



Novel, Potent, Selective, Small Molecule EP₄ Receptor Agonists Stimulate Rat Bone Marrow Stem Cell Differentiation

Stephen D. Barrett[†], Thomas A. Owen[§], Adam Uzieblo[†], Bradlee D. Germain[†], James B. Kramer[†], Andrei Kornilov[†], Joseph M. Colombo[†], Fred L. Ciske[†], Melissa C. Holt[†], Bill Ho[†], Adam S. Stein[†], M. Ines Morano[†]

[†]Cayman Chemical, Ann Arbor, Michigan www.caymanchem.com, [§]Ramapo College of New Jersey, Mahwah, NJ

Abstract

We have discovered a series of potent small molecule agonists selective for the EP₄ receptor through rational design that stimulate osteoblastic differentiation in rat bone marrow stem cells on par with or improved over PGE₂ and other known small molecule agonists. The SAR study was driven by compound synthesis and subsequent screening in competition-based binding assays and cell-based reporter assays. Computational modeling suggests binding pocket interactions that could further be exploited in future lead optimization work. The compounds have been designed to possess physical and pharmacokinetic property profiles amenable to matrix incorporation enabling local administration for bone fracture, spinal fusion, and other bone growth-promoting uses while minimizing systemic exposure and associated toxicities.

Test Compound Cellular Functional Activity

Compound Name	Compound Structure	Rat EP ₄ Activation in Transfected Cells	Rat Bone Marrow Stem Cell Differentiation
PGE ₂			
11-deoxy PGE ₂			
11-deoxy PGE ₁			
KMN-182			
KMN-165			
KMN-307			

Rat EP₄ 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 a T150 flask to allow recovery for 20-24 hours.
3. Cells were harvested from the flask and seeded on an EP₄ 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 with 5% CO₂ for 16-18 hours 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. PGE₂ 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, Item No. 600183**) was added to each well and SEAP activity was measured by reading the luminescent signal after a 10 minute incubation.
10. The EC₅₀ values for PGE₂ 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 1,000x 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 1.78x10⁵ cells/cm² in complete medium.
3. Cells were cultured (37°C, 5% CO₂, 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. EC₅₀ 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.

In Silico EP₄ Agonist Docking

Active-conformation, agonist-bound crystal structure of EP₃ (6AK3) was docked with KMN-80 and KMN-159 using an IFD protocol using Maestro 18-1 (Schrödinger NY, NY). Homology model of human EP₄ was generated from RaptorX structure prediction suite (<http://RaptorX.uchicago.edu/>). Binding site alignment of EP₃ active site and EP₄ active site was performed to nominate likely residues of importance within human EP₄.

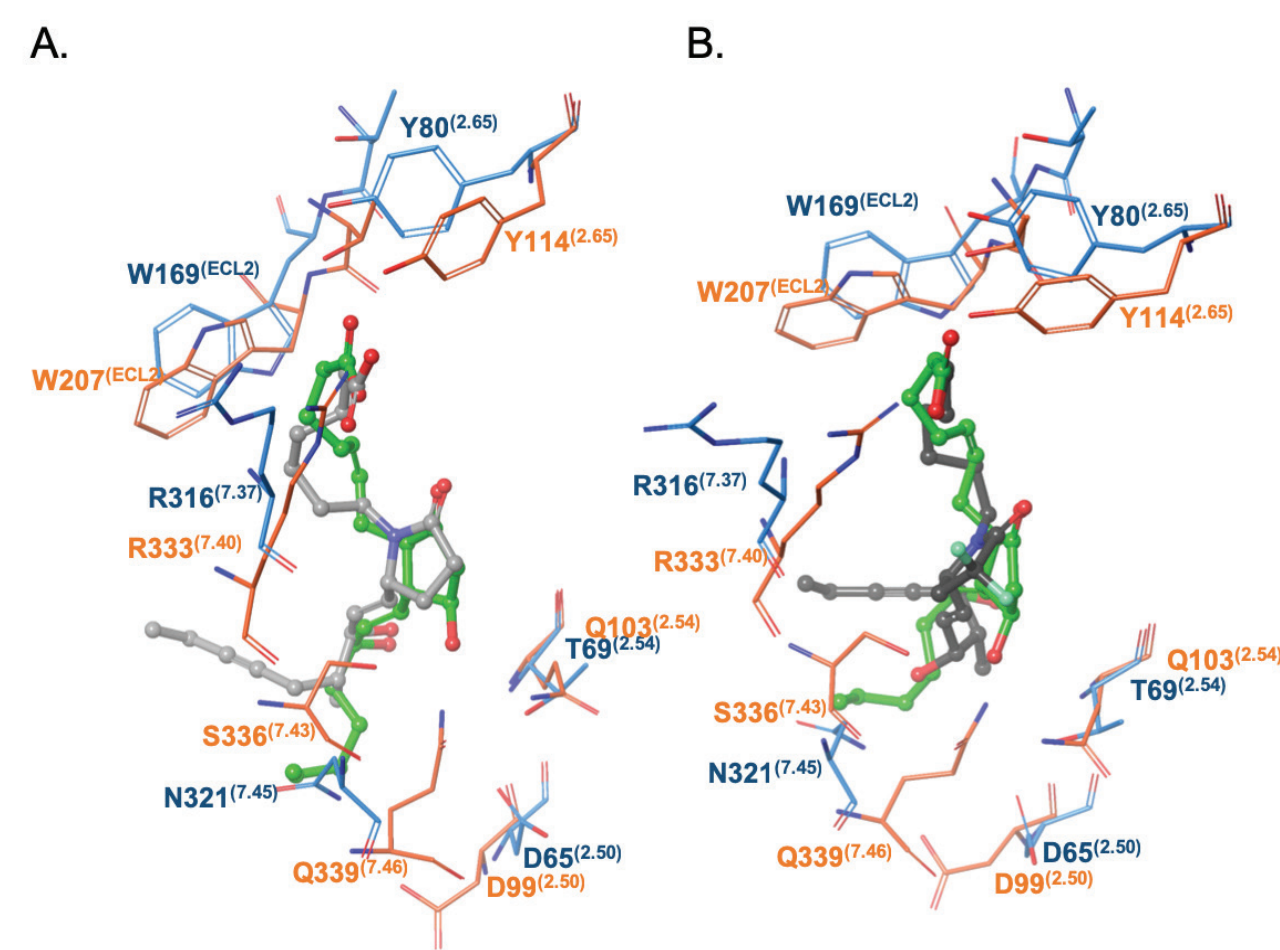


Figure 1. Crystal structure of PGE₂ (green stick & ball representation) bound to human EP₃ receptor, residues shown in line representation in orange (6AK3, 2.9Å). **A.** Docked pose of KMN-80 (gray stick & ball representation) in EP₃ receptor overlaid overtop of PGE₂. **B.** Docked pose of KMN-159 (black stick & ball representation) in EP₃ receptor overlaid on top of PGE₂. Binding site alignment with human EP₄ receptor performed with aligning residues shown in line representation in blue.

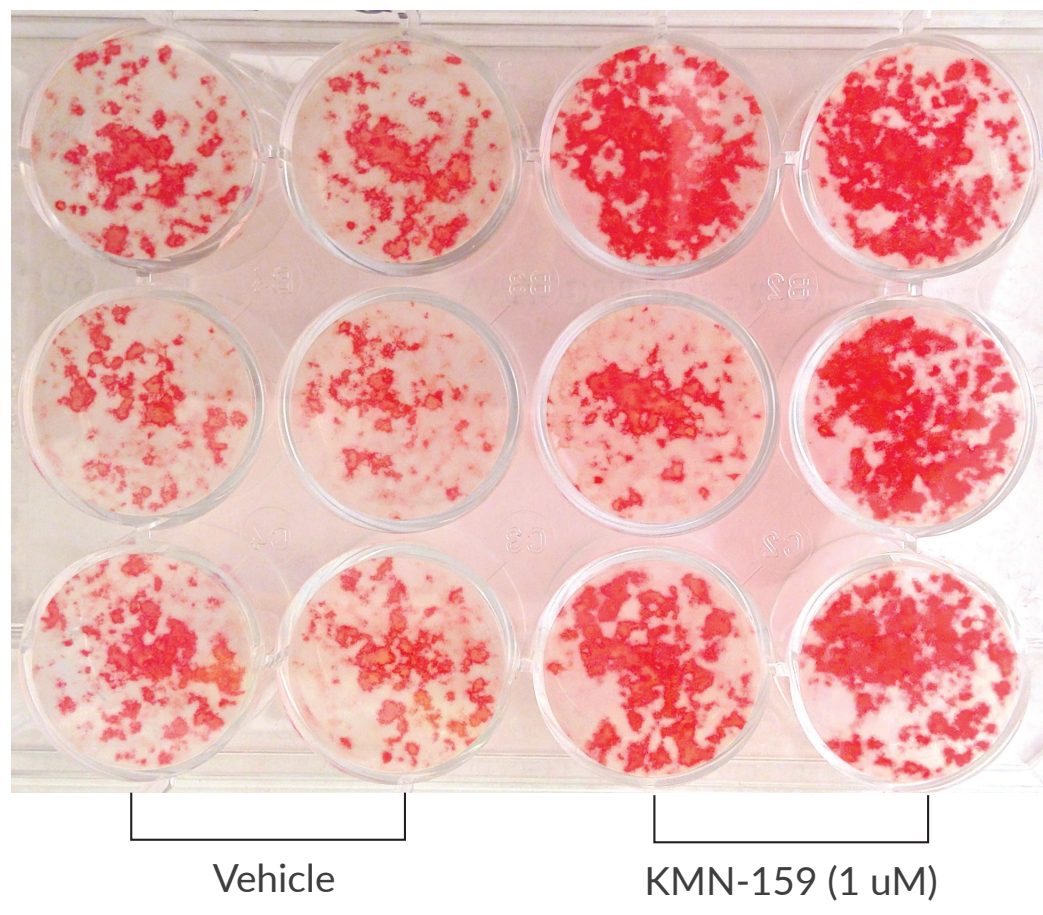


Figure 2. Enhanced differentiation of bone marrow stem cells into osteoblasts following treatment with KMN-159.

Test Compound Cellular Functional Activity

Compound Name	Compound Structure	Rat EP ₄ Activation in Transfected Cells	Rat Bone Marrow Stem Cell Differentiation
KMN-32			
KMN-80			
KMN-285			
KMN-293			
KMN-151			
KMN-123			
KMN-347			
KMN-159			
KMN-79			

Conclusions

- Novel γ-lactam EP₄-selective agonists possess picomolar activity in EP₄-overexpressed cells.
- Docking studies suggest KMN-80 and its gem-difluoro analog, KMN-159, bind to the receptor in a similar manner as the endogenous PGE₂ and make key interactions with a salt binding region near the ligand binding pocket.
- Nanomolar agonist concentrations promote rat bone marrow stem cell differentiation to osteoblasts.

United States Patents 9,180,116; 9,440,919; 9,487,478; 9,701,630; and others pending