Supporting Information for: Evolution of the Pyridylthiophene Class of Inhibitors of IKKβ: Part I – Hit to Lead Strategies

Authors:

1. **ATP Competition**
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ATP competition data for **compounds 2, 3, 20, 24, 36** and **38:**

ATP Competition Study Results

Relationship between IC50, substrate concentration and Ki

Competitive inhibitor

IC50 = 1/2 E + Ki + Ki/Km x [ATP]

if E<Ki, IC50= Ki + Ki/Km x [ATP]

Non-competitive inhibitor

IC50=1/2 E + Ki

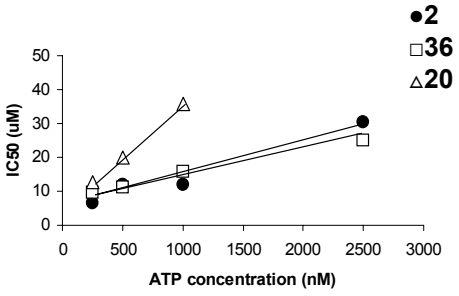
if E<Ki, IC50= Ki

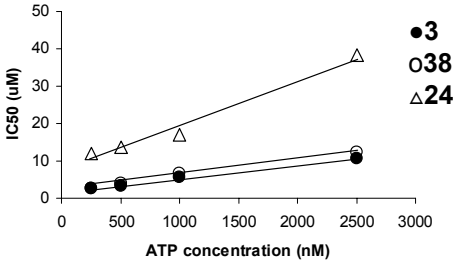
Experimental Protocol

standard assay conditions

4 [ATP] per plate in duplicate

1 compound per plate





Selectivity data for **3**, **20**, **24** and **38**:

Kinase assays:

SYK, ZAP-70, LYN, FYN, SRC, BTK, ITK, CDK1, CDK2, CDK4, VEGFR, IGFR1, FGFR1, EGFR, MKK1, MAPK2/ERK2, JNK1, SAPK2a/p38, SAPK2b/p38b2, SAPK3, SAPK4, MAPKAP-K1b, MAPKAP-K2, MSK1, PRAK, PKCα, PKBα, PDK1, SGK, p70 S6K, GSK3b, ROCK II, AMPK, CHK1, CK2, CSK, CDK2/Cyclin A, PKA*, PKC*, Ca^{2+}/Calmodulin-Dep. PK II*, EGFRTK*, ERK1*, HER2*, lck ($p56^{lck}$)*

Other protein targets*:

Calcineurin PP2B, CD45 Tyrosine Posphatase, PTP1B, PTP1C, T-Cell Tyrosine Phosphatase, Adrenergic α1, Adrenergic α2, Adrenergic β, calcium channel type L (benzothiazepine), calcium channel type L (dihydropyridine), calcium channel type L (phenylalkylamine), calcium channel type N, Dopamine D_1, Dopamine D_{2L}, Dopamine D_3, Dopamine $D_{4.2}$, Dopamine D_5, $GABA_A$ agonist site, $GABA_A$ chloride channel TBOB, $GABA_B$, Glutamate Non-selective, Histamine H_1, Histamine H_2, Muscarinic Non-selective, Nicotinic acetylcholine, opiate Non-selective, serotonin 5-HT_1 Non-selective, serotonin 5-HT_2, serotonin 5-HT_3, Sigma Non-selective, sodium channel site 2

*MDS Pan Labs

Summary of results for inhibitors against the panel of targets:

Compound **3**: no significant inhibition @ 10-100 μM except: BTK 15.1 μM

Compound **20, 24**: no significant inhibition @ 10-100 μM

Compound **38**: no significant inhibition @ 10-100 μM except: BTK 4.1 μM; ITK 7 POC @ 30 μM, PKA 27 μM

Cell Data and Assay Method for **2**, **3**, and **38**: ICAM-1 assay (n = 1):

2: 48% inhibition @ 10 μM

3: 42% inhibition @ 10 μM

38: 42% inhibition @ 10 μM

ICAM-1 Cell Assay Method: HeLa cells were seeded on 96 well tissue culture treated plates (Costar) in complete medium comprised of 10% decomplemented fetal bovine serum in RPMI1640 with gentamycin and L-glutamine and grown overnight to confluence. The following day, the media is changed and the wells were treated with test compounds. 10 mM DMSO stock solutions of compounds were serially diluted with 0.1% DMSO (final concentration)-screening media to 5 final concentrations (starting at 10 μM). Compounds were pre-incubated with cells for 30 min. followed by stimulation with TNFα (R&D Systems) for 5-6 hr. The adherent cells were then assayed for expression of intercellular adhesion molecule-1 (ICAM-1). Monolayers were washed three times with D-PBS (Gibco) and fixed for 10min at room temperature with 1% paraformaldehyde (Polysciences, Inc) diluted in D-PBS. After washing to remove fixative, the monolayers were blocked with 2% BSA-D-PBS overnight at 4^{o}C. 100 μL of anti-ICAM-1 mAB RR1-HRP (diluted 1:5000 in 2% BSA-DPBS; Zymed custom conjugate of BIPI mAB) was added for 1 hour at 37^{o}C. Wells were washed three times with D-PBS. 100 μL of ABTS substrate diluted in substrate buffer (Zymed) was added to each well. Optical absorbance was measured at 405 nm in a Thermomax microplate reader (Molecular Dynamics). Data was plotted as percent of control and IC_{50}s were determined using xlfit 4 model # 201 (dose response one site).

Cell Data and Assay Method for **2**, **3**, and **38**: Reporter Gene Assay: (n = 1)

2: 45% inhibition @ 10 μM

3: 20% inhibition @ 10 μM

38: 45% inhibition @ 10 μM

Reporter Gene Cell Assay Method: For the cell assay, compounds were tested in HeLa cells stably transfected with an NFκB-luciferase reporter gene. 10 mM DMSO stock solutions of compounds were serially diluted with screening media to 6 final concentrations (starting at either 10 or 50 μM). Compounds were pre-incubated with cells for 30 min. followed by stimulation with TNFα for 6 hr. Cells were then lysed using LucLite Reagent (Perkin-Elmer/Packard Bioscience). Luciferase activity was read on a Top Count scintillation counter and used to calculate EC_{50}'s, with unstimulated cell values as background and TNFα-stimulated cell values as control.

C, H, N Combustion Analyses

2 C 55.92, H 3.84, N 11.86, found C 56.09, H 3.69, N 11.72

3 C 60.53, H 4.62, N 12.83, found C 60.62, H 4.69, N 12.85

5 C 57.59, H 4.43, N 11.19, found C 57.26, H 4.43, N 10.87

6 C 59.07, H 4.96, N 10.60, found C 59.04, H 4.82, N 10.29

12 C 63.41, H 3.99, N 18.49, found C 63.10, H 3.82, N 18.39

16 C 64.50, H 4.34, N 13.52, found C 64.49, H 4.19, N 13.23

18 C 72.13, H 5.92, N 12.20, found C 72.17, H 5.93, N 12.28

20 C 59.11, H 4.46, N 20.68, found C 59.02, H 4.23, N 20.60

21 C 50.15, H 3.42, N 17.55, found C 50.40, H 3.09, N 17.25

22 C 56.65, H 4.75, N 18.02, found C 56.69, H 4.64, N 17.86

23 C 59.83, H 5.17, N 19.04, found C 59.80, H 5.35, N 18.91

24 C 48.72, H 2.97, N 15.49, found C 48.82, H 3.00, N 15.16

26 C 51.40, H 4.39, N 21.80, found C 51.22, H 4.44, N 21.62

27 C 50.54, H 3.39, N 17.68, found C 50.74, H 3.69, N 17.58

28 C 56.57, H 4.88, N 17.37, found C 56.64, H 4.75, N 17.19

29 C 60.82, H 5.10, N 19.34, found C 60.86, H 4.97, N 19.23

30 C 50.54, H 3.39, N 17.68, found C 50.79, H 3.65, N 17.66

32 C 39.58, H 2.16, N 13.32, found C 39.49, H 2.09, N 13.20

33 C 57.40, H 7.23, N 20.08, found C 57.52, H 7.05, N 19.69

35 C 51.23, H 3.98, N 26.56, found C 51.41, H 4.01, N 26.10

39 C 44.20, H 4.42, N 15.47, found C 44.53, H 4.39, N 15.21

50 C 55.76, H 5.95, N 13.01, found C 55.80, H 5.69, N 12.61

51 C 52.85, H 7.54, N 18.49, found C 53.15, H 7.54, N 18.42

52 C 62.81, H 6.85, N 21.97, found C 62.82, H 6.96, N 21.70