Supporting Information

Experimental Procedures

Reagents, antibodies, plasmids, and recombinant PTPs. *para*-nitrophenyl phosphate (*p*NPP), 3-O-methylfluorescein phosphate (OMFP), dithiothreitol (DTT), and sodium orthovanadate (Na_3VO_4) were purchased from Sigma-Aldrich. 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) was from Invitrogen, Inc. Biomol Green reagent was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Compounds for follow-up studies were purchased from Specs or ChemBridge. All compounds had a purity of >95% (verified by LC/MS and ^{1}H-NMR). All other chemicals and reagents were of the highest grade commercially available. All antibodies were from Cell Signaling Technology, Inc. Recombinant HePTP, MKP-3, VHR, and Lyp were expressed in *E. coli* and purified as described previously [i,ii,iii,iv]. Recombinant CD45, Shp2, TCPTP, LAR, and PTP1B were from Biomol Research Laboratories, Inc.

Chemical Library Screening for VHR inhibitors. VHR HTS was performed within the MLPCN network, PubChem AID 1654. A total of 291,018 compounds (comprising the full MLPCN library at the time of screening) were screened at a concentration of 13.3 μM. A colorimetric phosphatase assay was set up in 1536-well format, using the general phosphatase substrate *p*NPP [v]. The assay buffer contained 20 mM Bis-Tris (pH 6.0), 1 mM DTT, and 0.005% Tween-20. VHR working solution contained 40 nM recombinant VHR in assay buffer; *p*NPP working solution contained 1.474 mM *p*NPP in assay buffer; compound working solutions were 2 mM in DMSO. VHR and *p*NPP solutions were prepared fresh prior to use. The following protocol was used:

- Dispense 1.5 μL of assay buffer into columns 1 and 2 of a black, clear bottom 1536-well assay plate (Corning, #3891).
- Add 1.5 μL of *p*NPP working solution to all wells.
- Dispense 20 nL of compound working solutions to columns 5-48 using a HighRes Biosolutions pintool.
- Dispense 20 nL of DMSO to columns 1-4.
- Dispense 1.5 μL of VHR working solution to wells in columns 3 through 48.
- Incubate lidded plate for 1 hour at room temp.
- Add 3 μL of Biomol Green reagent to all wells.
- Incubate for 30 min to allow Biomol Green reagent signal to develop.

- Read plate on a Perkin Elmer Viewlux at 630 nm in absorbance mode (Ex1:2 = 630 DF10; Em1:564/250(absorbance); Em2:Clear; Light energy = 100000; measurement time = 5 sec; 1x binning)

Data analysis was performed using CBIS software (ChemInnovations, Inc). To quantitate the inhibitory efficacy of the library compounds, the ratio of inhibition in comparison to the negative control was determined. Every compound with >50% inhibition was cherry-picked and rescreened in triplicate to confirm it as a hit.

Single-concentration confirmatory assays for VHR hits using OMFP. Phosphatase activity was measured in triplicate in a 1536-well format assay system, using the fluoresceine-based phosphatase substrate OMFP. The assay buffer contained 20 mM Bis-Tris (pH 6.0), 1 mM DTT, and 0.005% Tween-20. VHR working solution contained 4 nM recombinant VHR in assay buffer; OMFP working solution contained 36 μM OMFP in assay buffer; compounds working solutions were 2 mM in DMSO. VHR and OMFP solutions were prepared fresh prior to use. The following protocol was used:

- Dispense 3 μL assay buffer in columns 1-2 of a black 1536-well plate (Corning, #3724).
- Dispense 3 μL of VHR working solution in columns 3-48.
- Pin 20 nL of compound solutions in triplicate wells per compound to columns 3-46 or DMSO to columns 1-2 and 47-48, using a HighRes Biosolutions pintool.
- Dispense 3 μL of OMFP working solution to wells in columns 1-48.
- Briefly spin plate down.
- Incubate lidded plate for 1 hour at room temp.
- Read plate on a Perkin Elmer ViewLux in fluorescence mode (ex: 480nm; 3m: 520nm; dichroic mirror is 515 nm).

Data analysis was performed using CBIS software (ChemInnovations, Inc). To quantitate the inhibitory efficacy of the library compounds, the ratio of inhibition in comparison to the negative control was determined. Compounds that demonstrated >50% average inhibition were defined as actives.

Dose-response confirmatory assays for VHR hits using OMFP. Phosphatase activity was measured in a 384-well format assay system. The assay buffer contained 20 mM Bis-Tris (pH 6.0), 1 mM DTT, and 0.005% Tween-20. VHR working solution contained 6 nM recombinant VHR in assay buffer; OMFP working solution contained 25 μM OMFP in assay buffer;

compound working solutions contained serially diluted compounds in DMSO. The compounds were tested in one or more concentration ranges (0-100/0-50/0-10/0-1 μM). All solutions were prepared fresh prior to use. The following protocol was used:

- Dispense 10 μL of OMFP working solution into all wells of a black 384-well small-volume plate (Greiner, #784076).
- Dispense 10 μL of assay buffer in columns 1-2.
- Add serially diluted compounds to columns 3-22 and DMSO to columns 1-2 and 23-24 (the final concentration of DMSO was 1.575%).
- Dispense 10 μL of VHR working solution to wells in columns 3 through 24.
- Incubate lidded plate for 1 hour at room temp.
- Read plate on a SpectraMax M5 plate reader (Molecular Devices) in fluorescence mode (ex: 480nm; 3m: 520nm; dichroic mirror is 515 nm).

Data analysis was performed using CBIS software (ChemInnovations, Inc), and the data were fitted to the Hill Equation.

Dose-response assays with HePTP and MKP-3 using OMFP. Phosphatase activity was measured in a 384-well format assay system, similar to the one described above. Compounds were 2-fold serially diluted in DMSO, before added to the reactions for a ten-point dose-response curve. The compounds were tested in one or more concentration ranges (0-100/0-50/0-10/0-1 μM). All solutions were prepared fresh prior to use. HePTP reactions contained 20 mM Bis-Tris, pH 6.0, containing 150 mM NaCl, 1 mM DTT, 0.005% Tween-20, 2.75 nM HePTP, and 300 μM OMFP. MKP-3 reactions contained 30 mM Tris, pH 7.0, 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.005% Tween-20, 1 mg/ml MKP-3, and 50 μM OMFP. Fluorescence intensity was measured over time to determine the slopes of progress curves using an Envision plate reader (PerkinElmer), an excitation wavelength of 485 nm, and an emission wavelength of 528 nm. The data were fitted to Hill Equation.

*Selectivity assays for compound **1** with HePTP, HePTP-K105A/T106A, CD45, Shp2, TCPTP, Lyp, LAR, and PTP1B.* The PTP-catalyzed hydrolysis of DiFMUP in the presence of compound was assayed at room temperatuer in a 60 μL 96-well format reaction system in 50 mM Bis-Tris, pH 6.0 assay buffer containing 1.7 mM DTT, 1% PEG, and 5% DMSO. At various concentrations of the compound, the initial rate at fixed DiFMUP concentration (equal to the corresponding K_m value for each PTP) was determined using a FLx800 micro plate reader (Bio-

Tek Instruments, Inc.), an excitation wave length of 360 nm and measuring the emission of the fluorescent reaction product 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) at 460 nm. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without addition of enzyme. The IC_{50} values of compound **1** for each enzyme were determined as described before [iii].

*Michaelis-Menten kinetic studies to determine the inhibition mode of compound **1**.* Competition inhibitory studies *versus* the OMFP substrate were performed in 50 mM Bis-Tris, pH 6.0, 1 mM DTT, 0.005% Tween-20, and 1.5 nM HePTP. OMFP substrate concentration was varied from 19 to 2400 µM, and the compound concentration was varied from 0.06 to 7.5 µM. Progress curves were monitored for each set of conditions, and the initial rates were determined by linear regression analysis. The global fit of the *V versus* [S] data to possible inhibition models was performed using GraPad Prism software (v. 5.02).

Generation of HePTP K105A/T106A double mutant. HePTP (residues 44–339) was subcloned into a derivative of the pET28a bacterial expression vector (Novagen) containing an N-terminal expression and hexahistidine purification tag (MGSDKIHHHHHH). Both the K105A and T106A mutations were incorporated into a single primer that was used for Quikchange site-directed mutagenesis (Stratagene) to generate the HePTP K105A/T106A expression plasmid. The protein was expressed and purified as previously described [vi].

In Silico Docking. Flexible ligand docking calculations were performed with the ICM docking algorithm [vii,viii] as implemented in the ICM-Pro program (v3.7-1g, Molsoft, LLC.). Calculations were run on a MacPro workstation. The coordinates of the 3D structure of HePTP (PDB code 3D44), VHR (PDB code 1J4X), and MKP-3 (PDB code 1MKP) were converted into ICM objects, charges were assigned, orientations of side chain amides were corrected, and hydrogen atoms added and their positions optimized by energy minimization using MMFF force field. Mutations in the crystal structures were corrected to wild-type amino acid sequences, and the side chains were optimized using the Optimize Side Chains tool as implemented in ICM-Pro. The binding pockets were defined as follows. HePTP and VHR: residues in an 8 Å radius around the *p*Tyr of the peptide ligand; MKP-3: residues in an 8 Å radius around the catalytic Cys-293. Energy-minimized 3D molecular models of the compounds were generated with the implemented routine in ICM. Each docking experiment was repeated three times; the ICM scores and docking poses with the lowest energy are reported.

TCR stimulation, cell lysis, and immunoblotting. Jurkat TAg T cells were treated with small-molecule compound or DMSO alone (vehicle control) for 45 min at 37^{o}C. Cells were then TCR-stimulated with an optimal dose of OKT3 (500 ng/ml) for 5 min. Thereafter, cells were lysed in lysis buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 1% Triton X-100, 50 mM NaF, 5 mM Na_3VO_4, 1 mM PMSF, 5 mM EDTA), proteins were resolved by SDS-PAGE, transferred onto PVDF membranes, and blotted with the indicated antibodies. These procedures were carried out as described previously [ix]. Quantification of the blots was done with the software ImageJ and values were normalized to loading.

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