Novel Potent Lactam Acetylene EP_4 Agonists Stimulate Alkaline Phosphatase Production and Differentiation in Bone Marrow Cells

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Abstract

The prostaglandin E G-protein coupled receptors (GPCR) subtypes 2 and 4 (EP_2 and EP_4 receptors, respectively) are stimulated by interaction with two eicosanoid products of the cyclooxygenase-catalyzed pathway, prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2), and signal by increasing intracellular cAMP levels.[1] Expression of EP_2 and EP_4 receptors in human bone and their roles in differentiation, growth, and remodeling have been previously investigated.[2] Although the presence and function of the EP_2 receptor remains ambiguous, the clear significance of the EP_4 receptor in bone anabolism has been demonstrated with both systemic and locally-administered selective EP_4 agonists. Most osteoporosis programs focused on generating agonists with high systemic exposure and selectivity for EP_2/EP_4 versus EP_1 and EP_3. Substituted γ-lactam (pyrrolidinone) derivatives that structurally mimic the carbocyclic prostaglandin scaffold were found to possess notable selectivity for EP_4 over the other EP receptor subtypes, especially EP_1. As the osteoporosis development programs were failing for safety reasons associated with high systemic exposures, attention shifted to localized delivery of anabolic agents to bone, resulting in the approval of the costly recombinant human bone morphogenetic protein 2 (BMP-2) for orthopedic indications. We have prepared novel γ-lactam derivatives designed to maximize selectivity for EP_4 versus EP_1, EP_3, and other prostanoid receptors and to minimize systemic exposure. These compounds were first screened in EP_4 and EP_2 transfected cells by measuring their effect on secreted alkaline phosphatase (SEAP) stimulation. Compounds of interest were then tested for their ability to stimulate osteoblastic differentiation of primary rat bone marrow cells.

IN VITRO SCREENING ASSAYS

RECEPTOR BINDING ASSAYS

EP_1-EP_4: [^{3}H]PGE_2 displacement/competition assay of human prostanoid $EP_{1\text{-}4}$ receptor in transfected HEK-293 cells (Cerep, Catalog Reference Nos. 0440, 1955, 2774, 0441, respectively)

FP: [^{3}H]PGF2a displacement/competition assay of human prostanoid FP receptor in transfected HEK-293 cells (Cerep, Catalog Reference No. 1979)

DP_1: [^{3}H]PGD2 displacement/competition assay of human prostanoid DP_1 receptor in transfected 1321N1 cells (Cerep, Catalog Reference No. 2517)

IP: [^{3}H]iloprost displacement/competition assay of human prostanoid IP receptor in transfected HEK-293 cells (Cerep, Catalog Reference No. 2230)

FUNCTIONAL CELL ASSAYS (STEP Plate Format)

Both SEAP activity and cAMP assays for EP_2 or EP_4 agonists were performed on EP_2 or EP_4 STEP (Surface Transfection and Expression Protocol) plates (from Cayman) which are coated with rat EP_2 or EP_4 receptor and secreted alkaline phosphatase (SEAP) -cyclic AMP response element (CRE) reporter constructs. Cells grown on the STEP complex will express EP_2 or EP_4 at the cell surface. Binding of agonists to EP_2 or EP_4 initiates a signal transduction cascade results in a transient increase in cAMP and an a prolonged increase in expression of SEAP which is secreted into the cell culture media. cAMP levels were then measured by ELISA and SEAP activity was measured with a luminescence-based alkaline phosphatase substrate.

EP_2 and EP_4Transfected HEK-293 Cell Assay (SEAP)

1. Cells were seeded on an EP_2 or EP_4 STEP plate at a density of 40,000 – 80,000 cells/well in 200 µL of reduced serum medium containing 0.5% FBS and incubated at 37 °C incubator with 5% CO_2 for 16-18 hour.

2. Culture media was aspirated and 200 µL of culture medium containing test compounds was added to wells. For each compound, an 8-point dose response curve (DRC) ranging from 10 µM to 0.001 pM was performed in triplicate. PGE_2 DRCs were run in parallel in all experiments (concentrations from 0-6,000 pM for EP_4 and 0-10,000 nM for EP_2)

3. After 6-8 hours of stimulation, 10 µL of media was transferred to a corresponding well of a 96-well solid black plate. The plate was heated at 65 °C for 30 minutes to inactivate endogenous alkaline phosphatase.

4. Luminescence-based alkaline phosphatase substrate (Michigan Diagnostics, LLC, Cat#SAP450101) was added to each well and SEAP activity was measured by reading the luminescent signal.

5. The EC_{50} values for PGE_2 and each test compound were calculated using GraphPad Prism 5.

EP_2 and EP_4Transfected HEK-293 Cell Assay (cAMP)

1. As above.

2. As above, except the culture medium contained 500 µM IBMX (an inhibitor of cAMP phosphodiesterase).

3. Cells were incubated at 37 °C for 30 minutes.

4. The plate was centrifuged at 1,000 x rpm for 10 minutes and the supernatant aspirated.

5. EIA assay buffer (100 µL) was added to each well and cells were lysed by freezing at -80 °C freezer for 1 hr followed by completely thawing at room temperature.

6. The plate was centrifuged at 1,000 x rpm for 10 minutes. The supernatant was assayed for cAMP by ELISA (Cayman Chemical item 581001).

7. The data were analyzed using GraphPad Prism 5 as above.

TP TRANSFECTED HEK-293 CELL ASSAY (intracellular [Ca^{2+}])

Cerep Catalog Reference No. 2059

TABLE 1 ω-Chain SAR

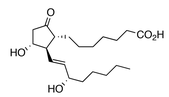
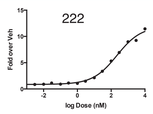
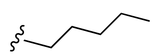
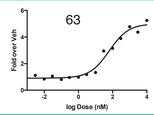
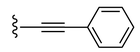
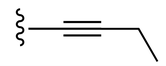
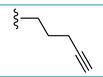
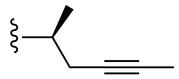
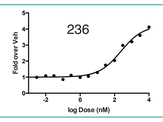
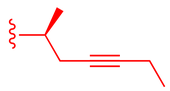
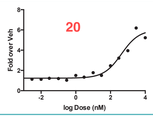
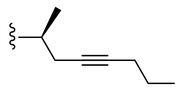
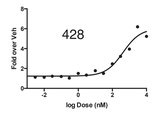
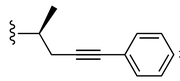
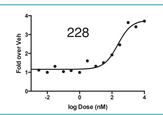
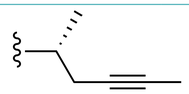
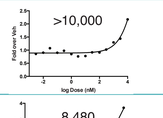
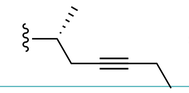
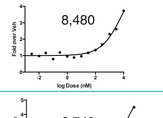
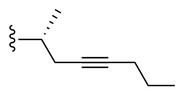
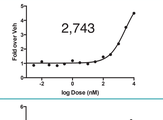
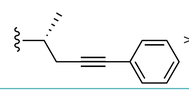
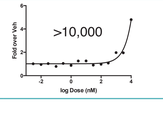
		PGE₂ Displacement Radioligand Binding EP_4 IC_{50}(nM)	HEK-293 Cells SEAP Stimulation EP_4 EC_{50} (nM)	Bone Marrow Cells EC_{50} (nM)
PGE_1			0.22 ± 0.04 (cAMP)	
KMN-182		2.0	0.71 (cAMP)	
KMN-42			23.5	
KMN-100			4.73	
KMN-64			371	
KMN-32		3.9	0.57	
KMN-80		**3.0**	**0.19 ± 0.07**	
KMN-285		54	0.65	
KMN-293		>100	0.33	
KMN-33			4.05	
KMN-79		6.9	1.14	
KMN-287		4.8	3.96	
KMN-295		>100	7.72	

TABLE 2 α-Chain SAR

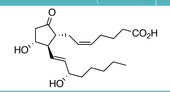
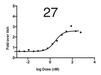
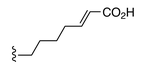
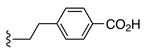
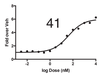
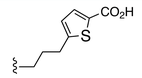
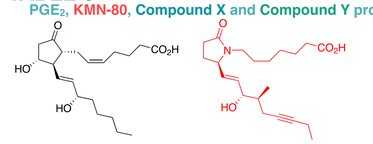
		PGE₂ Displacement Radioligand Binding EP_4 IC_{50} (nM)	HEK-293 Cells SEAP Stimulation EP_4 EC_{50} (nM)	Bone Marrow Cells EC_{50} (nM)
PGE_2		0.38 ± 0.07	0.05 ± 0.03	
KMN-151			2.57	
KMN-123		0.67	0.31	
KMN-212		6.1	1.54	

TABLE 3

PGE₂, KMN-80, Compound X and Compound Y prostanoid profiles



PGE₂ KMN-80 Compound X Compound Y

Radioligand Binding IC_{50} (nM)

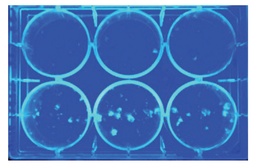
	EP_1	EP_2	EP_3	EP_4	FP	DP_1	IP
PGE_2	1.4	2.6	1	2.0	199 ± 12[3]	307 ± 106[3]	>10,000[3]
KMN-80	>10,000	>10,000	1,400	3.0	>10,000	>10,000	>10,000
Compound X	>10,000	9,000	430	1.3	>10,000	>10,000	>10,000
Compound Y	>10,000	120	400	0.74	>10,000	>10,000	>10,000

Functional EC_{50} (nM)

	EP_2	EP_4	TP
PGE_2	59 ± 17	0.05 ± 0.03	>20,000 (binding K_i)[3]
KMN-80	>1,000	0.19 ± 0.07	>10,000
Compound X	>1,000	0.04 ± 0.04	~10,000
Compound Y	394	0.01	>10,000

Bone Marrow Cells

	EC_{50} (nM)
PGE_2	27
KMN-000080	20
Compound X	3
Compound Y	0.29



PROGRAM GOALS

- **Novel, small molecule, potent EP_4** receptor agonist
- **Selectivity** for the EP_4 receptor versus other prostanoid receptors
- Suitable **physical property** and **pharmacokinetic profile** for **local administration**
- **Efficacy** at tolerated doses in combination with delivery matrix

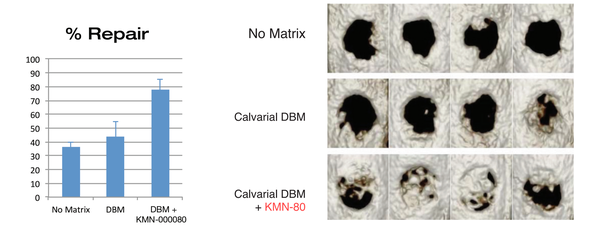
PROGRAM ACHIEVEMENTS

- KMN-80, Compound X, and Compound Y are potent in EP_4 receptor binding and functional/cellular assays.
- **Selective** for the EP_4 receptor against all other prostanoid receptors
- **Soluble** in local-administration matrices, and Compound Y is **highly cleared** (low systemic exposure at efficacious doses)
- **Efficacious** in key animal models; Compound Y is Ames negative

FIGURE 1

Rat Calvarial Defect treated with KMN-80 in demineralized bone matrix (DBM)

Rat Calvarial Defect (5 mm) • Week 6



RAT BONE MARROW CELL ASSAY

1. Extrude bone marrow from both tibia and femora of intact female rats between 6 and 12 weeks of age (Harlan) into complete cell culture medium (MEMa) supplemented with 15% fetal calf serum and antibiotics), filter through a 100 µm mesh filter, and count.

2. Plate cells into either 24-well (for alkaline phosphatase assay) or 6-well (for nodule formation) dishes at 1.78x10^5 cells/cm^2 in complete medium.

3. Culture cells (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, harvest cells at day 7 by incubation in alkaline phosphatase lysis buffer and store at -80 °C. Quantitate alkaline phosphatase activity by incubating 100 µL cell lysate with 50 µL pNPP substrate and reading product formation at 405 nm. Determine EC_{50} values were from the data using GraphPad Prism.

5. For nodule formation, feed cells every 3 days beginning at day 7 with MEMa supplemented with 10% FCS, antibiotics, 50 mg/ml ascorbic acid, 2 mM inorganic phosphate, and 1x10^{-8} M dexamethasone. At the last feeding on day 18, supplement the medium with calcein (1 mg/ml). Fix the cells in 10% buffered formalin on day 21 and photograph under UV light.

RAT CALVARIA *IN VIVO* ASSAY

Will be described in more detail in **Poster [MO0405]** at **Poster Session III** on **Monday, October 7.**

REFERENCES

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3. Young, R. *et al.*, *Heterocycles*, **2004**, *64*, 437-445. Receptor binding assays were performed using membranes from HEK-293ebna cells recombinantly expressing the corresponding human prostanoid cDNAs.