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Differential effects of formononetin and cladrin on osteoblast function, peak bone mass achievement and bioavailability in rats☆,☆☆

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Abstract

Dietary soy isoflavones including genistein and daidzein have been shown to have favorable effects during estrogen deficiency in experimental animals and humans. We have evaluated osteogenic effect of cladrin and formononetin, two structurally related methoxydaidzeins found in soy food and other natural sources. Cladrin, at as low as 10 nM, maximally stimulated both osteoblast proliferation and differentiation by activating MEK-Erk pathway. On the other hand, formononetin maximally stimulated osteoblast differentiation at 100 nM that involved p38 MAPK pathway but had no effect on osteoblast proliferation. Unlike daidzein, these two compounds neither activated estrogen receptor in osteoblast nor had any effect on osteoclast differentiation. Daily oral administration of each of these compounds at 10.0 mg kg^{-1} day^{-1} dose to recently weaned female Sprague–Dawley rats for 30 consecutive days, increased bone mineral density at various anatomic positions studied. By dynamic histomorphometry of bone, we observed that rats treated with cladrin exhibited increased mineral apposition and bone formation rates compared with control, while formononetin had no effect. Cladrin had much better plasma bioavailability compared with formononetin. None of these compounds exhibited estrogen agonistic effect in uteri. Our data suggest that cladrin is more potent among the two in promoting parameters of peak bone mass achievement, which could be attributed to its stimulatory effect on osteoblast proliferation and better bioavailability. To the best of our knowledge, this is the first attempt to elucidate structure–activity relationship between the methoxylated forms of daidzein and their osteogenic effects.

Keywords: Osteogenic; Proliferation; Differentiation; MAPK signaling; Peak bone mass

1. Introduction

The adult bone mass of an individual is critically dependent on the achievement of peak bone mass (PBM) during skeletal growth [1]. PBM is described as bone mass and strength achieved at the end of the growth period and is negatively correlated with the risk of osteoporosis fractures occurring after menopause. Phytoestrogens, found in many edible plants are diverse groups of biologically active compounds with structural similarity to estradiol [2]. The major estrogenic isoflavones, including daidzein, genistein and biochainin A, have been shown to have important role in reducing symptoms associated with estrogen deficiency disorders. These compounds may be protective against osteoporosis due to their ability to exert osteogenic and anti-resorptive actions on bone, particularly on bone turnover and growth [3]. High dietary intake of these isoflavones have been reported to increase bone mineral density (BMD) in lumbar spine of Japanese [4], Chinese [5] and American [6] postmenopausal women. Perinatal exposure to phytoestrogens has been reported to lead to a higher BMD later in life [1]. However, the effect of phytoestrogens on PBM achievement has not been investigated in detail.

Daidzein is an extensively studied phytoestrogen with respect to its skeletal effects. It promotes osteoblast functions and inhibits osteoclast functions in vitro [7]. These effects of daidzein are mediated via the estrogen receptors (ERs) [8]. Daidzein may also exhibit estrogenicity at the uterine level [9,10]. In addition to its direct effect,

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uterine estrogenicity of daidzein is partly contributed by its highly estrogenic metabolite, equol [10]. Ten weeks of daily injection of daidzein at 16.6 mg kg^{-1} dose to growing ovariectomized (Ovx) rats exhibited significant bone forming effects [11]. In adult Ovx mice on high calcium diet, daidzein at 100 mg kg^{-1} day^{-1} oral dose for 12 weeks favorably influenced both trabecular and cortical bone [12]. An intriguing report shows that daidzein fed immature mice exhibited sexually dimorphic skeletal effect with increased BMD and bone formation in males and decreased in females when compared with controls [13]. From these reports, it appears that daidzein could favorably affect PBM achievement, likely via its metabolite, equol [14].

Formononetin [7-hydroxy-3(4-methoxypheny)chromone or 4′-methoxy daidzein] is a soy isoflavonoid that is found abundantly in traditional Chinese medicine *Astragalus mongholicus* (Bunge) [15] and *Trifolium pretense* L. (red clover) [15], and in an Indian medicinal plant, *Butea monosperma* [16]. Crude extract of *Butea monosperma* is used for rapid healing of fracture in Indian traditional medicine [16]. In addition to formononetin, crude extracts of *Butea monosperma* abundantly contains another structurally related methoxylated daidzein, namely cladrin (3′,4′-dimethoxy daidzein). We hypothesized that formononetin and cladrin have osteogenic action and therefore investigated the effects of formononetin and cladrin in osteoblast functions in vitro and bone formation in vivo. Fundamental differences were apparent between these two methoxylated daidzeins and daidzein. Our study also reveals possible structural attributes and pharmacokinetic properties that may contribute to differences in their bone forming activity.

2. Materials and methods

2.1. Reagents and chemicals

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). All fine chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). p38 MAP kinase ELISA kit was purchased from Cell Signaling Technologies, Danvers, MA, USA. ECL kit was purchased from Amersham Pharmacia, USA. All antibodies for Western blot analysis were obtained from Cell Signaling Technologies. bromodeoxyuridine (BrdU) ELISA kit was procured from Roche (USA).

Reference standards of daidzein and equol were purchased from Indofine Chemical (Hillsborough, NJ, USA). 4-Hydroxymephenytoin (internal standard) was purchased from Sigma Aldrich. High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Sisco Research Laboratories (Mumbai, India). Diethyl ether was purchased from TKM Pharma (Hyderabad, India). Glacial acetic acid AR was purchased from E Merck (Mumbai, India). Milli-Q pure water was obtained from a Millipore Elix water purification system purchased from Millipore India (New Delhi, India). Heparin sodium injection i.p. (1000 IU/ml) was purchased from Gland Pharma (Hyderabad, India). Blank, drug free plasma samples were collected from adult, healthy female Sprague–Dawley rats at Division of Laboratory Animals of Central Drug Research Institute (Lucknow, India). Plasma was obtained by centrifuging the heparinised blood (25 IU/ml) at 13,000 rpm for 10 min. Prior approval from the Institutional Animal Ethics Committee was sought for maintenance, experimental studies, euthanasia and disposal of animal carcass.

HPLC system consisting of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin-Elmer instruments, Norwalk, CA, USA) was used to inject 10-μL aliquots of the processed samples on a Supelco Discovery C18 column. Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an electrospray ionization source.

2.2. Synthesis of compounds

Cladrin and formononetin, initially isolated from *Butea monosperma* [16], were subsequently synthesized in gram scale for all in vitro and in vivo studies. Cladrin and formononetin were synthesized by previously published protocol [17]. Synthesized compounds were matched with the data of the authentic samples and the purities of the compounds were confirmed by HPLC and nuclear magnetic resonance analytical methods [16]. Structures of the compounds are shown in Fig. 1.

2.3. In vitro studies with osteoblasts

2.3.1. Culture of calvarial osteoblasts

Rat calvarial osteoblasts were obtained following our previously published protocol of sequential digestion [18]. Briefly, calvaria from 1- to 2-day-old Sprague–Dawley rats (both sexes) were pooled. After surgical isolation from the skull and the removal of sutures and adherent mesenchymal tissues, calvaria were subjected to five sequential (10–15 min) digestions at 37°C in a solution containing 0.1% dispase and 0.1% collagenase P. Cells released from the second to fifth digestions were pooled, centrifuged, resuspended, and plated in T-25cm^2 flasks in α-minimum essential medium Eagle (MEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (complete growth medium).

2.3.2. Osteoblast proliferation

For the measurement of osteoblast proliferation, osteoblasts at ~80% confluence were trypsinized and 10^3 cells/well were seeded in 96-well plates. Cells were treated with different concentrations of the compounds for 24 h in α-MEM supplemented with 2% charcoal-treated FCS (osteoblast growth medium). After culturing for 22 h, the cells were pulsed with BrdU for 2 h, and the cell population entering S phase was determined by quantifying BrdU incorporation colorimetrically using a kit (Roche).

2.3.3. Osteoblast differentiation

For determination of alkaline phosphatase (ALP) activity, 2×10^3 cells/well were seeded in 96-well plates. Cells were treated with different concentrations of the compounds for 48 h in α-MEM supplemented with 5% charcoal treated FCS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 1% penicillin/streptomycin (osteoblast differentiation medium). At the end of incubation period, total ALP activity was measured using *p*-nitrophenylphosphate as substrate and quantitated colorimetrically at 405 nm [19].

2.3.4. Mineralization of bone marrow cells

For mineralization studies, bone marrow cells (BMCs) from female Sprague–Dawley rats weighing ~40 g were isolated and cultured according to a previously published protocol from our laboratory [18]. Briefly, the femurs were excised aseptically, cleaned of soft tissues, and washed 3×, 15 min each, in a culture medium containing 10 times the usual concentration of antibiotics as mentioned above. The epiphyses of femur were cut off and the marrow flushed out in 20 ml of culture medium consisting of α-MEM, supplemented with 15% charcoal treated 10% charcoal treated FCS, 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. Released BMCs were collected and plated (2×10^6 cells/well of six-well plate) in the culture medium, consisting of α-MEM, supplemented with 15% charcoal treated fetal calf serum, 10^{-7} M dexamethazone, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. Cells were cultured with and without the compounds for 21 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 48 h. After 21 days, the attached cells were fixed in 4% formaldehyde for 20 min at room temperature and rinsed once in phosphate-buffered saline. After fixation, the specimens were processed for staining with 40 mM Alizarin Red-S, which stains areas rich in nascent calcium.

For quantification of alizarin red-S staining, 800 μl of 10% (v/v) acetic acid was added to each well, and plates were incubated at room temperature for 30 min with shaking. The monolayer, now loosely attached to the plate, was then scraped with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5-ml tube. After vortexing for 30 s, the slurry was overlaid with 500 μl mineral oil (Sigma–Aldrich), heated to exactly 85°C for 10 min and transferred to ice for 5 min. The slurry was then centrifuged at 20,000×*g* for 15 min and 500 μl of the supernatant was removed to a new tube. Then 200 μl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. In some cases, the pH was measured at this point to ensure that it was between 4.1 and 4.5. OD (405 nm) of 150 μl aliquots of the supernatant were measured in 96-well format using opaque-walled, transparent-bottomed plates [16,20].

For studying cell signaling events, treatment of inhibitors (ICI182780, U0126, SB203580, SP600125 and LY294002) were given 30 min prior to the compound treatments.

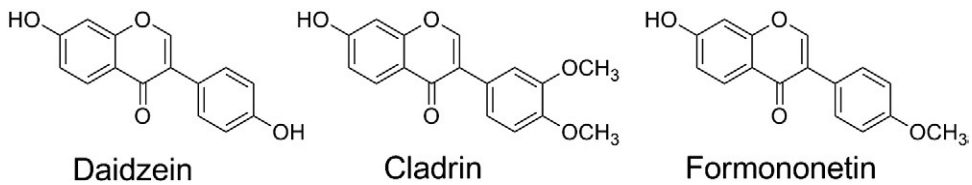

Fig. 1. Structures of daidzein, cladrin and formononetin.

2.3.5. p-38 MAPK ELISA

For measuring total and phospho-p38 mitogen activated protein kinese (MAPK), osteoblasts (20×10^3 cells/well) were seeded in six-well plates. Cells were exposed to formononetin for different time intervals (0, 15, 30, 60, 240 and 1440 min). Cells were lysed and protein quantification was made by Bradford method (Sigma, Aldrich). Total and phospho-p38 MAPK levels were determined by enzyme-linked immunosorbent assay (ELISA) kit (Cell Signaling Technologies) following manusfacturer's instruction. Inhibitor treatments were made as described in the figure legends [21].

2.3.6. Transfection assay

In order to validate whether or not the two compounds were able to activate ER-mediated transcription, a mammalian two-hybrid assay was performed. Huh7 (kind gift from Dr. Iannis Talianidis, Alexander Fleming Biomedical Sciences Research Center, Greece) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose) plus 10% charcoal treated FBS. Twenty-four hours before transfection, cells were seeded into 24-well plates and transfections with indicated DNAs were carried out with lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Sixteen hours after transfection, cells were treated with indicated amounts of compounds and ligands for 24 h, following which cells were lysed and luciferase and Green fluorescence protein (GFP;internal control) were measured. In all wells, total DNA was kept at 700 ng (including empty vectors). The data represent mean±S.E.M. of three independent experiments performed in duplicates.

2.3.7. Western blotting

Cells were grown to 60–70% confluence following which they were exposed to compounds for different time periods. The cells were then homogenized with triton lysis buffer (50 mM Tris–HCl, pH 8 containing 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide, 10 mM EDTA, 10 μg/ml aprotinin and 1 μg/ml aminoethylbenzenesulfonyl fluoride). Protein samples were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, proteins were transferred to PVDF membranes. The membranes were incubated with phospho and non-phospho Erk1/2 antibodies. The bands were developed using ECL kit.

2.4. In vivo experiments

The study was conducted in accordance with current legislation on animal experiments (Institutional Animal Ethical Committee) at C.D.R.I. 21d immature female Sprague–Dawley rats were used for the study [13]. All rats were housed at 21°C, in 12-h light:12-h dark cycles. Normal chow diet and water were provided ad libitum.

2.4.1. Assessment of various bone parameters

Rats were treated with 10.0 mg kg^{-1} body weight doses of individual compound or vehicle (gum acacia in distilled water) once daily for 30 consecutive days by oral gavage. Each animal received intraperitoneal injection of fluorochromes tetracycline (20 mg kg^{-1} body weight dose) and calcein (20 mg kg^{-1} body weight dose) on days 15 and 28 of treatment, respectively. At autopsy lumbar vertebrae, femur and tibia were dissected and separated from adjacent tissue, cleaned, fixed in 70% ethanol and stored at 4°C until bone strength and BMD measurements. Initial and final body weight and uterine weight were recorded. Uteri were carefully excised, gently blotted, weighed and fixed for histology and histomorphometry as we reported earlier [18].

BMD measurements of regions of interest were performed using a bone densitometer (Model 4500 Elite, Hologic) fitted with commercially available software (QDR 4500 ACCLAIM series). After BMD measurement, the bones were embedded in an acrylic material for the determination of bone formation rate (BFR), mineral

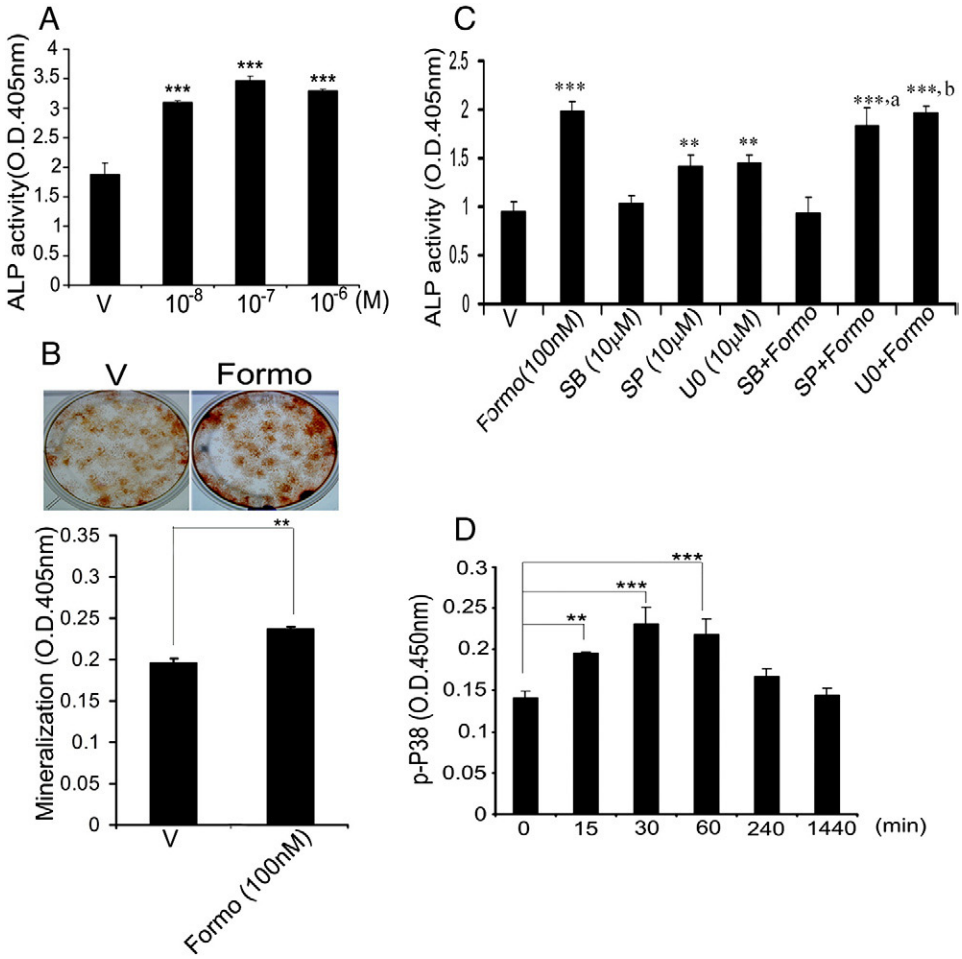

Fig. 2. Formononetin (Formo) stimulates osteoblast differentiation by p38MAPK pathway. (A) Effect of formononetin on osteoblast differentiation as assessed by ALP production by osteoblasts. Data shown as mean±S.E.M.; $n=3$; ***$P<.001$, compared with vehicle treated cells. (B) Bone marrow cells (25,000 cells/well) from rats were seeded into 12-well plates in differentiation medium and treated with formononetin (10^{-7} M) for 21 d (as described in the Materials and methods). At the end of the incubation, cells were stained with alizarin red-S. Stain was extracted, and optical density (OD) was measured colorimetrically. Data shown as mean±S.E.M.; $n=3$; **$P<.01$ compared with vehicle treated cells. (C) Formononetin induces osteoblast differentiation via p38 MAPK pathway. Osteoblasts were treated with various inhibitors alone or in combination with formononetin as described in the panel. Data are shown as mean±S.E.M.; $n=3$; **$P<.01$; ***$P<.001$ compared with vehicle treated cells; $^{a}P<.05$, SP-600125 vs. SP-600125 + formononetin and $^{b}P<.01$, U-0126 vs. U-0126+formononetin. (D) Osteoblasts were treated with 10^{-7} M formononetin for different time points, and p38 MAPK was measured by ELISA kit as described before. Data shown as mean±S.E.M.; $n=3$; **$P<.01$, ***$P<.001$.

appositional rate (MAR) and mineralization surface (MS). Sections 50 μm were made using Isomet Bone cutter, and photographs were taken under fluorescent microscope aided with appropriate filters. The calculations were done according to previous report [22]. Estrogen agonistic and antagonistic activities were evaluated as reported earlier from our laboratory [23].

2.4.2. Plasma pharmacokinetics

In-vivo oral pharmacokinetic study was performed in female Sprague–Dawley rats (n=3, weight range 200–220 g). Formononetin and cladrin were administered single oral dose of 50 and 10 mg/kg respectively to two separate sets of rats. Blood samples were collected from the retro-orbital plexus of rats under light ether anesthesia into microfuge tubes containing heparin as an anti-coagulant at 0.08, 0.25, 0.50, 1, 2, 4, 6, 8 and 10 h post-dosing. Plasma was harvested by centrifuging the blood at 13000 rpm for 10 min and stored frozen at −70±10°C until analysis. Plasma (100 μl) samples were spiked with IS (internal standard) and processed as described above. Along with the plasma samples, QC (quality control) samples were distributed among calibrators and unknown samples and analyzed by LC MS/MS.

2.5. Statistics

Data are expressed as mean±S.E.M. The data obtained in experiments with multiple treatments were subjected to one-way analysis of variance followed by post hoc Tukey test of significance using MINITAB 13.1 software. Student's t test was used to study statistical significance in experiments with only two treatments.

3. Results

3.1. Structure of the compounds

Fig. 1 shows structures of daidzein (4′,7-dihydroxyisoflavone), cladrin (7-hydroxy, 3′, 4′-dimethoxy isoflavone or 3′, 4′-dimethoxy daidzein) and formononetin (4′-methoxy-7-hydroxy isoflavone or 4′-methoxy daidzein).

3.2. Formononetin stimulates osteoblast differentiation by p38 MAPK pathway

Unlike cladrin, formononetin had no effect on the proliferation of osteoblasts (data not shown). However, formononetin (10^{-8}—10^{-6} M) stimulated osteoblast differentiation, assessed by osteoblast ALP production (Fig. 2A).The effective concentration was 10^{-7} M in comparison to 10^{-8} M required for cladrin to stimulate ALP production from osteoblasts. Furthermore, rat BMCs cultured in the presence of 100 nM formononetin for 21 d under osteoblast differentiation condition resulted in increased formation of mineralized nodules compared with vehicle (Fig. 2B), suggesting that formononetin stimulated formation of osteoporgenitor cells into mature osteoblasts in the bone marrow.

Treatment with p38 MAPK inhibitor, SB203580 (10.0 μM), but not JNK and Erk inhibitors, abolished formononetin-stimulated ALP production from osteoblasts (Fig. 2C). Consistent with this result, it was observed that formononetin rapidly activated phosphorylation of p38 MAPK in osteoblast, peaking at 30 min and declining at 240 min (Fig. 2D).

3.3. Cladrin stimulates osteoblast proliferation and differentiation by MEK-Erk pathway

From 10^{-9}—10^{-7} M, cladrin stimulated proliferation of rat neonatal calvarial osteoblasts, assessed by BrdU incorporation

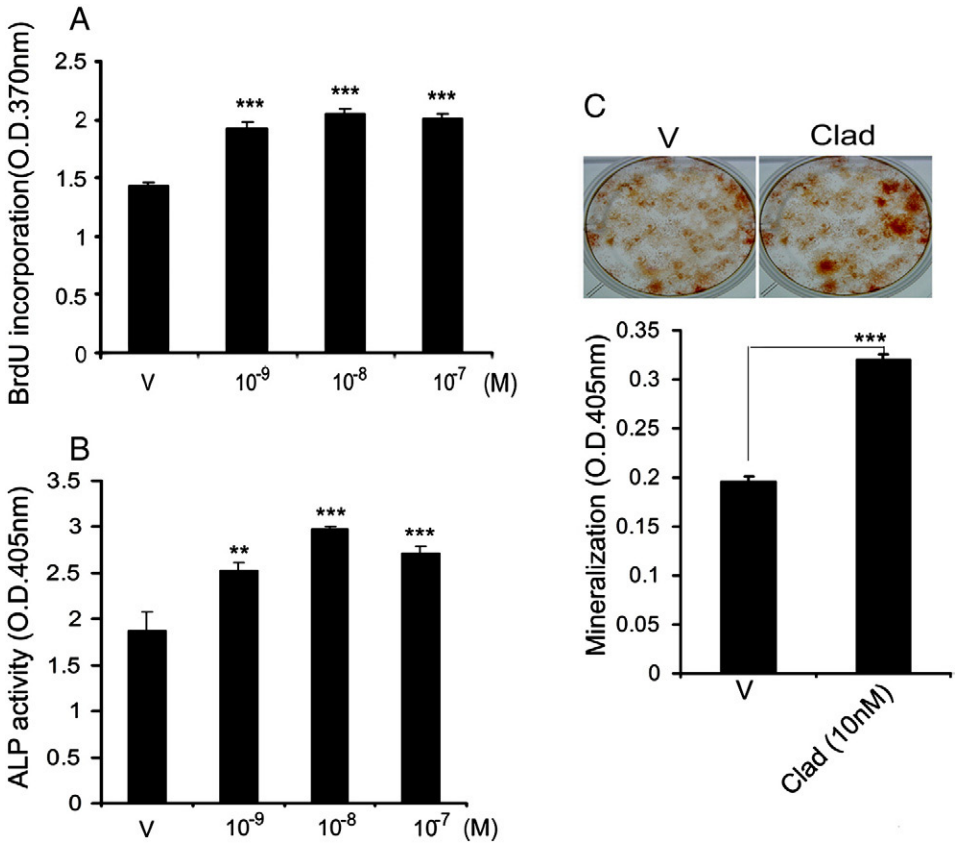

Fig. 3. Effects of cladrin on osteoblast proliferation, differentiation and mineralization of bone marrow osteoprogenitor cells. (A) Rat calvarial osteoblasts were exposed to various concentrations of cladrin (Clad) for 24 h and proliferation was determined by BrdU ELISA. Data are mean±S.E.M.; n=3; P<.001. (B) Cells were exposed to various concentrations of cladrin for 48 h, and ALP activity was determined as described in the Materials and methods. (C) Bone marrow cells (25,000 cells/well) from rats were seeded into 12-well plates in differentiation medium and treated with cladrin (10^{-8} M) for 21 days (as described in the Materials and methods). At the end of the incubation, cells were stained with alizarin red-S. Stain was extracted, and OD was measured colorimetrically. Data shown as mean±S.E.M.; n=3; **P<.01; ***P<.001 compared with vehicle-treated cells.

(Fig. 3A). In addition, cladrin (10^{-9}—10^{-7} M) stimulated osteoblast differentiation, assessed by osteoblast ALP production (Fig. 3B). Furthermore, rat BMCs cultured in presence of 10^{-8} M cladrin for 21 d under osteoblast differentiation condition resulted in increased formation of mineralized nodules compared with vehicle (Fig. 3C), suggesting that cladrin stimulates formation of osteoporgenitor cells into mature osteoblasts in the bone marrow.

The impact of cladrin-stimulated osteoblast proliferation (as assessed by BrdU incorporation) was abolished by U0126, a mitogen activated extracellular kinase (MEK)1/2 inhibitor whereas LY294002, an inhibitor of PI3 kinase had no effect, suggesting that cladrin stimulated osteoblast proliferation by activating MEK/Erk but not Akt pathway (Fig. 4A). U0126 also inhibited cladrin-stimulated production of ALP in osteoblasts, suggesting the involvement of MEK-Erk pathway in mediating cladrin action on osteoblast differentiation (Fig. 4B). We confirmed the inhibitor data by demonstrating that cladrin had a rapid (30 min) but sustained activation (up to 1440 min) on phosphorylation of Erk1/2 (Fig. 4C).

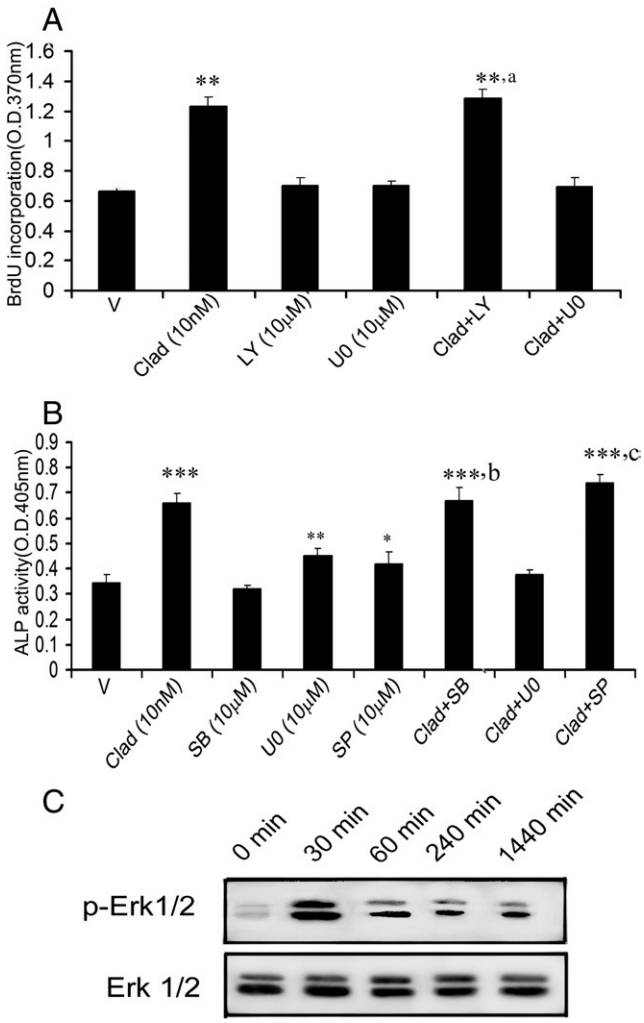

Fig. 4. Cladrin promotes osteoblast proliferation and differentiation by MEK-Erk1/2 pathway. (A) Effect of U0126 on osteoblast proliferation induced by cladrin. Cells were pre-treated with U0126 and Ly294002 (10 μM each) for 0.5 h followed by treatment with or without cladrin for 24 h. Results showed that U0126 abolished osteoblast proliferation induced by cladrin. Data shown as mean±S.E.M.; $n=3$; $^{**}P<.01$ compared with vehicle treated cells; $^{a}P<.01$, Ly-294002 vs. Ly-294002 +cladrin. (B) Effect of p38, JNK and Erk inhibitors on osteoblast cells induced by cladrin. Cells were pretreated with various inhibitors and then stimulated with 10^{-8} M cladrin for 48 h. Data shown as mean±S.E.M.; $n=3$; $^{*}P<.05$; $^{**}P<.01$; $^{***}P<.001$ compared with vehicle treated cells, $^{a}P<.01$ SB-203580 vs. SB-203580+cladrin, $^{b}P<.001$, SP-600125 vs. SP-600125+cladrin. (C) Cladrin stimulates Erk phosphorylation in osteoblasts. Cells were treated with 10^{-8} M cladrin and lysate were resolved on SDS-PAGE. Levels of Erk and their phosphorylated forms were determined by western blot analysis using specific antibody. Representative gel of three independent experiments with similar results.

3.4. Cladrin and formononetin act independent of ER

Reports show that isoflavones such as genistein and daidzein act via the ER in osteoblasts [24]. We tested whether cladrin or formononetin mediated their actions in osteoblasts through ER. Our data show that the presence of an ER antagonist, ICI-182780, failed to abolish cladrin- or formononetin-induced osteoblast functions such as proliferation and differentiation (Fig. 5A and B). Furthermore, in Huh 7 cells transfected with human ERα and ERβ, neither cladrin nor formononetin transactivated these reporter gene constructs (Fig. 5C and D), suggesting lack of ER-mediated signaling by these compounds.

3.5. Cladrin and formononetin differentially promote BMD and bone formation rate

We next assessed in vivo effect of cladrin and formononetin in growing rats where bone formation is the dominant event. Female Sprague–Dawley rats at weaning were given either cladrin or formononetin at 10.0 mg kg^{-1} day^{-1} dose by oral gavage for 30 consecutive days. Gum acacia was used as vehicle (control group).

Fig. 6 shows the effects of these compounds on BMD levels. For example, at 10.0 mg kg^{-1} day^{-1} dose of cladrin, BMD levels were increased in femur (midshaft), all regions of tibia and lumbar vertebra (global, L-1, L-2 and L-4). In case of formononetin, at 10.0 mg kg^{-1} day^{-1} dose, BMD levels were higher at femur (midshaft), all regions of tibia and all segments of lumbar vertebra. From these data, it appears that cladrin and formononetin are comparable in promoting BMD levels of growing rats compared with controls.

Dynamic histomorphometric studies by double fluorochrome (tetracycline-calcein) labeling experiment allowed determination of new bone formation during the period of administration of either cladrin or formononetin or vehicle (control). Fig. 7A and B show that while cladrin treatment to rats, increased MAR and BFR by >2.0-fold compared with control rats, formononetin treatment had comparables values with that of control. When compared with controls, cladrin or formononetin treatment modestly but significantly increased mineralizing surface (MS) (Fig. 7B).

3.6. Assessment of uterine estrogen agonistic/antagonistic activity of cladrin and formononetin

Daidzein is known to possess varied degrees of estrogenic or anti-estrogenic effects in vivo [25–29]. We tested whether cladrin or formononetin had such effect at the uterine level. Weaning (21-day-old) Sprague–Dawley rats were Ovx, followed by oral administration of cladrin or formononetin at 10 mg kg^{-1} day^{-1} dose for 3 consecutive days with or without 17β-E_2. Table 1 shows that both cladrin and formononetin had no estrogenic or anti-estrogenic effects as assessed by uterine wet weight. Furthermore, histological evaluation of uteri under various treatments revealed that whereas 17 β-E_2 treatment resulted in hypertrophy of luminal and glandular epithelium, neither of the two compounds had such effect (comparable to control) (Fig. 8).

3.7. Plasma pharmacokinetic studies

Pilot studies revealed that formononetin had substantially lesser oral bioavailability than cladrin (data not shown). Because

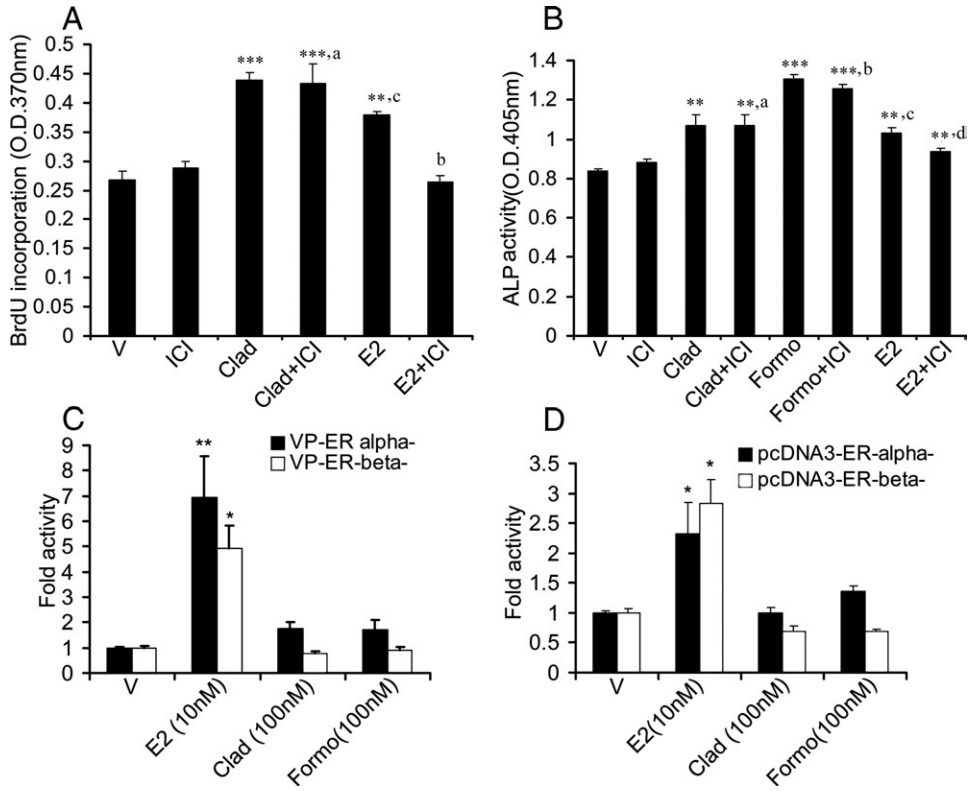

Fig. 5. Cladrin and formononetin do not signal via ER. (A) Effect of ICI-182780 (ICI) on osteoblast proliferation induced by cladrin (Clad). Cells were pre-treated with ICI-182780 (1.0 nM) for 0.5 h with or without various test compounds indicated and subsequently cultured for 24 h. Data shown as mean±S.E.M.; n=3; **P<.01; ***P<.001 compared with vehicle treated cells; [a]P<.01, ICI-182780 vs. ICI-182780+cladrin; [b]P<.001, ICI-182780 vs. ICI-182780+17β-E_2; [c]P<.01, ICI-182780 vs. 17β-E_2. (B) Effect of ICI-182780 on osteoblast differentiation induced by cladrin and formononetin (Formo). Cells were pre-treated with ICI-182780 for 0.5 h with or without the test compounds indicated and subsequently cultured for 48 h. Data show that ICI-182780 failed to abolish osteoblast differentiation induced by cladrin or formononetin. E_2 was taken as a positive control. Data shown as mean±S.E.M.; n=3; **P<.01; ***P<.001 compared with vehicle treated cells; [a]P<.01, ICI-182780 vs. ICI-182780+cladrin; [b]P<.001, ICI-182780 vs. ICI-182780+formononetin; [c]P<.05, ICI-182780 vs. 17β-E_2; [d]P<.05, ICI-182780 vs. ICI-182780+17β-E_2. (C) A mammalian two-hybrid assay was performed where, Huh7 cells in 24-well plates were co-transfected with 200 ng GAL4-UAS-Luc, 100 ng EGFPC1, 100 ng pMTIF-2 and 100 ng VP16-ER (α or −β) and treated with indicated compounds as described in materials and methods. Luciferase values were normalized with GFP values and are expressed as fold activity over vehicle treated cells. Data shown as mean±S.E.M.; n=3; *P<.05; **P<.01. (D) Huh7 cells were transfected with 200 ng ERE-Luc, 100 ng EGFPC1 and 40 ng pcDNA3 ER (α or −β) and treated with indicated compounds. Luciferase values were normalized with GFP values and are expressed as fold activity over vehicle treated cells. Data shown as mean±S.E.M.; n=3. *P<.05.

of this observation, we used 10.0 mg kg^{-1} dose for cladrin and 50.0 mg kg^{-1} dose for formononetin to investigate respective plasma bioavailability. Following single oral administration at the said doses of two compounds, plasma concentration-time profile of cladrin and formononetin was determined. Data show faster absorption of the cladrin (peak level at 0.5 h) compared with formononetin (peak level at 1.0 h) (Fig. 8A and B). Overall systemic availability [area under the curve (AUC)] of cladrin was 571.0±73.25 h*ng/ml, and it was detectable in plasma up to 24 h indicating its prolonged systemic availability (Fig. 8A). The absolute bioavailability of cladrin was found to be 13.7%. Neither daidzein nor equol formation was observed in the plasma samples of cladrin treated rats.

Plasma concentration-time profile of formononetin exhibited systemic availability of 278.30±38.50 hr*ng/ml (Fig. 8B), which is lower by half the value of cladrin despite input dose being 5 times higher than cladrin. At 50.0 mg kg^{-1} dose, formononetin plasma levels were detectable also up to 24 h. In addition, formononetin was metabolized to daidzein, having C_{max} (24 h) of 8.63±2.152 ng/ml with an AUC of 147.2488 h*ng/ml, thereby showing ~50% conversion of formononetin to daidzein (Fig. 8B). Interestingly, equol was also detected up to 28 h post dosing of 50 mg kg^{-1} dose of formononetin (data not shown). The absolute bioavailability of formononetin was found to be 4.294%, which is ~3.0-fold lesser than the bioavailability of cladrin.

4. Discussion

Daidzein affords bone-protective action by stimulation of osteoblast [30–32] and inhibition of osteoclast functions [33] through the ER. Daidzein also exhibits varying degrees of estrogenic and anti-estrogenic effects in species-specific manner [24]. Although, no human studies are available with daidzein, results from the studies with soy proteins (rich in daidzein among other isoflavones) in humans have been mixed, ranging from a modest impact to no effect [34]. That is because the efficacy of soy protein in bone health in humans may importantly be a function of the ability to biotransform soy isoflavones to the more potent estrogenic isoflavone, equol. Indeed, complementing the equol theory is a 2-year interventional trial, where 108 postmenopausal women who had taken 500 ml soy milk/day [35], had increased BMD and BMC in only those producing equol and not in those who did not produce equol. Daidzein has been synthetically modified to yield ipriflavone (IP). Although IP, has shown some promise in its ability to preserve bone in postmenopausal women [7], a definitive 3-year study on more than 400 postmenopausal women concluded that IP did not prevent bone loss [36]. From these reports, it appears that daidzein could be a suitable therapeutic “lead” molecule for menopausal osteoporosis if issues pertaining to its metabolism, estrogenicity, bioavailability and improvement of its potency in promoting osteoblast function are addressed.

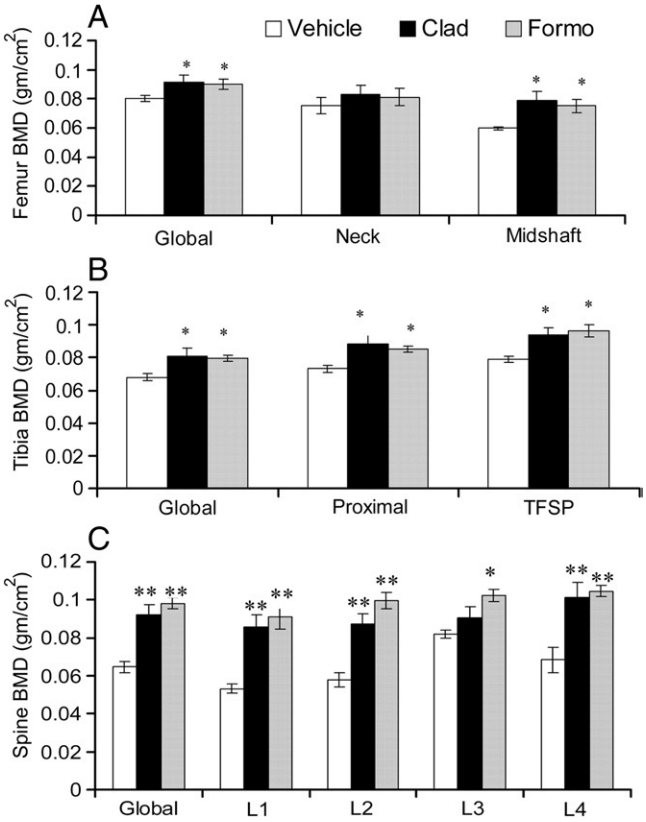

Fig. 6. Effect of cladrin and formononetin on BMD levels of various bones. (A) BMD of whole femur, femur neck (trabecular bone) and femur midshaft (cortical bone). (B) BMD of whole tibia, tibia proximal (trabecular bone) and tibia TFSP (tibio-fibula separating point; cortical bone) (C) BMD of whole vertebrae and L1–L4 vertebrae (all trabecular bones) with respect to vehicle. Data shown as mean±S.E.M.; $n=3$. *$P<.05$; **$P<.01$; ***$P<.001$.

We here studied the effects of two methoxylated daidzeins, cladrin and formononetin, present in various natural sources [37,38]. Cladrin has two and formononetin has one methoxyl groups. In vitro, both compounds promoted osteoblast functions with varying potency; maximal effect of cladrin on osteoblast mineralization was observed at 10 nM and that of formononetin at 100 nM. In addition, while cladrin stimulated osteoblast proliferation and differentiation via the activation of MEK-Erk pathway, formononetin exerted its differentiation promoting action by activating the p38 MAPK pathway. Formononetin has been shown to regulate secretion of interleukin-4 from activated T cells via p38 MAPK signaling [39]; however, there is no report on the specific MAP kinase associated with the signaling of cladrin. From our data, it appears that activation of MEK-Erk pathway in osteoblast by cladrin may contribute to its better potency over formononetin, which activates p38 MAPK pathway. Moreover, the methoxyl substitutions of daidzein may contribute to increased potency on osteoblast functions as the activity of both cladrin and formononetin were observed at concentrations 10–100-fold lower than that reported for daidzein [40]. Substitution of two methoxyl groups of daidzein as in case of cladrin may also contribute to its osteoblast-promoting actions (proliferation and differentiation) more than formononetin (differentiation), which has one methoxyl substitution.

Both cladrin and formononetin failed to activate transcriptional activity of human ERα or ERβ in Huh7 cells (Fig. 5C and D), thereby suggesting that unlike daidzein, its methoxyl derivatives signaled independent of ER. This was further confirmed when ICI-182780 failed to abrogate the stimulatory effects on osteoblast functions by cladrin or formononetin. In addition, unlike daidzein, cladrin and formononetin failed to inhibit osteoclastogenesis from BMCs (Supplement Figure 1). Therefore, it appears that methoxyl substitution of daidzein significantly alters its function; from being dually active (promoting osteoblast function and inhibiting osteoclast function) to being purely osteogenic. Finally, none of these compounds exhibited either estrogen agonistic or antagonistic effects in the uterus. Since daidzein is known to have uterine estrogen agonistic/antagonistic actions [41–44], it appears that methoxylation of daidzein abolishes its ER-"like" action of daidzein.

There is a well-recognized link between the prevalence of low PBM achievement and osteoporosis [45,46]. Growing animals represent bone modeling wherein bone formation is the predominant event. In rats, PBM is attained between 3–12 months of postnatal age [47]. We gave caldrin and formonetin treatments for 4 weeks after weaning when bones were yet to attain final PBM. Since isoflavones and their derivatives are considered as phytoestrogens, it was deemed reasonable to avoid estrogen action on growing skeleton by avoiding attainment of puberty in rats lest it could obscure the effects of cladrin and formononetin. "Vaginal opening" marks commencement of estrogen function in rats, and our study ensured termination before this event.

Under this circumstance of estrogen-independent skeletal growth, both cladrin and formononetin increased BMD at various anatomic sites equally. BMD depends upon bone mineral content and bone size [48]. Axial BMD increases more rapidly than appendicular BMD during skeletal growth [49]. Our BMD data demonstrate that cladrin and formononetin increased BMD levels of both cortical and trabecular bones with the exception of femur neck where BMD levels were comparable with control rats. Dynamic bone formation indices (MS, MAR and BFR) studied in femur using double fluorochrome labeling [22] revealed that both compounds significantly increased MS compared to control group. On the other hand, it was cladrin and not formononetin treatment of rats increased MAR and BFR over control. Since increase in MS is a function of increased osteoblast differentiation, formononetin treatment was expectedly found to increase this parameter over control. Increases in MAR and BFR as observed with cladrin treatment result from the expansion of osteoblast pool (by proliferation, recruitment and/or increased survival) in addition to increase in osteoblast differentiation. Thus, cladrin appears to favor modeling-directed bone formation allowing for rapid increase in cortical width.

Bioavailability is an important determinant of in vivo efficacy of any compound. Pharmacokinetic studies on methoxyisoflavones are scarce. The oral bioavailability is dependent on absorption and systemic clearance which in turn depend on lipophilicity of the molecules. It is reported that the *O*-methylation of free phenolic hydroxyl groups enhances the lipophilicity and leads to derivatives not susceptible to glucuronic acid or sulfate conjugation, resulting in increased metabolic stability. Improved transport through biological membranes, such as in intestinal absorption, and much increased oral bioavailability, diminished toxic side effects and improved health effects have also been observed with *O*-methylated flavones [50,51].

Both cladrin and formononetin are the *O*-methylated isoflavones. The increased lipophilicity of formononetin and cladrin results in their increased oral absorption, and therefore, the comparative profiling of their systemic bioavailability could shed light on the more potent action of cladrin than formononetin in bone formation in vivo. It was observed that approximately 50% of formononetin is converted to daidzein. This could be the reason for its lower absolute systemic bioavailability than cladrin. Indeed, cladrin exhibited ~3.0-fold greater absolute systemic bioavailability than formononetin, which correlates well with its improved pharmacodynamic response. Whether two methoxy groups in cladrin contributes to its better oral bioavailability than formononetin, which has one methoxy group

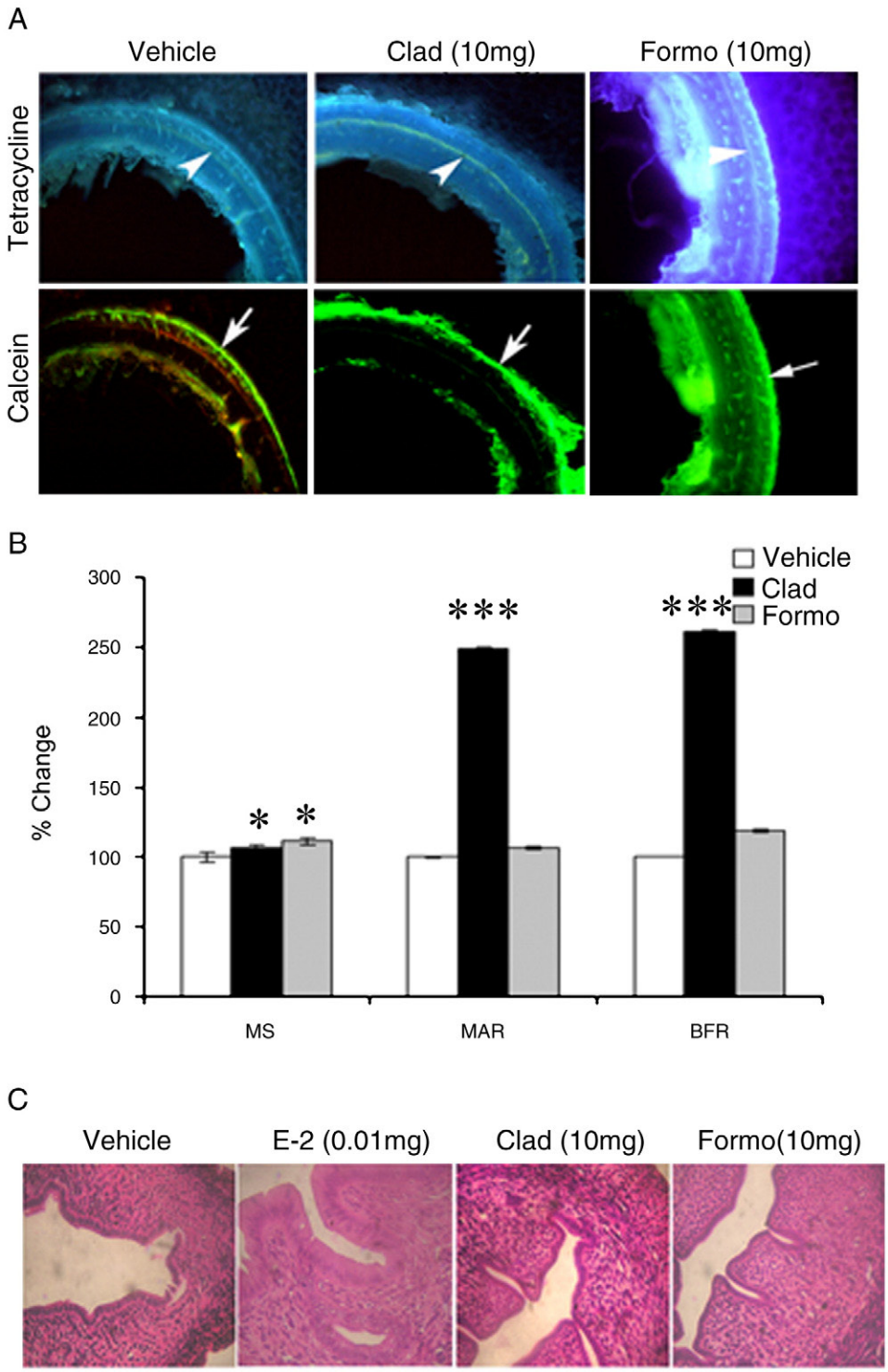

Fig. 7. (A) Dynamic histomorphometric studies by double fluorochrome (tetracycline-calcein) labeling experiment for determination of new bone formation. Representative images of transverse sections of tetracycline and calcein labeled femur diaphyses from rats after 30 days treatment with vehicle or cladrin (Clad) at 10.0 mg kg^{-1} day^{-1} dose or formononetin (formo) at 10.0 mg kg^{-1} day^{-1} dose. Tertracyclin (UV filter) and calcein (orange) labeling are shown. Arrow head indicates calcein; arrow, tetracyclin. (B) Histomorphometric parameters calculated from these labeling experiments. Data shown as mean±S.E.M.; $n=3$. *$P<.05$; ***$P<.001$. (C) Uteri were harvested from rats treated with vehicle or E_2 or cladrin (Clad) or formononetin (Formo). Cross-sections of uteri were stained with hematoxylin and eosin. Much thicker endometrium and greater epithelial cell heights were observed in the E_2-treated group while cladrin or formononetin exhibited comparable histological features with that of vehicle.

remain uncertain at present. Concerning the osteoblast-stimulating action in vitro, daidzein is less effective than formononetin [26,30]. In addition, there was trace amount of equol in the plasma of formononetin treated rats (data not shown), while equol was undetectable in the plasma of cladrin treated rats. Formation of equol appears to be a product of formononetin itself rather than from daidzein. This is because C_{max} for daidzein in formononetin treated rats is 24 h, suggesting an extra-intestinal conversion of daidzein from

Table 1
Estrogen agonistic and antagonistic effect of methoxyisoflavones in ovariectomized immature Sprague–Dawley rats

Treatment	Dose mg/kg	Estrogenic activity		Anti-estrogenic activity	
		Uterine weight	% Gain[a]	Uterine weight	% Inhibition[b]
Vehicle	-	14.7±2.2			
Ethynyl estradiol	0.01	98.3[c]±11.5	568		
Cladrin	10	14.9±1.0	1.3	91.2±5.0	7
Formononetin	10	17.5±2.0	19	97.4±4.8	1

Values are mean±S.E.M.
[a] Percent gain over corresponding vehicle control group.
[b] Percent inhibition in ethynylestradiol-induced uterine weight gain.
[c] $P<.001$ versus vehicle treated group.

formononetin whereas gut microflora is required to catalyze daidzein to equol conversion [52]. It is possible that formononetin's action in vivo is dampened by its conversions to daidzein and equol, as both are weaker than formononetin in stimulating osteoblast function [26,30]. Although equol has potent estrogen agonistic action, formononetin did not exhibit uterine estrogenicity. Estrogenic effect of formononetin by vaginal cytology in mice was observed at 40.0 mg kg^{-1} dose [53]. Therefore, it appears that the levels of circulating equol achieved by formononetin at its maximum dose (10.0 mg kg^{-1} day^{-1}) used by us for assessing in vivo bone forming action was too low to elicit uterine estrogenicity.

Collectively, we demonstrate that cladrin and formononetin have differential actions in modulating osteoblast functions in vitro and in acquisition of PBM in growing female rats in vivo. Structural differences in the methoxylation state of daidzein may contribute to significant alteration in biological effects between cladrin and formononetin. Apparently, methoxylation at two positions, as in the case of cladrin, may enormously enhance the osteogenic effect of daidzein. Together with its potent osteogenic effect, a good oral bioavailability and lack of uterine estrogenicity makes cladrin an attractive therapeutic candidate for menopausal osteoporosis.

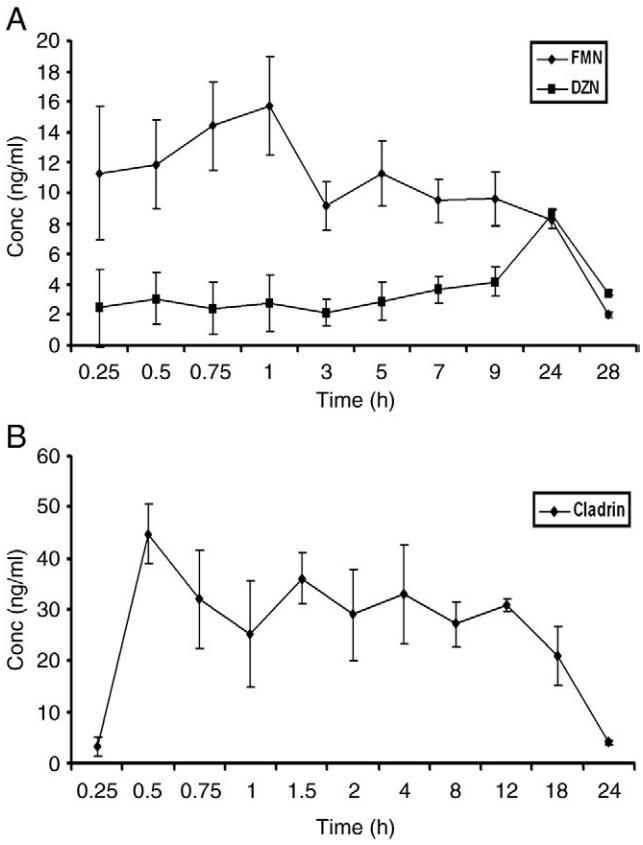

Fig. 8. Mean plasma concentration-time profile of cladrin (A) and, formononetin and daidzein (B) in female Sprague–Dawley rats after single oral dose treatments respectively of 10.0- and 50.0 mg kg^{-1}. Data are mean of three individual experiments. FMN, formononetin; DZN, daidzein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.02.010.

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