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Design, Synthesis, and Osteogenic Activity of Daidzein Analogs on Human Mesenchymal Stem Cells

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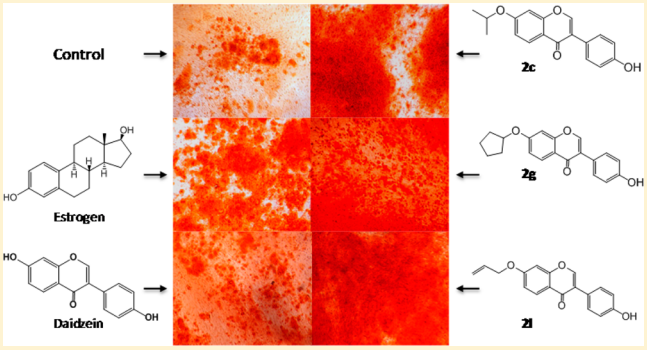
Supporting Information

ABSTRACT: Osteoporosis is caused by an overstimulation of osteoclast activity and the destruction of the bone extracellular matrix. Without the normal architecture, osteoblast cells are unable to rebuild phenotypically normal bone. Hormone replacement therapy with estrogen has been effective in increasing osteoblast activity but also has resulted in the increased incidence of breast and uterine cancer. In this study we designed and synthesized a series of daidzein analogs to investigate their osteogenic induction potentials. Human bone marrow derived mesenchymal stem cells (MSCs) from three different donors were treated with daidzein analogs and demonstrated enhanced osteogenesis when compared to daidzein treatment. The enhanced osteogenic potential of these daidzein analogs resulted in increased osterix (Sp7), alkaline phosphatase (ALP), osteopontin (OPN), and insulin-like growth factor 1 (IGF-1), which are osteogenic transcription factors that regulate the maturation of osteogenic progenitor cells into mature osteoblast cells.



KEYWORDS: *Daidzein analogs, mesenchymal stem cells, BMSCs, osteogenesis*

Osteoporosis, characterized by the loss of bone mass and strength that leads to fragility fractures, is a major clinical concern due to significant morbidity and mortality in postmenopausal women.[1] The prevalence of osteoporosis in the United States is estimated to increase from 10 million to 14 million people in 2020, increasing the economic burden from $17 billion to $25 billion.[2,3] The broad impact of osteoporosis across age, sex, race, ethnicity, and fracture site underscores the need for increased awareness of the disease and the development of novel therapeutic interventions for patients.[2]

The loss of bone mass results from an imbalance of two fundamental processes related to bone turnover: (1) excessive bone resorption, resulting in decreased bone mass and architectural deterioration of bone, and/or (2) inadequate formation in response to increased resorption during bone remodeling.[4] Excessive resorption without adequate bone formation can result in complete loss of trabecular structure, destroying the template for future bone formation. Inability to form bone during remodeling also contributes to the pathogenesis of osteoporosis. Estrogen deficiency during aging is likely to be the most important risk factor in the pathogenesis of bone fragility in women, as bone loss accelerates after menopause.[5,6] Estrogen therapy has been shown to prevent the early phases of bone loss and decrease the incidence of subsequent osteoporosis-related fractures by about 50%.[5] However, the incidence of endometrial cancer is increased with estrogen therapy and outweighs the benefits of reducing osteoporosis.[6] As alternatives, calcium, vitamin D, calcitonin, and bisphosphates have been investigated. The use of bisphosphates has shown significant improvement in bone density relative to controls, while higher concentrations and longer delivery period of calcium, vitamin D, and calcitonin only demonstrated modest improvements.[7] These bisphosphate compounds are carbon-substituted analogs of pyrophosphate, an endogenous physiologic inhibitor of bone mineralization, and are thus potent inhibitors of bone resorption.[7,8]

Nevertheless, concomitant delivery of drugs capable of increasing the number of osteoblasts or enhancing osteoblastic activity without increased cancer risk continues to be an attractive addition to bisphosphate therapy. Phytoestrogenic

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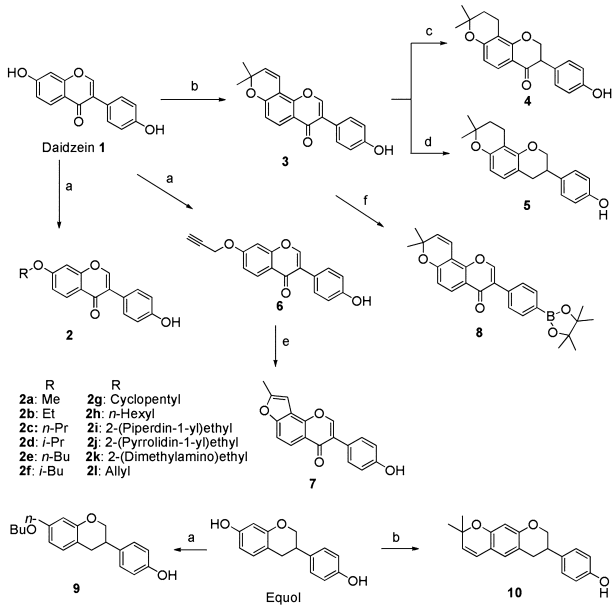
molecules, which mimic the effects of estrogen, have received a great deal of attention due to their potential to prevent hormone-related cancers and bone loss.[9−12] Isoflavones are found in abundance in soybeans and their derivative foods. *In vitro* studies have shown that daidzein and genistein have stimulatory effects on protein synthesis and on alkaline phosphatase (ALP) released by various types of osteoblast cells.[11] Recent studies have demonstrated that ovariectomy-induced bone loss in rats is rescued with the delivery of the isoflavones daidzein and genistein.[9,10] Epidemiological studies in humans have shown that high dietary phytoestrogen intake is associated with higher bone mineral density in postmenopausal women.[12]

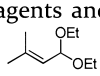
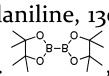
Daidzein is a potent estrogenic compound that has a beneficial effect on bone health,[11,12] but its clinical potential is limited by its low bioavailability, unfavorable metabolism, and uterine estrogenicity. A recent study reported improved functions of isoformononetin, a naturally occurring methoxydaidzein for the bone anabolic effect.[13] At the most effective osteogenic dose of isoformononetin, plasma and bone marrow levels were 90% isoformononetin and 10% daidzein. Also under these conditions, isoformononetin induced mesenchymal stem cell (MSC) mineralization and osteogenic gene expression in the calvaria of neonatal rats without causing uterine pathogenesis. More recently, Yadav et al. reported a series of synthetic daidzein analogs with both 7-OH and 4′-OH modified with various substitutions to exhibit a stronger osteoblast stimulating effect than daidzein.[14] Interestingly, a related isoflavone, genistein, which is a stronger estrogen than daidzein, was not as effective a bone loss inhibitor as daidzein,[10] suggesting that daidzein may be a better lead compound for pharmacophoric optimization of potential osteogenic therapeutic agents.

Previous studies found that replacing the 7-OH of daidzein with alkoxy groups attenuated the estrogenic potency to various degrees, with longer and bulkier substitutions having greater effects.[15] Moreover, some structural modifications on the 7-O position conferred a dramatic reversal from estrogenic to antiestrogenic property, suggesting the versatility of the daidzein structural motif could offer additional pharmacological functions that require an optimal balance of hormonal activities of the compounds. In this study, we investigate the utility of a series of daidzein analogs in promoting bone formation. The effects of structural variations of daidzein on the osteogenic induction of human bone marrow derived MSCs, which differentiate into osteoblasts under appropriate stimulation, were explored. Structural variations at the 7-OH position and the central daidzein moiety were made to test how the osteogenic activities varied as a result of such changes in substitution and the daidzein skeleton. Because it has been shown recently that equol, the metabolic product of daidzein, may be responsible for its superior bone-healing property compared to genistein and other isoflavones,[10] several racemic equol analogs were also synthesized and tested for potential gain of activity. We show that daidzein analogs can be potent stimulators of osteogenesis in MSCs in a dose-dependent manner. To understand how these daidzein analogs exert enhanced osteogenic potential in the MSCs, a relevant gene expression panel was analyzed and results were discussed for mechanistic interpretations.

As shown in Scheme 1, selective *O*-alkylation on the 7-hydroxyl group of daidzein (**1**) gave derivatives **2a−l** and **6**.[15] The derivative **3** was obtained by the reaction of **1** and 1, 1-diethoxy-3-methylbut-2-ene with picoline as the base under microwave irradiation. Further, the hydrogenation of **3** using 10% Pd/C resulted in the formation of derivative **4** or **5** as the major product, respectively, depending on the use of ethyl acetate or methanol as solvent. The derivative **7** was prepared by the successive Claisen rearrangement and cyclization of **6** in refluxing *N*,*N*-diethylaniline with CsF as the catalyst. Reacting with trifluoromethanesulfonic anhydride, **3** was converted into its triflate, which was the starting material for the synthesis of derivative **8** through the $PdCl_2$(dppf)-catalyzed borylation using the diboron reagent in the presence of KOAc. *O*-Butylation on the 7-hydroxyl group of equol gave the derivative **9**. The derivative **10** was obtained by the reaction of equol and 1,1-diethoxy-3-methylbut-2-ene in refluxing xylene with picoline as the base under microwave irradiation.

Scheme 1. Synthesis of Daidzein Derivatives and Analogs



Reagents and conditions: (a) K_2CO_3, DMSO, RBr or RCl, rt; (b)  , picoline, 130°C, microwave; (c) H_2, 10% Pd/C, ethyl acetate, rt; (d) H_2, 10% Pd/C, MeOH, rt; (e) CsF, *N*,*N*-diethylaniline, 130°C; (f) i). $(CF_3SO_2)O$, pyridine, CH_2Cl_2, 0°C - rt; ii).  , $PdCl_2$(dppf), KOAc, dioxane, 120°C.

Scheme 2 shows the synthetic route for compounds **11** and **12a−b**. The condensation of 2,4-dimethoxybenzoic acid and 4-anisidine or 4-((tetrahydro-2H-pyran-2-yl)oxy)aniline with DCC and DMAP as activating agents gave **12a** or 2,4-dimethoxy-*N*-(4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl) benzamide, respectively. Compound **11** was obtained by the deprotection of the intermediate benzamide in methanol with 4-toluenesulfonic acid. Finally, *O*-isobutylation of **11** in DMSO afforded compound **12b** in the presence of K_2CO_3.

Characterization of MSCs. MSCs undergo osteogenic differentiation under appropriate stimulation. MSCs were isolated from bone marrow aspirates from three donors and expanded separately prior to characterization. The demographic information for the three donors is provided in Supporting Information Table 1S. Each MSC donor (n = 3) was analyzed for the expression of cell surface markers and was positive for CD44, CD90, CD105, and CD166 and negative for CD34, CD45, and CD11b, determined with flow cytometry

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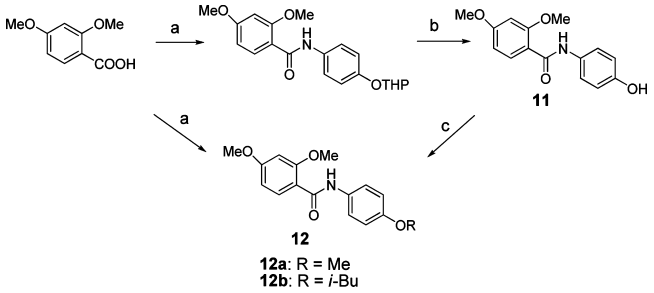
Scheme 2. Synthesis of 11, 12a, and 12b



Reagents and conditions: (a) 4-methoxy- or 4-THPO-aniline, DCC, DMAP, DMF, 0 °C to rt; (b) 4-toluenesulfonic acid, MeOH, reflux; (c) K_2CO_3, *i*-butylbromide, DMSO, rt.

(Supporting Information Figure S1). All MSCs were able to generate colony-forming units and undergo osteogenesis and adipogenesis (Supporting Information Figure S1). No differences were observed among the three MSC donors in cell surface marker profile, differentiation, or self-renewal capacity, as defined by colony forming units.

Estrogen and Daidzein Enhance Osteogenic Differentiation of MSCs. To investigate the osteogenic potential of estrogen, daidzein, and genistein, MSCs from three donors were individually treated with vehicle, estrogen, daidzein, or genistein, stained with Alizarin Red, and destained to quantify the amount of stain for each treatment group. Alizarin red is a good indicator of osteogenic differentiation, as it stains calcified extracellular matrix associated with bone formation. Estrogen enhanced osteogenic differentiation of MSCs by a fold of 1.5 ± 0.09, 1.5 ± 0.18, and 1.6 ± 0.09 relative to vehicle-treated MSCs from Donor 1, Donor 2, and Donor 3, respectively (P < 0.05; Figure 1), and daidzein enhanced the osteogenic differentiation of MSCs by 1.7 ± 0.19, 1.7 ± 0.01, and 1.6 ± 0.15 in MSCs from Donors 1 to 3, respectively (P < 0.05; Figure 1). Consistent with previous studies, MSCs can be induced to undergo osteogenic differentiation; in the presence of estrogen, daidzein, or genistein, the overall potential to undergo differentiation is enhanced.[9−12] Only estrogen and daidzein consistently enhanced osteogenic differentiation of MSCs in all three donors. Genistein enhanced the osteogenic differentiation of MSCs by (1.6 ± 0.19)-fold and (1.7 ± 0.02)-fold relative to vehicle-treated MSCs from Donor 1 and Donor 2, respectively (P < 0.05; Figure 1), but not in MSCs from Donor 3 (1.0 ± 0.15; P > 0.05; Figure 1). Our model based on the use of MSCs has thus verified the known osteogenic activities of estrogen, daidzein, and genistein, and will be next used to investigate the osteogenic potential of the synthetic daidzein analogs.

Daidzein Analogs Enhanced Osteogenic Differentiation of MSCs with Greater Efficacy than Estrogen or Daidzein. To investigate the efficacy of the daidzein analogs on osteogenic differentiation of MSCs, 23 daidzein analogs were screened to determine their ability to enhance osteogenic differentiation. Initially, MSCs from one donor (age: 21 yrs old; BMI: 22.7; race: Caucasian, gender: female) were treated with estrogen, daidzein, or daidzein analog in osteogenic differentiation media (ODM). After 14 days, estrogen and daidzein enhanced osteogenic differentiation of MSCs 1.7 ± 0.2 (P < 0.05; Figure 2A) and 1.7 ± 0.3 (P < 0.05; Figure 2A) relative to vehicle-treated MSCs in ODM, respectively. The effect of the daidzein analogs on osteogenic differentiation can be divided

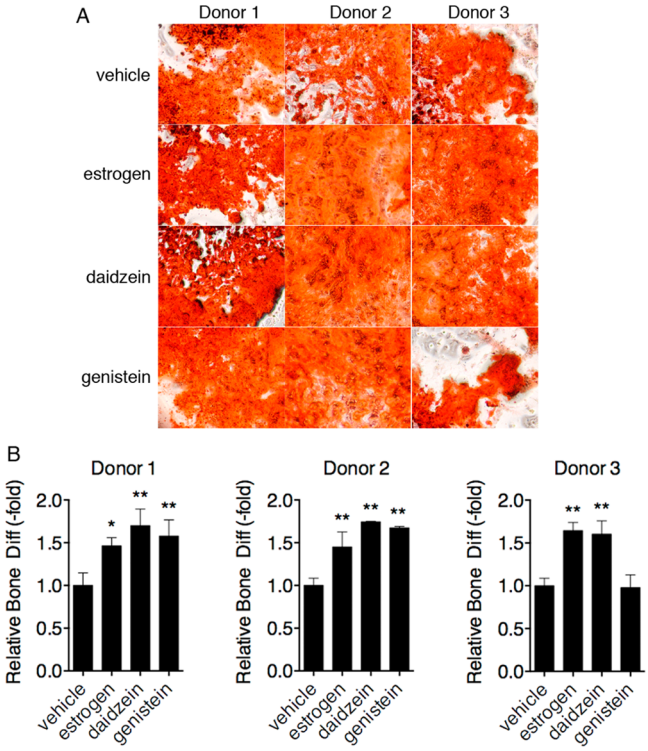


Figure 1. Phytoestrogens enhance osteogenesis of MSC. MSCs from three donors were plated and allowed to grow to 70% confluence before being induced to differentiate and being simultaneously exposed to estrogen (10 nM), daidzein (1 μM), and genistein (1 μM). MSCs induced to undergo osteogenesis for 14 days were stained with Alizarin Red S to detect calcium deposits, destained with cetylpyridinium chloride, and measured at 590 nm to quantify the amount of osteogenic differentiation. (A) Representative images of Alizarin Red-stained MSCs treated with vehicle, estrogen, daidzein, or genistein. Images were acquired at 4× magnification. (B) Bone differentiation was determined relative to vehicle-treated cells normalized to 1.0. *, P < 0.05; **, P < 0.01.

into three groups: no induction, low induction, and high induction. Sixteen of the 23 daidzein analogs enhanced osteogenic differentiation (P < 0.05; Figure 2A). Nine of the 23 daidzein analogs fall into the low induction category and induce osteogenic differentiation 1.6-fold to 2.0-fold relative to vehicle: **2d**, **2b**, **2a**, **4**, 7, **6**, **10**, **2g**, and **12b**, in ascending order. Seven of the daidzein analogs were high inducers of osteogenic differentiation (as high as >2.4-fold increase relative to vehicle): **2l**, **2f**, **12a**, **2i**, **2c**, **2h**, **2e**, in ascending order. When tested alongside the daidzein analogs, estrogen and daidzein were only low inducers of osteogenic differentiation.

To identify the most potent osteogenic daidzein analogs and determine the robustness of the daidzein analogs in inducing osteogenesis, the 10 osteogenic daidzein analogs that resulted in the highest levels of differentiation in Donor 1 were further examined after 14 days of differentiation in a total of three donors (mean age: 22.4 years old; mean BMI: 21.5; race: Caucasian; gender: female). Of the ten compounds investigated, **2i** and **2d** were cytotoxic to MSCs from Donor 2 and Donor 3 (Figure 2B). The other 8 compounds, **2c**, **2f**, **2g**, **2e**, **2h**, **2l**, **10**, **11**, and **12a**, enhanced osteogenesis in all three donors, increasing osteogenesis by 1.3−5.0-fold relative to vehicle-treated MSCs (P < 0.05). Additionally, it was observed that several daidzein analogs enhanced osteogenic differentiation significantly more in Donor 3, than in Donor 1 or

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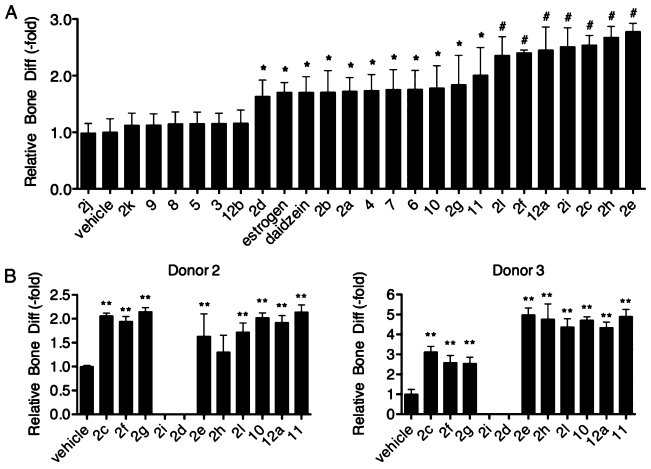

Figure 2. Screening of daidzein analogs identifies several molecules that show enhanced osteogenesis of MSCs compared to daidzein. MSCs from (A) Donor 1, (B) Donor 2, and Donor 3 were induced to undergo osteogenic differentiation and simultaneously exposed to vehicle (DMSO), estrogen (10 nM), daidzein (1 μM), or daidzein analogs (1 μM) at every medium change. After 14 days in osteogenic differentiation media, bone differentiation potential was determined relative to vehicle-treated cells normalized to 1.0. *, $P < 0.05$; #, $P < 0.01$; **, $P < 0.001$.

2. While no significant differences were observed through differentiation, self-renewal, or cell surface marker profile, Donors 1 and 2 are between the ages of 21 and 22, while Donor 3 is 33-years-old. It is possible that the enhanced osteogenic differentiation could be an age-related effect, but additional studies are necessary to determine the cause of the enhanced osteogenic differentiation in Donor 3.

Selected Daidzein Analogs Exhibit Low EC50 Concentrations in Stimulating Bone Differentiation. To further determine the dose-dependence of the daidzein analogs in osteogenic activity, the degree of bone differentiation of the MSCs was determined following treatment with varying concentrations of three selected daidzein analogs: **2c**, **2f**, **2g**, and **2l**. These analogs demonstrated enhanced differentiation at day 7 and more robust osteogenic stimulation (Figure 3). To test the dose response of these compounds on osteogenesis, cells were treated with 1 nM to 1 μM concentration of the daidzein analog to assess the EC50. As shown in Figure 4, the three analogs appear to have EC50 values in the low nanomolar to low micromolar range, depending on donor. While donor

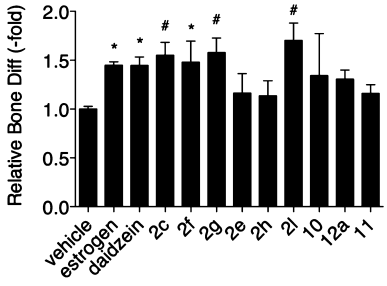

Figure 3. Daidzein analogs enhance osteogenesis of MSCs as early as Day 7. MSCs from three donors were induced to undergo osteogenic differentiation and simultaneously exposed to vehicle (DMSO), estrogen (10 nM), or daidzein analogs (1 μM) at every medium change for 7 days. The mean bone differentiation potential of the three donors is shown. *, $P < 0.05$; #, $P < 0.01$.

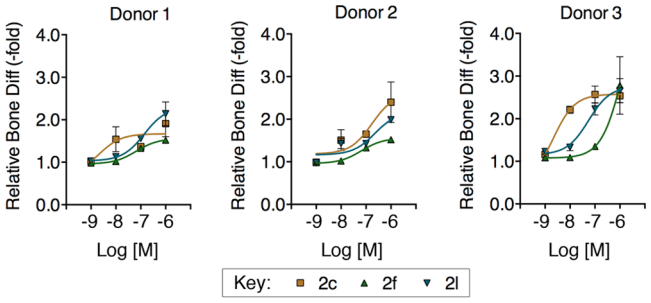

Figure 4. Enhanced osteogenic potential by the daidzein analogs is dose dependent. MSCs from three donors were induced to undergo osteogenic differentiation and simultaneously exposed to daidzein analogs **2c**, **2f**, or **2l** at 1 nM, 10 nM, 100 nM, or 1 μM concentrations at every medium change.

variability adds to the uncertainty in EC50 values, the order of osteogenesis potency for the three analogs in different donors appears to be consistent. For example, the EC50 for analog **2f** for Donor 1, Donor 2, and Donor 3 was 65.8 nM, 65.7 nM, and 1.38 μM, respectively, representing the least potent analog. In contrast, the EC50 for analog **2c** was 2.2 nM and 2.1 nM in the MSCs from Donor 1 and Donor 3, and 170 nM in Donor 2 cells, representing the most potent analog among the three ($P < 0.05$; Figure 4).

Daidzein Analogs Enhance Osteogenic Transcription Factor Expression in MSCs. To investigate the mechanism by which daidzein analogs enhance osteogenic differentiation of MSCs, cells treated with **2c** as a supplement to ODM for 14 days were compared to cells treated with vehicle in ODM. Cells treated with ODM containing **2c** expressed higher concentrations of ALP (2.45), Sp7 (20.08), OP (2.68), IGF1 (26.96), and Cola1 (8.00) relative to control ($P < 0.05$; Figure 5), while no significant differences were observed in cbfa-1, cFOS, Dlx5, or ON (Supporting Information Figure S2). Sp7 is one of the earlier genes necessary to commit progenitor stem cells or

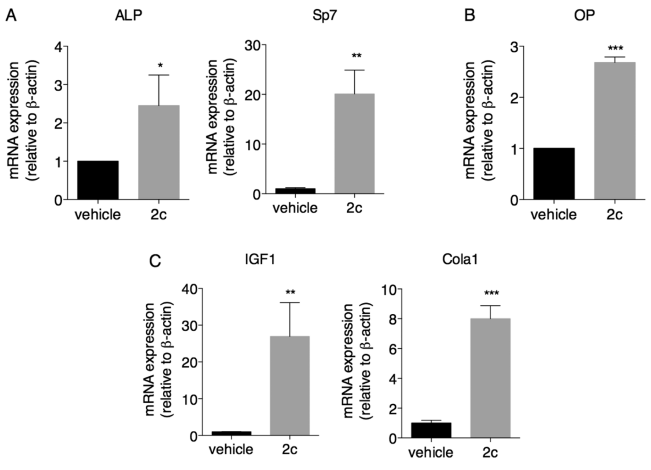

Figure 5. Enhanced osteogenic potential correlates with enhanced expression of osteogenic transcription factors. MSCs from three donors were pooled together, induced to undergo osteogenic differentiation, and simultaneously exposed to vehicle or daidzein analog **2c** (1 μM) at every medium change. After 14 days, cells were harvested, mRNA was isolated, and cDNA was synthesized from each sample. Real time RT-PCR of transcript levels of genes involved in (A) early, (B) middle, and (C) late osteogenesis was analyzed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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MSCs into the osteoblast lineage.[16,17] Following lineage commitment, osteoprogenitors undergo a proliferative stage and subsequently express genes, such as alkaline phosphatase (ALP), bone sialoprotein, and collagen type I, as they produce the osteogenic extracellular matrix. In the final stages of maturation, these osteoprogenitor cells mineralize the extracellular matrix and produce osteopontin (OP), osteocalcin, and collagen type 1 (Cola1). Once mature bone is formed, insulin-like growth factor 1 (IGF-1) regulates bone density to allow for strength and durability.[18,19] This highly regulated program of gene expression and cellular differentiation is governed by the expression and activity of a myriad of transcription factors. These factors act in conjunction with other transcription factors in an integrated and specific manner to allow for successful generation of bone.

Increased expression of Sp7, ALP, OP, IGF-1, and Cola1 in human MSCs treated with daidzein analog **2c** supporst increased bone formation observed phenotypically. Recent studies have described similar increases in ALP and IGF-1 expression in mouse osteoblast cells exposed to estrogen.[20,21] Additional studies on daidzein have investigated the upregulation of downstream receptors, such as estrogen receptor α (ERα) and estrogen receptor β (ERβ). These studies have demonstrated that daidzein increases not only ERα and ERβ expression but also vitamin D receptor synthesis, suggesting that daidzein promotes bone formation and maturation through these estrogen dependent pathways.[22,23] To determine the estrogenic or antiestrogenic activities that these daidzein analogs may have toward ER, we performed the ERE luciferase assays for 11 selected compounds (Supporting Information Table 2S). Most of the osteogenic analogs showed some level of diminished estrogenic activity compared to daidzein. Their antagonistic properties were also low to modest. Additional assays using the HEK293 cells transfected with ERα or ERβ revealed that these analogs were not selective for either estrogen receptor (Supporting Information Table 3S). While further mechanistic studies are necessary to delineate the exact mode of action and pathways involved, results obtained thus far suggest that these daidzein analogs are likely to be more efficient activators of the estrogen pathway, increasing the synthesis and maturation of bone.

Structure–Activity Relationships. Of the twelve **2** series daidzein analogs, **2a** and **2b** showed modest osteogenic activity comparable to estrogen and daidzein, while **2d** exhibited toxicity in two donor cells and modest osteogenesis in the third. Thus, methyl, ethyl, and *n*-propyl substitutions for the hydroxyl hydrogen in daidzein failed to bring about significantly enhanced osteogenic potential. When the substitution group was isopropyl (**2c**), *n*-butyl (**2e**), 2-butyl (**2f**), allyl (**2l**), *n*-hexyl (**2h**), or cyclopentyl (**2g**), marked enhancement of osteogenic potentials was observed. In fact, these analogs represent the most potent compounds in promoting bone formation in the stem cells of all three donors. Interestingly, analog **6**, in which the substitution is a propyl-1-yne, showed only modest osteogenic potential. When comparing the activities of **6**, **2d**, and **2l**, it is clear that the optimal structure for a three-carbon substitution is one containing a double bond, not a triple bond, nor a saturated σ bond. Also, in the **2** series, polar substitutions containing nitrogen (**2i**, **2j**, and **2k**) showed either little osteogenic effect (**2j** and **2k**), or toxicity (**2i**), indicating that increased polarity on the 7-O position may not be desirable for retaining osteogenic activity. Interestingly, in the reported work of Yadav et al.,[14] polar substitutions of the 7-O position, accompanied by transformation of the 4′-OH into a methoxy group, showed a strong osteoblast-stimulating effect in vitro, suggesting the important role of the 4′-O substitution in conferring the overall osteogenic activity.

Other structural modifications involving cyclization on the 7,8 position did not yield significant osteogenic potential. For example, of the three cyclic analogs (**3**, **4**, and **7**), analog **3** did not exhibit any osteogenic activity while **4** and **7** showed modest potential in promoting bone regeneration. When the daidzein structural moiety in the center was modified to that of racemic equol, a daidzein metabolite, there appeared to be a significant loss of activity. For example, the analog **2e** is one of the most potent compounds, promoting over 2-fold increase of bone formation in 14 days. Change of the central moiety of the molecule to that of equol (i.e., reductive removal of the carbonyl and the double bond on the central six-membered ring) results in analog **9**, which has no osteogenic activity in any of the three donor stem cells. In another comparable pair, the daidzein analog **4** showed modest osteogenic potential while the equol analog **5** had no discernible osteogenic activity.

In conclusion, we have designed and synthesized 23 daidzein analogs as potential osteogenesis agents, of which at least 16 showed significant bone differentiation activities in MSCs. Treatment with daidzein analogs resulted in increased osterix (Sp7), alkaline phosphatase (ALP), osteopontin (OPN), and insulin-like growth factor 1 (IGF-1), which are osteogenic transcription factors that regulate the maturation of osteogenic progenitor cells into mature osteoblast cells. These daidzein analogs thus represent novel organic chemical entities that could be further explored as promising therapeutic lead compounds for treatment of osteoporosis and for promoting bone formation.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for synthesis and characterization of the daidzein analogs, cell culture, and MSC characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ALP, alkaline phosphatase; BMI, body mass index; cbfa-1, runt-related transcription factor 2; cFOS, c-FBJ murine osteosacroma viral onogenic homologue; DCC, *N*,*N*′-dicyclohexylcarbodiimide; dppf, bis(diphenylphosphino)ferrocenepalladiuM(II)-dichloride; Dlx5, distal-less homeobox 5; DMAP, 4-dimethylaminopyridine; ER, estrogen receptor; IGF-1, insulin-like growth factor 1; MSC, mesenchymal stem cells; ODM, osteogenic

differentiation media; ON, osteonectin; OP, osteopontin; Sp7, osterix; THP, tetrahydropyranyl

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