Supporting Information

Fluorescence Linked Enzyme Chemoproteomic Strategy for Discovery of a Potent and Selective DAPK1 and ZIPK Inhibitor

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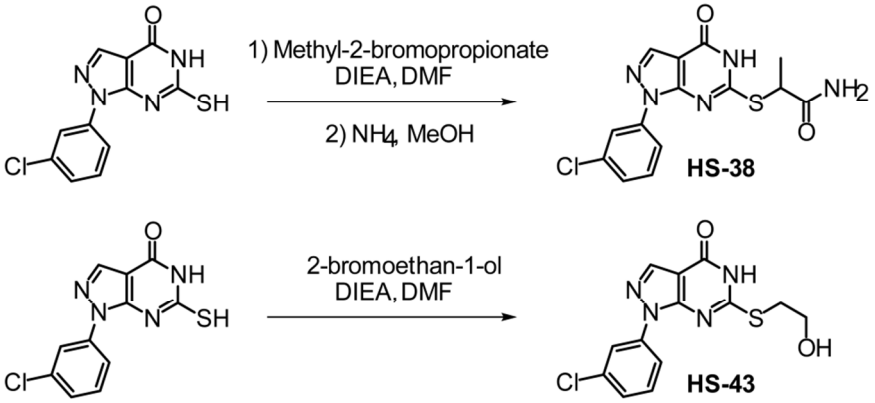
Methods

All reagents were purchased from commercial sources and used without further purification. ^{1}H NMR experiments were performed on a Varian 500 MHz instrument.



Supplementary Scheme 1. Synthetic scheme for preparation of HS38 and HS43

Synthesis of (2-((1-(3-chlorophenyl)-4-oxo-4,5-dihydro-1H-pyrazolo[3,4-*d*]pyrimidin-6-yl)thio)propanamide (HS38). 1-(3-chlorophenyl)-6-mercapto-1,3a,7,7a-tetrahydro-4H-pyrazolo[3,4-*d*]pyrimidin-4-one (500 mg, 1.8 mmol, Enamine EN300-27274) was dissolved in CH_2Cl_2 (5 mL) and treated with diisopropylethylamine (630 µL) and methyl 2-bromopropionate (329 mg, 1.97 mmol). After stirring for 24 h, the mixture was adsorbed onto silica (4 g) and added to a silica gel column (18 x 2.5 cm), flushed with CH_2Cl_2 (150 mL), and chromatographed (10% MeOH in CH_2Cl_2, 400 mL). The resulting solid was triturated with water and filtered to give methyl 2-((1-(3-chlorophenyl)-4-oxo-3a,4,7,7a-tetrahydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)propanoate (574 mg, 88%) as a white powder.

To the methyl ester (100 mg, 275 µmol) was added 7N ammonia in methanol (3 mL) and the mixture was heated to 65 ºC in a sealed vial. After 12 h, the reaction was condensed to dryness and the resulting solid was triturated with methanol in dichloroethane (1:1, 5 mL) and heated to 65 ºC. Upon cooling, the solid was filtered and washed with methanol (5 mL) to give HS38 (77 mg, 80%) as a white powder. Proton NMR matched that of the commercially available material HS38. ^{1}H NMR (500 MHz, dmso-d_6) δ: 12.84 (s, 1H), 8.28 (s, 1H), 8.15 (s, 2H), 7.76 (s, 1H), 7.60 (t, J = 8.0 Hz, 1H), 7.46 (d, J = 7.8 Hz, 1H), 7.29 (s, 1H), 4.48 (q, J = 7.0 Hz, 1H), 1.62 (d, J = 6.8 Hz, 3H).

Synthesis of 1-(3-chlorophenyl)-6-((2-hydroxyethyl)thio)-1,5-dihydro-4H-pyrazolo[3,4-*d*]pyrimidin-4-one (HS43). 1-(3-chlorophenyl)-6-mercapto-1,3a,7,7a-tetrahydro-4H-pyrazolo[3,4-*d*]pyrimidin-4-one (100 mg, 0.36 mmol, Enamine EN300-27274) was dissolved in DMF (1 mL) with DIEA (0.19 mL, 1.1 mmol, 3.0 eq.). To this was added 2-bromoethan-1-ol (30 µL, 0.39 mmol, 1.1 eq.) and the mixture was stirred for 12 h. The mixture was condensed, dissolved in DMSO/water (1:1) and purified by preparative HPLC (Agilent Prep C18 column, 21.2 mm I.D.) using 40-100% methanol in water (0.2% formic acid modifier) over 18 min to give HS43 (67 mg, 58% yield). ^{1}H NMR (500 MHz, dmso-d_6) δ: 8.25 (s, 1H), 8.20 (s, 1H), 8.09 (d, J = 7.5 Hz, 1H), 7.57 (t, J = 8.3 Hz, 1H), 7.43 (d, J = 7.5 Hz, 1H), 3.77 (t, J = 5.9 Hz, 3H), 3.33 (t, J = 5.9 Hz, 3H). ^{13}C NMR (500 MHz, dmso-d_6) δ: 161.93, 157.49, 151.93, 139.57, 136.51, 133.46, 130.96, 126.37, 120.26, 119.29, 104.85, 59.15, 33.73. HRMS m/z calcd. for $C_{13}H_{11}ClN_4O_2S$ $[M+H]^+$: 323.0364. Found: 323.0359.

Library Construction. A collection of 10000 compounds was initially selected from commercially available compounds using a previously described set of filters.[1] Two experienced medicinal chemists further refined the set down to 4000 compounds in order to remove structural liabilities not easily reduced to algorithmic analysis. Drug candidates (3379 compounds) were purchased from sources associated with emolecules.com and were dissolved in DMSO as 10 mM solutions. Copies of the libraries are available upon request to collaborators as 10 mM DMSO solutions in 96 well format.

Calculation of Z' factor for assay development. The average fluorescence signal from the 70 mM ATP control defined maximum ZIPKΔ-eGFP displacement from the ATP-Sepharose resin, and average of 10% DMSO in buffer controls defined minimum displacement. These parameters were used to determine the *Z'* factor:

$$Z'\,factor = 1 - \frac{3 \times (\sigma_p + \sigma_n)}{\left|\mu_p - \mu_n\right|}$$

where μ_p is the mean for maximum displacement, σ_p is standard deviation for maximum displacement, μ_n is the mean for minimum displacement, and σ_n is the standard deviation for minimum displacement. The *Z'* factor for this screen is 0.53, which is above the threshold (0.5) for an excellent assay[2].

Expression of GFP fusion proteins. HEK293 cells were plated in 15-cm tissue culture plates and incubated overnight at 37 °C under 5% CO_2. GFP fusion constructs were transfected into cells at a 1:3 ratio with Fugene HD (Roche) transfection reagent. After 24-48 hours, cell culture media was aspirated, and cells were scraped off the plate using PBS. Cells were pelleted by centrifugation (2,000 rpm, 2 min) and pellets were flash frozen in an EtOH/dry ice bath and stored at -80 °C. GFP fusion protein was extracted from cell pellets using mammalian lysis buffer (0.1% Triton; NaCl, 150 mM; $MgCl_2$, 60 mM; Tris·HCl, pH 7.5, 25 mM; Microcystin, 1 μM; protease inhibitor tablet) for 30 min over ice. Supernatant was isolated from cell debris by centrifugation (4,000 rpm, 5 min). The supernatant was then used for all subsequent screening and titration experiments.

Tissue Preparation and Force Measurements. All protocols and procedures for tissue harvest were carried out according to protocols approved by the Animal Care and Use Committees at the University of Virginia, Duke University, and the University of Calgary.

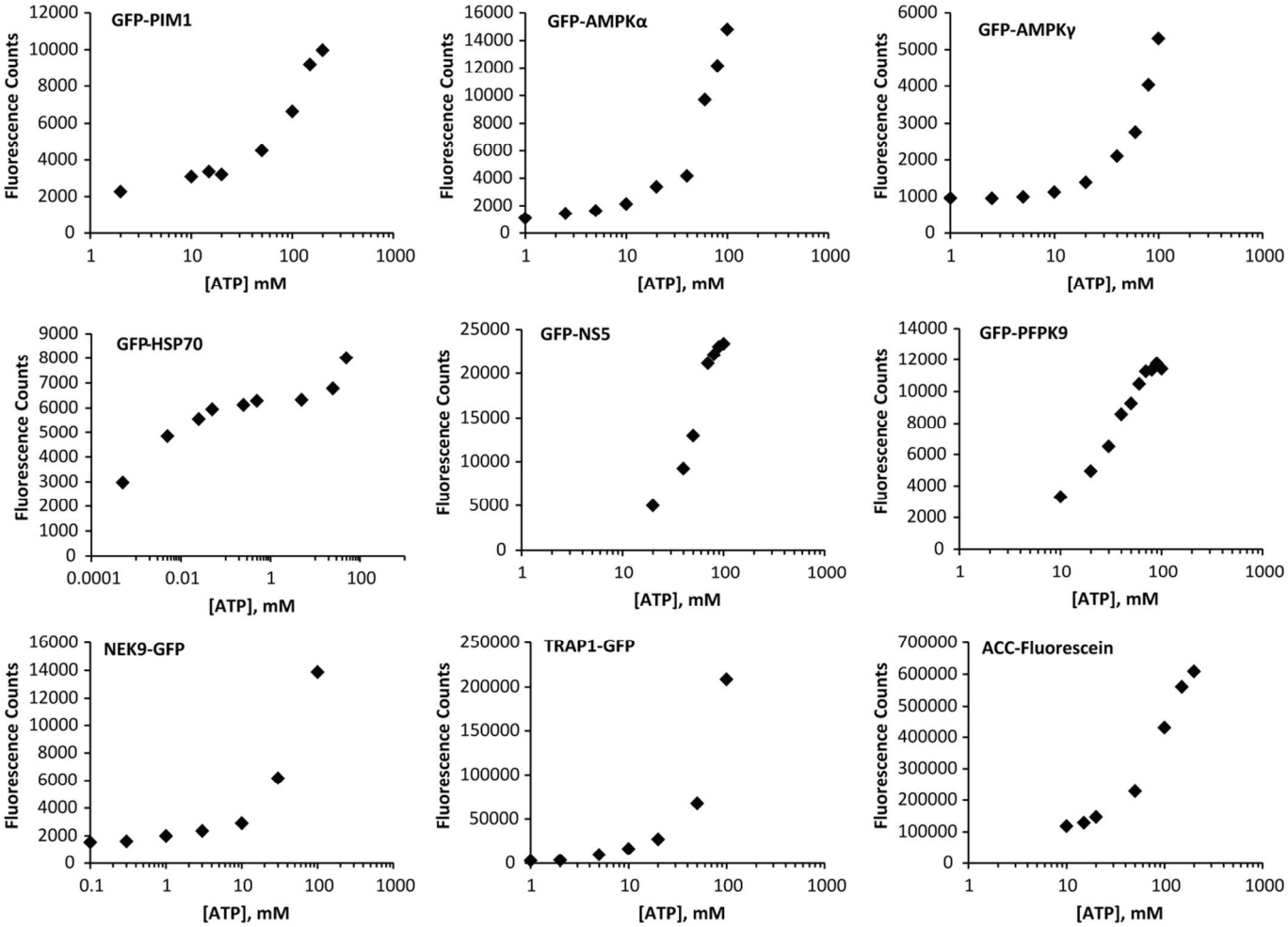
Rabbit Ileum. Helical strips of mouse aorta or strips of rabbit ileum were dissected, (~400 μm wide, 2 mm long) and mounted between two tungsten hooks on a bubble plate for force measurements.[3] Isometric force measurements were performed on mouse aortas as previously described.[4] Intact strips of mouse abdominal aorta were challenged with phenylephrine following pretreatment with HS38 or diluent, DMSO or HS38 and DMSO were added after maximal phenylephrine-induced force was achieved and force output measured. For evaluation of HS38 on Ca^{2+} sensitized force, after measurements of contractions induced by high K^+ (154 mM), ileum strips were permeabilized with either Staphylococcus aureus-toxin (500 U ml^{-1} for rabbit) for 40 min at room temperature in relaxing solution (G1) containing MgATP (4.5 mM) and EGTA (1 mM) as described previously[5] or b-escin (75 μM, Sigma-Aldrich) for 10 min. Ca^{2+} stores were depleted in muscle strips with the addition of Ca-ionophore A23187 (10 μM, Calbiochem) for 10 min in G1 solution. For Ca^{2+} activating solutions, EGTA (10 mM) and a calculated amount of Ca-methanesulfonate was added to give the desired free Ca^{2+} concentration.[3]

To examine the effect of ZIPK inhibition on sensitized force, muscle strips were stimulated with an intermediate calcium concentration solution (pCa 6.5). Once force reached a plateau, strips were Ca^{2+}-sensitized by the addition of the agonist carbachol (5 μM). Up to 2 μM of GTP was added together with the sensitizing agonist to compensate for possible loss of GTP during permeabilization. At the plateau of Ca-sensitized force (5-10 min upon stimulation), the ZIPK inhibitor, HS38 (50 μM) or diluent (0.1% DMSO) was added and the force relaxation time course was recorded. Tissue samples were frozen 20 min after the addition of HS38 for biochemical assays of RLC20 and MYPT1 phosphorylation.

To examine the Ca^{2+}-independent kinase activity of ZIPK, strips were pre-incubated with either HS38 (50 μM) or DMSO (0.1%) for 5 min in relaxing (G1) solution followed by addition of microcystin-LR (10 μM) for 35 min.

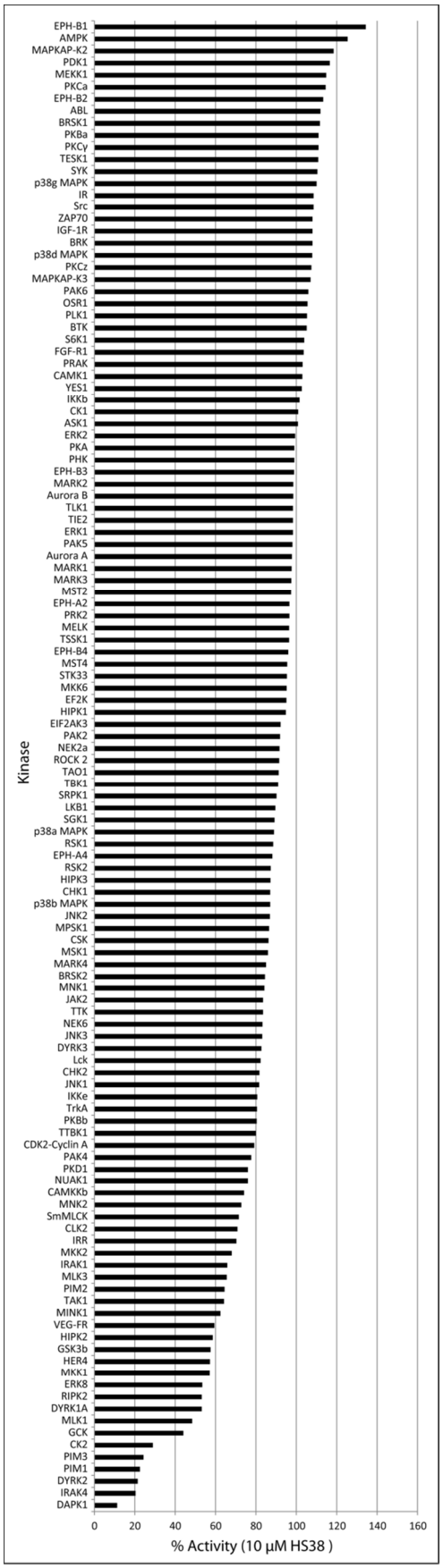
Rat Caudal Arteries. Caudal arteries were removed from male Sprague-Dawley rats (~300 g) that had been anesthetized and euthanized according to protocols approved by the University of Calgary Animal Care and Use Committee. The arteries were cleaned of excess adventitia, de-endothelialized and cut into helical strips (1.5 mm x 6 mm). Muscle strips were mounted on a Grass isometric force transducer (FT03C), and force was recorded as previously described.[6] Intact tissues were treated with calyculin A (0.5 μM) to stimulate Ca^{2+}-independent contractions. At selected sampling points, muscle strips were flash-frozen in 10% (w/v) TCA, 10 mM DTT in acetone followed by 3 x 10 s washes in 10 mM DTT

in acetone. Tissues were then lyophilized overnight. Protein was extracted from each arterial strip by incubation (16 h, 5 ºC) in 0.5 mL of SDS-PAGE sample buffer.

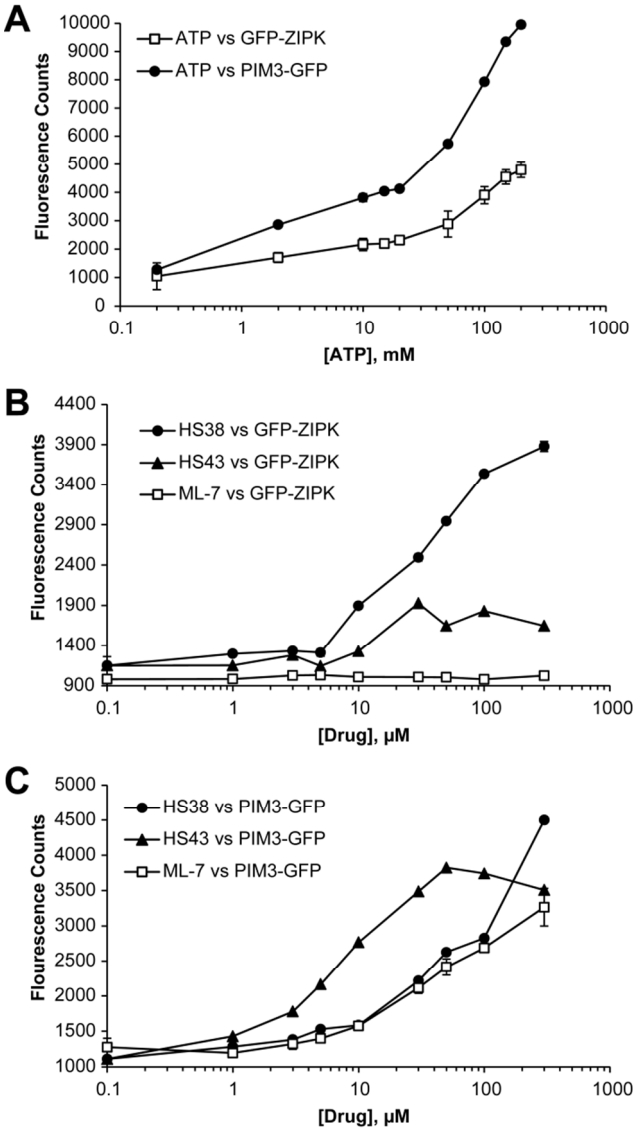


Supplementary Figure 1. Binding isotherms demonstrate the versatility of FLECS for the discovery of ATP competitive inhibitors of purine utilizing enzymes. Sigmoidal curves were obtained by elution of GFP fusion constructs of PIM1 (Addgene 23692), AMPK (α and γ subunits) (Data and protein provided by Raquel R. Bartz, Duke University, Durham, NC, USA), HSP70 (Addgene 15215; data provided by Matthew Howe, Duke University, Durham, NC, USA), NS5 (plasmid provided by David A. Jans, Monash University, Melbourne, Australia), PFPK9 (protein provided by Nisha Philip, Duke University, Durham, NC, USA), NEK9 (Origene, data provided by Nathan Cox, Duke University, Durham, NC, USA), and TRAP1 (plasmid provided by Leonard M. Neckers, National Cancer Institute, Rockville, MD, USA) as well as fluorescently labeled ACC (isolated from pig mammary gland provided by Lauretta A. Rund, University of Illinois, Urbana, IL, USA) from γ-linked ATP media with soluble ATP. Screening efforts are currently underway by multiple investigators to identify inhibitors of these enzymes within our chemical library.

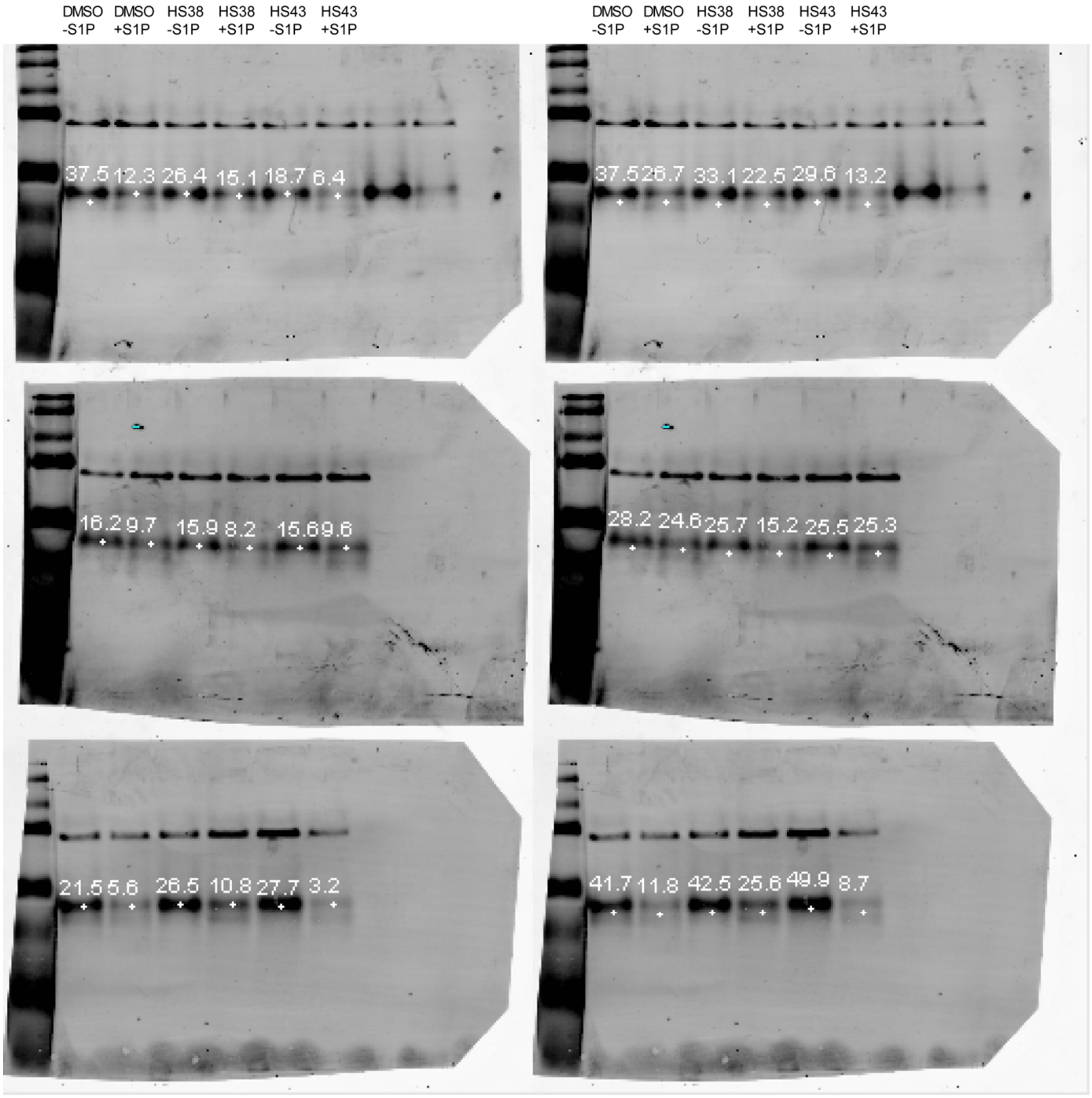
Supplementary Figure 2. Heat map with colored tiles representing relative fluorescence counts per well from each plate of drug candidates screened against GFP-ZIPK using FLECS. Drug candidate solutions (500 µM) were screened from columns 2-11 of 96-well plates with unique identifiers (e.g. THAS001002). Buffer (negative control) and ATP solutions (10 mM, 25 mM, and 50 mM) were screened in columns 1 and/or 12. Conditional color scale formatting was applied to each plate separately. All potential hits were verified by western blotting using anti-ZIPK. HS38 was located in THEN022002 well A03.



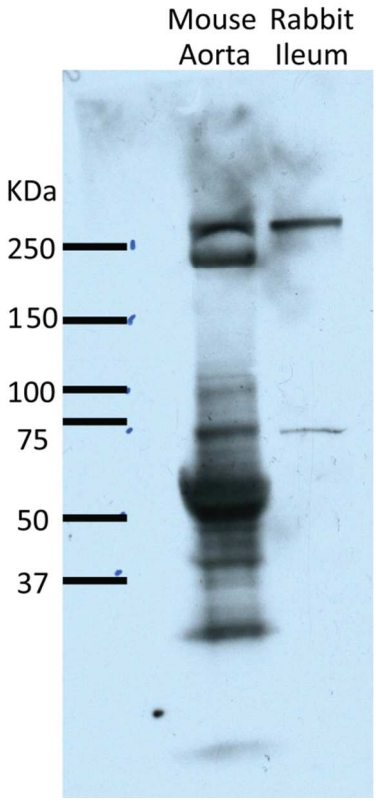
Supplementary Figure 3. The selectivity of HS38 represented as a bar graph. HS38 was evaluated against 124 purified protein kinases, using a ^{33}P-ATP filter-binding assay (International Centre for Kinase Profiling, University of Dundee).



Supplementary Figure 4. Titration curves generated by elution of GFP-ZIPK and PIM3-GFP from γ-linked ATP sepharose media by HS38, HS43, and ML-7. ML-7 was previously identified as a potential hit during the process of screen optimization while screening a truncated form of ZIPK, with C-terminal GFP tag (ZIPKΔ$^{13\text{-}289}$-GFP), against commercially available libraries (LopacTM Library, Sigma, 1280 members and BioFocusTM Library, BioFocus DPI, 3120 members). **A.** ATP elution curves. **B**. HS38 eluted GFP-ZIPK while HS43 eluted significantly less, and ML-7 eluted none. This suggests that, although ML-7 binds ZIPKΔ$^{13\text{-}289}$-GFP in an ATP competitive manner, this activity is not recapitulated with the full length construct. A possible explanation is that the presence of a C-terminal regulatory domain on full length ZIPK, which is essential for regulating and targeting its activity in vivo, but not present in the truncate, renders ML-7 affinity insufficient to displace full length ZIPK from immobilized ATP.[7] This finding demonstrates that full length constructs should be screened whenever possible. **C.** Elution of PIM3-GFP by HS38, HS43, and ML-7.



Supplementary Figure 5: Western blot data used to generate Figure 3B



Supplementary Figure 6. Western blot analysis of mouse aorta and rabbit ileum tissue against PIM3 Kinase (full length 326 AA, 35.8 kDa). PIM3 antibody (anti Human produced in mouse).

References

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