# Stimulation of Osteoblast Differentiation and Increased Rate of Bone Defect Repair by Novel Potent Lactam Acetylene $EP_4$ Receptor Agonists

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## Test Compound Cellular Functional Activity

| Compound Number | Compound Structure | Rat $EP_4$ Activation in Transfected Cells | Rat Bone Marrow Stem Cell Differentiation |
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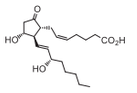
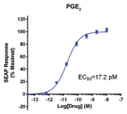
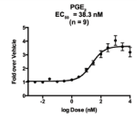
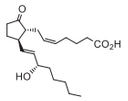
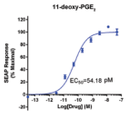
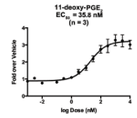
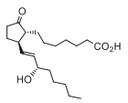
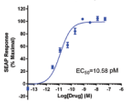
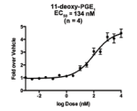
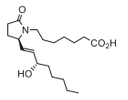
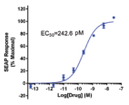
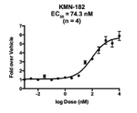
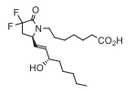
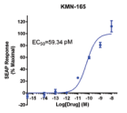
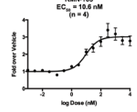
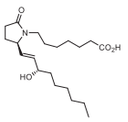
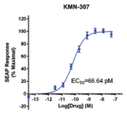
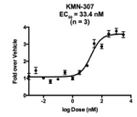
| $PGE_2$ |  |  |  |
| 11-Deoxy-$PGE_2$ |  |  |  |
| 11-Deoxy-$PGE_1$ |  |  |  |
| KMN-182 |  |  |  |
| KMN-165 |  |  |  |
| KMN-307 |  |  |  |

### RAT $EP_4$ ACTIVATION OF TRANSFECTED CELLS

1. Large batch of frozen HEK293T cells were prepared and stored in vapor phase of a liquid nitrogen vessel.
2. The day before plating cells on the reverse transfection plate, aliquot(s) of frozen HEK293T cells were thawed and plated onto T150 flask to allow recovery for 20-24 hours.
3. Cells were harvested from the flask and seeded on an $EP_4$ reporter reverse transfection plate at a density of 75,000 cells/well in 200 µL reduced serum medium containing 0.5% FBS.
4. Cells were incubated at 37 °C incubator with 5% $CO_2$ for 16-18 hour to allow expression of receptor target
5. Culture media was aspirated and replenished with 100 µL serum free culture medium.
6. Test compounds were prepared at 2x final concentration and added to wells. For each compound, an 8-point dose response curve (DRC) in 4-fold serial dilution was performed in triplicate. $PGE_2$ DRCs were run in parallel in all experiments (concentrations from 0-10 nM).
7. After 6 hours of stimulation, 10 µL of media was transferred to a corresponding well of a 96-well solid white plate.
8. The plate was heated at 65 °C for 30 minutes to inactivate endogenous alkaline phosphatase.
9. Luminescence-based alkaline phosphatase substrate (Cayman Chemical, Catalog #600183) was added to each well and SEAP activity was measured by reading the luminescent signal after a 10 minute incubation.
10. The $EC_{50}$ values for $PGE_2$ and each test compound were calculated using GraphPad Prism 6.

### RAT BONE MARROW STEM CELL DIFFERENTIATION

1. Bone marrow was extruded from both tibiae and femora of intact female SD rats between 6 and 12 weeks of age (Harlan) into complete cell culture medium (MEMα) supplemented with 15% fetal calf serum and antibiotics, filtered through a 100 µm mesh filter, and counted.
2. Cells were treated with test compounds from 1000x stocks made in 50% ethanol:50% PBS and immediately plated into either 24-well (for alkaline phosphatase assay) or 12-well (for nodule formation) dishes at $1.78x10^5$ cells/cm$^2$ in complete medium.
3. Cells were cultured (37 °C, 5% $CO_2$, humidified) for seven days with one media change on day four (50% media withdrawn and replaced with an equal volume of fresh media containing $2x10^{-8}$ M dexamethasone ($1x10^{-8}$ M final).
4. For the alkaline phosphatase assay, cells were harvested by adding 0.25 mL alkaline phosphatase lysis buffer per well and storing at -80 °C. Alkaline phosphatase activity was quantitated by incubating 150 µL cell lysate with 50 µl pNPP substrate and reading product formation at 405 nm. $EC_{50}$ values were determined from the data using GraphPad Prism 6.
5. For nodule formation, cells were fed as above and then every 3 days beginning at day 7 with MEMα supplemented with 10% FCS, antibiotics, 50 µg/ml ascorbic acid, 2 mM inorganic phosphate, and $1x10^{-8}$ M dexamethasone. On day 21, alkaline phosphatase positive cell nodules were visualized by histochemical staining.

### RAT CALVARIA IN VIVO ASSAY

Compounds were tested for *in vivo* activity by generating a critical calvarial defect in female SD rats and treating the defect with demineralized bone inserts treated with $EP_4$ agonist embedded inside calcium phosphate cement (CPC). Rats were imaged at weekly intervals and the size of the remaining defect measured from the cone-beam dental CT images (Vatech Pax Duo 3D).

**REFERENCES**

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2. Blackwell, K. A., Raisz, L. G., and Pilbeam, C. C., *Trends in Endocrinology & Metabolism*, 2010, 21, 294.
3. Ke, H. Z. et al., *Journal of Bone and Mineral Research*, 2006, 21, 565.
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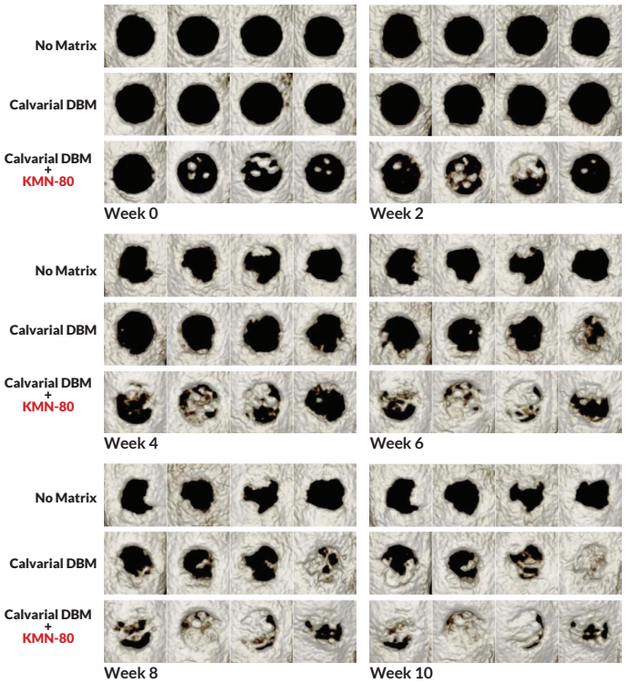
## ABSTRACT

Bone anabolic agents currently used in orthopedic and other local applications are typically recombinant proteins that are costly, require special storage conditions, and possess potential safety liabilities due to their morphogenic activity. Small molecule agents offer a combination of much lower cost of goods together with good chemical stability, longer anticipated shelf lives, and lack of morphogenic activity. Endogenous prostaglandins $PGE_1$ and $PGE_2$ interact with the G-protein coupled receptors $EP_2$ and $EP_4$ which signal by increasing intracellular cAMP (1). Both $EP_2$ and $EP_4$ are expressed in bone and their roles in metabolism have been investigated (2). The bone anabolic effects of $EP_4$ have been clearly demonstrated using selective $EP_4$ agonists (3,4). We have prepared a novel series of substituted γ-lactam (pyrrolidinone) derivatives that mimic the carbocyclic prostaglandin scaffold structure and are potent, highly-selective $EP_4$ agonists. Since these molecules will be developed for local administration, we designed them for rapid metabolic elimination ensuring minimal systemic exposure. Members of this chemical series can stimulate osteoblastic differentiation in bone marrow stem cells using both alkaline phosphatase (AP) activity and mineralized nodule endpoints. These compounds are also able to increase the rate of bone healing in the rat calvarial defect model when administered as a single dose in a slow-release matrix.

**Figure 1.** Accelerated rat calvaria defect repair by application of **KMN-80** loaded CPC plugs in void-filling demineralized bone



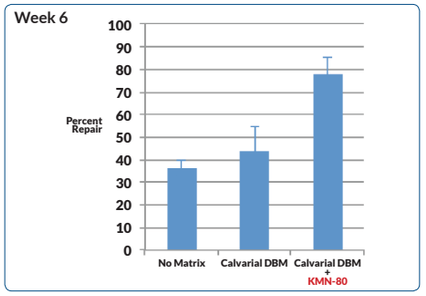
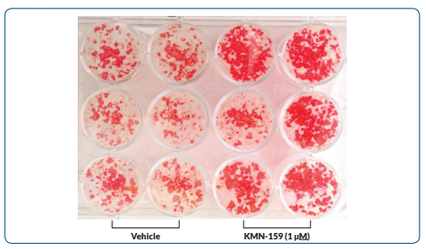




**Figure 2.** Enhanced differentiation of bone marrow stem cells into osteoblasts following treatment with KMN-159




## Test Compound Cellular Functional Activity

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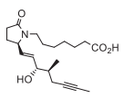
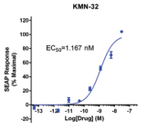
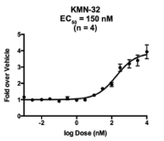
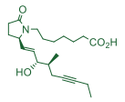
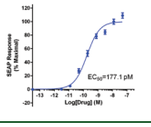
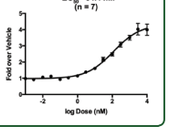
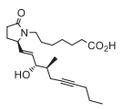
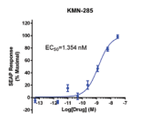
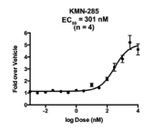
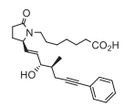
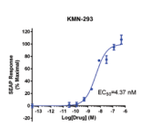
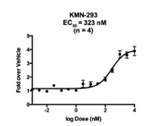
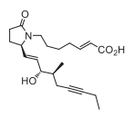
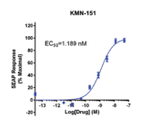
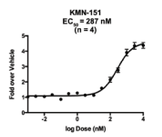
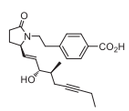
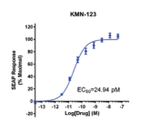
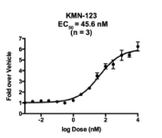
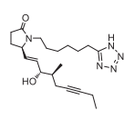
| KMN-32 |  |  |  |
| **KMN-80** |  |  |  |
| KMN-285 |  |  |  |
| KMN-293 |  |  |  |
| KMN-151 |  |  |  |
| KMN-123 |  |  |  |
| KMN-347 |  |  |  |
| **KMN-159** |  |  |  |
| KMN-79 |  |  |  |

## CONCLUSIONS

- We have designed and prepared two novel small-molecule $EP_4$ receptor agonist compound series, the KMN-80 and the parallel difluoro KMN-159 series, which demonstrate robust structure-activity relationship.
- KMN-80, KMN-159, and their analogs demonstrate cellular potencies and activities comparable or superior to those of the now-discontinued big pharma programs.
- Both KMN-80 and KMN-159, as mixtures with CPC slow-release matrix in void-filling osteoconductive demineralized bone, demonstrate efficacy *in vivo* comparable to that of BMP-2 but with a significantly lower cost of goods, stability, and ease of storage.

**Next steps:**

1. We will add rat studies to optimize KMN-159 dose and slow-release α/β-CPC composition ratio with demineralized bone matrix (DBM).
2. We seek to dose KMN-159 in rabbit and/or dog spinal fusion and fracture repair models.

**PATENTS PENDING**