Effects of a Novel EP_4 Agonist on Bone Metabolism *in vitro*

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Abstract

Prostaglandin E_2 (PGE_2) is known to be a crucial regulator of bone remodeling, helping to maintain the balance between bone formation and resorption. These biologic effects of PGE_2 are primarily mediated through its interaction with the G protein-coupled receptor EP_4, a potential target for bone anabolic therapies. We have developed a potent, novel small molecule EP_4 receptor agonist (KMN-159) with high EP_4 selectivity and excellent chemical stability. The present study is aimed at investigating the impact of KMN-159 on bone metabolism *in vitro*. We utilized human bone marrow-derived mesenchymal stem cells (hBM-MSCs) to determine the influence of KMN-159 on osteogenic and adipogenic differentiation and utilized RAW 264.7 cells to evaluate the effect of KMN-159 on osteoclastogenesis. For the osteogenesis studies, hBM-MSCs were treated with 100 nM KMN-159 in the presence of osteogenic medium (10 mM β-glycerophosphate, 10 nM dexamethasone, and 100 µM L-ascorbic acid 2-phosphate). Alkaline phosphatase activity and mineralization of hBM-MSCs were significantly induced by KMN-159, demonstrating the stimulation of osteoblastic differentiation by KMN-159. Consistently, KMN-159 upregulated the mRNA levels of osteoblast marker genes including Runx2, alkaline phosphatase, type I collagen, and osterix. Furthermore, the mRNA expression level of osteoprotegerin (OPG) was decreased, whereas receptor activator of NF-κB ligand (RANKL) was increased in hBM-MSCs (21 days) by KMN-159, indicating that KMN-159 enhances osteoclast formation. Our investigation of the effects of KMN-159 on osteoclastogenesis in RAW 264.7 cells showed that 100 nM KMN-159 significantly increases osteoclast formation in the presence of RANKL. Moreover, Oil Red O staining showed that KMN-159 moderately inhibited adipocyte differentiation of hBM-MSCs in the presence of osteogenic medium but not in adipogenic medium (AM). Overall, this study demonstrated that KMN-159, our novel EP_4 receptor agonist, is able to enhance the osteoblastic differentiation of human MSCs *in vitro*, strongly suggesting that it may increase bone formation *in vivo* and therefore be a potential candidate for augmentation of bone mass and enhancing fracture repair.

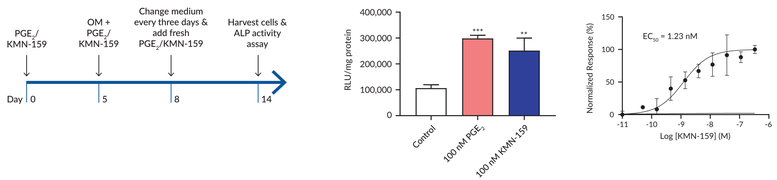
Introduction

PGE_2 is a potent endogenous molecule that is synthesized by inducible and constitutively expressed PGE synthase. PGE_2 exerts various biological effects by binding to prostaglandin receptors EP_1 – EP_4. Of the four EP receptors, PGE_2 is thought to mediate its bone anabolic effects through its binding to the EP_4 receptor. EP_4 has been found to be involved in bone formation by osteoblasts and resorption of existing bone by osteoclasts. When the mesenchymal stem cells undergo differentiation into osteoblasts, there is an upregulation of osteoblast marker genes, especially alkaline phosphatase. Increased alkaline phosphatase enzyme activity and mineralization is observed in actively growing bone. Bone resorption is another important component to bone remodeling and repair after a fracture. Osteoblasts secrete several factors, especially RANKL, to stimulate differentiation of macrophages into multinucleated osteoclasts. Increased expression of markers including tartrate-resistant acid phosphatase (TRAP) and cathepsin K is observed when cells undergo osteoclastogenesis. We have synthesized and developed a series of novel difluorolactam small molecule EP_4 receptor agonists.[1] KMN-159, the lead compound from that series, is shown in the work presented here to stimulate the osteogenic differentiation of hBM-MSCs. Cells were stimulated with KMN-159 in the presence of osteogenic media and then analyzed for alkaline phosphatase activity, mRNA levels of osteoblast phenotype markers, and mineralization. We investigated the effect of KMN-159 on osteoclastic differentiation of RAW 264.7 cells by TRAP staining and quantifying the expression of osteoclast marker genes. Additionally, the effect of KMN-159 on adipogenesis in hBM-MSCs was also studied by Oil Red O staining.

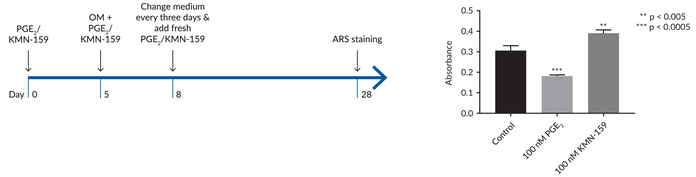
Methods & Results

KMN-159 increases alkaline phosphatase (ALP) activity and mineralization of hBM-MSCs in the presence of osteogenic media

To determine the effect of KMN-159 on osteogenesis, hBM-MSCs were plated in mesenchymal stem cell basal media with growth supplements (ATCC) in the presence of either 100 nM KMN-159 or 100 nM PGE_2 (or vehicle). Beginning on day 5, cells were fed with the compounds in basal growth medium plus 10 mM β-glycerophosphate (βGP), 10 nM dexamethasone, and 100 µM L-ascorbic acid 2-phosphate (osteogenic medium, OM). The cells were harvested on day 14 and ALP activity was assessed. KMN-159 significantly increased ALP activity in hBM-MSCs with efficacy similar to PGE_2. The EC_{50} value for KMN-159 was determined to be 1.23 nM.



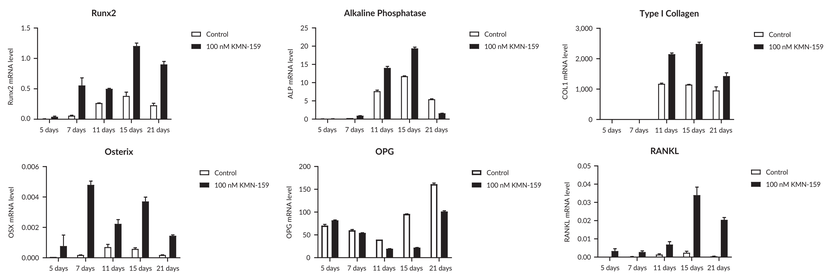
To determine the effect of KMN-159 on mineralization, hBM-MSCs were plated and fed with 100 nM KMN-159 or 100 nM PGE_2 and stained with Alizarin Red S (ARS) on day 28. PGE_2 inhibited mineralization, whereas KMN-159 was found to significantly increase mineralization in hBM-MSCs.



KMN-159 upregulates the mRNA levels of osteogenic marker genes in hBM-MSCs

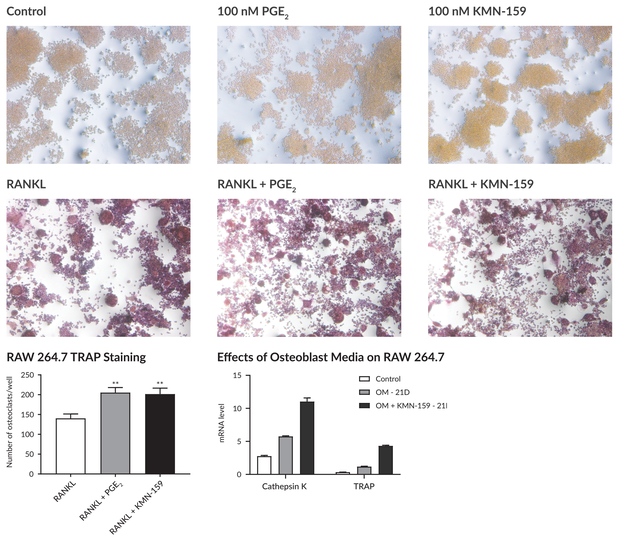
hBM-MSCs were plated and treated with 100 nM KMN-159 as above and, beginning on day five, fed every three days with 100 nM KMN-159 in osteogenic medium. Cells were harvested on days 5, 7, 11, 15, and 21 and mRNA levels of osteogenic marker genes were determined by RT-qPCR using β-actin as a reference gene.

All early-stage osteoblastic markers including runt-related transcription factor 2 (Runx2), alkaline phosphatase, type I collagen, and osterix were found to be upregulated by KMN-159 compared to vehicle control. Runx2 and osterix mRNA were detectable as early as day 5 and alkaline phosphatase and type I collagen mRNA were detectable by day 7 or 11, respectively. Furthermore, osteoprotegerin (OPG), an osteoclastogenesis inhibitory factor, was down-regulated by KMN-159, whereas receptor activator of NF-κB ligand RANKL was significantly upregulated, suggesting that KMN-159 stimulates hBM-MSCs to upregulate genes necessary for crosstalk in promoting osteoclast differentiation.



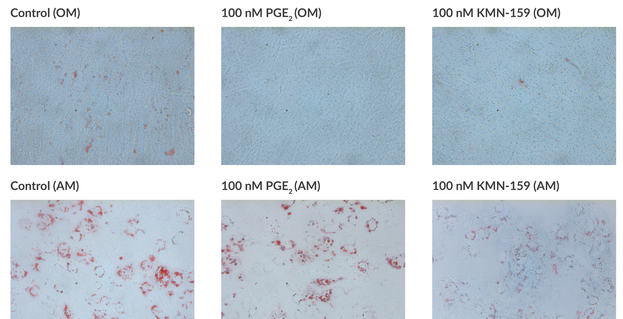
Effect of KMN-159 on osteoclastogenesis

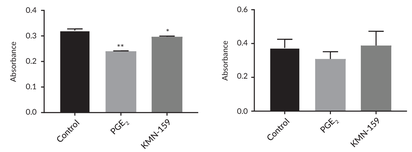
RAW 264.7 cells were treated with 100 nM PGE_2 or 100 nM KMN-159 together with 50 ng/ml RANKL for five days to induce the osteoclastic differentiation of the cells. After five days, the cells were stained for TRAP activity using the acid phosphatase, leukocyte kit (Sigma-Aldrich) to detect osteoclasts. As expected, osteoclastic differentiation of RAW 264.7 cells was not stimulated by PGE_2 or KMN-159 in the absence of RANKL. In the presence of RANKL, the cells underwent osteoclastogenesis and both PGE_2 and KMN-159 treatment showed an increased number of TRAP-positive cells as compared to RANKL alone. Moreover, when RAW 264.7 cells were cultured in media collected at 21 days from hBM-MSC treated with KMN-159 in the presence of RANKL, there was a significant upregulation of mRNA levels of cathepsin K and TRAP, suggesting that KMN-159 enhances osteoclast formation in RAW 264.7 cells.



KMN-159 moderately inhibits adipocyte differentiation of hBM-MSCs in the presence of osteogenic media but not in adipogenic media

To study the effect of KMN-159 on adipogenesis, hBM-MSCs were treated with 100 nM PGE_2 or 100 nM KMN-159 for five days. The cells were then fed every three days with either OM for 28 days or AM for 18 days in the presence of 100 nM PGE_2, KMN-159, or vehicle (control). Fresh compound was added each time the media was changed. The cells were then fixed for 15 minutes at room temperature and stained with Oil Red O. The Oil Red O stain was eluted from the fixed cells by a ten-minute elution in 100% isopropanol and quantified by absorbance at 500 nm. KMN-159 (100 nM) slightly inhibited adipocyte differentiation of hBM-MSCs in osteogenic media but had no effect on adipogenesis when cells were incubated in AM.





Conclusions

- KMN-159 significantly enhances the osteoblastic differentiation of hBM-MSCs under osteogenic culture conditions as measured by induction of alkaline phosphatase activity.
- In contrast to PGE_2, KMN-159 stimulates mineralization in hBM-MSCs after 28 days in osteogenic culture medium.
- Osteoblast phenotype marker genes including Runx2, alkaline phosphatase, type I collagen, and osterix are expressed at higher levels in hBM-MSCs treated with KMN-159 as compared to the vehicle-treated controls.
- KMN-159 significantly enhances osteoclast formation in RAW 264.7 cells under osteoclastogenic culture conditions as measured by the increase in TRAP-positive multinucleated cells.
- KMN-159 increases RANKL and decreases OPG gene expression in hBM-MSCs, demonstrating that it stimulates hBM-MSCs to secrete the factors necessary for regulating osteoclastogenesis.
- The mRNA expression levels of both cathepsin K and TRAP, well-known osteoclast phenotype markers, increase in RAW 264.7 cells following exposure of the cells to conditioned OM collected from hBM-MSCs treated with KMN-159 for 21 days.
- KMN-159 has no effect on adipogenesis of hBM-MSCs in the adipogenic media.

Overall, this study demonstrated that KMN-159, our novel EP_4 receptor agonist, is able to enhance the osteoblastic differentiation of human hBM-MSCs *in vitro*, strongly suggesting that it may increase bone formation *in vivo* and therefore be a potential candidate for augmentation of bone mass.

Future Directions

- Compare the effects of a single exposure of hBM-MSCs to KMN-159 to those following the chronic treatment with the compound reported here.
- Treat whole human bone marrow with KMN-159 to determine if cells in addition to the MSCs are involved in the osteogenic response to EP_4 agonists.
- Determine the mechanism of action of KMN-159 by studying the signaling pathways downstream of EP_4.

Reference

1. Barrett, S.D., Holt, M.C., Kramer, J.B., *et al.* Difluoromethylene at the γ-lactam α-position improves 11-deoxy-8-aza-PGE_1 series EP_4 receptor binding and activity: 11-deoxy-10,10-difluoro-8-aza-PGE_1 analog (KMN-159) as a potent EP_4 agonist. *J. Med. Chem.* **62(9)**, 4731-4741 (2019).