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Discovery of a tetrazolyl β -carboline with *in vitro* and *in vivo* osteoprotective activity under estrogen-deficient conditions†

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β -Carbolines have been assessed for osteoclastogenesis. However, their effect on osteoblasts during estrogen deficiency is still unclear. Here, a series of novel piperazine and tetrazole tag β -carbolines have been synthesized and examined for osteoblast differentiation *in vitro*. *In vitro* data suggest that compound **8g** is the most promising osteoblast differentiating agent that was evaluated for *in vivo* studies. Compound **8g** promoted osteoblast mineralization, stimulated Runx2, BMP-2 and OCN expression levels, increased BrdU incorporation and inhibited generation of free radicals as well as nitric oxide. Since a piperazine group is involved in bone repair activity and β -carboline in I κ B kinase (IKK) inhibition, compound **8g** inhibited tumor necrosis factor α (TNF α) directed I κ B α phosphorylation, preventing nuclear translocation of NF- κ B thereby alleviating osteoblast apoptosis. *In vivo* studies show that compound **8g** was able to restore estrogen deficiency-induced bone loss in ovariectomized rats without any toxicity, thus signifying its potential in bone-protection chemotherapy under postmenopausal conditions.

Introduction

Aging and postmenopausal osteoporosis are the most frequent and extensive metabolic bone diseases characterized by low bone mass and structural deterioration of bone tissue. They result from a disproportion of the bone-remodeling process, with bone resorption exceeding bone formation. Bone resorption is a multistep process which ultimately requires secretion of hydrochloric acid by osteoclasts.^{1–3} Global bone loss with reduced mineral density, bone destruction, and imbalance of bone remodeling are pathological hallmarks of osteoporosis, inflammatory joint disease, and rheumatoid ar-

thritis (RA).^{4–6} Selective estrogen receptor antagonists and hormone replacement therapy (HRT) act by inhibiting bone resorption and are effective in the prevention and treatment of osteoporosis associated with estrogen deficiency. Hormone replacement therapy has been associated with an increased risk for cancer. Thus, HRT is no longer recommended for fracture prevention for postmenopausal women.^{7–9} The array of existing treatments for the disease characterized by amplified osteoclast activity, therefore, falls into comparatively few mechanistic classes, highlighting the need to identify new classes of bone anabolic agents. Natural products isolated from nature have been verified to be rich sources of disease-modulating drugs throughout the history of medicinal chemistry and pharmaceutical drug development.^{10,11} Therefore, there is a need for the advancement of efficient synthesis methods which give access to natural product derived and inspired compound collections. In view of the foregoing facts, the search for innovative drugs based on new molecular scaffolds that target the specific metabolic pathway of the bone should be highly prioritized, which in turn requires new medicinal chemistry approaches to discover novel lead compounds that might populate a pipeline of new therapeutics. An attractive concept of hybridization for osteoporosis is becoming popular as an emerging structural modification tool to design new molecules with improved bioactivity when compared to the parent compounds. In this context, biology-oriented synthesis (BIOS) of natural product inspired scaffolds

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and their hybrids may show promising results in finding new lead structures for chemical biology and medicinal chemistry research.¹² Furthermore, bioactive small molecule natural products possess potency, selectivity and pharmacokinetic properties, an important paradigm for drug-like molecules.¹³ However, the number of natural products is limited; hence incorporating either different natural products or drug fragments may provide millions of combinations which will have enriched biological activity and less toxicity.

β -Carboline is a building block of naturally occurring alkaloids and is utilized as a drug-like scaffold in several natural products (harmine, harmane and harmol), as it possesses a wide range of biological activities including antitumor, anti-convulsant, antiviral, anti-inflammatory, analgesic, antimicrobial, antifungal, antimalarial, antidiabetic and cytotoxicity.^{14–16} However, little is known about the effects of β -carboline alkaloids on bone metabolism.¹⁷ β -Carboline alkaloids are also known for their anti-inflammatory activity. Principally, β -carboline inhibits I κ B kinase (IKK), which has long been required as a specific regulator of NF- κ B.¹⁸

On the other hand, tetrazole and acids are bioisosteres. Tetrazole plays a similar role to acids in biological systems; acid components are commonly present in drugs prescribed for osteoporosis.¹⁹ Recently, piperazine derivatives have appeared with bone repair activity.²⁰ They were also useful in treating bone fracture or deficiency, osteolytic bone disease, metastatic bone disorder, post dental implantation and post-prosthetic joint surgery.²¹ On the basis of this reasoning and as a part of our ongoing interest in the design and synthesis of novel heterocycles as bone anabolic agents, we have synthesized β -carboline, tetrazole, and piperazine hybrids *via* IMCR chemistry as discussed above with different possible functionalities (Fig. 1). To the best of our knowledge, the hy-

bridization of these bioactive pharmacophores and their bone protection activity have never been reported to date. Through these processes, we have discovered novel scaffolds with potent bone protection activity with a high selectivity index which is comparable or superior to that of 17 β -estradiol. The compound which has a combination of high efficacy and no cytotoxicity with a high value of the selectivity index was tested further for *in vivo* studies in an ovariectomized (Ovx) rodent model.

Chemistry

The detailed synthetic route to synthesize compounds **8a–p** is outlined in Schemes 1 and 2. The first objective of this study was to access methyl or ethyl 1-formyl-9H- β -carboline-3-carboxylate. Our method commenced with the Pictet–Spengler reaction of tryptophan methyl or ethyl ester with dimethoxyglyoxal (60% solution in water) to afford the tetrahydro- β -carboline derivative **2** in a yield of 96% and in good purity (Scheme 1). Oxidation of **2** with KMnO₄ at room temperature overnight resulted in acetal **3** (90%). Deprotection of the formyl group with aqueous AcOH gave the required methyl 1-formyl-9H- β -carboline-3-carboxylate in 86% yield. More importantly, in this three-step synthesis, at no stage was any purification required and reactions could be scaled-up to obtain 10 g of the aldehyde.²² The next step of our methodology is a Ugi-MCR using methyl or ethyl 1-formyl-9H- β -carboline-3-carboxylate **4** with sodium azide, amines and isocyanides for the synthesis of β -carbolines with tethered tetrazole **8** in good to excellent yields (Scheme 2). All the final compounds (**8a–8p**), as well as the intermediates (**4a–4b**), were afforded in excellent yields (Table 1). All the compounds were characterized using ¹H NMR, ¹³C NMR, 2D

Fig. 1 A natural product inspired molecular hybridization approach for the synthesis of tetrazole tag β -carbolines.

Scheme 1 Synthesis of the 1-formyl-9H-β-carbolines: i) 2–5% TFA in CH₂Cl₂, **2d–3d**. ii) KMnO₄, dry THF, room temp., **3d–5d**. iii) AcOH/H₂O (2 : 3), 120 °C, 45 min.

Scheme 2 Synthesis of tetrazole tethered β-carbolines by isocyanide-based condensation.

NMR, mass spectrometry and IR spectroscopy. Additionally, the structure of a representative compound (**8b**) was unambiguously confirmed by single crystal X-ray analysis (CCDC deposit No.: 1542868; refer to the ESI† for details). The target compounds and substructures presented herein are not associated with commonly encountered PAINS,²³ ESI† Table S1, (http://www.cbiligand.org/PAINS/search_struct.php).

Results

In vitro studies

The β-carboline intermediates (**4a**, **4b**) and final hybrid compounds (tetrazole tethered β-carbolines: **8a–8p**) were screened for alkaline phosphatase (ALP, a marker of osteoblast differentiation) activity in primary rat calvarial osteoblasts (RCOs). Cells were treated with the synthesized compounds and evaluated for ALP activity at concentrations ranging from 1 pM to 1 μM. The ALP activity was assessed spectrophotometrically, and the optical density was measured at 405 nm (Fig. 2A). Table 2 shows the % ALP activity together with EC₅₀ and *E*_{max} values for all the synthesized compounds. Out of the eighteen derivatives, compounds **4b**, **8g**, **8i** and **8j** significantly stimulated ALP activity in osteoblasts. β-Carboline intermediate **4b** exhibited maximum ALP activity at 100 pM (~65%, *p* < 0.001). However, the synthesized derivatives **8g** (~89%, *p* < 0.001) and **8j** (~80%, *p* < 0.001) showed higher activity at the same concentration, and compound **8i** exhibited higher activity at 10 nM (~75%, *p* < 0.001). This experiment noticeably demonstrated that β-carboline derivatives were found to be more potent leads for osteoblast differentiation.^{24,25} Structure–activity relationship (SAR) analysis showed that piperazine derivatives with an *N*-methyl group are the most effective

molecules. An ethyl ester with a β-carboline ring also increases the ALP activity in osteoblasts. Fragment-based SAR analysis demonstrated that tetrazole and piperazine substitutions were required for higher activity (Fig. 2B).

The toxicity of these four active compounds was tested on RCOs using the MTT assay.²⁶ Apparently, these compounds showed no toxicity to the osteoblast cells compared to the control group (untreated cells) at doses ranging from 1 pM to 1 μM (Fig. 2C).

Compounds that were found active for ALP activity (**4b**, **8g**, **8i**, and **8j**) were further evaluated for their osteogenic activity by a mineralization assay. RCOs were cultured with or without the compounds for 21 days, and at the end of the differentiation process, the cells were fixed and stained with alizarin red-S.^{24,27} Alizarin red-S stains the newly formed mineralized nodules that deposit calcium. Compounds **8g**, **8i** and **8j** showed significantly increased mineralizing ability compared to the control (untreated) cells (Fig. 3A). Quantification of mineralization by optical density measurement of alizarin red-S extracted from stained cultures showed a significant increase in mineralization of osteoblasts cultured in the presence of these compounds (Fig. 3B). Data suggest that compound **8g** (at 100 pM) was found to be the most potent mineralizing agent (*P* < 0.001 vs. the corresponding control group) and showed ~70% increased mineralization compared to control (untreated) cells. This is followed by compounds **8i** (*p* < 0.01) at 10 nM, **8j** (*p* < 0.01) at 100 pM and **4b** (*p* < 0.05) at 100 pM, which showed ~52%, ~48% and ~20% increased mineralization compared to control (untreated) cells, respectively.

Table 1 Synthesis of β -carbolines with tethered tetrazole by utilizing a Ugi-MCR

Entry	R	R ₁	R ₂	Yield (%)
8a	CH ₃		<i>tert</i> -Butyl	78
8b	CH ₃		<i>tert</i> -Butyl	92
8c	CH ₃		<i>tert</i> -Butyl	87
8d	CH ₃		<i>tert</i> -Butyl	89
8e	CH ₃		<i>tert</i> -Butyl	87
8f	C ₂ H ₅		<i>tert</i> -Butyl	91
8g	C ₂ H ₅		<i>tert</i> -Butyl	88
8h	C ₂ H ₅		<i>tert</i> -Butyl	85
8i	C ₂ H ₅		<i>tert</i> -Butyl	92
8j	CH ₃		<i>tert</i> -Butyl	89
8k	C ₂ H ₅		<i>tert</i> -Butyl	77
8l	C ₂ H ₅		<i>tert</i> -Butyl	79
8m	C ₂ H ₅		<i>tert</i> -Butyl	92
8n	C ₂ H ₅		<i>tert</i> -Butyl	89
8o	CH ₃		Cyclohexyl	86
8p	CH ₃		<i>tert</i> -Butyl	84

We next studied the effect of the active compounds (**4b**, **8g**, **8i**, and **8j**) on the expression of various osteogenic genes

including Runx-2, BMP-2, and osteocalcin (OCN) in RCOs by qPCR (Fig. 3C). The housekeeping gene GAPDH was used as the internal control in this study. Runx-2, a bone-specific transcription factor, is a key regulator of osteoblast differentiation and is important for the expression of ALP and collagen I (a biomarker of osteoblast differentiation).^{28,29} Furthermore, we also studied the effect of potent compounds on the relative expression of the bone morphogenetic protein (BMP-2) which is an estrogen-regulated gene involved in osteoblast differentiation. The transcript levels of Runx-2, BMP-2, and OCN were assessed by qPCR after treatment with the compounds, and the results were expressed as the fold change over untreated cells. Our data show that the expression of osteogenic genes after treatment with the compounds increased from ~1.8- to ~4.6-fold, with the maximum increase being with compound **8g** at 100 pM. As an intermediate derivative, compound **4b** showed a similar effect to that of the control. We performed a western blot for the three active compounds to check the expression pattern of various important genes (BMP-2, Runx-2, and OCN) at the protein levels (Fig. 3D). Densitometry analysis showed that compounds **8g**, **8i** and **8j** significantly up-regulated the marker proteins (ESI,† Fig. S33). Thus, taken together, the active compounds increase the differentiation and mineralization of calvarial osteoblasts and up-regulated the osteogenic genes at both RNA and protein levels in the following order: compound **8g** > compound **8j** > compound **8i** > compound **4b**.³⁰ The BrdU incorporation assay showed that compound **8g** exhibited significant proliferation of osteoblastic cells at concentrations ranging from 1 pM to 1 μ M with 100 pM showing maximum proliferation compared with its control (Fig. 3E). Altogether, our results demonstrated that compound **8g** significantly increased the alkaline phosphatase activity, mineralization, and the expression of various key osteogenic genes.

TNF α is the active mediator of generation of intracellular ROS in Ovx animals and results in ROS mediated osteoblast apoptosis.^{31,32} The effect of compound **8g** on scavenging intracellular ROS was evaluated by measuring the oxidation of DCFH-DA using a fluorescence reader (wavelength Ex 485 nm/Em 535 nm),³³ and the results showed that it markedly reduced ($P < 0.001$) intracellular ROS production induced by TNF α (Fig. 4A). N-Acetyl cysteine (NAC) was used as the positive control.

Free radicals and reactive oxygen species (ROS) damage cells, which leads to pathophysiological conditions. Antioxidants with their free radical scavenging ability can delay or inhibit cellular damage and may prevent the pathophysiological conditions. Antioxidants are small in size, have low molecular weight, can interact with free radicals and terminate (partially eradicate) the chain reaction.

Antioxidants are produced in the body and taken through diet as well.³⁴ We assessed the antioxidant activity of compound **8g** at various concentrations using DPPH (Fig. 4B).³⁵ The DPPH (1,1-diphenyl-2-picryl-hydrazyl) violet solution was reduced in the presence of different concentrations of compound **8g**. DPPH itself will exhibit no scavenging activity (100% free radicals or 0% scavenging) and was used as the

Fig. 2 Activity and cell toxicity of the potent derivatives in RCOs. (A) RCOs were treated with derivatives at various concentrations as assessed by alkaline phosphatase (ALP) activity. At the end of the experiment, the ALP activity was measured colorimetrically at 405 nm. NCC: No cell control. (B) Fragment-based structure–activity relationship (SAR) analysis. (C) Cells were cultured in differentiation medium and treated with various concentrations of the compound ranging from 1 pM to 1 μM for 48 h, and cell viability was assessed by the MTT assay. The percent viable cells were calculated compared to untreated cells taken as control. Data represent the mean $\pm$ SEM; $n = 5$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ compared with untreated cells taken as control.

control. The free radical scavenging activity of compound **8g** at concentrations ranging from $1.95 \mu\text{g ml}^{-1}$ to $1000 \mu\text{g ml}^{-1}$ showed that **8g** is active at all concentrations. The maximum free radical scavenging activity of **8g** was 37.79%, 43.54%, and 47.84% at $250 \mu\text{g ml}^{-1}$, $500 \mu\text{g ml}^{-1}$ and $1000 \mu\text{g ml}^{-1}$, respectively, in a concentration-dependent manner. However, its minimum scavenging activity was 3.34% and 3.82% at $1.95 \mu\text{g ml}^{-1}$ and $3.90 \mu\text{g ml}^{-1}$, respectively.

NAC was used as a reference. The NO level was found to be elevated in the Ovx rat model.³⁶ We observed the maxi-

mum nitric oxide scavenging activity of **8g** to be 19.28%, 30.71% and 31.36% at $250 \mu\text{g ml}^{-1}$, $500 \mu\text{g ml}^{-1}$ and $1000 \mu\text{g ml}^{-1}$, respectively. The minimum nitric oxide inhibition was observed to be 4.07% at a lower concentration (Fig. 4C).

β -Carbolines have been reported to be potent inhibitors of IKK.¹⁸ It has been reported that stimulation with TNF α induces excessive generation of ROS to activate NF- κ B signaling pathways. Activation of NF- κ B resulted in nuclear β -catenin degradation and hampered osteoblast differentiation and mineralization.³⁷ **E2** prevented NF- κ B activation and β -catenin

Table 2 Alkaline phosphatase activity of synthesized derivatives

Compound	$E_{\max}$	EC ₅₀ (nM)	ALP activity, %
8a	100 pM	468	29
8b	—	Inactive	Inactive
8c	10 nM	Inactive	Inactive
8d	—	Inactive	Inactive
8e	—	Inactive	Inactive
8f	—	Inactive	Inactive
8g	100 pM	900	89 ($p < 0.001$)
8h	100 nM	936	14
8i	10 nM	1200	75 ($p < 0.001$)
8j	100 pM	3270	80 ($p < 0.001$)
8k	—	Inactive	Inactive
8l	—	Inactive	Inactive
8m	—	Inactive	Inactive
8n	—	Inactive	Inactive
8o	10 pM	530	30
8p	—	Inactive	Inactive
4a	1 nM	545	24
4b	100 pM	613	65 ($p < 0.001$)

Data represent the mean $\pm$ SEM; $n = 5$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ compared with untreated cells taken as control.

degradation by preventing I κ B α phosphorylation.³⁸ Therefore, we examined whether compound 8g has an effect on the NF- κ B signaling pathways induced by TNF α . The results showed that compound 8g decreases the activation of NF- κ B induced by TNF α . Compound 8g markedly suppressed the nuclear levels of p65, nuclear β -catenin degradation and phosphorylation of I κ B α in the presence of TNF α (Fig. 4D and E). Immunofluorescence microscopy showed that compound 8g treatment prevented the TNF α mediated nuclear translocation of NF- κ B and was comparable with E2 treatment (Fig. 4F).

It has been reported that TNF α activation of NF- κ B results in down-regulation of BCL-2 and Bcl-xl and up-regulation of Bax which are involved in the apoptotic pathway.³⁹ E2 treatment has shown up-regulation of Bcl-2 and down-regulation of Bax.^{40,41} Compound 8g significantly prevented the down-regulation of Bcl-2 as well as Bcl-xl and the up-regulation of Bax induced by TNF α (Fig. 4E). FACS analysis showed that compound 8g prevents osteoblast cells from undergoing TNF α induced apoptosis by $\sim 47\%$ (Fig. 4G and H). Therefore, compound 8g is used for further detailed *in vivo* evaluation in ovariectomized rats.

In vivo studies

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India and approved by the Institutional Animal Ethical Committee (IAEC) at the Central Drug Research Institute (CDRI), Lucknow, India. The trabecular bone is easily deteriorated in the Ovx condition; hence, it was interesting to study if compound 8g is able to prevent the deterioration of the trabecular microarchitecture

as a result of Ovx. The Ovx + vehicle (1% gum acacia dissolved in distilled water) group showed a significant decrease in BMD levels by $\sim 64\%$ in the distal femoral (trabecular bone) compared with the sham + vehicle (1% gum acacia dissolved in distilled water) group (Fig. 5A). Compound 8g at both doses, 1 mg kg⁻¹ and 5 mg kg⁻¹, significantly restored the trabecular BMD levels in the femur by $\sim 46\%$ compared with the Ovx + vehicle group. Fig. 5B shows representative femoral metaphysis reconstructed 3D μ CT images of trabecular microarchitectures among the various treatment groups. It is evident from the images that when compared with the sham group, the Ovx group had a significantly deteriorated bone microarchitecture. Treatment with compound 8g significantly restored the microarchitecture at both doses (1 and 5 mg kg⁻¹ per day) with improved bone parameters.^{42–45} Table 3 summarizes the microarchitectural parameters of the bone as assessed by μ CT.^{46–48} Ovx is typically associated with a reduction in trabecular thickness, number, connectivity, and “plate-like” structures. Data revealed that Ovx resulted in significant microarchitectural deterioration as indicated by reduced bone volume/trabecular volume (BV/TV) and trabecular number (Tb.N), together with increased trabecular separation (Tb.Sp) and structural model index (SMI). The SMI is used to assess “plate-like” trabeculae. Higher SMI values indicate deterioration of the trabeculae from plate-like to “rod-like” structures. The treatment of Ovx rats with compound 8g for 12 weeks restored the microarchitectural parameters at both doses, suggesting its potent bone restoring action. Compound 8g at a dose of 1 mg kg⁻¹ increased the BV/TV ($p < 0.001$) and Tb.N ($p < 0.001$) values and decreased the Tb.Sp. ($p < 0.001$), Tb.Pf ($p < 0.001$) and SMI ($p < 0.001$) values. Similar to the 1 mg kg⁻¹ per day dose, the dose of 5 mg kg⁻¹ per day also restored the bone microarchitectural parameters. Interdose comparison shows that the lower dose of 1 mg kg⁻¹ per day exhibited the best response and was comparable to 17 β -estradiol which improved the trabecular microarchitecture parameters BV/TV ($p < 0.001$) and Tb.N ($p < 0.001$) and decreased the Tb.Sp. ($p < 0.001$), Tb.Pf ($p < 0.001$), SMI ($p < 0.001$) values compared with Ovx + V animals. At the end of the 12-week treatment with compound 8g, BMCs were harvested from the long bones that showed more mineralized nodules in sham rats when compared with Ovx rats (Fig. 5C). Quantification of mineralization showed that Ovx resulted in $\sim 60\%$ reduction compared with sham rats (Fig. 5D). Compound 8g treatment of Ovx rats at both doses increased mineralization by ~ 1.5 to 1.4 fold. Ovx induced bone loss is characterized by a higher bone turnover rate. As expected, the bone resorption marker (serum CTX) level is elevated (Fig. 5E) and the bone formation marker (serum PINP) is reduced (Fig. 5F) in the Ovx + V group compared with the Sham + V group.⁴⁹ The treatment of Ovx rats with compound 8g significantly lowered the CTX levels while it stimulated the PINP levels in serum. This indicates attenuation of bone resorption while stimulating bone formation. The serum PINP levels of Ovx rats treated with 1.0 and 5.0 mg kg⁻¹ of compound 8g were comparable to those of the

Fig. 3 Effect of potent derivatives on osteoblast differentiation and mineralization. (A) RCOs were cultured in osteoblast differentiation medium maintained for 21 days in the presence of derivatives at their active concentrations. At the end of the experiment, cells were stained with alizarin red S. Photomicrographs show increased formation of mineralized osteoblast nodules by various treatments compared with untreated cells taken as control. (B) Quantification of mineralization by extraction of alizarin red S dye is shown. (C) Active compounds increased the expression of osteogenic genes BMP-2, Runx-2, and OCN in treated ROCs. Expression was normalized to GAPDH as an internal control. (D) ROCs were treated with or without various compounds for 48 h. Proteins extracted from cell lysates were transblotted onto a membrane, probed with primary antibodies, followed by the corresponding secondary antibodies, and normalized with β -actin. (E) Cell proliferation was measured in RCOs by the BrdU incorporation assay at 48 h after compound **8g** treatment. Values represent mean $\pm$ SEM from three independent experiments: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ compared with untreated cells taken as control.

sham + vehicle group whereas the CTX serum levels were significantly reduced at both doses, 1 and 5 mg kg⁻¹ per day, of compound **8g**. The CTX levels were not different between the sham + V and E2 groups. Overall data suggest that compound **8g** could decrease the Ovx induced bone turnover rate that is characteristically increased under estrogen deficiency.²⁴

As BMP-2 plays an important role in osteoblast differentiation and since β -carboline derivatives have been found to be potent BMP-2 up-regulating agents,⁵⁰ we also studied the expression of this gene using our compound **8g**. Femoral bones from Ovx + vehicle rats exhibited decreased mRNA levels of osteoblast-specific genes including those for Runx-2, BMP-2, type I collagen ($p < 0.05$) and OCN ($p < 0.01$) compared with the sham group (Fig. 6A). Compound **8g** treatment of Ovx rats resulted in an increase in the mRNA levels of all key genes at both doses. At 1 mg kg⁻¹ per day, compound **8g** increased the expressions of these osteogenic genes which were

significantly higher than those of the sham + V, Ovx + V, and Ovx + E2 groups.⁵¹ Interdose comparison showed that the 1 mg kg⁻¹ dose exhibited a better response than the 5 mg kg⁻¹ dose ($p < 0.001$).

To further validate the bone anabolic effect of compound **8g**, we measured the parameters of new bone formation by dynamic histomorphometry in femur diaphysis (Fig. 6B). As expected, both the mineral apposition rate (MAR) ($p < 0.001$) and bone formation rate (BFR) ($p < 0.001$) were reduced in Ovx rats compared with those in the sham group, as assessed by bone histomorphometry (Table 3). Compound **8g** at doses 1 and 5 mg kg⁻¹ per day exhibited an increase in BFR and MAR ($p < 0.01$) compared with Ovx. The bone formation parameters were comparable to treatment with Ovx + 17 β -estradiol ($p < 0.001$). Statistical analysis of the data showed that the 1 mg kg⁻¹ per day dose has the best response to the MAR and BFR compared to the 5 mg kg⁻¹ per day dose. The 1

Fig. 4 Active compound **8g** shows anti-oxidant potential and prevents TNF α mediated osteoblast inhibition. (A) The effect of compound **8g** on ROS levels in the presence of TNF α was measured by DCFH-DA fluorimetry analysis (wavelength Ex 485 nm/Em 535 nm) after 24 h with NAC as the positive control. (B) The free radical scavenging activity of compound **8g** at various concentrations was assessed by using the cell-free DPPH assay and absorbance at 517 nm. (C) The SNP induced NO radical scavenging activity of compound **8g** was assessed by using a Griess reagent and absorbance at 546 nm. Values represent mean $\pm$ SEM; $n = 8$. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ compared with untreated cells taken as control. (D and E) Western blot analysis to check the effect of compound **8g** on TNF α mediated osteoblast inhibition and densitometric analysis was performed to check the fold change in the expression of the proteins using β -actin as the loading control. Values represent mean $\pm$ SEM; NE = nuclear extract, CE = cytosolic extract. (F) Immunofluorescence analysis for NF- κ B in the presence of compound **8g** was performed to check its nuclear translocation mediated by TNF α . (G) The anti-apoptotic effect of compound **8g** in osteoblasts was analyzed by using a Becton Dickinson FACS and FL-H channel (annexin-V) and an FL2-H channel (PI). Data show that compound **8g** treatment inhibited apoptosis of osteoblast cells as shown in representative dot plots. (H) Quantification of flow cytometry data is shown as the percent of apoptotic cells. Values represent mean $\pm$ S. E. from three independent experiments ($n = 3$). * $p < 0.05$ compared with the TNF α treated group.

mg kg⁻¹ per day dose showed a comparable MAR and BFR with E2 and suggested the efficacy of a lower dose of compound **8g**. The biomechanical strength of the cortical bone in

the femur was measured by using the three-point bending test. As shown in Table 3, the OvX + vehicle group reduced the bone strength at the femoral diaphysis in rats by

Fig. 5 Active compound **8g** treatment of OvX rats restored bone microarchitectural parameters. (A) μ CT assessment of BMD from femora was performed in various groups. (B) Representative 3D μ CT images of femoral epiphyseal regions of various experimental groups. Refer to Table 3 for the analysis of trabecular parameters. Values are expressed as mean $\pm$ SEM ($n = 10$ rats per group). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the OvX + vehicle (1% gum acacia dissolved in distilled water) group. (C) Representative images of mineralized nodule formation stained with alizarin red-S. (D) Quantification of alizarin dye at 405 nm. Values are represented in relative fold change. (E and F) Bone turnover markers, serum CTX-1 and serum OCN were determined by ELISA, respectively. Values are expressed as mean $\pm$ SEM ($n = 10$ rats per group). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the OvX + vehicle (1% gum acacia dissolved in distilled water) group.

Table 3 Active compound **8g** treatment prevented microarchitectural deterioration of the femur and promoted new bone formation in OvX rats

Parameters	Sham + V	OvX + V	OvX + 8g (1 mg kg ⁻¹)	OvX + 8g (5 mg kg ⁻¹)	OvX + E2 (100 μg kg ⁻¹)
Assessment of trabecular microarchitecture					
BV/TV (%)	13.88 $\pm$ 0.74***	4.68 $\pm$ 0.58	10.55 $\pm$ 1.34***	10.09 $\pm$ 0.57***	11.09 $\pm$ 0.47***
Tb.N (mm ⁻¹)	1.99 $\pm$ 0.11***	0.56 $\pm$ 0.06	1.34 $\pm$ 0.20***	1.38 $\pm$ 0.15***	1.55 $\pm$ 0.05***
Tb.Sp (mm)	0.52 $\pm$ 0.06***	1.31 $\pm$ 0.06	0.73 $\pm$ 0.11***	0.87 $\pm$ 0.06***	0.68 $\pm$ 0.02***
Tb.Pf (mm ⁻¹)	5.81 $\pm$ 0.38***	10.81 $\pm$ 0.68	5.67 $\pm$ 0.57	5.45 $\pm$ 0.22***	5.99 $\pm$ 0.39***
SMI	1.58 $\pm$ 0.03***	2.07 $\pm$ 0.06	1.66 $\pm$ 0.04***	1.61 $\pm$ 0.05***	1.60 $\pm$ 0.02***
Dynamic histomorphometry					
MAR (μm per day)	1.271 $\pm$ 0.07***	0.169 $\pm$ 0.004	1.062 $\pm$ 0.05**	0.92 $\pm$ 0.05*	1.06 $\pm$ 0.06**
BFR (μm ³ μm ⁻² per day)	1.601 $\pm$ 0.07***	0.171 $\pm$ 0.005	1.032 $\pm$ 0.05**	0.98 $\pm$ 0.03**	1.04 $\pm$ 0.03**
Femoral bone strength					
Energy, (mJ)	66.63 $\pm$ 1.44*	46.27 $\pm$ 4.36	70.55 $\pm$ 3.45*	60.87 $\pm$ 4.45*	62.77 $\pm$ 1.1*
Stiffness (N mm ⁻¹)	186.67 $\pm$ 11.98*	122.07 $\pm$ 12.18	151.3 $\pm$ 5.67*	146.6 $\pm$ 6.67*	171.75 $\pm$ 1.38*

Quantification of μ CT data of various trabecular parameters of femora is presented as BV/TV, Tb.N, Tb.Sp, Tb.Pf and SMI, respectively. Histomorphometric parameters calculated from double fluorochrome labeling experiments showed an increase in MAR (mineral apposition rate) and BFR (bone formation rate) with compound **8g** treatment. The compound **8g** treated group showed improved femoral strength represented by energy and stiffness. Values are expressed as mean $\pm$ SEM ($n = 10$ rats per group). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the OvX + vehicle (1% gum acacia dissolved in distilled water) group.

Fig. 6 Active compound **8g** treatment of OvX rats supported osteogenesis and showed no toxicity. (A) Expression of osteogenic genes BMP-2, OCN, Runx-2, and Col-1 in the femur of various treatment groups was studied using qPCR analysis. Values are expressed as mean $\pm$ SEM. Each assay was performed in triplicate and $n = 3$ separate determinations. (B) Representative images of transverse sections (50 μ m) of tetracycline and calcein-labeled femur diaphysis from rats after 12 weeks of treatment. Values are expressed as mean $\pm$ SEM ($n = 10$ rats per group). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the OvX + vehicle (1% gum acacia dissolved in distilled water) group.

decreasing the energy by $\sim 30\%$ for breaking and stiffness by $\sim 35\%$ compared with the sham + vehicle group. The OvX + E2 group increased both the breaking energy and stiffness by $\sim 27\%$ and $\sim 30\%$ compared with the OvX + vehicle group, respectively. Compound **8g** increased the breaking energy and stiffness in OvX rats, and the values were comparable to those of the E2-treated group in both doses.

Finally, for preliminary safety evaluation of the lead compound, rats were treated with 1, 5 mg kg^{-1} per day of compound **8g** for 12 weeks orally. Livers and uterus from the rats were harvested, and a histological examination was performed using 5 μ m thick sections.^{52,53} The liver histomorphology of the treated and untreated animals was similar, and no morphological changes were observed (ESI,† Fig. S34). Uterine histomorphometric data in Table 4 show that as expected, the total uterine area, luminal area, and lu-

minal epithelial height were reduced in the OvX + vehicle group compared with those in the sham + vehicle group. E2 treatment of OvX rats resulted in an increase in the total uterine area, luminal area, and luminal height by $\sim 80\text{--}85\%$. There were no differences in these three parameters between the OvX + vehicle group and the OvX + **8g** group at both doses. This preliminary study suggested that compound **8g** would be safer for use in postmenopausal osteoporosis.

Discussion

A biology-oriented synthesis (BIOS) of natural product inspired tetrazole tethered β -carboline derivatives was documented. We have also established an inimitable example of a pre-Ugi-multicomponent reaction modification. The β -Carboline alkaloid has been shown to suppress the NF- κ B

Table 4 Compound **8g** treatment did not show uterine estrogenicity in ovariectomized rats

Parameters	Sham + vehicle	Ovx + vehicle	Ovx + 8g (1 mg kg ⁻¹)	Ovx + 8g (5 mg kg ⁻¹)	Ovx + E2 (100 µg kg ⁻¹)
Uterine weight, g	0.74 ± 0.04***	0.12 ± 0.02	0.13 ± 0.04	0.12 ± 0.03	0.45 ± 0.09***
Total uterine area, mm ²	1.86 ± 0.02***	0.11 ± 0.003	0.14 ± 0.005	0.13 ± 0.004	0.87 ± 0.04***
Luminal area, mm ²	0.043 ± 0.002***	0.010 ± 0.0004	0.012 ± 0.001	0.012 ± 0.003	0.054 ± 0.003***
Luminal epithelial height, µm	27.29 ± 0.96***	3.46 ± 0.24	3.70 ± 0.26	3.61 ± 0.25	17.80 ± 0.59***

Measurement of histomorphometric parameters such as total uterine area, luminal area, and luminal epithelial height. The compound **8g** treated group had histological features comparable to those of the Ovx group. Values are expressed as mean ± SEM (*n* = 10 rats per group). ****p* < 0.001 compared with the Ovx + vehicle (1% gum acacia dissolved in distilled water) group.

signaling pathway through inhibition of IKK activity in LPS-stimulated RAW 264.7 cells.⁵⁴ A β-carboline is an alkaloid that inhibits osteoclast differentiation and bone resorption *in vitro* and *in vivo*.¹⁷ However, the effect of β-carboline on osteoblasts is still unclear. Molecular hybridized β-carbolines were screened for their osteogenic potential in primary RCOs. *In vitro* data suggest that compound **8g** promoted osteoblast differentiation by enhancing ALP and mineralization, increasing nodule formation in Alizarin red staining. Compound **8g** is found to be devoid of toxic effects and promote osteoblast growth. Compound **8g** treatment of osteoblast cells up-regulated the bone formation genes Runx-2, BMP-2, Col-1 and OCN at both RNA and protein levels.

The accelerated bone loss that occurs in postmenopausal women has been linked to oxidative stress and increased free radicals.⁵⁵ Oxidative stress due to reactive oxygen species (ROS) can cause oxidative damage to cells. Ovx induces oxidative stress and impairs the bone antioxidant system in adult rats as there is a deficiency of estrogen.⁵⁶ TNFα is the active mediator of generation of intracellular ROS in Ovx animals and results in ROS mediated osteoblast apoptosis.^{31,32} In our study, compound **8g** markedly reduced the intracellular ROS production induced by TNFα and the effect was comparable to that of E2. Compound **8g** was able to scavenge free radicals as evaluated by the DPPH assay and free nitric oxide radicals thereby implicating its anti-oxidant potential. The maximum scavenging was noticed at 1000 µg ml⁻¹, and above this, all the concentrations were showing almost the same percentage inhibition. However, below this, all the concentrations were showing decreasing scavenging % with concentration.

It has already been reported that the loss of E2 results in increased serum TNFα levels and oxidative stress which results in inhibition of osteoblast differentiation and promotion of apoptosis.^{57,58} TNFα plays a key role in skeletal disease in which it promotes reduced bone formation by mature osteoblasts and increased osteoclastic resorption.⁵⁹ Activation of several factors such as caspase and NF-κB resulting in transcription of pro-inflammatory cytokines, chemokines, and anti-apoptotic molecules is mediated by activation of the TNF receptor 1 (TNFR1) death domain.⁶⁰ TNFα inhibits differentiation of osteoblasts from precursor cells by phosphorylation of IKK. However, in its inactive state, a complex consisting of NF-κB with IκB protein is retained within the cytoplasm. Upon TNFα stimulation, the two catalytic compo-

nents of IKK, IKKα, and IKKβ work together, resulting in proteasomal degradation of IκB, which subsequently leads to nuclear translocation of NF-κB p65/RelA to modulate the transcription of genes further downstream. Moreover, osteoporotic bone loss induced by Ovx in adult mice was prevented by the inhibition of IKK/NF-κB in differentiated osteoblasts.⁶¹ The data showed that compound **8g** prevented TNFα directed IκBα phosphorylation and prevented nuclear translocation of NF-κB/p65. Moreover, the immunofluorescence staining confirmed the inhibitory effects of compound **8g** on the nuclear translocation of p65 *in vitro*, thus preventing nuclear β-catenin degradation and promoting osteoblast differentiation. These data thus indicate that IKK might be the molecular target of compound **8g**. Our findings point toward the direct inhibitory effect of compound **8g** on TNFα mediated NF-κB activation and prevention of osteoblast apoptosis by up-regulating anti-apoptotic Bcl-2 and Bcl-xl and down-regulating pro-apoptotic Bax.

The bone sparing action of compound **8g** was further evaluated by *in vivo* studies using the Ovx rat model. Overall, the data indicate that in comparison with Ovx rats (after estrogen withdrawal) treated with the vehicle, compound **8g** was nontoxic and at a lower dose of 1 mg kg⁻¹ to Ovx rats, the treatment resulted in improved trabecular microarchitectures of the long bones, decreased bone resorption marker CTX levels and stimulated bone formation marker PINP levels, increased expression of skeletal osteogenic genes (BMP-2, Runx-2, and OCN), and promotion of new bone formation by increasing the BFR and MAR. *In vivo* toxicity assessment using liver histomorphology data showed that compound **8g** did not exhibit toxicity after oral administration. This study confirmed that compound **8g** exhibits an osteoanabolic effect due to inhibition of IKKB. The exquisite potency and structural novelty of **8g** suggest that it may serve as a valuable lead in the development of a new class of bone anabolic agents.

Conclusions

Thus, our findings clearly demonstrate the osteoprotective effects of compound **8g** that were comparable to the known 17β-estradiol which is a natural bone sparing hormone and prevents osteoblast apoptosis. TNFα mediated IKK/NF-κB activation during the absence of E2 is inhibited by compound **8g**. As 17β-estradiol is no longer in use for clinical purposes as it is associated with the risk of developing breast cancer,⁶²

being orally efficacious, compound **8g** could be an attractive therapeutic option for the treatment of postmenopausal osteoporosis; however, further studies are needed to advance this compound for preclinical studies.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

- J. R. Center, T. V. Nguyen, D. Schneider, P. N. Sambrook and J. A. Eisman, *Lancet*, 1999, 353, 878–882.
- T. J. Martin, *J. Bone Miner. Metab.*, 2014, 21, 8–20.
- O. Johnell and J. A. Kanis, *Osteoporosis Int.*, 2006, 17, 1726–1733.
- E. Romas, M. T. Gillespie and T. J. Martin, *Bone*, 2002, 30, 340–346.
- S. Tanaka, *Am. J. Nephrol.*, 2007, 27, 466–478.
- N. C. Walsh and E. M. Gravallese, *Curr. Opin. Rheumatol.*, 2004, 16, 419–427.
- G. E. Fuleihan, *N. Engl. J. Med.*, 1997, 337, 1686–1687.
- V. Beral and C. Million Women Study, *Lancet*, 2003, 362, 419–427.
- V. Beral, D. Bull, G. Reeves and C. Million Women Study, *Lancet*, 2005, 365, 1543–1551.
- L. A. Marcaurelle and C. W. Johannes, *Prog. Drug Res.*, 2008, 66(187), 189–216.
- L. O. Haustedt, C. Mang, K. Siems and H. Schiewe, *Curr. Opin. Drug Discovery Dev.*, 2006, 9, 445–462.
- S. Wetzel, R. S. Bon, K. Kumar and H. Waldmann, *Angew. Chem., Int. Ed.*, 2011, 50, 10800–10826.
- J. Svenda, M. Sheremet, L. Kremer, L. Maier, J. O. Bauer, C. Strohmann, S. Ziegler, K. Kumar and H. Waldmann, *Angew. Chem., Int. Ed.*, 2015, 54, 5596–5602.
- A. Al-Shamma, S. Drake, D. L. Flynn, L. A. Mitscher, Y. H. Park, G. S. Rao, A. Simpson, J. K. Swayze, T. Vaysoglu and S. T. Wu, *J. Nat. Prod.*, 1981, 44, 745–747.
- R. Kumar, S. Khan, A. Verma, S. Srivastava, P. Viswakarma, S. Gupta, S. Meena, N. Singh, J. Sarkar and P. M. Chauhan, *Eur. J. Med. Chem.*, 2010, 45, 3274–3280.
- J. Yasuhara-Bell and Y. Lu, *Antiviral Res.*, 2010, 86, 231–240.
- T. Yonezawa, S. Hasegawa, M. Asai, T. Ninomiya, T. Sasaki, B. Y. Cha, T. Teruya, H. Ozawa, K. Yagasaki, K. Nagai and J. T. Woo, *Eur. J. Pharmacol.*, 2011, 650, 511–518.
- A. C. Castro, L. C. Dang, F. Soucy, L. Grenier, H. Mazdiyasni, M. Hottelet, L. Parent, C. Pien, V. Palombella and J. Adams, *Bioorg. Med. Chem. Lett.*, 2003, 13, 2419–2422.
- W. H. Song, M. M. Liu, D. W. Zhong, Y. L. Zhu, M. Bosscher, L. Zhou, D. Y. Ye and Z. H. Yuan, *Bioorg. Med. Chem. Lett.*, 2013, 23, 4528–4531.
- L. Sun, M. C. Qiu, L. L. Weng, H. Zheng and J. S. Liu, *Acta Pharmacol. Sin.*, 2000, 21, 200–204.
- C. Ruan, N. Hu, Y. Hu, L. Jiang, Q. Cai, H. Wang, H. Pan, W. W. Lu and Y. Wang, *Polymer*, 2014, 55, 1020–1027.
- O. Profant, J. Roth, Z. Bures, Z. Balogova, I. Liskova, J. Betka and J. Syka, *Clin. Neurophysiol.*, 2017, 128, 1946–1953.
- J. B. Baell and G. A. Holloway, *J. Med. Chem.*, 2010, 53, 2719–2740.
- R. Trivedi, S. Kumar, A. Kumar, J. A. Siddiqui, G. Swarnkar, V. Gupta, A. Kendurker, A. K. Dwivedi, J. R. Romero and N. Chattopadhyay, *Mol. Cell. Endocrinol.*, 2008, 289, 85–93.
- P. Kushwaha, V. Khedgikar, N. Ahmad, A. Karvande, J. Gautam, P. Kumar, R. Maurya and R. Trivedi, *Eur. J. Pharmacol.*, 2016, 788, 65–74.
- A. Sharma, B. Chakravarti, M. P. Gupt, J. A. Siddiqui, R. Konwar and R. P. Tripathi, *Bioorg. Med. Chem.*, 2010, 18, 4711–4720.
- A. Karvande, V. Khedgikar, P. Kushwaha, N. Ahmad, P. Kothari, A. Verma, P. Kumar, G. K. Nagar, P. R. Mishra, R. Maurya and R. Trivedi, *J. Pharm. Pharmacol.*, 2017, 69, 1381–1397.
- C. Manferdini, E. Gabusi, F. Grassi, A. Piacentini, L. Cattini, N. Zini, G. Filardo, A. Facchini and G. Lisignoli, *J. Cell. Physiol.*, 2011, 226, 2675–2682.
- P. Kushwaha, V. Khedgikar, D. Sharma, T. Yuen, J. Gautam, N. Ahmad, A. Karvande, P. R. Mishra, P. K. Trivedi, L. Sun, S. K. Bhadada, M. Zaidi and R. Trivedi, *J. Biol. Chem.*, 2016, 291, 3959–3966.
- B. Bhargavan, A. K. Gautam, D. Singh, A. Kumar, S. Chaurasia, A. M. Tyagi, D. K. Yadav, J. S. Mishra, A. B. Singh, S. Sanyal, A. Goel, R. Maurya and N. Chattopadhyay, *J. Cell. Biochem.*, 2009, 108, 388–399.
- A. R. de Medeiros, A. Z. Lamas, I. F. Caliman, P. L. Dalpiaz, L. B. Firmes, G. R. de Abreu, M. R. Moyses, E. M. Lemos, A. M. dos Reis and N. S. Bissoli, *Regul. Pept.*, 2012, 179, 55–60.
- M. N. Weitzmann and R. Pacifici, *J. Clin. Invest.*, 2006, 116, 1186–1194.
- A. R. Rosenkranz, S. Schmaldienst, K. M. Stuhlmeier, W. Chen, W. Knapp and G. J. Zlabinger, *J. Immunol. Methods*, 1992, 156, 39–45.
- F. Alam, Q. N. Saqib and M. Ashraf, *BMC Complementary Altern. Med.*, 2017, 17, 3.

- 35 Y. Lu, Y. Xue, S. Chen, H. Zhu, J. Zhang, X. N. Li, J. Wang, J. Liu, C. Qi, G. Du and Y. Zhang, *Sci. Rep.*, 2016, **6**, 22909.
- 36 S. A. Ronchetti, L. I. Machiavelli, F. A. Quinteros, B. H. Duvilanski and J. P. Cabilla, *PLoS One*, 2016, **11**, e0162455.
- 37 J. Chang, F. Liu, M. Lee, B. Wu, K. Ting, J. N. Zara, C. Soo, K. Al Hezaimi, W. Zou, X. Chen, D. J. Mooney and C. Y. Wang, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 9469–9474.
- 38 K. Fujimoto, T. Sakata, K. Kurata, Y. Okabe and K. Arase, *Eur. J. Pharmacol.*, 1988, **147**, 477–479.
- 39 K. V. Ramana, A. Bhatnagar and S. K. Srivastava, *FEBS Lett.*, 2004, **570**, 189–194.
- 40 L. Fan, S. C. Pandey and R. S. Cohen, *J. Neurosci. Res.*, 2008, **86**, 3655–3664.
- 41 M. S. Bynoe, C. M. Grimaldi and B. Diamond, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 2703–2708.
- 42 R. Trivedi, A. Kumar, V. Gupta, S. Kumar, G. K. Nagar, J. R. Romero, A. K. Dwivedi and N. Chattopadhyay, *Mol. Cell. Endocrinol.*, 2009, **302**, 86–91.
- 43 K. Verdelis, L. Lukashova, E. Atti, P. Mayer-Kuckuk, M. G. Peterson, S. Tetradis, A. L. Boskey and M. C. van der Meulen, *Bone*, 2011, **49**, 580–587.
- 44 C. H. Turner, *J. Musculoskeletal Neuronal Interact.*, 2002, **2**, 527–528.
- 45 C. H. Turner, *Osteoporosis Int.*, 2002, **13**, 97–104.
- 46 J. Gautam, P. Kushwaha, G. Swarnkar, V. Khedgikar, G. K. Nagar, D. Singh, V. Singh, M. Jain, M. Barthwal and R. Trivedi, *Phytomedicine*, 2012, **19**, 1134–1142.
- 47 V. Khedgikar, J. Gautam, P. Kushwaha, A. Kumar, G. K. Nagar, P. Dixit, R. Chillara, S. Voruganti, S. P. Singh, W. Uddin, G. K. Jain, D. Singh, R. Maurya, N. Chattopadhyay and R. Trivedi, *Menopause*, 2012, **19**, 1336–1346.
- 48 A. Kumar, G. K. Gupta, V. Khedgikar, J. Gautam, P. Kushwaha, B. Changkija, G. K. Nagar, V. Gupta, A. Verma, A. K. Dwivedi, N. Chattopadhyay, P. R. Mishra and R. Trivedi, *Eur. J. Pharm. Biopharm.*, 2012, **82**, 508–517.
- 49 H. Cui, Y. Zhu, Q. Yang, W. Zhao, S. Zhang, A. Zhou and D. Jiang, *Sci. Rep.*, 2016, **6**, 33803.
- 50 T. Yonezawa, J. W. Lee, A. Hibino, M. Asai, H. Hojo, B. Y. Cha, T. Teruya, K. Nagai, U. I. Chung, K. Yagasaki and J. T. Woo, *Biochem. Biophys. Res. Commun.*, 2011, **409**, 260–265.
- 51 K. Sharan, J. S. Mishra, G. Swarnkar, J. A. Siddiqui, K. Khan, R. Kumari, P. Rawat, R. Maurya, S. Sanyal and N. Chattopadhyay, *J. Bone Miner. Res.*, 2011, **26**, 2096–2111.
- 52 N. Ahmad, R. Chillara, P. Kushwaha, V. Khedgikar, A. Karvande, D. Choudhary, S. Adhikary, R. Maurya and R. Trivedi, *Biomed. Pharmacother.*, 2017, **88**, 804–813.
- 53 M. P. Melancon, T. Appleton Figueira, D. T. Fuentes, L. Tian, Y. Qiao, J. Gu, M. Gagea, J. E. Ensor, N. M. Munoz, K. L. Maldonado, K. Dixon, A. McWatters, J. Mitchell, M. McArthur, S. Gupta and A. L. Tam, *Radiology*, 2017, **286**, 149–157.
- 54 J. W. Yoon, J. K. Kang, K. R. Lee, H. W. Lee, J. W. Han, D. W. Seo and Y. K. Kim, *J. Toxicol. Environ. Health, Part A*, 2005, **68**, 2005–2017.
- 55 S. B. Doshi and A. Agarwal, *J. Midlife Health*, 2013, **4**, 140–146.
- 56 S. Muthusami, I. Ramachandran, B. Muthusamy, G. Vasudevan, V. Prabhu, V. Subramaniam, A. Jagadeesan and S. Narasimhan, *Clin. Chim. Acta*, 2005, **360**, 81–86.
- 57 C. Roggia, Y. Gao, S. Cenci, M. N. Weitzmann, G. Toraldo, G. Isaia and R. Pacifici, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 13960–13965.
- 58 S. Khosla, M. J. Oursler and D. G. Monroe, *Trends Endocrinol. Metab.*, 2012, **23**, 576–581.
- 59 L. Gilbert, X. He, P. Farmer, S. Boden, M. Kozlowski, J. Rubin and M. S. Nanes, *Endocrinology*, 2000, **141**, 3956–3964.
- 60 D. J. MacEwan, *Br. J. Pharmacol.*, 2002, **135**, 855–875.
- 61 J. Chang, Z. Wang, E. Tang, Z. Fan, L. McCauley, R. Franceschi, K. Guan, P. H. Krebsbach and C. Y. Wang, *Nat. Med.*, 2009, **15**, 682–689.
- 62 E. D. Staren and S. Omer, *Am. J. Surg.*, 2004, **188**, 136–149.