Identification of Novel 2-((1-(Benzyl(2-hydroxy-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic Acid Analogues as BMP-2 Stimulators

Vishal M. Balaramnavar,[†] Imran A. Khan,[†] Jawed Akhtar Siddiqui,[‡] Mohd Parvez Khan,[‡] Bandana Chakravarti,[‡] Kunal Sharan,[‡] Gaurav Swarnkar,[‡] Namrata Rastogi,[‡] H. H. Siddiqui,[§] Durga Prasad Mishra,[‡] Naibedya Chattopadhyay,[‡] and Anil K. Saxena*,[†]

[†]Division of Medicinal and Process Chemistry and [‡]Division of Endocrinology, CSIR—Central Drug Research Institute, Lucknow, 226001, India

[§]Department of Pharmacy, Integral University, Lucknow, India

Ⓢ *Supporting Information*

ABSTRACT: The synthesis and SAR studies of 10 new chemical entities (NCEs) that have shown BMP-2 stimulation and osteoblast differentiation are reported. Among these, 2-((1-(benzyl(2-hydroxy-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid (**11**) was the most effective while its analogue **13** also showed good activity in inducing osteoblast BMP-2 production. Compound **11** induced osteoblast differentiation in vitro, and this effect was abrogated by a physiological BMP-2 inhibitor, noggin. It also exhibited dose dependent increase in nascent bone formation (2.16- and 3.12-fold more than the control at 1 and 5 mg/kg dose, respectively) at the fracture site in rats. At the maximum osteogenic concentration, compound **11** significantly inhibited osteoblastic proteosomal activity. This compound was safe, as it had no effect on BMP synthesis in cardiovascular tissue.
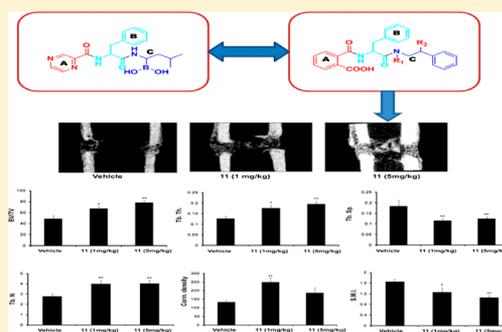
■ INTRODUCTION

Osteoporosis is a leading cause of fractures in adults. It is characterized by progressive bone loss with deterioration of bone microarchitecture.[1−3] In pathophysiological terms, increased bone loss in osteoporosis is coupled with reduced formation of new bone.[4] Osteoporosis associated with estrogen deficiency after menopause is the most common cause of age-related bone loss,[1] affecting at least one-quarter of all postmenopausal women (70% of women in United States) with increased proportion to women older than 80 years. Hormone replacement therapy (HRT) effectively prevents postmenopausal osteoporosis and reduces the incidence of fractures.[2] However, HRT also increases the risk of breast and endometrial cancer, in addition to other undesirable side effects.

Agents for the treatment of osteoporosis are classified as antiresorptive and anabolic. The first line of treatment for osteoporosis in the clinic is by antiresorptive agents, which prevent bone loss by inhibiting the activity of osteoclasts. Anabolic agents act by stimulating the formation of new bone and thus provide an additional option for osteoporosis patients and represent a major advancement in the treatment of osteoporosis. Anabolic agents are capable of increasing bone mass to a greater degree than antiresorptive agents with the capacity to improve both the bone quality and strength.[5−10] Parathyroid hormone PTH (1−34) and PTH (1−84) are the

only anabolic agent available for clinical use, which not only increase bone mass but also bone quality and strength by improving microarchitecture and geometry.[11,12]

Recently, PTH (1−84, Preos) has been launched in Europe, including U.K., and is awaiting FDA approval in the U.S. However, PTH is the therapy of last resort and is recommended for a maximum of only 24 months in the U.S. and in Europe.[13] Major drawbacks associated with PTH are its parenteral route of administration and the risk of developing osteogenic sarcoma.

The bone morphogenetic proteins (BMPs) are secreted signaling molecules belonging to the transforming growth factor β (TGF-β) superfamily. They have been shown to augment fracture healing in several animal models and clinical cases.[14,15] BMPs are typically delivered to the fracture site as recombinant proteins, but there are several limitations with their use. Recombinant proteins are rapidly resorbed and redistributed from the fracture site. Their synthesis and purification are expensive, and their requirement in large doses for treatment increases the probability of side effects.[16,17] Moreover, multiple treatments are required to obtain sustained concentrations at the fracture site that are inconvenient and may reduce treatment adherence.

Scheme 1[a]
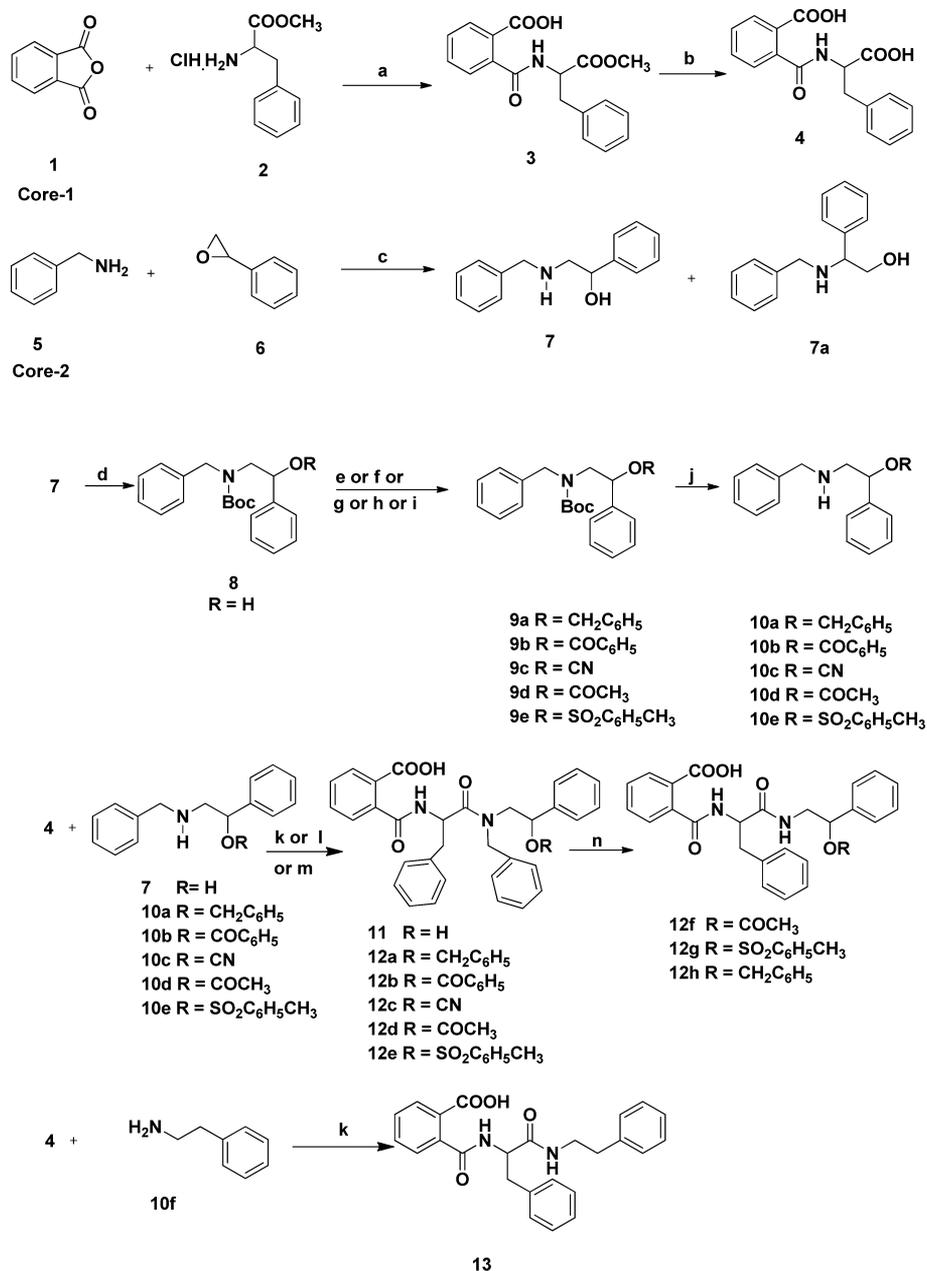
[a]Reagents and conditions: (a) dried DCM, TEA (1.2 equiv) (0 °C to rt) for 12 h; (b) LiOH (4 equiv), THF/MeOH/H$_2$O (4:4:1) for 2 h; (c) SiO$_2$, N$_2$ atm for 24 h; (d) 1.6 equiv of Boc anhydride, 1.2 equiv of TEA, DCM, for 6 h; (e) dry DCM, TEA (1.2 equiv), benzyl chloride, rt for 12 h; (f) dry DCM, TEA (1.2 equiv), benzoyl chloride for 12 h; (g) TsIm/TBAI/TEA, DMF, reflux, 3–6 h; (h) Ac$_2$O/pyridine (2:6) for 12 h; (i) dry DCM, TEA (1.2 equiv), tosyl chloride, rt, for 12 h; (j)TFA/DCM (50%:50%) for 12 h; (k) COCl$_2$, THF, 0 °C, 1 h, TEA, 2 equiv for 2 h; (l) DCC coupling; (m) BOP coupling; (n) debenzylation.

BMP-2, a member of the BMP family, is synthesized by osteoblasts and stimulates osteoblast differentiation and bone generation and regeneration in different animal models.[18] Recombinant adenoviral transfer of BMP-2 c-DNA in osteoporotic sheep model induces fracture healing.[19] This approach represents a significant advancement in bone repair and received FDA approvals for use in orthopedics.[20] However, systemic infusion of BMP-2 has no significant osteogenic effect because of poor bioavailability, and considering the cost of synthesis and purification of the recombinant protein,[21] the selective augmentation of BMP-2 in osteoblast by small molecules that are orally efficacious may be considered as a

significant advancement in this field. Such molecules may provide new therapeutic agents for the treatment of fracture healing, osteoporosis, and bone loss conditions in the future.

Statins have been shown to stimulate bone formation, and the effect is associated with increased expression of BMP-2 in bone cells.[22] Previous reports have shown that the BMP-2 gene is a transcriptional target of estrogen receptor, and estrogens including various phytoestrogens stimulate BMP-2 synthesis in osteoblasts.[23,24] Piceatannol stimulates osteoblast differentiation and is associated with the increase in BMP-2 production.[25] Recently, some proteasome inhibitors, viz. epoximycin (a natural product) and bortezomib, showed increased bone
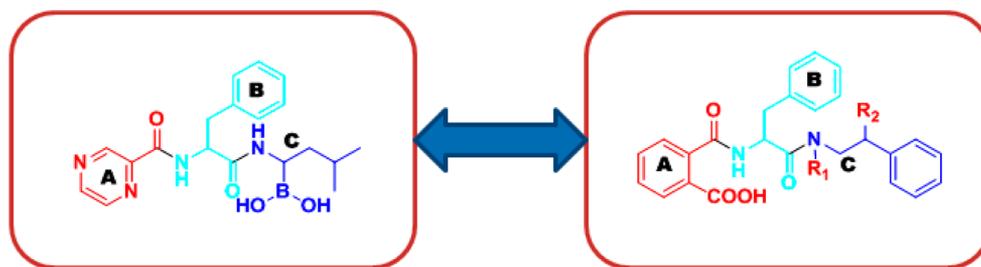
Figure 1. Strategy employed for the design of NCEs based on known reference compound.

Table 1. Structures and Effect of Compounds on BMP-2 Secretion in RCOs[a]

| | | | BMP-2 level in the conditioned medium | | | | |
compd	R_1	R_2	0.1 nM	1 nM	10 nM	100 nM	1000 nM
11	-CH$_2$C$_6$H$_5$	-OH	140 ± 3.4**	159.5 ± 2.3***	155.6 ± 4.1**	137.2 ± 3.2**	133 ± 3.1*
12a	-CH$_2$C$_6$H$_5$	-OCH$_2$C$_6$H$_5$	89.8 ± 3.5	100.66 ± 2.2	98.34 ± 3.1	103.27 ± 2.2	92.6 ± 2.2
12b	-CH$_2$C$_6$H$_5$	-OCOC$_6$H$_5$	98.4 ± 2.3	107.69 ± 1.9	103.15 ± 3.1	103.15 ± 3.1	112 ± 3.5
12c	-CH$_2$C$_6$H$_5$	-CN	83.6 ± 3.6	97.35 ± 2.6	89.28 ± 3.7	96.69 ± 4.1	92.6 ± 1.6
12d	-CH$_2$C$_6$H$_5$	-OCOCH$_3$	88.7 ± 4.2	89.49 ± 4.2	83.45 ± 3.1	98.76 ± 3.7	100.6 ± 1.4
12e	-CH$_2$C$_6$H$_5$	-OSO$_2$C$_6$H$_5$CH$_3$	122 ± 3.2	141.67 ± 3.2**	109.39 ± 3.1	138.25 ± 1.4**	111 ± 2.5
12f	H	-OCOCH$_3$	106 ± 2.4	98.67 ± 3.1	98.33 ± 4.2	96.03 ± 2.6	103 ± 1.6
12g	H	-OSO$_2$C$_6$H$_5$CH$_3$	119 ± 3.1	131.75 ± 2.9**	111.78 ± 1.3	114.88 ± 2.8	104 ± 1.8
12h	H	-OCH$_2$C$_6$H$_5$	87.8 ± 1.9	107.27 ± 1.1	101.32 ± 6.2	117.2 ± 5.2	93.6 ± 2.3
13	H		111 ± 1.6	122.8 ± 4.2	114.24 ± 5.2	166.11 ± 7.3**	132 ± 4.4*

[a]Each assay was performed in triplicate, and results are expressed as the mean ± SEM of percentage of control (100%) after normalization of total protein content/well: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs control.

formation rates in vitro, with the participation of BMP-2 signaling in osteoblasts.[26−29] Recently two series of substituted benzothiophene and benzofuran derivatives have been reported.[30,31] as novel classes of BMP-2 up-regulators.

Ten compounds belonging to the prototype incorporating pharmacophoric features of the bortezomib core were prioritized with the aid of preliminary modeling studies that are not presented in the manuscript. The synthesis and biological evaluation of these compounds as BMP-2 promoters are presented in this study.

CHEMISTRY

The synthesis of designed molecules (11, 12a−h, 13) was accomplished by the method outlined in Scheme 1 from two key intermediates 4 (2-((1-carboxy-2-phenylethyl)carbamoyl)-benzoic acid) and 7 (2-(benzylamino)-1-phenylethanol). The key intermediate 4 was synthesized by the hydrolysis of 3 by lithium hydroxide. The 3 was prepared by the reaction of phthalic anhydride (1) with phenylalanine methyl ester hydrochloride (2) in dry DCM using TEA as acid scavenger. The other key intermediate 7 was synthesized by silica catalyzed regioselective epoxide opening of (±)-styrene oxide (6) by nucleophilic attack of benzylamine (5) to give other key intermediate (7) in 88% along with its corresponding regioisomer 2-(benzylamino)-2-phenylethanol (7a).[32] The desired β-aminol (7) was used for the synthesis of the prioritized molecule through other intermediates (10a−e). The target molecules (11, 12a−h, 13) were prepared by the reaction of the key intermediate 4 with other key intermediates

7, 10a−e through amide coupling. In view of the high cost of BOP/PyBOP and sluggish working up procedure for DCC the discriminated reactivity of aliphatic acid over aromatic acid was used for the activation of aliphatic acid selectively through in situ generation of acyl chloride.

SAR STUDY

The series of the compounds represented by the prototype was designed in such a manner that structural modifications were in parts A, B, and C of the template/reference molecule bortezomib (Figure 1). All the compounds were tested on BMP-2 secretion assay using primary calvarial osteoblast cultures from rats (RCO) (Table 1) with respect to different R_2 substitutions. Among all these compounds, compound 11 (R_2 = OH) showed maximum effect on BMP-2 secretion at lower concentrations (up to 0.1 nM). The substitution of the −OH functionality by different bulkier groups starting from acetyl (12d), cyano (12c), benzyl (12a), benzoyl (12b) resulted in the reduction in the activity. The tosyl derivative (12e) seemed to have reasonable activity showing that the free −OH at the R_2 position is optimum for the activity. The next important variation was carried out at the R_1 portion in the compounds, viz. 12f, 12g, 12h, and 13 where the activity improved in case of N-debenzylated analogue of acetylated derivative 12f compared to the benzyl substituted acetylated analogue 12d. However, this trend was not observed in the debenzylated analogue 12e or 12d. The substitution of the bulky groups at the R_2 position by hydrogen in the debenzylated analogue (12f−h) led to the compound 13
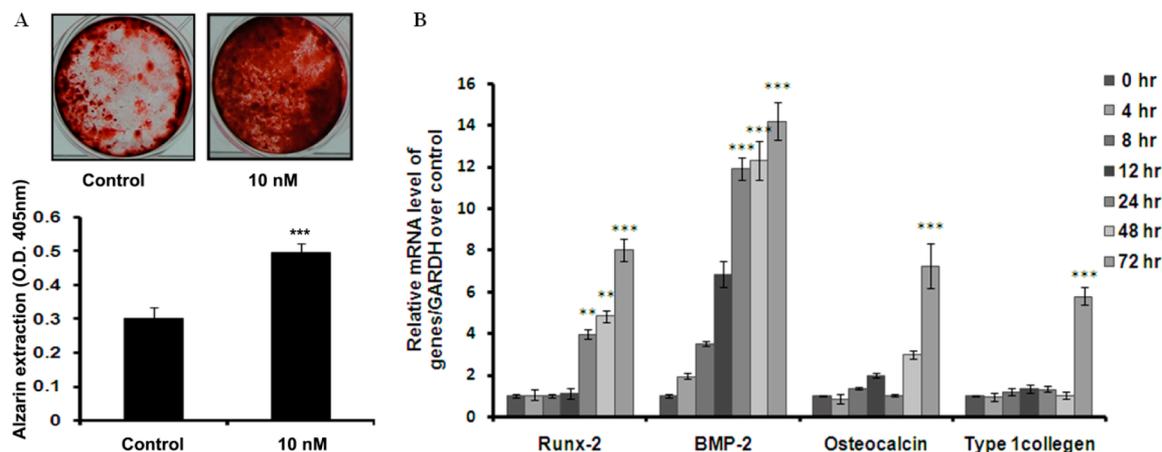
Figure 2. Compound **11** increases osteoblast function. (A) RCOs were cultured in osteoblast differentiation medium as described above in the presence of compound **11** (10 nM) or vehicle (control). Cells were stained with Alizarin Red S. Photomicrographs show increased formation of mineralized nodules by compound **11** treatment of osteoblasts compared with vehicle treated osteoblasts (upper panel). Quantification of mineralization by extraction of Alizarin Red S dye is shown (lower panel). (B) qPCR analysis of various osteogenic genes, Runx-2, osteocalcin, BMP-2 and type I collagen in osteoblasts treated with compound **11** (10 nM) at different time intervals as indicated. Each assay was performed in triplicate, and results are represented as the mean $\pm$ SEM of percentage/fold change: $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$.

which showed significant increase in BMP-2 secretion. Most of the compounds did not show an increase in BMP-2 secretion in a dose/concentration dependent manner, possibly because of copious production of BMP-2 by osteoblasts at the basal level that negatively impacts the assay sensitivity with respect to the determination of concentration−response relation by ELISA. Hence, we confirmed the effect of the most effective compounds **11** and **13** at BMP-2 mRNA levels in osteoblasts at 48 h. The qPCR quantification revealed that relative to the control RCOs, cells exposed to increasing concentrations of compounds **11** and **13** responded with increased BMP-2 mRNA levels (Table 1, Supporting Information). The absolute BMP-2 levels (pg/mL) in the conditioned medium were included in Table 2 in Supporting Information.

■ DETAILED BIOLOGICAL EVALUATION OF THE LEAD

As the ability of any agent/compound to induce BMP-2 production in osteoblast has the potential to promote osteoblast differentiation, we assessed the effect of compound **11** on RCO differentiation. As BMP-2 stimulatory effect of compound **11** was maximum at 10 nM, we used this concentration to study its impact on osteoblast mineralization. In comparison to the control RCOs, compound **11** stimulated the formation of mineralized nodules ($P < 0.001$) (Figure 2A).

Furthermore, the effect of compound **11** was investigated on the expression of various osteogenic genes including Runx-2, BMP-2, and osteocalcin, and type I collagen by qPCR and the data showed a time-dependent increase in their mRNA levels over control (Figure 2B). Out of these four genes, Runx-2 and BMP-2 mRNA levels were significantly elevated over control at as early as 24 h. Stimulation of mRNA levels of Runx-2 and BMP-2 genes by compound **11** continued up to 72 h. Since BMP-2 is a potent stimulator of osteoblast differentiation, we investigated whether stimulation of osteoblast differentiation by compound **11** was mediated via the increased production of BMP-2. To that effect, RCOs were treated with compound **11** with or without noggin. Figure 3 shows that the induction of Runx-2 and osteocalcin mRNA levels in the RCOs by compound **11** was brought back to the basal (unstimulated)
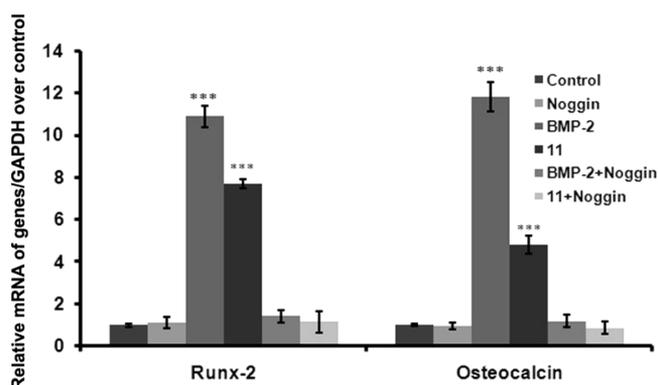
Figure 3. Effect of BMP-2 neutralization on osteoblast differentiation by compound **11**. Cells were treated for 72 h with compound **11** (10 nM), BMP-2 (100 ng/mL), and noggin (50 ng/mL) either alone or in combination as indicated. mRNA analysis of Runx-2 and osteocalcin genes were performed by qPCR. Data represent the mean $\pm$ SEM from three independent experiments: $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$.

levels when noggin was present in the culture medium. Together, these data indicate that compound **11** exerts an osteogenic effect in an autocrine/paracrine mode by stimulating osteoblastic BMP-2 production.

Effect of Compound 11 on Fracture Healing in the Drill-Hole Fracture Model in Rats. BMPs have been shown to enhance fracture healing in several animal models and clinical cases.[33,34] BMP-2 and -7 are usually delivered to the fracture site as recombinant proteins to accelerate healing. Because compound **11** stimulated BMP-2 production to promote osteoblast function, we tested whether it could enhance fracture healing. Figure 4 shows that compared to the control (rats receiving vehicle), compound **11** dose-dependently increased osteoblasts recruitment, callus regeneration, and osteoblasts formation (measured from the intensity of calcein labeling) in the femur drill-hole. Compared to control rats, new bone formation was 2.16- and 3.12-fold more at 1.0 and 5.0 mg/kg dose, respectively.
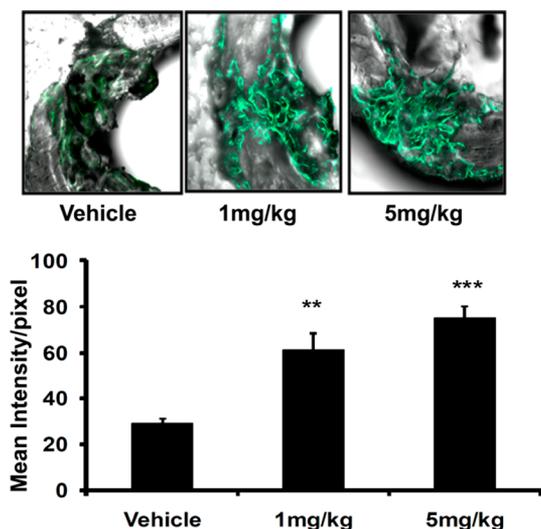
Figure 4. Compound **11** promotes fracture healing in the drill-hole in rats. Representative confocal images of calcein labeling are shown in the drill-hole of various groups 2 weeks after drill-hole injury. Lower panel shows quantification of the mean intensity of calcein label. Values are expressed as the mean $\pm$ SEM (n = 10 rats/group): $\ast\ast$, $P <$ 0.01; $\ast\ast\ast$, $P <$ 0.001.

Assessment of internal microstructure of the mineralized tissue in the bony hole was analyzed by 3-D μCT (micro-computed tomography). At the drill-hole site, compound **11**

treatment to rats at both doses resulted in higher bone volume/trabecular volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), connection density (conn.D), lower trabecular spacing (Tb.sp), and structure model index (SMI) compared to the vehicle treated groups (Figure 5). These data indicated that compound **11** treatment promoted fracture healing by stimulating the formation of new bone at the site of the drill-hole.

Assessment of Off-Target Effect of Compound 11. The osteoinductive potential of BMP-2 could lead to not only stimulated bone formation in the skeleton but also extraskeletal tissues such as heart and aorta.[35] Therefore, we investigated whether compound **11** has an off-target effect, i.e., induction of BMP-2, -4, and -7 mRNAs in cardiovascular tissues. While compound **11** at 1.0 and 5.0 mg/kg doses enhanced fracture healing, the expression levels of BMP-2, -4, and -7 in the aorta (Figure 6A) and cuspid valves of the heart (Figure 6B) of these rats were comparable to controls.

Proteosome Inhibition Assay. On the basis of the close structural analogy to bortezomib, compound **11** was analyzed for its possible mechanism of action through proteosomal inhibition. The proteosomal inhibition of this compound was evaluated using the 20S proteasome activity assay kit (Chemicon International, U.S.). Compound **11** significantly inhibited proteosomal activity in a concentration dependent fashion from 0.1 to 10 nM. However, at 100 nM, compound **11** had no proteosome inhibitory action. Lactacystin (25 μM), a standard proteosome inhibitor, was used for comparison
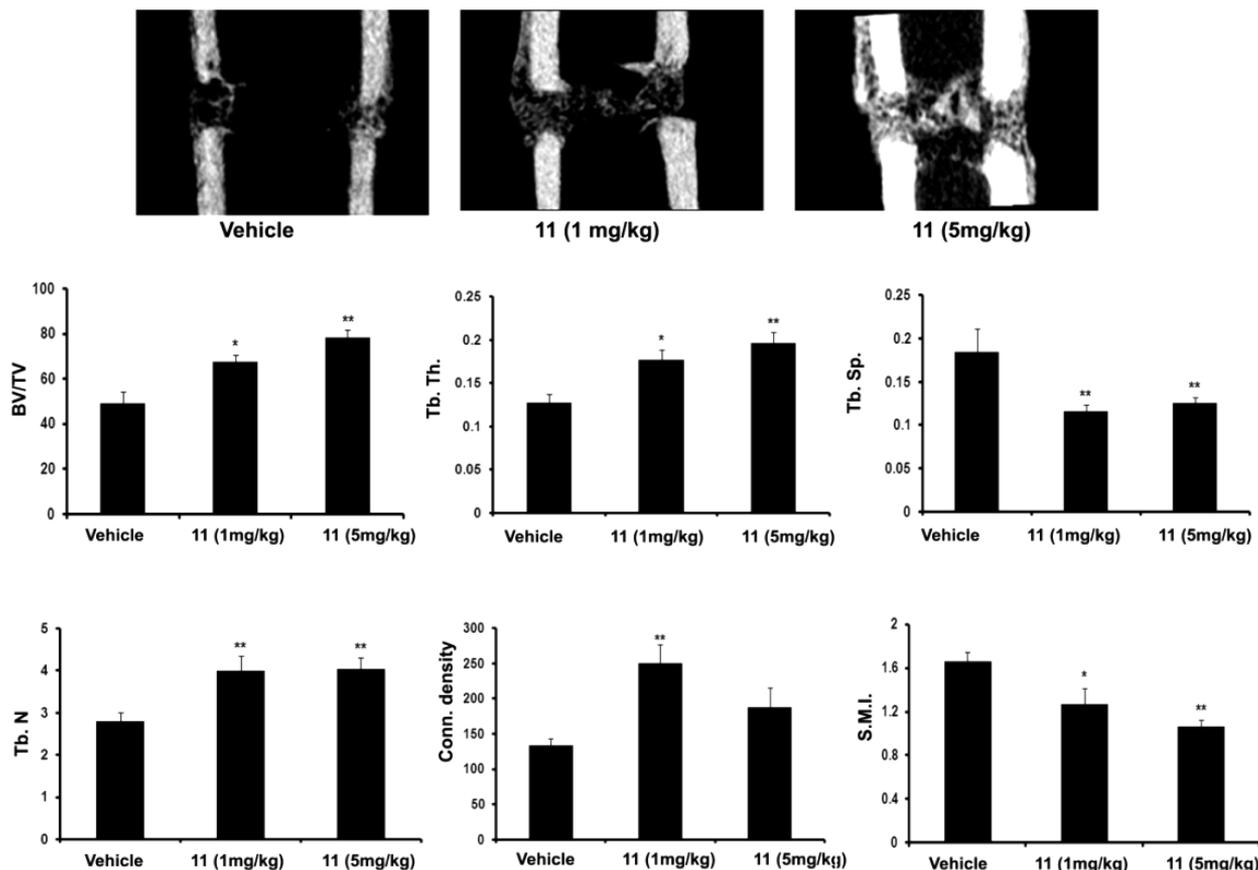
Figure 5. Representative μCT images from the center of the bony hole (upper panel). μCT analysis shows BV/TV, bone volume/tissue volume (%); Tb.N, strut number (1/mm); Tb.Th, strut thickness (mm); Tb.Sp, strut spacing (mm); Conn.Dens, connection density (1/mm³); SMI, structure model index. All values are expressed as the mean $\pm$ SEM (n = 10 rats/group): $\ast$, $P <$ 0.05; $\ast\ast$, $P <$ 0.01; $\ast\ast\ast$, $P <$ 0.001.
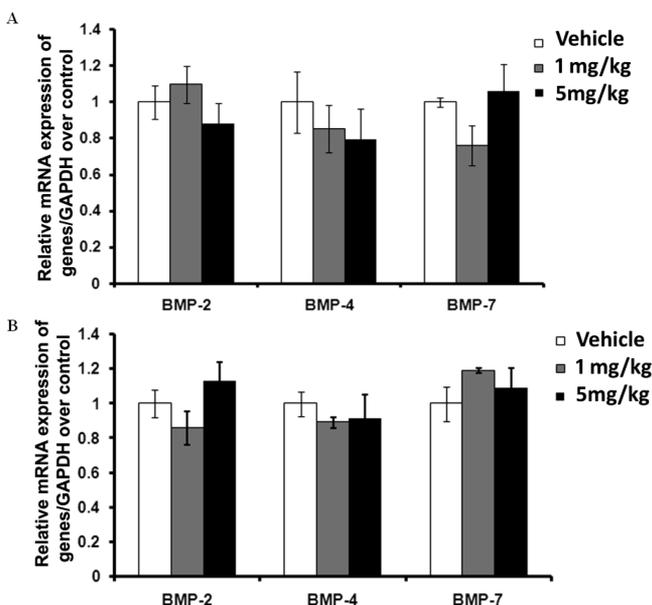
Figure 6. Compound **11** has no effect on BMP expression in cardiovascular tissues (A, B). Total RNA was extracted from aorta and cuspid valves of heart following 2 weeks of **11** treatment and qPCR was performed for the analysis of BMP-2, BMP-4, and BMP-7 mRNA levels. Data represent the mean ± SEM ($n = 10$ rats/group).

(Figure 7). Because inhibition of proteosome activity by bortezomib has been reported to induce osteoblast differ-
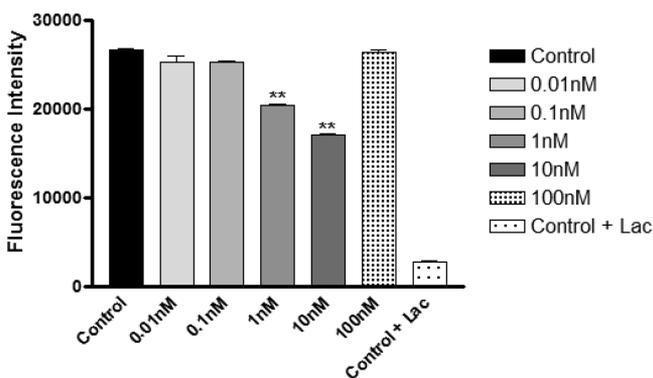
Figure 7. Compound **11** showed reduction in the proteasome activity. Mouse calvarial cell line MC3T3-E1 was incubated with compound **11** for 48 h, and proteasome activity was measured. Lac is lactacystin (25 μM). Data represent the mean ± SEM from three independent experiments: ∗∗, $P < 0.01$.

entiation by augmenting BMP-2 production,[36] compound **11** may serve to act similarly and in the process accelerate BMP-2 mediated fracture healing in vivo.

CONCLUSION

There is no oral drug available for accelerating fracture repair. Human recombinant BMP-2 and BMP-7 have been approved by U.S. FDA for local application at the site of fracture, including compound fracture (spinal fusion or open tibial surgery). Clearly, this is an interventional approach having limited use.

In our studies, compound **11** was found to potently stimulate BMP-2 and osteoblast differentiation from a series of structurally similar compounds. BMP-2 synthesized by

osteoblasts accumulates in the extracellular matrix and acts as a factor promoting osteoblast function.[37] BMP-2 signals via BMP-2 receptors and promotes osteoblast differentiation by an autocrine/paracrine mechanism.[38,39] Our data showing abolition of osteoblast differentiation by compound **11** in the presence of noggin suggest that its osteogenic effect is mediated via the activation of autocrine/paracrine loop of BMP-2. Enhanced calcification, a major risk factor for cardiovascular diseases, is associated with the emergence of osteoblast-like cells in the vascular tissues due to increased BMP production.[40] Despite having a BMP-2 stimulatory effect in osteoblasts, compound **11** has no effect on the expression of BMPs in cardiovascular tissues, indicating cardiovascular safety.

BMP-2 is required for normal fracture healing, as its absence results in the failure of mesenchymal progenitors to differentiate at the fracture site leading to a failed healing response.[41] Since compound **11** stimulated osteoblast differentiation via BMP-2 production, we studied its effect on the bone healing process in a drill-hole injury model of long bone.[42−44] This model seems suitable for analyzing the healing process quantitatively and useful for investigating osteoblast differentiation in vivo.[42,44] Our data showed that treatment of rats with compound **11** significantly increased the fracture healing process by stimulating new bone formation at the drill-hole injury site compared to the vehicle treated group. This finding was complemented by μCT analysis showing greater newly generated bone in the drill-hole of compound **11** treated groups compared with vehicle. Increased BV/TV, Tb.Th, and Tb.N by the treatment of compound **11** indicated structurally more robust growth of bone and compact assembly of trabecular bones at the drill-hole site compared to controls. The stability of the newly formed bone is importantly dependent on structural parameters determined by Conn.D, SMI, and Tb.pf.[45] Higher Conn.D, preferred platelike structure (lower SMI), and more concave trabecular surface (lower Tb.pf) presented a more compact bone in rats treated with compound **11** compared to control. These results suggested that the impact of compound **11** treatment on the structure (connectivity) and microarchitecture (bone geometry) of new bone in the drill-hole was better than control. Our results suggest that compound **11** stimulates BMP-2 production in osteoblasts and promotes new bone formation at the fracture site. Being orally efficacious, compound **11** could be an attractive strategy to shorten the healing period of fracture in the clinical setting.

EXPERIMENTAL SECTION

Biological 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). High-performance liquid chromatography grade acetonitrile was obtained from Merck India Ltd. (Mumbai, India). Heparin sodium injection (1000 IU/mL IP) was purchased from Gland Pharma (Hyderabad, India).

In Vitro Studies. Culture of Calvarial Osteoblasts. Rat calvarial osteoblasts (RCOs) were obtained following previously published protocol of sequential digestion.[46−48] Briefly, calvaria from 1- to 2-day-old Sprague−Dawley rats (both sexes) were pooled. After surgical isolation from the skull and the removal of sutures and adherent mesenchymal tissues, calvaria were subjected to five sequential (10−15 min) digestions at 37 °C in a solution containing 0.1% Dispase and 0.1% collagenase P. Cells released from the second to fifth digestions were pooled, centrifuged, resuspended, and plated in T-25 cm² flasks

in α-MEM containing 10% FCS and 1% penicillin/streptomycin (complete growth medium).

Osteoblast Differentiation. For the measurement of alkaline phosphatase (ALP) activity, RCOs at ~80% confluence were trypsinized and 2×10^3 cells/well were seeded in 96-well plates. Cells were treated with different concentration of compound for 48 h in α-MEM supplemented with 5% charcoal treated FCS, 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid, and 1% penicillin/streptomycin (osteoblast differentiation medium). At the end of the incubation period, total ALP activity was measured using p-nitrophenylphosphate (PNPP) as substrate and absorbance was read at 405 nm.[49]

BMP-2 ELISA. For measurement of BMP-2 production from osteoblasts, 5×10^3 cells/well were seeded in 24-well plates. Cells were exposed to given concentrations of compounds for 48 h in α-MEM medium supplemented with 10% charcoal-stripped FCS, 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid. At the end of incubation, supernatants were collected for determination of BMP-2 by ELISA according to the manufacturer's instructions (Quantikine, R&D Systems).

Mineralization of Calvarial Osteoblasts. Mineralization of RCOs was performed following a previously published protocol.[50] Briefly, RCOs were cultured until 80% confluence and were trypsinized and plated in the differentiation medium (25 000 cells/well in a 12-well plate), consisting of complete growth medium with ascorbic acid (50 μg/mL) and β-glycerophosphate (100 mM). The medium was changed every other day up to 21 days. The treatment group contained a similar medium with **11** (10nM). At the end of the experiment, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. The fixed cells were stained with 40 mM (pH 4.5) Alizarin Red S for 30 min followed by washing with water.[51]

For quantification of Alizarin Red S staining, 800 μL of 10% (v/v) acetic acid was added to each well, and plates were incubated at room temperature for 30 min with shaking. The monolayer, now loosely attached to the plate, was then scraped with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5 mL tube. After the mixture was vortexed for 30s, the slurry was overlaid with 500 μL of 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 20000g 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) and 150 μL aliquots of the supernatant were measured in 96-well format using opaque-walled, transparent-bottomed plate.[46,48,52]

Quantitative Real-Time Polymerase Chain Reaction (qPCR). qPCR reaction was performed for quantitative comparative measurement of the expression of osteoblast specific genes Runx-2, BMP-2, osteocalcin, and collagen type-1 following our optimized protocol.[45,48,53] The housekeeping gene GAPDH was used as the internal control in this study. Primers were designed using the Universal ProbeLibrary (Roche Applied Sciences) for the following genes: BMP-2, 5′-CGG CTGCGGTCTCCTAA-3′ (sense), 5′-GGGAAGCAG-CAACACTAGA-3′ (antisense); osteocalcin, 5′-ATA-GACTCCGGCGCTACCTC-3′ (sense), 5′-CCAGGGGATCTGGG-TAGG-3′ (antisense); Runx-2, 5′- CCACAGAGCTATTAAAGTGA-CAGTG-3′ (sense), 5′-AACAAACTAGGTTTAGAGTCATCAAGC-3′ (antisense); GAPDH, 5′-CAGCAAGGATAC TGAGAGCAAGAG-3′ (sense), 5′-GGATGGAATTGTGAGGGAGATG-3′ (antisense). For real-time PCR, cDNA was synthesized with a Revert Aid cDNA synthesis kit (Fermentas, Austin, TX, U.S.) using 2.0 μg of total RNA. SYBR green chemistry was used to perform quantitative determination of the relative expression of transcripts for all genes. All genes were analyzed using the Light Cycler 480 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.) real time PCR machine.

In Vivo Studies. Fracture Healing in Drill-Hole Injury at Femur. Thirty adult female Sprague−Dawley rats (200 ± 20 g each) were taken for the study. Drill-hole injury was created in femur as

described before.[45] The front skin of the mid-femur in rats was incised longitudinally in a straight manner at 1 cm in length under anesthesia. After splitting the muscle, we stripped the periosteum to expose the femoral bone surface. A drill-hole injury was made by inserting a drill bit with a diameter of 0.8 mm in the anterior portion of the diaphysis of the bilateral femurs, 2 cm above the knee joint. Treatments started from the next day of injury and continued for 2 weeks. For the various treatments, rats were divided into three equal groups (10 rats/group) as follows: vehicle (gum acacia in distilled water), **11** (1.0 mg·kg⁻¹·day⁻¹) and **11** (5.0 mg·kg⁻¹·day⁻¹). Each animal received intraperitoneal administration of fluorochrome calcein (20 mg kg⁻¹) 2 days before autopsy. After 2 weeks of various treatments described above, all rats were euthanized and autopsied to collect their heart and aorta for safety measurement and to collect the femur for the measurement of bone microarchitectural parameters in the drill-hole as described below. Bones were embedded in an acrylic material. Sections of 50 μm were made using an Isomet bone cutter, and photographs were taken under a confocal microscope (Carl Zeiss LSM 510 Meta) aided with appropriate filters. The intensity of calcein binding, which is an indication of the amount of new mineral depositions, was calculated using Carl Zeiss AM 4.2 image analysis software.

Statistical Analysis. Data are expressed as the mean ± SEM. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by post hoc Newman−Keuls multiple comparison test of significance using GraphPad Prism 5.04 software.[54]

Proteasome Activity Assay. Isolated 20S proteasome activity was evaluated using proteasome activity assay kit (Chemicon International, U.S.) according to the manufacturer's instructions. Mouse calvarial cell line MC3T3-E1, having a phenotypic response comparable to that of its rat counterpart, was seeded in six-well plates at a density of 2×10^6 per well and treated with compound **11** for 48 h. Both treated and untreated cells were harvested by scraping followed by washing twice with ice cold PBS and resuspending in lysis buffer (50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, and 1% Triton X-100). Cells were incubated for 30 min on ice with regular vortexing for 15 s after every 10 min of incubation. Lysate was centrifuged for 15 min at 4 °C at 14000g, and protein was estimated using the Bradford assay. Aliquots of 10 μg of isolated proteasome were incubated with fluorogenic substrate in 1× assay buffer provided with the kit for 1 h in the dark at 37 °C. Fluorescence intensity was measured by fluorimeter (BMG Fluorostar Omega) with a 380/460 nm filter set.

Synthetic Methods. General Chemistry. All the chemicals were purchased from different chemical suppliers. All the reactions were monitored by TLC (thin layer chromatography). The purity of all the compounds was determined by a high-pressure liquid chromatography (HPLC) by lachrome and was >95% using a diode array detector. The eluting process was performed on Chirapak 18 columns using an acetonitrile/methanol (90:10% v/v for compounds) system. ¹H NMR spectra of all the synthesized compounds were recorded by using a Bruker Spectrospin spectrometer at 300 MHz with tetramethylsilane used as internal standard. Mass spectra were recorded on a Jeol (Japan) SX 102/DA-6000 mass spectrometer. IR spectroscopy was carried out using a Perkin-Elmer 881 spectrophotometer, and the values are expressed as ν_{max}, cm⁻¹. Elemental analyses were performed on a Carlo Erba model EA-1108 elemental analyzer, and data for C, H, and N are indicated.

Synthesis of 2-((1-Methoxy-1-oxo-3-phenylpropan-2-yl)-carbamoyl)benzoic Acid (3). Anhydrous phthalic anhydride (0.495 mg, 3.352 mmol, 1.2 equiv) was added to a stirred mixture of methyl ester of phenylalanine (500 mg, 2.791 mmol) and triethylamine (0.3384 mg, 3.35 mmol, 1.2 equiv) at 0 °C in dry DCM (dichloromethane). The mixture was stirred for 12 h. After the completion of the reaction the reaction mixture was treated with 1 N HCl (5 mL) and then diluted with water (10 mL) and extracted with ethyl acetate (2 × 25 mL). The extracts layer of ethyl acetate was dried by using sodium sulfate and was concentrated in vacuo to give the title product **3** (1.04 g, 95.41%). ¹H NMR (CDCl₃, 300 MHz), δ 11.586 (1H, s,COOH), 7.8325−7.6548 (4H, m, CH), 7.4984 (5H, m, CH),5.822 (1H, bs, NH), 4.91725 (1H, d, J = 2.13 Hz, CH), 3.702 (3H, s, CH₃), 3.4285 (2H, m, CH₂). Chemical formula: $C_{18}H_{17}NO_5$.

FTIR (KBr): cm^{-1} 3415, 3312, 3031, 2829, 2620, 1715, 1480, 1320, 1200, 1120, 760, 751, 669. EIMS: m/z 328.2 (M + 1). Elemental analysis: C, 65.90; H, 5.21; N, 4.27.

Synthesis of 2-((1-Carboxy-2-phenylethyl)carbamoyl)-benzoic Acid (4). 2-((1-Methoxy-1-oxo-3-phenylpropan-2-yl)-carbamoyl)benzoic acid (500 mg,1.53 mmol) acid was stirred in a solvent mixture of tetrahydrofuran/methanol/water (4:4:1). To the stirring solution was subsequently added lithium hydroxide (0.1464 mg, 6.11 mmol, 4 equiv), and the mixture was stirred for 2 h at room temperature. The milky suspension was then treated with 1 N HCl (5 mL) and diluted with water (10 mL) and extracted with ethyl acetate. The ethyl acetate layer was dried over sodium sulfate and concentrated in vacuo to give the title product **4** (0.4 g, 85.10%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 11.36 (2H, s, COOH), 7.7984 (4H, m, CH), 7.3725−7.1328 (5H, m, CH), 5.6312 (1H, bs, NH), 4.9251 (1H, d, J = 2.10 Hz, CH), 3.1245(2H, m, CH$_2$). Chemical formula: C$_{17}$H$_{15}$NO$_5$. FTIR (KBr): cm^{-1} 3415, 3312, 3031, 2829, 2620, 1715, 1480, 1320, 1200, 1120, 760, 751, 669. EIMS: m/z 314.2 (M + 1). Elemental analysis: C, 65.10; H, 4.81; N, 4.46.

Synthesis of 2-(Benzylamino)-1-phenylethanol (7). Benzyl-amine (0.5 g, 4.66 mmol) was added to a stirred solution of styrene oxide (0.56 g, 4.67 mmol) and SiO$_2$ (30%) in nitrogen atmosphere for 24 h. To the resulting suspension was added dichloromethane to dissolve the solid product. The solution was filtered and concentrated under pressure to obtain a solid product to give the title product **7** (1.02 g, 97.14%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.21−7.33 (10H, m, CH), 4.9825 (1H, d, J = 2.23 Hz, CH), 3.824 (2H, bs, CH$_2$), 3.2413 (2H, m, CH$_2$), 1.78 (1H, s, NH). Chemical formula: C$_{15}$H$_{17}$NO. FTIR (KBr): cm^{-1} 3445, 2314, 1336, 1218, 1148, 771, 669. EIMS: m/z 228.2 (M + 1). Elemental analysis: C, 79.19; H, 7.52; N, 6.14.

Synthesis of *tert*-Butyl Benzyl(2-hydroxy-2-phenylethyl)-carbamate (8). *tert*-Butyl formate (0.66 g, 3.522 mmol, 1.6 equiv) was added to a stirred mixture of 2-(benzylamino)-1-phenylethanol (0.5 g, 2.201 mmol) and triethylamine (0.266 g, 2.633 mmol, and 1.2 equiv) and stirred for 6 h. After 6 h, the reaction mixture was diluted with water (10 mL) and extracted with ethyl acetate (3 × 25 mL) and was concentrated under vacuum and kept at 0 °C for crystallization to give the title product **8** (0.65 g, 90.27%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.23−7.38 (10H, m, CH), 5.2625 (1H, bs, CH), 4.24 (2H, bs, CH$_2$), 3.37 (2H, m, CH$_2$), 1.52 (9H, s, CH$_3$). Chemical formula: C$_{20}$H$_{25}$NO$_3$. FTIR (KBr): cm^{-1} 3462, 2362, 1654, 1303, 1147, 1057, 1398, 1346, 1302, 1147, 1057, 738, 697. EIMS: m/z 328.2 (M + 1). Elemental analysis: C, 73.34; H, 7.68; N, 4.27.

Synthesis of 2-(Benzyl(*tert*-butoxycarbonyl)amino)-1-phenylethyl Acetate (9a). *tert*-Butyl benzyl(2-hydroxy-2- phenylethyl)-carbamate (0.4 g, 1.222 mmol) was added to a stirred solution of acetic anhydride and pyridine in a proportion of 2:6 and stirred for 12 h. After completion of reaction the reaction mixture was worked up with 1 N HCl (5 mL) and diluted with water (10 mL) and extracted with DCM (2 × 25 mL). The organic layer was dried over sodium sulfate and concentrated under vacuum to give the title product **9a** (0.39 g, 86.66%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.3725−7.1328 (10H, m, CH), 5.7414 (1H, dd, J = 2.16 Hz, CH), 4.3070 (2H, s, CH$_2$), 3.6539 (2H, m, CH$_2$), 2.1985 (3H, s, CH$_3$), 1.52 (9H, s, CH$_3$). Chemical formula: C$_{22}$H$_{27}$NO$_4$. FTIR (KBr): cm^{-1} 3062, 2933, 1654, 1562, 1335, 1229, 1258, 1087, 824, 784, 745, 699. EIMS: m/z 370.2 (M + 1). Elemental analysis: C, 71.49; H, 7.35; N, 3.78.

Synthesis of *tert*-Butyl Benzyl(2-(benzyloxy)-2-phenylethyl)-carbamate (9b). To a stirred solution of *tert*-butyl benzyl(2-hydroxy-2-phenylethyl)carbamate (0.4 g, 1.222 mmol) in dry dichloromethane and triethylamine (0.148 g, 1.467 mmol, 1.2 equiv) was added benzyl chloride (0.154 g, 1.222 mmol, 1 equiv), and the mixture was stirred for 12 h. The reaction was monitored by TLC and after completion of reaction worked up with water (10 mL) and extracted in DCM (2 × 25 mL), dried over sodium sulfate, and concentrated under vacuum to give the title product **9b** (0.48 g, 94.12%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.3725−7.1328 (15H, m, CH), 4.7211 (1H, m,CH), 4.6213 (2H, s, CH$_2$), 4.5808 (2H, s, CH$_2$), 3.7494 (2H, s, CH$_2$), 1.52 (9H, s, CH$_3$). Chemical formula: C$_{27}$H$_{31}$NO$_3$. FTIR (KBr): cm^{-1} 3062, 2933,

1654, 1562, 1335, 1229, 1258, 1087, 824, 784, 745, 699. EIMS: m/z 418.2 (M + 1). Elemental analysis: C, 77.60; H, 7.40; N, 3.34.

Synthesis of *tert*-Butyl Benzyl(2-cyano-2-phenylethyl)-carbamate (9c). To a stirred solution of *tert*-butyl benzyl(2-hydroxy-2-phenylethyl)carbamate (0.4 g, 1.222 mmol) in dry dichloromethane and triethylamine (0.247 g, 2.445 mmol, 2 equiv) were added TsIm (0.325 g, 1.466 mmol, 1.2 equiv), NaCN (0.119 g, 2.42 mmol, 2 equiv), and a catalytic amount of TBAI (0.045 g, 0.1222 mmol) in DMF (30 mL). The reaction mixture was refluxed for 6 h.[55] The solvent was evaporated under vacuum, and the remainder was diluted with DCM and washed with water (2 × 100 mL). The organic layer was dried over sodium sulfate and evaporated. The crude compound **9c** was purified by using column chromatography by silica gel, eluting with *n*-hexane/EtOAc (10:1). Yield (0.283 g, 69.02%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.3725−7.1328 (10H, m, CH), 4.4322 (1H, dd, J = 2.16 Hz, CH), 4.3070 (2H, s, CH$_2$), 3.75−3.59 (2H, m, CH$_2$), 1.52 (9H, s, CH$_3$). Chemical formula: C$_{21}$H$_{24}$N$_2$O$_2$. FTIR (KBr): cm^{-1} 3050, 2347, 1741, 1721, 1688, 1360, 1290, 1141, 1030, 786, 734, 634. EIMS: m/z 337.2 (M + 1). Elemental Analysis: C, 74.90; H, 7.17; N, 8.30.

Synthesis of 2-(Benzyl(*tert*-butoxycarbonyl)amino)-1-phenylethyl Benzoate (9d). To a stirred solution of *tert*-butyl benzyl(2-hydroxy-2-phenylethyl)carbamate (0.4 mg,1.222 mmol) in dry dichloromethane and triethylamine (0.148 mg, 1.467 mmol, 1.2 equiv) was added benzoyl chloride (0.179 g, 1.22 mmol, 1 equiv), and the mixture was stirred for 12 h. The reaction was monitored by TLC and after completion of reaction worked up with water (10 mL) and extracted in DCM (2 × 25 mL), dried over sodium sulfate, and concentrated under vacuum to give the title product **9d** (0.49 g, 94.23%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.96−7.92 (2H, d, J = 7.5), 7.3725−7.1328 (13H, m, CH), 5.9239 (1H, d, J = 7.18,CH), 4.1633 (2H, m, CH), 3.4156 (2H, m, CH$_2$), 1.52 (9H, s, CH$_3$). Chemical formula: C$_{27}$H$_{29}$NO$_4$. FTIR (KBr): cm^{-1} 3020, 2927, 1731, 1741, 1698, 1340, 1230, 1161, 1040, 789, 735, 635. EIMS: m/z 432.2 (M + 1). Elemental analysis: C, 75.10; H, 6.75; N, 3.24.

Synthesis of 2-(Benzyl(*tert*-butoxycarbonyl)amino)-1-phenylethyl 4-Methylbenzenesulfonate (9e). To a stirred solution of *tert*-butyl benzyl(2-hydroxy-2-phenylethyl)carbamate (0.4 mg, 1.222 mmol) in dry dichloromethane and triethylamine (0.148 mg, 1.467 mmol, 1.2 equiv) was added 4-methylbenzene-1-sulfonyl chloride (0.2322 g. 1.222 mmol, 1 equiv). The reaction was monitored by TLC and after completion of reaction worked up with water (10 mL) and extracted in DCM (2 × 25 mL), dried over sodium sulfate, and concentrated under vacuumto give the title product **9e** (0.55 g, 94.82%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.76−7.74 (2H, d, J = 7.5, CH), 7.4725−7.232 (12H, m, CH), 6.75 (1H, m, CH), 4.22 (2H, s, CH$_2$), 3.2659 (2H, m, CH$_2$), 2.37 (3H, s, CH$_3$), 1.52 (9H, s, CH$_3$). Chemical formula: C$_{27}$H$_{31}$NO$_5$S. FTIR (KBr): cm^{-1} 3485, 1658, 1504, 1453, 1204, 1130. EIMS: m/z 482.2 (M + 1). Elemental analysis: C, 67.39; H, 6.47; N, 2.90.

Synthesis of 2-(Benzylamino)-1-phenylethyl Acetate (10a). The compound was synthesized by stirring 2-(benzyl(*tert*-butoxycarbonyl)amino)-1-phenylethyl acetate (0.3 g, 0.8134 mmol) in trifluoroacetic acid (0.1111 g, 0.9745 mmol, 1.2 equiv) and dichloromethane (50:50%) and for 2 h. After the completion of the reaction, to the reaction mixture was added sodium bicarbonate until the solid was separated out to give the title product **10a** (0.18 g, 76.1%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.23−7.38 (10H, m, CH), 5.7414 (1H, dd, J = 2.16 Hz, CH), 3.90 (2H, s, CH$_2$), 3.2519 (2H, m, CH$_2$), 2.1985 (3H, s, CH$_3$), 1.84 (1H, s, NH). Chemical formula: C$_{17}$H$_{19}$NO$_2$. FTIR (KBr): cm^{-1} 3412, 2363, 2101, 1740, 1637, 1320, 1219, 771, 671, 552. EIMS: m/z 270.2 (M + 1). Elemental analysis: C, 75.79; H, 7.09; N, 5.18.

Synthesis of *N*-Benzyl-2-(benzyloxy)-2-phenylethanamine (10b). The compound was synthesized by stirring *tert*-butyl benzyl(2-(benzyloxy)-2-phenylethyl)carbamate (0.3 g, 0.9458 mmol) and trifluoroacetic acid (0.123 g, 1.135 mmol, 1.2 equiv) and dichloromethane (50:50%) for 2 h, using the same protocol used for compound **10a** to give the title product **10b** (0.18 g, 81.81%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.23−7.48 (15H, m, CH), 4.60 (2H, s,

CH_2), 4.34 (1H, m, CH), 3.13 (2H, m, CH_2), 3.82 (2H, s, CH_2), 1.87 (1H, s, NH). Chemical formula: $C_{22}H_{23}NO$. FTIR (KBr): cm^{-1} 3430, 2930, 1400, 1340, 1240, 1100, 780, 769. EIMS: *m/z* 318.2 (M + 1). Elemental analysis: C, 83.20; H, 7.28; N, 4.39.

Synthesis of 3-(Benzylamino)-2-phenylpropanenitrile (10c). The compound was synthesized by stirring *tert*-butyl benzyl(2-cyano-2-phenylethyl)carbamate (0.3 g, 0.813 mmol), trifluoroacetic acid (0.110 g, 0.975 mmol, 1.2 equiv), and dichloromethane (50:50%) for 2 h, using the same protocol used for compound **10a** to give the title product **10c** (0.16gm, 76.19%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.23−7.38 (10H, m, CH), 4.2334 (1H, dd, *J* = 2.16 Hz, CH), 4.1002 (2H, s, CH_2), 3.7559 (2H, m, CH_2), 1.89 (1H, s, NH). Chemical formula: $C_{17}H_{16}N_2O_2$. FTIR (KBr): cm^{-1} 3409, 2353, 2121, 1730, 1622, 1310, 1229, 765, 651, 562. EIMS: *m/z* 237.2 (M + 1). Elemental analysis: C, 81.25; H, 6.80; N, 11.86.

Synthesis of 2-(Benzylamino)-1-phenylethyl Benzoate (10d). The compound was synthesized by stirring 2-(benzyl(*tert*-butoxycarbonyl)amino)-1-phenylethyl benzoate (0.3 g, 0.6952 mmol) and trifluoroacetic acid (0.0951 g, 0.8342 mmol, 1.2 equiv) and dichloromethane (50:50%) for 2 h using the same protocol used for compound **10a** to give the title product **10d** (0.17 g, 73.91%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 8.02 (2H, d, *J* = 7.5, CH), 7.56−7.23 (13H, m, CH), 5.49 (1H, d, *J* = 7.18, CH), 3.91 (2H, s, CH), 3.23 (2H, m, CH_2), 1.96 (1H, s, NH). Chemical formula: $C_{22}H_{21}NO_2$. FTIR (KBr): cm^{-1} 3442, 3023, 1640, 1492, 1341, 1215, 1031, 762, 669, 559. EIMS: *m/z* 332.2 (M + 1). Elemental analysis: C, 79.69; H, 6.37; N, 4.23.

Synthesis of 2-(Benzylamino)-1-phenylethyl 4-Methylbenzenesulfonate (10e). The compound was synthesized by stirring 2-(benzyl(*tert*-butoxycarbonyl)amino)-1-phenylethyl 4-methylbenzenesulfonate (0.3 g, 0.6229 mmol), trifluoroacetic acid (0.085 g, 0.7454 mmol, 1.2 equiv), and dichloromethane (50:50%) for 2 h using the same protocol used for compound **10a** to give the title product **10e** (0.19 g, 82.60%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 7.74−7.232 (14H, m, CH), 6.40 (1H, m, *J* = 6.39 Hz, CH), 3.92 (2H, s, CH_2), 3.2659 (2H, m, CH_2), 2.34 (3H, s, CH_3), 1.82 (1H, s, NH). Chemical formula: $C_{22}H_{23}NO_3S$. FTIR (KBr): cm^{-1} 3429, 3022, 1638, 1337, 1217, 1152, 769,671. EIMS: *m/z* 382.2 (M + 1). Elemental analysis: C, 69.20; H, 6.05; N, 3.65.

Synthesis of 2-((1-(Benzyl(2-hydroxy-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic Acid (11). To a stirring suspension of 2-((1-carboxy-2-phenylethyl)carbamoyl)benzoic acid (**4**) (0.5 g, 1.5959 mmol) was added 4 equiv of oxylyl chloride (0.8102 g, 6.38 mmol, 4 equiv) at 0 °C to form clear solution. The mixture was stirred for 30 min to form 2-((1-chloro-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid, which was then concentrated in vacuum to remove excess of oxylyl chloride. Then in another flask of stirred 2-(benzylamino)-1-phenylethanol (**7**) (0.434 g, 1.195 mmol, 1.2 equiv) in THF was added 4 equiv of triethylamine (0.644 g, 6.3836 mmol, 4 equiv) at 0 °C, and the mixture was stirred for 30 min. Finally the 2-((1-chloro-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic derivative was added to this system and mixture was allowed to come to room temperature and stirred for 5−6 h. After the completion of reaction the reaction was worked up with 1 N HCl (10 mL) and extracted with dichloromethane (4 × 25 mL). After vigorous shaking, the organic layer was separated and dried over sodium sulfate and concentrated over vacuum to give the title product **11** (0.79 g, 94.78%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 9.8921 (1H, brs, COOH), 7.5465 (1H, d, *J* = 3.33 Hz, CH), 7.5274 (1H, d, *J* = 3.06 Hz, CH), 7.0561 (17H, m, CH), 5.022 (1H, bs, NH), 4.9091 (1H, d, *J* = 18 Hz, CH), 4.6442 (1H, d, *J* = 18 Hz, CH), 3.9494 (2H, s, CH_2), 3.6502 (1H, s, OH), 3.7804 (2H, m, CH_2), 3.2177 (2H, m, CH_2) . ^{13}C NMR (CDCl$_3$, 300 MHz): 170.65, 167.25, 167.20, 142.06, 137.02, 136.90, 135.62, 133.67,131.10, 129.25, 128.70, 128.61, 128.49, 128.32, 128.07,128.02, 128.04, 127.71, 126.95, 126.84, 125.82, 125.65, 125.47, 125.39, 125.29, 125.26, 123.19, 73.46, 56.42, 52.55, 52.41, 35.27. $C_{32}H_{30}N_2O_5$. FTIR (KBr): cm^{-1} 3708, 3426, 3246, 2928, 1690, 1625, 1520, 1472, 1472, 1322, 1230, 1162, 1001,775, 739. EIMS: *m/z* 523.2 (M + 1). Elemental analysis: C, 73.50; H, 5.77; N, 5.38.

Synthesis of 2-((1-(Benzyl(2-(benzyloxy)-2-phenylethyl)-amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic Acid (12a). This product was synthesized by using the same method described for compound **11** by stirring a suspension of 2-((1-carboxy-2-phenylethyl)carbamoyl)benzoic acid (**4**) (0.5 g, 1.5959 mmol) with 4 equiv of oxylyl chloride (0.8102 g, 6.38 mmol, 4 equiv) to form 2-((1-chloro-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid. Then *N*-benzyl-2-(benzyloxy)-2-phenylethanamine (0.607 g, 1.192 mmol, 1.2 equiv) in THF and 4 equiv of triethylamine (0.6447 g, 6.383 mmol) were added at 0 °C. After completion of the reaction the final product was treated with 1 N HCl (10 mL) and extracted by the same protocol described for compound **11** to give the title product **12a** (0.82 g, 83.93%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 11.36 (1H, s, COOH), 7.9812−7.8111 (2H, m, CH), 7.4984 (4H, m, CH), 7.3725−7.1328 (18H, m, CH), 5.022 (1H, bs, NH), 4.3808 (1H, d, *J* = 10.02 Hz, CH), 4.1271 (1H, m, CH), 3.9494 (2H, s, CH_2), 3.5395 (2H, s, CH_2), 3.5051 (2H, m, CH_2), 3.0094 (2H, t, *J* = 5.79 Hz, 6.21 Hz, CH_2). ^{13}C NMR (CDCl$_3$, 300 MHz), δ 171.51, 167.55, 167.32, 158.15, 146.12, 145.21, 138.50, 137.65, 137.42, 136.99, 135.55, 134.04, 129.4, 128.87, 128.51, 128.16, 128.38, 127.36, 127.30, 127.26, 127.34, 127.21, 127.14, 127.10, 126. 76, 126.54, 126.32, 125.55, 124.54, 124.37,123.39, 74.66, 53.32, 51.54, 48.40, 45.99, 34.53, 30.33, 27.70. Chemical formula: $C_{39}H_{36}N_2O_5$. FTIR (KBr): cm^{-1} 3755, 3460, 3090, 2925, 2368, 1686, 1643, 1447, 1346, 1158, 1062, 707, 697. EIMS: *m/z* 613.2 (M + 1). Elemental analysis: C, 76.40; H, 5.90; N, 4.55.

Synthesis of 2-((1-((2-(Benzoyloxy)-2-phenylethyl)(benzyl)-amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic Acid (12b). This product was synthesized by using the same method described for compound **11** by stirring a suspension of 2-((1-carboxy-2-phenylethyl)carbamoyl)benzoic acid (0.5 g, 1.5959 mmol) with 4 equiv of oxylyl chloride (0.8102 g, 6.38 mmol, 4 equiv) to form 2-((1-chloro-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid. Then 2-(benzylamino)-1-phenylethyl benzoate (0.6347 g, 1.9150 mmol, 1.2 equiv) in THF and 4 equiv of triethylamine (0.6447 g, 6.383 mmol) were added at 0 °C. After completion of the reaction the final product was treated with 1 N HCl (10 mL) and extracted by the same protocol described for compound **11** to give the title product **12b** (0.86 g, 86%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 11.48 (1H, s, COOH), 8.0278 (1H, s, NH), 7.7593 (1H, d, *J* = 1.94 Hz, CH), 7.74215 (1H, d, CH, *J* = 3.45 Hz), 7.6766−7.1197 (22H, m, CH), 5.6014 (1H, dd, *J* = 4.89 Hz, CH), 5.4727 (1H, t, *J* = 8.43 Hz, *J* = 7.7 Hz, CH), 4.4321 (2H, s, CH_2), 3.775 (2H, d, *J* = 6.8 Hz, *J* = 8.87 Hz, CH_2), 3.598 (2H, t, *J* = 6.87 Hz, CH_2). ^{13}C NMR (CDCl$_3$, 300 MHz), δ 170.24, 167.42, 166.34, 138.21, 137.67, 136.64, 136.50, 134.32, 133.56, 132.45, 130.63, 130.34, 128.42, 129.86, 129.75, 129.40, 128.67, 128.63, 128.55, 128.46, 128.39, 128.33, 128.30, 128.25,128.13, 127.82, 127.78, 127.74, 127.65, 127.60, 127.52, 127.43, 127.37, 125.43, 72.03, 56.62, 52.67, 49.03, 37.17. Chemical formula: $C_{39}H_{34}N_2O_6$. FTIR (KBr): cm^{-1} 3781, 3426, 3034, 2982, 1773, 1716, 1630, 1448, 1381, 1336, 1235, 1088,875, 746, 718, 699. EIMS: *m/z* 627.2 (M + 1). Elemental analysis: C, 74.70; H, 5.48; N, 4.46.

Synthesis of 2-((1-(Benzyl(2-cyano-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic Acid (12c). This product was synthesized by using the same method described for compound **11** by stirring a suspension of 2-((1-carboxy-2-phenylethyl)carbamoyl)benzoic acid (0.5 g,1.5959 mmol) with 4 equiv of oxylyl chloride (0.8102 g, 6.38 mmol, 4equiv) to form 2-((1-chloro-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid. Then 3-(benzylamino)-2-phenylpropanenitrile (0.4522 g, 1.915 mmol, 1.2 equiv) in THF and 4 equiv of triethylamine (0.6447 g, 6.383 mmol) were added at 0 °C. After completion of the reaction the final product was treated with 1 N HCl (10 mL) and extracted by the same protocol described for compound **11** to give the title product **12c** (0.67 g, 78.98%). ^{1}H NMR (CDCl$_3$, 300 MHz), δ 11.4820 (1H, s, COOH), 9.2741 (1H, s, NH), 8.3159 (1H, d, *J* = 7.7 Hz CH), 7.866 (2H, d, *J* = 8.34 Hz, CH), 7.6594 (2H, dd, *J* = 7.56, 9.9 Hz, CH), 7.4347 (14H, m, CH), 5.2878 (1H, m, CH), 5.0953 (1H, m, CH), 4.4321 (2H, s, CH_2), 3.5101 (2H, t, *J* = 7.27 Hz, CH_2), 3.1221 (2H, t, *J* = 7.6 Hz, CH_2). ^{13}C NMR (CDCl$_3$, 300 MHz), δ 173.21, 168.12, 168.01, 137.84, 137.63, 137.45, 134.56, 132.48, 131.56, 130.42, 130.02, 128.76, 128.63, 128.57,

128.43, 128.32, 127.91, 127.88, 127.84, 127.80, 127.72, 127.69, 127.67, 127.59, 127.43, 127.39, 127.21, 127.11, 57.21, 51.09, 49.03, 37.89, 29.84. Chemical formula: $C_{33}H_{29}N_3O_4$. FTIR (KBr): cm^{-1} 3624, 3326, 3054, 2382, 1763, 1716, 1640, 1438, 1281, 1236, 1225, 1088, 875, 746, 718, 699. EIMS: *m/z* 532.2 (M + 1). Elemental analysis: C, 74.35; H, 5.48; N, 7.89.

Synthesis of 2-((1-((2-Acetoxy-2-phenylethyl)(benzyl)-amino)-1-oxo-3-phenylpropan-2-Yl)carbamoyl)benzoic Acid (12d). This product was synthesized by using same method described for compound **11** by stirring a suspension of 2-((1-carboxy-2-phenylethyl)carbamoyl)benzoic acid (0.5 g, 1.5959 mmol) with 4 equiv of oxylyl chloride (0.8102 g, 6.38 mmol, 4equiv) to form 2-((1-chloro-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid. Then 2-(benzylamino)-1-phenylethyl acetate (0.5158 g, 1.915 mmol, 1.2 equiv) in THF and 4 equiv of triethylamine (0.6447 g, 6.383 mmol) were added at 0 °C. After completion of the reaction the final product was treated with 1 N HCl (10 mL) and extracted by the same protocol described for compound **11** to give the title product **12d** (0.76 g, 86.44%). ¹H NMR (CDCl₃, 300 MHz), δ 11.24 (1H, s, COOH), 7.70515 (2H, dd, J = 2.7 Hz, CH), 7.88 (1H, bs, NH), 7.7898 (2H, m, J = 3.1587, CH), 7.6998 (2H, t, J = 3.51 Hz, J = 2.95 Hz, CH) 7.5753 (1H, t, J = 3.51 Hz, CH), 7.3291−7.1821 (12H, m, CH), 5.8770 (1H, bs, NH), 5.7414 (1H, dd, J = 2.16 Hz, CH), 4.9361 (1H, dd, J = 2.22 Hz, CH), 4.4070 (2H,s, CH₂), 3.8403 (2H, dd, J = 5.43, 5.07, CH₂), 3.2832 (2H, m, CH₂), 2.1985 (3H, s, CH₃). ¹³C NMR, δ 170.22, 170.01, 167.63, 167.55, 138.52, 137.97, 136. 43, 136.67, 134.01, 132.08, 130.24, 130.12,129.24, 128.81,128.69, 128.65, 128.66, 128.60, 128.55, 128.54, 127.95, 127.76, 127.16, 127.39, 127.22, 127.16, 127.10, 125.9, 72.7,55.6, 52.5, 49.6, 37.7, 21.0. Chemical formula: $C_{34}H_{32}N_2O_6$. FTIR (KBr): cm^{-1} 3781, 3426, 3035, 2927, 1960, 1773, 1716, 1629, 1447, 1381, 1235, 1093, 874, 717. EIMS: 565.2 (M + 1). Elemental analysis: C, 72.29; H, 5.69; N, 4.94.

Synthesis of 2-((1-(Benzyl(2-phenyl-2-(tosyloxy)ethyl)-amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic Acid (12e). This product was synthesized by using same method described for compound **11** by stirring a suspension of 2-((1-carboxy-2-phenylethyl)carbamoyl)benzoic acid (0.5 g,1.5959 mmol) with 4 equiv of oxylyl chloride (0.8102 g, 6.38 mmol, 4 equiv) to form 2-((1-chloro-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid. Then 2-(benzylamino)-1-phenylethyl 4-methylbenzenesulfonate (0.0.73 g, 1.9150 mmol,1.2 equiv) in THF and 4 equiv of triethylamine (0.6447 g, 6.383 mmol) were added at 0 °C. After completion of the reaction the final product was treated with 1 N HCl (10 mL) and extracted by the same protocol described for compound **11** to give the title product **12e** (0.85 g, 78.70%). ¹H NMR (CDCl₃, 300 MHz), δ 10.824 (1H, s, COOH), 9.2741 (1H, s, NH), 8.7712 (d, 1H, 8.67 Hz, CH), 7.9501 (1H, d, 4.5 Hz, CH), 7.4907 (21H, m, CH), 6.7947 (1H, m, J = 6.39 Hz, CH), 4.912 (1H, t, J = 6.66, 4.86 Hz, CH), 4.6691 (2H,s, CH₂), 3.8324 (2H, m, CH₂), 3.2659 (2H, m, CH₂), 2.1929 (3H, s, CH). ¹³C NMR (CDCl₃, 300 MHz): 170.01, 167.63, 167.57, 144.43, 140.60, 140.53, 134.0, 132.0, 137.91, 137.82 136.44, 136.36, 130.10, 130.51, 130.45, 128.42, 128.40, 128.36, 129.28, 128.19,128.15, 128.12, 128.10, 128.09,128.03, 128.02, 127.1, 127.9, 127.7,127.1, 127.9, 127.0, 125.9, 72.7, 49.9, 55.6, 52.2, 37.7, 21.3. Chemical formula: $C_{39}H_{36}N_2O_6S$. FTIR (KBr): cm^{-1} 3393, 3064, 1750, 1740, 1493, 1451, 1373, 1336, 1157, 1241, 1102, 1041, 903, 824, 760, 700,616. EIMS: *m/z* 677.2 (M + 1). Elemental analysis: C, 69.16; H, 5.35; N, 4.14.

Debenzylation of Compounds 12f−h. The title products **12f**, **12g**, and **12h** were synthesized by debenzylation using palladium on carbon (10%) in methanol with 12 h of stirring in a Parr assembly with a pressure of 40 par. The title products were residues there were chromatographed on silica gel column using ethyl acetate/hexane (20:80).

Synthesis of 2-((1-((2-Acetoxy-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic Acid (12f). The product was synthesized by using 2-((1-((2-acetoxy-2-phenylethyl)-(benzyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid (0.2 g, 0.3546 mmol) as the initial compound and was debenzylated and converted to its corresponding product by a debenzylation procedure as described above to give the title product **12f** (0.13 g, 81.25%). ¹H NMR (CDCl₃, 300 MHz), δ 10.8630 (1H, s, 1COOH), 8.2062 (1H, d, J = 9.03 Hz, CH), 7.9827 (1H, bs, NH), 7.3915 (10H, m, CH), 7.1410 (3H, m, CH), 5.0206 (1H, dd, J = 2.19, 2.29 Hz, CH), 4.91725 (1H, d, J = 2.13 Hz, CH), 3.7967 (2H, dd, J = 5.43, 5.07 Hz, CH₂), 3.2413 (2H, m, CH₂), 2.0968 (3H, s, CH₃). ¹³C NMR (CDCl₃, 300 MHz), δ 171.71, 170.22, 167.60, 167.53, 130.11, 137.90, 138.52, 134.0,136.67, 132.10, 128.63, 129.23, 128.92, 128.65, 128.69, 128.56, 128.54, 128.50, 127.26,127.24, 127.22, 125.69, 74.91, 57.80, 44.13, 37.44, 21.50. Chemical formula: $C_{27}H_{26}N_2O_6$. FTIR (KBr): cm^{-1} 3779, 3656, 3379, 2927, 1755, 1698, 1599, 1420, 1389, 1309, 1163, 1083, 838, 766, 722, 699, 660. EIMS: 475.2 (M + 1). Elemental analysis: C, 68.30; H, 5.50; N, 5.88.

Synthesis of 2-((1-Oxo-3-phenyl-1-((2-phenyl-2-(tosyloxy)-ethyl)amino)propan-2-yl)carbamoyl)benzoic Acid (12g). The product was synthesized by using 2-((1-(benzyl(2-phenyl-2-(tosyloxy)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)-benzoic acid (0.2 g, 0.2955 mmol) as the initial compound and was debenzylated and converted to its corresponding product by a debenzylation procedure as described above to give the title product **12g** (0.14 g, 82.35%). ¹H NMR (CDCl₃, 300 MHz), δ 10.820 (1H, s, 1COOH), 9.2741 (1H, s, NH), 8.3159 (1H, s, NH), 7.8664 (4H, m, CH), 7.6594 (4H, m, CH), 7.4479 (10H, m, CH), 6.4739 (1H, d, J = 7.38, CH), 4.8333 (1H, m, CH), 3.4156 (2H, dd, J = 5.8, 4.99 Hz, CH₂), 3.2638 (2H, dd, J = 6.5, 6.49 Hz, CH₂), 2.1929 (3H, s, CH₃). ¹³C NMR (CDCl₃, 300 MHz), δ 171.72, 167.64, 167.52, 144.47, 140.65, 140.43, 140.26, 137.59, 136.66, 136.56, 134.80, 132.10,130.31, 130.75, 129.42, 128.79, 128.46, 128.23, 128.16, 127.71, 127.67, 127.61, 128.59, 128.56, 127.47, 127.36, 125.29, 74.82, 57.80, 44.01, 37.14, 21.53. Chemical formula: $C_{32}H_{30}N_2O_6S$. FTIR (KBr): cm^{-1} 3441, 3022, 1740, 1637, 1543, 1630, 1455, 1367, 1217, 1160, 768, 701, 669, 607. EIMS: 587.2 (M + 1). Elemental analysis: C, 65.49; H, 5.13; N, 4.76.

Synthesis of 2-((1-((2-(Benzyloxy)-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic Acid (12h). The product was synthesized by using 2-((1-(benzyl(2-(benzyloxy)-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid (0.2 g, 0.3167 mmol) as initial compound and debenzylated to its corresponding product by a debenzylation procedure as described above to give the title product **12h** (0.12gm, 70.58%). ¹H NMR (CDCl₃, 300 MHz), δ 12.7012 (1H, s, COOH), 9.2741 (1H, s, NH), 8.0670−8.0544 (1H, J = 1.83 Hz,1.95 Hz, CH), 8.0382 (1H,t, J = 8.43, 2.3 Hz, CH), 7.9078−7.1965 (17H, m, CH), 5.4727 (1H, s, NH), 5.0627 (1H, d, J = 7.17 Hz, CH), 4.2136 (1H, t, J = 15.93 Hz, CH), 4.6321 (2H, s, CH₂), 3.8241 (2H, dd, J = 7.83, 7.56 Hz, CH₂),3.775 (2H, m, CH₂). ¹³C NMR, δ 171.72, 167.72, 167.64, 147.06, 142.11, 137.9, 136.67, 134.40, 132.30, 130.11, 129.63, 128.56, 128.46, 128.40, 128.36, 128.34, 128.29, 128.25, 127.87, 127.80, 127.76, 127.74,127.64, 127.60, 127.54, 127.51, 125.50, 86.31, 72.10, 57.84, 43.92, 37.41. Chemical formula: $C_{32}H_{30}N_2O_5$. FTIR (KBr): cm^{-1} 3751, 3506, 3027, 2926, 1605, 1531, 1651, 1349, 1217, 1159, 1095, 937, 764, 699. EIMS: *m/z* 523.2 (M + 1). Elemental analysis: C, 73.50; H, 5.77; N, 5.36.

Synthesis of 2-((1-Oxo-1-(phenethylamino)-3-phenylpropan-2-yl)carbamoyl)benzoic Acid (13). This product was synthesized by using same method as described for compound **11** by stirring a suspension of 2-((1-carboxy-2-phenylethyl)carbamoyl)-benzoic acid (0.5 g, 1.5959 mmol) with 4 equiv of oxylyl chloride (0.8102 g, 6.38 mmol, 4 equiv) to form 2-((1-chloro-1-oxo-3-phenylpropan-2-yl)carbamoyl)benzoic acid. Then 2-phenylethanamine (0.2320 g, 1.5959 mmol, 1.2 equiv) in THF and 4 equiv of triethylamine (0.6447 g, 6.383 mmol) were added at 0 °C, and extraction was by the same protocol as described for compound **11** to give the title product **13** (0.52 g, 78.79%). ¹H NMR (CDCl₃, 300 MHz), δ 12.7012 (1H, s, COOH), 7.7054 (4H, m, CH), 7.2805 (1H, s, NH), 7.1339 (10H, m, CH), 6.1225 (1H, m, CH), 5.0698 (1H, dd, J = 6.96 Hz, 6.99 Hz, CH), 3.283 (2H, t, CH₂), 2.8134 (2H, t, J = 6.81, 6.87, CH₂), 2.938 (2H, d, J = 6.816, CH₂). ¹³C NMR (CDCl₃, 300 MHz), δ 171.94, 168.46, 167.93, 151.80, 138.58, 136.71, 134.28, 131.34, 130.21, 129.13, 128.81, 128.77, 128. 64, 128.53, 126.93, 126.46, 123.51, 119.27, 117.80, 117.50, 116.87, 55.93, 40.94, 35.37,

34.69. Chemical formula: $C_{25}H_{24}N_2O_4$. FTIR (KBr): cm^{-1} 3440, 3023, 2923, 2821, 1491, 1299, 1031, 747, 687, 636. EIMS: 417.2 (M + 1). Elemental analysis: C, 72.10; H, 5.81; N, 6.73.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MTT assay for cell viability at 1, 10, and 100 nM compounds and ALP production by osteoblasts for the selected compound **11** at 10 nM (Figure 1); relative BMP-2 transcript level in RCOs (Table 1); absolute BMP-2 levels (pg/mL) in the conditioned medium (Table 2). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +91 522 2624273. Fax: +91 522 2623405. E-mail: anilsak@gmail.com.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS USED

BMP-2, bone morphogenetic protein 2; HRT, hormone replacement therapy; PTH, parathyroid hormone; TGF-β, transforming growth factor β; SAR, structure−activity relationship

■ REFERENCES

(1) Kanis, J. A.; Stevenson, J. C. Effect of estrogen therapy on bone density in elderly women. *N. Engl. J. Med.* **1994**, *330*, 715−716.

(2) Lindsay, R.; Hart, D. M.; Clark, D. M. The minimum effective dose of estrogen for prevention of postmenopausal bone loss. *Obstet. Gynecol.* **1984**, *63*, 759−763.

(3) Trivedi, R.; Kumar, S.; Kumar, A.; Siddiqui, J. A.; Swarnkar, G.; Gupta, V.; Kendurker, A.; Dwivedi, A. K.; Romero, J. R.; Chattopadhyay, N. Kaempferol has osteogenic effect in ovariectomized adult Sprague-Dawley rats. *Mol. Cell. Endocrinol.* **2008**, *289*, 85−93.

(4) Wong, G. L.; Kent, G. N.; Ku, K. Y.; Cohn, D. V. The interaction of parathormone and calcium on the hormone-regulated synthesis of hyaluronic acid and citrate decarboxylation in isolated bone cells. *Endocrinology* **1978**, *103*, 2274−2282.

(5) Deal, C. Potential new drug targets for osteoporosis. *Nat. Clin. Pract. Rheumatol.* **2009**, *5*, 20−27.

(6) Bilezikian, J. P. Combination anabolic and antiresorptive therapy for osteoporosis: opening the anabolic window. *Curr. Osteoporosis Rep.* **2008**, *6*, 24−30.

(7) Garrett, I. R. Anabolic agents and the bone morphogenetic protein pathway. *Curr. Top. Dev. Biol.* **2007**, *78*, 127−171.

(8) Girotra, M.; Rubin, M. R.; Bilezikian, J. P. Anabolic agents for osteoporosis: What is their likely place in therapy? *Treat. Endocrinol.* **2006**, *5*, 347−358.

(9) Audran, M. Drug combination strategies for osteoporosis. *Jt., Bone, Spine* **2006**, *73*, 374−378.

(10) Khan, A. W.; Khan, A. Anabolic agents: a new chapter in the management of osteoporosis. *J. Obstet. Gynaecol. Can.* **2006**, *28*, 136−141.

(11) Goltzman, D. Discoveries, drugs and skeletal disorders. *Nat. Rev. Drug Discovery* **2002**, *1*, 784−796.

(12) Maniatopoulos, C.; Sodek, J.; Melcher, A. H. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res.* **1988**, *254*, 317−330.

(13) Zanchetta, J. R.; Bogado, C. E.; Cisari, C. Treatment of postmenopausal women with osteoporosis with PTH(1-84) for 36 months: treatment extension study. *Curr. Med. Res. Opin.* **2010**, *26*, 2627−2633.

(14) Friedlaender, G. E.; Perry, C. R.; Cole, J. D.; Cook, S. D.; Cierny, G.; Muschler, G. F.; Zych, G. A.; Calhoun, J. H.; LaForte, A. J.; Yin, S. Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibialnonunions. *J. Bone Jt. Surg. Am.* **2001**, *83A*, S151−158.

(15) Govender, S.; Csimma, C.; Genant, H. K.; Valentin-Opran, A.; Amit, Y.; Arbel, R.; Aro, H.; Atar, D.; Bishay, M.; Börner, M. G.; Chiron, P.; Choong, P.; Cinats, J.; Courtenay, B.; Feibel, R.; Geulette, B.; Gravel, C.; Haas, N.; Raschke, M.; Hammacher, E.; van der Velde, D.; Hardy, P.; Holt, M.; Josten, C.; Ketterl, R. L.; Lindeque, B.; Lob, G.; Mathevon, H.; McCoy, G.; Marsh, D.; Miller, R.; Munting, E.; Oevre, S.; Nordsletten, L.; Patel, A.; Pohl, A.; Rennie, W.; Reynders, P.; Rommens, P. M.; Rondia, J.; Rossouw, W. C.; Daneel, P. J.; Ruff, S.; Ruter, A.; Santavirta, S.; Schildhauer, T. A.; Gekle, C.; Schnettler, R.; Segal, D.; Seiler, H.; Snowdowne, R. B.; Stapert, J.; Taglang, G.; Verdonk, R.; Vogels, L.; Weckbach, A.; Wentzensen, A.; Wisniewski, T. BMP-2 evaluation in surgery for tibial trauma (BESTT) study group. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. *J. Bone Jt. Surg. Am.* **2002**, *84A*, 2123−2134.

(16) Baltzer, A. W.; Lattermann, C.; Whalen, J. D.; Ghivizzani, S.; Wooley, P.; Krauspe, R.; Robbins, P. D.; Evans, C. H. Potential role of direct adenoviral gene transfer in enhancing fracture repair. *Clin. Orthop. Relat. Res.* **2000**, *379*, S120−S125.

(17) Huang, X.; Yang, Y. Innate immune recognition of viruses and viral vectors. *Hum. Gene Ther.* **2009**, *20*, 293−301.

(18) Riley, E. H.; Lane, J. M.; Urist, M. R.; Lyons, K. M.; Lieberman, J. R. Bone morphogenetic protein-2: biology and applications. *Clin. Orthop. Relat. Res.* **1996**, *324*, 39−46.

(19) Egermann, M.; Baltzer, A. W.; Adamaszek, S.; Evans, C.; Robbins, P.; Schneider, E.; Lill, C. A. Direct adenoviral transfer of bone morphogenetic protein-2 cDNA enhances fracture healing in osteoporotic sheep. *Hum. Gene Ther.* **2006**, *17*, 507−517.

(20) McKay, W. F.; Peckham, S. M.; Badura, J. M. A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE bone graft). *Int. Orthop.* **2007**, *31*, 729−734.

(21) Zerath, E.; Holy, X.; Noel, B.; Malouvier, A.; Hott, M.; Marie, P. J. Effects of BMP-2 on osteoblastic cells and on skeletal growth and bone formation in unloaded rats. *Growth Horm. IGF Res.* **1998**, *8*, 141−149.

(22) Mundy, G.; Garrett, R.; Harris, S.; Chan, J.; Chen, D.; Rossini, G.; Boyce, B.; Zhao, M.; Gutierrez, G. Stimulation of bone formation in vitro and in rodents by statins. *Science* **1999**, *286*, 1946−1949.

(23) Zhou, S.; Turgeman, G.; Harris, S. E.; Leitman, D. C.; Komm, B. S.; Bodine, P. V.; Gazit, D. Estrogens activate bone morphogenetic protein-2 gene transcription in mouse mesenchymal stem cells. *Mol. Endocrinol.* **2003**, *17*, 56−66.

(24) Jia, T. L.; Wang, H. Z.; Xie, L. P.; Wang, X. Y.; Zhang, R. Q. Daidzein enhances osteoblast growth that may be mediated by increased bone morphogenetic protein (BMP) production. *Biochem. Pharmacol.* **2003**, *65*, 709−715.

(25) Chang, J. K.; Hsu, Y. L.; Teng, I. C.; Kuo, P. L. Piceatannol stimulates osteoblast differentiation that may be mediated by increased bone morphogenetic protein-2 production. *Eur. J. Pharmacol.* **2006**, *551*, 1−9.

(26) Garrett, I. R.; Chen, D.; Gutierrez, G.; Zhao, M.; Escobedo, A.; Rossini, G.; Harris, S. E.; Gallwitz, W.; Kim, K. B.; Hu, S.; Crews, C. M.; Mundy, G. R. Selective inhibitors of the osteoblast proteasome stimulate bone formation in vivo and in vitro. *J. Clin. Invest.* **2003**, *111*, 1771−1782.

(27) Babatunde, O. I.; Ross, G.; Anjana, G.; Alda, F.; Javier, E.; Steve, M.; Ming, Z.; Mundy, G. R. Stimulation of new bone formation by the proteasome inhibitor, bortezomib: implications for myeloma bone disease. *Br. J. Haematol.* **2007**, *139*, 434−438.

(28) Roodman, D. G. Bone building with bortezomib. *J. Clin. Invest.* **2008**, *118*, 462−464.

(29) Fujii, M.; Takeda, K.; Imamura, T.; Aoki, H.; Sampath, T. K.; Enomoto, S.; Kawabata, M.; Kato, M.; Ichijo, H.; Miyazono, K. Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. *Mol. Biol. Cell* **1999**, *10*, 3801−3813.

(30) Liu, Z. Y.; He, X. B.; Yang, Z. Y.; Shao, H. Y.; Li, X.; Guo, H. F.; Zhang, Y. Q.; Si, S. Y.; Li, Z. R. Synthesis and evaluation of 1-(benzo[*b*]thiophen-2-yl)ethanone analogues as novel anti-osteoporosis agents acting on BMP-2 promotor. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4167−4170.

(31) Guo, H. F.; Shao, H. Y.; Yang, Z. Y.; Xue, S. T.; Li, X.; Liu, Z. Y.; He, X. B.; Jiang, J. D.; Zhang, Y. Q.; Si, S. Y.; Li, Z. R. Substituted benzothiophene or benzofuran derivatives as a novel class of bone morphogenetic protein-2 up-regulators: synthesis, structure−activity relationships, and preventive bone loss efficacies in senescence accelerated mice (SAMP6) and ovariectomized rats. *J. Med. Chem.* **2010**, *53*, 1819−1829.

(32) Sharma, S.; Pandey, A. K.; Shukla, P. K.; Saxena, A. K. Synthesis and 2D QSAR of O- sulphonated *β*-aminols derivatives as novel antifungal and antibacterial agents. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6476−6481.

(33) Kloen, P.; Di Paola, M.; Borens, O.; Richmond, J.; Perino, G.; Helfet, D. L.; Goumans, M. J. BMP signaling components are expressed in human fracture callus. *Bone* **2003**, *33*, 362−371.

(34) Garrison, K. R.; Shemilt, I.; Donell, S.; Ryder, J. J.; Mugford, M.; Harvey, I.; Song, F.; Alt, V. Bone morphogenetic protein (BMP) for fracture healing in adults. *Cochrane Database Syst. Rev.* **2010**, *6*, CD006950.

(35) Shao, J. S.; Cai, J.; Towler, D. A. Molecular mechanisms of vascular calcification: lessons learned from the aorta. *Arterioscler., Thromb., Vasc. Biol.* **2006**, *26*, 1423−1430.

(36) De Matteo, M.; Brunetti, A. E.; Maiorano, E.; Cafforio, P.; Dammacco, F.; Silvestris, F. Constitutive down-regulation of osterix in osteoblasts from myeloma patients: in vitro effect of bortezomib and lenalidomide. *Leuk. Res.* **2010**, *34*, 243−249.

(37) Martinovic, S.; Borovecki, F.; Miljavac, V.; Kisic, V.; Maticic, D.; Francetic, I.; Vukicevic, S. Requirement of a bone morphogenetic protein for the maintenance and stimulation of osteoblast differentiation. *Arch. Histol. Cytol.* **2006**, *69*, 23−36.

(38) Rawadi, G.; Vayssiere, B.; Dunn, F.; Baron, R.; Roman-Roman, S. BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J. Bone Miner. Res.* **2003**, *18*, 1842−1853.

(39) van der Horst, G.; van Bezooijen, R. L.; Deckers, M. M.; Hoogendam, J.; Visser, A.; Lowik, C. W.; Karperien, M. Differentiation of murine preosteoblastic KS483 cells depends on autocrine bone morphogenetic protein signaling during all phases of osteoblast formation. *Bone* **2002**, *31*, 661−669.

(40) Touyz, R. M.; Montezano, A. C. Vascular smooth muscle cells sense calcium: a new paradigm in vascular calcification. *Cardiovasc. Res.* **2009**, *81*, 237−239.

(41) Tsuji, K.; Bandyopadhyay, A.; Harfe, B. D.; Cox, K.; Kakar, S.; Gerstenfeld, L.; Einhorn, T.; Tabin, C. J.; Rosen, V. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nat. Genet.* **2006**, *38*, 1424−1429.

(42) Tanaka, K.; Tanaka, S.; Sakai, A.; Ninomiya, T.; Arai, Y.; Nakamura, T. Deficiency of vitamin A delays bone healing process in association with reduced BMP2 expression after drill-hole injury in mice. *Bone* **2010**, *47*, 1006−1012.

(43) Nagashima, M.; Sakai, A.; Uchida, S.; Tanaka, S.; Tanaka, M.; Nakamura, T. Bisphosphonate (YM529) delays the repair of cortical bone defect after drill-hole injury by reducing terminal differentiation of osteoblasts in the mouse femur. *Bone* **2005**, *36*, 502−511.

(44) Uchida, S.; Doi, Y.; Kudo, H.; Furukawa, H.; Nakamura, T.; Fujimoto, S. Transient expression of activinbeta A mRNA on osteoprogenitor cells in rat bone regeneration after drill-hole injury. *Bone* **2000**, *27*, 81−90.

(45) Sharan, K.; Mishra, J. S.; Swarnkar, G.; Siddiqui, J. A.; Khan, K.; Kumari, R.; Rawat, P.; Maurya, R.; Sanyal, S.; Chattopadhyay, N. A novel quercetin analogue from a medicinal plant promotes peak bone mass achievement and bone healing after injury and exerts an anabolic effect on osteoporotic bone: the role of aryl hydrocarbon receptor as a mediator of osteogenic action. *J. Bone Miner. Res.* **2011**, *26*, 2096−2111.

(46) Trivedi, R.; Kumar, S.; Kumar, A.; Siddiqui, J. A.; Swarnkar, G.; Gupta, V.; Kendurker, A.; Dwivedi, A. K.; Romero, J. R.; Chattopadhyay, N. Kaempferol has osteogenic effect in ovariectomized adult Sprague-Dawley rats. *Mol. Cell. Endocrinol.* **2008**, *289*, 85−93.

(47) Wong, G. L.; Kent, G. N.; Ku, K. Y.; Cohn, D. V. The interaction of parathormone and calcium on the hormone-regulated synthesis of hyaluronic acid and citrate decarboxylation in isolated bone cells. *Endocrinology* **1978**, *103*, 2274−2282.

(48) Siddiqui, J. A.; Swarnkar, G.; Sharan, K.; Chakravarti, B.; Gautam, A. K.; Rawat, P.; Kumar, M.; Gupta, V.; Manickavasagam, L.; Dwivedi, A. K.; Maurya, R.; Chattopadhyay, N. A naturally occurring rare analog of quercetin promotes peak bone mass achievement and exerts anabolic effect on osteoporotic bone. *Osteoporosis Int.* **2011**, *22*, 3013−3027.

(49) Ishizuya, T.; Yokose, S.; Hori, M.; Noda, T.; Suda, T.; Yoshiki, S.; Yamaguchi, A. Parathyroid hormone exerts disparate effects on osteoblast differentiation depending on exposure time in rat osteoblastic cells. *J. Clin. Invest.* **1997**, *99*, 2961−2970.

(50) Siddiqui, J. A.; Swarnkar, G.; Sharan, K.; Chakravarti, B.; Sharma, G.; Rawat, P.; Kumar, M.; Khan, F. M.; Pierroz, D.; Maurya, R.; Chattopadhyay, N. 8,8″-Biapigeninyl stimulates osteoblast functions and inhibits osteoclast and adipocyte functions: osteoprotective action of 8,8″-biapigeninyl in ovariectomized mice. *Mol. Cell. Endocrinol.* **2010**, *323*, 256−267.

(51) Prabhakar, U.; James, I. E.; Dodds, R. A.; Lee-Rykaczewski, E.; Rieman, D. J.; Lipshutz, D.; Trulli, S.; Jonak, Z.; Tan, K. B.; Drake, F. H.; Gowen, M. A novel human bone marrow stroma-derived cell line TF274 is highly osteogenic in vitro and in vivo. *Calcif. Tissue Int.* **1998**, *63*, 214−220.

(52) Gregory, C. A.; Gunn, W. G.; Peister, A.; Prockop, D. J. An alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal. Biochem.* **2004**, *329*, 77−84.

(53) Bandyopadhyay, S.; Romero, J. R.; Chattopadhyay, N. Kaempferol and quercetin stimulate granulocyte-macrophage colony-stimulating factor secretion in human prostate cancer cells. *Mol. Cell. Endocrinol.* **2008**, *287*, 57−64.

(54) *GraphPad Prism*, version 5.04 for Windows; GraphPad Software: La Jolla, CA, U.S.

(55) Soltani Rad, M. N.; Khalafi-Nezhad, A.; Behrouz, S.; Faghihi, M. A. A simple one-pot procedure for the direct conversion of alcohols into alkyl nitriles using TsIm. *Tetrahedron Lett.* **2007**, *48*, 6779−6784.