Biochemical and Molecular Characterization of the Osteoblastic Differentiation of Rat Bone Marrow Stem Cells Treated with KMN-159, a Novel Selective EP_4 Prostaglandin Receptor Agonist

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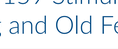
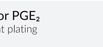
Introduction

In many animals, including humans, PGE_2 causes bone formation with its anabolic actions primarily due to its binding to the GPCR PTGER4 (EP_4).[1] However, systemic PGE_2 administration causes undesired side effects such as pain, lethargy, and diarrhea due to its binding to the EP_1 and EP_3 receptors.[2] The current bone anabolic therapeutic agents are primarily protein based, requiring appropriate storage conditions and having relatively short shelf lives. They are also expensive, and the morphogenic activity associated with many of them raises safety concerns and limits their potential use in orthopedic and other local applications. To alleviate these issues in a potential therapeutic, we developed a series of novel difluorolactam EP_4 receptor agonists.[3] The work presented here is from the ongoing characterization of our lead compound, KMN-159, which shows high selectivity for EP_4 binding, excellent potency for EP_4 activation, potent stimulation of osteoblastic differentiation in unfractionated bone marrow cells (BMCs), and remarkable chemical stability.

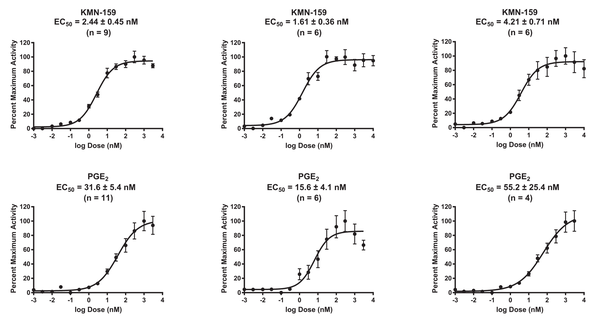
KMN-159 is a Highly Selective and Potent Activator of the Rat EP_4 Receptor

While the natural ligand PGE_2 is a potent activator of both the rat EP_2 and EP_4 receptors, KMN-159, the lead compound in our novel difluorolactam series, demonstrates potency for binding, stimulation of cAMP production, and activation of a CRE-SEAP reporter that is equivalent to PGE_2 but does so in a clearly EP_4-selective manner.

Compound	Structure	rat EP_2 receptor binding K_i (nM)	rat EP_4 receptor cAMP formation EC_{50} (nM)	rat EP_4 receptor CRE activation EC_{50} (nM)	rat EP_4 receptor cAMP formation EC_{50} (nM)	rat EP_4 receptor CRE activation EC_{50} (nM)
PGE_2		0.19	88.4 ± 4.2 (n = 5)	2.8 ± 0.7 (n = 9)	0.48 ± 0.09 (n = 5)	0.03 ± 0.002 (n = 56)
KMN-159		0.24	>10,000 (n = 3)	>10,000 (n = 3)	0.50 ± 0.08 (n = 4)	0.03 ± 0.003 (n = 7)

Binding cAMP measurement and CRE activation assays were all performed as in Barrett *et al., J. Med. Chem.*, 2019.[3]

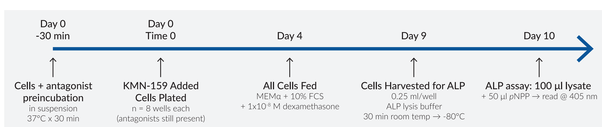
KMN-159 Stimulates Osteoblastic Differentiation in Young and Old Female and Young Male Rat BMCs

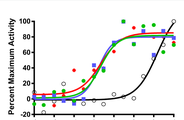


Young Female (<6 months) | Old Female (>12 months) | Young Male (<6 months)

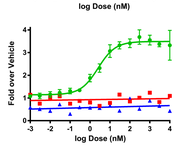
Stimulation of Osteoblastic Differentiation in Rat Bone Marrow Cells Occurs *via* EP_4 Activation

These data demonstrate that KMN-159 stimulates osteogenesis *via* activation of the EP_4 receptor; specific agonists for the related EP_2 and EP_3 receptors do not stimulate osteogenesis and only an EP_4-selective antagonist blocks the osteogenesis induced by KMN-159.



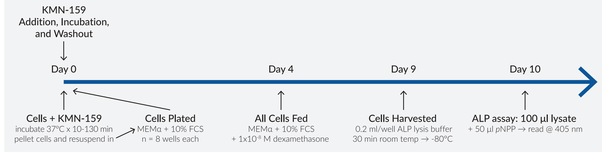


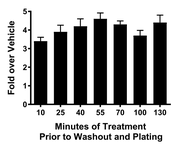
Cells were stimulated with the indicated doses of KMN-159 following a 30-minute pretreatment with either vehicle (●); ONO-8711, an EP_1 selective antagonist (●); PF-04418948, an EP_2 selective antagonist (■); or ONO-AE3-208, an EP_3 selective antagonist (O). Cells were cultured as above and alkaline phosphatase activity was measured on day 9 after plating.



In order to further demonstrate that the induction of osteoblastic differentiation in rat bone marrow cells occurs *via* the EP_4 receptor, we first stimulated cells isolated from young female rats with the indicated doses of either Butaprost, an EP_2 selective agonist (■); Sulprostone, an EP_3 selective agonist (▲); or our EP_4 selective agonist, KMN-159 (●).

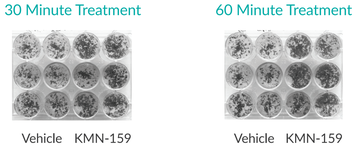
Commitment to Osteogenic Differentiation Requires Only a Short Exposure to KMN-159





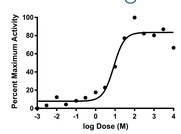
Alkaline Phosphatase (Day 9)

These data show that rat BMCs exposed to KMN-159 for as little as 10 minutes are committed to induction of alkaline phosphatase measured 9 days later. While only 30- and 60-minute exposures of the cells to KMN-159 were tested for mineralization, it is clear that these short exposure times lead to the commitment of stem cells within the bone marrow to the complete program of osteogenesis. This is demonstrated by increased mineralization with 1,000 nM KMN-159 treatment (right half of each plate) as compared to vehicle treatment (left half of each plate). This is consistent with the typical transient activation of a GPCR such as EP_4.



Mineralization (Day 21)

KMN-159 Stimulates ALP Activity Even After Dry Plate Storage at Room Temperature

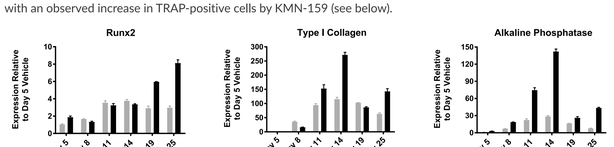


KMN-159 dilutions were made in ethanol and 0.5 ml of each dilution was added to a well of a 24-well plate (n = 8 wells/dilution). Plates were dried overnight in a cell culture hood and then stored dry, in room light at room temperature for 24 days. Freshly isolated rat BMCs were plated into the wells containing the dried dilutions of KMN-159 and cultured for 9 days as above. ALP activity was assayed, and the EC_{50} for KMN-159 under these conditions was determined to be 8.4 nM.

KMN-159 was active in stimulating ALP enzyme activity in BMCs with an EC_{50} value equivalent to that seen in cells treated directly with compound. This demonstrates that the compound is stable at room temperature and retains its potency for at least 24 days.

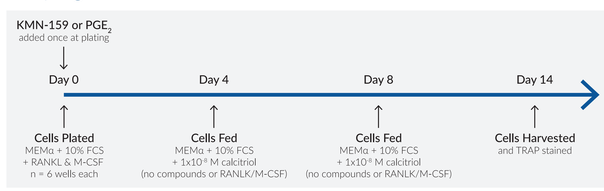
KMN-159 Stimulates Expression of Osteoblast Phenotype Marker Genes

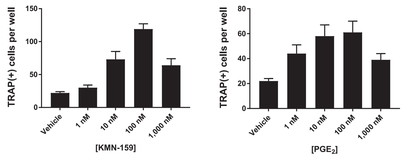
Phenotype marker genes were expressed in a manner consistent with the stimulation of osteoblastic differentiation by KMN-159. RANKL expression was also stimulated, followed by a later increase in OPG expression, consistent with an observed increase in TRAP-positive cells by KMN-159 (see below).



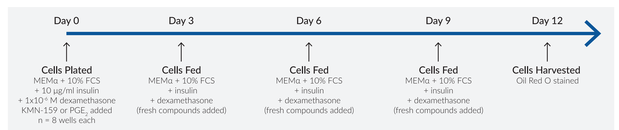
Rat BMCs were treated with 1,000 nM KMN-159 or ethanol vehicle and plated in 12-well dishes as above. Cells were fed every 3 days with osteogenic medium beginning on day 7. RNA was prepared on the indicated days and used as a template for RT-qPCR using GAPDH as a reference gene. (vehicle = ■ KMN-159 = ■)

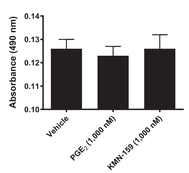
KMN-159 Stimulates Osteoclastogenesis but not Adipogenesis





These data suggest that KMN-159, like the natural ligand PGE_2, induces osteoclast formation. It is not known if this is a direct effect on the mononuclear precursor cells or an indirect effect *via* the stimulation of RANKL production by osteoblasts in the culture during their differentiation.





These data demonstrate that neither KMN-159 nor the natural EP_4 ligand PGE_2 affect adipogenesis under bone marrow cell culture conditions which promote adipocyte formation.

Conclusions

- KMN-159, a novel difluorolactam EP_4 receptor agonist, specifically binds to and activates the rat EP_4 receptor with potency similar to the natural ligand PGE_2.
- KMN-159 stimulates osteoblastic differentiation in BMCs regardless of the age or sex of the donor rats.
- When dried on the surface of cell culture wells and stored at room temperature for at least 24 days, KMN-159 retains the potency and efficacy equivalent to that seen when cells are treated with freshly prepared stocks.
- Consistent with the EP_4 receptor being a GPCR, BMCs need to be exposed to KMN-159 for less than 30 minutes to fully initiate osteogenesis as measured by ALP and mineralization.
- The osteoblastic differentiation induced by KMN-159 occurs solely through stimulation of the EP_4 receptor as demonstrated by receptor-specific agonist and antagonist studies.
- Osteoblast phenotype marker genes including Runx2, type I collagen, ALP, and OC are expressed at higher levels in the KMN-159 treated cells than in the vehicle treated controls.
- The expression of RANKL is increased by KMN-159.
- KMN-159 and PGE_2 both increased the number of osteoclasts but not adipocytes.

Significance and Future Directions

- The work presented here used whole rat bone marrow to explore the action of KMN-159. This complex mixture of cells is similar to the milieu in which the compound would act as a therapeutic agent for fracture therapy.
- Complete stimulation of osteogenic differentiation in bone marrow cells by a single dose of KMN-159 with an exposure time of less than 30 minutes suggests that a combination of this compound with a surgically appropriate matrix will allow a single clinical application to be performed. This will provide advantages over current therapies including long-term shelf stability and a greatly reduced cost of goods.
- The combination of potency of KMN-159 and its selectivity for EP_4, as shown by several measures, should decrease the risk of off-target activity following release from a matrix in a local administration modality.
- Application of KMN-159 or similar compounds in a local manner to increase the rate of bone formation should have many clinical uses including the augmentation of bone mass prior to implant, spinal fusion, repair of non-union fractures, bridging long bone critical defects, coating implants, and craniofacial repair.

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References

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2. Li, M., Thompson, D.D., and Paralkar, V.M. *Int. Orthop.* **31(6)**, 767-772 (2007).
3. Barrett, S.D., Holt, M.C., Kramer, J.B., *et al. J. Med. Chem.* **62(9)**, 4731-4741 (2019).