Supporting Information

Pyrrolopyridine Inhibitors of Mitogen Activated Protein Kinase-Activated Protein Kinase 2 (MK-2)

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Table of Contents

Table S1. Combustion Analysis

Compd	Formula	Anal. Calcd	Found
8	$C_{12}H_{11}N_3O\cdot CF_3CO_2H\cdot 0.2H_2O$	C, 50.82; H, 3.78; N, 12.70	C, 50.77; H, 3.61; N, 12.49
9	$C_{18}H_{15}N_3O\cdot CF_3CO_2H\cdot H_2O$	C, 57.01; H, 4.31; N, 9.97	C, 57.01; H, 4.29; N, 9.83
10	$C_{17}H_{14}N_4O\cdot 2.6CF_3CO_2H$	C, 45.44; H, 2.85; N, 9.55	C, 45.27; H, 2.80; N, 9.40
11	$C_{17}H_{14}N_4O\cdot 2CF_3CO_2H\cdot 0.8H_2O$	C, 47.34; H, 3.33; N, 10.52	C, 47.39; H, 3.11; N, 10.42
12	$C_{16}H_{13}N_5O\cdot CF_3CO_2H$	C, 53.33; H, 3.48; N, 17.27	C, 53.30; H, 3.75; N, 17.40
13	$C_{16}H_{13}N_3OS\cdot CF_3CO_2H\cdot 1.4H_2O$	C, 49.74; H, 3.89; N, 9.66	C, 49.80; H, 3.76; N, 9.51
14	$C_{22}H_{17}N_3O\cdot 1.3CF_3CO_2H\cdot 0.5H_2O$	C, 59.49; H, 3.92; N, 8.46	C, 59.79; H, 3.98; N, 8.06
15	$C_{20}H_{15}N_3OS\cdot CF_3CO_2H\cdot 0.3H_2O$	C, 56.84; H, 3.60; N, 9.04	C, 56.83; H, 3.68; N, 8.90
16	$C_{21}H_{16}N_4O\cdot 2.8CF_3CO_2H$	C, 48.43; H, 2.87; N, 8.49	C, 48.12; H, 3.09; N, 8.66
17	$C_{18}H_{15}N_3O_2\cdot CF_3CO_2H\cdot 2.3H_2O$	C, 52.13; H, 4.50; N, 9.11	C, 51.97; H, 4.11; N, 8.99
18	$C_{18}H_{13}N_3O_2\cdot CF_3CO_2H\cdot 2H_2O$	C, 52.75; H, 4.42; N, 9.22	C, 52.70; H, 4.33; N, 9.11
19	$C_{18}H_{15}N_3O_2\cdot CF_3CO_2H\cdot 1.9H_2O$	C, 52.95; H, 4.39; N, 9.26	C, 52.95; H, 4.11; N, 9.09
20	$C_{18}H_{14}ClN_3O\cdot CF_3CO_2H\cdot 2H_2O$	C, 50.70; H, 4.04; N, 8.87	C, 50.69; H, 4.00; N, 8.86
21	$C_{18}H_{14}ClN_3O\cdot 1.3CF_3CO_2H\cdot 0.4H_2O$	C, 51.63; H, 3.39; N, 8.77	C, 51.78; H, 3.36; N, 8.79
22	$C_{18}H_{14}ClN_3\cdot CF_3CO_2H\cdot H_2O$	C, 52.70; H, 3.76; N, 9.22	C, 52.84; H, 3.54; N, 9.20
23	$C_{18}H_{14}FN_3O\cdot CF_3CO_2H\cdot 1.2H_2O$	C, 54.23; H, 3.96; N, 9.49	C, 54.24; H, 3.90; N, 9.30
24	$C_{18}H_{14}FN_3O\cdot CF_3CO_2H\cdot 1.4H_2O$	C, 54.23; H, 3.96; N, 9.49	C, 54.14; H, 3.79; N, 9.50
25	$C_{18}H_{14}FN_3O\cdot CF_3CO_2H\cdot 1.1H_2O$	C, 54.45; H, 3.93; N, 9.52	C, 54.43; H, 3.79; N, 9.45
26	$C_{19}H_{14}N_3OF_3\cdot CF_3CO_2H\cdot H_2O$	C, 51.54; H, 3.50; N, 8.59	C, 51.29; H, 3.41; N, 8.53
27	$C_{19}H_{14}N_4O\cdot CF_3CO_2H\cdot 1.5H_2O$	C, 55.39; H, 3.98; N, 12.30	C, 55.43; H, 4.26; N, 12.05
28	$C_{20}H_{17}N_3O_2\cdot 1.4CF_3CO_2H\cdot 0.9H_2O$	C, 53.99; H, 4.01; N, 8.28	C, 54.13; H, 3.89; N, 8.34
29	$C_{19}H_{17}N_3O_2\cdot 1.3CF_3CO_2H\cdot 1.0H_2O$	C, 53.43; H, 4.21; N, 8.65	C, 53.49; H, 4.09; N, 8.65

30	$C_{18}H_{16}N_4O\cdot1.2CF_3CO_2H\cdot1.3H_2O$	C, 52.17; H, 4.18; N, 11.81	C, 52.71; H, 4.15; N, 12.00
31	$C_{19}H_{15}N_3O_3\cdot1.2CF_3CO_2H\cdot1.6H_2O$	C, 51.51; H, 3.91; N, 8.42	C, 51.59; H, 3.95; N, 8.44
32	$C_{22}H_{20}N_4O_2\cdot CF_3CO_2H\cdot1.6H_2O$	C, 55.94; H, 4.73; N, 10.87	C, 55.94; H, 4.43; N, 10.95
33	$C_{24}H_{24}N_4O_2\cdot1.2CF_3CO_2H\cdot0.6H_2O$	C, 57.85; H, 4.85; N, 10.22	C, 57.91; H, 5.18; N, 9.88
34	$C_{25}H_{26}N_4O_2\cdot CF_3CO_2H\cdot2.1H_2O$	C, 57.25; H, 5.55; N, 9.89	C, 57.29; H, 5.49; N, 9.92
35	$C_{26}H_{22}N_4O_2\cdot CF_3CO_2H\cdot H_2O$	C, 60.64; H, 4.54; N, 10.10	C, 60.62; H, 4.65; N, 9.91
36	$C_{27}H_{24}N_4O_2\cdot CF_3CO_2H\cdot0.7H_2O$	C, 61.85; H, 4.72; N, 9.94	C, 61.79; H, 4.79; N, 9.96
37	$C_{27}H_{24}N_4O_2\cdot CF_3CO_2H\cdot1.6H_2O$	C, 60.12; H, 4.90; N, 9.67	C, 60.04; H, 4.60; N, 9.69

Kinase Selectivity Assays

CDK2. Active cyclin dependent kinase 2 (CDK2) activity was determined by measuring the phosphorylation of histone type 3, in the presence of [γ-^{33}P]ATP. Active CDK2 (2 nM enzyme) was incubated for 20 minutes in the presence of inhibitor in a solution containing 25 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 18 mM DTT, 2% DMSO, and 0.05% BSA. Compounds were evaluated as potential inhibitors of the CDK2 kinase by measuring their effect on CDK2 phosphorylation of the histone type 3 substrate. Compounds were tested in triplicate at six concentrations in 10-fold serial dilutions beginning at 200 µM. The reaction was carried out at 0.4 µM ATP (~K_m levels) and 10 µM histone type 3.

ERK2. Extracellular signal-regulated kinase, ERK2, activity was determined by measuring the phosphorylation of the epidermal growth factor receptor peptide substrate, EGFRP (KKRELVEPLTPSGEAPNQALLR), in the presence of [γ-^{33}P]ATP. Compounds were evaluated as potential inhibitors of the ERK2 kinase by measuring their effect on ERK2 induced phosphorylation of the peptide substrate. Compounds were tested in triplicate at six concentrations in 10-fold serial dilutions beginning at 200 µM. Reactions were carried out for 60 minutes in a reaction mixture containing 25 mM HEPES pH 7.5, 4% glycerol, 0.04% bovine serum albumin, 1 mM Na_3VO_4, 10 mM magnesium acetate, 500 µM EGFRP as substrate, 50 µM ATP (~K_m levels), 0.075 µCi [γ-^{33}P]ATP, and 10% DMSO. The assay was initiated by addition of 15 nM activated ERK2 and allowed to proceed for one hour at room temperature.

IKK2. IkappaB kinase-2, IKK2, activity was determined by measuring the phosphorylation of a peptide substrate, biotinylated IkappaB (IκB) in the presence of [γ-^{33}P]ATP. Compounds were tested using 10-fold serial dilutions over (0.002 µM to 200 µM) in 2% final concentration of DMSO. Each concentration of inhibitor was tested in triplicate. Kinase reactions were carried out for 30 minutes at room temperature. Compounds were evaluated in 50 µL reaction volumes in the presence of 25 mM HEPES pH 7.5, 10 mM magnesium acetate, 4% glycerol, 0.4% bovine serum albumin, 0.8 mM DTT, 0.1 µCi [γ-^{33}P]ATP, 5 µM biotinylated IκB peptide and 1 µM unlabeled ATP (~K_m levels).

JNK2. c-Jun NH2-terminal kinase 2 (JNK2) activity was determined by following the phosphorylation of a specific substrate GST-cJun (c-Jun 1-79). Compounds were evaluated for JNK inhibition by measuring their effects on phosphorylation of GST-cJun. Compounds were tested in triplicate at six concentrations in 10-fold serial dilutions beginning at 200 µM. Reactions mixtures included 25 mM HEPES pH 7.5, 4% glycerol, 0.04% bovine serum albumin, 1 mM Na_3VO_4, 10 mM magnesium acetate, 10 µM GST-cJun, 50 µM ATP (~K_m levels), and 0.11 µCi [γ-^{33}P]ATP. Reactions were initiated by the addition of 150 nM JNK2, previously activated by a constitutively active form of the upstream kinase GST-MKK7b, and allowed to proceed for 60 minutes. The JNK isoforms were activated by incubation at 30 ^{o}C for one hour in the presence of 50 µM ATP and a molar ratio of GST-MKK7b:JNK of 1:50.

MK-3. Mitogen activated protein kinase activated protein kinase-3, MK-3, activity was determined by following the phosphorylation of serine residues in a peptide substrate (KKKALSRNLSVAA), in the presence of [γ-^{33}P]ATP. Compounds were evaluated as potential inhibitors of the MK-3 kinase by measuring their effects on MK-3 phosphorylation of the peptide substrate. Compounds were tested in triplicate at six concentrations in 10-fold serial dilutions beginning at 200 µM. Reaction mixtures included 50 mM β-glycerophosphate pH 7.5, 4% glycerol, 0.04% bovine serum albumin, 1 mM Na_3VO_4, 10 mM magnesium acetate, 30 µM unlabeled ATP (~K_m levels), 500 µM peptide substrate, 0.1 µCi [γ-^{33}P]ATP, 800 µM DTT and 2% DMSO. Reactions were carried out for 15 minutes and were initiated by addition of 12 nM MK-3, which had been previously activated by incubation with activated p38α kinase (p38α:MK3, 1:50) at 30 ^{o}C for two hours in the presence of 250 µM ATP.

MNK1. Mitogen activated protein kinase-interacting kinase, MNK1, activity was determined by measuring the phosphorylation of myelin basic protein, MBP, in the presence of [γ-^{33}P]ATP. Compounds were evaluated as potential inhibitors of the MNK1 kinase by measuring their effects on MNK1 phosphorylation of MBP. Compounds were tested in triplicate at six concentrations in 10-fold serial dilutions beginning at 200 µM. The assay was allowed to proceed for one hour at room temperature using reagent mixtures containing 50 mM β-glycerophosphate pH 7.5, 270 µM MBP, 500 µM ATP (~K_m levels), 0.1 µCi [γ-^{33}P] ATP, 800 µM DTT, and 2% DMSO. Reactions were initiated by the addition of 100 nM MNK1, which had been previously activated using activated p38α kinase (p38α:MNK, 1:100) at 30 °C for one hour in the presence of 500 µM ATP.

MSK1. Mitogen/stress kinase 1, MSK1, activity was determined following the phosphorylation of serine residues of a 13 amino acid peptide substrate (KKKALSRNLSVAA), in the presence of [γ-^{33}P]ATP. Compounds were evaluated as potential inhibitors of MSK1 kinase by measuring their effects on MSK1 phosphorylation of the peptide substrate. Compounds were tested in triplicate at six concentrations in 10-fold serial dilutions beginning at 200 µM. The assay was allowed to proceed for 30 minutes at room temperature using reagent mixtures containing 50 mM β-glycerophosphate pH 7.5, 4%

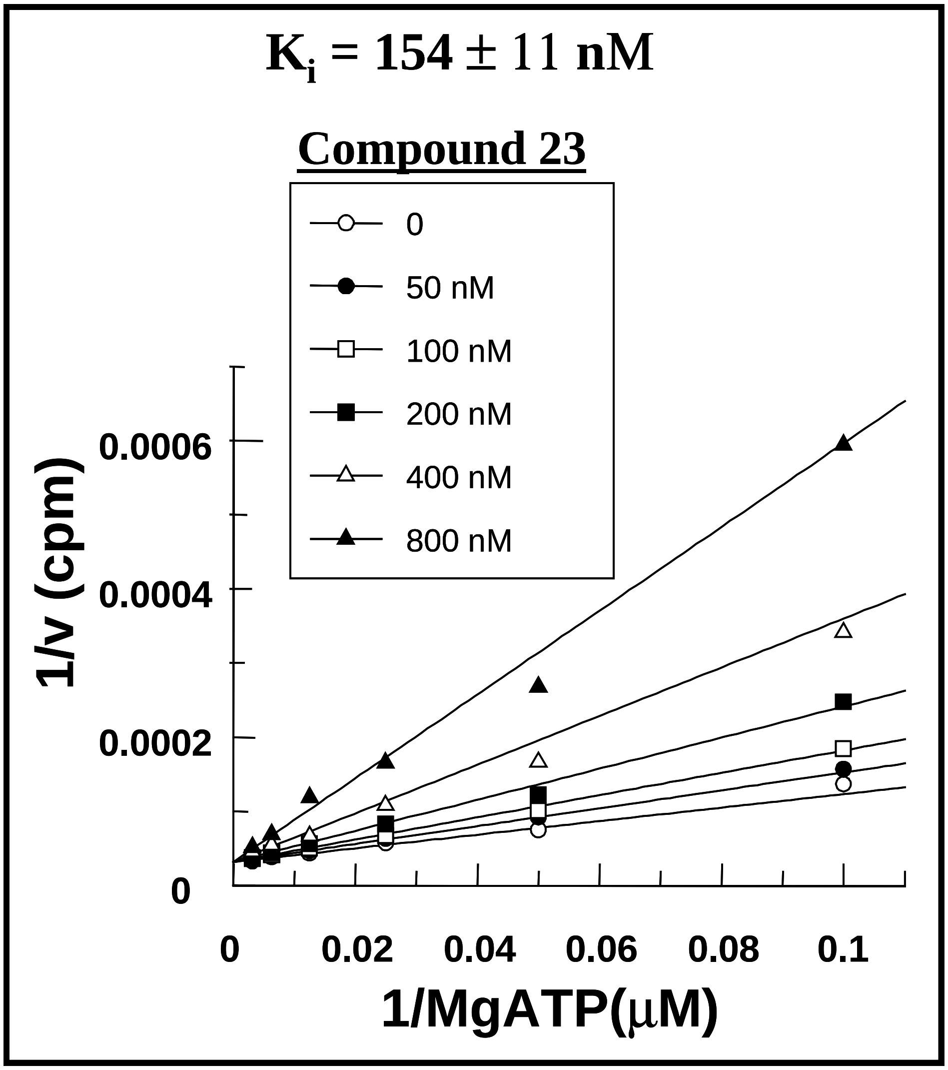
glycerol, 0.04% bovine serum albumin, 1 mM Na_3VO_4, 10 mM magnesium acetate, 300 µM unlabeled ATP (~K_m levels), 200 µM peptide substrate, 0.5 µCi [γ-^{33}P]ATP, 800 µM DTT and 2% DMSO. Reactions were initiated by addition of 25 nM MSK1, which had been previously activated by incubation with activated p38α kinase (p38α:MSK1, 1:50) at 30 °C for one hour in the presence of 250 µM ATP.

MSK2. Mitogen/stress kinase 2, MSK2, activity was determined following the phosphorylation of serine residues of a 13 amino acid peptide substrate (KKKALSRNLSVAA), in the presence of [γ-^{33}P]ATP. Compounds were evaluated as potential inhibitors of MSK2 kinase by measuring their effects on MSK2 phosphorylation of the peptide substrate. Compounds were tested in triplicate at six concentrations in 10-fold serial dilutions beginning at 200 µM. The assay was allowed to proceed for 60 minutes at room temperature using reaction mixtures containing 50 mM β-glycerophosphate pH 7.5, 4% glycerol, 0.04% bovine serum albumin, 1 mM Na_3VO_4, 10 mM magnesium acetate, 160 µM unlabeled ATP (~K_m levels), 120 µM peptide substrate, 0.5 µCi [γ-^{33}P]ATP, 800 µM DTT and 2% DMSO. Reactions were initiated by addition of 125 nM MSK2, which had been previously activated by incubation with activated p38α kinase (p38α:MSK2, 1:25) at 30 °C for one hour in the presence of 250 µM ATP.

p38α. p38α kinase activity was determined by monitoring the phosphorylation of the EGFRP substrate peptide in the presence of [γ-^{33}P]ATP. Compounds were tested in triplicate at six concentrations in 10-fold serial dilutions beginning at 200 µM in 10% DMSO. The assay was allowed to proceed for 60 minutes at room temperature using reaction mixtures containing 25 mM HEPES pH 7.5, 4% glycerol, 0.04% bovine serum albumin, 1 mM Na_3VO_4, 10 mM magnesium acetate, 50 µM unlabeled ATP (~K_m levels), 200 µM EGFRP, and 0.05 µCi [γ-^{33}P]ATP. Reactions were initiated by the addition of 20 nM p38 kinase previously activated with GST-MKK6 (p38:MKK6,100:1) for one hour at 30°C in the presence of 50 µM ATP.

PRAK. p38-Regulated/activated protein kinase, PRAK, activity was determined by measuring the phosphorylation at serine residues of a peptide substrate (KKKALSRNLSVAA), in the presence of [γ-^{33}P]ATP. Compounds were evaluated as potential inhibitors of PRAK by measuring their effects on PRAK phosphorylation of the peptide substrate. Compounds were tested in triplicate at six concentrations in 10-fold serial dilutions beginning at 200 µM. Reaction mixtures included 50 mM β-glycerophosphate pH 7.5, 4% glycerol, 0.04% bovine serum albumin, 1 mM Na_3VO_4, 10 mM magnesium acetate, 50 µM unlabeled ATP (~K_m levels), 500 µM peptide substrate, 0.1 µCi [γ-^{33}P]ATP, 800 µM DTT and 2% DMSO. Reactions were carried out for 15 minutes and were initiated by addition of 10 nM PRAK, which had been previously activated by incubation with p38α kinase (p38α:PRAK, 1:100) at 30 °C for one hour in the presence of 250 µM ATP.

Figure S1: Lineweaver-Burke Plot Showing Compound 23 is Competitive with ATP for MK-2 Kinase Inhibition



Competitive inhibition with ATP as the varied substrate was measured as described[S1] using HSP27-peptide held constant at 200 μM. Compound **23** inhibitor concentrations varied 0-800 nM as indicated in the figure legend**.**

Permeability Studies in Caco-2 Cells

The ability of **23** to traverse a human intestinal cell membrane (A→B) was assessed in Caco-2 cell monolayers at 37°C under ambient oxygenation. Prior to each experiment, the confluent cell monolayers (24-well inserts, 0.33 cm^2) were washed and equilibrated for 30 minutes with transport media (Hank's balanced salt solution (HBSS) containing 10 mM of N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) and 10 mM of glucose, pH 7.4). The experiment was initiated by adding a solution containing the test compound (1, 10 and 100 µM) to the apical compartment. At two hours, aliquots of receiving solution were withdrawn from the basolateral side and subjected to liquid chromatography/tandem mass spectrometry for quantitation of **23**. The unidirectional permeability (Papp, A→B) of **23** was determined via the following relationship,

$$P_{app} = \frac{1}{A \bullet C_D\ (0)} \bullet \mathrm{dMR}/dt$$

where A is the surface area of the cell monolayer (0.33 cm^2), $C_D(0)$ is the initial concentration of **23** in the apical compartment and dM_R/dt is the rate of appearance of **23** in the receiver compartment.

Binding of Compound 23 to Sprague-Dawley Rat Plasma Proteins and Cell Media

Delipidized plasma from Sprague-Dawley rats was thawed and fortified with compound **23** (2.5 µg/ml). The fortified plasma was then equilibrated at 37°C. An aliquot of the initial fortified plasma was reserved for liquid chromatography/mass spectrometric analysis (i.e., confirm total concentration of **23**). The remaining plasma samples were then subjected to ultracentrifugation (125,000 x g, fixed angle Beckman rotor) in polycarbonate tubes at 37° C for 15 hours. The resulting supernatants were analyzed via liquid chromatography/mass spectrometry to ascertain free concentrations of **23**. All samples were subjected to acetonitrile precipitation prior to liquid chromatography/mass spectrometric analysis. The following relationship was used to determine the fraction of **23** bound to plasma proteins (*fb*):

$$\mathrm{fb} = (\mathrm{Conc}_{\mathrm{total}} - \mathrm{Conc}_{\mathrm{free}})/\mathrm{Conc}_{\mathrm{total}}$$

$$\%\mathrm{free} = (1-\mathrm{fb}) \cdot 100$$

In a typical cell media binding protocol, the media used in the U937 cell assay was fortified with **23** (2.5 µg/mL) and equilibrated at 37°C. The extent of binding of **23** in this media was determined as described above.

References

S1. Anderson, D. R.; Hegde, S.; Reinhard, E.; Gomez, L.; Vernier, W. F.; Lee, L.; Liu, S.; Sambandam, A.; Snider, P. A.; Masih, L., Aminocyanopyridine inhibitors of mitogen activated protein kinase-activated protein kinase 2 (MK-2). *Bioorganic & Medicinal Chemistry Letters* **2005,** 15, (6), 1587-1590.