

Supporting Information

Discovery of small molecule antagonists of chemokine receptor CXCR6 that arrest tumor growth in SK-HEP-1 mouse xenografts as a model of hepatocellular carcinoma

Satyamaheshwar Peddibhotla^{a*}, Paul M. Hershberger^a, R. Jason Kirby^a, Eliot Sugarman^a, Patrick R. Maloney^a, E. Hampton Sessions^a, Daniela Divilanska^a, Camilo J. Morfa^a, David Terry^a, Anthony B. Pinkerton^a, Layton H. Smith^a and Siobhan Malany^{a,b*}

^aConrad Prebys Center for Chemical Genomics, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California 92037, USA.

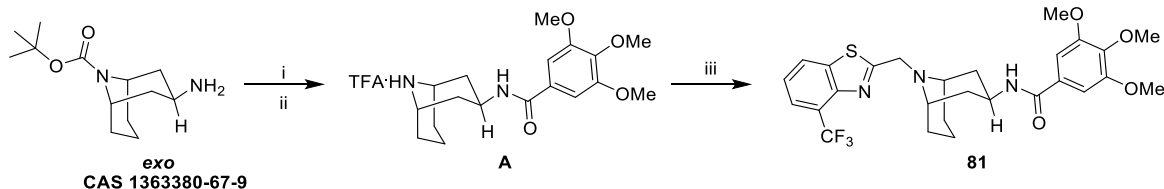
^bCollege of Pharmacy, University of Florida, 1345 Center Drive, Gainesville, FL 32610, USA

General Considerations.....	S2
Synthesis of Compound 81.....	S3
Spectra of Compound 81.....	S5
Supplementary Table 1.....	S7
Supplementary Table 2.....	S8
Supplementary Table 3.....	S9
Supplementary Figure 1.....	S10
Material and Methods.....	S11
References.....	S17

General Considerations

Small molecule derivatives were synthesized and purified at the CPCCG of SBP Medical Discovery Institute as described. ^1H and ^{13}C NMR spectra were recorded on Bruker 500 MHz and 126 MHz spectrometer. Data for ^1H NMR (CDCl_3 referenced at δ 8.07) and ^{13}C NMR (CDCl_3 referenced at δ 77.16) reported as followings: multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, hept = heptet, dd = doublet of doublets, and m = multiplet), integration, coupling constant (Hz), and chemical shift (ppm). Low resolution mass spectrometry (LRMS) data were recorded on an instrument by electrospray ionization (ESI). All reactions were monitored by thin-layer chromatography (TLC) on silica gel (Merck, 60 Å F-254) TLC plates using UV light as the visualizing agent. Flash column chromatography were performed on silica gel (Merck, 60 Å 0.049-0.063 mm). Other starting compounds were purchased from Sigma-Aldrich (St. Louis, MO).

Synthesis of Compound 81.



Supplemental Scheme 1: Synthesis of **81**, conditions: i) 3,4,5-trimethoxybenzoyl chloride, Et₃N, CH₂Cl₂, 2 h, r.t.; ii) trifluoroacetic acid, CH₂Cl₂, 1 h, r.t.; iii) 2-(chloromethyl)-4-(trifluoromethyl)benzo[d]thiazole, K₂CO₃, acetonitrile, 70 °C, 5h 66%.

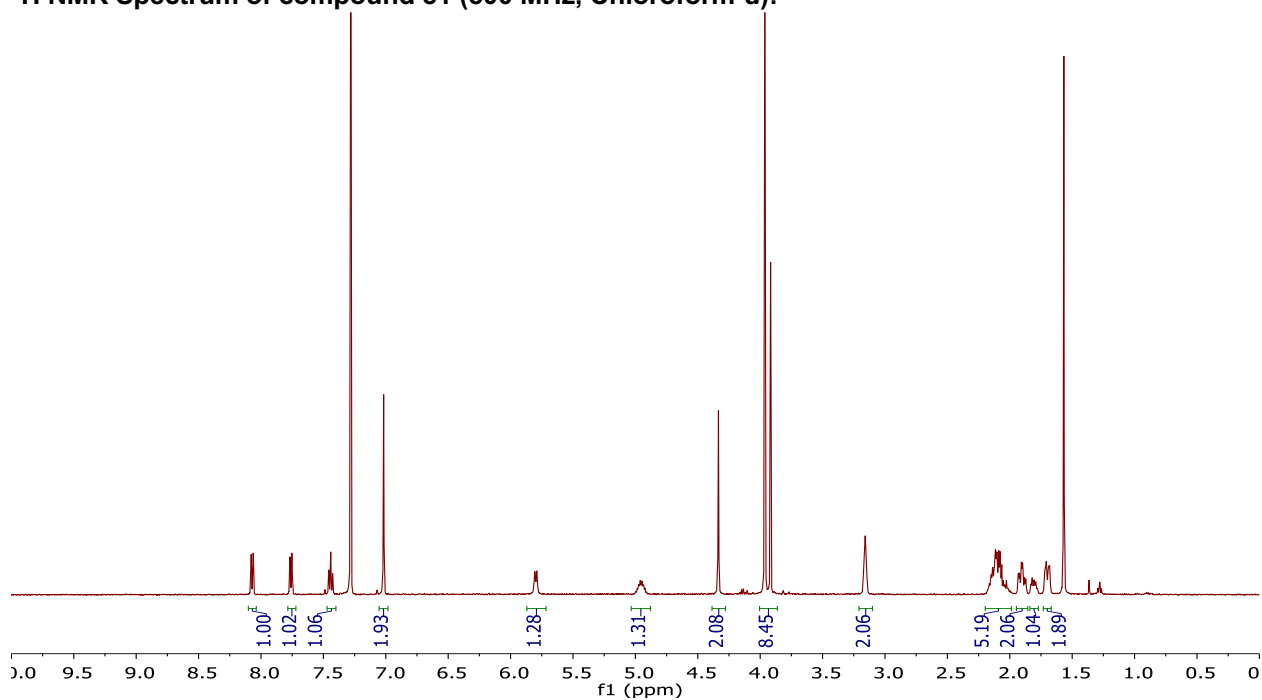
Exo-3-Amino-*N*-Boc-9-azabicyclo[3.3.1]nonane (CAS #1363380-67-9, 150 mg, 0.62 mmol) was dissolved in 5 mL of dichloromethane before charging with triethylamine (0.26 mL, 1.87 mmol) and 3,4,5-trimethoxybenzoyl chloride (173 mg, 0.75 mmol). After 2 h, the volatiles were evaporated *in vacuo* and the residue treated with 5 mL of water and extracted with ethyl acetate. Concentration of the organics returned crude *exo*-((1R,3S,5S)-*tert*-butyl 3-(3,4,5-trimethoxybenzamido)-9-azabicyclo[3.3.1]nonane-9-carboxylate. ¹H NMR (500 MHz, CDCl₃) δ 6.97 (s, 2H), 5.70 (d, *J* = 8.1 Hz, 1H), 5.00 (dq, *J* = 12.4, 6.1 Hz, 1H), 4.40 (br. s, 2H), 3.94 (s, 6H), 3.90 (s, 3H), 3.13 (qd, *J* = 7.3, 4.8 Hz, 1H), 2.16 (dd, *J* = 13.1, 5.9 Hz, 2H), 2.11 – 1.98 (m, 1H), 1.88 (tt, *J* = 13.3, 5.9 Hz, 2H), 1.80 – 1.63 (m, 4H), 1.50 (s, 9H). MS (ESI+ve): Calculated for C₂₃H₃₅N₂O₆, [M+H] = 435.24, observed [M+H] = 435.29. *Exo*-((1R,3S,5S)-*tert*-butyl 3-(3,4,5-trimethoxybenzamido)-9-azabicyclo[3.3.1]nonane-9-carboxylate., (0.62 mmol from the previous step) was dissolved in 4 mL of dichloromethane and treated with 4 mL of trifluoroacetic acid. After stirring for 1 h, the mixture was concentrated to afford *exo*-*N*-((1R,3S,5S)-9-azabicyclo[3.3.1]nonan-3-yl)-3,4,5-trimethoxybenzamide (**A**), as a colorless oil. The material was understood to be the trifluoroacetate salt, partly

contaminated with residual trifluoroacetic acid, and was carried forward without purification. ^1H NMR (500 MHz, CDCl_3) δ 7.08 (s, 2H), 5.10 (dd, $J = 13.3, 6.4$ Hz, 1H), 3.98 – 3.90 (m., 2H), 3.93 (s, 9H), 3.24 (qd, $J = 7.3, 4.8$ Hz, 1H), 2.50 – 2.27 (m, 4H), 2.27 – 2.03 (m, 4H), 2.03 – 1.85 (m, 1H). MS (ESI+ve): Calculated for $\text{C}_{18}\text{H}_{27}\text{N}_2\text{O}_4$, $[\text{M}+\text{H}] = 335.41$, observed $[\text{M}+\text{H}] = 335.22$.

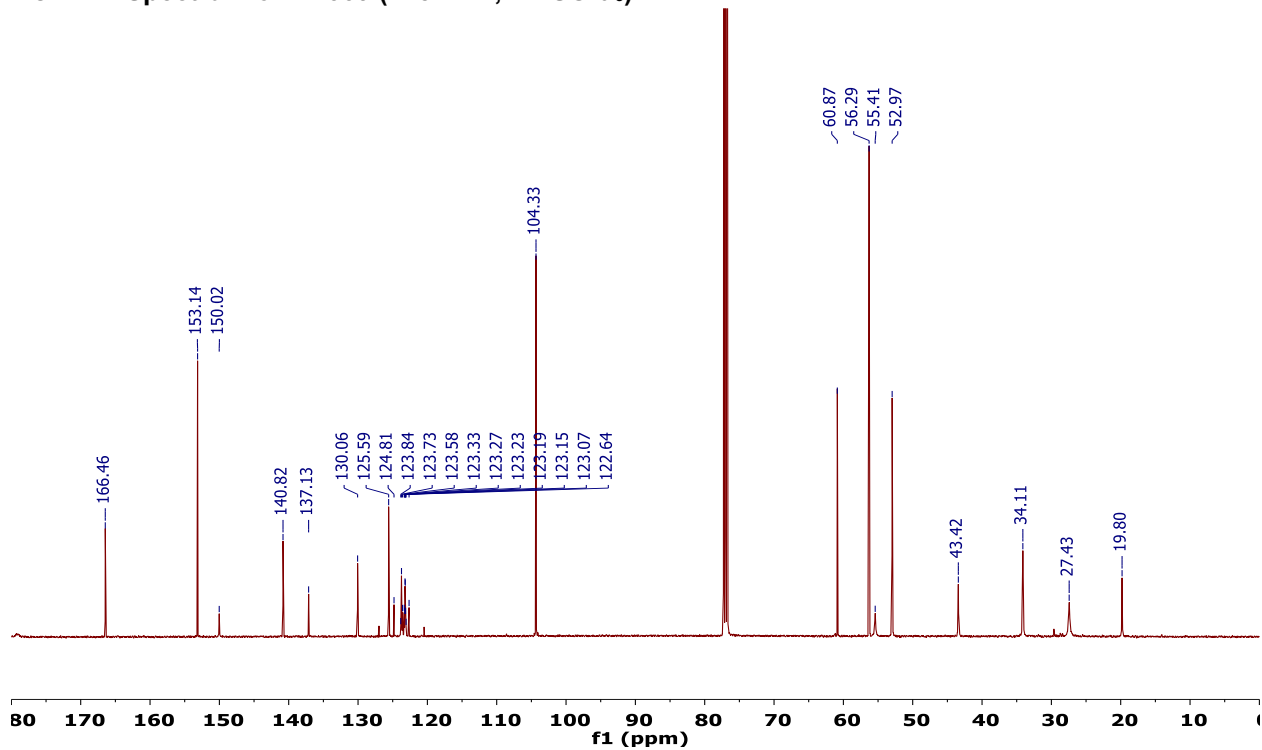
exo-N-((1R,3S,5S)-9-azabicyclo[3.3.1]nonan-3-yl)-3,4,5-trimethoxybenzamide (**A**) from the previous step (max 25 mg, 58 μmol) was dissolved in 1.5 mL of acetonitrile before charging with K_2CO_3 (24 mg, 174 μmol) and 2-(chloromethyl)-4-(trifluoromethyl)benzo[d]thiazole (CAS #1363380-67-9, 15 mg, 61 μmol). After stirring for 5 h at 70 $^\circ\text{C}$, the mixture was partitioned between water and ethyl acetate. The volatile organics were evaporated under vacuum and the residue was purified via preparative silica gel TLC, eluting with 0-40% ethyl acetate / hexane, to afford 3,4,5-trimethoxy-*N*-((1R,3s,5S)-9-((4-(trifluoromethyl)benzo[d]thiazol-2-yl)methyl)-9-azabicyclo[3.3.1]nonan-3-yl)benzamide, **81** as a white solid (21 mg, 66 % overall yield for the 3 step sequence). ^1H NMR (500 MHz, Chloroform-*d*) δ 8.07 (d, $J = 8.0$ Hz, 1H), 7.76 (d, $J = 7.6$ Hz, 1H), 7.44 (t, $J = 7.8$ Hz, 1H), 7.02 (s, 2H), 5.80 (d, $J = 8.0$ Hz, 1H), 5.01 – 4.89 (m, 1H), 4.33 (s, 2H), 3.96 (s, 6H), 3.92 (s, 3H), 3.16 (s, 2H), 2.20 – 1.98 (m, 5H), 1.90 (td, $J = 12.3, 5.0$ Hz, 2H), 1.84 – 1.77 (m, 1H), 1.75 – 1.66 (m, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ 166.45, 153.11, 150.00, 140.77, 137.10, 130.04, 126.97, 125.58 (CH), 124.80, 123.71 (CH), 123.53, 123.27, 123.19 (CH; q, $J_{\text{C-F}} = 4.8$ Hz), 123.02, 122.63, 120.46, 104.34 (CH), 60.85 (OCH₃), 56.25 (OCH₃), 55.36 (CH₂), 52.95 (CH), 43.41 (CH), 33.99 (CH₂), 27.47 (CH₂), 19.79 (CH₂). HRMS (ESI+ve): Calculated for $\text{C}_{27}\text{H}_{30}\text{F}_3\text{N}_3\text{O}_4\text{S}$, $[\text{M}+\text{Na}] = 572.1807$, observed $[\text{M}+\text{Na}] = 572.1786$.

Spectra of compound 81

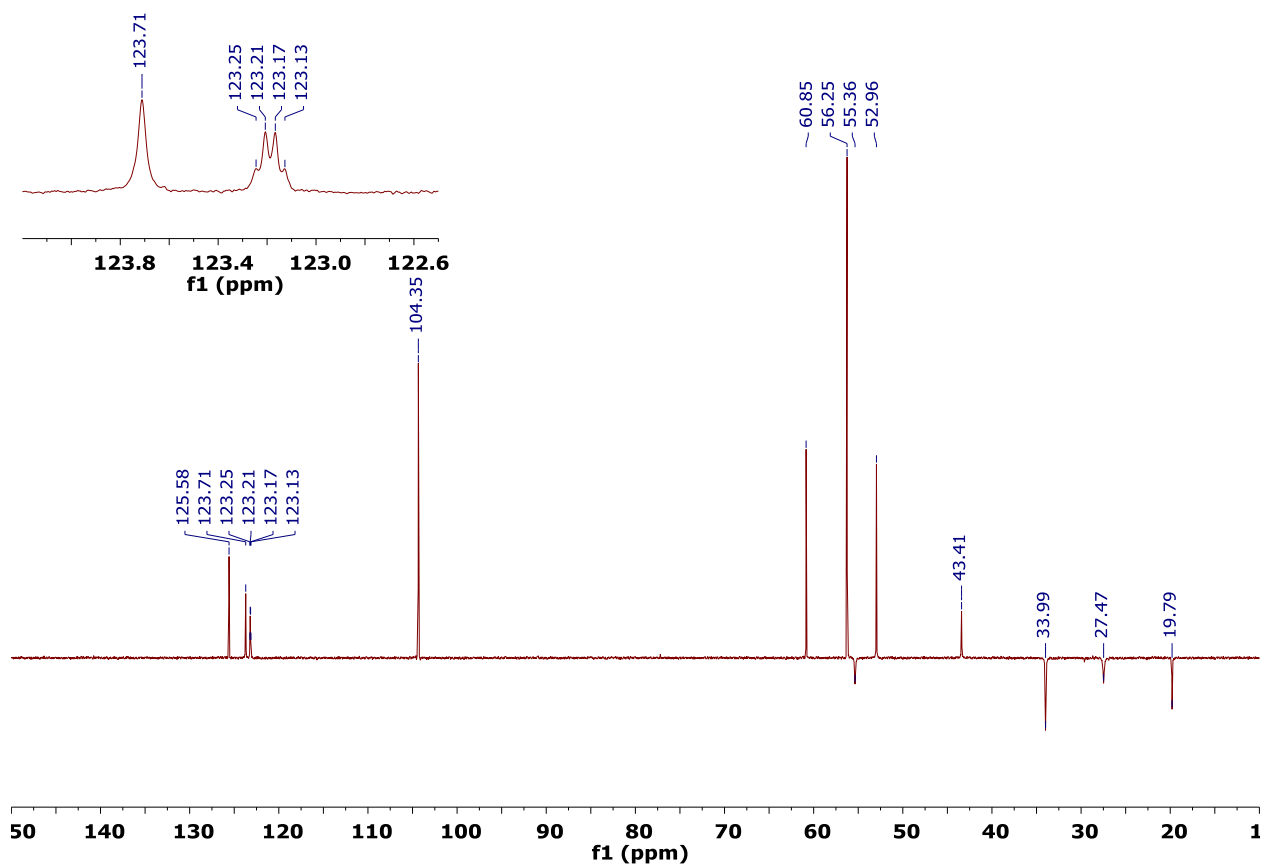
^1H NMR Spectrum of compound 81 (500 MHz, Chloroform- d):

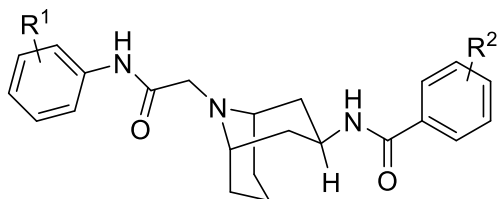


^{13}C NMR Spectrum of ML339 (125 MHz, DMSO- d_6):



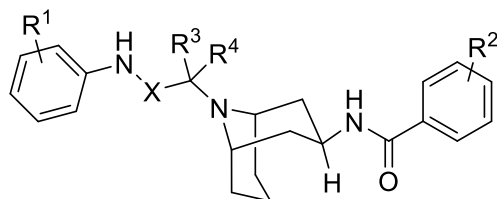
DEPT 135 (CH, CH3 up; CH2 down)





Supplementary Table 1. SAR studies of monomethoxy substituents and electron-withdrawing groups

Entry	R ¹	R ²	CXCR6:β-arrestin		CXCR6: cAMP	
			IC ₅₀ (μM)	% response	IC ₅₀ (μM)	% response
37	2-Cl	2-OMe	>50	NA	15.1	103
38	2-Cl	3-OMe	>50	NA	33.4	60
39	2-Cl	4-OMe	>50	NA	43.7	50
40	H	2-OMe	>40	NA	50.0	48
41	2-F	2-OMe	>40	NA	39.6	64
42	2-Br	2-OMe	>40	NA	27.7	77
43	2-CF ₃	2-OMe	>40	NA	11.5	114
44	3-Cl	2-OMe	>40	NA	29.0	73
45	4-Cl	2-OMe	>40	NA	5.2	111
46	4-Cl	2-Me	>40	NA	6.1	95
47	4-Cl	2-F	>40	NA	5.9	106
48	4-Cl	2-Cl	>40	NA	4.2	96
49	4-Cl	3-Cl	>40	NA	3.5	93

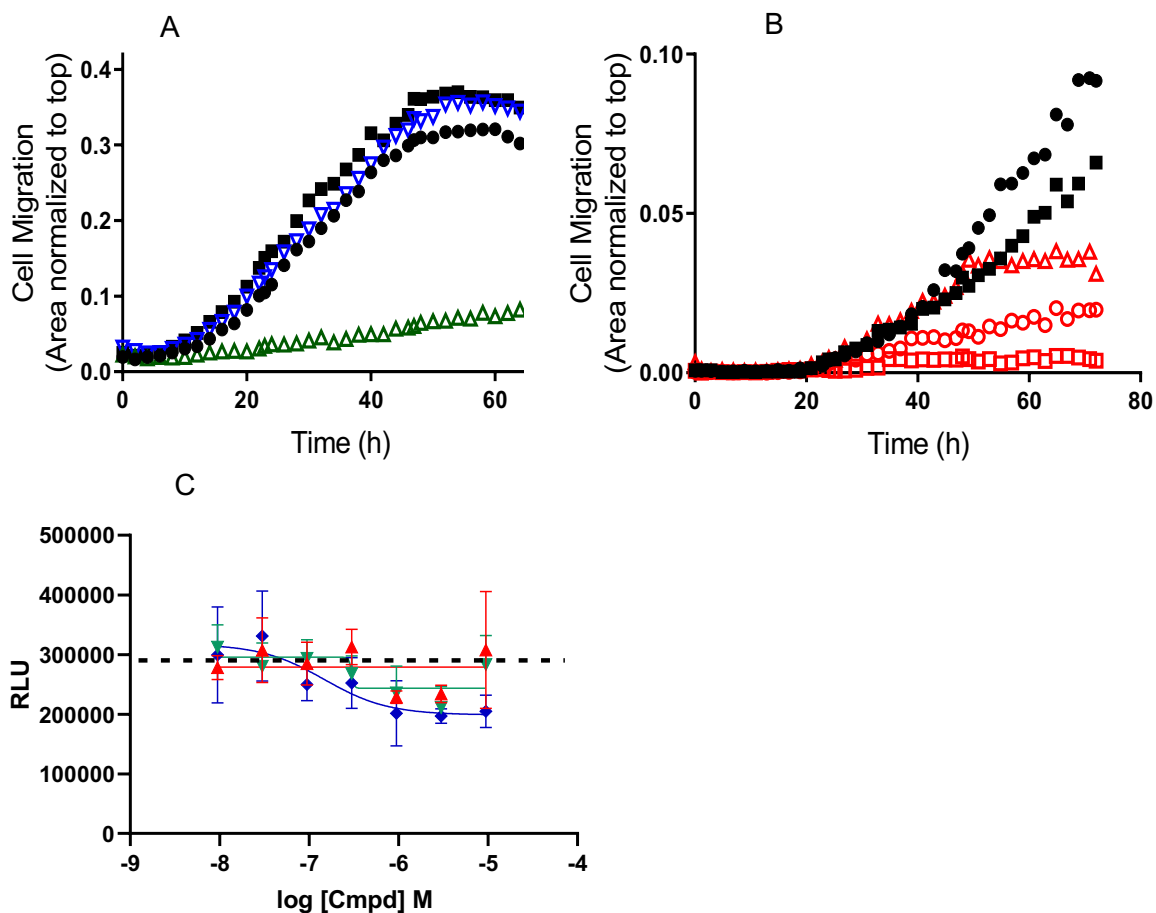


Supplementary Table 2: SAR studies of the glycine linker

Entry	R ¹	R ²	R ³	R ⁴	X	CXCR6: β -arrestin		CXCR6: cAMP	
						IC ₅₀ (μ M)	E _{max} (%)	IC ₅₀ (μ M)	E _{max} (%)
50	2-Cl	3,4,5-trimethoxy	H	H	CH ₂	26.5	64	>50	NA
51	2-Cl	3,4,5-trimethoxy	Me	Me	CO	>50	NA	12.3	76
52	2-Cl	2-OMe	Me	Me	CO	>40	NA	6.27	109.2
52	2-Cl	3-OMe	Me	Me	CO	>40	NA	40.4	58
53	2-Cl	4-OMe	Me	Me	CO	>40	NA	50.0	58
54	3-Cl	2-OMe	Me	Me	CO	>40	NA	>50	>40
55	4-Cl	2-OMe	Me	Me	CO	>40	NA	>50	>40

Supplementary Table 3. ADME profile of compound 81

Aqueous Solubility in 1x PBS, pH 7.4 ($\mu\text{g/mL}$) [μM] ^a		1.0 [1.82]
PAMPA Permeability , P_e ($\times 10^{-6}$ cm/s) Donor pH: 5.0 / 6.2 / 7.4 Acceptor pH: 7.4		7504/0/0
Plasma Protein Binding (% Bound)	Human 1 μM / 10 μM	99.8 / 99.7
	Mouse 1 μM / 10 μM	99.7 / 99.8
Plasma Stability (%Remaining at 3 hrs) Human/Mouse		100.3 / 91.6
Hepatic Microsome Stability (% Remaining at 1hr) Human/Mouse		0.2 / 0.3
Toxicity Towards Fa2N-4 Immortalized Human Hepatocytes LC_{50} (μM)		25.0



Supplementary Figure 1. Compound 73 and 81 inhibit migration of SK-HEP-1 cells.

SK-HEP-1 (100 k cells/well) migration over time in matrigel-coated transwell assay in the presence of A) media (circles), CXCL16 (squares) at 0.6 $\mu\text{g/ml}$, compound **73** (Table 3, green triangles) at 10 μM and compound **6** (Table 1, blue triangles) at 10 μM and B) in the presence of various concentrations of compound **81** (Table 3) at 2.5 (red triangles), 5 (red circles) and 10 (red squares) μM . C) Cell viability after incubation of SK-HEP-1 cells plated at (100 k cells/well) in 96 well in the presence of compound **73** (green triangles), **6** (blue diamonds), and **81** (red triangles) for 60 hr as determined by ATPLite luminescence. Dotted line denotes cells treated with DMSO only ($289,000 \pm 48,000$) for 100 % viability. No cells or 0% viability = $2,800 \pm 1,000$ RLU.

Materials and Methods

Reagents and Drug Treatment Protocol. Small molecule derivatives were synthesized and purified at the CPCCG of SBP Medical Discovery Institute as described. Synthesis of compound **81** is shown in Scheme S1 and outlined in Supplemental Material. ¹H NMR, ¹³C NMR and LC-MS spectra of compound **81** is included in Supplemental Material. Other starting compounds were purchased from Sigma-Aldrich (St. Louis, MO). All compounds were maintained as 10 mM DMSO stocks. To determine the effect of selected compounds and derivatives, cells were plated at the required density in 1536-well white, solid-bottom, tissue culture-treated plates (Corning; CLS3727) and grown overnight at 37 °C; 5%CO₂. Test compounds at different concentrations were added by acoustic dispensing (ECHO 555 liquid handler, Labcyte, INC, San Jose, CA) to the plates. DMSO concentration was kept constant across all wells and did not exceed 0.3%.

Cell Culture. Human and mouse cell lines expressing CXCR6 receptor were cultured in growth media consisting of F12 Ham's medium (Invitrogen; #11765-054) supplemented with 10% Fetal Bovine Serum (HyClone; SH30396.03), 100 IU penicillin, 100 mg/ml streptomycin sulfate (Invitrogen; #15140122), 1% L-Glutamine (Invitrogen; #25030081), 800 ug/mL G418 (ThermoFisher; #10131035) and 300 ug/mL Hygromycin B (Invitrogen; #10687-010). SK-HEP-1 cell line (ATCC HTB-52) were maintained as a monolayer culture in Eagle's Minimum Essential Medium (Corning; MT10009CV) supplemented with 10% Fetal Bovine Serum. Cell cultures were maintained in cell culture incubator at 37 °C in 5% CO₂ and were routinely subcultured twice weekly by trypsin-EDTA treatment (0.05% Trypsin-EDTA). The cells in an exponential growth phase were harvested and counted for cell signaling assays or tumor inoculation.

B-Arrestin Activity. CXCR6 β -arrestin cells (DiscoverRx; Cat#93-0250C2) were resuspended in assay media (growth media without G418 or Hygromycin B) at a density of 2.7×10^5 cells/mL and 3 μ L were plated in culture plates (800 cells/well) and grown overnight at 37 °C; 5% CO₂ covered with Kalypsys metal lids. 40 nL test compounds in DMSO were added to cells using ECHO acoustic dispenser. CXCL16 (R&D Systems; 976-cx) was prepared as stock solution at 4.9 μ M in PBS with 0.1% BSA. CXCL16 stock was diluted in assay media to 25 nM and 2 μ L was added to columns 3-4. 2 μ L media only was dispensed to columns 1-2. Plates were spun at 1000 rpm for 30 sec and incubated for 90 min at 37 °C; 5% CO₂ covered with Kalypsys lids. 3 μ L PathHunter Detection Reagent (DiscoverRx; #93-0001) prepared according to manufacturer's protocol were added to all wells and luminescence signal was detected on an EnVision detector (PerkinElmer). Wells treated with 0.1% DMSO only serve as negative controls (columns 1 + 2) and wells treated with DMSO and 20 nM CXCL16 (columns 3 + 4) serve as positive controls.

cAMP Production. Antagonism of CXCR6-mediated inhibition of cAMP production was measured in CXCR6 β -arrestin cells (DiscoverRx; #93-0250C2) using Lance Ultra cAMP assay (Perkin Elmer; TRF0263). Cells were suspended in assay media (OptiMEM; 2% FBS; 1X pen/strep) at a cell density of 2.5×10^5 cells/mL and 2 μ L (500 cells/well) were plated to all wells and grown overnight. 40nL test compounds are added to cells. 2 μ L 2.5mM forskolin prepared in stimulation buffer (HBSS; 5mM HEPES; 0.1% BSA; 0.1mM RO-20-1724) with or without 20nM CXCL16 transferred to wells using BioRAPTR. Wells treated with forskolin and CXCL16 serve as negative controls (columns 3-4) and wells treated with forskolin only serve as positive controls (columns 1-2). Cells are incubated

at room temperature for 30 min followed by addition of 2 μ L each EU-cAMP tracer and *ULight* anti-cAMP Ab prepared in detection buffer according to manufacturer's instruction. After 30 min at room temperature, TR-FRET was measured using an EnVision detector.

Migration Assay. SK-HEP-1 cells were plated at 100k/well cells in 0.2% fetal bovine serum in the upper chamber of a 96-well ClearView Chemotaxis Plate (InCucyte, Ann Arbor, MI) pre-coated with 50 μ l/well matrigel (250 μ g/ml) (BD Biosciences, San Jose, CA). CXCL16 was added to the lower chamber and test compounds were added to the upper and lower chambers. Plates were allowed to equilibrate at 37 °C in 5% CO₂ within the IncuCyte® for a minimum of 15 minutes before the first scan. The software was set to scan the experiment every hour. The data was analyzed by confluence calculated by custom algorithms that are part of the IncuCyte™ software package. For ZOOM Processing, an image collection was created using three to five representative phase contrast images. A cell type-specific Processing Definition was then used to analyze experimental data.

Microsome Stability Assay. Hepatic microsomes stability assays were performed as reported before¹. Briefly, 3 μ L of 25 μ M compound solution in acetonitrile were incubated with 123 μ L of mouse, human or rat liver microsomes (Xenotech, Kansas City, KS). After preincubation at 37 °C for 10 min, enzyme reactions were initiated by adding 120 μ L of NADPH-generating system (2 mM Nicotinamide adenine dinucleotide phosphate (NADP⁺), 10 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 5 mM MgCl₂) in the presence of 100 mM potassium phosphate buffer (pH 7.4). The final concentration of each model compound used was 1 μ M. The microsomal concentrations used were 1.0 mg/mL. Compounds were incubated in microsomes for 0,

5, 15, 30 and 60 min. Cytotoxicity Fa2N-4 immortalized human hepatocytes (Xenotech) or HREC cells were grown to subconfluency in wells of a 96-well plate and exposed to different concentrations of compounds for 24 h after which the cells were washed and the media and compounds replaced. The cells were returned to the incubator for another 24 h. The extent of cell death (cell lysis) was determined by ATP-Lite reagent (Perkin-Elmer).

Pharmacokinetic Studies. Mice were manipulated and housed according to the protocols approved by the Shanghai Medical Experimental Animal Care Commission. DMPK Studies were performed at WuXi AppTec (Shanghai) Co., Ltd. The test article was accurately weighed and mixed with DMSO:Tween80:Water (1:1:8) and sonicated. The formulation was prepared fresh daily and animals (3 in each group, Male C57BL/6, 7-9 weeks) were dosed 2 hr after the formulation was prepared. Aliquots of each formulation was dose validated by LC/MS/MS. PO dosing was administered via oral gavage. The dose volume was determined by the animals' body weight collected on the morning of the dosing day. Serial bleeding (about 30 μ l blood per time point) was collected from submandibular or saphenous vein. All blood samples were transferred to microcentrifuge tube containing 2 μ l of K₂EDTA (0.5 M) as anti-coagulant and placed on ice until processed for plasma. Blood samples were processed for plasma by centrifugation at 3000 g, quick frozed and stored -70 °C until LC/MS analysis.

Tumor Model. Altogen Labs received IACUC study LC03279 protocol approval on April 28, 2017 (IACUC protocol 4-05752). Twenty (20) NU(NCr)-Foxn1nu nude mice were purchased from the Harlan Laboratories (ENVIGO) and went through mandatory 1-week acclimatization per IACUC guidelines. All animal procedures and maintenance were conducted in accordance with the institutional guidelines. Mice were housed at Altogen

Labs animal facility and carcasses frozen and disposed at the end of the study per institutional guidelines. Each mouse was inoculated subcutaneously at the flank region with SK-HEP-1 tumor cells (1.0×10^7) in 0.1 ml of 1X PBS mixed with Matrigel (1:1) for tumor development (Day 1). The treatments were started on Day 4 (5 animals in each group, female NOD/SCID, 8-10 weeks). The test article was accurately weighed and mixed with DMSO:Tween80:Water (1:1:8) and sonicated. The date of tumor cell inoculation is denoted as day 1. After tumor cells inoculation, the animals were checked daily for morbidity and mortality. At the time of routine monitoring, the animals were checked for any adverse effects of tumor growth and treatments on normal behavior such as mobility, visual estimation of food and water consumption, body weight gain/loss, eye/hair matting and any other abnormal effects. There was no animal death and no clinical signs or behavioral phenotype observed within the study. No BWL > 20% were observed in any of the groups. Tumor volumes were measured two times a week in two dimensions using an electronic caliper, and the volume data are expressed in mm^3 using the formula: $V = 0.5 a \times b^2$ where a and b are the long and short diameters of the tumor, respectively. Tumor tissues were harvested, fixed in 10% neutral buffered formalin and stored at 4°C . Dosing and tumor volume measurement procedures were conducted in a Laminar Flow Cabinet according to Altogen Labs IACUC regulations.

Curve Fit and Statistical Analysis. All concentration response curves were analyzed to determine EC_{50} and E_{max} using the following equation:

$$Y = \frac{100}{1 + 10^{\frac{(\text{LogEC}_{50} - X)}{\times \text{Hillslope}}}}$$

where Y = the normalized response (0–100%), X is the log of concentration of compound tested, and the Hill slope is set equal to 1. 0% Activity refers to activity induced by EC80 concentration of CXCL16 alone; 100% Activity refers to wells untreated with CXCL16 ligand. $Z' > 0.5$ was observed between 0% and 100% control wells (64 wells each). All experiments were repeated at least twice on different days from different stock dilutions. Results are averaged pIC_{50} values and standard deviations. Non-linear regression curve fit and statistical analyses was performed using Prism 7 (GraphPad Software, Inc., La Jolla, CA). For *in vivo* results, the mean and the standard error of the mean (SEM), are provided for the tumor volume of each group at each time point. Statistical analysis of difference in tumor volume among the groups and the analysis of drug interaction were conducted on the data obtained after the final dose.

REFERENCES

1. Peddibhotla S, Hedrick MP, Hershberger P, et al. Discovery of ML314, a Brain Penetrant Non-Peptidic β -Arrestin Biased Agonist of the Neurotensin NTR1 Receptor. *ACS Med Chem Lett.* 2013;4(9): 846-851.