A Specific Inhibitor of Hematopoietic Protein Tyrosine Phosphatase Augments ERK1/2 and p38 Activation *in vivo*

Eduard Sergienko[2,5], Jian Xu[3,5], Wallace H. Liu[1], Russell Dahl[2], David A. Critton[4], Ying Su[2], Brock T. Brown[2], Xochella Chan[2], Li Yang[2], Ekaterina V. Bobkova[2], Stefan Vasile[2], Hongbin Yuan[2], Justin Rascon[2], Sharon Colayco[2], Shyama Sidique[2], Nicholas D.P. Cosford[2], Thomas D.Y. Chung[2], Tomas Mustelin[1], Rebecca Page[4], Paul J. Lombroso[3,6] & Lutz Tautz[1]

Supporting Information

1. SUPPLEMENTARY METHODS

Recombinant proteins

HePTP (residues 44-339), MKP-3 (full-length), and VHR (full-length) were expressed in *E. coli* and purified as described previously [1,2,3]. *PTP1B*. PTP1B (residues 1–298) was subcloned into a derivative of the pET28a bacterial expression vector (Novagen) containing an N-terminal expression and hexahistidine purification tag (MGSDKIHHHHHHMAS) [4]. The construct was verified by sequencing (SeqWright). The expression plasmid was transformed into BL21-CodonPlus (DE3)-RIL (Stratagene) cells and expression carried out in LB medium containing kanamycin and chloramphenicol. Cell cultures were grown at 37°C with vigorous shaking to an OD_{600} of 0.8, at which point the cells were incubated on ice for 1 h. Expression was induced by the addition of IPTG (1 mM final concentration) and the cultures grown for an additional 18 h at 18°C with vigorous shaking. The cells were harvested by centrifugation and resuspended in extraction buffer (50 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100, and EDTA-free protease inhibitor tablets, Roche) and lysed by high pressure cell homogenization (Avestin C3 Emulsiflex). The cell debris was removed by centrifugation (35,000 g/30 min/4°C). The filtered supernatant was loaded onto a HisTrap HP column (GE Healthcare) equilibrated with 50 mM Tris pH 8.0, 5 mM imidazole and 500 mM NaCl and eluted with a 5–300 mM imidazole gradient. Fractions corresponding to purified PTP1B were pooled, concentrated and further purified using size exclusion chromatography (Superdex75 26/60, GE Healthcare) equilibrated in protein buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.2 mM EDTA, 3 mM DTT). Fractions corresponding to PTP1B were pooled, concentrated, and frozen in liquid nitrogen and stored at –80°C until needed. *PTP-SL*. PTP-SL (residues 332–655) was subcloned into the same vector, expressed and purified using identical methods to that for PTP1B. The only difference was the SEC protein buffer (10 mM HEPES, pH 7.5, 150 mM NaCl and 0.5 mM TCEP). *STEP.* STEP (residues 244–539) was cloned into a derivative of the pET28a bacterial expression vector (Novagen) containing an N-terminal expression tag, a hexahistidine purification tag and a TEV protease cleavage sequence (MGSDKIHHHHHHENLYFQGH) [4]. The protein was expressed and purified as described for PTP1B with the following differences. After his-tag purification, the protein was pooled and dialyzed overnight with TEV protease (25mM Tris pH 7.5, 35mM NaCl, 0.5 mM TCEP, 4°C). Following cleavage, the protease was separated from STEP using a 5 ml HiTrap Q-FF column. In a final

step, the protein was purified using SEC (26/60 Superdex 75 in 25 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 0.1 mM EDTA). *LYP*. LYP (residues 2–301) was subcloned into pET23a (Novagen) with a C-terminal his_6-tag. The protein was expressed and purified as for PTP1B with the following changes. Following his-tag purification, LYP was purified by anion exchange (HiTrap Q-FF) and finally SEC (26/60 Superdex 75 in 25 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM TCEP, 0.1 mM EDTA).

Chemical library screening and dose-response assays

HePTP was screened within the Molecular Library Screening Center Network (MLSCN, http://mli.nih.gov/mli/secondary-menu/mlscn/) by the San Diego Center for Chemical Genomics (SDCCG, now Conrad Prebys Center for Chemical Genomics, CPCCG). The screening data was deposited at the PubChem website, AID 521. A total of 112,438 compounds (comprising the MLSCN library at the time of screening and 50,000 compounds of the DIVERSet library from ChemBridge) were screened at a concentration of 20 μM. A colorimetric phosphatase assay was set up in 384-well format, using the general phosphatase substrate *p*NPP [5]. The assay buffer contained 50 mM Bis-Tris, pH 6.0, 2.5 mM DTT, 0.0125% Tween 20. HePTP working solution contained 7 nM recombinant HePTP in assay buffer; *p*NPP working solution contained 1 mM *p*NPP in Milli-Q water. Solutions were prepared fresh prior to use. Vanadate (a general PTP inhibitor) working solution contained 45 mM Na_3VO_4 in 10% DMSO. Solutions were dispensed using either a Multidrop microplate dispenser (Thermo Scientific) or a WellMate bulk dispenser (Matrix Technologies). The following protocol was used for high-throughput screening (HTS): 4 μL of 100 μM compound solution in 10% DMSO were dispensed in columns 3–24 of Greiner 384-well clear microtiter plates (#781101). 4 μL 10% DMSO was dispensed in column 1 (negative control). 4 μL of Na_3VO_4 working solution was dispensed in column 2 (positive control). 8 μL of HePTP working solution was added to the entire plate. 8 μL of *p*NPP working solution was added to the entire plate. Final concentrations of the components in the assay were as follows: 20 mM Bis-Tris, pH 6.0, 1.0 mM DTT, 0.005% Tween 20; 2.75 nM HePTP (columns 1-24); 0.4 mM *p*NPP (columns 1-24); 9 mM Na_3VO_4 (column 2); 2% DMSO (columns 1-24); 20 μM compounds (columns 3-24). Plates were incubated for 1 h at room temperature, before 40 μL Biomol Green reagent was added to the entire plate. For the development of the

green colored complex, plates were incubated for 30 min at room temperature. Absorbance at 620 nm was measured using an Envision plate reader (PerkinElmer). Data analysis was performed using CBIS software (ChemInnovations, Inc). To quantify the inhibitory efficacy of the library compounds, the ratio of inhibition compared to the positive control was determined. Compounds with >50% inhibition were cherry-picked and rescreened to confirm as hits. For dose-response experiments, compounds were 2-fold serially diluted in DMSO, and assays were performed as described above. Ten-point dose-response curves were analyzed using the Hill Equation.

Generation of HePTP K105A/T106A double mutant and H237A mutant proteins

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 HePTP H237A expression plasmid was generated using similar protocols. Both mutants were verified by sequencing (Agencourt), and the proteins were expressed and purified as previously described [6].

PK analysis

To determine metabolic stability, pooled rat liver microsomes (BD Biosciences) in phosphate-buffered saline (PBS) at pH 7.4 were preincubated with compound **1**, along with a compound of known microsomal half-life (verapamil, microsomal $t_{1/2}$ = 11 min), at 37.5 °C for 5 min. After addition of NADPH (Sigma), the mixtures were incubated for 0-15-30-45 min. The final concentrations were 4 μM compound **1**, 2 mM NADPH, and 1 mg/mL (total protein) liver microsomes. Aliquots of 100 μL of the reaction mixture were withdrawn at the time points stated, and immediately combined with 200 μL of acetonitrile/methanol containing an internal standard. After mixing, the samples were centrifuged at 13,000 rpm for 12 min, and the supernatant was analyzed using LC/MS-MS (Shimadzu 2010EV) in multiple-reaction-monitoring (MRM) mode. The change of the AUC (area under the curve) of the parent compound in function of time was used as a measure of microsomal stability, which is expressed as a percentage of the remaining

compound compared to the initial concentration and presents the average ± standard deviation of the test run in triplicate. Microsomal half-life was calculated using linear regression and the program Prism (v5.0b GraphPad Software, Inc.).

To determine the achievable concentration and clearance of compound **1** *in vivo*, mice were injected intraperitoneally (i.p.) with the inhibitor at 3 mg kg^{-1} and blood serum was collected after 5-20-60-120 min. Using a calibration curve with known inhibitor concentrations and LC/MS analysis as described above, serum concentrations of compound **1** were determined. Compound concentrations in the spleen and the brain were determined similarly after homogenizing the tissue using a glass dounce homogenizer on ice and ice-cold PBS solution, and extracting the compound with a water:acetonitrile (25:75) solution containing 1% formic acid. The samples were vortexed for 3 min and centrifuged for 15 min (7,500xg), before the supernatants were collected, diluted in a water:acetonitrile (50:50) solution, and filtered prior to LC/MS analysis.

2. SUPPLEMENTARY TABLE

Table S1: Primary and secondary antibodies used in western blotting experiments with mouse tissue samples

Antibody	Format	Immunogen	Host	Dilution	Source
anti-*p*ERK1/2	whole IgG, unconjugated	human synthetic phosphopeptide	rabbit	1:1000	Santa Cruz Biotechnology
anti-ERK2	whole IgG, unconjugated	C-terminus of rat sequence	rabbit	1:5000	Santa Cruz Biotechnology
anti-*p*p38	whole IgG, unconjugated	human synthetic phosphopeptide	rabbit	1:500	Cell Signaling Technologies
anti-p38	whole IgG, unconjugated	C-terminus of mouse sequence	rabbit	1:1000	Santa Cruz Biotechnology
anti-*p*Akt	whole IgG, unconjugated	mouse synthetic phosphopeptide	rabbit	1:1000	Cell Signaling Technologies
anti-Akt	whole IgG, unconjugated	C-terminus of mouse sequence	rabbit	1:1000	Cell Signaling Technologies
anti-HePTP	whole IgG, unconjugated	human synthetic peptide	rabbit	1:500	Abgent
anti-GAPDH	IgG_1, unconjugated	Rabbit muscle GAPDH	mouse	1:5000	Millipore
anti-rabbit	whole IgG peroxidase-conjugated	rabbit Fc	donkey	1:10,000	Amersham Biosciences
anti-mouse	whole IgG peroxidase-conjugated	mouse Fc	sheep	1:10,000	Amersham Biosciences

3. SUPPLEMENTARY FIGURES

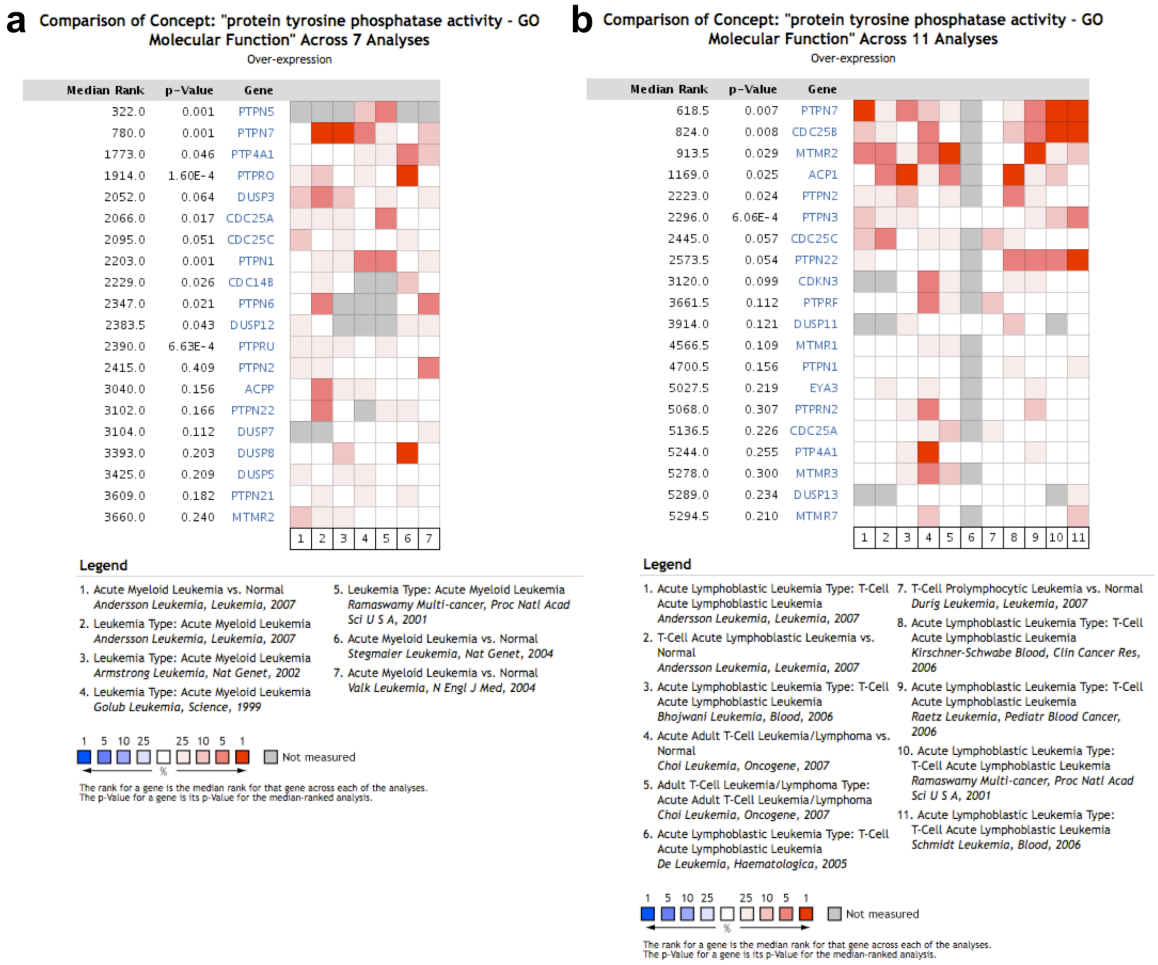

Figure S1. Oncomine™ database search for PTPs overexpressed in acute leukemia. HePTP (*PTPN7*) mRNA levels are significantly upregulated in both AML (**a**) and T-ALL (**b**). (http://www.oncomine.org)

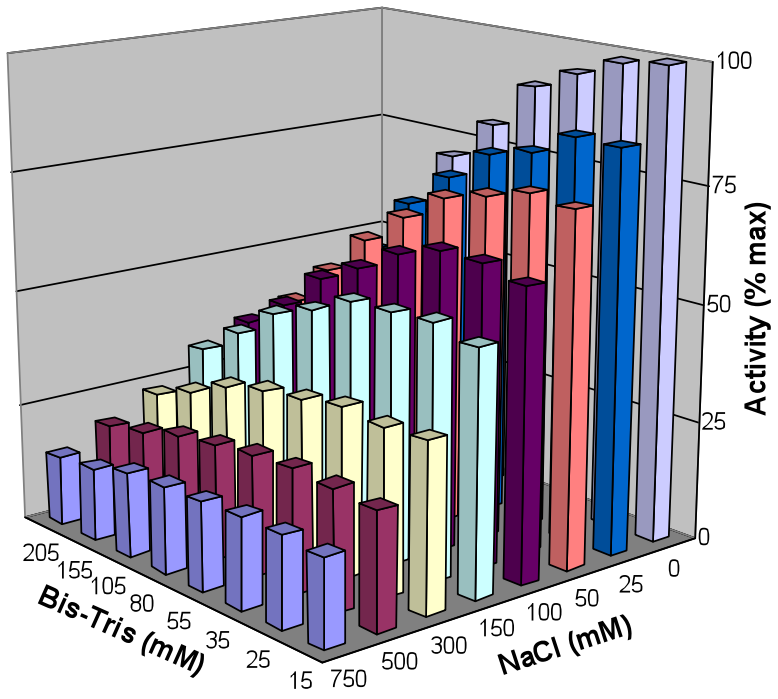

Figure S2. Buffer optimization of the HePTP colorimetric assay in 384-well format. Using the small molecule substrate *p*-nitrophenyl phosphate (*p*NPP), dephosphorylated *p*-nitrophenol was detected using absorbance at 405 nm after quenching the reaction with NaOH. Activity of HePTP at a concentration of 50 nM was tested in the presence of 1.3 mM *p*NPP, 1 mM DTT, 0.005% Tween 20, and various concentrations of Bis-Tris, pH 6.0, and NaCl, over a reaction time of 1 h. The optimal buffer under HTS conditions was 20 mM Bis-Tris, pH. 6.0, 1 mM DTT, and 0.005% Tween 20. The K_m value for *p*NPP was 0.4 mM. The reaction demonstrated linearity of the progress curves over a period of 2 hours. Enzymatic activity was proportional to enzyme concentration. Assay performance was confirmed using the general PTP inhibitor orthovanadate, the IC_{50} value of which was 150 μM.

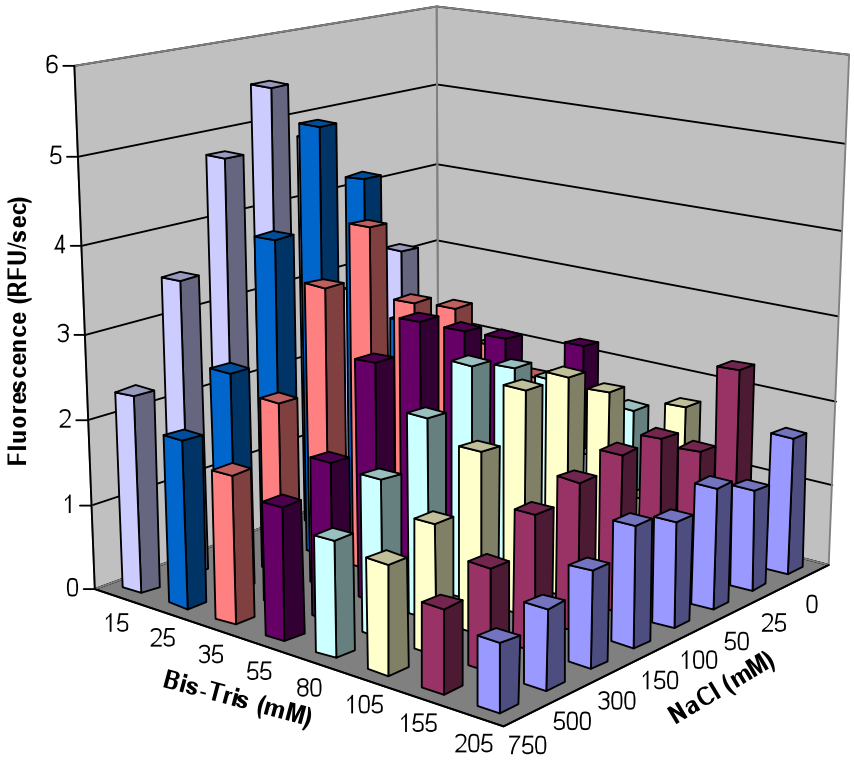

Figure S3. Buffer optimization for the HePTP fluorescent assay in 384-well format. The assay utilized 3-O-methylfluorescein phosphate (OMFP), a fluorogenic substrate of protein phosphatases. Activity of HePTP at a concentration of 6.25 nM was tested in the presence of 0.5 mM OMFP, 1 mM DTT, 0.005% Tween 20, and various concentrations of Bis-Tris (pH 6.0) and NaCl. Fluorescence intensity was measured in kinetic mode using fluorescein optics. The optimal buffer condition was 20 mM Bis-Tris, pH 6.0, 150 mM NaCl, 1 mM DTT, and 0.005% Tween 20. The K_m value for OMFP in the optimized buffer was 0.3 mM.

4. SUPPLEMENTARY REFERENCES

1. Mustelin, T., Tautz, L., and Page, R. (2005) Structure of the hematopoietic tyrosine phosphatase (HePTP) catalytic domain: structure of a KIM phosphatase with phosphate bound at the active site, *J Mol Biol 354*, 150-163.
2. Tierno, M. B., Johnston, P. A., Foster, C., Skoko, J. J., Shinde, S. N., Shun, T. Y., and Lazo, J. S. (2007) Development and optimization of high-throughput in vitro protein phosphatase screening assays, *Nat Protoc 2*, 1134-1144.
3. Wu, S., Vossius, S., Rahmouni, S., Miletic, A. V., Vang, T., Vazquez-Rodriguez, J., Cerignoli, F., Arimura, Y., Williams, S., Hayes, T., Moutschen, M., Vasile, S., Pellecchia, M., Mustelin, T., and Tautz, L. (2009) Multidentate small-molecule inhibitors of vaccinia H1-related (VHR) phosphatase decrease proliferation of cervix cancer cells, *J Med Chem 52*, 6716-6723.
4. Peti, W., and Page, R. (2007) Strategies to maximize heterologous protein expression in Escherichia coli with minimal cost, *Protein Expr Purif 51*, 1-10.
5. Tautz, L., and Mustelin, T. (2007) Strategies for developing protein tyrosine phosphatase inhibitors, *Methods 42*, 250-260.
6. Critton, D. A., Tortajada, A., Stetson, G., Peti, W., and Page, R. (2008) Structural basis of substrate recognition by hematopoietic tyrosine phosphatase, *Biochemistry 47*, 13336-13345.