

RESEARCH ARTICLE

Mollugin enhances the osteogenic action of BMP-2 via the p38–Smad signaling pathway

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Abstract Bone morphogenetic protein 2 (BMP-2) has been used clinically to encourage bone regeneration; although, there can be major side effects with larger doses. Therefore, there is a need to identify new small molecules to potentiate the osteogenic action of BMP-2. In this study, we investigated the effect of mollugin on bone formation in murine bi-potential mesenchymal progenitor C2C12 cells by combination with BMP-2. We found mollugin could enhance the BMP-2-mediated osteoblast differentiation of C2C12 cells. This was accompanied by the induction of other osteogenic BMPs. We also found the enhancing potential of mollugin may involve activation of the p38–Smad1/5/8 signaling axis. Furthermore, mollugin promoted skeletal development in zebrafish. The combination of BMP-2 with small molecules, including mollugin, could minimize its clinical limitations, and these molecules might lead to the development of effective stem cell stimulants for bone regeneration and fracture healing.

Keywords Mollugin · BMP-2 · p38 · Smad · Osteoblast · Bone formation

Introduction

Bone morphogenetic proteins (BMPs) are most potent growth factors that promote osteogenesis (Cheng et al. 2003). Commercially available BMP-2 has been used as a clinical therapy for spinal injuries, bone grafts, and other orthopedic surgeries to encourage bone regeneration in patients (McKay et al. 2007; Benglis et al. 2008; Irad and Einhorn 2009). Recombinant human BMP-2 (1.5 mg/ml) has enhanced bone repair in patients with open tibia fractures in a human trial study (Alt et al. 2009). Globally, the percentage of the elderly that require various bone-related healthcare products continues to grow. The “Transparency Market Research” report expects the global BMP market to rise up to \$587 million by 2022. However, the clinical application of BMP-2 in high doses can cause life-threatening side effects, including cervical spine swelling or even tumors (James et al. 2016). Thus, there is a need to identify small molecules to enhance the osteogenic action of BMPs. The combination of BMP-2 and these small molecules could lower the doses of BMP-2, and thus, the risk of its side effects and the potential cost of BMP-2-based clinical treatment in the future (Cao et al. 2014; Fan et al. 2016).

Natural products are promising resources for identifying bioactive molecules. Since natural products provide novel molecular scaffolds and chemistry, many therapeutics including anti-cancer drugs are natural product-based/inspired (Mondal et al. 2012). In fact, in the field of bone biology, many bioactive natural products have been identified (Weaver et al. 2012), and several phytochemicals enhance the osteogenic action of BMP-2. More

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interestingly, phytochemicals, such as licochalcone A and tanshinone IIA, also exhibited the anti-osteoclastogenic activity (Kwak et al. 2006; Kim et al. 2008, 2012; Kim and Kim 2010).

Mollugin (naphthohydroquinone; Fig. 1a) is a non-mutagenic constituent isolated from the root of *Rubia tinctorum* in Europe, *Rubia akane* in Taiwan, or *Rubia cordifolia* in India (Kawasaki et al. 1992). It has exhibited several biological activities including neuroprotective (Jeong et al. 2011), anti-viral (Ho et al. 1996), anti-inflammatory (Kim et al. 2009; Zhu et al. 2013), anti-adipogenic (Jun et al. 2011), and anti-cancer activities (Do et al. 2013; Lee et al. 2013). Interestingly, anti-osteoclastogenic activity of mollugin was recently reported (Baek et al. 2015), but its action in bone formation has not yet been evaluated. In this study, the effect of mollugin on bone formation was investigated in murine bi-potential mesenchymal progenitor C2C12 cells by combination with BMP-2. The BMP-2-

induced commitment of C2C12 cells into osteoblasts is a well-defined in vitro bone formation model.

Materials and methods

Materials

Mollugin was synthesized as described previously (Lee et al. 2007). Recombinant human BMP-2 (rhBMP-2) and SB202190 (p38 inhibitor) were purchased from R&D Systems (Minneapolis, MN, USA) and Calbiochem (San Diego, CA, USA), respectively. All cell culture materials were purchased from HyClone (Logan, UT, USA). Antibodies against phospho(p)-p38, p38, p-c-Jun N-terminal kinase (p-JNK), JNK, p-extracellular-signal-regulated kinase (p-ERK), ERK, p-Sma and Mad related proteins 1/5/8 (p-Smad1/5/8), and Smad were purchased from Cell

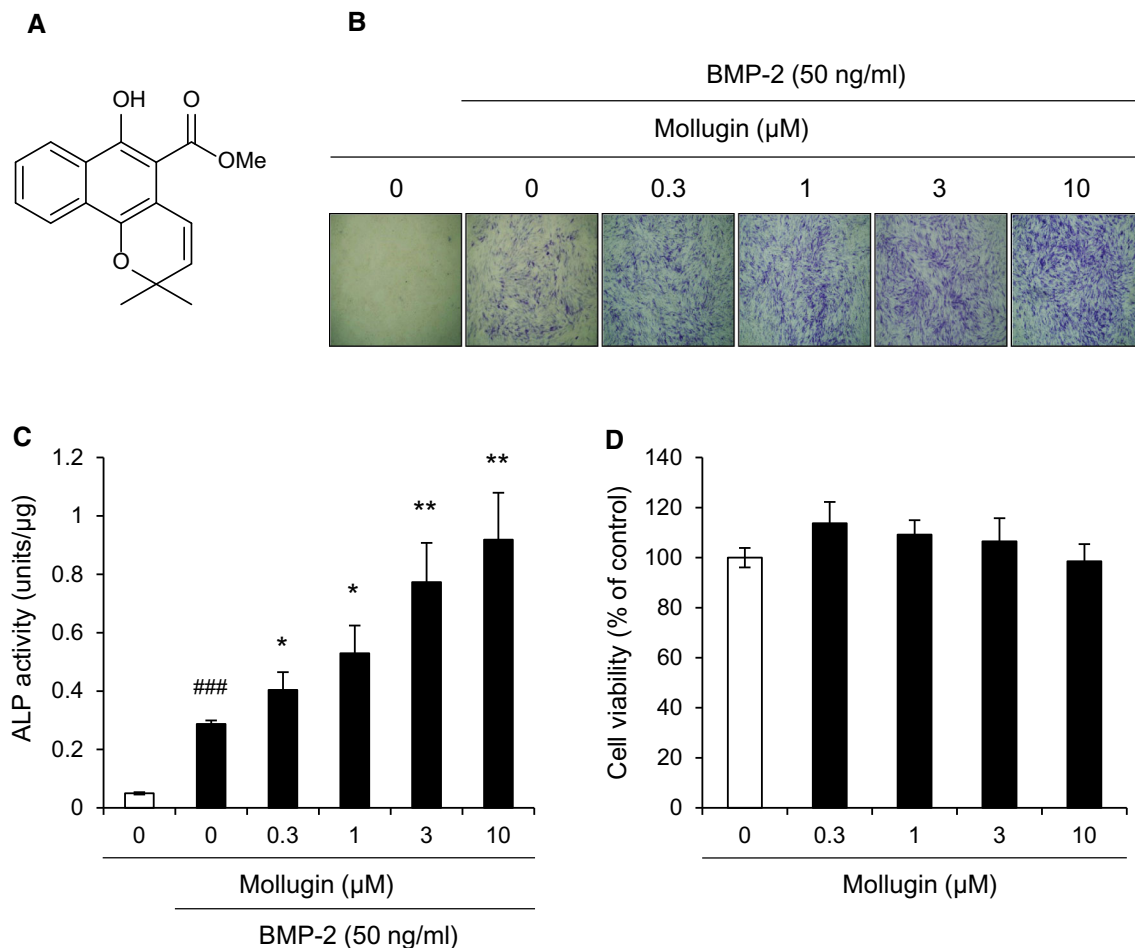


Fig. 1 Mollugin enhances BMP-2-induced ALP. **a** Chemical structure of mollugin. The effect of mollugin on BMP-2-induced osteoblast differentiation was evaluated by visualizing ALP staining **b** and measuring ALP activity **c** in C2C12 cells. **d** The effect of mollugin on the viability of C2C12 cells was evaluated using the CCK-8 assay. DMSO (0.1%) was used as the vehicle control. ### $p < 0.01$ (vs the vehicle control group); * $p < 0.05$; ** $p < 0.01$ (vs the BMP-2-treated group)

Signaling Technology Inc. (Danvers, MA, USA). The antibody against actin and the secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell culture and differentiation

The C2C12 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were maintained in growth medium (GM; Dulbecco's Modified Eagle's Medium [DMEM] containing 10% fetal bovine serum [FBS], 100 U/ml of penicillin, and 100 µg/ml streptomycin). Cells were seeded and, after 1 day, differentiated by replacing the medium with differentiation medium (DM; DMEM containing 5% FBS and 50 ng/ml rhBMP-2).

Alkaline phosphatase (ALP) staining and activity assay

Alkaline phosphatase (ALP), an early biomarker of osteoblastogenesis, was assayed as described previously (Kim and Kim 2010). Briefly, C2C12 cells (4×10^3 cells/well) were seeded in a 96-well plate, and after 24 h, the medium was replaced with DM in the absence or presence of mollugin. After 3 days, cells were washed twice with phosphate-buffered saline (PBS), fixed with 10% formalin in PBS for 30 s, rinsed with deionized water, and stained using the Alkaline Phosphatase Kit (Sigma, St. Louis, MO, USA). To measure ALP activity, cells were washed twice with PBS, and 100 µl of the 1-Step PNPP (*p*-nitrophenyl phosphate) substrate solution (Thermo Fisher Scientific, Waltham, MA, USA) was added to each well. Then, cells were incubated for 15 min at room temperature and the reaction was stopped by adding 50 µl of 2 N NaOH. Then, absorbance was measured at 405 nm using a Wallac EnVision microplate reader (PerkinElmer, Waltham, MA, USA).

Cell viability assay

Cells were seeded in a 96-well plate at 4×10^3 cells/well. After 24 h, cells were incubated with mollugin for 3 days, and then cell viability was evaluated in triplicate using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA) according to the manufacturer's protocol. Absorbance was measured at 450 nm using a Wallac EnVision microplate reader.

Evaluation of mRNA expression

The mRNA expression level was evaluated as described previously (Kim and Kim 2010). Briefly, primers used in

this study were designed using an online primer design program (Rozen and Skaletsky 2000; Table 1). Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) and the first strand cDNA was synthesized using 1 µg of total RNA, 1 µM of oligo-dT₁₈ primer, 10 units of the RNase inhibitor, RNasin (Promega, Madison, WI, USA), and Omniscript Reverse Transcriptase (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Then, a SYBR green-based quantitative polymerase chain reaction (PCR) was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) in a 20 µl reaction mixture containing 10 µl of TOPreal qPCR 2× PreMIX (Enzynomics, Daejeon, Korea), 2 pmol of forward primer, 2 pmol of reverse primer, and 2 µl of 1:10-diluted first strand cDNA. Amplification parameters consisted of an initial denaturation at 95 °C for 10 min and 40 cycles of three-step PCR (denaturation at 94 °C for 40 s, annealing at 53 °C for 40 s, and extension at 72 °C for 1 min). All reactions were run in triplicate and data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the control gene. Significance was determined with GAPDH-normalized $2^{-\Delta\Delta CT}$ values.

Western blot analysis

Cells were lysed with cold lysis buffer (0.2 M Tris-HCl, pH 7.5, 150 mM NaCl, 1% deoxycholic acid, 1% NP-40, and 1 mM EDTA) containing 1× protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL, USA). Cell lysates were subsequently incubated at 4 °C for 20 min and then centrifuged at $14,000 \times g$ for 15 min. The protein concentration of the supernatants was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (20 µg) in sample buffer (100 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6) were denatured at 95 °C for 15 min and loaded on 10% polyacrylamide gels. Electrophoresis was performed with the Mini Protean 3 Cell (Bio-Rad) and the resolved proteins were transferred by electroblotting to polyvinylidene difluoride (PVDF) membrane (Millipore, Temecula, CA, USA). Membranes were incubated with TBST buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat dry milk and incubated with diluted primary antibodies (1:1000) overnight at 4 °C. The membranes were washed with TBST at room temperature and incubated with diluted secondary antibodies (1:5000) for 1 h. After washing with TBST three times (15 min each), the membranes were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and evaluated

Table 1 Primer sequences used in this study

Target gene	Forward primer (5'–3')	Reverse primer (5'–3')
<i>ALP</i>	ATGGGCGTCTCCACAGTAAC	TCACCCGAGTGGTAGTCACA
<i>BMP-2</i>	GGGACCCGCTGTCTTAGT	TCAACTCAAATTCGCTGAGGA
<i>BMP-4</i>	CCTGGTAACCGAATGCTGAT	AGCCGGTAAAGATCCCTCAT
<i>BMP-6</i>	CAACGCCCTGTCCAATGAC	ACTCTTGCGGTTCAAGGAGTG
<i>BMP-7</i>	CGATACCACCATCGGGAGTTC	AAGGTCTCGTTGTCAAATCGC
<i>BMP-9</i>	CAGGTGAGAGCCAAGAGGAG	CCTTGTGGGGAACCTTGAGA
<i>GAPDH</i>	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

with the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Zebrafish skeleton development

As described previously (Moon et al. 2015), the skeleton development of zebrafish was visualized by alizarin red S according to the standard protocol approved by the Institutional Animal Care and Use Committees of Chungnam National University (CNU-00393). Briefly, zebrafish embryos were added to a 24-well plate (5/well) 5 days after fertilization and maintained in 1 ml of buffered medium (sea salt, 0.06 mg/l) with or without mollugin. The medium was changed every day. At 8 days after fertilization, embryos were fixed in 4% paraformaldehyde for 1 h, washed 3 times with PBS containing 0.1% Tween 20 (PBST) at 10 min intervals, and treated with a bleaching solution (PBST with 1% KOH and 3% H₂O₂) at room temperature until pigmented cells were removed. After the pigmented cell removal was complete, embryos were washed 3 times with PBST at 10 min intervals and treated with 1 ml of alizarin red S staining buffer (pH 4.2). After destaining with 1% KOH, embryos were treated successively with KOH solutions that contained 20% glycerol, 50% glycerol, and 80% glycerol. Images were acquired using a Leica microscope (Wetzlar, Germany).

Statistical analysis

All quantitative values are presented as the mean \pm SD. Each experiment was performed three times, and results from one representative experiment are presented here. Statistical differences were analyzed using Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Mollugin enhances BMP-2-induced ALP

Biological activity of mollugin in the process of bone formation was evaluated in C2C12 cells by ALP staining

and its activity measurement. Since mollugin per se did not show any osteogenic action without BMP-2 (data not shown), its enhancing effect on the BMP-2-induced differentiation of C2C12 cells was evaluated. As shown in Fig. 1b and c, 50 ng/ml of BMP-2 significantly induced the activity of ALP, the representative marker for osteoblast differentiation, and its induction was enhanced by mollugin in a dose-dependent manner. Up to 10 μ M, mollugin did not exhibit any cytotoxicity under the growth culture conditions (Fig. 1d). In addition, mollugin significantly enhanced the BMP-2-induced mRNA expression of ALP at 3–10 μ M (Fig. 2).

Mollugin enhances BMP-2-induced mRNA expression of osteogenic BMPs

The effect of mollugin on the mRNA expression levels of BMPs was investigated by real-time PCR analysis. As shown in Fig. 2, BMP-2 significantly induced the mRNA expressions of BMP-2, 4, 6, 7, and 9 in C2C12 cells, and those inductions were dramatically enhanced by mollugin; especially, at 10 μ M.

Mollugin induces the phosphorylation of p38 and Smad1/5/8

To elucidate the cellular mechanism of osteo-enhancing mollugin, Western blot analysis was performed to evaluate its effect on the activation of mitogen-activated protein (MAP) kinases and Smad. As shown in Fig. 3a, mollugin induced the phosphorylation of p38 in a dose-dependent manner, but not JNK and ERK, in C2C12 cells in the presence of BMP-2. The phosphorylation of Smad1/5/8 was also induced by mollugin (Fig. 3b). As the biological activity of mollugin has been linked to the regulation of JAK signaling as well as MAP kinases (Zhu et al. 2013), the effect of mollugin on the activity of JAK2 was also evaluated by a kinase activity assay. We found mollugin did not regulate the activity of JAK2 (data not shown).

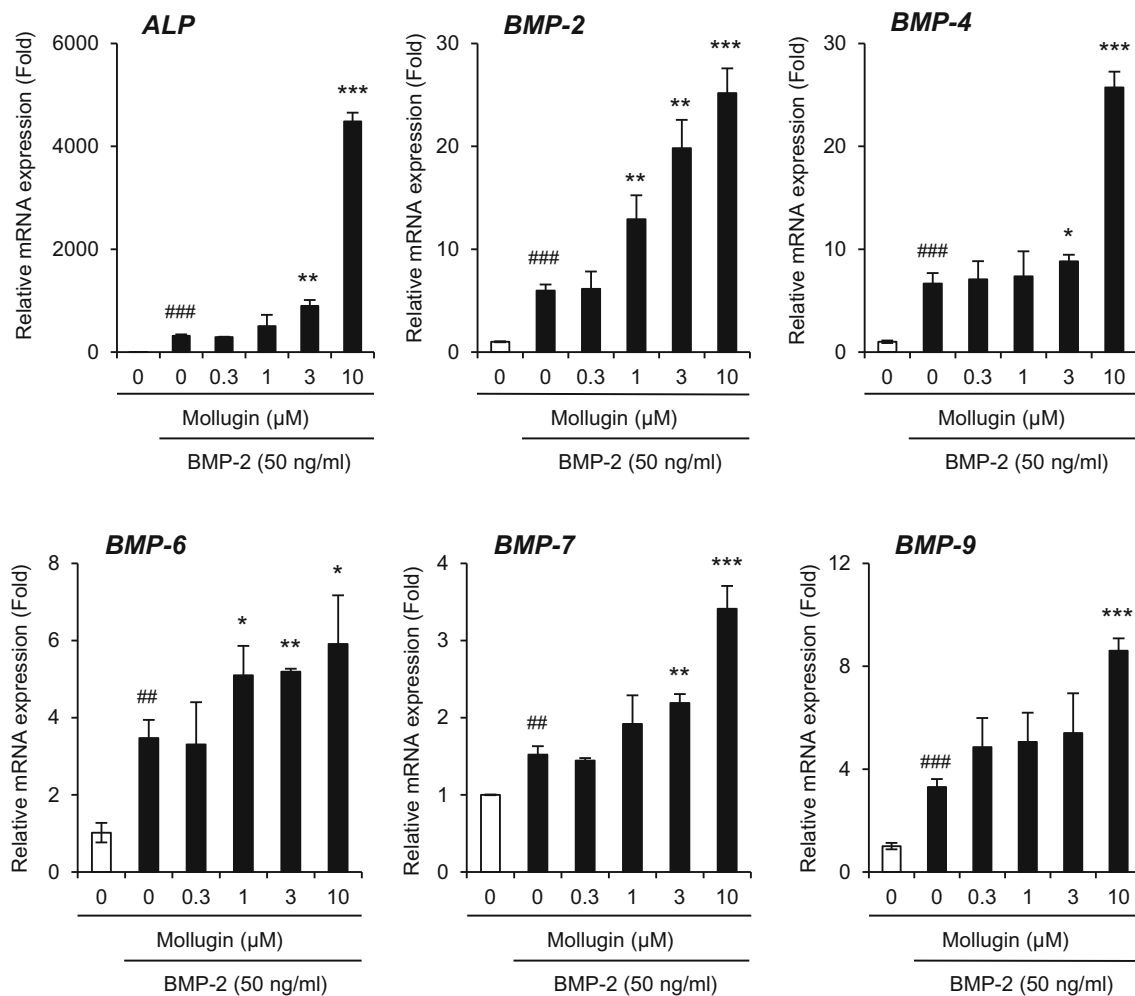


Fig. 2 Mollugin enhances BMP-2-induced mRNA expression of ALP and osteogenic BMPs in C2C12 cells. Cells were treated with the vehicle (0.1% DMSO), BMP-2 (50 ng/ml), or BMP-2 and mollugin for 3 days. Then, mRNA expression levels were evaluated by real-time PCR. Relative GAPDH-normalized expression levels of genes to the control are presented. ## $p < 0.01$; ### $p < 0.001$ (vs the vehicle control group); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (vs the BMP-2-treated group)

SB202190 inhibits mollugin-enhanced phosphorylation of p38 and Smad1/5/8, and induction of ALP activity

The involvement of the p38–Smad signaling pathway was validated by a pharmacological inhibition study. Consistent with the results shown in Fig. 3, mollugin induced the phosphorylation of both p38 and Smad1/5/8 in C2C12 cells in the presence of BMP-2 (Fig. 4a), but those inductions were prevented, in a dose-dependent manner, by pretreatment with SB202190, a p38 specific inhibitor (Fig. 4a). In C2C12 cells, mollugin-induced ALP activity in the presence of BMP-2 was also significantly prevented by SB202190 in a dose-dependent manner (Fig. 4b).

Mollugin enhances the skeletal development of zebrafish

Furthermore, the in vivo effect of mollugin on the skeletal development of zebrafish was investigated, similar to previous studies on several osteo-enhancing compounds (Kim et al. 2012; Baek et al. 2015; Moon et al. 2015). As shown in Fig. 5, mollugin enhanced the development of vertebrae in zebrafish, with the number of alizarin red S-stained vertebrae higher in mollugin-treated zebrafish than the control.

Discussion

In this study, we found that mollugin enhanced the BMP-2-mediated commitment of C2C12 cells into osteoblasts and enhanced other BMPs at the transcript level. Like BMP-2,

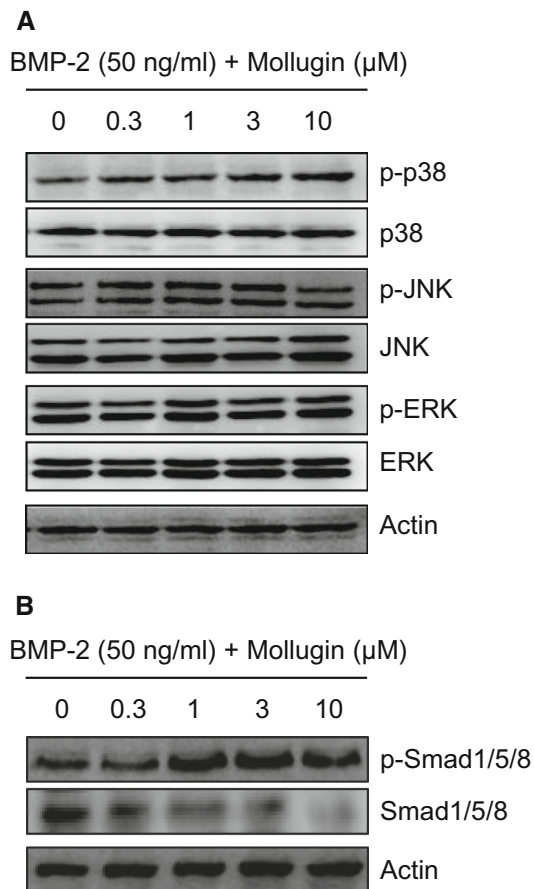


Fig. 3 Mollugin induces the phosphorylation of p38 and Smad1/5/8. The effect of mollugin on the activations of MAP kinases and Smad1/5/8 were evaluated by Western blot analysis. Briefly, C2C12 cells (2×10^5 cells/well) were seeded in a 6-well plate and cultured in GM. After reaching confluence, cells were incubated with DM in the absence or presence of mollugin for 10 min (MAP kinases; **a**) or 3 days (Smad1/5/8; **b**). Western blot analysis was then performed as described in the “Materials and methods”

the other BMPs investigated in this study also exhibited *in vitro* and *in vivo* osteogenic activities (Gitelman et al. 1995; Yamaguchi et al. 1996; Yeh et al. 2002; Li et al. 2003). In addition, the ability of BMP-2 to enhance the gene expression of osteogenic BMPs may play a critical role in bone formation. BMP-2 enhanced the gene expression of other BMPs during osteoblast differentiation (Chen et al. 1997), and the rapid bone formation by the gene transfer of BMP-2 and BMP-7 was also accompanied by increased expression of endogenous BMP-4 (Kawai et al. 2006). These results suggested that the mollugin-enhanced expression of other osteogenic BMPs in the presence of BMP-2 might act synergistically to promote the commitment of C2C12 cells into osteoblasts.

MAP kinases and Smad1/5/8 are essential signaling molecules for BMP-2-induced osteoblast differentiation (Gallea et al. 2001; Lee et al. 2002; Nöth et al. 2003). Here,

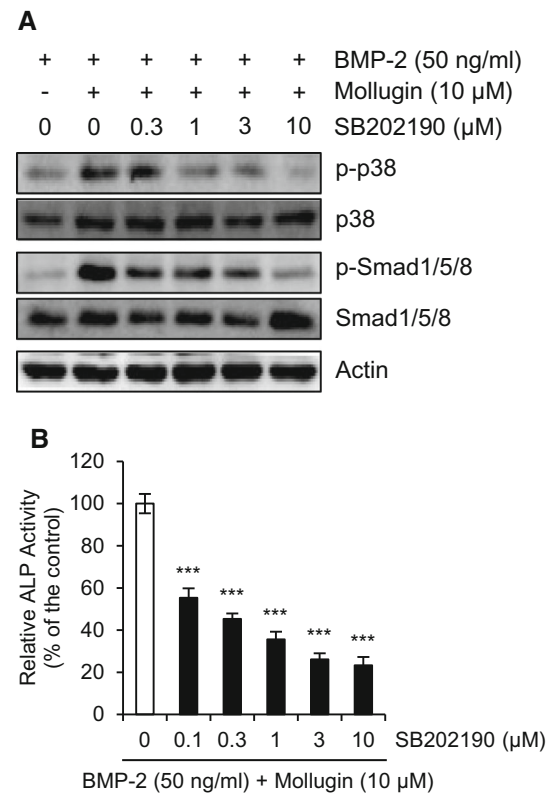


Fig. 4 SB202190 inhibits mollugin-enhanced phosphorylation of p38 and Smad1/5/8, and induction of ALP activity. The inhibitory effects of SB202190 on mollugin-enhanced phosphorylation of p38 and Smad1/5/8 (**a**), and induction of ALP activity (**b**) were evaluated in C2C12 cells. Briefly, cells were pre-incubated with SB202190 for 2 h and then cultured with DM in the absence or presence of mollugin for 2 days (for Western blot analysis, **a**) or 3 days (for ALP activity assay, **b**). *** $p < 0.001$ (vs the BMP-2 and mollugin-treated group)

mollugin enhanced the BMP-2-mediated commitment of C2C12 cells into osteoblasts by accelerating the activation of p38 and Smad1/5/8. In addition, a pharmacological inhibition study confirmed the involvement of the p38–Smad1/5/8 signaling axis in mollugin’s enhancement of the osteogenic activity of BMP-2. The p38 inhibitor, SB202190, hindered the mollugin-enhanced phosphorylation of p38 and Smad1/5/8, and induction of ALP activity in C2C12 cells. The activation of p38 controls the expression of ALP during the osteoblastic differentiation of osteoblast-like cells or stromal cells (Suzuki et al. 2002; Nishikawa et al. 2014). BMP-2 can trigger the osteogenic signaling through the action of Smad, and the phosphorylation of Smad then induces the expression of genes related to osteoblast differentiation (Nishimura et al. 1998; Yamachika et al. 2009).

The *in vitro* osteo-enhancing activity of mollugin further led us to investigate its *in vivo* effect on the skeletal development of zebrafish. The mammalian bone forming pathway has been suggested to be conserved during the

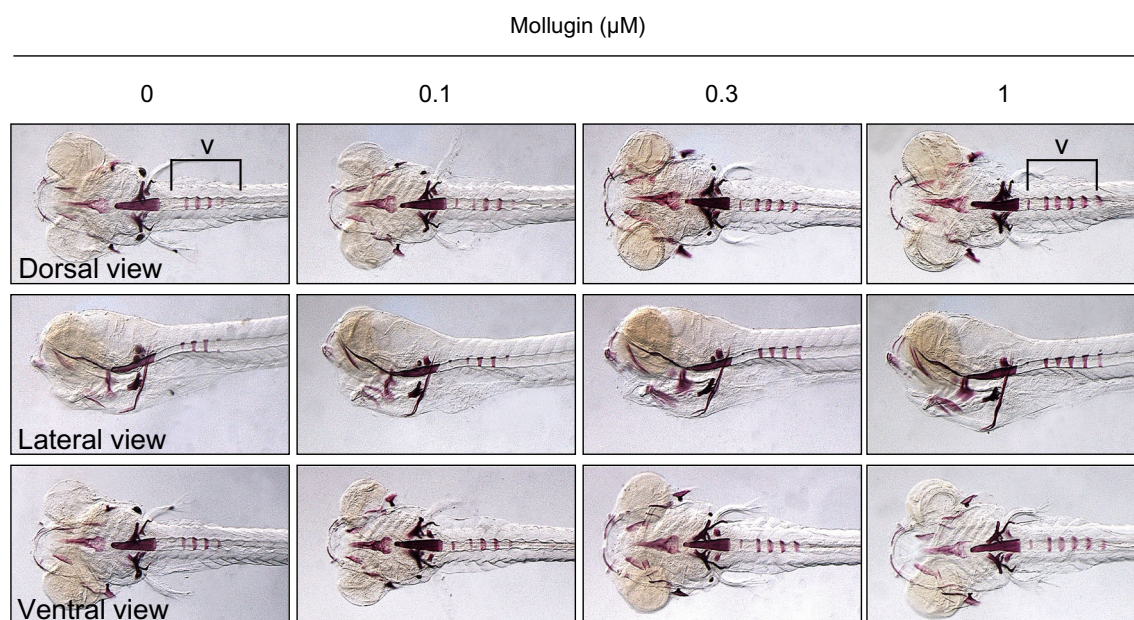


Fig. 5 Mollugin enhances the skeletal development of zebrafish. In vivo osteogenic activity of mollugin was investigated in zebrafish as described in the “Materials and methods”. Briefly, zebrafish (5 days after fertilization) were treated with mollugin for 3 days, fixed, and stained with alizarin red S. The appearance of vertebrae (v) is indicated

skeletal development of zebrafish (Li et al. 2009), and zebrafish are successful models for identifying bone-forming agents (Du et al. 2001; Fleming et al. 2005). When zebrafish embryos were treated with mollugin, it promoted the rate of vertebral development.

In summary, mollugin has the potential to enhance the osteogenic activity of BMP-2 in C2C12 cells by accelerating the activation of the p38–Smad1/5/8 signaling axis. In addition, mollugin promoted skeletal development in zebrafish. When considering the anti-osteoclastic activity of mollugin (Baek et al. 2015), its dual action in inhibiting bone resorption and stimulating bone formation might be beneficial in treating bone-related disorders. Moreover, the combination of BMP-2 with osteogenic small molecules could minimize the limitations associated with BMP-2. Furthermore, since chemical biologists in stem cell research are trying to identify bioactive small molecules to direct stem cells to a lineage-specific patterns (differentiation), mollugin might lead to the development of effective stem cell stimulants for tissue repair, especially bone regeneration and fracture healing (Hong et al. 2011; Choi and Nam 2012).

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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