Supplementary Information

Selective MAP kinase activity sensors through the application of directionally-programmable D domain motifs

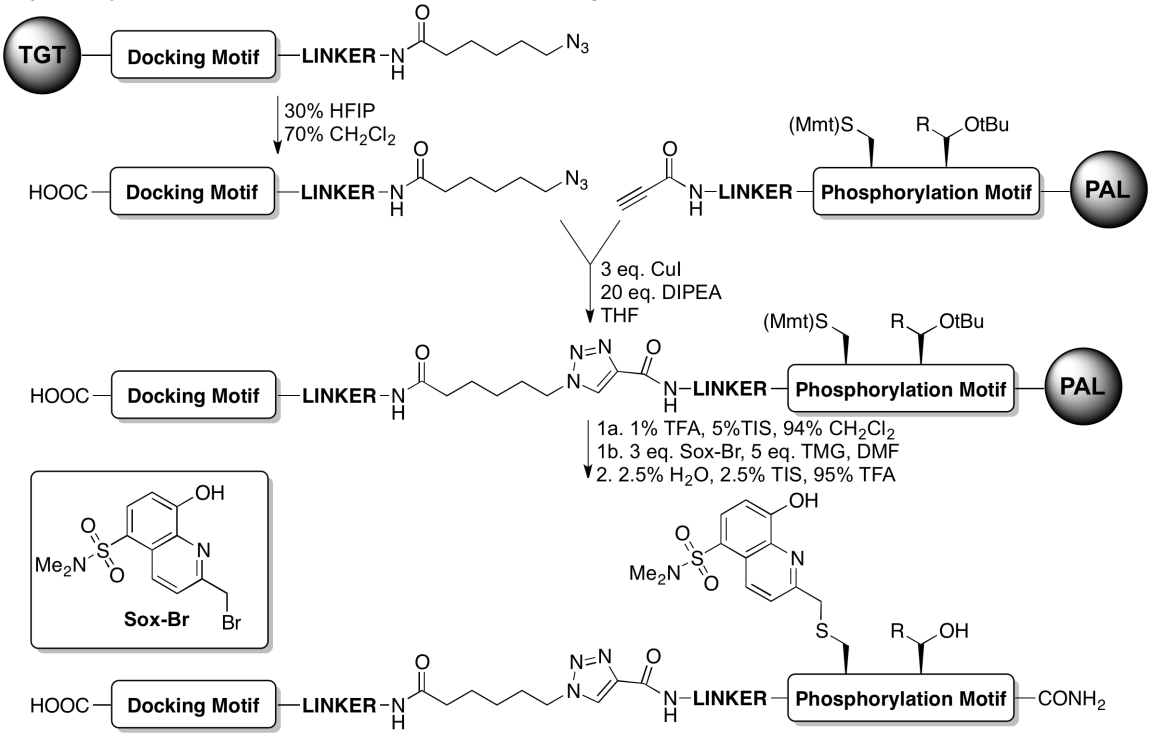
Laura B. Peterson[1], Michael B. Yaffe[2], and Barbara Imperiali[1,*]

[1]Departments of Chemistry and Biology

[2]Departments of Biology and Biological Engineering, David H. Koch Institute for Integrative Cancer Research

Massachusetts Institute of Technology, Cambridge, MA, 02139, USA

Peptide synthesis of sensors with reversed docking domains.



The reversed docking motifs, for inclusion in the p38 and ERK sensors, were synthesized via standard SPPS on the mild-acid labile resin, Fmoc-Gly-NovaSyn-TGT (Millipore). To the docking motif, PEG_2-propionic acid (AAPPTec) and 6-azido-hexanoic acid were then coupled via standard conditions. The phosphorylation motifs were prepared via SPPS on PAL-PEG-PS (Invitrogen). Following synthesis of the phosphorylation motif through the PEG_2 moiety, propiolic acid was coupled: The dried resin was swelled in anhydrous CH_2Cl_2 and subjected to a mixture of propiolic acid (3 eq.) and EEDQ (3.1 eq.) in anhydrous CH_2Cl_2 (1.0 mL per 100 mg resin). The reaction was allowed to proceed for 3 h. at 25 °C. The resin was then washed 5 X CH_2Cl_2, 5 X DMF, 5 X CH_2Cl_2 and 5 X MeOH. The resin was then dried under vacuum.

Following the synthesis of the docking motif, the docking motif peptide was cleaved fully protected in 30% hexafluoroisopropanol (HFIP) in CH_2Cl_2, 4 x 10 min. The resin was then washed with CH_2Cl_2 (4 x 10 min) and washes were combined with cleaved peptide. Solvent was removed in vacuo and the peptide was dried under vacuum.

The crude docking motif (30 µmol) was then dissolved in anhydrous THF and mixed with CuI (3 eq.), DIPEA (20 eq.), and THF (0.1 M). The peptide mixture was then introduced to the on-resin phosphorylation motif peptide (20 µmol) that had been swelled in THF and the click reaction was allowed to proceed for 14 h. Following the reaction, the resin was washed 5 X DMF, 10 X 10 min with 0.5% sodium diethyldithiocarbamate and 0.5% DIPEA in DMF, 5 X DMF, 5 X CH_2Cl_2, and 5 X MeOH. The peptide was then modified with Sox-Br and cleaved as previously described.

Preparation of phospho-peptides.

In order to determine V_{max} and k_{cat}, preparation of the phosphorylated peptides was necessary, which allows for the conversion of fluorescence units to absolute product formation. Phospho-peptides were produced enzymatically with the kinase of interest. A 50 nmol aliquot of peptide was incubated at 30 °C with 100 ng of the appropriate kinase for 72-120 h in 500 µL of assay buffer containing 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 0.01% Brij-35, and 1 mM ATP. The reaction was monitored by reversed phase HPLC-MS. Following complete conversion of the starting material, the

phosphopeptides were purified via HPLC and characterized as described for the unphosphorylated peptides.

Characterization of purified peptides.

The purity of the synthetic peptides was assessed by reversed-phase HPLC and identity was confirmed by ESI-MS.

Table 1. Sequence and molecular formula for each peptide sensor and corresponding phospho-peptide product.

Sensor	Peptide Sequence	Mol. Formula
JNK1/2/3	ERPSRDHLYLPLEP-PEG_2-SANLLSP-Csox-PA	$C_{137}H_{213}N_{37}O_{43}S_2$
pJNK1/2/3	ERPSRDHLYLPLEP-PEG_2-SANLL(p)SP-Csox-PA	$C_{137}H_{214}N_{37}O_{46}PS_2$
p38α/β	GKKRRKLLLPNSADEIKKIKI-PEG_2-Triazole-PEG_2-QP-Csox-ASPVV	$C_{179}H_{307}N_{51}O_{49}S_2$
p-p38α/β	GKKRRKLLLPNSADEIKKIKI-PEG_2-Triazole-PEG_2-QP-Csox-A(p)SPVV	$C_{179}H_{308}N_{51}O_{52}PS_2$
ERK1/2	GLKRVRRQALISSEIPKLQP-PEG_2-Triazole-PEG_2-VP-Csox-LTPGGRR	$C_{180}H_{307}N_{57}O_{49}S_2$
pERK1/2	GLKRVRRQALISSEIPKLQP-PEG_2-Triazole-PEG_2-VP-Csox-L(p)TPGGRR	$C_{180}H_{308}N_{57}O_{52}PS_2$

Table 2. M/S characterization data for each peptide sensor.

Sensor	$[M+xH]^{x+}$ Calc.	$[M+xH]^{x+}$ Obsvd.
JNK1/2/3	$[M+2H]^{2+}$: 1565.26	1565.40
pJNK1/2/3	$[M+2H]^{2+}$: 1605.24	1605.53
p38α/β	$[M+3H]^{3+}$: 1340.77	1341.00
p-p38α/β	$[M+3H]^{3+}$: 1367.41	1367.33
ERK1/2	$[M+3H]^{3+}$: 1372.76	1373.80
pERK1/2	$[M+3H]^{3+}$: 1399.41	1400.33

Determination of kinetic parameters.

Kinetic parameters were derived as described in the Experimental Methods sections and as previously described.[1]

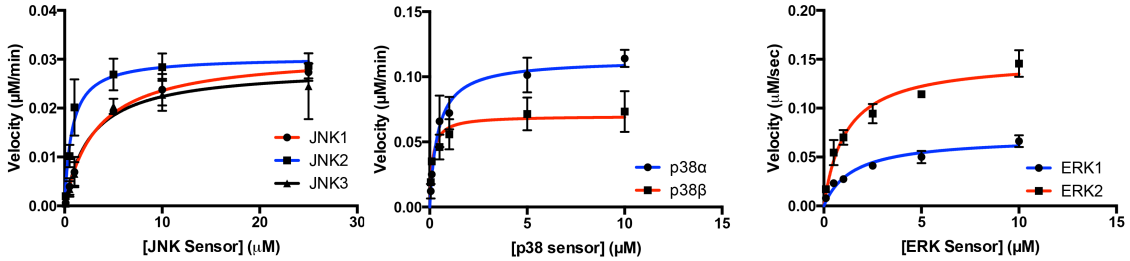


Figure 1. Michaelis Menten plots for each MAP kinase activity sensor.

Antibodies. Antibodies used in western blot analysis are as follows:
p-JNK: Cell Signaling, #9251
p-ERK: Cell Signaling, #4377
p-p38: Cell Signaling, #9215
Tubulin: Cell Signaling, #3873

MAP Kinase subfamily homology. Sequence alignments were determined within each MAP kinase subfamily using ClustalO. Phylogenic tree for the MAP kinase family was determined by sequence alignment of all MAPK isoforms. The MAP kinase isoforms used are as follows:

Kinase	Accession Number	Kinase	Accession Number	Kinase	Accession Number
JNK1	P45983.2	p38α	NP_001306.1	ERK1	P27361.4
JNK2	P45984.2	p38β	NP_002742.3	ERK2	P28482.3
JNK3	P53779.2	p38δ	NP_002745.1	ERK5	Q13164.2
		p38γ	NP_002960.2		

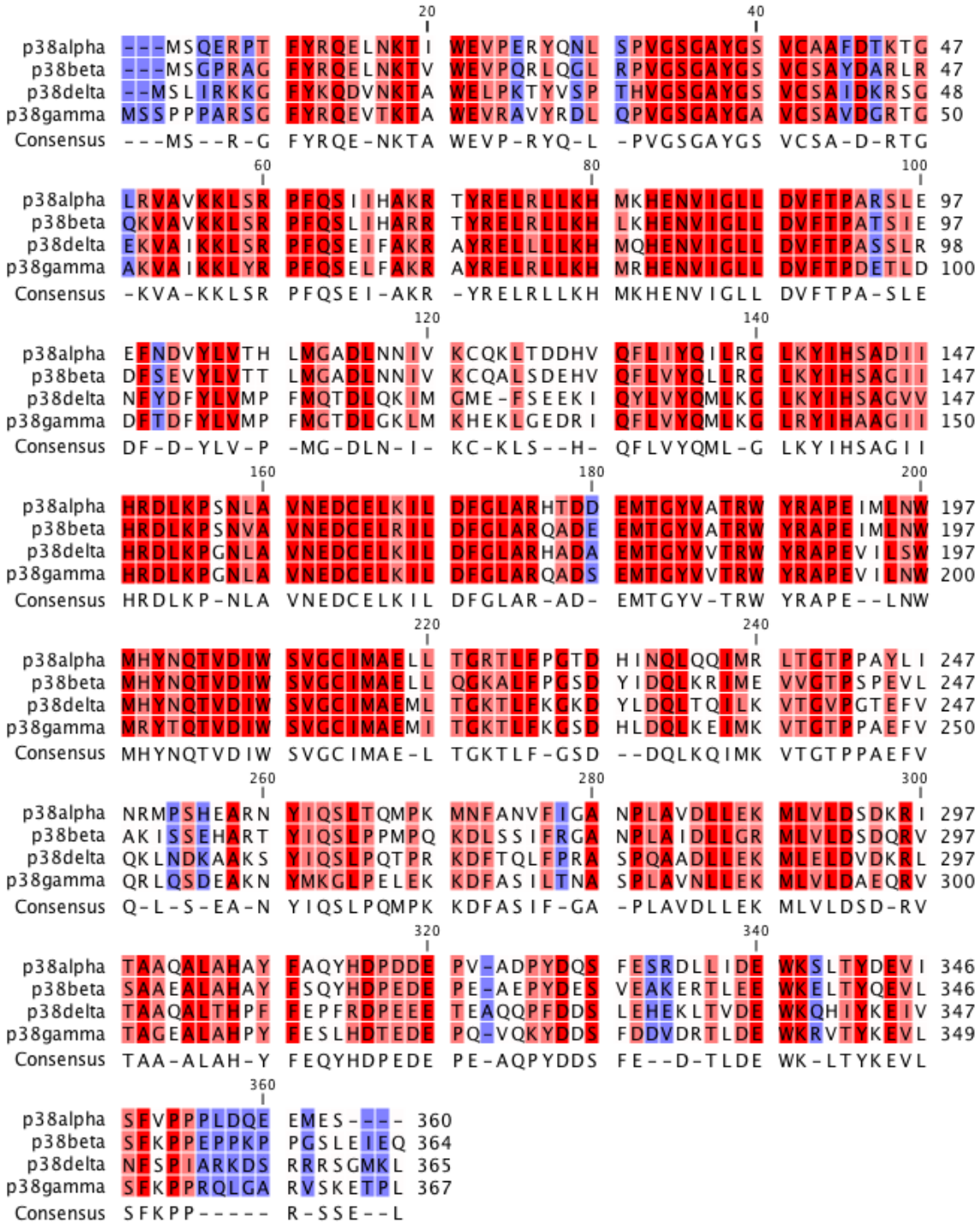


Figure 2. Sequence alignment for each p38 MAP kinase.

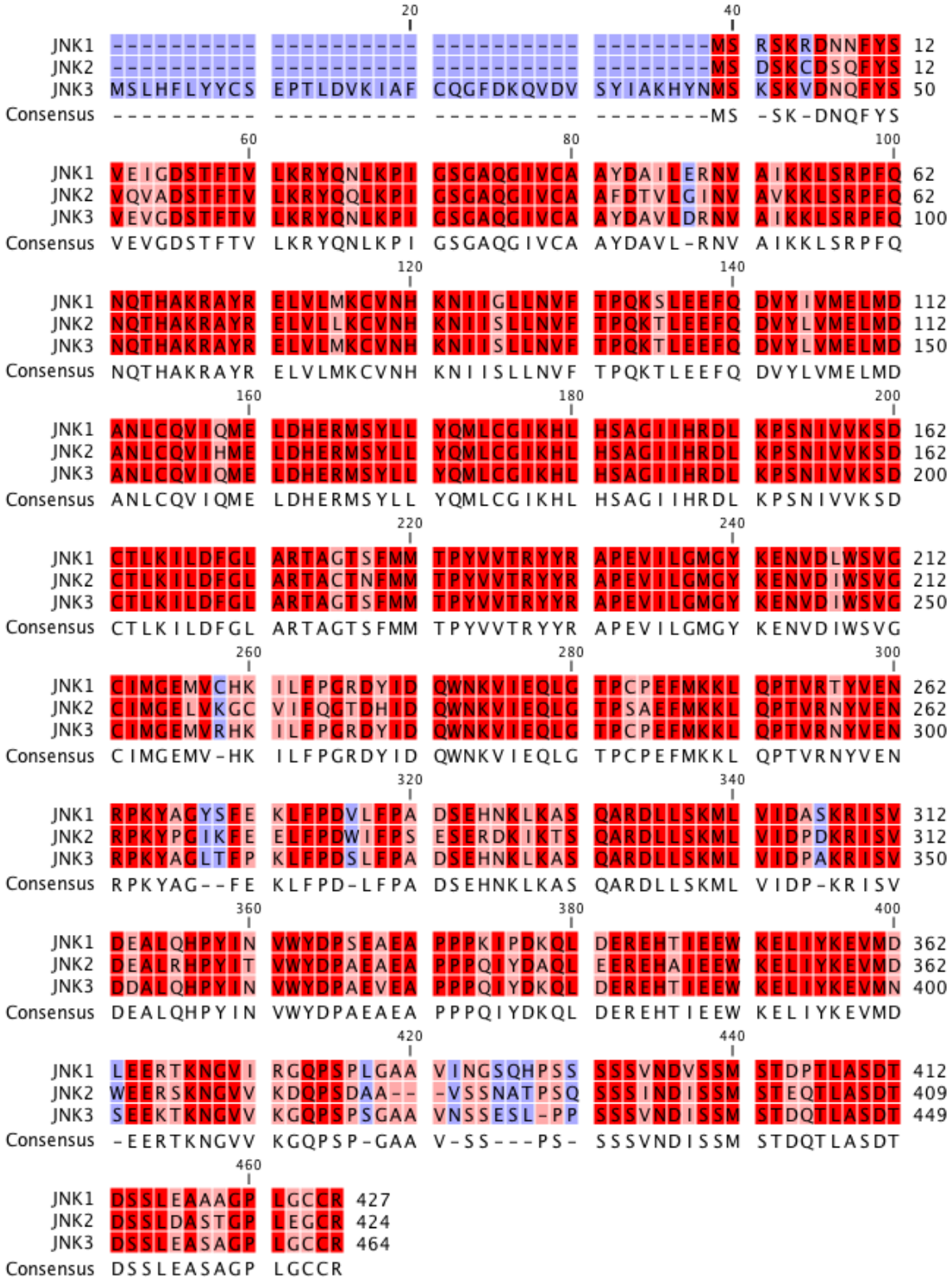


Figure 3. Sequence alignment for each JNK MAP kinase isoform.

Figure 4. Sequence alignment for each ERK MAP kinase.

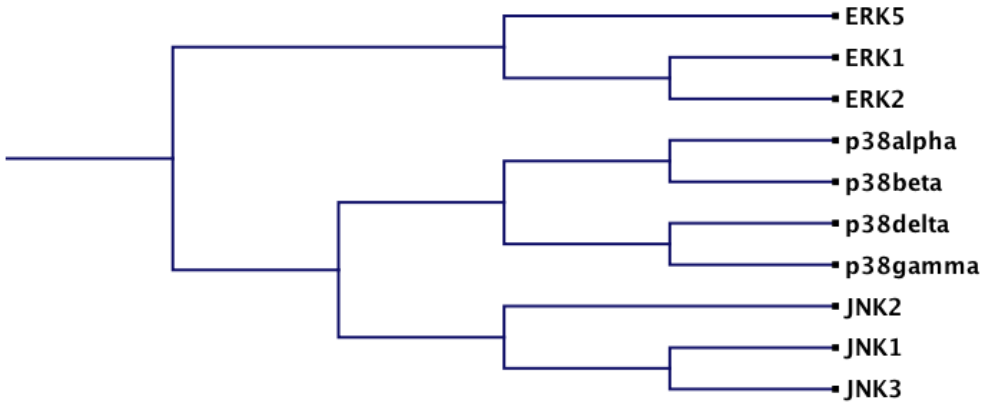


Figure 5. Phylogenic tree for the MAP kinase family.

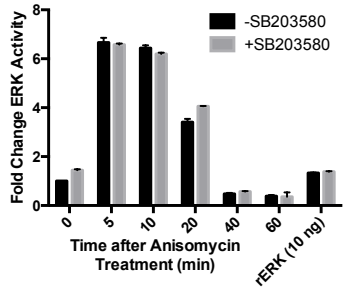


Figure 6. Activity of ERK1/2 in HeLa cell lysates following treatment with 1 μM anisomycin. Assays were run in the presence and absence of the p38 inhibitor, SB203580 (500 nM). Values are plotted as fold change from the 0 min control and are representative of triplicate measurements performed in duplicate

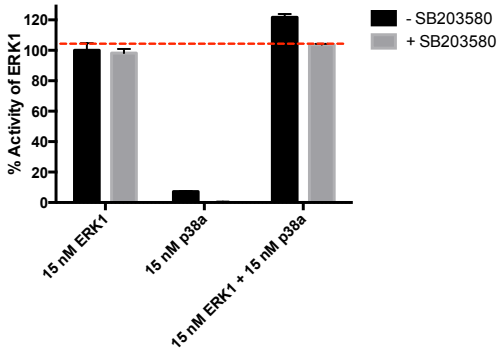


Figure 7. Activity of ERK1, p38α, or a combination of ERK1 and p38α as detected using the ERK1/2 sensor. p38α activity can be effectively diminished with the addition of 500 nM SB203580, a selective p38 inhibitor.

Assays with Kinase Inhibitors.

Assays were performed as described in the Experimental Section, but in the presence of selective kinase inhibitors. Inhibitor stocks were made 100X in DMSO. Recombinant kinases or lysates were incubated with inhibitors for 30 min at 30 °C at 1X inhibitor concentration (final concentration of DMSO <1%). Substrate peptides in assay buffer were also incubated with 1X inhibitor prior to kinase/lysate addition. IC_{50}'s were derived by plotting the %Activity of the DMSO (vehicle) control versus inhibitor concentration and curve fit using nonlinear regression analysis was performed in GraphPad Prism to calculate IC_{50}s.

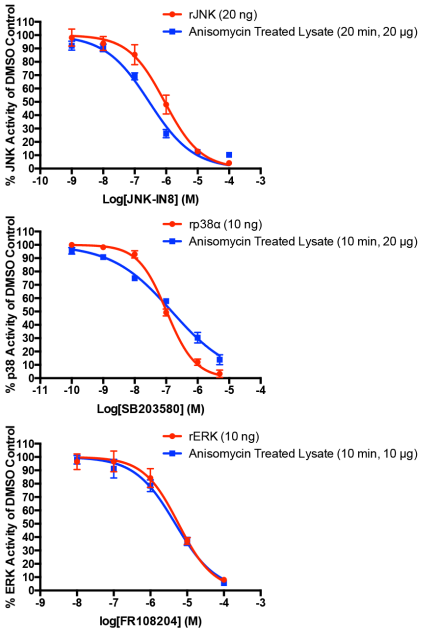


Figure 8. Dose-response curves for each selective MAP kinase subfamily inhibitor in both cell lysates and against recombinant kinase.

References

(1) Luković, E.; González-Vera, J. A.; Imperiali, B. *J. Am. Chem. Soc.* **2008**, *130*, 12821.