Identification of Pirin as a Molecular Target of the CCG-1423/CCG-203971 Series of Anti-Fibrotic and Anti-Metastatic Compounds

Erika M. Lisabeth[1], Dylan Kahl[2], Indiwari Gopallawa[1], Sarah E. Haynes[3], Sean A. Misek[1], Phillip L. Campbell[4], Thomas S. Dexheimer[1], Dinesh Khanna[4], David A. Fox[4], Xiangshu Jin[5], Brent R. Martin[3], Scott D. Larsen[2,6] Richard R. Neubig[1*]

[1]Department of Pharmacology & Toxicology, [5]Department of Biochemistry, Michigan State University, East Lansing, MI, USA [2]Department of Medicinal Chemistry, [3]Department of Chemistry [4]Department of Internal Medicine, Division of Rheumatology, [6]Vahlteich Medicinal Chemistry Core, College of Pharmacy, University of Michigan, Ann Arbor, MI, USA

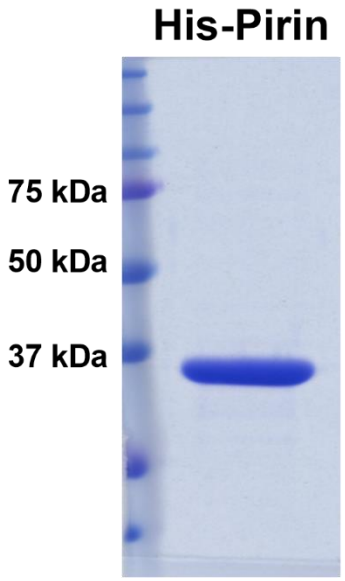
*to whom correspondence should be addressed

Supplementary Materials Table of Contents

40 pages

I. Supplemental Figures

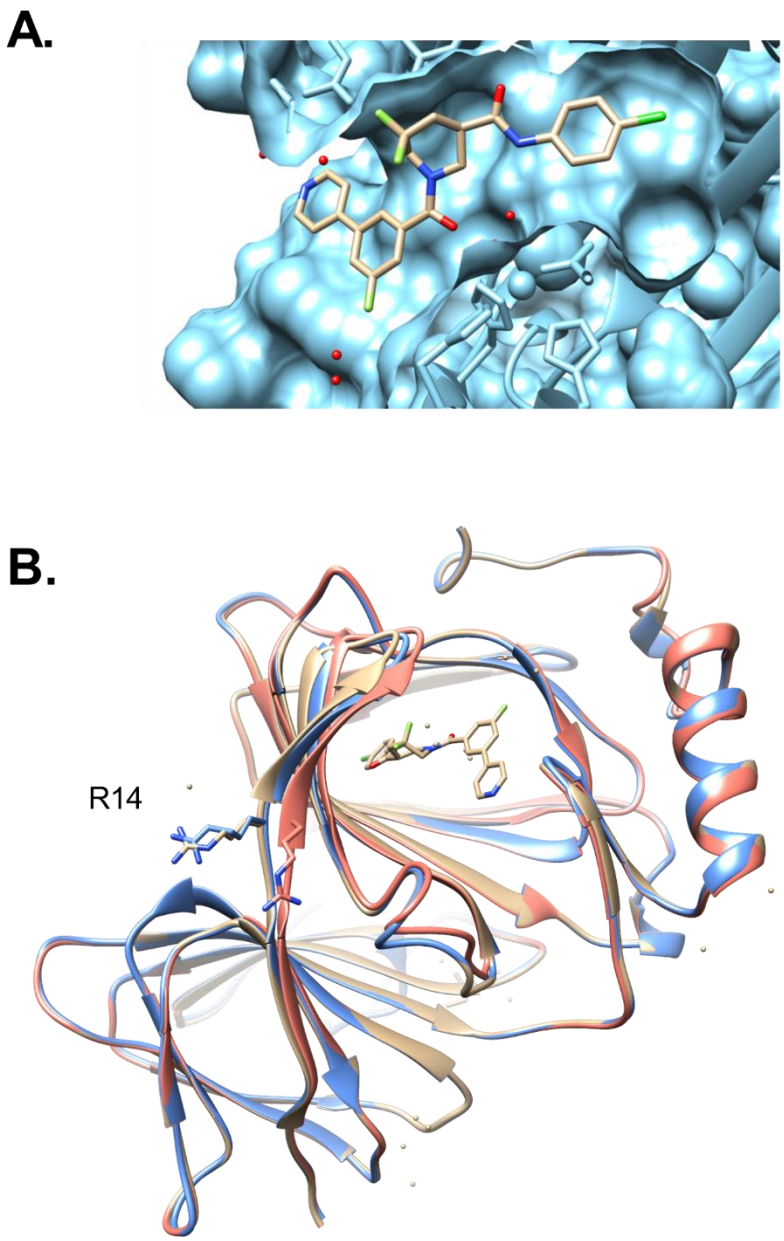
Supplemental Figure 1.



Supplemental Figure 1. Coomassie gel of recombinant His-pirin.

10 μg of purified pirin was loaded onto a 12% SDS-PAGE and was shown to be >90% pure by Coomassie staining

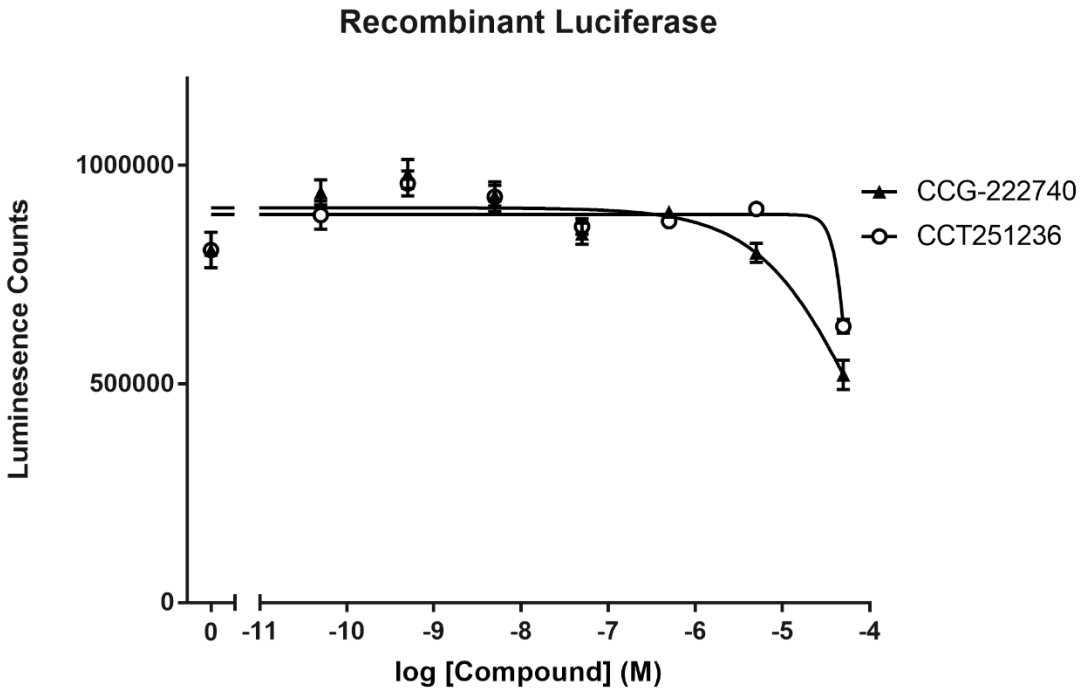
Supplemental Figure 2.



Supplemental Figure 2. CCG-257081 Bound Pirin is Similar to Inactive Pirin

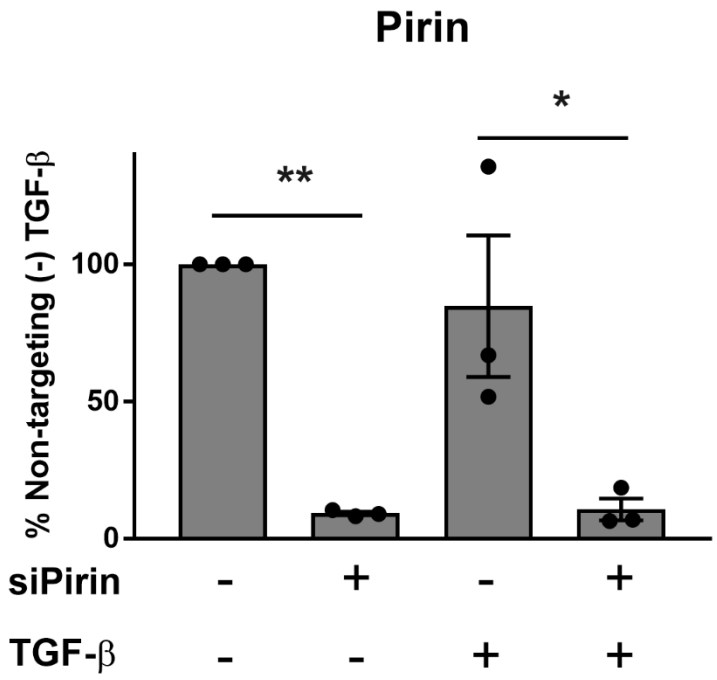
(A) Surface rendering of pirin and the groove where CCG-257081 binds (B) Structural alignment between inactive pirin (Fe^{2+} bound, PDB ID: 1j1l, blue), active pirin (Fe^{3+} bound, PDB ID: 4gul, red) and CCG-257081 bound pirin (PDB TBD, tan). Overall, the structures align quite well, but the β2 sheet in the CCG-257081 bound pirin structure more closely aligns with the inactive pirin, rather than the active pirin, as well as R14.

Supplemental Figure 3.



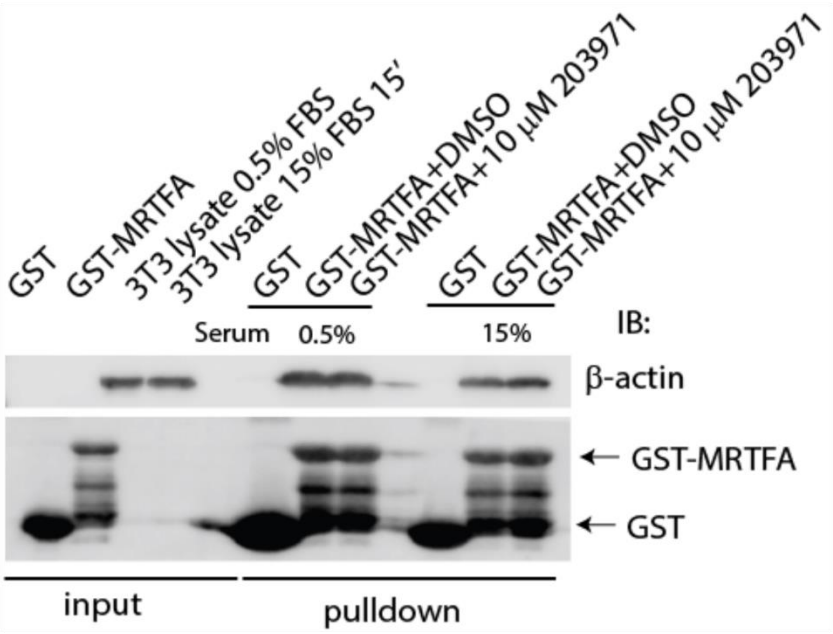
Supplemental Figure 3. CCG-222740 and CCT251236 have minimal effects on recombinant luciferase activity. Recombinant firefly luciferase was incubated with varying concentrations of CCG-222740 or CCT251236. Neither inhibited luciferase activity with 100% efficacy, and CCG-222740 both have an $IC_{50}>10^{-4}$ M. n=2

Supplemental Figure 4.



Supplemental Figure 4. Pirin mRNA is reduced in siPirin primary dermal fibroblasts. qPCR analysis of pirin mRNA showed that they were reduced approximately 90% after siRNA targeting pirin treatment. Data are expressed as an overall mean ± SEM (bar graphs) as well as individual values (circles) *p<0.05, **p<0.01 as determined by One-way ANOVA with Tukey's multiple comparisons test (n=3)

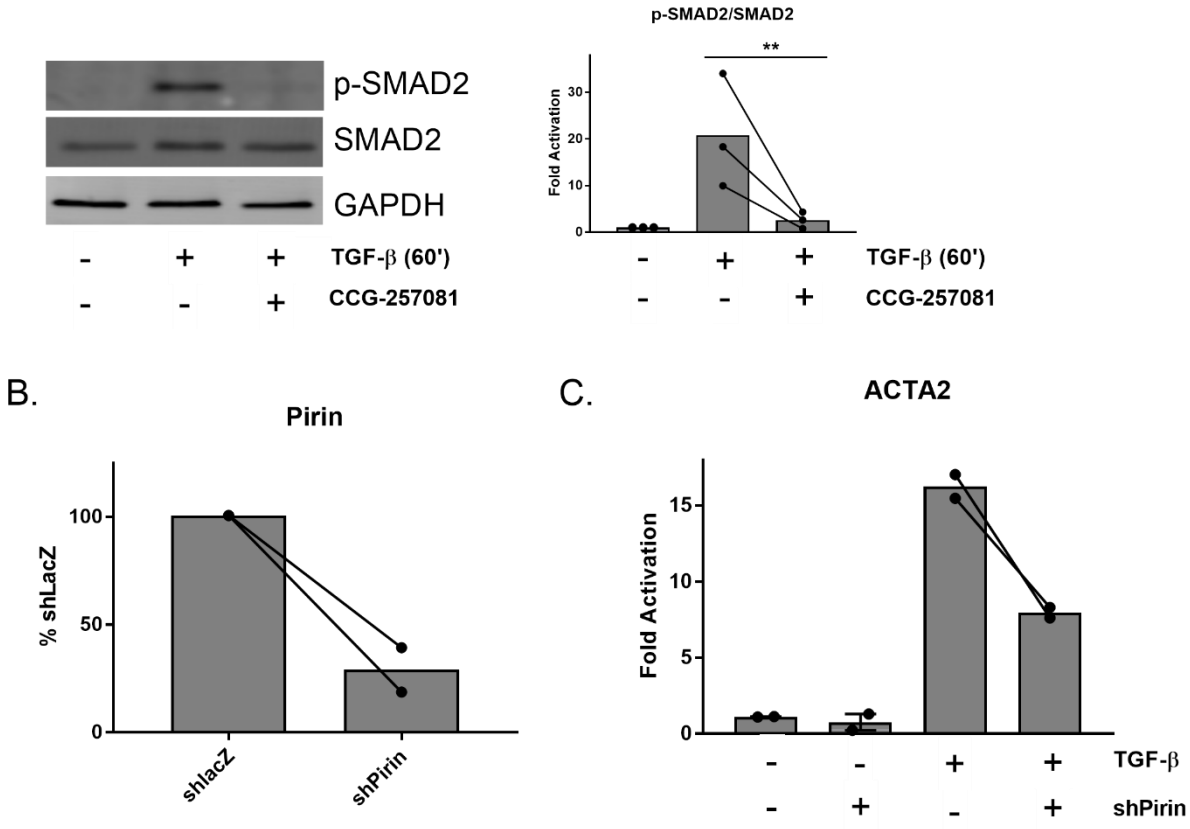
Supplemental Figure 5.



Supplemental Figure 5. CCG-203971 does not disrupt the MRTFA-RPEL:actin interaction

GST-MRTFA (AA 10-142), was used to pull down actin from 3T3 cells in the presence or absence of serum stimulation. During the pulldown, 10 μM 203971 was added to the binding reaction, but the amount of actin pulled down did not decrease significantly, suggesting that these compounds do not directly bind the RPEL domain of MRTFA.

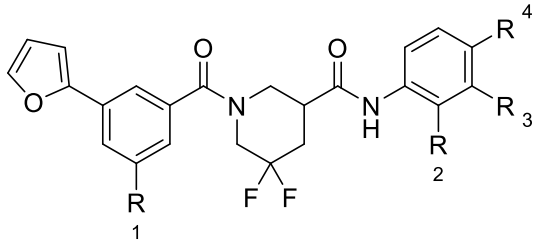
Supplemental Figure 6.



Supplemental Figure 6. CCG compounds and pirin affect the TGF-β signaling pathway. (A) Primary human dermal fibroblasts were pretreated with 10 μM CCG-257081 and then stimulated with 10 ng/mL TGF-β for 60'. Phospho-SMAD2 levels were determined by western blot (left panel) and quantified (right panel). Results are expressed as the overall mean (bar graphs) as well as individual paired mean values, n=3, **p<0.01 as determined by a ratio paired t-test. (B) Determination of pirin mRNA levels after expression of shRNA against pirin determined by qPCR. Results are expressed as the overall mean (bar graph) as well as mean experimental values, normalized to the shLacZ control, n=2 (C) Primary human dermal fibroblasts expressing shLacZ or shPirin were stimulated with 10 ng/mL TGF-β for 24 hours, and then ACTA2 mRNA levels were determined by qPCR. Results are expressed as the overall mean (bar graph) as well as individual experimental mean values, n=2.

II. Supplemental Tables

Supplemental Table I.



Compound	R1	R2	R3	R4	SRE.L IC50 (uM)	Max Efficacy
CCG-222740	H	H	H	Cl	0.4	100
1a	OMe	H	H	Cl	2	100
1b	H	OMe	H	Cl	0.83	100
1c	H	H	OMe	Cl	1.1	100
1d	H	H	Cl	OMe	0.58	88
1e	H	H	H	OMe	5.6	100
2a	$O(CH_2)_2OMe$	H	H	Cl	2.3	90
2b	H	$O(CH_2)_2OMe$	H	Cl	2.5	68
2c	H	H	$O(CH_2)_2OMe$	Cl	1.6	99

Supplemental Table I: Exemplary probe development SAR.

Supplemental Table II

Excel file of proteomic results.

Supplemental Table III.

Data collection and refinement statistics

Data collection		
	Compound 257081	Compound 222740
Resolution range	35.75 - 1.46 (1.51 - 1.46)	29.86 - 1.79 (1.83 - 1.79)
Space group	P $2_12_12_1$	P $2_12_12_1$
Unit cell	42.25 67.10 107.26 90 90 90	42.36 67.54 107.65 90 90 90
Total reflections	333430 (28611)	173434 (55946)
Unique reflections	53779 (5202)	34483 (1695)
Multiplicity	6.2 (5.5)	5.0 (5.0)
Completeness (%)	99.57 (97.87)	99.1 (100)
Mean I/sigma(I)	22.7 (2.0)	24.7 (3.1)
Wilson B-factor	16.41	22.81
R-merge	0.077 (0.496)	0.092 (0.589)
R-meas	0.084 (0.548)	0.103 (0.66)
R-pim	0.033 (0.229)	0.044 (0.291)
Refinement		
Reflections used in refinement	53776 (5202)	34449 (3220)
Reflections used for R-free	2709 (277)	1760 (184)
R-work	0.1774 (0.2610)	0.1709 (0.2292)
R-free	0.2089 (0.2929)	0.2052 (0.2837)
Number of non-hydrogen atoms	2713	2565
macromolecules	2259	2251
ligands	50	40
solvent	404	274
Protein residues	288	288
RMS(bonds)	0.006	0.007
RMS(angles)	0.88	0.84
Average B-factor	20.61	25.95
macromolecules	18.70	24.90
ligands	26.12	30.13
solvent	30.62	33.99

Statistics for the highest-resolution shell are shown in parentheses.

Supplemental Table IV.

Intersection between CCG-1423 and siPirin	
TOP2A	Topoisomerase
CDK1	Cyclin dependent kinase 1
KIF20A	Kinesin Family Member 20A
DLGAP5	Discs Large Homolog Associated Protein
SGOL2	Shugoshin-2
NDC80	NDC80, Kinetochore Complex Component)
TTK	a key spindle assembly checkpoint protein
DTL	Denticleless protein homolog
PBK	PDZ binding kinase
ANLN	Anillin Actin Binding Protein
NUF2	Kinetochore protein
FAM83D	Family With Sequence Similarity 83 Member D
CASC5	Kinetochore Scaffold 1
NUSAP1	Nucleolar And Spindle Associated Protein 1
KIF15	Kinesin Family Member 15
SHCBP1	SHC Binding And Spindle Associated 1
CENPU	Centromere Protein U
BRIP1	RecQ DEAH helicase family and interacts with the BRCT repeats of breast cancer, type 1 (BRCA1).
SPC25	SPC25, NDC80 Kinetochore Complex Component
CEP55	Centrosomal Protein 55
NCAPG	Non-SMC Condensin I Complex Subunit G,
ZNF367	Zinc Finger Protein 367,
NEK2	NIMA (Never In Mitosis Gene A)-Related Kinase 2
BIRC5	Baculoviral IAP Repeat Containing 5
KIF11	Kinesin Family Member 11
BUB1	BUB1 Mitotic Checkpoint Serine/Threonine Kinase
OIP5	Opa Interacting Protein 5
TK1	Thymidine Kinase 1
HJURP	Holliday Junction Recognition Protein
POLE2	DNA Polymerase Epsilon 2
CENPA	Centromere Protein A
AURKB	Aurora Kinase B
CDCA7	Cell Division Cycle Associated 7
CCNA2	Cyclin A2
MAD2L1	Mitotic Arrest Deficient 2 Like 1
HMMR	Hyaluronan Mediated Motility Receptor

GINS1	DNA Replication Complex GINS Protein PSF1
CCNB2	Cyclin B2
ZWINT	ZW10 Interacting Kinetochore Protein,
RFC3	Replication Factor C Subunit 3
KIF2C	Kinesin Family Member 2C
CENPE	Centromere Protein E
CDCA3	Cell Division Cycle Associated 3
CDKN3	Cyclin Dependent Kinase Inhibitor 3

A list of the 44 genes that are shared between the top 100 downregulated genes (based on fold change) upon CCG-1423 treatment in PC3 cells (GSE30188) and after siPirin treatment in WM-266 cells (GSE17551).

Supplemental Table V.

Gene signatures tested and overlap with CCG-1423 gene signature. Excel File attached.

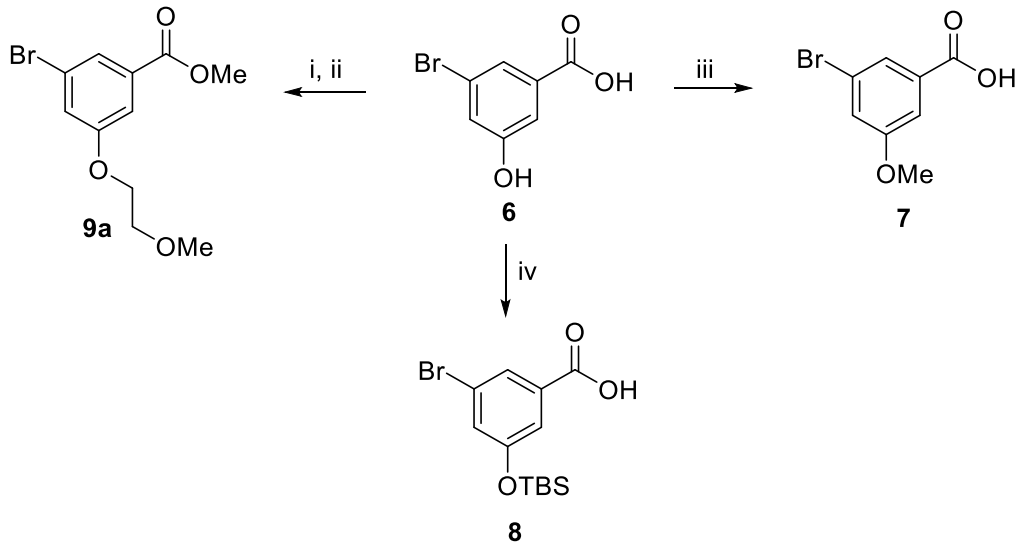
Supplemental Table VI.

Intersection between CCG-1423 and MRTF	
KIF11	Kinesin Family Member 11
BUB1	BUB1 Mitotic Checkpoint Serine/Threonine Kinase
KIF23	Kinesin Family Member 23
TOP2A	Topoisomerase
HMMR	Hyaluronan Mediated Motility Receptor
NUASP1	Nucleolar And Spindle Associated Protein 1
PBK	PDZ Binding Kinase
TK1	Thymidine Kinase 1
CENPF	Centromere Protein F
CDKN3	Cyclin Dependent Kinase Inhibitor 3
CCNB2	Cyclin B2
TTK	a key spindle assembly checkpoint protein
TPX2	TPX2, Microtubule Nucleation Factor
UBE2C	Ubiquitin Conjugating Enzyme E2 C
NEK2	NIMA Related Kinase 2
CENPA	Centromere Protein A
KIF2C	Kinesin Family Member 2C
KIF20A	Kinesin Family Member 20A
BIRC5	Baculoviral IAP Repeat Containing 5
RRM	RNA recognition motif
AURKB	Aurora Kinase B

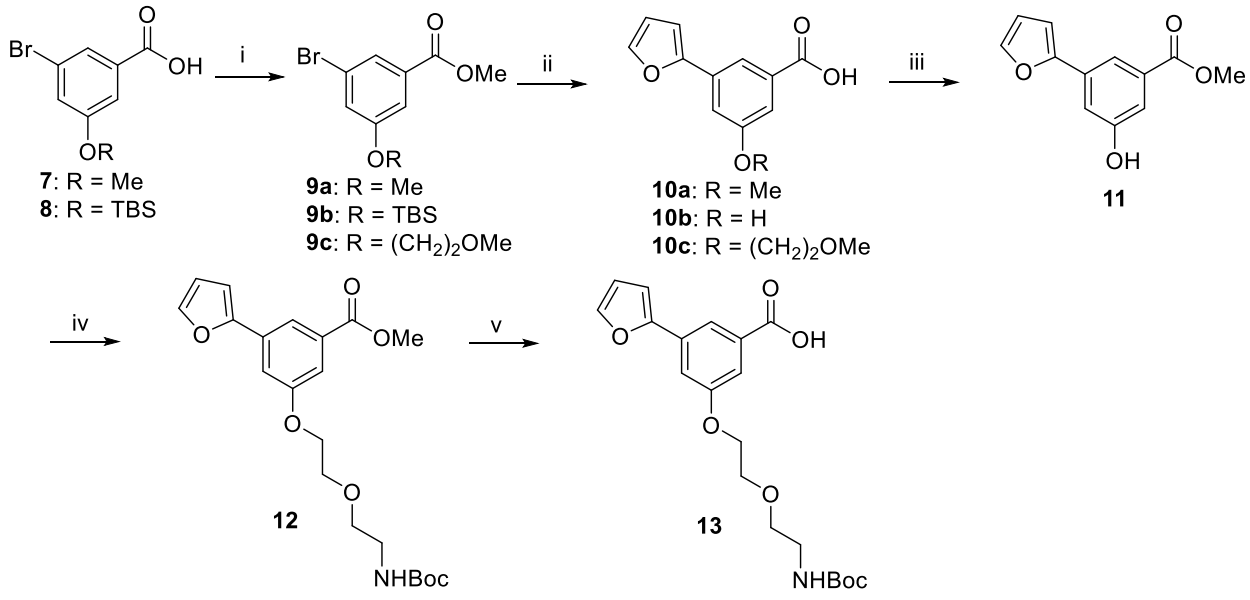
A list of the 21 genes that are shared between the top 100 downregulated genes (based upon fold change) between CCG-1423 treatment in PC3 cells (GSE30188) and the entire MRTF gene signature.

III. Materials and Methods

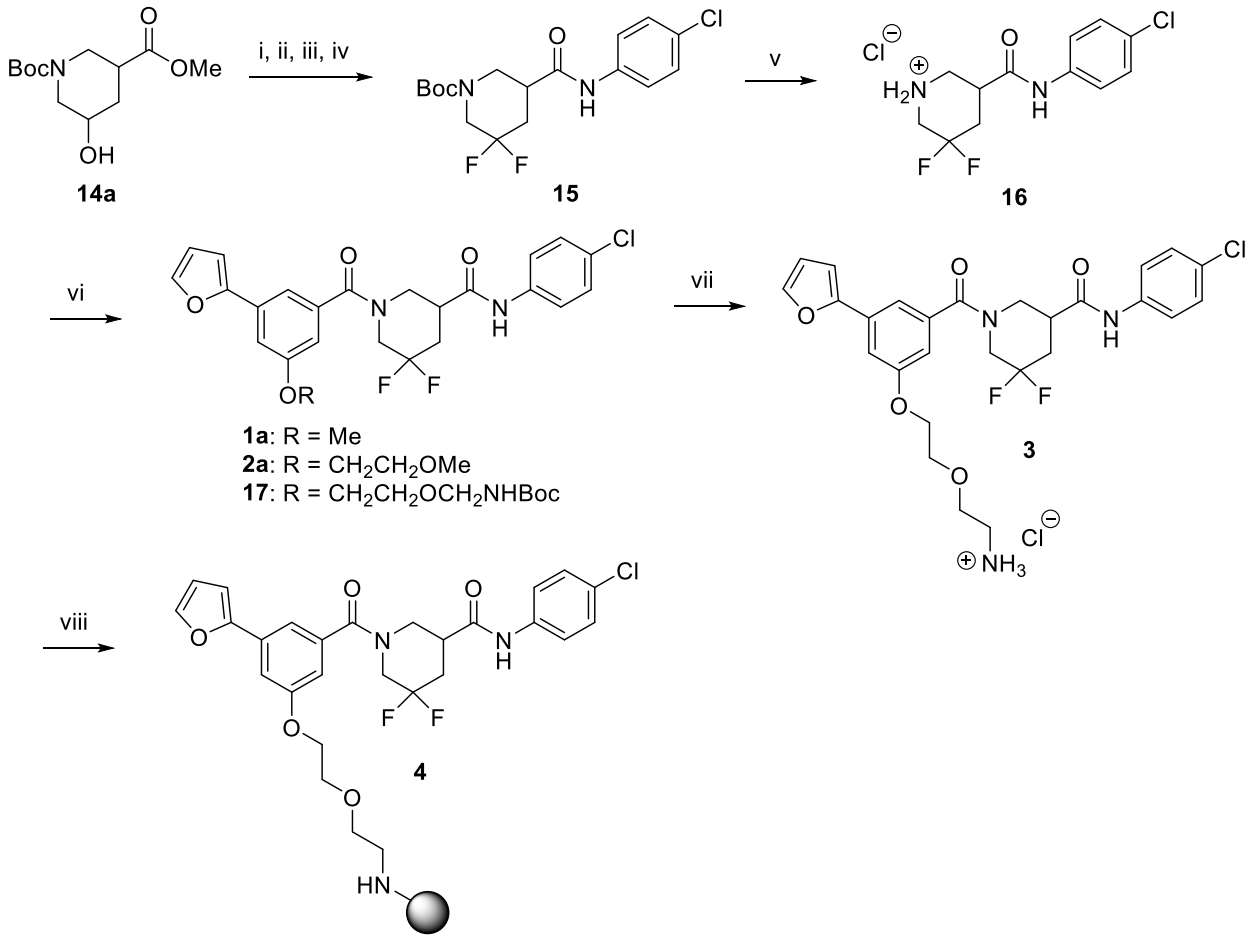
Synthesis of **1a**, **2a**, and **4**:



Scheme 1. Conditions: (i) H_2SO_4/MeOH/reflux; (ii) $BrCH_2$ CH_2OMe/K_2CO_3/DMF/90 °C[1]; (iii) Cs_2CO_3/MeI/DMF/rt[2]; (iv) TBS-Cl/imidazole/DMF/rt[3]

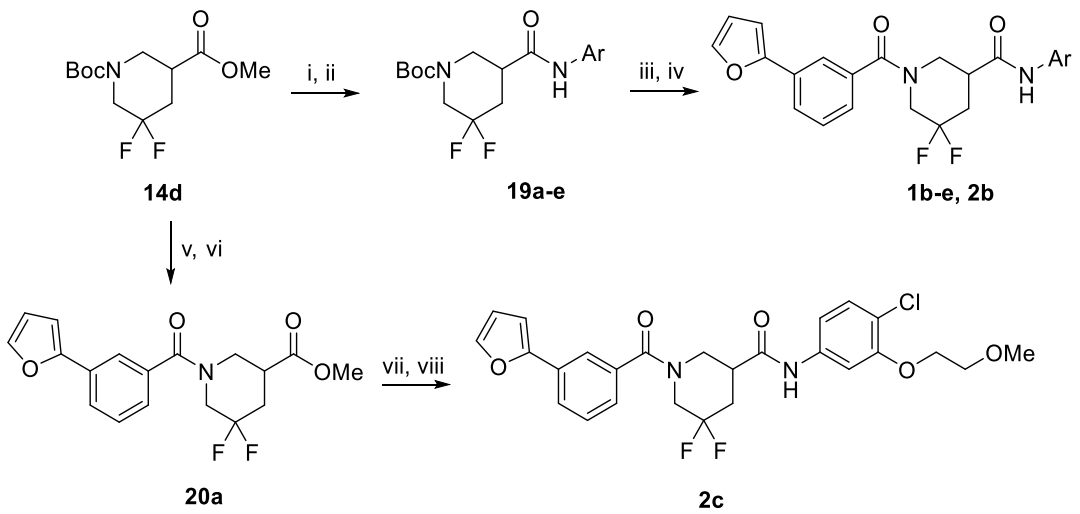


Scheme 2. Conditions: (i) H_2SO_4/MeOH/reflux; (ii) furan-2-ylboronic acid/Na_2CO_3/$Pd(PPh_3)_4$/H_2O/DME/100 °C; (iii) H_2SO_4/MeOH/reflux; (iv) $BocHN(CH_2)_2O(CH_2)_2OMs/Cs_2CO_3$/DMF/75 °C; (v) 1M NaOH/THF/rt.



Scheme 3. Conditions: (i) $(COCl)_2$/DMSO/DCM/-78 °C to rt; (ii) DAST/DCM/-78 °C to rt; (iii) 1M NaOH/THF/rt; (iv) HATU/4-chloroaniline/DIPEA/DMF/rt[4]; (v) 4M HCl in Dioxanes/rt; (vi) HATU/**10a,b** & **13**/DIPEA/DMF/rt; (vii) 4M HCl in Dioxanes/rt; (viii) NHS-activated agarose resin, DMSO, PBS, followed by ethanolamine.

Synthesis of **1b-e** and **2b,c**



Scheme 4. Conditions: (i) 1M NaOH/THF/rt; (ii) HATU/**18a-e**/DIPEA/DMF/rt; (iii) 4M HCl in Dioxanes/rt; (iv) HATU/3-(furan-2-yl)benzoic acid/DIPEA/DMF/rt; (v) 4M HCl in Dioxanes/rt; (vi) HATU/3-(furan-2-yl)benzoic acid/DIPEA/DMF/rt; (vii) 1M NaOH/THF/rt; (viii) HATU/**18f**/DIPEA/DMF/rt.

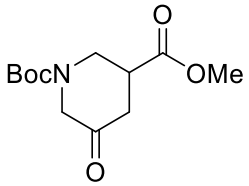
Experimentals:

Chemistry General Information: All reagents were used without further purification as received from commercial sources unless noted otherwise. ^{1}H NMR spectra were taken in DMSO-*d*6, MeOD, or $CDCl_3$ at room temperature on Varian Inova 400 MHz or Varian Inova 500 MHz instruments. Reported chemical shifts for the ^{1}H NMR spectra were recorded in parts per million (ppm) on the δ scale from an internal standard of residual tetramethylsilane (0 ppm). Mass spectrometry data were obtained on either a Micromass LCT or Agilent Q-TOF. An Agilent 1100 series HPLC with an Agilent Zorbax Eclipse Plus−C18 column was used to determine purity of biologically tested compounds. All tested compounds were determined to be >95% pure using a 6-minute gradient of 10-90% acetonitrile in water followed by a 2-minute hold at 90% acetonitrile with detection at 254 nm. Purification of some final compounds was performed using a Waters semipreparative HPLC with a Vydac protein and peptide C18 reverse phase column, using a linear gradient of 0% solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in water) to 100% solvent B in solvent A at a rate 1% per minute and monitoring UV absorbance at 230 nm.

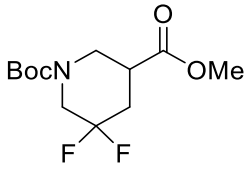
General Procedure for Methyl Ester Hydrolysis (Procedure A): In a round-bottomed flask, the desired methyl ester (1.15 mmol) was dissolved in THF (3 mL). 1 M NaOH (3 mL) was added and the reaction was stirred at 25 °C for 1 hr. The THF was evaporated *in vacuo,* and then the solution was acidified with 1 N HCl (7 mL). The product was extracted with EtOAc (3 x 20 mL), washed with brine (1 x 15 mL), dried with $MgSO_4$, and concentrated in vacuo. Unless otherwise noted, the residue was crystalized with EtOAc/Hex, producing a white precipitate that was filtered and dried under vacuum.

General Procedure for Amide Coupling (Procedure B): In a round bottomed flask, the carboxylic acid (0.19 mmol) was dissolved in DMF (2 mL), and HATU (0.38 mmol), desired aniline (0.28 mmol), and DIPEA (0.38 mmol) were added, respectively. The reaction was stirred at 25 °C for 16 hr, and then quenched with brine (10 mL). The product was extracted with EtOAc (3 x 15 mL), dried with $MgSO_4$, and concentrated in vacuo. The subsequent oil was subjected to silica gel chromatography eluting with 15% EtOAc: 85% Hex. The fractions containing product were concentrated in vacuo.

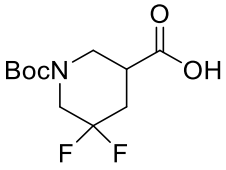
General Procedure for Suzuki Cross Coupling (Procedure C): In a round bottomed flask, methyl 3-bromo-5-methoxybenzoate (2.24 mmol), furan-2-ylboronic acid (2.69 mmol), Tetrakis® (0.11 mmol), and Na_2CO_3 (5.61 mmol) were dissolved in DME (10 mL) and H_2O (10 mL). The solution was degassed, and then heated at 100 °C for 16 hr under Ar_2. The solution was filtered through a pad of Celite® to remove particulates. The filtrate was acidified with 1N HCl (15 mL), and the product was extracted with EtOAc (3 x 15 mL), washed with brine (1 x 15 mL), dried with $MgSO_4$, and concentrated *in vacuo*. The subsequent oil was subjected to silica gel chromatography eluting with 5% MeOH: 95% DCM. The fractions containing product were concentrated in vacuo.



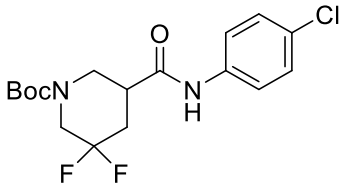
1-tert-butyl 3-methyl 5-oxopiperidine-1,3-dicarboxylate (14b). In a flame dried 50-mL round bottomed flask, $(COCl)_2$ (0.71 g, 0.49 mL, 5.6 mmol) was dissolved in DCM (15 mL) and cooled to -78 °C. DMSO (0.87 g, 0.79 mL, 11.2 mmol) was added and the solution was stirred for 15 min. 1-tert-butyl 3-methyl 5-hydroxypiperidine-1,3-dicarboxylate (**14a**, 1.0 g, 3.86 mmol) dissolved in DCM (5 mL) was added dropwise, and the reaction was stirred at -78 °C for 15 min. Et_3N (1.76 g, 2.42 mL, 17.4 mmol) was added, the reaction stirred at -78 °C for 30 min, and then at 25 °C for 2 hr. The reaction was quenched with sat. $NaHCO_3$ (20 mL). The product was extracted with DCM (3 x 20 mL), washed with brine (2 x 15 mL), dried with $MgSO_4$, and concentrated in vacuo. The oil was subjected to silica gel chromatography eluting with 25% EtOAc: 75% Hex. The fractions containing product were concentrated to produce yellow solid. Yield=85%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 4.01 (s, 2H) 3.88-3.77 (m, 2H) 3.73 (s, 3H) 3.07 (p, 6.1 Hz, 1H) 2.74 (dd, 16.9, 7.4 Hz, 1H) 2.63 (dd, 16.9, 5.8 Hz, 1H) 1.46 (s, 9H); MS (ESI+) *m/z*: 312.1 $[M+MeOH+Na]^+$.



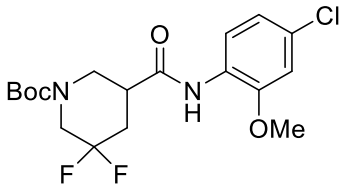
1-tert-butyl 3-methyl 5,5-difluoropiperidine-1,3-dicarboxylate (14c). In a flame dried 50-mL round bottomed flask, 1-tert-butyl 3-methyl 5-oxopiperidine-1,3-dicarboxylate (**14b**, 0.84 g, 3.28 mmol) was dissolved in DCM (15 mL) and cooled to -78 °C. DAST (1.06 g, 0.87 mL, 6.56 mmol) was added and the solution was stirred for 1 hr, and then at 25 °C for 1 hr. The reaction was quenched with sat. $NaHCO_3$ (20 mL). The product was extracted with DCM (3 x 20 mL), washed with brine (2 x 15 mL), dried with $MgSO_4$, and concentrated in vacuo. The oil was subjected to silica gel chromatography eluting with 15% EtOAc: 85% Hex. The fractions containing product were concentrated to produce yellow solid. Yield=83%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 4.49-4.22 (m, 2H) 3.72 (s, 3H) 3.09-2.90 (m, 2H) 2.85-2.75 (m, 2H) 2.00 (dtd, 31.5, 13.0, 4.8 Hz, 1H) 1.47 (s, 9H); MS (ESI+) *m/z*: 302.1 $[M+Na]^+$.



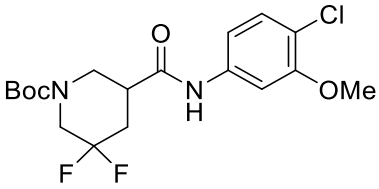
1-(tert-butoxycarbonyl)-5,5-difluoropiperidine-3-carboxylic acid (14d). Prepared according to Procedure A starting from **14c** to afford 0.24 g (79% yield) of title compound as white powder. Yield=79%. MS (ESI-) *m/z*: 264.1 $[M-H]^-$.



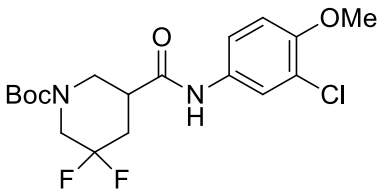
tert-butyl 5-((4-chlorophenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (15). Prepared according to Procedure B using 4-chloroaniline and **14d** to afford 0.05 g (64% yield) of title compound as white solid. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ 8.16 (br.s, 1H) 7.51 (d, J = 8.7 Hz, 1H) 7.29 (d, J = 8.6, 2H) 4.05 (br. s, 2H) 3.41-3.30 (m, 2H) 2.80-2.75 (m, 1H) 2.51-2.30 (m, 2H) 1.48 (s, 9H); MS (ESI+) *m/z*: 397.1 $[M+Na]^+$; HPLC Ret: 8.06 min.



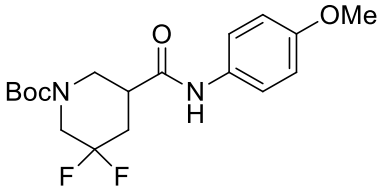
tert-butyl 5-((4-chloro-2-methoxyphenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (19a). Prepared according to Procedure B using 4-chloro-2-methoxyaniline (**18a**) and **14d** to afford 0.064 g (84% yield) of title compound as yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 9.61 (br.s, 1H) 7.87 (d, J = 8.6 Hz, 1H) 7.12 (d, J = 2.3 Hz, 1H) 6.97 (dd, J = 8.5, 2.3 Hz, 1H) 4.12 (br. s, 2H) 3.85 (s, 3H) 3.76 (s, 2H) 3.10-3.02 (m, 1H) 2.35-2.10 (m, 2H) 1.42 (s, 9H); MS (ESI+) *m/z*: 405.1 $[M+H]^+$; HPLC Ret: 8.17 min.



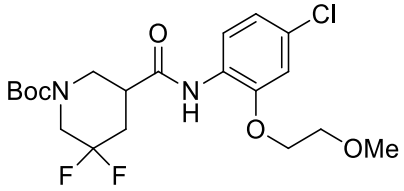
tert-butyl 5-((4-chloro-3-methoxyphenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (19b). Prepared according to Procedure B using 4-chloro-3-methoxyaniline (**18b**) and **14d** to afford 0.048 g (63% yield) of title compound as white solid. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 10.35 (br.s, 1H) 7.51 (d, J = 2.3 Hz, 1H) 7.34 (d, J = 8.6 Hz, 1H) 7.17 (dd, J = 8.7, 2.2 Hz, 1H) 4.14 (br. s, 2H) 3.81 (s, 3H) 3.32 (s, 2H) 2.85-2.80 (m, 1H) 2.35-2.10 (m, 2H) 1.42 (s, 9H); MS (ESI+) *m/z*: 405.1 $[M+H]^+$; HPLC Ret: 7.95 min.



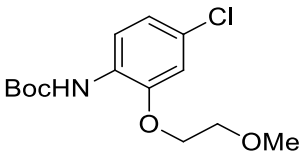
tert-butyl 5-((3-chloro-4-methoxyphenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (19c). Prepared according to Procedure B using 3-chloro-4-methoxyaniline (**18c**) and **14d** to afford 0.06 g (88% yield) of title compound as white solid. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 7.92 (br.s, 1H) 7.64 (br. s, 1H) 7.38 (dd, J = 8.8, 1H) 6.88 (d, J = 8.8 Hz, 1H) 4.04 (br. s, 2H) 3.88 (s, 3H) 3.40-3.32 (m, 2H) 2.79-2.73 (m, 1H) 2.48-2.28 (m, 2H) 1.48 (s, 9H); MS (ESI+) *m/z*: 427.1 $[M+Na]^+$; HPLC Ret: 7.82 min.



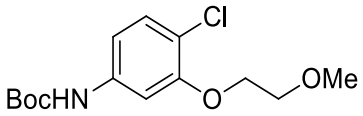
tert-butyl 3,3-difluoro-5-((4-methoxyphenyl)carbamoyl)piperidine-1-carboxylate (19d). Prepared according to Procedure B using 4-methoxyaniline (**18d**) and **14d** to afford 0.066 g (95% yield) of title compound as white solid. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 7.70 (br. s, 1H) 7.43 (d, J = 8.0, 2H) 6.86 (d, J = 8.1 Hz, 2H) 4.10 (br. s, 2H) 3.79 (s, 3H) 3.29-3.19 (m, 2H) 2.78-2.74 (m, 1H) 2.47-2.29 (m, 2H) 1.48 (s, 9H); MS (ESI+) *m/z*: 393.2 $[M+Na]^+$; HPLC Ret: 7.39 min.



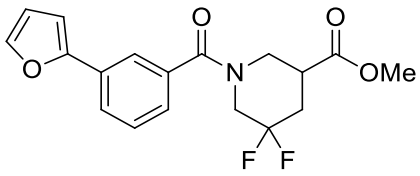
tert-butyl 5-((4-chloro-2-(2-methoxyethoxy)phenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (19e). Prepared according to Procedure B using tert-butyl (4-chloro-2-(2-methoxyethoxy)phenyl)carbamate (**18e**) and **14d** to afford 0.062 g (73% yield) of title compound as white solid. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 8.48 (br. s, 1H) 8.28 (d, J = 8.7 Hz, 1H) 7.03-6.94 (m, 2H) 4.48-4.30 (m, 2H) 4.14 (t, J = 4.5 Hz, 2H) 3.74 (t, J = 4.5 Hz, 2H) 3.48 (s, 3H) 3.02-2.75 (m, 4H) 2.42-2.18 (m, 2H) 1.48 (s, 9H); MS (ESI+) *m/z*: 449.2 $[M+H]^+$; HPLC Ret: 8.23 min.



tert-butyl (4-chloro-2-(2-methoxyethoxy)phenyl)carbamate (18e). tert-butyl (4-chloro-2-hydroxyphenyl)carbamate (0.3 g, 1.23 mmol) was dissolved in DMF (6.0 mL), and then K_2CO_3 (0.34 g, 2.46 mmol) and 1-bromo-2-methoxyethane (0.22 g, 1.60 mmol, 0.16 mL) were added. The reaction was stirred at 90 ^oC for 1 hr. After the reaction cooled to 25 ^oC brine was added and the product was extracted with DCM (3 x 20 mL). The organic layers were combined, washed with brine (3x 10 mL), dried with $MgSO_4$, and concentrated *in vacuo*. The subsequent oil was subjected to silica gel chromatography eluting with 15% EtOAc: 85% Hex. The fractions containing product were concentrated in vacuo to produce red oil. Analogous analogs were made in a similar fashion. Yield = 43%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 8.04 (s, 1H) 7.28 (s, 1H) 6.95 (dd, J = 8.7, 2.3 Hz, 1H) 6.88 (s, 1H) 4.14 (t, J = 4.5 Hz, 2H) 3.73 (t, J = 4.5 Hz, 2H) 3.46 (s, 3H) 1.52 (s, 9H); MS (ESI+) *m/z*: 324.1 $[M+Na]^+$.

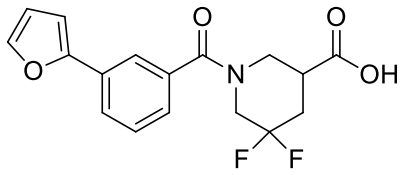


tert-butyl (4-chloro-3-(2-methoxyethoxy)phenyl)carbamate (18f). Same procedural steps as **18e** except tert-butyl (4-chloro-3-hydroxyphenyl)carbamate (0.3 g, 1.23 mmol) was used. White solid. Yield = 44%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 7.31 (s, 1H) 7.22 (d, J = 8.6 Hz, 1H) 6.70 (dd, J = 8.6, 2.4 Hz, 1H) 6.46 (s, 1H) 4.19 (t, J = 4.5 Hz, 2H) 3.80 (t, J = 4.5 Hz, 2H) 3.48 (s, 3H) 1.51 (s, 9H); MS (ESI+) *m/z*: 324.1 $[M+Na]^+$.

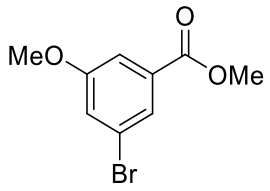


methyl 5,5-difluoro-1-(3-(furan-2-yl)benzoyl)piperidine-3-carboxylate (20a). In a 10 mL round bottomed flask, 1-tert-butyl 3-methyl 5,5-difluoropiperidine-1,3-dicarboxylate (0.123 g, 0.44 mmol) was dissolved in 4 M HCl/Dioxanes (2.0 mL) and the reaction was stirred at 25 °C for 2 hr. The solvents were evaporated in vacuo, and the subsequent solid was taken into the next step without further purification. 3-(furan-2-yl)benzoic acid (0.08 g, 0.44 mmol) was dissolved in DMF (3 mL) and HATU (0.34 g, 0.88 mmol), methyl 5,5-difluoropiperidine-3-carboxylate, HCl, and DIPEA (0.17 g, 0.23 mL, 1.32 mmol) were added. The reaction was stirred at 25 °C for 16 hr and brine (10 mL) was added. The product was extracted with EtOAc (3 x 15 mL), dried with $MgSO_4$, and concentrated in vacuo. The subsequent oil was subjected to silica gel chromatography eluting with 40% EtOAc: 60% Hex. The fractions containing product were concentrated in vacuo to produce white solid. Yield=84%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ

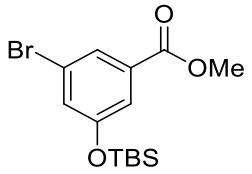
ppm 7.79-7.70 (m, 2H) 7.51-7.41 (m, 2H) 7.33-7.23 (m, 1H) 6.71 (d, J = 3.4 Hz, 1H) 6.49 (dd, J = 3.4, 1.8 Hz, 1H) 4.97 (br. s, 1H) 3.74 (br. s, 3H) 3.32-3.15 (m, 2H) 3.08-2.88 (m, 2H) 2.65-2.51 (m, 2H); MS (ESI+) *m/z*: 350.1 $[M+H]^+$; HPLC Ret: 7.02 min.



5,5-difluoro-1-(3-(furan-2-yl)benzoyl)piperidine-3-carboxylic acid (20b). Prepared according to Procedure A using **20a** to afford 0.12 g (100% yield) of title compound as white powder. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 7.79-7.71 (m, 2H) 7.51-7.42 (m, 2H) 7.33-7.23 (m, 1H) 6.71 (d, J = 3.4 Hz, 1H) 6.49 (dd, J = 3.4, 1.8 Hz, 1H) 4.94 (br. s, 1H) 3.35-3.21 (m, 2H) 3.10-2.92 (m, 2H) 2.67-2.55 (m, 2H); MS (ESI-) *m/z*: 334.1 $[M-H]^-$; HPLC Ret: 6.24 min.

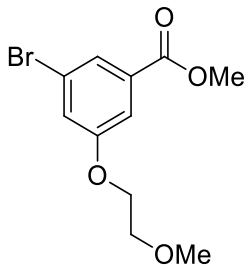


methyl 3-bromo-5-methoxybenzoate (9a). In a 50 mL round bottomed flask, 3-bromo-5-hydroxybenzoic acid (**6**, 0.5 g, 2.3 mmol) was dissolved in DMF (10 mL). Cs_2CO_3 (2.25 g, 6.91 mmol) and (after 10 mins stirring) MeI (0.82 g, 0.36 mL, 5.76 mmol) were added. The reaction took place at 25 °C for 16 hr. The product was extracted with EtOAc (3 x 20 mL), washed with brine (2 x 15 mL), and concentrated in vacuo to afford brown oil. The resulting oil (**7**) was subjected to General Procedure A to afford the title compound. Yield = 97% (2-steps). ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 7.76 (t, 1.5 Hz, 1H) 7.49 (dd, J = 2.5, 1.3 Hz, 1H) 7.24 (t, J = 2.1 Hz, 1H) 3.94 (s, 3H) 3.84 (s, 3H); MS (ESI+) *m/z*: 247.0 $[M+H]^+$; HPLC Ret: 7.64 min.

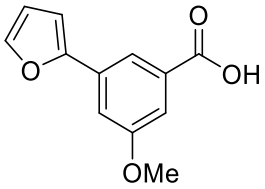


methyl 3-bromo-5-((tert-butyldimethylsilyl)oxy)benzoate (9b). Methyl 3-bromo-5-hydroxybenzoate (**6**, 0.47 g, 2.03 mmol) was dissolved in DMF (5.0 mL), and then ImH (0.18 g, 2.64 mmol) and TBS-Cl (0.34 g, 2.24 mmol) were added. The reaction was stirred at 25 ^{o}C for 16 hr. The reaction was partitioned between H_2O and 1:1 EtOAc/Hex, and the product was

extracted with 1:1 EtOAc/Hex (3 x 20 mL). The organic layers were combined, washed with brine (3x 10 mL), dried with $MgSO_4$, and concentrated *in vacuo* to produce orange oil. The resulting oil (**8**) was subjected to General Procedure A to afford the title compound. Yield = 87% (2-steps) ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 7.77 (t, J = 1.5 Hz, 1H) 7.41 (dd, J = 2.3, 1.3 Hz, 1H) 7.18 (dd, J = 4.2, 0.7 Hz, 1H) 3.91 (s, 3H) 0.99 (s, 9H) 0.22 (s, 6H); MS (ESI+) *m/z*: 347.1 $[M+H]^+$.

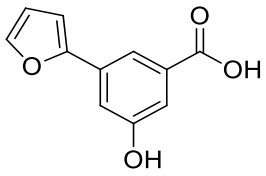


methyl 3-bromo-5-(2-methoxyethoxy)benzoate (9c). 3-bromo-5-hydroxybenzoic acid (**6**, 1.0 g, 4.61 mmol) was dissolved in 20 mL MeOH. Sulfuric acid (0.59 g, 0.3 mL, 5.99 mmol) was added dropwise and the reaction mixture was heated at reflux (95 °C) for 16 hr. MeOH was removed *in vacuo* and the residue was diluted in 15 mL H_2O. The product was extracted with EtOAc (3x 15 mL), washed with brine (2 x 10 mL), dried with $MgSO_4$, and concentrated *in vacuo*. The resulting oil was triturated with EtOAc/Hex to produce pale orange powder. The freshly synthesized methyl 3-bromo-5-hydroxybenzoate (0.2 g, 0.87 mmol) was dissolved in DMF (4.0 mL), and then K_2CO_3 (0.24 g, 1.73 mmol) and 1-bromo-2-methoxyethane (0.16 g, 1.13 mmol, 0.11 mL) were added. The reaction was stirred at 90 ^{o}C for 1 hr. After the reaction cooled to 25 ^{o}C, brine was added and the product was extracted with DCM (3 x 20 mL). The organic layers were combined, washed with brine (2 x 10 mL), dried with $MgSO_4$, and concentrated *in vacuo*. The subsequent oil was subjected to silica gel chromatography eluting with 100% EtOAc. The fractions containing product were concentrated in vacuo to produce orange oil. Yield = 85%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 7.77 (t, J = 1.5 Hz, 1H) 7.52 (dd, J = 2.5, 1.3 Hz, 1H) 7.29 (dd, J = 2.5, 1.3 Hz, 1H) 4.15 (t, J = 4.5 Hz, 2H) 3.91 (s, 3H) 3.76 (t, J = 4.5 Hz, 2H) 3.45 (s, 3H); MS (ESI+) *m/z*: 289.0 $[M+H]^+$; HPLC Ret: 7.37 min.

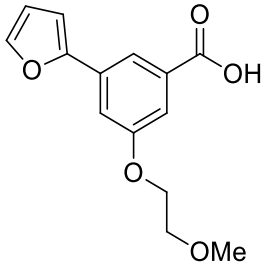


3-(furan-2-yl)-5-methoxybenzoic acid (10a). (Prepared according to Procedure C to afford 0.28 g (1.30 mmol, 58% yield) of title compound as white solid). In a 50 mL round bottomed flask, methyl 3-bromo-5-methoxybenzoate (**9a**, 0.55 g, 2.24 mmol), furan-2-ylboronic acid (0.30 g, 2.69 mmol), Tetrakis® (0.13 g, 0.11 mmol), and Na_2CO_3 (0.60 g, 5.61 mmol) were dissolved in DME (10 mL) and H_2O (10 mL). The solution was degassed and heated at 100 °C for 16 hr under Ar_2. The solution was filtered through a pad of Celite® to remove particulates. The filtrate was acidified with 1N HCl (15 mL) and the product was extracted with EtOAc (3 x 15 mL),

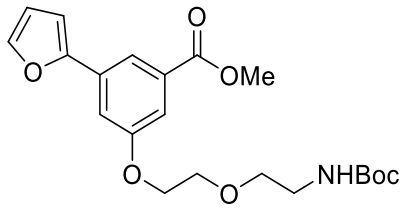
washed with brine, dried with $MgSO_4$, and concentrated in vacuo. The subsequent oil was subjected to silica gel chromatography eluting with 5% MeOH: 95% DCM. The fractions containing product were concentrated in vacuo to produce white solid. Analogous compounds were synthesized using this procedure. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 13.18 (br. s, 1H) 7.84 (t, 1.5 Hz, 1H) 7.79 (dd, J = 1.8, 0.7 Hz, 1H) 7.48 (dd, J = 2.6, 1.6 Hz, 1H) 7.36 (dd, J = 2.6, 1.4 Hz, 1H) 7.11 (dd, J = 3.4, 0.7 Hz, 1H) 6.63 (dd, J = 3.4, 1.8 Hz, 1H) 3.86 (s, 3H); MS (ESI-) *m/z*: 217.1 $[M-H]^-$; HPLC Ret: 6.35 min.



3-(furan-2-yl)-5-hydroxybenzoic acid (10b). Prepared according to Procedure C from **9b** to afford 0.26 g (1.30 mmol, 74% yield) of title compound as white solid) White solid. Yield = 74%. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 13.00 (br. s, 1H) 9.93 (s, 1H) 7.76 (dd, J = 1.8, 0.7 Hz, 1H) 7.69 (t, J = 1.5 Hz, 1H) 7.34-7.23 (m, 2H) 6.98 (dd, J = 3.4, 0.8 Hz, 1H) 6.60 (dd, J = 3.4, 1.8 Hz, 1H); MS (ESI-) *m/z*: 203.0 $[M-H]^-$; HPLC Ret: 5.42 min.

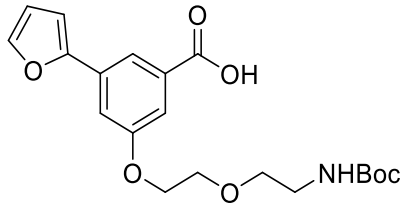


3-(furan-2-yl)-5-(2-methoxyethoxy)benzoic acid (10c). Prepared according to Procedure C to from **9c** afford 0.18 g (0.68 mmol, 98% yield) of title compound as white solid) White solid. Yield = 98%. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 13.18 (br. s, 1H) 7.84 (t, 1.4 Hz, 1H) 7.79 (d, J = 1.8 Hz, 1H) 7.51-7.49 (m, 1H) 7.43 (dt, J = 8.7, 2.2 Hz, 1H) 7.35 (dd, J = 2.5, 1.4 Hz, 1H) 7.12 (d, J = 3.4 Hz, 1H) 6.62 (dd, J = 3.4, 1.7 Hz, 1H) 4.20 (t, J = 4.5 Hz, 2H) 3.67 (t, J = 4.5 Hz, 2H) 3.32 (s, 3H); HPLC Ret: 6.17 min.

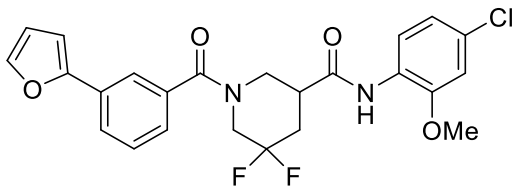


methyl 3-(2-(2-((tert-butoxycarbonyl)amino)ethoxy)ethoxy)-5-(furan-2-yl)benzoate (12). 3-(furan-2-yl)-5-hydroxybenzoic acid (**10b**, 0.15 g, 0.74 mmol) was dissolved in 5 mL MeOH.

Sulfuric acid (0.09 g, 0.05 mL, 0.96 mmol) was added dropwise, and the reaction mixture was heated at reflux (95 °C) for 16 hr. MeOH was removed *in vacuo* and the residue was diluted in 15 mL H_2O. The product was extracted with EtOAc (3x 15 mL), washed with brine (2 x 10 mL), dried with $MgSO_4$, and concentrated *in vacuo*. The resulting oil was triturated with EtOAc/Hex to produce white powder. The resulting solid (**11**, 0.05 g, 0.23 mmol) was dissolved in 1 mL DMF, and freshly prepared 2-(2-((tert-butoxycarbonyl)amino)ethoxy)ethyl methanesulfonate[5] (0.06 g, 0.21 mmol) and Cs_2CO_3 (0.1 g, 0.31 mmol) were added. The reaction was stirred at 75 °C for 2 hr. The reaction was diluted with 10 mL brine and extracted with DCM (3 x 15 mL). The organic layers were combined, dried with $MgSO_4$, and evaporated. The subsequent oil was subjected to silica gel chromatography eluting with 30% EtOAc: 70% Hex. The fractions containing product were concentrated in vacuo to produce pale yellow oil. Yield=86%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 7.94 (t, 1.4 Hz, 1H) 7.51-7.42 (m, 2H) 6.72 (dd, J = 3.5, 0.8 Hz, 1H) 6.51-6.46 (m, 1H) 4.97 (br. s, 1H) 4.22 (t, J = 5.7 Hz, 2H) 3.94 (s, 3H) 3.85 (t, J = 5.5 Hz, 2H) 3.62 (t, J = 5.2 Hz, 2H) 3.36 (q, J = 5.5 Hz, 2H) 1.43 (s, 9H); MS (ESI+) *m/z*: 406.2 $[M+H]^+$; HPLC Ret: 7.89 min.

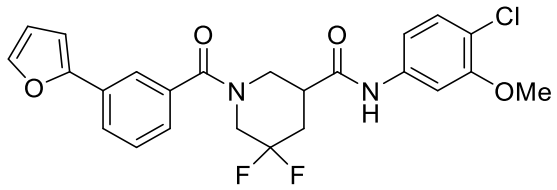


3-(2-(2-((tert-butoxycarbonyl)amino)ethoxy)ethoxy)-5-(furan-2-yl)benzoic acid (13). (Prepared according to Procedure A to afford 0.068 g (0.17 mmol, 96% yield) of title compound as white solid). In a 10 mL round-bottomed flask, methyl 3-(2-(2-((tert-butoxycarbonyl)amino)ethoxy)ethoxy)-5-(furan-2-yl)benzoate (**12**, 0.07 g, 0.18 mmol) was dissolved in THF (1.0 mL). 1 M NaOH (1.0 mL) was added to the solution and the reaction was stirred at 25 °C for 1 hr. The THF was evaporated *in vacuo* and then the solution was acidified with 1 N HCl (7 mL). The product was extracted with EtOAc (3 x 15 mL), washed with brine, dried with $MgSO_4$, and concentrated in vacuo. Yield=96%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 8.00 (s, 1H) 7.55-7.45 (m, 3H) 6.74 (d, J = 3.3 Hz, 1H) 6.49-6.47 (m, 1H) 5.00 (br. s, 1H) 4.23 (d, J = 5.6 Hz, 2H) 4.12 (t, J = 7.1 Hz, 2H) 3.64 (t, J = 5.2 Hz, 2H) 3.37-3.32 (m, 2H) 1.44 (s, 9H); MS (ESI+) *m/z*: 391.0 $[M+H]^+$; HPLC Ret: 6.80 min.

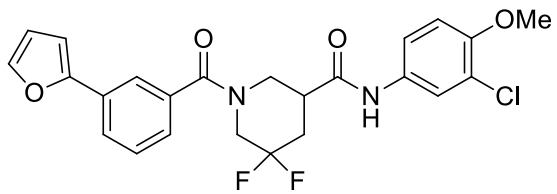


N-(4-chloro-2-methoxyphenyl)-5,5-difluoro-1-(3-(furan-2-yl)benzoyl)piperidine-3-carboxamide (1b). In a 10 mL round bottomed flask tert-butyl 5-((4-chloro-2-methoxyphenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (0.064 g, 0.16 mmol) was dissolved in 4M HCl/Dioxane solution (1.5 mL), and the reaction was stirred at 25 °C for 2 hr.

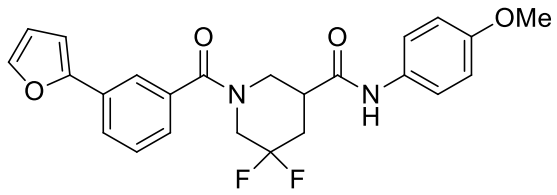
The solvents were evaporated in vacuo. The subsequent oil was taken into the next step without further purification. N-(4-chloro-2-methoxyphenyl)-5,5-difluoropiperidine-3-carboxamide, HCl was dissolved in DMF (0.5 mL) and added to HATU (0.12 g, 0.32 mmol) and 3-(furan-2-yl)benzoic acid (0.03 g, 0.16 mmol) dissolved in DMF (1.0 mL). DIPEA (0.06 g, 0.08 mL, 0.48 mmol) was added and the reaction was stirred at 25 °C for 16 hr. Brine (15 mL) was added and the product was extracted with EtOAc (3 x 20 mL), dried with $MgSO_4$, and concentrated in vacuo. The subsequent oil was subjected to silica gel chromatography eluting with 35% EtOAc: 65% Hex. The fractions containing product were concentrated in vacuo to produce white powder. Analogous compounds were produced using this procedure. Yield=65%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 8.25 (br.s, 1H) 8.00 (br. s, 1H) 7.79-7.76 (m, 2H) 7.50-7.41 (m, 2H) 7.31 (d, J = 7.7 Hz, 1H) 6.94 (d, 8.7 Hz, 1H) 6.87 (br. s, 1H) 6.70 (d, J = 3.4 Hz, 1H) 6.49 (dd, J = 3.4, 1.8 Hz, 1H) 4.12 (br. s, 2H) 3.89 (br. s, 3H) 3.43-3.24 (m, 1H) 3.10-2.92 (m, 2H) 2.48-2.29 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 171.12, 168.19, 152.76, 148.63, 142.63, 134.93, 131.44, 129.39, 129.15, 125.88, 125.48, 122.59, 120.84, 120.78, 120.69, 111.84, 110.85, 106.07, 60.38, 55.96, 44.39, 41.40, 35.38, 21.00; MS (ESI+) *m/z*: 475.1 $[M+H]^+$; HPLC Ret: 7.98 min; 96 % pure.



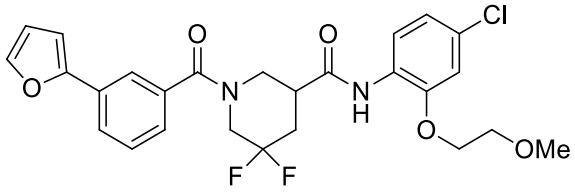
N-(4-chloro-3-methoxyphenyl)-5,5-difluoro-1-(3-(furan-2-yl)benzoyl)piperidine-3-carboxamide (1c). Same procedural steps as **1b** except tert-butyl 5-((4-chloro-3-methoxyphenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (0.05 g, 0.12 mmol) was used. White powder. Yield=88%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 9.53 (br.s, 1H) 7.78-7.72 (m, 2H) 7.51-7.42 (m, 3H) 7.30 (d, J = 7.6 Hz, 1H) 7.20 (d, 8.5 Hz, 1H) 6.87 (d, J = 8.5 Hz, 1H) 6.68 (d, J = 3.4 Hz, 1H) 6.47 (dd, J = 3.4, 1.8 Hz, 1H) 4.04 (br. s, 2H) 3.83 (br. s, 3H) 3.43 (dd, J = 26.5, 14.0, 1H) 3.22 (t, J = 12.1 Hz, 1H) 2.96 (dq, J = 11.1, 6.3, 5.3 Hz, 1H) 2.52-2.30 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 171.20, 168.57, 155.03, 152.36, 142.78, 137.88, 134.59, 131.76, 129.85, 125.78, 125.40, 122.17, 118.83, 117.45, 112.31, 111.92, 106.34, 104.36, 60.42, 56.04, 44.49, 40.90, 34.99, 21.05; MS (ESI+) *m/z*: 475.1 $[M+H]^+$; HPLC Ret: 7.80 min; 98 % pure.



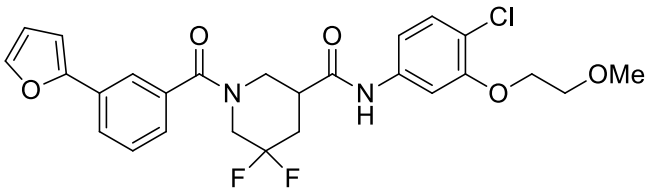
N-(3-chloro-4-methoxyphenyl)-5,5-difluoro-1-(3-(furan-2-yl)benzoyl)piperidine-3-carboxamide (1d). Same procedural steps as **1b** except tert-butyl 5-((3-chloro-4-methoxyphenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (0.06 g, 0.16 mmol) was used. White powder. Yield=86%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 9.25 (br. s, 1H) 7.75 (br. s, 2H) 7.54 (br. s, 1H) 7.49-7.42 (m, 2H) 7.32 (dd, 25.3, 8.2 Hz, 2H) 6.79 (d, J = 8.9 Hz, 1H) 6.69 (d, J = 3.4 Hz, 1H) 6.48 (dd, J = 3.3, 1.8 Hz, 1H) 4.84 (s, 1H) 4.02 (br. s, 1H) 3.83 (s, 3H) 3.43 (dd, J = 26.3, 14.0, 1H) 3.22 (t, J = 10.5 Hz, 1H) 2.94 (td, J = 10.7, 5.2 Hz, 1H) 2.55-2.30 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 168.31, 152.48, 151.88, 142.71, 134.64, 131.67, 129.33, 125.71, 125.49, 122.47, 122.21, 119.64, 118.86, 112.03, 111.87, 106.26, 60.41, 56.31, 44.44, 40.69, 34.92, 21.05; MS (ESI+) *m/z*: 475.1 $[M+H]^+$; HPLC Ret: 7.67 min; 97 % pure.



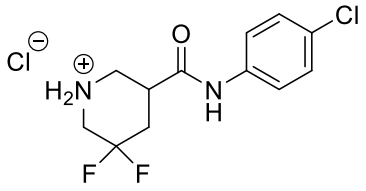
5,5-difluoro-1-(3-(furan-2-yl)benzoyl)-N-(4-methoxyphenyl)piperidine-3-carboxamide (1e). Same procedural steps as **1b** except tert-butyl 5-((3-methoxyphenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (0.06 g, 0.18 mmol) was used. White powder. Yield=84%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 8.81 (br.s, 1H) 7.78-7.71 (m, 2H) 7.49-7.39 (m, 4H) 6.69 (d, J = 3.4 Hz, 1H) 6.48 (dd, J = 3.3, 1.7 Hz, 1H) 6.30 (d, J = 7.7 Hz, 1H) 4.81 (s, 1H) 4.01 (br. s, 1H) 3.76 (s, 3H) 3.42 (dd, J = 26.4, 14.0, 1H) 3.24 (br. s, 1H) 2.93 (br. s, 1H) 2.57-2.33 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 168.15, 156.51, 152.60, 142.67, 131.56, 129.25, 125.68, 125.62, 122.36, 121.74, 114.05, 111.85, 106.19, 60.40, 55.43, 44.45, 40.76, 21.04; MS (ESI+) *m/z*: 441.2 $[M+H]^+$; HPLC Ret: 7.30 min; 97 % pure.



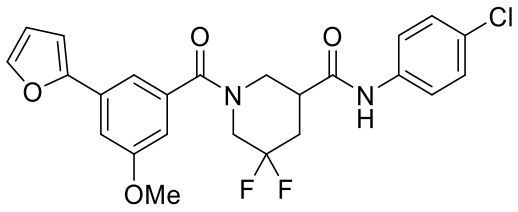
N-(4-chloro-2-(2-methoxyethoxy)phenyl)-5,5-difluoro-1-(3-(furan-2-yl)benzoyl)piperidine-3-carboxamide (2b). Same procedural steps as **1b** except tert-butyl 5-((4-chloro-2-(-2-methoxyethoxy)phenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (0.05 g, 0.13 mmol) was used. White powder. Yield=61%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 8.71 (br. s, 1H) 8.28 (br. s, 1H) 7.78-7.71 (m, 2H) 7.51-7.42 (m, 2H) 7.31 (d, J = 7.6 Hz, 1H) 7.02-6.93 (m, 2H) 6.71 (d, J = 3.4 Hz, 1H) 6.49 (dd, J = 3.4, 1.8 Hz, 1H) 4.95 (br. s, 1H) 4.19-4.00 (m, 3H) 3.79-3.72 (m, 2H) 3.50 (s, 3H) 3.41-3.22 (m, 1H) 2.99 (br. s, 1H) 2.53-2.28 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 171.47, 167.02, 152.78, 142.61, 131.45, 129.13, 129.04, 125.49, 121.25, 111.82, 106.06, 77.20, 70.62, 60.33, 59.06, 53.02, 44.33, 39.21, 34.87, 21.02; MS (ESI+) *m/z*: 519.2 $[M+H]^+$; HPLC Ret: 8.02 min; 98% pure.



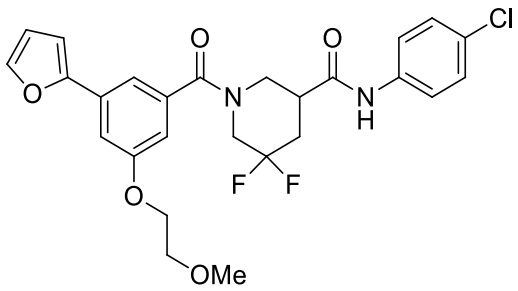
N-(4-chloro-3-(2-methoxyethoxy)phenyl)-5,5-difluoro-1-(3-(furan-2-yl)benzoyl)piperidine-3-carboxamide (2c). In a 10 mL round bottomed flask tert-butyl (4-chloro-3-(2-methoxyethoxy)phenyl)carbamate (**18f**, 0.03 g, 0.10 mmol) was dissolved in 4 M HCl/Dioxanes (1.0 mL) and the reaction was stirred at 25 °C for 3 hr. The solvents were evaporated in vacuo, and the subsequent solid was taken into the next step without further purification. 5,5-difluoro-1-(3-(furan-2-yl)benzoyl)piperidine-3-carboxylic acid (**20b**, 0.03 g, 0.09 mmol) was dissolved in DMF (1 mL) and HATU (0.07 g, 0.18 mmol), 4-chloro-3-(2-methoxyethoxy)aniline, HCl (0.02 g, 0.09 mmol) and DIPEA (0.035 g, 0.05 mL, 0.27 mmol) were added. The reaction was stirred at 25 °C for 16 hr and brine (10 mL) was added. The product was extracted with EtOAc (3 x 15 mL), dried with $MgSO_4$, and concentrated in vacuo. The subsequent oil was subjected to silica gel chromatography eluting with 0-30% EtOAc/Hex. The fractions containing product were concentrated in vacuo to produce colorless oil. Yield=52%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 9.45 (br. s, 1H) 7.75 (br. s, 2H) 7.49-7.39 (m, 3H) 7.35-7.18 (m, 2H) 6.95-6.89 (m, 1H) 6.68 (d, J = 3.4 Hz, 1H) 6.48 (dd, J = 3.4, 1.8 Hz, 1H) 4.84 (d, J = 13.2 Hz, 1H) 4.16-4.08 (m, 3H) 4.03 (br. s, 1H) 3.81-3.74 (m, 2H) 3.48-3.41 (m, 5H) 3.21 (t, J = 12.2 Hz, 1H) 2.93 (dt, J = 11.3, 4.2 Hz, 1H) 2.52-2.30 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 172.20, 168.49, 154.40, 152.40, 142.77, 137.73, 134.56, 131.71, 130.48, 129.35, 125.77, 125.48, 122.21, 118.12, 112.71, 111.90, 108.39, 106.32, 105.77, 70.71, 68.76, 60.41, 59.38, 52.83, 44.52, 40.86, 34.94, 21.05; MS (ESI+) *m/z*: 519.2 $[M+H]^+$; HPLC Ret: 7.79 min; 96.6% pure.



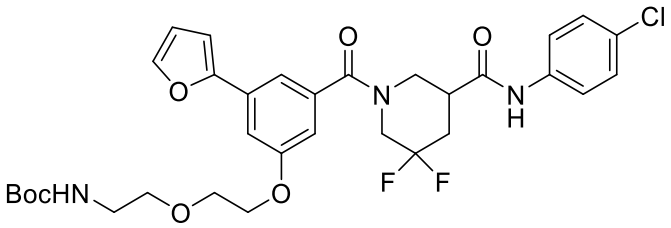
N-(4-chlorophenyl)-5,5-difluoropiperidine-3-carboxamide, HCl (16). In a 10 mL round bottomed flask, tert-butyl 5-((4-chlorophenyl)carbamoyl)-3,3-difluoropiperidine-1-carboxylate (0.045 g, 0.12 mmol) was dissolved in 4 M HCl/Dioxanes (1 mL), and the reaction was stirred at 25 °C for 1 hr. The solvents were evaporated in vacuo, and the subsequent solid was taken into the next step without further purification (HPLC = 4.86 min)



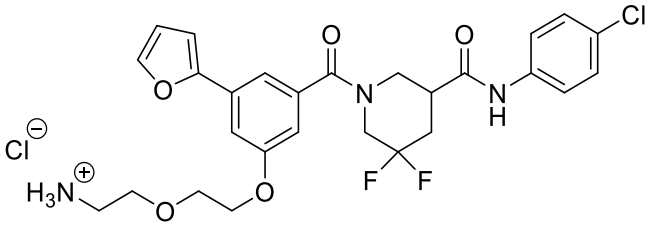
N-(4-chlorophenyl)-5,5-difluoro-1-(3-(furan-2-yl)-5-methoxybenzoyl)piperidine-3-carboxamide (1a). (Prepared according to Procedure B to afford 0.09 g (0.19 mmol, 83% yield, 98% pure) of title compound as white solid). 3-(furan-2-yl)-5-methoxybenzoic acid (0.05 g, 0.23 mmol) was dissolved in DMF (2 mL) and HATU (0.17 g, 0.46 mmol), N-(4-chlorophenyl)-5,5-difluoropiperidine-3-carboxamide (0.07 g, 0.23 mmol), and DIPEA (0.09 g, 0.12 mL, 0.69 mmol) were added. The reaction was stirred at 25 °C for 18 hr and brine (20 mL) was added. The product was extracted with EtOAc (3 x 20 mL), dried with $MgSO_4$, and concentrated in vacuo. The subsequent oil was subjected to silica gel chromatography eluting with 2% MeOH: 98% DCM. The fractions containing product were concentrated in vacuo to produce white solid. Analogous compounds made in similar fashion. Yield=83%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 9.26 (br. s, 1H) 7.55-7.45 (m, 3H) 7.32-7.20 (m, 4H) 6.83 (s, 1H) 6.67 (d, J = 3.4 Hz, 1H) 6.48 (dd, J = 3.4, 1.8 Hz, 1H) 4.04 (br. s, 2H) 3.84 (s, 3H) 3.45-3.39 (m, 1H) 3.24 (t, J = 11.9 Hz, 1H) 2.95-2.88 (m, 1H) 2.51-2.30 (m, 2H) ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 168.36, 160.20, 152.41, 142.72, 128.93, 126.98, 125.47, 121.14, 120.97, 118.78, 116.62, 114.68, 111.89, 106.51, 104.25, 60.41, 55.81, 44.47, 40.79, 35.60, 21.14; MS (ESI+) *m/z*: 475.1 $[M+H]^+$; HPLC Ret: 8.00 min; 98% pure.



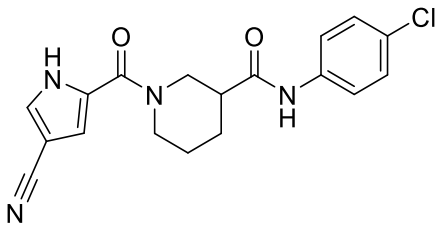
N-(4-chlorophenyl)-5,5-difluoro-1-(3-(furan-2-yl)-5-(2-methoxyethoxy)benzoyl)piperidine-3-carboxamide. (2a). Prepared according to Procedure B using **10c** to afford 0.06 g (0.11 mmol, 83% yield, 97% pure) of title compound as yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 9.12 (br. s, 1H) 7.53-7.44 (m, 3H) 7.35-7.29 (m, 2H) 7.27-7.20 (m, 2H) 6.85 (s, 1H) 6.66 (d, J = 3.4 Hz, 1H) 6.48 (dd, J = 3.4, 1.8 Hz, 1H) 4.13 (t, J = 4.5 Hz, 2H) 3.92 (br. s, 2H) 3.74 (t, J = 4.6 Hz, 2H) 3.45 (s, 3H) 3.30-3.20 (m, 2H) 2.95-2.90 (m, 2H) 2.49-2.35 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 168.50, 159.38, 152.30, 142.74, 136.59, 135.74, 133.00, 129.25, 128.83, 122.15, 121.23, 120.06, 114.79, 112.01, 111.65, 106.56, 70.81, 70.62, 67.89, 67.61, 59.19, 44.42, 40.72, 34.95; MS (ESI+) *m/z*: 519.2 $[M+H]^+$; HPLC Ret: 7.88 min; 96.6% pure.



tert-butyl (2-(2-(3-(5-((4-chlorophenyl)carbamoyl)-3,3-difluoropiperidine-1-carbonyl)-5-(furan-2-yl)phenoxy)ethoxy)ethyl)carbamate (17). Prepared according to Procedure B using **13** to afford 0.06 g (0.09 mmol, 52% yield, 95% pure) of title compound as white solid. 3-(2-(2-((tert-butoxycarbonyl)amino)ethoxy)ethoxy)-5-(furan-2-yl)benzoic acid (0.068 g, 0.17 mmol) was dissolved in DMF (2 mL) and HATU (0.13 g, 0.35 mmol), N-(4-chlorophenyl)-5,5-difluoropiperidine-3-carboxamide, HCl (0.05 g, 0.17 mmol), and NMM (0.05 g, 0.06 mL, 0.52 mmol) were added. The reaction was stirred at 25 °C for 16 hr, and then brine (20 mL) was added. The product was extracted with EtOAc (3 x 15 mL), dried with $MgSO_4$, and concentrated in vacuo. The subsequent oil was subjected to silica gel chromatography eluting with 50% EtOAc: 50% Hex. The fractions containing product were concentrated in vacuo to produce white amorphous solid. Yield=51.5%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ ppm 8.66 (br. s, 1H) 7.56-7.43 (m, 3H) 7.36-7.20 (m, 4H) 6.88 (br. s, 1H) 6.68 (d, J = 3.4 Hz, 1H) 6.48 (br. s, 1H) 4.96 (br. s, 1H) 4.59 (br. s, 1H) 4.19-4.10 (m, 2H) 3.85-3.79 (m, 2H) 3.64-3.59 (m, 2H) 3.58-3.25 (m, 6H) 2.60-2.34 (m, 2H) 1.43 (s, 9H); ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 168.55, 159.37, 156.06, 152.35, 142.74, 136.59, 135.80, 132.95, 129.30, 128.83, 121.25, 118.89, 114.87, 111.95, 106.56, 79.38, 70.45, 69.33, 68.00, 67.75, 44.43, 40.79, 40.37, 35.03, 28.40; MS (ESI+) *m/z*: 648.2 $[M+H]^+$; HPLC Ret: 8.12 min.



1-(3-(2-(2-aminoethoxy)ethoxy)-5-(furan-2-yl)benzoyl)-N-(4-chlorophenyl)-5,5-difluoropiperidine-3-carboxamide, HCl (3). In a 10 mL round bottomed flask, tert-butyl (2-(2-(3-(5-((4-chlorophenyl)carbamoyl)-3,3-difluoropiperidine-1-carbonyl)-5-(furan-2-yl)phenoxy)ethoxy)ethyl)carbamate (0.02 g, 0.03 mmol) was dissolved in 0.5 mL 4M HCl in 1,4-dioxane. The reaction was stirred at 25 ^{o}C for 1 hr upon which the solvents were evaporated. The product was subjected to high vacuum overnight to produce white oil. Yield=100%. ^{1}H NMR (400 MHz, 80 ^{o}C, DMSO-d_6) δ ppm 10.28 (s, 1H) 7.98 (br. s, 3H) 7.71 (dd, J = 1.8, 0.8 Hz, 1H) 7.65-7.57 (m, 2H) 7.42-7.25 (m, 4H) 7.00 (dd, J = 3.4, 0.7 Hz, 1H) 6.88 (dd, J = 2.6, 1.4 Hz, 1H) 6.58 (dd, J = 3.4, 1.8 Hz, 1H) 4.20-4.30 (m, 3H) 3.87-3.70 (m, 6H) 3.53-3.20 (m, 2H) 3.02-2.98 (m, 3H) 2.55-2.28 (m, 1H); ^{13}C NMR (500 MHz, $CDCl_3$-*d*) δ ppm 169.77, 159.47, 152.63, 143.89, 143.54, 138.23, 137.74, 132.62, 129.09, 128.64, 121.93, 115.24, 114.78, 112.60, 111.92, 107.72, 69.42, 68.31, 67.23, 66.88, 39.16, 35.38; MS (ESI+) *m/z*: 548.2 $[M+H]^+$; HPLC Ret: 5.98 min; 92% pure.



N-(4-chlorophenyl)-1-(4-cyano-1H-pyrrole-2-carbonyl)piperidine-3-carboxamide (5). To a solution of 4-cyano-1H-pyrrole-2-carboxylic acid (50 mg, 0.38 mmol) in THF (2 mL) was added N-(4-chlorophenyl)piperidine-3-carboxamide, HCl (**16**, 88 mg, 0.38 mmol) followed by EDC (106 mg, 0.55 mmol), DIPEA (0.160 ml, 0.92 mmol), and DMAP (9 mg, 0.07 mmol). The product was extracted with DCM (3 x 15 mL), washed with sat. $NaHCO_3$ and brine, dried with $MgSO_4$, and concentrated *in vacuo*. The subsequent oil was subjected to silica gel chromatography eluting with 0%-5% MeOH: DCM. The fractions containing product were concentrated in vacuo to produce white amorphous solid. Yield = 74%. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 12.43 (s, 1H) 10.14 (br. s, 1H) 7.75-7.60 (m, 3H) 7.36 (d, J = 5.6 Hz, 2H) 6.99 (s, 1H) 4.39 (br. s, 1H) 4.25 (d, J = 13.2 Hz, 1H) 3.36 (s, 1H) 2.51 (s, 1H) 2.03 (d, J = 12.5 Hz, 1H) 1.84-1.69 (m, 2H) 1.59-1.39 (m, 1H); ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm 172.12, 160.60, 138.42, 129.26, 129.02, 127.22, 126.43, 121.21, 116.43, 114.28, 92.24, 43.67, 40.39, 40.22, 40.79, 28.15, 24.86; QTOF ES+ MS: (M+H) 357.11; HPLC Ret: 6.37 min; 97% pure.

NHS-agarose linking (**4**)

A 10 mM solution of CCG-262545 was made in 100% DMSO and diluted to 600 μM in PBS. 160 mg of dry NHS-activated agarose (Thermo-Fisher Cat# 26197) was mixed with 2 mL of compound and PBS solution and rotated for 2 hours at room temperature. Beads were washed twice with PBS and the remaining NHS sites were blocked with 1 mL of 1 M ethanolamine. Beads were washed again with PBS and resuspended in 2 mL of PBS + 0.05% Na Azide.

Cell Lysate Preparation

HEK293T cells were passaged in DMEM (Gibco, cat#11995065) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco) at 37^{0}C in 5% CO_2. Cells were grown in 15 cm plates and lysed in PBS with protease inhibitors (Roche, cat #11836145001) and sonicated. The lysates were spun at 20,000 rpm for 45' at 4^{0}C and the supernatant was snap frozen with liquid nitrogen and stored at -80^{0}C.

Agarose Bead Pulldown

Supernatants were separated into two groups, each approximately 1 mg/mL total protein concentration. One group received 30 μM CCG-222740, and the other received DMSO control. Technical duplicates were incubated on ice for 30' and then 750 μL of each sample was applied to a spin column (Pierce, cat #69705) loaded with prepared agarose resin. Lysates and beads

were incubated at room temperature with rotation for 1 hour, and immediately washed 3 times with 1 mL chilled PBS. Beads were collected and snap frozen and sent on dry ice for liquid chromatography-mass spectrometry proteomics analysis.

Mass Spectrometry

Proteomics Sample Preparation. Reagents were obtained from Fisher unless otherwise noted. On-bead digests were performed by resuspending each sample in 200 µL of phosphate-buffered saline (PBS) containing 6M urea. To each sample, dithiothreitol (DTT) was added to a final concentration of 5 mM and allowed to sit at room temperature for 30 minutes. Iodoacetamide (Sigma) was then added to a final concentration of 30 mM and incubated in the dark at room temperature for 30 minutes. Samples were diluted to a final concentration of 1.2 M urea and digested overnight with Trypsin-LysC mix (Promega). Following digestion, samples were centrifuged at 1000 rpm for 1 minute (Eppendorf Centrifuge 5424) and the supernatant was collected. To remove any remaining beads, supernatants were filtered through polypropylene Micro Bio-Spin Columns (BioRad) by centrifugation at 5000 rpm with two 200 µL washes with PBS. These washes were combined and desalted with Waters Oasis HLB Prime µElution Plate according to the manufacturer's protocol. Samples were then dried on a Savant SPD1010 SpeedVac Concentrator.

Liquid Chromatography-Mass Spectrometry. Desalted, dried peptides were resuspended in LC-MS grade water with 3% acetonitrile and 0.1% formic acid. Three LC-MS injections (technical replicates) were made for each biological replicate. LC-MS analysis was performed using a Waters nanoAcquity UPLC system fitted with a 5 µm Symmetry C18 trap column (180 µm x 20 mm) and a 1.8 µm HSS T3 analytical column (75 µm x 250 mm). Tryptic peptides were loaded onto the trap column for a 10 minute wash, followed by analytical separation over a 110 minute gradient (3% acetonitrile to 40% acetonitrile over 92 minutes) at a flow rate of 0.5 µL / min. Eluting peptides were introduced into the Synapt-G2S HDMS traveling wave ion mobility quadrupole time-of-flight mass spectrometer (Waters) by electrospray ionization using a pre-cut uncoated 10 µm ID SilicaTip nanospray emitter (New Objective). The Synapt-G2S auxiliary pump was used to deliver the lock mass compounds [Glu1]-Fibrinopeptide B (100 fmol / µL; Waters) at 400 nL / min through the reference sprayer of the nanoLockSpray source. The nanoESI source was set to 70°C with a 3 kV applied potential. Data-independent acquisition was performed in positive mode. The quadrupole mass analyzer was set to transmit all precursor ions (50-2000 m/z) through to the TOF mass analyzer. Precursor ions traveled through the quadrupole mass analyzer, and were then separated in the traveling wave device

using the manufacturer recommended constant wave height of 40 V and variable wave velocity of 1000-600 m/s. Low and elevated-energy scans were alternated every 0.5 seconds (0.05 s interscan delay) to acquire sequential precursor and product ion data. Collision energy was applied after ion mobility separation in the transfer cell. In the low-energy mode, data were collected at constant collision energy (CE) of 4 eV across all 200 drift bins (TOF scans). In high-energy mode, collision energy ramping was applied as follows: 0 eV for bins 1-19; 16.4-58.5 eV for bins 20 – 119; and 58.9 – 63.9 eV for bins 120 – 200. Wave velocities in the trap and transfer cell were set to 311 m / s and 380 m / s, respectively, with wave heights of 4 V. In the trap cell, the N2 gas flow rate was 2.0 mL / min. In the ion mobility cell, the He and N2 flow rates were set to 180 and 90 mL / min, respectively. The mass spectrometer was operated in Resolution mode (V-mode), with a typical resolving power of at least 20,000 FWHM. Data were mass corrected post-acquisition by comparison to the doubly-charged monoisotopic peak of [Glu1]-Fibrinopeptide B (m/z = 785.843), which was sampled every 30 seconds during acquisition.

Proteomics Data Analysis. Raw data were analyzed with ProteinLynx Global SERVER version 3.0.2 (PLGS, Waters Corporation), searching against the Uniprot database of the human proteome. The following criteria were applied to perform the search: (i) trypsin as digestion enzyme, (ii) one missed cleavage allowed, (iii) carbamidomethyl cysteine as a fixed modification and methionine oxidation as a variable modification, (iv) a minimum of two identified fragment ions per peptide and a minimum of five fragments per protein, and (v) at least two identified peptides per protein. The global protein false discovery rate (FDR) was set at 1% using a reversed sequence database. Any identified peptides with calculated mass error greater than 10 ppm were not considered. Protein abundance fold differences and p-values between active / blocked lysate were calculated based on label-free peptide abundances using only unique peptides.

Gene Expression Analysis

Differential gene expression (based on fold change) was calculated for Pirin knockdown (GSE 17551) and CCG-1423 treatment (GSE 30188). The top 100 downregulated genes were used to create gene signatures from the Pirin microarray dataset and the CCG-1423 microarray dataset. The C1-C6 and H gene set collections were dowloaded from MSigDB (accessed 09/2017). The hypergeometric distribution between the CCG-1423 gene set and all other gene sets was

calculated in R to identify statistically significant gene set overlaps. The pirin target gene set has the most significant overlap with the CCG-1423 dataset.

SRE.L Luciferase Assay

HEK293T (7500 cells/well) cells were plated in each well of a 384 well plate in 0.5% FBS+DMEM and transfected using Lipofectamine 2000 (Invitrogen) with 2 ng of a constitutively active $G\alpha_{12}$ (Q231L) and 4 ng of a SRE.L luciferase reporter[6]. Five hours after transfection, compounds were added using a robotic pin tool and cells were incubated overnight at 37^0C. The next day, cell viability was measured using a Gly-Phe-AFC peptide (MP Biochemicals) and luciferase activity was measured using Steady Glo (Promega). Fluorescence (excitation: 390nm, emission: 505 nm) and luminescence was read on a Synergy Neo plate reader (Biotek). IC_{50} values were calculated as a percent of DMSO control using GraphPad Prism. For pirin overexpression, pirin was amplified from pQStrep2-PIR (Addgene plasmid #31570, a gift from Konrad Buessow) using ATTB flanked primers and ligated into pcDNA3.2/V5-DEST using Gateway Cloning (ThermoFisher) and luminescence counts were normalized to cell viability.

Protein Purification

Pirin protein purification protocol was adapted from[7]. Using site-directed mutagenesis, a stop codon was introduced in front of the streptavidin tag and confirmed by sequencing. Pirin plasmid was transformed into BL21 DE3 One Shot cells (Novagen). A single colony was grown in 50 mL LB with 100 ug/mL ampicillin overnight at 37^0C with shaking and transferred to 1 L LB containing 100 ug/mL ampicillin the next day. Cells were grown until the OD_{600} reached 0.5 and then cells were induced with 0.7 mM IPTG for 5 hours at 37^0C. Cells were lysed in 50 mL of 20 mM Tris-HCl pH 8.0, 150 mM NaCl with protease and phosphatase inhibitors (Roche) using sonication. Lysates were spun down at 12,000 rpm for 40' at 4^0C and the supernatent was loaded onto a pre-equilibrated Ni^{2+} column (equilibrated with 50 mL of 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM Imidazole). The column was washed with equilibration buffer including 40 mM Imidazole, and eluted with equilibration buffer with 150 mM Imidazole. The elution was then further purified on a Superdex 200 Prep Grade column (GE) in 20 mM Tris pH 8.0, 150 mM NaCl and analyzed by SDS-PAGE.

Isothermal Titration Calorimetry

Thermodynamic measurements were performed with a VP-ITC Microcalorimeter (MicroCal). Purified and concentrated pirin (100 μM) was loaded into the injection syringe in gel filtration buffer with addition of DMSO (0.1%). The CCG compounds were also dissolved in matched gel filtration buffer at 10 μM and loaded into the cell. Both protein and compounds were degassed for 5 minutes under vacuum. The first injection of protein was 0.8 μL, all subsequent injections were 10 μL, duration 1.5 or 20 seconds, with spacing of 120 or 240 seconds and 29 injections total. A DMSO control experiment was subtracted from each experiment, and data were analyzed by a one-site binding model in VP Viewer 200, using the plotting software ORIGIN 7 (Origin Lab Corp).

Protein Crystallization

Pirin was crystallized by concentrating to 56 mg/mL and setting up hanging drop trays in 24 well trays (Hampton Research). Hanging drops were set up with 1 μL of protein and 1 μL of reservoir solution (0.1 M Hepes, pH 7.5, 8% Ethylene Glycol, 20% PEG 8000)[8] Crystals grew in less than 1 week at 25^0C and then were soaked in 250 μM CCG-257081 or CCG-222740. The diffraction data were collected at the Life Sciences Collaborative Access Team beamline 21-ID-D and 21-ID-G at the Advanced Photon Source, Argonne National Laboratory. Data were processed using the HKL2000 package, and iterative model building and refinement were carried out using Coot[9] and Phenix[10], to yield the final refined structures whose statistics are detailed in Supplemental Table II. Structure figures were generated using UCSF Chimera.

Cell Culture

All human subject studies were reviewed and approved by the Institutional Review Board of the University of Michigan. Primary dermal fibroblasts were isolated from healthy donors and were used before passage ten. Human primary dermal fibroblasts were grown in DMEM (Gibco) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco) at 37^0C in 5% CO_2. All fibroblasts were grown in 6-well (for siRNA studies) or 96-well plates (for compound studies). For compound studies, the cells were stimulated with TGF-β (10 ng/mL, R&D cat #240-B-002) and co-treated with compounds for 24 hours before lysis.

siRNA-mediated gene silencing

ON-TARGET plus SMART pools were commercially synthesized by Dharmacon against human pirin (GE, cat # L-012613-00-0005) or non-targeting pool control (GE, cat# D-001810-10-05) and were used according to the manufacturer's recommendations. Briefly, normal primary dermal fibroblasts (2×10^5) were plated in 6-well culture plates with DMEM containing 10% FBS and 1% 100 units/mL antibiotics/antimycotics. The next day, 25 nmol/L siRNA smart pools were transfected using DharmaFECT 1 (GE, cat# T-2001-01) dissolved in Opti-MEM media. After 24 hours of transfection, the media on the cells was changed to DMEM containing 10% FBS and 1% 100 units/mL antibiotics/antimycotics and were kept for an additional 24 hours before changing the media to 0.5% FBS+DMEM and treating with the stimulus.

To measure the effect of downregulating pirin on SRE.L Luciferase, cells were treated as above to transfect siRNA against pirin. After 48 hours of siRNA treatment, cells were trypsinized, plated into a 384 well plate in 0.5% FBS+DMEM and transfected with 2 ng/well MRTF-A and 56 ng/well of SRE.L Luciferase using Lipofectamine LTX and Plus Reagent (Invitrogen) and read 24 hours later. Luciferase values were normalized to cell viability as measured by using a Gly-Phe-AFC peptide (MP Biochemicals).

RT-PCR

For Figure 4A and B, 10,000 cells were plated in each well of a 96 well plate for 24 hours before changing the media to DMEM containing 0.5% FBS and concurrently adding TGF-β as well as compound or DMSO control. The cells were lysed in iScript RT-qPCR lysis buffer (Biorad, cat #170-8898), and the iTaq Universal SYBR Green One Step Kit was used (BioRad, cat #172-5151) following the manufacturer's instructions. 1 uL of template lysate was added to the RT-qPCR master mix. For Figure 4C and D, RNA was isolated using the RNeasy Kit (Qiagen) following the manufacturer's protocol. RNA (1 μg) was used as a template for synthesizing complementary DNA utilizing the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). SYBR green qPCR (Thermo Fisher Scientific) was performed using a Stratagene Mx3000P (Agilent Technologies) and C_t values were analyzed relative to *GAPDH* expression. Primer sequences used were as follows: *GAPDH*: 5′-GAAGGTGAAGGTCGGAGTCA-3′, 3′-CTGGGGAAGTAACTGGAGTT-5′; *CTGF*: 5′-CAGAGTGGAGCGCCTGTT-3′, 3′-

CTGCAGGAGGCGTTGTCA-5′; *ACTA2:* 5′-AGCCCAGCCAAGCACTG-3′, -3′-GAGACATTCCGGCCGAAAC-5′ and *PIRIN*: 5′-GTGGAGCCTCAGTACCAGGA-3′, 3′-AAATGTGAGCGTGTGGTTGG-5′

Western blotting

Cells were lysed with RIPA buffer containing 10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 140 mM NaCl, protease inhibitor cocktail (Roche, cat# 11873580001). Cell lysates were sonicated for 10 min and centrifuged to pellet insoluble material. The supernatant was transferred to labeled Eppendorf tubes and protein concentrations were quantitated using a BCA protein assay (Pierce BCA Protein Assay, Thermo Scientific). Protein lysates (40 µg) were then run on 10% polyacrylamide gels and transferred to PVDF membranes, and blocked in LI-COR buffer for one hour. Membranes were incubated in 1:1000 diluted primary antibodies pirin (Thermo Fisher Scientific, cat # PA5-51441), p-SMAD2 (S465/467) (Cell Signaling, cat # 3108T), total SMAD2 (Cell Signaling, cat # 5339T) and GAPDH (Santa Cruz, cat # SC-365062) overnight at 4°C and were incubated in 1:5000 diluted secondary antibodies for one hour at 25°C the next day. Blots were visualized using a LI-COR Odyssey Fc and quantified using Image Studio software.

shRNA Virus Production and Infection

HEK-293T cells were seeded into 10-cm plates and were allowed to attach overnight. The next day, the cells were transfected with a plasmid cocktail containing 5000 ng of the pLKO plasmid containing shRNA sequences, 3750 ng of psPAX2, 1250 ng of pMD2.G, and 20 uL of Lipofectamine 2000 in 400 uL of OptiMEM. The next morning, the media was changed to 10 mL of fresh culture medium, and the next day each plate was supplemented with an additional 5 mL of culture medium. After 24 h, the culture medium was harvested and filtered through a 0.45-micron syringe filter.

Primary human dermal fibroblasts were seeded into 6 well plates and allowed to attach overnight. The next day, 1 mL of virus was added to the appropriate well, along with 4 ug/mL polybrene. The next day, cells were split into 10 cm plates and selected with 5 ug/mL puromycin. Validation of shRNA knockdown efficiency was done by qPCR for pirin.

shPirin_1

Top: CCGGACTCGCACACCAACCTTATATCTCGAGATATAAGGTTGGTGTGCGAGTTTTTTG

shPirin_1

Bottom: AATTCAAAAAACTCGCACACCAACCTTATATCTCGAGATATAAGGTTGGTGTGCGA
GT

shLacZ

Top: CCGGCGCTAAATACTGGCAGGCGTTCTCGAGAACGCCTGCCAGTATTTAGCGTTTTT

shLacZ Bottom:

AATTAAAAACGCTAAATACTGGCAGGCGTTCTCGAGAACGCCTGCCAGTATTTAGCG

Immunocytochemistry

10,000 primary dermal fibroblasts infected with virus against LacZ or Pirin were plated onto 8 well chamber slides (Lab_Tek, cat #154534). The next day, cells were washed 1x with PBS and then treated with 10 ng/mL TGF-β for 24 hours in DMEM containing 0.5% FBS and antibiotics. Cells were then fixed with 4% formaldehyde and permeabilized with 0.25% Triton-X. Several drops of Image-It Fx Signal Enhancer (Life Technologies, cat #136933) were added to each well for 30', and then cells were blocked with 10% goat serum for 1 hour. Cells were then incubated with anti-MRTF-A antibody (Cell Signaling, cat #14760S) using an antibody dilution of 1:100 in 2% BSA, PBS, and 0.1% Triton-X overnight at 4^0C. After washing with PBS, cells were incubated with anti-rabbit Alexa-Fluor 488 conjugated secondary antibody (Thermofisher, cat #A11034). Cells were counterstained and mounted using Pro-long Gold antifade reagent with DAPI (Thermofisher, cat # P36395). Images were obtained using an Evos microscope at 20x magnification, and were randomized and blinded before subcellular localization was assigned using Image J. Post-image processing using Image J and Powerpoint used the same parameters for all images.

Bleomycin skin fibrosis

Twenty-four C57BL/6J mice were ordered from Jackson labs for delivery at 15-16 weeks of age. Mice were preconditioned with supplemental chow for two weeks prior to start of experiment. Mice were randomized by weight into three groups (vehicle, CCG-257081, PBS). Each group consisted of two cages with four mice per cage. Drug stock solutions were aliquoted and stored at 4^oC for the duration of the study. Aliquots were warmed to 37^oC and briefly vortexed prior to filling the gavage syringes. Vehicle and CCG-257081 groups received daily bleomycin injections (0.1mL, ID) each afternoon in a defined area of shaved dorsal skin. PBS control group received daily injections (0.1mL, ID) of PBS in the afternoon. Mice were anesthetized with

ketamine/xylazine for ID injections. CCG-257081 (50mg/kg) and the vehicle (20% DMSO/50% PEG-400/30% PBS) were administered (PO) in the morning by the University of Michigan's Unit for Laboratory Animal Medicine In-Vivo Animal Core. Mice were weighed daily to determine gavage dosage volume. Mice were euthanized after fourteen days of treatment by CO_2 inhalation/thoracotomy. Skin from the defined area was excised for biochemical and histological analysis. A portion of the skin was fixed in neutral buffered formalin (10%), washed in 70% ethanol, and paraffin-embedded for Masson's trichrome histological staining. Another portion of the skin was snap frozen for hydroxyproline measurement.

Histological Analysis.

Fixed skin was paraffin embedded and sectioned at the University of Michigan Comprehensive Cancer Center Histology Core. Skin sections were stained with Masson's trichrome (Sigma-Aldrich). Stained sections were analyzed with an Olympus BX51\DP72 microscope. Dermal thickness was determined by measuring the maximal distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction. Three measurements were averaged from each skin section. The measurement was performed using the measurement tool in the cellSens imaging software package (Olympus).

Hydroxyproline Assay.

Skin sections were weighed and hydrolyzed in 6M HCl at 120^{o}C for three hours. Hydrolyzed skin supernatant and hydroxyproline standards (Sigma-Aldrich) were transferred to a microplate and dried at 60^{o}C. Samples and standards were oxidized with Chloramine T oxidation buffer for 5 minutes at room temperature. 4-(Dimethylamino) benzaldehyde was added to the wells and incubated at 60^{o}C until the standard was well-defined (approximately 90 minutes). Absorbance was measured at 560 nm using a Synergy HT microplate reader (BioTek Instruments). Hydroxyproline values were normalized to tissue weight.

GST-MRTF-A Actin Pulldown

GST-MRTFA 10-142 plasmid (in pGEX-4T-2 vector) was transformed into BL21 DE3 One Shot cells (Novagen). A single colony was grown in 50 mL LB with 100 ug/mL ampicillin overnight at 37^{0}C with shaking and transferred to 1 L LB containing 100 ug/mL ampicillin the next day. Cells were grown until the OD_{600} reached 0.5 and then cells were induced overnight at 25^{0}C. Cell pellets were resuspended in 50 mL of 50 mM Tris pH 8.0, 300 mM NaCl, 1% Triton-X, 1 mM

DTT, 1 mM EDTA and protease inhibitors (Roche). Pellets were sonicated, and lysate was centrifuged at 33,000 rpm for 20' @ 4^0C. Soluble lysate was bound to Glutathione beads overnight, and washed with 25 mL lysis buffer without Triton-X and eluted with lysis buffer including 40 mM reduced glutathione. Actin lysate was collected from 2 confluent plates of NIH-3T3 cells that were lysed in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 3 mM MgCl2, 0.2 mM EGTA, 0.2 mM ATP, 1 mM DTT, 0.5% Triton-X and protease inhibitors.

Recombinant Luciferase Assay

Recombinant Firefly luciferase (QuantiLum, North American firefly, Photinus pyralis) was purchased from Promega (Cat #E1701) and diluted to 20 nM in PBS. 30 μL of diluted luciferase was dispensed into a 384 well opaque white plate and 150 nL of compound or DMSO control was added to each well with a robotic pintool. Luciferase was incubated with compounds for 10 minutes and then 10 μL of Steady-Glo was added and plates were read on a Synergy Neo Plate Reader.

Statistical Analysis

All statistical analyses were performed in GraphPad Prism, version 7.04. For experiments with multiple comparisons, One-way ANOVA was performed with post-hoc multiple comparisons tests. For experiments with a single comparison, unpaired t-tests were used for comparisons using normalized data. Ratio paired t-tests were used for non-normalized comparisons due to the substantial variability in the TGF-β stimulation between experiments. Differences were considered significant if the P value was <0.05. Asterisks were used as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

References

1. Davenport, A. J.; Braeuer, N.; Fischer, O. M.; Rotgeri, A.; Rottmann, A.; Neagoe, I.; Nagel, J.; Godinho-Coelho, A.-M. 1,3-Thiazol-2-ylbenzamides as P2X purinoceptor antagonists and their preparation. WO2016091776A1, 2016.
2. Belanger, D. B.; Flohr, S.; Gelin, C. F.; Jendza, K.; Ji, N.; Liu, D.; Lorthiois, E. L. J.; Karki, R. G.; Mainolfi, N.; Powers, J. J.; Randl, S. A.; Rogel, O.; Vulpetti, A.; Yoon, T. Preparation of aminomethyl-biaryl derivatives as complement alternative pathway modulators for treatment of opthalmic and other diseases. WO2015009977A1, 2015.
3. Feutrill, J.; Leriche, C.; Middlemiss, D. Preparation of aniline derivatives as EP2 agonists useful in treating glaucoma. WO2013037705A2, 2013.
4. Hutchings, K. M.; Lisabeth, E. M.; Rajeswaran, W.; Wilson, M. W.; Sorenson, R. J.; Campbell, P. L.; Ruth, J. H.; Amin, A.; Tsou, P. S.; Leipprandt, J. R.; Olson, S. R.; Wen, B.; Zhao, T.; Sun, D. X.; Khanna, D.; Fox, D. A.; Neubig, R. R.; Larsen, S. D., Pharmacokinetic optimitzation of CCG-203971: Novel inhibitors of the Rho/MRTF/SRF transcriptional pathway as potential antifibrotic therapeutics for systemic scleroderma. *Bioorg Med Chem Lett* **2017,** *27* (8), 1744-1749.
5. Lercher, L.; McGouran, J. F.; Kessler, B. M.; Schofield, C. J.; Davis, B. G., DNA Modification under Mild Conditions by Suzuki-Miyaura Cross-Coupling for the Generation of Functional Probes. *Angew Chem Int Edit* **2013,** *52* (40), 10553-10558.
6. Evelyn, C. R.; Wade, S. M.; Wang, Q.; Wu, M.; Iniguez-Lluhi, J. A.; Merajver, S. D.; Neubig, R. R., CCG-1423: a small-molecule inhibitor of RhoA transcriptional signaling. *Mol Cancer Ther* **2007,** *6* (8), 2249-60.
7. Zeng, Q.; Li, X.; Bartlam, M.; Wang, G.; Pang, H.; Rao, Z., Purification, crystallization and preliminary X-ray analysis of human pirin. *Acta Crystallogr D Biol Crystallogr.* **2003,** *59* (Pt 8), 1496-8. Epub 2003 Jul 23.
8. Cheeseman, M. D.; Chessum, N. E.; Rye, C. S.; Pasqua, A. E.; Tucker, M. J.; Wilding, B.; Evans, L. E.; Lepri, S.; Richards, M.; Sharp, S. Y.; Ali, S.; Rowlands, M.; O'Fee, L.; Miah, A.; Hayes, A.; Henley, A. T.; Powers, M.; Te Poele, R.; De Billy, E.; Pellegrino, L.; Raynaud, F.; Burke, R.; van Montfort, R. L.; Eccles, S. A.; Workman, P.; Jones, K., Discovery of a Chemical Probe Bisamide (CCT251236): An Orally Bioavailable Efficacious Pirin Ligand from a Heat Shock Transcription Factor 1 (HSF1) Phenotypic Screen. *J Med Chem.* **2017,** *60* (1), 180-201. doi: 10.1021/acs.jmedchem.6b01055. Epub 2016 Dec 22.
9. Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K., Features and development of Coot. *Acta Crystallogr D Biol Crystallogr.* **2010,** *66* (Pt 4), 486-501. doi: 10.1107/S0907444910007493. Epub 2010 Mar 24.
10. Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H., PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr.* **2010,** *66* (Pt 2), 213-21. doi: 10.1107/S0907444909052925. Epub 2010 Jan 22.