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Methoxylated Isoflavones, Cajanin and Isoformononetin, Have Non-Estrogenic Bone Forming Effect Via Differential Mitogen Activated Protein Kinase (MAPK) Signaling

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ABSTRACT

Following a lead obtained from stem-bark extract of *Butea monosperma*, two structurally related methoxyisoflavones; cajanin and isoformononetin were studied for their effects in osteoblasts. Cajanin had strong mitogenic as well as differentiation-promoting effects on osteoblasts that involved subsequent activation of MEK-Erk and Akt pathways. On the other hand, isoformononetin exhibited potent anti-apoptotic effect in addition to promoting osteoblast differentiation that involved parallel activation of MEK-Erk and Akt pathways. Unlike genistein or daidzein, none of these two compounds appear to act via estrogen receptors in osteoblast. Once daily oral (by gavage) treatment for 30 consecutive days was given to recently weaned female Sprague–Dawley rats with each of these compounds at 10.0 mg kg^{-1} day^{-1} dose. Cajanin increased bone mineral density (BMD) at all skeletal sites studied, bone biomechanical strength, mineral apposition rate (MAR) and bone formation rate (BFR), compared with control. BMD levels at various anatomic positions were also increased with isoformononetin compared with control however, its effect was less potent than cajanin. Isoformononetin had no effect on the parameters of bone biomechanical strength although it enhanced MAR and BFR compared with control. Isoformononetin had very mild uterotrophic effect, whereas cajanin was devoid of any such effect. Our data suggest that cajanin is more potent than isoformononetin in accelerating peak bone mass achievement. To the best of our knowledge, this work represents the first attempt to elucidate structure-activity relationship between the two methoxylated isoflavones regarding their effects in osteoblasts and bone formation. J. Cell. Biochem. 108: 388–399, 2009.

KEY WORDS: OSTEOGENIC; APOPTOSIS; CAJANIN; ISOFORMONONETIN; MAPK SIGNALING; PEAK BONE MASS

High dietary intake of isoflavones has been reported to increase BMD levels in lumbar spine in Japanese [Somekawa et al., 2001], Chinese [Mei et al., 2001], and US [Kritz-Silverstein and Goodman-Gruen, 2002] postmenopausal women than the control group. Soy isoflavones including genistein and daidzein have been widely investigated for having beneficial effects on bone [Setchell and Lydeking-Olsen, 2003]. At micromolar concentrations in vitro, genistein and daidzein promote osteoblast functions via estrogen

Biju Bhargavan, Abnish Kumar Gautam, and Divya Singh contributed equally to this work.

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receptor (ER)-dependent mechanism [Morito et al., 2002]. Oral or subcutaneous injections of genistein and daidzein inhibit bone loss in ovariectomized or orchidectomized mice [Ishimi et al., 2002]. Daidzein exerts sexually dimorphic effect on bone formation in growing rats [Fujioka et al., 2007]. Both isoflavones appear to exert effects on skeleton via equol, a highly estrogenic metabolite [Phrakonkham et al., 2007].

There is a well-recognized link between the prevalence of low peak bone mass (PBM) attainment and osteoporosis. In humans, gender differences in the acquisition of PBM are well recognized and may substantially contribute to the increased prevalence of fragility fractures in women compared with men [Orwoll et al., 2001]. Effect of phytoestrogen on PBM achievement has not been studied systematically.

Recently, we reported that the crude extract of *Butea monosperma* exhibited in vitro bone forming (osteoblast mineralization) activity [Maurya et al., 2009]. Several methoxyisoflavones were found to be abundantly present in the crude extract of *B. monosperma* [Maurya et al., 2009]. We hypothesized that in vitro bone forming activity of the extract could be mediated by the methoxyisoflavones. We selected cajanin, a derivative of genistein (2′-hydroxy-7-methoxy genistein) and isoformononetin, a derivative of daidzein (7-methoxy daidzein) and tested their possible effects in osteoblasts. The rational for selecting cajanin and isoformononetin out of other methoxyisoflavones present in *B. monosperma* is because of their structural similarity; cajanin and isoformononetin contain methoxyl group at C-7 position of genistein and daidzein, respectively. Our study reveals differential effects and signaling mechanisms of these two compounds on osteoblasts in vitro as well as in bone formation.

MATERIALS AND METHODS

REAGENTS AND CHEMICALS

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). All fine chemicals were purchased from Sigma-Aldrich (St. Louis, MO). ECL kit was purchased from Amersham Pharmacia. Annexin-PI kit was procured from Sigma-Aldrich. All antibodies for western blot analysis were obtained from Cell Signaling Technologies (USA). BrdU ELISA kit was procured from Roche (USA).

SYNTHESIS OF COMPOUNDS

Cajanin and isoformononetin, initially isolated from *B. monosperma* [Maurya et al., 2009] were synthesized in gram scale for detailed in vitro and in vivo studies. Cajanin was synthesized in four steps as described before [Miller et al., 2003]. Isoformononetin was synthesized by a previously published protocol [Bass, 1976]. The synthesized compounds were matched with the data of the authentic samples and the purities of the compounds were confirmed by HPLC and NMR analytical methods [Maurya et al., 2009]. Structures of the compounds are shown in Figure 1.

CULTURE OF CALVARIAL OSTEOBLASTS

Rat calvarial osteoblasts were obtained following our previously published protocol of sequential digestion [Trivedi et al., 2008].

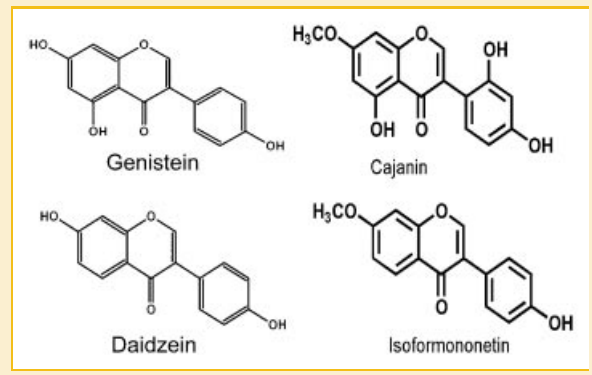

Fig. 1. Structure of genistein, daidzein, cajanin and isoformononetin.

Briefly, calvaria from ten to twelve 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 collected, centrifuged, resuspended, and plated in T-25 cm^2 flasks in α-MEM containing 10% FCS and 1% penicillin/streptomycin (complete growth medium).

For studying the involvements of various MAP kinase pathways and estrogen signaling, that are described subsequently, osteoblasts were routinely treated with inhibitors (U0126, SB203580, SP600125, LY294002, ICI182780) given 30 min prior to the treatments of cajanin or isoformononetin.

OSTEOBLAST DIFFERENTIATION

For alkaline phosphatase (ALP) activity measurement, osteoblasts at ~80% confluence were trypsinized and 2×10^3 cells/well were seeded in 96-well plates. Cells were treated with different concentrations of isoflavones for 48 h in α-MEM supplemented with 5% 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 (PNPP) as substrate and quantitated colorimetrically at 405 nm [Ishizuya et al., 1997].

MINERALIZATION OF BONE MARROW CELLS (BMCS)

For mineralization studies, BMCs from female Sprague–Dawley rats weighing ~40 g were isolated and cultures prepared according to a previously published protocol from our laboratory [Trivedi et al., 2008]. Briefly, the femora were excised aseptically, cleaned of soft tissues, and washed 3 times, 15 min each, in a culture medium containing 10 times the usual concentration of antibiotics as mentioned above. The epiphyses of femora were cut off and the marrow flushed out in 20 ml of culture medium consisting of α-MEM, supplemented with 15% fetal bovine serum, 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 6-well plate) in the culture medium, consisting of α-MEM, supplemented with 15% fetal bovine serum, 10^{-7} M dexamethasone,

50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Cells were cultured with and without isoflavones 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 PBS. After fixation, the specimens were processed for staining with 40 mM Alizarin Red-S, which stains areas rich in nascent calcium.

For quantification of 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 from the plate 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 [Gregory et al., 2004; Maurya et al., 2009].

CELL PROLIFERATION AND CELL CYCLE ANALYSIS

Bromodeoxy uridine (BrdU) cell proliferation assay was performed as per the protocol of kit manufacturer's at 50–60% confluency. For cell cycle analysis, osteoblast cells were cultured in T75 culture flasks in complete growth media. When the cells attained 50–60% confluency, isoflavone treatments was given for 24 h. On termination cells were harvested in PBS and stained with propidium iodide (PI) stain and cell cycle analysis was carried out in a FACS machine.

ANALYSIS OF APOPTOSIS BY ANNEXIN-PI STAINING

For apoptosis study, osteoblast cells were grown to ~50–60% confluency, serum was withdrawn from the culture for 2 h and then exposed to isoflavones at effective dose (ED) for 24 h in α-MEM containing 0.5% FCS. Annexin-PI staining for FACS analyses was carried out according to kit manufacturer's instructions.

TRANSFECTION ASSAY

In order to validate whether the two methoxyisoflavones 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 DMEM (high glucose) plus 10% 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 ligands for 24 h, following which cells were lysed and luciferase and GFP (internal control) were measured. In all wells, total DNA was kept at 700 ng (including empty vectors). The data represent mean ± SEM of three independent experiments performed in duplicates.

WESTERN BLOTTING

Cells were grown to 60–70% confluence following which they were exposed to isoflavones for different time intervals. The cells were then homogenized with 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% SDS–PAGE gel. After electrophoresis proteins were transferred to a PVDF membrane. The membranes were incubated with phospho and non-phospho Erk1/2 and phospho and non-phospho Akt antibodies. The bands were developed using ECL kit.

IN VIVO EXPERIMENTS

The study was conducted in accordance with current legislation on animal experiments [Institutional Animal Ethical Committee (IAEC)] at C.D.R.I. 21-day immature female Sprague–Dawley rats were used for the study [Fujioka et al., 2007]. All rats were housed at 21°C, in 12-h light/12-h dark cycles. Normal chow diet and water were provided ad libitum.

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 intra-peritoneal 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 mechanical testing and bone mineral density (BMD) measurement. 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 [Trivedi et al., 2008].

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 bone formation rate (BFR), mineral appositional rate (MAR), and mineralization surface (MS) measurements. Fifty micron sections were cut using Isomet Bone cutter and photograph was taken under fluorescent microscope aided with appropriate filters. The calculations were done according to previous report [Hara et al., 2002]. Measurement of bone strength was done with 3 point bending strength of femur using Bone strength tester Model TK 252C as we reported earlier [Trivedi et al., 2008]. Following endplate removal, the third lumbar vertebrae (LV3) from each rat was isolated for compression testing [Trivedi et al., 2008]. Estrogen agonistic and antagonistic activities were evaluated as reported earlier from our laboratory [Srivastava et al., 2007].

STATISTICS

Data are expressed as mean ± SEM. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA 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.

RESULTS

STRUCTURE OF THE COMPOUNDS

As described in the Materials and Methods Section, both cajanin and isoformononetin were synthesized for this study. Figure 1 shows structures of cajanin (2′,4′,5-trihydroxy-7-methoxy isoflavone or 2′-hydroxy-7-methoxy genistein), a methoxy-derivative of genistein and isoformononetin (4′-hydroxy-7-methoxy isoflavone or 7-methoxy daidzein), a methoxy-derivative of daidzein.

CAJANIN STIMULATED OSTEOBLAST PROLIFERATION AND DIFFERENTIATION: SUBSEQUENT ACTIVATION OF MEK-ERK AND AKT PATHWAYS

At concentrations ranging from 10^{-12} to 10^{-10} M, cajanin stimulated osteoblast proliferation, as assessed by BrdU incorporation (Fig. 2A). In subsequent experiments, we used 10^{-11} M cajanin. Cell cycle analysis shows reduced population of osteoblasts in G_0/G_1 phase due to cajanin treatment; $78.34 \pm 1.45\%$ in control (10% FCS + vehicle) versus $64 \pm 1.73\%$ in the presence of 10^{-11} M cajanin (Fig. 2B). On the other hand, cajanin treatment resulted in 3.0-fold increase in osteoblast population at G_2/M phase; $1.5 \pm 0.26\%$ in control (10% FCS + vehicle) versus $5.07 \pm 0.13\%$ in the presence of cajanin (Fig. 2B). Osteoblasts at S phase were marginally increased ($27.35 \pm 1.16\%$) by cajanin treatment compared with cells treated with 10% FCS ($20.16 \pm 1.22\%$) (Fig. 2B). Together, these data suggested that cajanin stimulated osteoblast proliferation by enhancing osteoblasts mitosis G_2/M- and S phase of the cell cycle.

In addition, cajanin treatment led to increased osteoblast differentiation as assessed by osteoblast ALP production at 10^{-11} M (Fig. 2C). At the same concentration, cajanin increased mRNA levels of ALP in osteoblasts (Supplementary Fig. 1). Furthermore, we observed that cajanin, in addition to calvarial

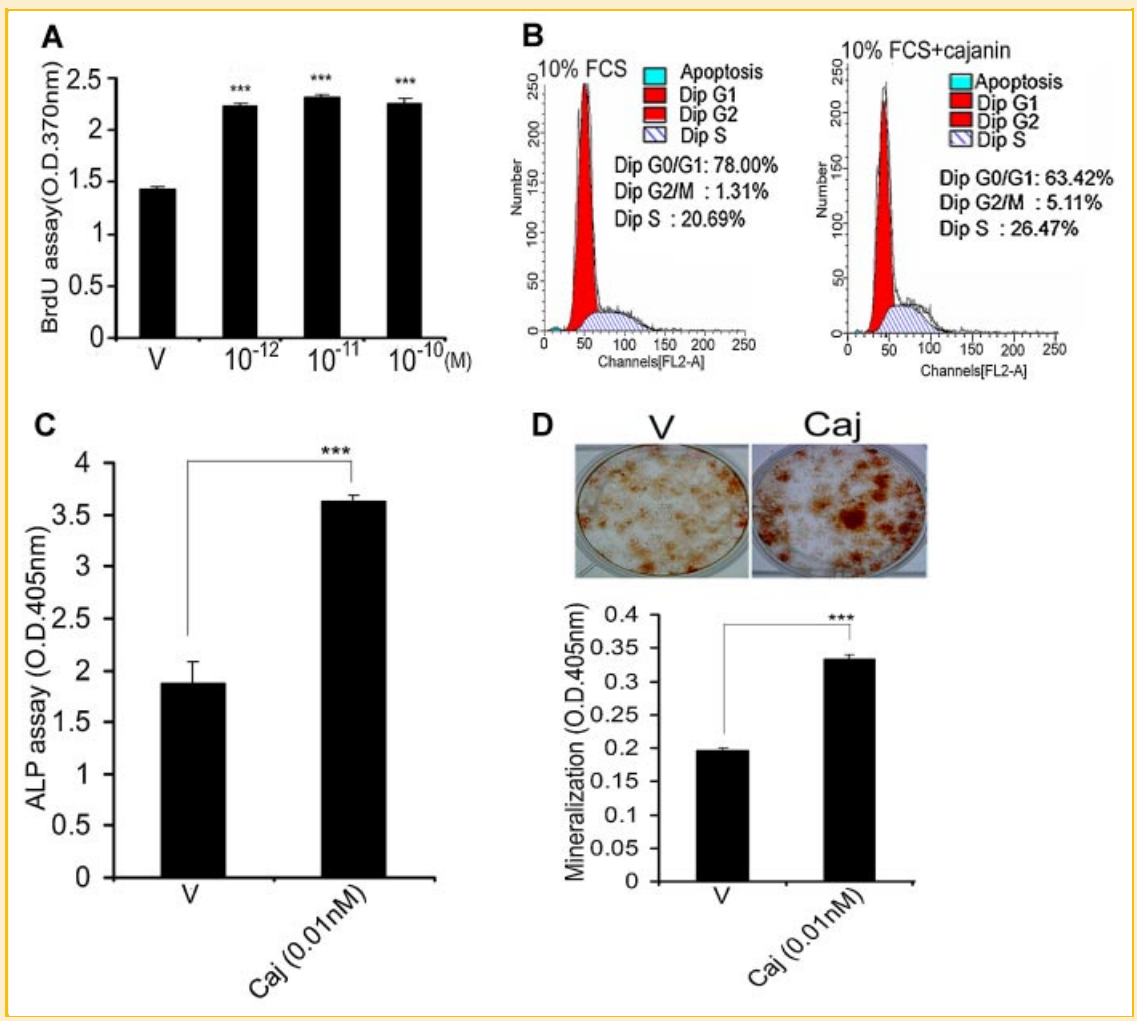

Fig. 2. Effects of cajanin on osteoblast proliferation, differentiation, and mineralization of bone marrow osteoprogenitor cells. A: Calvarial osteoblasts were exposed to various concentrations of cajanin (Caj) for 24 h and proliferation was determined by BrdU ELISA. Data are mean ± SEM; n = 3; $P < 0.001$. B: Osteoblasts were treated with 10% FCS or 10% FCS + cajanin (10^{-11} M) for 24 h and stained with propidium iodide (PI) for FACS analysis. C: Cells were exposed to cajanin (10^{-11} M) for 48 h and ALP activity was determined as described in the Materials and Methods Section. D: Bone marrow cells (25,000 cells/well) from rats were seeded into 12-well plates in differentiation medium and treated with cajanin (10^{-11} M) for 21 days (as described in the Materials and Methods Section). At the end of the incubation, cells were stained with alizarin red-S. Stain was extracted and OD measured colorimetrically. Data shown as mean ± SEM; n = 3; $^{**}P < 0.01$, $^{***}P < 0.001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

osteoblasts, promoted differentiation of BMCs. To this end, rat BMCs were induced for mineralization for 21 days under osteoblast differentiation condition, whereby cajanin was found to stimulate increased formation of mineralized nodules of BMCs compared with control cells (Fig. 2D).

The impact of cajanin on the stimulation of osteoblast proliferation, assessed by BrdU incorporation, was abolished by U0126, a MEK1/2 inhibitor (Fig. 3A). In addition, Figure 3A shows that osteoblast proliferation was also dependent on Akt signaling, a cellular survival pathway, as the specific inhibitor, LY-294002 (10.0 μM) completely abrogated cajanin-stimulated BrdU incorporation to osteoblasts. Together, these data suggested activation of MEK-Erk and subsequent Akt pathways in regulating cajanin-stimulated osteoblast proliferation. In case of stimulation of osteoblast differentiation by cajanin, it was however, observed that U0126 completely inhibited ALP production by osteoblast, in support of above data, suggesting involvement of MEK-Erk pathway in this process (Fig. 3B).

Western blot data were in conformity with the inhibitor data as cajanin-induced phosphorylation of both Erk1/2 and Akt (Fig. 3C). Pre-treatment with LY294002 had no effect on the phosphorylation of Erk1/2 by cajanin (Fig. 3D), whereas presence of U0126 completely abolished phosphorylation of Akt induced by cajanin (Fig. 3E), suggesting sequential activation of MEK-Erk and Akt pathways by cajanin in promoting osteoblast functions.

ISOFORMONONETIN STIMULATED OSTEOBLAST SURVIVAL AND DIFFERENTIATION: SIMULTANEOUS ACTIVATION OF MEK-ERK AND AKT PATHWAYS

In pilot study we observed that unlike cajanin, isoformononetin (10^{-9}–10^{-7} M) had no effect on BrdU incorporation (data not shown). Next, calvarial osteoblasts induced for apoptosis by serum deprivation was tested for possible protection by cajanin or isoformononetin, assessed by FACS using annexin-PI staining. We observed that cajanin failed to afford any protection to osteoblast apoptosis under serum deprivation (data not shown). In

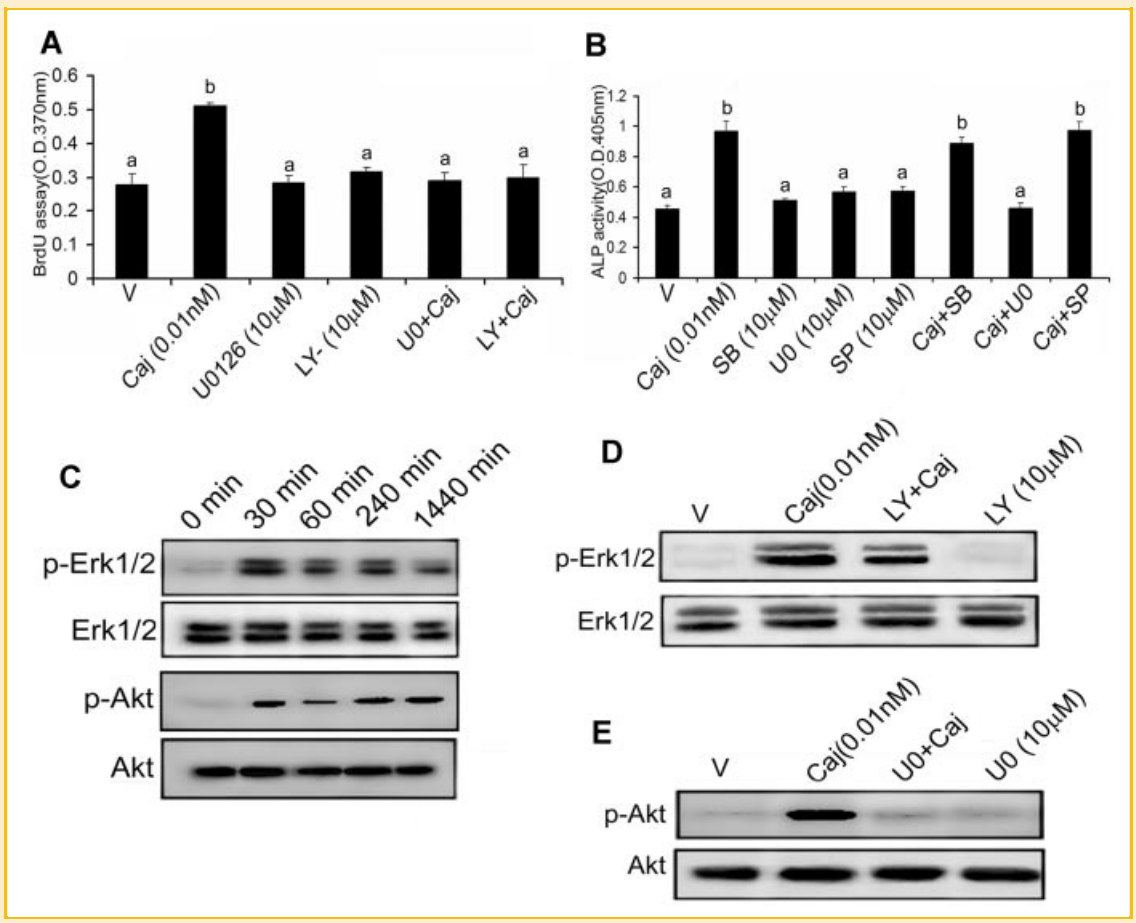

Fig. 3. Stimulation of proliferation and differentiation of osteoblasts by cajanin are mediated by MEK/Erk–Akt pathway. A: Treatment of osteoblasts with U0126 (10 μM) and LY294002 (10 μM) significantly abolished cajanin (Caj)-induced osteoblast proliferation assessed by BrdU ELISA. Data are mean ± SEM; n = 3. Different alphabets denote significant differences amongst individual mean at $P < 0.001$. B: Osteoblasts were incubated for 48 h with cajanin in the presence or absence of various MAPK inhibitors, viz. U0126, SB203580 and SP600125. ALP production from osteoblasts was measured as described before. Data are mean ± SEM; n = 3. Different alphabets denote significant differences amongst individual mean at $P < 0.001$. C: Activation of Erk and Akt pathways by cajanin in osteoblasts. Cells were treated with 10^{-11} M cajanin and cell lysates were collected at different time points. Lysates were resolved on SDS–PAGE and the blots were probed with specific antibodies as indicated in the figure. D: Pre-treatment with LY294002 had no effect on Erk1/2 phosphorylation, while pre-treatment with U0126 (E) inhibited the phosphorylation of Akt induced by cajanin. Panels C–E are representative gels of three independent experiments with similar results.

contrast, isoformononetin protected osteoblasts from serum deprivation-induced apoptosis (Fig. 4). Osteoblasts cultured in 10% FCS, contained 18% apoptotic cells compared with 56.6% apoptotic cells when cultured under serum-deprived condition (0.5% FCS) (Fig. 4). When isoformononetin (10^{-8} M) was added to osteoblast cultures under serum-deprived condition only 24.6% cells were found to be apoptotic (Fig. 4). These data indicate that isoformononetin inhibits osteoblast apoptosis by ~2.0-fold under serum deprivation.

Calvarial osteoblasts, when treated with LY294002 alone under serum deprived condition had 48% apoptotic cells (not shown in Fig. 4) compared with 36.6% in cells treated with isoformononetin + LY294002 (Fig. 4). From these data it appears that the anti-apoptotic effect of isoformononetin in osteoblasts is partly dependent on Akt pathway. In addition, when osteoblasts were treated with U0126 alone under serum deprived condition exhibited 45% apoptotic cells (not shown in Fig. 4) compared with 37.6% in cells treated with isoformononetin + U0126 (Fig. 4). These data suggest that in addition to PI3K-Akt pathway, the anti-apoptotic effect of isoformononetin is partly mediated by MEK1/2 pathway. When treated with both LY294002 and U0126, the effect of isoformononetin on osteoblast survival was completely abolished (Fig. 4), suggesting synergistic activation of MEK-Erk and Akt pathways by isoformononetin in affording osteoblast survival under serum deprivation condition.

In addition to osteoblast survival, isoformononetin promoted osteoblast differentiation and mineralization. Treatment of calvarial osteoblast cells with isoformononetin from 10^{-9} to 10^{-7} M resulted in enhanced ALP production (Fig. 5A). At the same concentration, isoformononetin increased mRNA levels of ALP in osteoblasts (Supplementary Fig. 1). Furthermore, we observed that isoformononetin, in addition to calvarial osteoblasts, promoted differentiation of BMCs. To this end, rat BMCs were induced for mineralization for 18–21 days under osteoblast differentiation condition, whereby isoformononetin was found to stimulate increased formation of mineralized nodules of BMCs compared with control cells (Fig. 5B).

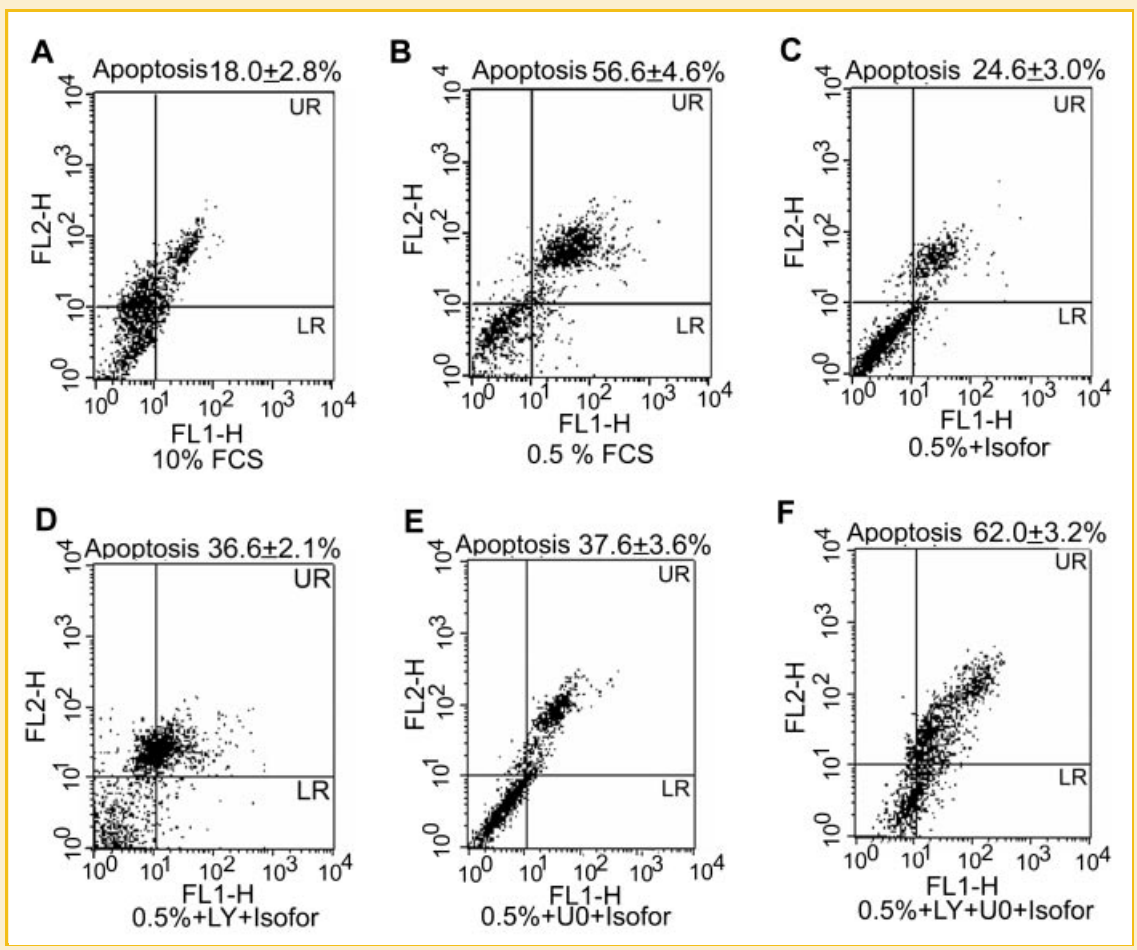

Fig. 4. Isoformononetin exerts anti-apoptotic effect in osteoblasts via the activation of Erk1/2- and Akt pathways. Osteoblasts were treated under various conditions and stained with annexin-V and PI for FACS analysis. A: Osteoblasts cultured in complete growth medium (10% FCS), (B) after culturing in 10% FCS, osteoblasts were exposed to serum-deprived condition (0.5% FCS) for 24 h, which induced osteoblast apoptosis, (C) osteoblasts cultured in 0.5% FCS in the presence of isoformononetin (Isofor, 10^{-8} M) for 24 h, (D) osteoblasts cultured in 0.5% FCS in the presence of isoformononetin (10^{-8} M) and LY294002 for 24 h, (E) osteoblasts cultured in 0.5% FCS in the presence of isoformononetin (10^{-8} M) and U0126 for 24 h and (F) osteoblasts cultured in 0.5% FCS in the presence of isoformononetin (10^{-8} M) and both LY294002 and U0126 for 24 h. Data are mean ± SEM; n = 3.

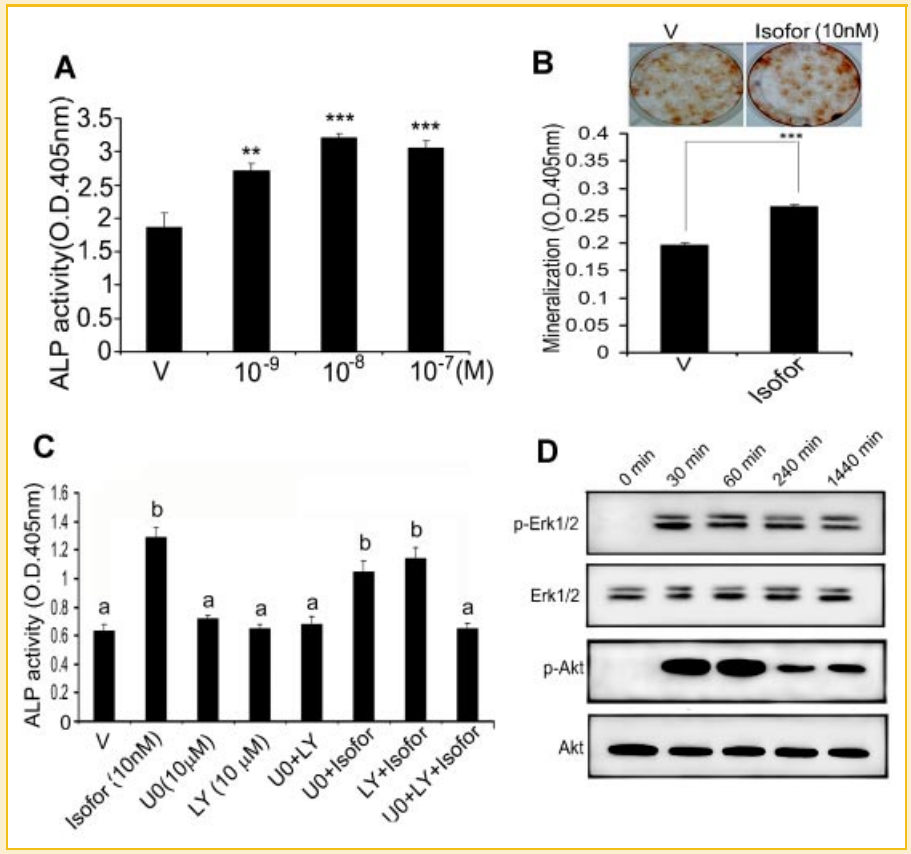

Fig. 5. Effect of isoformononetin on differentiation of calvarial osteoblasts and mineralization of bone marrow osteoprogenitor cells. A: Osteoblasts were exposed to various concentrations of isoformononetin (Isofor) for 48 h and ALP activity was determined as described in the Materials and Methods Section. Data are mean ± SEM; n = 3; $^{**}P < 0.01$, $^{***}P < 0.001$. B: Bone marrow cells (25,000 cells/well) from rats were seeded into 12-well plates in differentiation medium and treated with isoformononetin (10^{-8} M) for 21 days (as described in the Materials and Methods Section). At the end of the incubation, cells were stained with alizarin red-S. Stain was extracted and OD measured colorimetrically. Data are mean ± SEM; n = 3; $^{***}P < 0.001$. C: Osteoblasts were incubated for 48 h with isoformononetin (10^{-8} M) in the presence or absence of U0126 or LY294002. Data are mean ± SEM; n = 3. Different alphabets denote significant differences amongst individual mean at $P < 0.001$. D: Time course of activation of Erk and Akt by isoformononetin. The figure is a representative gel of three independent experiments with similar results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Effect of isoformononetin on osteoblast ALP production was inhibited by U0126 or LY-294002, albeit both partially (Fig. 5C). Since, co-treatment of U0126 and LY-294002 completely abolished ALP production stimulated by isoformononetin, suggested parallel activation of MEK-Erk and Akt pathways by isoformononetin in stimulating osteoblast ALP production (Fig. 5C). These results were confirmed by western blotting which show that isoformononetin stimulated phosphorylation of both Erk1/2 and Akt in osteoblasts (Fig. 5D).

CAJANIN AND ISOFORMONONETIN ACT INDEPENDENT OF ESTROGEN RECEPTOR (ER)

Reports show that isoflavones such as genistein and daidzein act via the ER in osteoblasts [Choi et al., 2008]. Hence, in order to test whether cajanin or isoformononetin mediated their actions in osteoblasts through ER, we used anti-estrogen, ICI-182780. Our data show that ICI-182780 had no effect on cajanin- or isoformononetin-induced osteoblast functions such as proliferation and differentiation (Fig. 6A,B). Furthermore, in Huh7 cells transfected with human ERα and ERβ neither cajanin nor isoformononetin transactivated these reporter gene constructs (Fig. 6C,D), suggesting lack of ER-mediated signaling by these compounds.

CAJANIN AND ISOFORMONONETIN DIFFERENTIALLY PROMOTE ACHIEVEMENT OF PBM

We next assessed the in vivo effects of cajanin and isoformononetin in growing female rats where bone formation is the dominant event. Female Sprague–Dawley rats at weaning were given individual treatment of either cajanin or isoformononetin at 10.0 mg kg^{-1} day^{-1} dose by oral gavage for 30 consecutive days. Since, pilot studies at lower doses than 10.0 mg kg^{-1} day^{-1} dose had no effect, we selected this dose. Gum acacia was used as vehicle (control group).

Figure 7 shows the effects of these isoflavones on BMD levels. Cajanin robustly increased BMD levels in all anatomical regions of the skeleton studied when compared with control. Isoformononetin treated rats, when compared with control, also exhibited significant increases in BMD levels at all anatomical regions of skeleton except

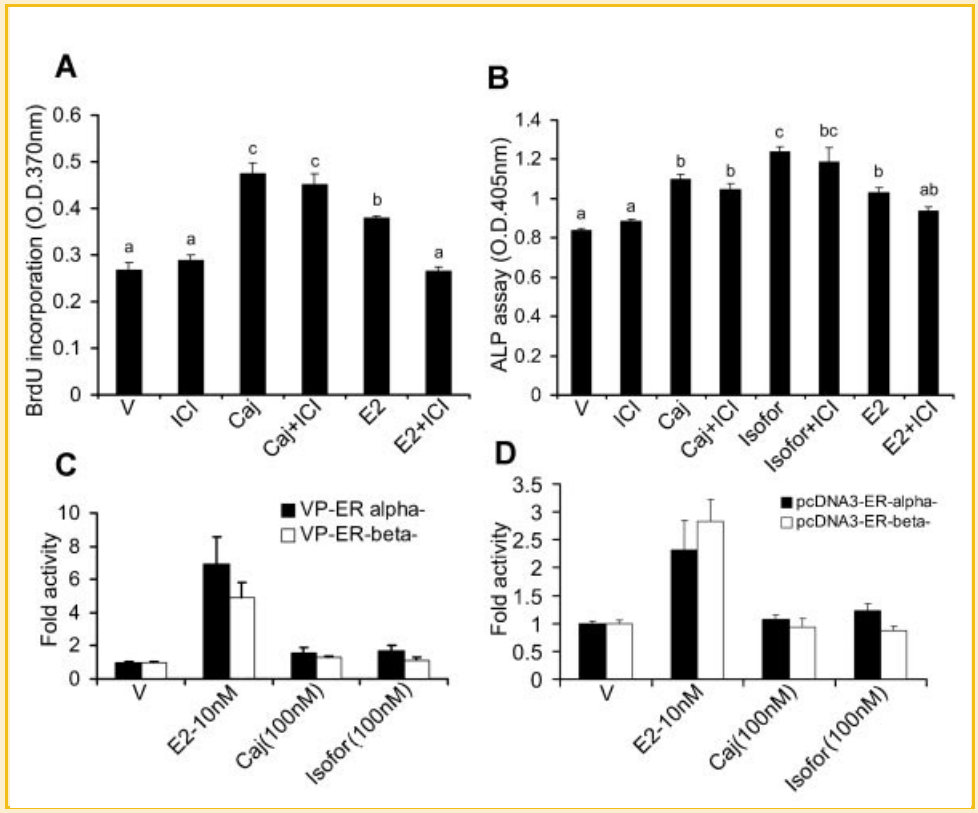

Fig. 6. Cajanin and isoformononetin do not signal via ER. A: Effect of ICI-182780 on osteoblast proliferation induced by cajanin (Caj). Cells were pre-treated with ICI 182780 (1 nM) for 30 min with or without various test compounds indicated and subsequently cultured for 24 h. Data show that ICI 182780 failed to abolish osteoblast proliferation induced by cajanin. Data are mean ± SEM; n = 3. Different alphabets denote significant differences amongst individual mean at $P < 0.001$. E2 was taken as a positive control. B: Effect of ICI-182780 on osteoblast differentiation induced by cajanin and isoformononetin (Isofor). Cells were pre-treated with ICI 182780 (1 nM) for 30 min 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 cajanin or isoformononetin. E2 was taken as a positive control. Data are mean ± SEM; n = 3. Different alphabets denote significant differences amongst individual mean at $P < 0.001$. 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 Section. Luciferase values were normalized with GFP values and are expressed as fold activity over vehicle treated cells. 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.

femur neck, however, the extent of increase was less robust than that obtained with cajanin treatment.

Bone strength is the measure of the quality of bone. Table I shows that force and stiffness of femur were significantly higher in rats treated with cajanin compared with controls, correlating with femoral BMD data. Isoformononetin had no effect on femoral force or stiffness, although it significantly increased BMD of midshaft. Our data suggest that a *P*-value less than 0.001 in BMD levels of femoral midshaft, as in the case of cajanin correlates with increased femoral biomechanical strength. Compressive energy measurement of lumbar vertebra 3 (LV3) also was in agreement with the BMD data. Cajanin treatment but not isoformononetin showed a higher compressive energy for breaking LV3 when compared with vehicle group. Thus, cajanin treatment not only increased the BMD but also improved the quality of bone.

Dynamic histomorphometric studies by double fluorochrome (tetracycline–calcein) labeling experiment allowed determination of new bone formation during the period of administration of a given agent. Figure 8A show that both cajanin and isoformononetin significantly increased mineral apposition rate (MAR) and BFR compared with control.

ASSESSMENT OF UTERINE ESTROGEN AGONISTIC/ANTAGONISTIC ACTIVITY OF CAJANIN AND ISOFORMONONETIN

Isoflavones are known to possess varied degrees of estrogenic or anti-estrogenic effects [Hopert et al., 1998]. We observed that in vitro, none of these compounds appear to signal via ERs. Here, we studied whether cajanin and isoformononetin have estrogen agonistic or antagonistic effects in uterus. Weaning (21 days old) Sprague–Dawley rats were ovariectomized (OVx) and oral administration of cajanin or isoformononetin were given at 10 mg kg^{-1} day^{-1} dose for 3 consecutive days with or without 17β-E_2. Table II shows that while cajanin did not exhibit neither estrogenic nor anti-estrogenic effects as assessed by uterine wet weight, isoformononetin was mildly uterotropic as it increased uterine weight by ~88% in OVx rats over the control. As expected, 17β-E_2 treatment (0.01 mg kg^{-1} day^{-1}) to OVx rats exhibited ~5.7-fold increase in uterine wet weight compared with controls (Table II).

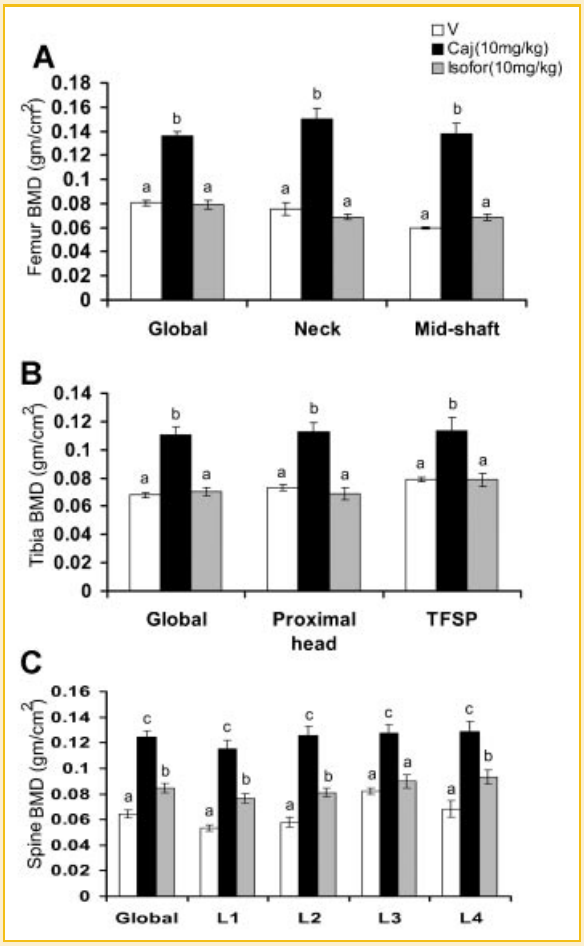

Fig. 7. Effect of cajanin and isoformononetin on BMD levels of various bones. A: BMD of whole femur, femur neck (trabecular bone) and femur midshaft (cortical bone). B: BMD of whole tibia, tibia proximal (trabecular bone) and tibia tibio-fibula separating point (TFSP; cortical bone) (C) BMD of whole vertebrae and L1–L4 vertebrae (all trabecular bones) with respect to vehicle. Data represented as mean ± SEM. Different alphabets denote significant differences amongst individual mean at $P < 0.001$.

Furthermore, histological evaluation of uteri with various treatments show that whereas E_2 treatment resulted in characteristic hypertrophy of luminal and glandular epithelium compared with control, cajanin and isoformononetin treated rats exhibited comparable histological features with that of controls (Fig. 8B). Thus, while cajanin was devoid of any estrogenic or anti-estrogenic effects, isoformononetin exhibited a mild uterotropic effect.

DISCUSSION

We have recently shown that the crude extract of the stem-bark of *B. monosperma* promote osteoblast mineralization in vitro [Maurya et al., 2009]. Employing activity-guided fractionation of the crude extract using ALP production by calvarial osteoblasts as bioassay (data not shown) yielded two methoxyisoflavones, that is, cajanin and isoformononetin [Maurya et al., 2009]. Cajanin and isoformononetin, respectively, are methoxyl derivatives of genistein and daidzein having methoxyl group at C-7 position of both the molecules. Although the effects of genistein and daidzein on osteoblats have been well reported [Anderson and Garner, 1998], effects of cajanin and isoformononetin are unknown. In order to study the effects of cajanin and isoformononetin in osteoblasts and bone formation in vivo, we synthesized both compounds.

Isoflavones such as genistein, daidzein (the soy isoflavones) and myricetin are known to have bone-protective action by virtue of their estrogen-"like" effect [Chrzan and Bradford, 2007; Sharan et al., 2009]. However, results of the clinical and epidemiological studies on the effects of consumption of soy foods enriched in soy isoflavones on bone health are not convincing in affording bone-conserving effects in menopausal osteoporosis, thereby leaving the scope for identifying better forms of isoflavones for promoting bone health [Geller and Studee, 2006]. Examples of other methoxylated isoflavones include biochanin A and glycetin, which exhibit ER-dependence in promoting osteoblast function in vitro [Peterson et al., 1998; Song et al., 1999; Yoshida et al., 2001] however, in vivo studies are lacking. Higher acquisition of PBM correlates with better bone health after menopause [Sowers, 2000]. The only study addressing the effect of soy isoflavones in increasing PBM is inconclusive [Fujioka et al., 2007]. Herein, we demonstrate that two methoxyisoflavones, cajanin and isoformononetin, have differential modes of action in osteoblasts and on bone formation and the effects are ER independent.

In vitro, cajanin and isoformononetin promoted different osteoblast functions with varying potency. Cajanin exerts mitogenic effect by increasing the number of osteoblasts at S- and G_2/M phases of cell cycle. Cajanin also stimulates osteoblast differentiation evident from increased ALP mRNA levels (Supplementary Data 1) and activity as well as formation of mineralized nodules from BMCs. These effects of cajanin are regulated by sequential activation of MEK-Erk and Akt pathways in osteoblasts. On the other hand, isoformononetin has strong anti-apoptotic effect in osteoblasts, suggesting that isoformononetin promotes osteoblast survival. This

TABLE I. Effect of Isoflavones on Bio-Mechanical Properties of Isolated Right Femur and Third Vertebrae

		Bending force, right femur bone		Compression, Third Vertebrae (LV3)	
Treatment	Dose (mg/kg)	Ultimate force (N)	Stiffness (N/mm)	Energy (mJ)	Stiffness (N/mm)
Vehicle	10	33.8 ± 1.70	47.8 ± 4.13	301.56 ± 20.27	394.55 ± 15.70
Cajanin	10	44.0 ± 1.72[a]	75.8 ± 9.71[a]	549.33 ± 29.50[b]	485.07 ± 14.45[b]
Isofor	10	32.0 ± 1.33	51.0 ± 3.4	384.42 ± 22.58[a]	455.78 ± 15.88[a]

Values represent mean±SEM of eight independent sets of bones in each treatment group.
[a]$P < 0.05$,
[b]$P < 0.01$versus corresponding vehicle control group.
Isofor-Isoformononetin.

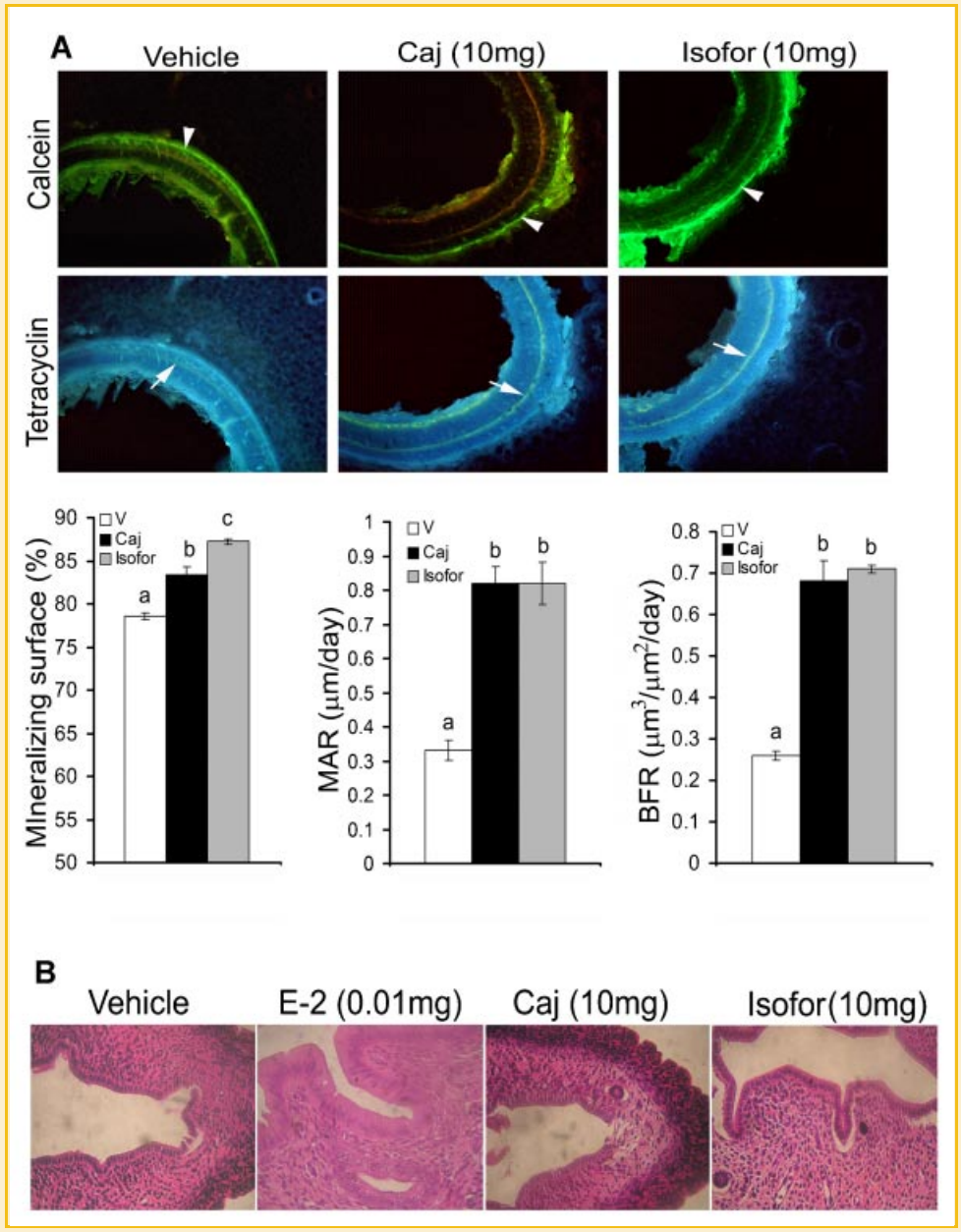

Fig. 8. Cajanin and isoformononetin promote bone formation and do not exhibit uterine estrogenicity. A: Representative images of transverse sections of tetracycline and calcein labeled femur diaphyses from rats after 30 days treatment with vehicle or cajanin (Caj) at 10.0 mg kg^{-1} day^{-1} dose or isoformononetin (isofor) at 10.0 mg kg^{-1} day^{-1} dose. Tertracyclin (UV filter) and calcein (orange) labeling are shown. Arrow head—calcein, arrow—tetracyclin. Bone histomorphometric parameters calculated from these labeling experiments are shown in lower panel. Different alphabets denote significant differences amongst individual mean at $P < 0.001$. B: Uteri were harvested from rats treated with vehicle or E_2 or cajanin (Caj) or isoformononetin (Isofor). Cross-sections of uterine tissues were stained with H&E. Much thicker endometrium and greater epithelial cell heights were observed in the E_2 treated group while cajanin or isoformononetin exhibited comparable histological features with that of vehicle. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

effect of isoformononetin is partially mediated by MEK-Erk and Akt pathways. In addition, both MEK-Erk and Akt pathways regulate isoformononetin-induced differentiation of osteoblast. Finally, cajanin at 0.01 nM stimulated formation of mineralized nodules from the BMCs compared to 10 nM required for isoformononetin for the same effect. As the concentration of cajanin required for stimulating various osteoblast functions in vitro was less than that required by isoformononetin, it appears that cajanin is more potent than isoformononetin. Sequential activation of MEK-Erk and Akt pathways by cajanin compared with simultaneous activation of both these pathways by isoformononetin may be attributed to more potent effect of cajanin over isoformononetin. It should be noted that the effects of cajanin and isoformononetin on osteoblasts are observed at concentrations that are 100- to 1,000-fold less than that reported for genistein and daidzein [Yamaguchi and Sugimoto, 2000]. Therefore, it appears that methoxylation enhances activities

TABLE II. Estrogen Agonistic and Antagonistic Effect of Methoxyisoflavones in Ovariectomized Immature Sprague-Dawley Rats

		Estrogenic activity		Anti-estrogenic activity	
Treatment	Dose (mg/kg)	Uterine weight	% Gain[a]	Uterine weight	% Inhibition[b]
Vehicle	10	14.7 ± 2.20			
Ethynylestradiol	0.01	$98.3^{c} \pm 11.50$	568		
Cajanin	10	16.0 ± 2.20	9	94.8 ± 3.10	4
Isofor	10	$27.7^{d} \pm 3.00$	88	92.8 ± 15.50	6

Isofor, isoformononetin.
Values are mean ± SEM.
[a]Percent gain over corresponding vehicle control group.
[b]Percent inhibition in ethynylestradiol induced uterine weight gain.
[c]$P < 0.001$ versus vehicle treated group.
[d]$P < 0.05$ versus vehicle treated group.

of genistein (as in case of cajanin) as well as daidzein (as in case of isoformononetin).

We observed that neither cajanin nor isoformononetin activated transcriptional activity of human ERα or ERβ in Huh7 cells, suggesting lack of ER-mediated signaling by these compounds. ER independent effect was further confirmed when ICI-182780 failed to abrogate the stimulatory effects of osteoblast proliferation and differentiation by cajanin and isoformononetin, respectively. Therefore, our data suggest that unlike genistein and daidzein, their methoxylated forms have no ER dependence in vitro. Daidzein and genistein are also known to have uterine estrogen agonistic/antagonistic actions [Farmakalidis et al., 1985; Shao et al., 1998; De Wilde et al., 2004; Matsumura et al., 2005]. In contrast, cajanin and isoformononetin did not exhibited estrogen-"like" effect in uterus. Although cajanin has been reported to have anti-proliferative effect in MCF-7 cells, which is an anti-estrogenic effect [Umehara et al., 2008], we failed to observe such effect of cajanin or isoformononetin in rat uterus. Considering ER dependence of biochanin A (methoxyl group at C-6 position) and glycetin (methoxyl group at C-4′ position) [Choi et al., 2008] and ER independence of cajanin and isoformononetin (in both cases methoxyl group at C-7 position), it appears that the site in which methoxyl substitution of isoflavones take place could be a determinant of estrogenicity. Lack of estrogen agonistic or antagonistic effects of cajanin and isoformononetin allows scope for their use in menopausal osteoporosis treatment.

In vivo, compared with control rats, both cajanin and isoformononetin treatment resulted in significant increases in the BMD and bone biomechanical strengths in growing rats, albeit with varying efficacy. Cajanin was more effective than isoformononetin in increasing BMD levels, which was true at all anatomic sites of the skeleton. Cajanin and not isoformononetin increased bone strength in femur and spine. It was interesting to note that despite increased BMD levels by isoformononetin at various skeletal sites compared with controls, bone strengths of isoformononetin-treated rats were unchanged from the control rats. It appears that increases in the femoral biomechanical strengths in cajanin correlates with much higher diaphyseal BMD levels achieved by cajanin treatment (132%, $P < 0.001$) compared with isoformononetin treatment (15%, $P < 0.05$), over control. The same explanation may be applied in case of vertebral compression as cajanin treatment resulted in 55% ($P < 0.001$) increase in the BMD levels of third lumbar spine compared to no changes in the BMD levels of the same spine in isoformononetin treated rats compared with control rats.

Dynamic histomorphometric studies [Hara et al., 2002] in femur revealed that both cajanin and isoformononetin to a comparable extent increased mineralizing surface compared to control group. Both compounds increased MAR and BFR by ~2.5-fold over control. Increases in MAR and BFR result from the expansion of osteoblast pool (by proliferation and/or increased survival) and increase in osteoblast differentiation. Cajanin and isoformononetin stimulate osteoblast proliferation, survival and differentiation, thereby explaining increased MAR and BFR by these two compounds over control. However, our data revealed that assessments of BMD and bone strengths did not correlate with histomorphometric parameters, as cajanin exhibited more robust effects in increasing BMD and bone strength compared with isoformononetin yet mineralizing surface, MAR and BFR values were comparable between cajanin and isoformononetin. Bone strength is determined by multiple factors including bone geometry, trabecular bone morphology, and intrinsic properties of bony tissues. From our study, we speculate that cajanin and not isoformononetin treatment may favorably affect the factors influencing bone strength thereby contributing to increased bone strength by cajanin.

Collectively, we demonstrate that cajanin and isoformononetin have differential actions in osteoblasts and parameters of acquisition of PBM in growing female rats. Structural differences in methoxylation and hydroxylation of genistein and daidzein may contribute to significant alteration in biological effects. Given their osteogenic activity and lack of estrogen-"like" effect, cajanin and isoformononetin could be better prophylactic options over genistein or daidzein and also hold therapeutic promise in menopausal osteoporosis. In this regard, cajanin appears to be the more suitable candidate.

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