Discovery and Hit-to-Lead Optimization of Non-ATP Competitive (MAPKAPK2) MK2 Inhibitors

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Supporting Information

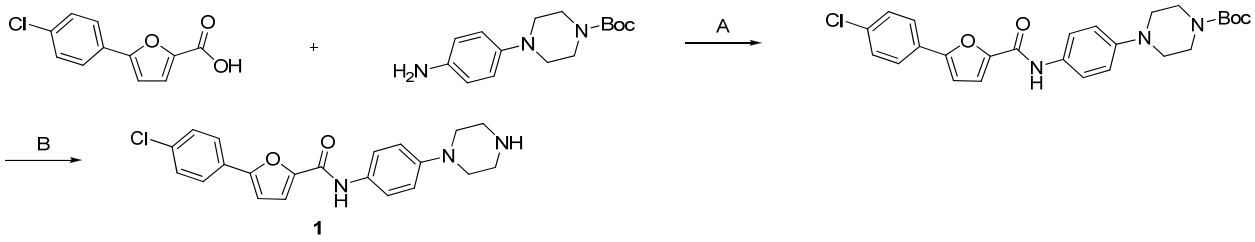
Commonly used abbreviations

ACN	Acetonitrile
AcOH	Acetic acid
DCM	Dichloromethane
DIEA	Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
EtOH	Ethanol
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
Hex	hexanes
HPLC	High pressure liquid chromatography
LCMS	Liquid chromatography mass spectrometry
NMR	Nuclear magnetic resonance
RT	Room temperature
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
hIRAK4	Human interleukin-1 receptor-associated kinase 4
hCAMk4	Human calcium/calmodulin-dependent protein kinase IV
hCHK1	Human checkpoint homolog kinase 1
hMET	Human met proto-oncogene kinase
hMST2	Human serine/threonine kinase 3
hTSSk2	Human testis-specific serine kinase 2
hROCK2	Human Rho-associated coiled-coil containing protein kinase 2
hIGF1R	Human insulin-like growth factor 1 receptor
hAKT1	Human v-akt murine thymoma viral oncogene homolog 1 (also known as PKB)
hIkkB	Human inhibitor of nuclear factor kappa-B kinase subunit beta
hLCK	Human Lymphocyte specific protein tyrosine kinase
hCSNk1D	Human casein kinase 1, delta

Section I: Synthetic Chemistry and Key Compound Characterization

The purity of all the compounds in the manuscript was ≥95%, determined by NMR, LC/MS.

(A) Synthesis of compound **1**:



1

Reagents and conditions: (A) HATU, DIEA, DMF, rt; (B) TFA.

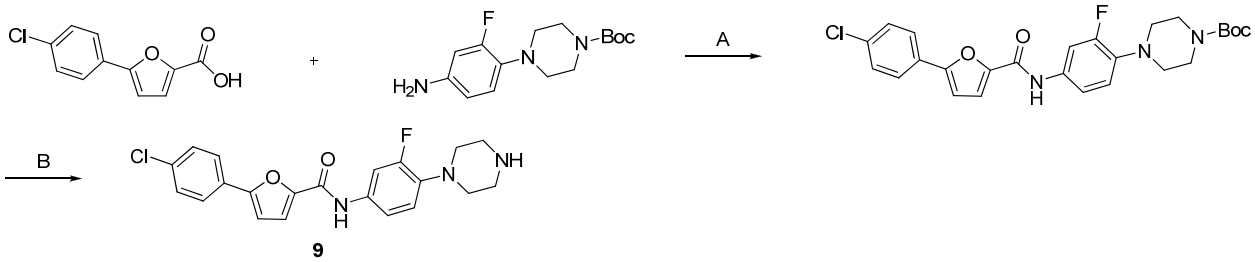
Step A

To a mixture of 5-(4-chlorophenyl)furan-2-carboxylic acid (0.22 g, 1.0 mmol) and HATU (0.38 g, 1.0 mmol) in DMF (3 mL) was added DIEA (0.35 mL, 2.0 mmol) and *tert*-butyl 4-(4-aminophenyl)piperazine-1-carboxylate (0.42 g, 1.5 mmol). The reaction mixture was stirred at room temperature for overnight, and concentrated. The residue was purified by chromatography on silica gel column. *tert*-Butyl 4-(4-(5-(4-chlorophenyl)furan-2-carboxamido)phenyl)piperazine-1-carboxylate (0.48 g, 99% yield) was eluted with a solvent mixture of hexane and EtOAc (1:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.19 (s, 1H), 7.64 (d, *J* = 9.0 Hz, 2H), 7.57 (d, *J* = 9.0 Hz, 2H), 7.35 (d, *J* = 8.5 Hz, 2H), 7.24 (d, *J* = 3.5 Hz, 1H), 6.90 (d, *J* = 9.0 Hz, 2H), 6.72 (d, *J* = 3.5 Hz, 1H), 3.56 (t, *J* = 5.0 Hz, 4H), 3.08 (t, *J* = 5.0 Hz, 4H), 1.48 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.0, 154.8, 154.6, 148.5, 147.3, 134.6, 130.3, 129.2, 128.1, 125.8, 121.7, 117.23, 117.16, 108.1, 80.0, 49.8, 29.8, 28.5.

Step B

tert-Butyl 4-(4-(5-(4-chlorophenyl)furan-2-carboxamido)phenyl)piperazine-1-carboxylate (0.1 g, 0.2 mmol) was stirred in TFA (neat, 1 mL) at room temperature for 15 minutes. The solution was concentrated. The residue was purified by reverse phase HPLC to give compound **1** (75 mg, 95% yield) as an HCl salt. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.22 (s, 1H), 9.50 (br s, 2H), 8.00 (dd, *J* = 6.5, 2.0 Hz, 2H), 7.69 (d, *J* = 9.0 Hz, 2H), 7.56 (dd, *J* = 7.0, 2.0 Hz, 2H), 7.42 (d, *J* = 4.0 Hz,1H), 7.22 (d, *J* = 3.5 Hz,1H), 7.07 (d, *J* = 9.0 Hz, 2H), 3.23 (s, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 155.8, 153.9, 147.1, 145.9, 133.2, 131.8, 129.0, 128.3, 126.3, 121.9, 116.8, 116.7, 108.6, 46.2, 42.4. Mass calcd for $C_{21}H_{21}ClN_3O_2^+$ $(M+H)^+$ 382.13168; Found 382.13141.

(B) Synthesis of compound **9**:



Reagents and conditions: (A) HATU, DIEA, DMF, rt; (B) TFA.

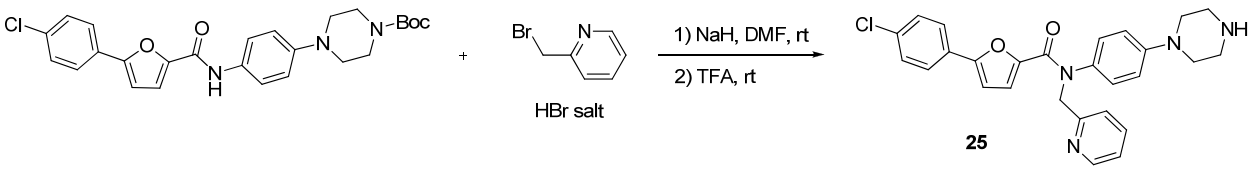
Step A

To a mixture of 5-(4-chlorophenyl)furan-2-carboxylic acid (0.22 g, 1.0 mmol) and HATU (0.42 g, 1.1 mmol) in ACN (3 mL) was added DIEA (0.35 mL, 2.0 mmol) and *tert*-butyl 4-(4-amino-2-fluorophenyl)piperazine-1-carboxylate (0.35 g, 1.2 mmol). The reaction mixture was stirred at room temperature for overnight. The precipitates were filtered, washed with ACN and dried in *vacuo*. The product, *tert*-butyl 4-(4-(5-(4-chlorophenyl)furan-2-carboxamido)-2-fluorophenyl)piperazine-1-carboxylate, was obtained as a white solid (0.44 g, 87% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 (s, 1H), 7.65 (d, J = 8.5 Hz, 2H), 7.58 (dd, J = 14.0, 2.5 Hz, 1H), 7.39 (d, J = 8.5 Hz, 2H), 7.28 (d, J = 3.5 Hz, 1H), 7.30–7.25 (m, 1H), 6.90 (t, J = 9.0 Hz, 1H), 6.76 (d, J = 3.5 Hz, 1H), 3.59 (t, J = 5.0 Hz, 4H), 3.00 (t, J = 5.0 Hz, 4H), 1.48 (s, 9H). ^{19}F NMR (470 MHz, $CDCl_3$) δ -121.04 (dd_{H-F}, J = 13.6, 9.4 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.6, 156.1, 155.01, 154.97, 154.7, 147.0, 137.0, 136.9, 135.0, 132.8, 132.7, 129.4, 128.1, 126.0, 119.53, 119.50, 117.82, 116.21, 116.18, 109.5, 109.3, 108.4, 80.1, 50.9, 28.7, observed complexity due to C-F coupling.

Step B

tert-butyl 4-(4-(5-(4-chlorophenyl)furan-2-carboxamido)-2-fluorophenyl)piperazine-1-carboxylate (80 mg, 0.16 mmol) was stirred in TFA (neat, 1 mL) at room temperature for 15 minutes. The solution was concentrated. The residue was purified by reverse phase HPLC to give compound **9** (64 mg, 100% yield) as an HCl salt. ^{1}H NMR (500 MHz, CD_3OD) δ 7.94–7.90 (m, 2H), 7.69 (dd, J = 14.5, 2.5 Hz, 1H), 7.50–7.47 (m, 1H), 7.47–7.44 (m, 2H), 7.36 (d, J = 4.0 Hz, 1H), 7.13 (t, J = 9.5 Hz, 1H), 7.01 (d, J = 4.0 Hz, 1H), 3.43–3.39 (m, 4H), 3.37–3.32 (m, 4H). ^{19}F NMR (470 MHz, CD_3OD) δ -123.73 (dd_{H-F}, J = 14.1, 9.4 Hz). ^{13}C NMR (125 MHz, DMSO-d_6) δ 155.9, 155.3, 154.2, 153.3, 146.7, 134.7, 134.6, 134.34, 134.26, 133.3, 129.1, 128.2, 126.3, 119.6, 117.4, 116.7, 109.0, 108.8, 108.7, 47.3, 42.8, observed complexity due to C-F coupling. Mass calcd for $C_{21}H_{20}ClFN_3O_2^+$ $(M+H)^+$ 400.12226; Found 400.12215.

(C) Synthesis of compound **25**:



To a solution of *tert*-butyl 4-(4-(5-(4-chlorophenyl)furan-2-carboxamido)phenyl)piperazine-1-carboxylate (0.12 g, 0.24 mmol) in DMF (1 mL) at room temperature was added sodium

hydride (21 mg, 0.53 mmol, 60% in mineral oil), followed by the HBr salt of 2-(bromomethyl)pyridine (72 mg, 0.29 mmol). The reaction mixture was stirred at room temperature for overnight. HPLC analysis of the crude reaction mixture showed clean conversion of starting material to the desired product. The mixture was concentrated. To the residue was added TFA (neat, 1 mL). The reaction mixture was stirred at room temperature for 15 minutes. The solution was concentrated. The residue was purified by reverse phase HPLC to give compound **25** (98 mg, 87% yield) as an HCl salt. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.60 (br s, 2H), 8.80 (d, J = 5.0 Hz, 1H), 8.48–8.41 (m, 1H), 8.05 (d, J = 8.0 Hz, 1H), 7.87 (t, J = 6.5 Hz, 1H), 7.47-7.43 (m, 2H), 7.41–7.33 (m, 4H), 7.07–7.02 (m, 3H), 6.49 (d, J = 3.5 Hz, 1H), 5.33 (s, 2H), 3.46–3.40 (m, 4H), 3.19 (br s, 4H).^{13}C NMR (125 MHz, DMSO-d_6) δ 158.5, 153.6, 153.0, 149.8, 146.0, 142.6, 133.8, 133.3, 129.0, 128.8, 127.9, 125.8, 125.5, 125.4, 120.1, 116.3, 108.2, 52.0, 45.1, 42.5. Mass calcd for $C_{27}H_{26}ClN_4O_2^+$ $(M+H)^+$ 473.17388; Found 473.17361.

Section II: Compound **25** kinase counter screen

(A) Against CarnaBiosciences kinases[a]

Kinase	% activity remaining	Kinase	% activity remaining	Kinase	% activity remaining
Abl(h)	134	GSK3β(h)	130	PKCδ(h)	88
ACK1(h)	109	Haspin(h)	60	PKCι(h)	103
ALK(h)	92	Hck(h)	112	PKCμ(h)	88
ALK4(h)	97	HIPK1(h)	114	PKCθ(h)	117
Arg(h)	58	HIPK3(h)	119	PKCζ(h)	98
ARK5(h)	100	IKKα(h)	119	PKD2(h)	95
ASK1(h)	110	IR(h)	97	PKG1α(h)	128
Aurora-A(h)	111	IRR(h)	117	PKG1β(h)	142
Axl(h)	110	Itk(h)	115	Plk1(h)	115
Bmx(h)	102	JAK2(h)	127	Plk3(h)	84
BRK(h)	117	JAK3(h)	106	PRK2(h)	101
BrSK1(h)	126	JNK3(h)	145	PrKX(h)	102
BrSK2(h)	118	KDR(h)	117	PTK5(h)	95
CaMKI(h)	79	LOK(h)	104	Pyk2(h)	121
CaMKIIβ(h)	79	MAPK1(h)	92	Ret(h)	106
CaMKIIγ(h)	125	MAPKAP-K2(h)	8	RIPK2(h)	113
CaMKIIδ(h)	100	MARK1(h)	107	Ron(h)	105
CDK2/cyclinA(h)	97	MELK(h)	112	Ros(h)	113
CDK7/cyclinH/MAT1(h)	101	Mer(h)	98	Rse(h)	99
CDK9/cyclin T1(h)	119	MINK(h)	103	Rsk1(h)	107
CHK2(h)	95	MLCK(h)	93	Rsk1(r)	75
CK1γ1(h)	105	MLK1(h)	106	Rsk2(h)	119
CK1γ2(h)	120	Mnk2(h)	101	Rsk3(h)	96
CK1γ3(h)	30	MRCKα(h)	96	Rsk4(h)	136
CK1δ(h)	105	MSK1(h)	103	SAPK2a(h)	117
CK2(h)	60	MSSK1(h)	88	SGK(h)	90
CLK2(h)	91	mTOR(h)	110	SGK2(h)	125
cKit(h)	73	MuSK(h)	137	SGK3(h)	135

CSK(h)	107	NEK2(h)	115	SIK(h)	134
c-RAF(h)	97	NEK11(h)	102	SRPK1(h)	120
DAPK1(h)	132	NLK(h)	121	SRPK2(h)	101
DAPK2(h)	92	p70S6K(h)	93	STK33(h)	76
DRAK1(h)	131	PAK2(h)	109	Syk(h)	137
DYRK2(h)	103	PAR-1Bα(h)	97	TAK1(h)	112
EGFR(h)	103	PASK(h)	61	TAO1(h)	86
EphA1(h)	100	PDK1(h)	110	TAO3(h)	121
EphB1(h)	91	PhKγ2(h)	98	TBK1(h)	117
ErbB4(h)	100	PI3 Kinase (p110β/p85α)(h)	102	Tec(h) activated	80
FAK(h)	119	PI3 Kinase (p120γ)(h)	93	Tie2(h)	98
FGFR1(h)	113	PI3 Kinase (p110δ/p85α)(h)	88	TLK2(h)	100
FGFR1(V561M)(h)	90	PI3 Kinase (p110α/p85α)(m)	100	TrkA(h)	105
FGFR2(h)	104	Pim-1(h)	57	TrkB(h)	101
FGFR2(N549H)(h)	112	Pim-2(h)	133	TSSK1(h)	101
FGFR3(h)	95	Pim-3(h)	91	TSSK2(h)	118
Flt4(h)	120	PKA(h)	86	ULK2(h)	110
GCK(h)	126	PKBα(h)	105	WNK2(h)	118
GRK5(h)	87	PKBβ(h)	116	VRK2(h)	93
GRK6(h)	100	PKBγ(h)	120	ZIPK(h)	80
GRK7(h)	110	PKCα(h)	100		
GSK3α(h)	109	PKCβI(h)	105		

[a] Screen was performed in duplicate @ 10 μM.

(B) In-house kinase assays

Kinase	IC50 (nM)
AKT1	>30000
CaMKIV(h)	>30000
CDK2/cycA	>30000
CHK1(h)	>30000
CSNK1D	>30000
EGFR	>30000
EPHB4	>30000
ERK2	>30000
FLT3	>30000
GSK3B	>30000
IGF-1R(h)	>30000
IKKβ(h)	>30000
IRAK4(h)	>30000
JNK1α1(h)	>30000
KDR	>30000
Lck(h)	>30000
Met(h)	>30000
MST1(h)	>30000
MST2	>30000

NEK2	>30000
PKCA	>30000
PLK3	>30000
ROCK-II(h)	>30000
RSK2	>30000
TSSK2(h)	>30000

Section III: Compound **25** CYP (cytochrome p450) inhibition data and experimental protocol

CYP 3A4	Co. IC50 > 20 μM
	Pre. IC50 > 20 μM
CYP 2D6	Co. IC50 > 20 μM
	Pre. IC50 > 20 μM
CYP 2C9	Co. IC50 > 20 μM
	Pre. IC50 > 20 μM

In order to assess the potential for inhibition of CYPs, human liver microsomes were incubated with several concentrations of tested articles (0.2, 2 and 20 μM), 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) and substrates for various CYPs at 37 ºC. The substrate concentration was kept near the Km value for each CYP reaction. They were 16 μM dextromethorphan (*O*demethylase reaction) for CYP2D6, 100 μM testosterone (6β-hydroxylase reaction) for CYP3A4 and 200 μM tolbutamide (4-hydroxylase reaction) for CYP2C9. The concentrations of the metabolites formed from each substrate after incubation were determined by LCMS/MS using a standard curve. The concentrations at which 50% of the initial enzyme activity was inhibited (IC_{50}) were determined from the graph of concentrations versus percent of inhibition.

To evaluate time-dependent inhibition, compound, at the stated concentrations (0.2, 2 and 20 μM), was preincubated with human liver microsomes for 30 min at 37 ºC in the presence of NADPH and in the absence of substrates. After the pre-incubation step, the CYP substrates were added at the previously stated concentrations and the reactions were allowed to proceed as indicated in the previous paragraph.

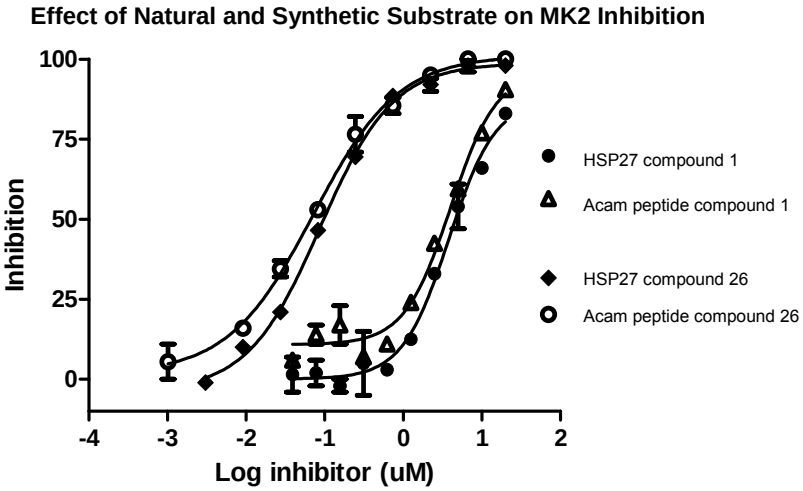
Section IV: Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA) Enzyme Assay Protocol

All steps are carried out at room temperature. Active MK2 kinase (UPSTATE # 14-337) diluted into 2X reaction buffer* to prepare a 5 nM kinase concentration was combined in black polypropylene 96 well plates with compound prepared in 25% DMSO. Following a 30 minute pre-incubation, 20 μL of 6 μM Acam peptide (1411B Autocam Biotinylated Peptide; Cell Signaling Technologies) was added and the reaction is allowed to proceed for 10 minutes. The reaction was terminated by transfer of 10 microliters into 190 microliters of DELFIA Assay buffer, 10 mM in EDTA (Perkin-Elmer # 4002-0010) in Streptavidin plates (Roche Diagnostics Corp. #117347760001). After 1 hour of shaking the plates were washed (DELFIA wash buffer 4010-0010) and detection antibodies (Cell Signaling Technologies #9386; Perkin Elmer # AD0124) diluted into DELFIA Assay buffer were added to wells for 1 hour incubation. Following the antibody incubation the plates were washed a second time. Enhancement

solution (Perkin Elmer# 4001-0010) was added to the wells and after 10 minutes of shaking, activity (Europium fluorescence) read on a Wallac Victor 1420 plate reader.
*FINAL 1X REACTION BUFFER:
20 mM HEPES pH 7.3
2/100 µM ATP
50 mM NaCl
10 mM $MgCl_2$
1 mM dithiothreitol

Section V: Effect of Synthetic and Natural Substrate on MK2 Inhibition

To confirm the equivalence in enzymatic IC50 results using either synthetic substrate, Acam peptide, or natural substrate, HSP27, ALIS hit compound **1** and optimized analog **26** were assayed in DELFIA using both substrates, and the results were shown below. The resemblance in both the IC50 value and curve shape demonstrated equivalent inhibitor activity against an artificial peptide substrate and a natural substrate.



	Compound 1		Compound 26	
	HSP27	Acam	HSP27	Acam
IC50 (µM)	3.81	4.01	0.088	0.072
HillSlope	1.47	1.48	0.94	0.80

HSP27 Natural Substrate Assays

DELFIA assays carried out with biotinylated HSP27 as substrate were performed according to the standard assay procedure. Biotinylated HSP27 DELFIA assay substrate was prepared by biotinylation of HSP27 recombinant protein (RayBiotech, Inc.# IP-16-027P) with biotinylation kit #21955 from Thermo Scientific Research.

Section VI: Assay of HSP27 phosphorylation in SW1353 cells and SW1353 cell viability

Changes in IL-1β-stimulated HSP27 phosphorylation in SW1353 chondrosarcoma cells were measured using an in-cell ELISA method. SW1353 cells (ATCC; Rockville, MD) were routinely

grown in a medium consisting of DMEM containing 10% FBS, 2 mM GlutaMAX I (Invitrogen), 1X penicillin-streptomycin (Gibco #15070; Invitrogen) and 0.1 mM non-essential amino acids at 37 ºC in 5% atmospheric CO_2. One day prior to assay, cells were collected and suspended in serum-free medium and added to a 96-well assay culture plate at $6.0x10^3$ cells/100 uL/well and the plate incubated overnight at 37 ºC and 5% CO_2. Cells were pre-treated with serially diluted test compounds for 30 min. prior to stimulation with IL-1β (100 pg/mL) for 27 min. Growth medium was then immediately aspirated off the cells and they were fixed with 4% formaldehyde in PBS for 20min. Specific detection of HSP27 serine 209 phosphorylation was then performed using the HSP27 FACE Kit #48350 (Active Motif; Carlsbad, CA) and specifically following the manufacturer's protocols. Cell viability after compound exposure was determined indirectly by measuring cellular total ATP content in a sister plate set up identically to the SW1353 HSP27 phosphorylation assay plate. Cellular ATP content was measured by employing a commercially available ATP-dependent luciferase-based luminescent assay kit and following the manufacturer's protocol (CellTiter-Glo; Promega # G7571).

Section VII: Assay of MMP-13 secretion from SW1353 cells and human primary osteoarthritis-derived chondrocytes.

Cells in DMEM containing 10% FBS, 2 mM GlutaMAX I (Invitrogen), 1X penicillin-streptomycin (Gibco #15070; Invitrogen) and 0.1 mM non-essential amino acids were distributed at $5.0x10^3$ cells/100µl/well in a 96-well plate and cultured overnight at 37 ºC in 5% atmospheric CO_2. The following day the culture medium in all wells was exchanged with 100 uL of the same supplemented with 0.5% lactalbumin hydrolysate (Sigma-Aldrich; Cat. No. L9010) and then cells were further cultured for 6 hours before compound addition. After 30 minutes of treatment with varying concentrations of compound **25** the cells were stimulated with IL-1β (20 ng/mL) for 24 hours and then MMP-13 in the supernatant was specifically measured by ELISA using a commercial kit and protocol as described by the manufacturer (GE Healthcare; R&D Systems; Cat. No. RPN2621). Effect of compound **25** on MMP-13 secreted from human primary chondrocytes was assessed by the same method as used for SW1353 cells. The primary human adult chondrocytes were obtained as cryopreserved samples from a commercial vendor that obtained them from needle biopsies of patients undergoing knee replacement surgery.

Section VIII: Assay of TNFα and IL6 secretion from THP1 cells.

THP1 cells were obtained from ATCC (Cat. No. TIB-202) and cultured in medium consisting of RPMI1640 supplemented with 2mM L-glutamine, 1.5g/L sodium bicarbonate, 4.5 g/L glucose, 10% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate and 50 µM 2-mercapatoethanol. Cells were plated into the wells of 96-well culture plates at 0.5×10^5 and 0.5×10^4 cells/well for TNFα and IL6 assays, respectively , then cultured for 60 mins to allow pre-equilibration, and the treated with varying concentrations of compound **25** for 30 mins prior to addition of LPS (Sigma-Aldrich, Cat. No. L2654) at 1 ug/mL. After 3 and 18 hours of culture for TNFα and IL6 assays, respectively, supernatants were removed and secreted cytokines were measured using commercially available kits and manufacturer's protocols. Secreted TNFα was specifically measured by an ELISA method (R&D Systems; Cat. No. DTA00C)

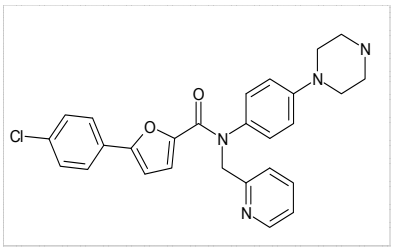
whereas IL6 was specifically measured by an immunoabsorbant-based electrochemical luminescence assay (MesoScale Discovery, Cat. No. K151AKB-2).

Section IX: Enzymatic Analysis of Mode of Inhibition Assays

Mode of inhibition assays were performed according to the standard assay protocol with the following exceptions. The ATP concentration was 100 μM and the Acam peptide concentration was varied from 0 to 200 μM. Stopped reaction aliquots containing more than 3 μM in Acam peptide were diluted to 3 μM concentration prior to addition to a streptavidin plate. Fluorescence values obtained from these wells were corrected for dilution prior to analysis.
Methods:
Compound **1** inhibition data fit to noncompetitive inhibition model with Graph Pad Prizm 5.02.
Global evaluation of inhibition models:
Goodness of fit to kinetic models was evaluated by the method of Akaike[1] using the Sigma Plot Enzymes Kinetics Module.

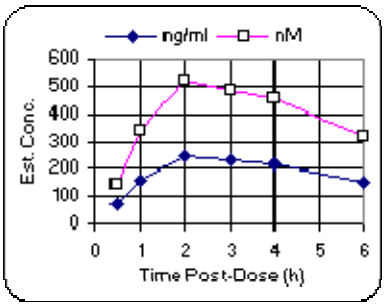
Section X: Compound **25** PK data[2]

HYDROCHLORIDE salt



Formulation: 20% HPBCD

Est. AUC (0-6 h)			(ng.h/ml) 1104	(nM.h) 2334
Pl. Conc.@ h			(ng/ml)	(nM)
		0.5	69	146
Pl.	LOQ: 10 (ng/ml)	1	162	342
		2	246	520
		3	228	481
Liver.	1000 (ng/g)	4	216	457
		6	150	317
	Liver.(ng/g)	19721	**Liver/Pl.**	131.5



%C6/Cmax = 61

HPBCD: hydroxypropyl-β-cyclodextrin.
LOQ: limit of quantification.

Reference

(1) Akaike, H. An information criterion (AIC). *Math. Sci.* **1976**, *14*, 5.
(2) Mei, H.; Korfmacher, W.; Morrison, R. Rapid in vivo oral screening in rats: reliability, acceptance criteria, and filtering efficiency. *The AAPS Journal* **2006**, *8*, E493.