Supplemental Material For

Identification of cosalane as an inhibitor of human and murine CC-Chemokine Receptor 7 signaling via a high throughput screen

Emily A Hull-Ryde, Melissa A Porter, Kenneth A Fowler, Dmitri Kireev, Kelin Li, Catherine D Simpson, Maria F Sassano, Mark J Suto, Kenneth H Pearce, William Janzen, James M Coghill

Address correspondence to:

James M. Coghill, MD

Lineberger Comprehensive Cancer Center

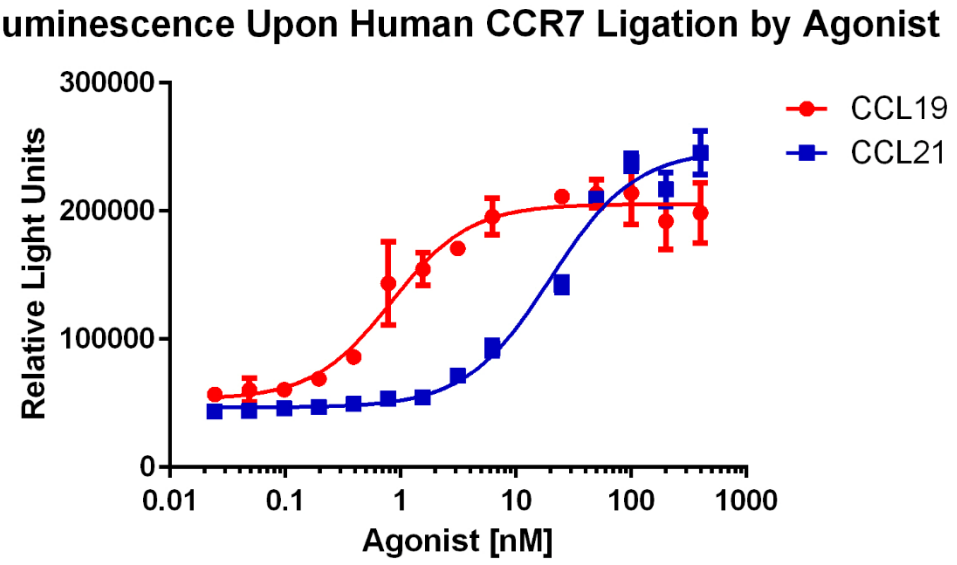
University of North Carolina at Chapel Hill

Chapel Hill, North Carolina 27599

Email: jcoghill@email.unc.edu

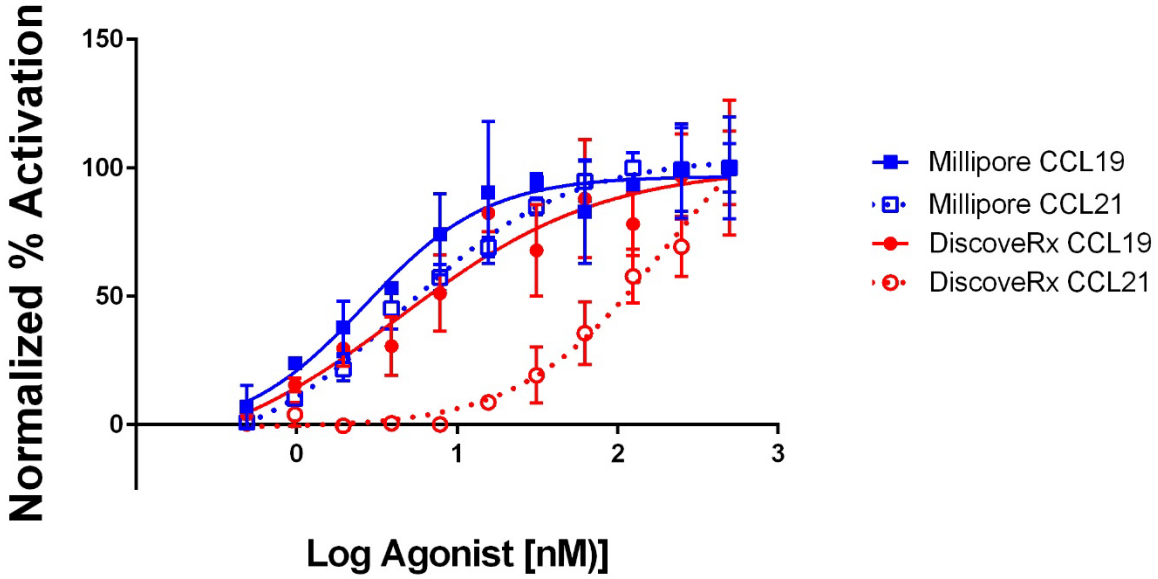
Phone: 919-843-7237

Supplemental Figure 1



Supplemental Figure 1: Representative Chemi-luminescence Curves for DiscoveRx PathHunter® β-Arrestin CHO K1 cells expressing human CCR7 with increasing concentrations of agonist. Typical EC80s for hCCR7/CCL19 and hCCR7/CCL21 were 7nM and 25nM respectively.

Supplemental Figure 2



	Millipore CCL19	DiscoveRx CCL19	Millipore CCL21	DiscoveRx CCL21
EC50 (nM)	2.656	4.386	4.540	192.9

Supplemental Figure 2: The potencies of CCL19 and CCL21 for G protein associated calcium flux vary by cell type. Calcium flux in response to CCL19 and CCL21 was evaluated using FLIPR detection in Chem-1 CCR7 cells (Millipore) expressing high levels of the recombinant promiscuous G protein Gα15 and in PathHunter® CHO-K1 CCR7 β-Arrestin cells (DiscoveRx) expressing native G protein. EC50s for each cell type according to agonist are depicted.

Supplemental Methods:

Human Chem-1 cells were purchased from Millipore in ready to use format for calcium flux assays (Catalogue number HTS012RTA; Burlington, Massachusetts).

PathHunter® CHO-K1 CCR7 β-Arrestin cells (Catalogue number 93-0195C2) were obtained from DiscoveRx (Fremont, California).

Cells were plated into Poly-L-Lysine (PLL) coated 384-well black clear bottom cell culture plates in plating media supplemented with 1% dialyzed FBS at a density of 12,000 cells in 30µl per well for each cell line and cultured overnight before assay. One hour prior to assay, media was aspirated from the wells and 20µl of Fluo-4 Direct® dye-loading solution (Invitrogen; Carlsbad, California) prepared according to the manufacturer's instructions were added per well. After a one hour incubation at room temperature, a FLIPR$^{TETRA®}$ system (Molecular Devices; Sunnyvale, California) was used to read fluorescence (excitation wavelength: 470-495 nm, emission wavelength: 515-575 nm) to establish an individual baseline reading for each well (average value of 10 readings before ligand addition). This initial fluorescence intensity was used to correct each kinetic trace for well-to-well variability in loading of the cells. Next, the FLIPR$^{TETRA®}$ transferred 10 µl of the ligand solution, CCL19 or CCL21, from the drug plate to the cell plate. Readings were made every 1 second for 5 minutes. Data was collected using FLIPR's ScreenWorks® software (Molecular Devices) and exported with the built-in batch export function. Maximal fluorescence intensity readings (RFU, Relative Fluorescence Unit) were determined within a minute after ligand addition as agonist activity, and values were exported to MicroSoft Excel as "fold of basal". Each well was transformed using the equation ((well value – basal)/(max – basal) *100) equals % normalized activation where basal levels are transformed to 0% and 'max' is the maximum reference agonist value transformed to 100% for each cell line. Average

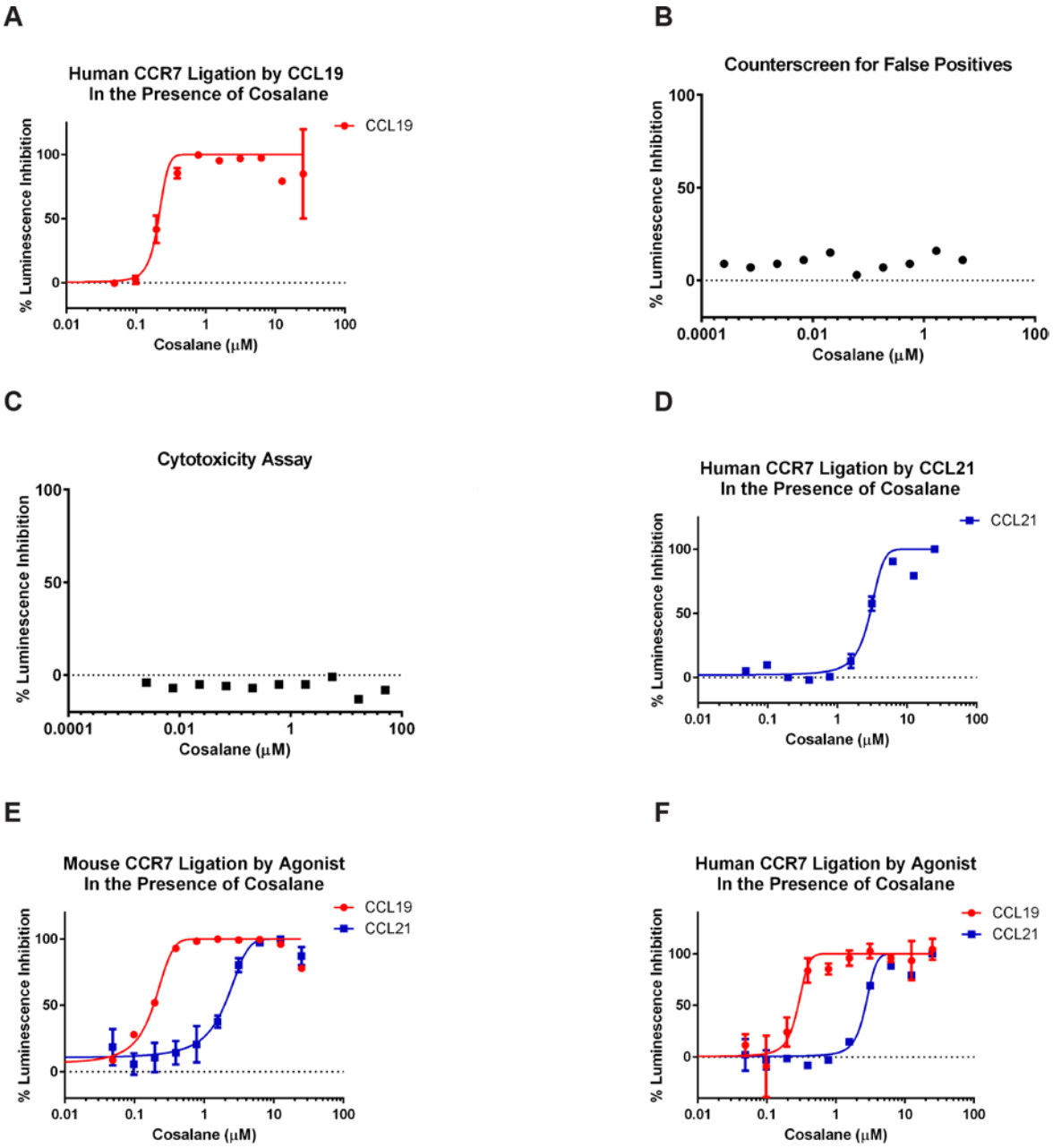
values (n=3) with standard deviation error bars were plotted with Prism and fit with 4-parameter curves for EC_{50} determinations.

Supplemental Table

Compound Name	Lot Number	hCCR7/ CCL19 IC50 (μM)	Counterscreen	Cytotoxicity (μM)	hCCR7/ CCL21 IC50 (μM)	mCCR7/ CCL19 IC50 (μM)	mCCR7/ CCL21 IC50 (μM)
UNC10150582 **"Cosalane"**	SRI-18287	0.207	Non-Reactive	>50	2.66	0.193	1.98
UNC10150759	SRI-19093	8.9	Non-Reactive	>50	7.6	9.3	NP
UNC10155741	SRI-10238	7.9	Non-Reactive	>50	5.4	11	NP
UNC10146458	SRI-26043	7.6	Non-Reactive	>50	7	8.3	NP
UNC10151089	SRI-6596	9	Non-Reactive	>50	4.2	7.6	NP
UNC10219981	SRI-25714	8.4	Non-Reactive	>50	6.4	10.7	NP
UNC10225619	LCGC-91877	1.8	Non-Reactive	>100	4.1	NP	NP
UNC10242795	LCGC-011_168832	4.4	Non-Reactive	>100	2.8	5.2	NP
UNC10242782	LCGC-009_279471	6.8	Non-Reactive	>100	2	3.9	NP
UNC10242786	LCGC-010_190415	10	Non-Reactive	>100	8.6	18.2	NP
UNC10242753	LCGC-007_11213	5.2	Non-Reactive	>100	2.7	4.6	NP
UNC10220221	LCGC-010_189652	9.3	Non-Reactive	>100	8.1	10.4	NP
UNC10242764	LCGC-008_140602	8.2	Non-Reactive	>100	6.7	27	NP
UNC10242794	LCGC-011_168049	10.3	Non-Reactive	>100	4.3	8	NP

Supplemental Table: IC50s for 14 confirmed/progressed hits against human and where indicated mouse CCR7 in DiscoveRx PathHunter® β-Arrestin CHO K1 cells are depicted. NP = Not Performed.

Supplemental Figure 3



Supplemental Figure 3: Primary Bio-Luminescence Data for Cosalane During Compound Progression (A) Dose response curve for cosalane (Southern Research)

against hCCR7/CCL19 in DiscoveRx PathHunter® β-Arrestin CHO K1 cells. Y-axis represents bioluminescence percent inhibition by cosalane compared to cells not exposed to inhibitor (B) Counterscreen for cosalane against hCCR7/CCL19 in DiscoveRx PathHunter® β-Arrestin CHO K1 cells in which cosalane was added to cell cultures after the agonist incubation period had already been completed. (C) HeLA cells were incubated with cosalane for 72 hours and subsequently lysed. The amount of liberated ATP was then determined using a commercially available bioluminescence assay. (D) Dose response curve for cosalane against hCCR7/CCL21 in DiscoveRx PathHunter® β-Arrestin CHO K1 cells. (E) Dose response curve for cosalane against mCCR7/CCL19 and mCCR7/CCL21 in DiscoveRx PathHunter® β-Arrestin CHO K1 cells. (F) Dose response curve for a separate batch of cosalane synthesized at UNC against hCCR7/CCL19 and hCCR7/CCL21 in DiscoveRx PathHunter® β-Arrestin CHO K1 cells