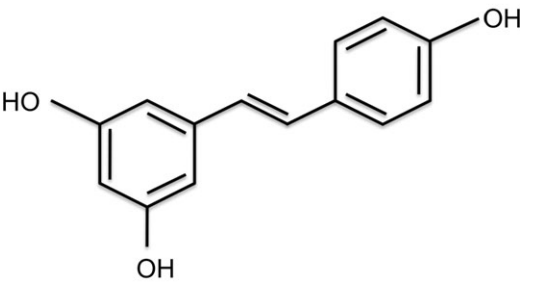

Figure 1. Resveratrol, also known as *trans*-resveratrol, 3,5,4′-trihydroxy-*trans*-stilbene, is produced by grape vines in response to fungal infections. It is induced by stress, injury, or ultraviolet irradiation. Resveratrol is also found in *Polygonum cuspidatum* and several other plant species. Resveratrol has anti-inflammatory, anticarcinogenic, antimutagenic, antineoplastic, and antioxidant properties.

use of HRT to an increase in a woman's risk of depression and cardiovascular sequelae including stroke.[12] Many experts recommend that long-term estrogen replacement therapy only be considered for women with a significant risk for osteoporosis that outweighs the risks of taking HRT. Like other drugs and hormone treatments, estrogen has side effects and should be taken at the lowest dose and for the shortest possible duration. Women who experience side effects from taking estrogen are advised to seek help from their medical practitioner.

Phytoestrogens and resveratrol

The side effects of estrogen therapy highlight a medical need for safer treatments for bone mineral loss and associated bone and joint disorders. As suggested earlier, the term *estrogen* refers to a large number of steroidal and nonsteroidal molecules, many of which naturally exist in our food and the environment. Phytoestrogens are plant-derived compounds found in a wide variety of foods. They are capable of substituting for estrogens[13] and may be implicated in lowering the risk of osteoporosis, heart disease, breast cancer, and menopausal symptoms.[14] Phytoestrogens possess estrogenic and antiestrogenic effects, and some of them are considered to be endocrine disruptors with the potential to cause adverse health effects.[14] Some phytoestrogens can enhance bone formation, increase bone mineral density, and increase the expression of bone markers including alkaline phosphatase, osteocalcin, osteopontin, and type I collagen.[15] Resveratrol, also known as 3,5,4′-trihydroxy-*trans*-stilbene, is a polyphenolic phytoestrogen and phytoalexin present in plants such as grapes, berries, and peanuts (Fig. 1). It has a variety of potentially beneficial health effects and has been reported to possess anti-inflammatory, immunomodulatory, and antioxidative capabilities.[16] The subsequent sections highlight selected research articles on the effects of resveratrol on bone.

In vitro and *in vivo* effects of resveratrol on bone

Resveratrol has been shown to stimulate bone cell proliferation and differentiation. One of the first studies of resveratrol action on bone cells was carried out using osteoblastic MC3T3-E1 cells. This study demonstrated the ability of resveratrol to directly stimulate the proliferation and differentiation of osteoblasts.[17] Resveratrol dose dependently increased DNA synthesis and alkaline phosphatase (ALP) and prolyl hydroxylase activities in MC3T3-E1 cells. The effects of resveratrol were antagonized by the antiestrogen drug tamoxifen. Turner *et al.* carried out an *in vivo* study published in 1999 to determine if resveratrol can act as an estrogen agonist in growing rats.[18] Their 6-day study in weanling rats aimed to determine the dose response of orally administered resveratrol on estrogen target tissues. They used resveratrol concentrations including 1, 4, 10, 40, and 100 μg/day. Using 10% ethanol as a solvent had no significant effect on any of the measurements. They found that resveratrol had no effect

on body weight, uterine wet weight, uterine epithelial cell height, cortical bone histomorphometry, or serum cholesterol. Based on the *in vivo* data obtained, they proposed that resveratrol has little or no estrogen agonism on reproductive and nonreproductive estrogen target tissues and may be an estrogen antagonist. Similar work carried out by Durbin *et al.* has shown that resveratrol supplementation of 6-month-old Brown Norway male rats had no bone protective effects and may even have detrimental bone effects.[16] In contrast, work by Liu *et al.* has shown that resveratrol from *Polygonum cuspidatum* increases bone mineral density in the epiphysis of the ovariectomized female rat model.[19] This model is much more relevant to hormone-dependent osteoporosis and provides more tangible evidence for using resveratrol for protecting against bone loss induced by estrogen deficiency.

These studies highlight the fact that *in vitro* and *in vivo* data are not always directly comparable. Also, data from animal models, especially rodents, are often conflicting and the outcomes depend on the type of model that was employed. Although rodent data may not always be directly translatable to humans, the ovariectomized female rat is probably the most suitable animal model for research on drugs and natural compounds for treating osteoporosis.

More recent data from our own work suggest that resveratrol affects sirtuin 1 (Sirt1). The *Sirt1* gene encodes a member of the sirtuin family of proteins, homologs to the yeast Sir2 protein, which are known to regulate epigenetic gene silencing. Members of the sirtuin family are histone deacetylases characterized by a sirtuin core domain. Sirt1 is a NAD^+-dependent histone deacetylase. Sirtuins are believed to function as intracellular regulatory proteins with mono-ADP–ribosyltransferase activity. The effects of resveratrol on Sirt1 influence its interactions with receptor activator of NF-κB ligand (RANKL) and the bone-specific transcription factor Runx2, in bone-derived cells and mesenchymal stem cells (MSCs), respectively.[20,21]

The RANKL/RANK/osteoprotegerin (OPG) system plays an important role in the regulation of bone resorption.[22] RANKL is a cytokine and a member of the TNF superfamily. It stimulates osteoclast differentiation and augments bone loss. We recently used high-density bone cultures to investigate the effects of resveratrol on RANKL during bone morphogenesis *in vitro*.[20] We observed that RANKL induced formation of tartrate-resistant acid phosphatase–positive multinucleated cells that exhibited morphological features of osteoclasts. RANKL also induced NF-κB activation. RANKL upregulated the expression of p300 (a histone acetyltransferase), which, in turn, promoted acetylation of NF-κB. However, in cultures pretreated with resveratrol, this activation was inhibited. Resveratrol also suppressed the activation of IκBα kinase and the phosphorylation and degradation of IκBα. Resveratrol inhibited RANKL-induced acetylation and nuclear translocation of NF-κB in a time- and concentration-dependent manner. In addition, activation of Sirt1 by resveratrol induced Sirt1–p300 association in both bone-derived and preosteoblastic cells, leading to deacetylation of RANKL-induced NF-κB, inhibition of NF-κB transcriptional activation, and osteoclastogenesis. Cotreatment with resveratrol activated the bone transcription factor Cbfa1 and Sirt1 and induced the formation of Sirt1–Cbfa1 complexes. This *in vitro* study demonstrated that resveratrol-activated Sirt1 plays important roles in regulating the balance between bone resorption and bone production. This was the first study that highlighted the mechanisms underlying the therapeutic potential of resveratrol for treating osteoporosis and arthritis-related bone loss.[20]

In a more recent study, we examined whether activation of Sirt1 by resveratrol affects osteogenic differentiation. We employed monolayer and high-density cultures of MSCs and preosteoblastic cells and treated them with an osteogenic induction medium with or without the Sirt1 inhibitor nicotinamide and/or resveratrol in a concentration-dependent manner. MSCs and preosteoblastic cells differentiated into osteoblasts when exposed to osteogenic induction medium. Osteogenesis was blocked by nicotinamide, resulting in adipogenic differentiation and expression of the adipose transcription regulator PPARγ (peroxisome proliferator–activated receptor). However, in nicotinamide-treated cultures, pretreatment with resveratrol significantly enhanced osteogenesis by increasing expression of Runx2 (a transcription factor that encodes a nuclear protein with a Runt DNA-binding domain), and decreased the expression of PPARγ. Activation of Sirt1 by resveratrol in MSCs increased its binding to PPARγ and repressed PPARγ activity by involving its cofactor nuclear receptor corepressor (NCoR). The

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modulatory effects of resveratrol on nicotinamide-induced expression of PPARγ and its cofactor NCoR were found to be partly mediated by the association between Sirt1 and Runx2 and by the deacetylation of Runx2. Knockdown of Sirt1 protein expression by antisense oligonucleotides abolished the inhibitory effects of resveratrol, namely, nicotinamide-induced Sirt1 suppression and Runx2 acetylation, suggesting that the acetylation of Runx2 is related to down-regulated Sirt1 expression. This study suggests that Runx2 acetylation/deacetylation is important during osteogenic differentiation in MSCs.[21]

Synergistic actions of resveratrol and curcumin

Combinations of phytochemicals and phytoestrogens are thought to exert synergistic effects *in vitro* and *in vivo*. Recently, we have critically reviewed the scientific evidence and rationale for the development of phytochemicals such as curcumin and resveratrol as nutraceutricals for joint health.[23] Curcumin and resveratrol have the capacity to target NF-κB signaling and inflammation in osteoarthritis. Recent studies from our laboratories have focused on the synergistic anti-inflammatory effects of curcumin and resveratrol on cartilage cells (chondrocytes), when these agents are used in combination. *In vitro*, resveratrol and curcumin have been shown to inhibit IL-1β–induced apoptosis in chondrocytes, by inhibition of caspase 3 and downregulation of the NF-κB pathway.[24–27] Resveratrol and curcumin have also been shown to suppress NF-κB–dependent proinflammatory mediators such as PGE_2, leukotriene B_4 (LTB_4), COX2, MMP-1, MMP-3, and MMP-13. These studies highlight the fact that combinations of these phytochemicals may be more effective than the individual compounds by themselves. Treatment with curcumin and resveratrol suppresses the expression of NF-κB–regulated gene products involved in inflammation (e.g., COX2, MMP-3, MMP-9, and vascular endothelial growth factor [VEGF]).[25] Combinations of curcumin and resveratrol inhibit apoptosis and prevent activation of caspase 3.[25] Closer examination of the signaling pathway has shown that IL-1β–induced NF-κB activation can be suppressed directly by mixtures of curcumin and resveratrol through inhibition of IKK and proteasome activation, inhibition of IκBα phosphorylation and degradation, and inhibition of nuclear translocation of NF-κB. Combining curcumin and resveratrol also activates MEK/Erk signaling. The mitogen-activated protein kinase (MAPK) pathway is stimulated in differentiated chondrocytes and is an important signaling cascade for the maintenance of the chondrocyte phenotype. Activation of this pathway is thought to be required for the maintenance of chondrocyte differentiation and survival. These observations support the enhanced potential of combination therapy, with both anti-inflammatory and antiapoptotic capabilities mediated by inhibition of multiple components of the NF-κB pathway, to treat osteoarthritis and osteoporosis.

Recent work suggests that resveratrol has osteogenic effects on MSCs.[13] For example, resveratrol promotes osteogenesis of human MSCs by upregulating Runx2 gene expression by activating the Sirt1–FOXO3a axis.[28] Resveratrol enhances the canonical Wnt signaling pathway, thus promoting osteoblastic differentiation of MSCs.[28] Resveratrol also enhances osteoblastic differentiation in MSCs via Erk1/2 activation.[29]

In addition, *in vitro* combinations of resveratrol and curcumin have the synergistic potential to promote chondrogenic and osteogenic differentiation of MSCs by targeting NF-κB. For example, treating MSC cultures with curcumin has been shown to suppress NF-κB, thus establishing a microenvironment in which the effects of proinflammatory cytokines are antagonized.[30] This facilitates the chondrogenesis of MSC-like progenitor cells co-cultured with primary chondrocytes.[30] The use of this strategy *in vitro* may support the regeneration of articular cartilage in cell-based cartilage repair techniques, such as autologous chondrocyte implantation (ACI), since cell-based repair of lesions in articular cartilage will be compromised in already inflamed joints. Resveratrol-mediated modulation of Sirt1 and Runx2 promotes osteogenic differentiation of MSCs.[21] Our work also suggests that acetylation and deacetylation of Runx2 are critical for osteogenic differentiation.[21]

Based on these results, we (and others) have proposed that combining these natural compounds may potentially be a more useful strategy for supporting cartilage and bone health than using each individual compound alone. The data available suggest that combinations of phytochemicals work well in culture, but further research is required in animal models and human subjects.

Resveratrol, sirtuins, and mitochondrial function

The identification of naturally occurring compounds capable of altering mitochondrial function could complement strategies to reduce cartilage degradation in osteoarthritis.[31] Similar approaches may support bone turnover in osteoporosis. Regulating cartilage and bone metabolism, autophagy, and apoptosis may be achieved naturally, through pharmacological and physiological modulation of sirtuins. As described earlier, sirtuins are a family of seven NAD^+-dependent deacetylases that may be activated by NAD^+ and the antioxidant phytochemical resveratrol.[32] Resveratrol has been shown to protect chondrocytes against oxidant injury and apoptosis through its effects on mitochondrial repolarization and ATP production.[33] The authors have recently reviewed the potential benefits of resveratrol for enhancing chondrocyte function.[23,34] Dietary supplementation with resveratrol and related antioxidant phytoestrogens may be another important nutritional preventive strategy for osteoarthritis and osteoporosis, especially in people with compromised antioxidant systems. Indeed, it has been reported that resveratrol, as a natural polyphenolic compound, can protect various tissues against oxidative damage: its chemical structure contains electron donors that can prevent hydroxyl radical and superoxide anion formation, thereby suppressing lipid peroxidation, protein oxidation, and DNA damage.[35,36]

Conclusions

The aim of this article was to review the effects of resveratrol on bone cells *in vitro* and highlight the osteogenic and osteoinductive effects of this compound in studies of bone cells in culture. Dietary resveratrol may have potential benefits for modulating bone resorption in age-related, hormone-dependent, and postmenopausal osteoporosis. Cross-sectional studies have demonstrated a positive association between higher fruit intake and higher bone mineral density.[37,38] Therefore, there is considerable interest in finding natural alternatives in foods and combining them with vitamins for further synergistic action on bone.[39] This highlights the potential of natural osteoinductive phytoestrogens such as resveratrol for the nutritional

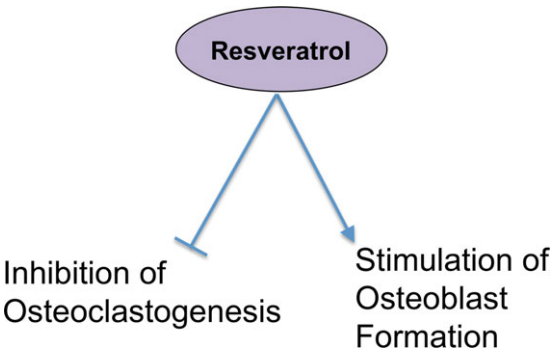

Figure 2. **The effects of resveratrol on osteoclastogenesis and osteoblast formation.**

support of bone. The schematics in Figures 2 and 3 summarize the key effects of resveratrol on bone cells (osteoblasts and osteoclasts) and MSCs. The currently available *in vitro* data support the role for resveratrol in bone health in general. However, it is important to note that the potential for using resveratrol in the treatment of osteoporosis is currently underdeveloped and requires further investigation and extensive clinical trials. The major challenges facing this area of research are the safety and bioavailability of resveratrol.

According to the data available on TOXNET (http://toxnet.nlm.nih.gov/), safety issues related to the consumption of natural forms of resveratrol are relatively minor. Pregnant women and nursing mothers are advised to avoid the use of resveratrol-containing supplements; they should also avoid the use of wine as a primary source of resveratrol. There are no reported safety concerns for older women. However, closer examination of the evidence available suggests that the issue of the safety of resveratrol (and its analogues) is controversial. In 2010, the pharmaceutical company GlaxoSmithKline (GSK) suspended a clinical trial of SRT501 (http://www.clinicaltrials.gov/show/NCT00920556), a proprietary form of resveratrol, due to safety concerns, and terminated the study. The pharmacokinetics, bioavailability, and safety profile of *trans*-resveratrol have recently been studied in healthy human volunteers.[40] The study was a double-blind, randomized, placebo-controlled investigation of increasing concentrations of *trans*-resveratrol (25, 50, 100, or 150 mg), given orally, six times a day, for a maximum of 13 doses. Peak plasma concentrations of

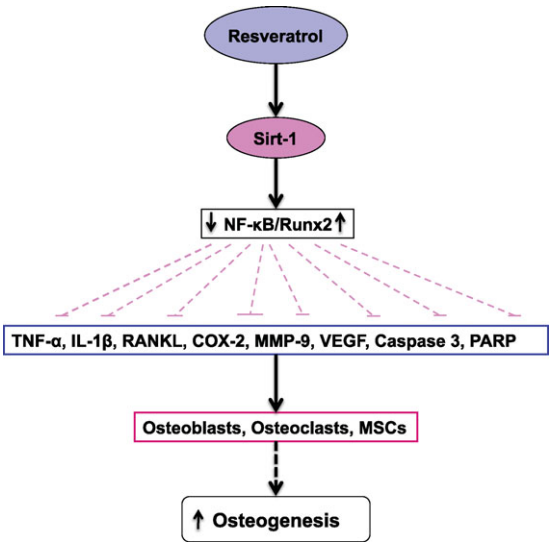

Figure 3. Proposed signaling pathway summarizing the molecular targets of resveratrol in bone and its effects on osteogenesis.

trans-resveratrol were reached at 0.8–1.5 h after oral ingestion, and these were higher after morning administration. Although repeated oral administration of high doses with short dosing intervals was well tolerated, this approach produced relatively low plasma concentrations of *trans*-resveratrol; 150 mg of *trans*-resveratrol resulted in a peak plasma concentration (C_{max}) of 63.8 ng/mL.

Another underdeveloped area relating to research on resveratrol is the paucity of clinical trials. No convincing randomized clinical trials have been conducted to test the *in vivo* efficacy and safety of resveratrol thus far. The poor bioavailability of resveratrol is a major problem affecting the design of clinical trials of various oral formulations. Once this issue has been satisfactorily addressed, large-scale clinical trials will be needed to determine whether resveratrol supplementation will prove beneficial for bone health in estrogen-deficient young patients and cohorts of elderly men and women over 75 years of age. Finally, resveratrol analogues act as antagonists of osteoclast activity and promoters of osteoblast function.[41] Despite the suspension of the clinical trial of SRT501, there are opportunities for the pharmaceutical industry for developing resveratrol analogues with enhanced gastrointestinal absorption and bioavailability for modulating bone remodeling. Future work will need to evaluate the risk–benefit ratio of resveratrol supplementation and to consider the cost of supplements. We and other investigators will be evaluating the emerging evidence for the potential beneficial effects of resveratrol on bone health (as we have recently done for glucosamine[42]) and will propose new strategies for the design of clinical trials aimed at identifying beneficial physiological effects on bone.

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Conflicts of interest

The authors declare no conflicts of interest.

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