# Chapter-7

BIOLOGICAL EVALUATION OF DESIGNED LEADS

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7.1.1 Biological testing of designed leads.

The selected 10 compounds were biologically tested for the BMP-2 production in vitro using the BMP-2 ELISA kit. The biological data of these synthesized compounds also fortify the in-silico observations in terms of docking and pharmacophore analysis. Five compounds 11, 13, 12b, 12g and 12e out of 10 were able to activate the BMP-2 production. (i) The BMP-2 production was observed with compound 11 at all concentrations (1nM, 10nM and 100nM) studied when compared to control (cell receiving vehicle) (Table 6). (ii) The compounds 13, 12b, 12g and 12e also increased BMP-2 production, compared to control however, significant loss of osteoblast viability was observed with 12b, 12g and 12e (Fig 6). (iii) Therefore 13 and 11 were considered as ‘leads’ for stimulating/promoting BMP-2 secretion from osteoblasts. (iv) Compound 11 was the most potent and showed consistent effect at all concentrations. The rest of the molecules from Core II (Compound 16) and the core III (Compounds 21-40) were unable to show BMP-2 stimulation significantly and therefore not studied further for this target.

7.1.2 SAR study

The series of the compounds represented by the prototype was designed in such a manner that structural modifications were in part 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 R2 substitutions. Among all these compounds the compound 11 (R2=OH) showed maximum effect on BMP-2 secretion at lower concentrations (up to 0.1nM). 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 R2 position is optimum for the activity. The next important variation was carried out at R1 portion in the compounds viz. 12f, 12g, 12h and 13 where the activity improved in case of N-debenzylated analog of acetylated derivative 12f as compared to the benzyl substituted acetylated analog 12d. However this trend was not observed in the debenzylated analog 12e of 12d. The substitution of the bulky groups at R2 position by hydrogen in the debenzylated analog (12f-12h) led to the compound 13 which showed significant increase in BMP-2 secretion. As most of the compounds did not show increase in BMP-2 secretion in dose/concentration dependent manner, possibly due to copious production of BMP-2 by
osteoblasts at the basal level which 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 7.1). The absolute BMP-2 levels (pg/ml) in the conditioned medium were included in the table 7.2 and table 7.3.

Table 7.1. The structures and effect of compounds on BMP-2 secretion in RCOs.

![Chemical Structure]

<table>
<thead>
<tr>
<th>Comp.</th>
<th>R₁</th>
<th>R₂</th>
<th>BMP-2 levels in the conditioned medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1nM</td>
</tr>
<tr>
<td>11</td>
<td>-CH₂C₆H₅</td>
<td>-OH</td>
<td>140±3.4**</td>
</tr>
<tr>
<td>12a</td>
<td>-CH₂C₆H₅</td>
<td>-OCH₂C₆H₅</td>
<td>89.8±3.5</td>
</tr>
<tr>
<td>12b</td>
<td>-CH₂C₆H₅</td>
<td>-OCOC₆H₅</td>
<td>98.4±2.3</td>
</tr>
<tr>
<td>12c</td>
<td>-CH₂C₆H₅</td>
<td>-CN</td>
<td>83.6±3.6</td>
</tr>
<tr>
<td>12d</td>
<td>-CH₂C₆H₅</td>
<td>-OCOCH₃</td>
<td>88.7±4.2</td>
</tr>
<tr>
<td>12e</td>
<td>-CH₂C₆H₅</td>
<td>-OSO₂C₆H₅CH₃</td>
<td>122±3.2</td>
</tr>
<tr>
<td>12f</td>
<td>H</td>
<td>-OCOCH₃</td>
<td>106±2.4</td>
</tr>
<tr>
<td>12g</td>
<td>H</td>
<td>-OSO₂C₆H₅CH₃</td>
<td>119±3.1</td>
</tr>
<tr>
<td>12h</td>
<td>H</td>
<td>-OCH₂C₆H₅</td>
<td>87.8±1.9</td>
</tr>
<tr>
<td>13</td>
<td>H</td>
<td>-</td>
<td>111±1.6</td>
</tr>
<tr>
<td>14</td>
<td>H</td>
<td>-OH</td>
<td>224±3.4**</td>
</tr>
</tbody>
</table>
Each assay was performed in triplicate and results are expressed as mean ± S.E.M. of percentage of control (100%) after normalization of total protein content/well. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. control.

7.1.3 MTT assay

The MTT assay for cell viability at 1 nM, 10 nM and 100 nM of compounds and ALP production by osteoblasts for the selected compound 11 at 10 nM (Figure 7.1). Relative BMP-2 transcript level in RCOs (Table 7.2). Absolute BMP-2 levels (pg/ml) in the conditioned medium (Table 7.3).

MTT Assay for Cell Viability

Calvaria derived primary osteoblasts cells were plated in a 96-well plate at 2x10^3 cells/well. When they attained ~60% confluence, they were pre-starved for about 3 h in 0.5% FCS medium and incubated with different concentrations (1nM, 10nM and 100nM) of compounds or vehicle in 2% FCS phenol red free DMEM for 24 hr. On termination of incubation, 5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) solution was added and incubated for another 4 hr. Formazan crystals were dissolved in DMSO and optical density (OD) was measured at 550 nm on an ELISA reader. The results of the MTT assay were represented in the Figure 7.1.
Figure 7.1. Cytotoxicity evaluation of compounds. Osteoblasts purified from neonatal calvariae of rats (RCO) were plated directly in 96-well plates after collagenase digestion at 2 x 10^3/well and cultured for 3–5 d. At ~70% confluence, they were pre-starved for about 3 h in 0.5% FCS medium and incubated with different concentrations (1-, 10- and 100nM) of compounds (11, 13, 12b, 12g and 12e) or vehicle in 2% FCS containing phenol red free DMEM for 24 h. Cell viability was measured with MTT assay as described in Materials and Methods. Each assay was performed in triplicate and results are represented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
Table 7.2. Relative BMP-2 transcript level in RCOs.

<table>
<thead>
<tr>
<th>Comp.</th>
<th>0.1nM</th>
<th>1nM</th>
<th>10nM</th>
<th>100nM</th>
<th>1000nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>2.50±0.91</td>
<td>5.07±3.01*</td>
<td>10.81±1.19***</td>
<td>14.63±1.01***</td>
<td>18.27±1.45***</td>
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<tr>
<td>13</td>
<td>0.96±0.61</td>
<td>1.16±0.31</td>
<td>1.44±0.15</td>
<td>1.58±0.54</td>
<td>2.27±0.28*</td>
</tr>
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</table>
## Table 7.3. Absolute BMP-2 levels (pg/ml) in the conditioned medium

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Control</th>
<th>0.1nM</th>
<th>1nM</th>
<th>10nM</th>
<th>100nM</th>
<th>1000nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>119.14±4.7</td>
<td>166.79±4.6**</td>
<td>189.43±3.2***</td>
<td>185.38±5.3**</td>
<td>163.46±4.1**</td>
<td>158.45±3.9*</td>
</tr>
<tr>
<td>12a</td>
<td>119.14±4.7</td>
<td>106.98±3.9</td>
<td>119.14±3.8</td>
<td>117.11±3.4</td>
<td>122.95±3.1</td>
<td>110.32±3.1</td>
</tr>
<tr>
<td>12b</td>
<td>119.14±4.7</td>
<td>117.23±6.3</td>
<td>127.47±2.9</td>
<td>122.71±4.7</td>
<td>122.49±6.4</td>
<td>133.43±5.4</td>
</tr>
<tr>
<td>12c</td>
<td>119.14±4.7</td>
<td>99.60±3.8</td>
<td>115.92±4.9</td>
<td>106.36±5.6</td>
<td>115.19±6.4</td>
<td>110.32±5.8</td>
</tr>
<tr>
<td>12d</td>
<td>119.14±4.7</td>
<td>105.67±4.6</td>
<td>106.55±6.9</td>
<td>99.42±4.6</td>
<td>117.13±5.3</td>
<td>119.85±4.7</td>
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<tr>
<td>12e</td>
<td>112.76±3.9</td>
<td>137.56±3.5</td>
<td>158.99±4.6**</td>
<td>122.90±5.7</td>
<td>155.60±2.2**</td>
<td>125.16±5.2</td>
</tr>
<tr>
<td>12f</td>
<td>112.76±3.9</td>
<td>119.52±6.3</td>
<td>111.26±5.4</td>
<td>110.87±6.9</td>
<td>108.28±4.7</td>
<td>116.14±4.6</td>
</tr>
<tr>
<td>12g</td>
<td>112.76±3.9</td>
<td>134.18±3.9</td>
<td>148.56±4.9**</td>
<td>126.04±5.3</td>
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<td>12h</td>
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<td>99.00±4.5</td>
<td>120.65±3.5</td>
<td>113.88±6.5</td>
<td>131.92±5.8</td>
<td>105.54±6.3</td>
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<tr>
<td>13</td>
<td>112.76±3.9</td>
<td>125.16±4.8</td>
<td>137.56±5.2</td>
<td>128.54±5.7</td>
<td>187.18±7.2**</td>
<td>148.84±5.5*</td>
</tr>
</tbody>
</table>

Each assay was performed in triplicate and results are expressed as mean ± S.E.M. *P < 0.05; **P < 0.01; ***P < 0.001 vs. control.
7.1.4 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 RCOs 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 7.2A).

**Figure 7.2.** Compound 11 increases osteoblast function.(A) RCO were cultured in osteoblast differentiation medium as described above in the presence of compound 11 (10nM) 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 (lower panel). (B) qPCR analysis of various osteogenic genes, Runx-2, osteocalcin, BMP-2 and type I collagen in osteoblasts treated with compound 11 (10nM) at different time intervals as indicated. Each assay was performed in triplicate and results are represented as mean ± SEM of percentage/fold change. *P < 0.05; **P < 0.01; ***P < 0.001.

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 7.2B). 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 7.3 shows that the induction of Runx-2 and osteocalcin mRNA levels in the RCOs by compound 11 was brought back to the basal (unstimulated) 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.

![Figure 7.3](image)

**Figure 7.3.** Effect of BMP-2 neutralization on osteoblast differentiation by compound 11. Cells were treated for 72 h with compound 11 (10nM), BMP-2 (100ng/ml) and noggin (50ng/ml) either alone or in combination as indicated. mRNA analysis of Runx-2 and osteocalcin genes were performed by qPCR. Data represent mean ± SEM from three independent experiments; *P < 0.05; **P < 0.01; ***P < 0.001.

### 7.1.5 Effect of Compound 11 on Fracture Healing

BMPs have been shown to enhance fracture healing in several animal models and clinical cases. 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 7.4 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 1 and 3.12 folds more at 1.0 and 5.0 mg/kg dose respectively.

![Figure 7.4](image_url)

**Figure 7.4.** Compound 11 promotes fracture healing in the drill-hole in rats. Representative confocal images of calcein labeling shown in the drill-hole of various groups 2 weeks after drill-hole injury. Lower panel showing quantification of the mean intensity of calcein label. Values are expressed as mean ± SEM (n =10 rats/group); **P < 0.01, ***2 P < 0.001.

Assessment of internal microstructure of the mineralized tissue in the bony hole was analyzed by 3-D μCT (microcomputed 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 7.5). These data indicated that compound 11 treatment promoted fracture healing by stimulating the formation of new bone at the site of drill hole.

Figure 7.5. Representative µCT images from the center of the bony hole (upper panel). µCT analysis showing 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 mean ± SEM (n =10 rats/group); *P < 0.05, **P < 0.01, ***2 P < 0.001.
7.1.6 Assessment of Off-Target Effect of Compound 11

The osteo-inductive potential of BMP-2 could lead not only to a stimulated bone formation in the skeleton but also extra-skeletal tissues such as heart and aorta. Therefore, we investigated whether compound 11 has off-target effect, i.e. induction of BMP-2, -4 and -7 mRNAs in cardiovascular tissues. Whilst compound 11 at 1.0- and 5.0 mg/kg doses enhanced fracture healing, however, the expression levels of BMP-2, -4 and -7 in the aorta (Figure 7.6A) and cuspid valves of the heart (Figure 7.6B) of these rats were comparable to controls.

![Graph A](image)

![Graph B](image)

**Figure 7.6.** Compound 11 has no effect on BMPs expression in cardio-vascular tissues. (A and B) Total RNA was extracted from aorta and cuspid valves of heart following 2 weeks of 11 treatment and qPCR were performed for the analysis of BMP-2, BMP-4 and BMP-7 mRNA levels. Data represented as mean ± SEM (n=10 rats/group).
7.1.7 Proteosome Inhibition Assay

Based on 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 26S proteasome activity assay kit (Chemicon International, USA). Compound 11 significantly inhibited proteosomal activity in a concentration dependent fashion from 0.1-10nM. However, at 100nM, compound 11 had no proteosome inhibitory action. Lactacystin (25µM), a standard proteosome inhibitor was used for comparison (Figure 7.7). Because, inhibition of proteosome activity by Bortezomib has been reported to induce osteoblast differentiation by augmenting BMP-2 production, compound 11 may serve to act similarly, and in the process accelerate BMP-2 mediated fracture healing in vivo.

![Figure 7.7.](image)

**Figure 7.7.** 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 – lactacystin (25µM). Data represent mean ± SEM from three independent experiments, **P < 0.01.**
7.1.8 Conclusion

There is no oral drug available for accelerating fracture repair. Human recombinant BMP-2 and BMP-7 have been approved by U.S. F.D.A. 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.\(^5\) BMP-2 signals via BMP-2 receptors and promotes osteoblast differentiation by an autocrine/paracrine mechanism.\(^6\)\(^-\)\(^7\) 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.\(^8\) Inspite of having BMP-2 stimulatory effect in osteoblasts compound 11 has no effect on the expression of BMPs in cardio-vascular tissues, indicating cardio-vascular 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.\(^9\) 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.\(^10\)\(^-\)\(^12\) This model seems suitable for analyzing the healing process quantitatively and useful for investigating osteoblast differentiation in vivo.\(^10\)\(^,\)\(^12\) Our data showed that treatment of rats with compound 11 significantly increased the fracture healing process by stimulating new bone formation at drill hole injury site compared to the vehicle treated group. This finding was complimented by µCT analysis showing greater newly generated bone in the drill-hole of compound 11 treated groups compared 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.\(^13\) Higher Conn.D, preferred plate-like 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 stimulate BMP-2 production in osteoblasts and promote 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.

7.2 EXPERIMENTAL SECTION

7.2.1 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 (1,000 IU/mL IP) was purchased from Gland Pharma (Hyderabad, India).

7.2.2 In Vitro Studies

7.2.2.1 Culture of Calvarial Osteoblasts:

Rat calvarial osteoblasts (RCOs) were obtained following previously published protocol of sequential digestion.14-16 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-25cm² flasks in α-MEM containing 10% FCS and 1% penicillin/streptomycin (complete growth medium).

7.2.2.2 Osteoblast Differentiation:

For the measurement of alkaline phosphatase (ALP) activity, RCOs at ~80% confluence were trypsinized and 2 ×10³ 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 mMβ-glycerophosphate, 50 μg/ml ascorbic acid and 1% penicillin/streptomycin (osteoblast
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differentiation medium). At the end of incubation period, total ALP activity was measured using p-nitrophenylphosphate (PNPP) as substrate and absorbance was read 405 nm.\(^\text{17}\)

### 7.2.2.3 BMP-2 ELISA:

For measuring BMP-2 production from osteoblasts, \(5 \times 10^3\) cells/well were seeded in 24-well plates. Cells were exposed to given concentration of compounds for 48 h in \(\alpha\)-MEM media supplemented with 10% charcoal-stripped FCS, 10mM \(\beta\)-glycerophosphate and 50 \(\mu\)g/ml ascorbic acid. At the end of incubation, supernatants were collected for determination of BMP-2 by ELISA as per the manufacturer’s instructions (Quantikine, R&D systems).

#### 7.2.2.4 Mineralization of Calvarial Osteoblasts:

Mineralization of RCOs was performed following previously published protocol.\(^\text{18}\) Briefly, RCOs were cultured until 80% confluence were trypsinized and plated in the differentiation medium (25,000 cells/well in 12-well plate), consisting of complete growth medium with ascorbic acid (50 \(\mu\)g/ml) and \(\beta\)-glycerophosphate (100 mM). The medium was changed every alternate day up to 21 d. 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.\(^\text{19}\)

For quantification of alizarin red-S staining, 800\(\mu\)l of 10% (v/v) acetic acid was added to each well, and plates were incubated at room temperature for 30 min with shaking. The monolayer, now loosely attached to the plate, was then scraped with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5-ml tube. After vortexing for 30s, the slurry was overlaid with 500\(\mu\)l mineral oil (Sigma–Aldrich), heated to exactly 85\(^\circ\)C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000\(\times\)g for 15 min and 500\(\mu\)l of the supernatant was removed to a new tube. Then 200\(\mu\)l of 10% (v/v) ammonium hydroxide was added to neutralize the acid. In some cases, the pH was measured at this point to ensure that it was between 4.1 and 4.5. OD (405 nm) of 150\(\mu\)l aliquots of the supernatant were measured in 96-well format using opaque-walled, transparent-bottomed plate.\(^\text{14, 18, 20}\)

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7.2.2.5 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.\textsuperscript{15,18,21} The house keeping gene GAPDH was used as the internal control in this study. Primers were designed using the Universal ProbeLibrary (Roche Applied sciences) for genes BMP-2 5′-CGG CTGCGGTCTCCTAA-3′(sense), 5′-GGGAAGCAGCAACACTAGA-3′ (antisense), Osteocalcin—5′-ATAGACTCCGCGCTACCTC-3′ (sense), 5′-CCAGGGGATCTGGGTAGG-3′(antisense), Runx-2—5′- CCACAGAGCTATTAAAGTGACAGTG-3′(sense), 5′-AACAAACTAGGTTTAGAGTCATCAAGC-3′ (antisense), GAPDH 5′-CAGCAAGGATAC TGAGAGCAAGA-3′ (sense), 5′-GGATGGAATTGTGAGGGAGATG-3′ (antisense). For real-time PCR, cDNA was synthesized with a Revert Aid cDNA synthesis kit (Fermentas, Austin, USA) using 2.0 μg of total RNA. SYBR green chemistry was used to perform quantitative determination of relative expression of transcripts for all genes. All genes were analyzed using the Light Cycler 480 (Roche Molecular Biochemicals, Indianapolis, Indiana, USA) real time PCR machine.

7.3 In Vivo Studies

7.3.1 Fracture Healing in Drill-Hole Injury at Femur:

Thirty adult female \textit{Sprague Dawley} rats (200±20g each) were taken for the study. Drill-hole injury was created in femur as described before.\textsuperscript{13} The front skin of the mid-femur in rats was incised straightly and longitudinally 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 3 equal groups (10 rats/group) as follows: vehicle (gum acacia in distilled water), 11 (1.0 mg.kg\textsuperscript{-1}.day\textsuperscript{-1}) and 11 (5.0 mg.kg\textsuperscript{-1}.day\textsuperscript{-1}). Each animal received intraperitoneal administration of fluorochrome calcine (20mg kg\textsuperscript{-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, femur for the measurement of bone micro architectural parameters in the drill-hole as described below. Bones were embedded in an acrylic material. Fifty µm sections were made using Isomet Bone cutter and photographs were taken under 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 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 3.02 software.

7.3.2 Proteasome activity assay

Isolated 26S proteasome activity was evaluated using Proteasome activity Assay Kit (Chemicon International, USA) according to manufactures instruction. Mouse calvarial cell line, MC3T3-E1 having comparable phenotypic response to its rat counterpart, was seeded in 6 well plates at a density of 2x10⁶ 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 secs after every 10 min of incubation, lysate was centrifuged for 15 min at 4⁰C at 14,000 x g and protein was estimated using Bradford assay. Aliquots of 10µg isolated proteasome was incubated with fluorogenic substrate in 1x assay buffer provided with the kit for 1 h in dark at 37⁰ C. Fluorescence intensity was measured by fluorimeter (BMG Fluorostar Omega) with a 380/460 nm filter set.
7.4 References


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