Release of the EP_4 Receptor Agonist KMN-159 from Scaffolds *in vitro*

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KEY FINDING

Active KMN-159 is rapidly released without causing ectopic bone formation.

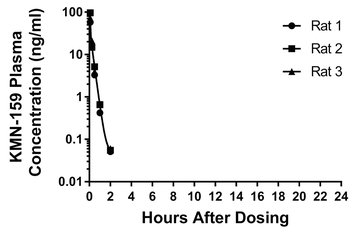
Introduction

Prostaglandin E_2 (PGE_2) is known to cause bone formation with its activation of the EP_4 receptor being primarily responsible for its anabolic actions (Pagkalos *et al.*, *Curr. Mol. Pharmacol.*, 2012). Current bone anabolic therapies for local application are typically protein-based and have relatively short shelf lives. In addition to being expensive, safety concerns are raised by the morphogenic activity associated with some of these therapies, limiting their potential use in orthopedic and other local applications. We have developed a series of novel difluorolactam EP_4 receptor agonists in order to alleviate these issues in a potential therapeutic agent (Barrett *et al.*, *J. Med. Chem.*, 2019). The work presented here is part of the ongoing characterization of our lead compound, KMN-159, which shows high selectivity and excellent potency for EP_4 binding and activation, potent stimulation of osteoblastic differentiation in bone marrow stem cells, and good efficacy in rat calvarial and femoral critical defect models. Our aims were to demonstrate initial safety parameters of KMN-159, determine its half-life *in vivo*, measure its release from three different scaffolds *in vitro*, demonstrate the osteogenic activity of the released compound on bone marrow-derived cell cultures, and test whether KMN-159 demonstrates any morphogenetic properties in an ectopic bone formation model.

Access to Barrett *et al.*, *J. Med. Chem.*, 2019
https://pubs.acs.org/doi/pdf/10.1021/acs.jmedchem.9b00336

Methods and Results

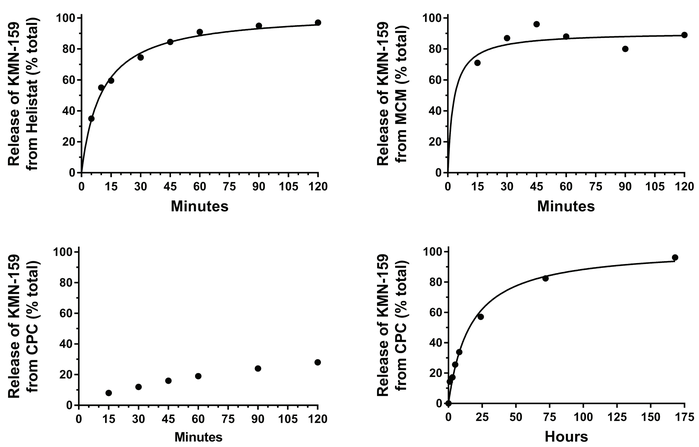
KMN-159 Has a Favorable Genotoxicity Profile and a Short *in vivo* Half-Life Following i.v. Dosing in Rats



The Ames test for mutagenicity, the CHO cell micronucleus test for clastogenicity, a bacterial cytotoxicity assay, and cytochrome P450 inhibition assays were performed by Eurofins Pharma Discovery Services (Bois-l'Évêque, France) and previously reported by Barrett *et al.* (*J. Med. Chem.*, 2019). In all cases, KMN-159 was negative. The *in vivo* half-life of KMN-159 was calculated by measuring its concentration in plasma samples from 3 male SD rats over 24 hours after bolus i.v. administration of 0.5 mg/kg and determined to be 14.6 ± 1.3 minutes. The plasma concentration of KMN-159 was lower than the limit of quantification (0.05 ng/ml) after 2 hours.

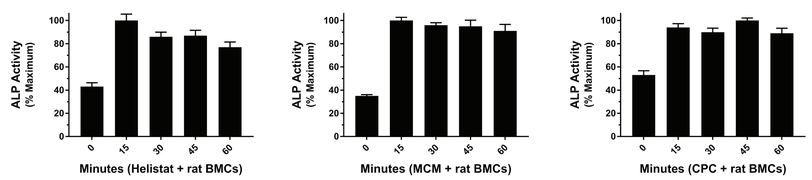
KMN-159 has favorable drug-like properties and is rapidly cleared from circulation, suggesting a lower potential for undesired effects in a local delivery scenario.

KMN-159 is Rapidly Released from Absorbable Collagen Matrix (Helistat) and Mineralized Collagen Matrix (MCM) but Slowly Released from Calcium Phosphate Cement (CPC)



1 mg KMN-159 was dissolved in 0.2 ml 100% ethanol and loaded by absorption onto a 7 x 7 x 7 mm Helistat collagen sponge (Integra LifeSciences Corp). 2 mg KMN-159 was dissolved in 0.2 ml 100% ethanol and loaded by absorption onto an MCM or a CPC matrix. Matrices were air dried at room temperature overnight in a biosafety cabinet and stored at -20°C until used (>10 weeks for MCM and CPC). To test KMN-159 release, matrices were incubated in MEMα + 10% FCS in a 37°C/5% CO_2 incubator with either media alone (25 ml; Helistat) or with primary rat bone marrow cells (7.1 x 10^7 cells/100 ml; MCM and CPC). For the Helistat experiment, the 25 ml media was replaced at each timepoint while for MCM and CPC, 4.5 ml of media and cells were removed at each timepoint. KMN-159 was extracted from cell culture media by protein precipitation using acetonitrile. A specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was used to detect and quantify KMN-159 in the extract using its abundant transition (398.21 *m/z* →207.12 *m/z*). KMN-159 was rapidly released from both Helistat and MCM matrices with ~90% of the total released within 60 minutes. In contrast, KMN-159 was slowly released from the CPC matrix, with maximum release not achieved for several days.

KMN-159 Released from Matrices Induces Osteogenic Differentiation in Rat Bone Marrow Cells



KMN-159 was dissolved and loaded onto the indicated matrices as described above. Each matrix was incubated with 7.1 x 10^7 primary rat bone marrow cells in 100 ml MEMα + 10% FCS in a 1,000 ml flat-sided bottle on a rocker platform (30 rpm) in a 37°C/5% CO_2 incubator. At the indicated times, 4.5 ml was removed and plated in a 24-well dish (0.5 ml/well; n = 8 wells). On day 4, cells were fed with plating medium supplemented with 3 x 10^{-8} M dexamethasone (0.25 ml/well = 1 x 10^{-8} M dexamethasone final) and an extra 50 µg/ml ascorbic acid. On day 9, cultures were lysed, and ALP activity measured.

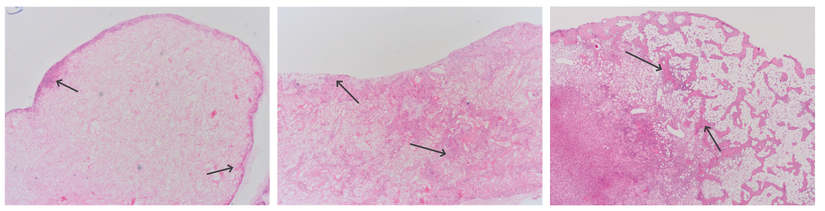
For all matrices tested, a sufficient amount of KMN-159 was released by 15 minutes to achieve a concentration greater than that necessary for the maximal stimulation of ALP activity in the primary rat bone marrow culture system (1 µM). The similar activity profiles achieved with all three matrices was also an indication of the stability of KMN-159 loaded onto a matrix (>10 weeks for MCM and CPC).

Helistat Sponges Support ALP (+) and Mineralizing Colonies

Helistat sponges were loaded with either vehicle or KMN-159 (1 mg) as described above and dried overnight. Primary rat bone marrow cells (1 x 10^7 cells in 0.25 ml MEMα + 10% FCS) were then added to each sponge, which was then placed into a T25 flask standing on end with 8 ml media. Cells were fed on day 4 as above and then fed every 3 days with this medium now also supplemented with 10 mM β-glycerophosphate. On the indicated days, sponges were fixed in 2% paraformaldehyde and stained for either ALP activity or von Kossa-stained for mineralization.

By day 9, ALP-positive colonies were seen in the sponges, by day 12, ALP activity spread throughout the sponges, and by day 20, mineral deposition was observed. While these are not quantitative assays, it appears that sponges loaded with KMN-159 had increased ALP activity and mineralization.

KMN-159 Does Not Induce Ectopic Bone Formation



The in-life portion of this study was performed by T3 Labs (Atlanta, GA). Helistat-absorbable collagen sponges (12.7 x 5 x 7 mm) were loaded with either 0.25 ml 100% ethanol (**vehicle**); 1 mg **KMN-159** (dissolved in 0.25 ml 100% ethanol); or 50 µg recombinant human bone morphogenic protein-2 (**rhBMP-2**; GenScript; dissolved in 0.25 ml 5 mM HCl + 0.1% BSA). An implant was placed in each hind limb of 10-week-old male Sprague-Dawley rats midway between the hip and the knee (6 implants total per treatment). Rats were sacrificed 28 days after surgery and implants were fixed in 10% neutral buffered formalin.

Histological analysis of the implants was performed by Histion (Everett, WA). Specimens were decalcified in formic acid and embedded in paraffin. Three sections were made from each implant and H&E stained. In the representative sections of vehicle or KMN-159 implants (above; 20x original magnification), the arrows indicate areas of inflammatory response, while the arrows in the representative rhBMP-2 section indicate areas of mineralized bone.

				Osteoinductivity (OI) Index Scores	
Group	Treatment	Time Point	N	Median	SE
1	Collagen Sponge + Ethanol Vehicle	28 days	6	0.00	0.00
2	Collagen Sponge + rhBMP-2	28 days	6	3.17	0.17
3	Collagen Sponge + KMN-159	28 days	6	0.00	0.00

All sections were evaluated blind to treatment by a veterinary pathologist and an osteoinductivity (OI) score assigned based on the percentage of the implant area occupied by calcified lamellar or woven bone, bone marrow (fatty or cellular), and bone-forming elements including cartilage, osteoid, and chondrocytes. Osteoblasts were determined for each section.

These semiquantitative data show a median OI index score of 3.17 for rhBMP-2, indicating that 51% to >75% of the implant area was involved in new bone formation. Both the vehicle and KMN-159 groups had individual and median OI scores of zero and had a similar macrophage and giant cell response associated with residual collagen. Collectively, these results indicate that the criteria for the model were met and that KMN-159 does not induce ectopic bone formation.

Conclusions

- KMN-159 has favorable drug-like properties, has passed initial toxicity testing, and has a short *in vivo* half-life.
- KMN-159 is rapidly released from collagen and MCM matrices, and the released drug induces rat BMC osteoblastic differentiation.
- Helistat collagen sponges support rat BMC osteoblastic differentiation with KMN-159 appearing to increase differentiation in the sponges.
- KMN-159 does not induce ectopic bone formation in the rat muscle pouch model.
- The combination of KMN-159 plus matrix gives a cost-effective and chemically stable alternative to protein-based therapeutics.

Acknowledgments

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