Identification and structure–function analyses of an allosteric inhibitor of the tyrosine phosphatase PTPN22

Kangshuai Li #1, Xuben Hou #2,4, Ruirui Li #1, Wenxiang Bi #1, Fan Yang #, Xu Chen #, Peng Xiao #, Tiantian Liu #, Tiange Lu #, Yuan Zhou #, Zhaomei Tian #, Yue Mao Shen #, Yingkai Zhang #5, Jiayun Wang #, Hao Fang #, Jinpeng Sun*, #1, Xiao Yu*, #3

1. Key Laboratory Experimental Teratology of the Ministry of Education and Department of Biochemistry and Molecular Biology, School of Medicine, Shandong University, Jinan, Shandong 250012, China.
2. Department of Medicinal Chemistry and Key Laboratory of Chemical Biology of Natural Products (MOE), School of Pharmacy, Shandong University, Jinan, Shandong 250012, China.
3. Department of Physiology, School of Medicine, Shandong University, Jinan, Shandong 250012, China.
4. Department of Chemistry, New York University, New York, New York 10003 United States.
5. NYU-ECNU Center for Computational Chemistry at NYU Shanghai, Shanghai 200062, China.
6. Laboratory of Quantum Biophysics and Laboratory of RNA Biology, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, 100101, China.
7. Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, Jinan, Shandong 250012, China.

# These authors contributed equally to this work.

* Address correspondence and reprint requests to:
Xiao Yu, Department of Physiology, School of Medicine, Shandong University, Jinan, Shandong 250012, China. E-mail: yuxiao@sdu.edu.cn;
Jinpeng Sun, Key Laboratory Experimental Teratology of the Ministry of Education and Department of Biochemistry and Molecular Biology, Shandong University, School of Medicine, Jinan, Shandong, 250012, China. E-mail: sunjinpeng@sdu.edu.cn.

Keywords: protein tyrosine phosphatase non-receptor type 22 (PTPN22); tyrosine phosphatase inhibitor; autoimmunity; WPD loop; lymphoid-specific tyrosine phosphatase (LYP)

Abstract

Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) is a lymphoid-specific tyrosine phosphatase (LYP), and mutations in the PTPN22 gene are highly correlated with a spectrum of autoimmune diseases. However, compounds and mechanisms that specifically inhibit LYP enzymes to address therapeutic needs to manage these diseases remain to be discovered. Here, we conducted a similarity search of a commercial database for PTPN22 inhibitors and identified several LYP inhibitor scaffolds, which helped identify one highly active inhibitor, NC1. Using noncompetitive inhibition curve and phosphatase
assays, we determined NC1’s inhibition mode toward PTPN22 and its selectivity toward a panel of phosphatases. We found that NC1 is a noncompetitive LYP inhibitor and observed that it exhibits selectivity against other protein phosphatases and effectively inhibits LYP activity in lymphoid T cells and modulates T-cell receptor signaling. Results from site-directed mutagenesis, fragment-centric topographic mapping, and MD simulation experiments suggested that NC1, unlike other known LYP inhibitors, concurrently binds to a “WPD” pocket and a second pocket surrounded by a LYP-specific insert, which contributes to its selectivity against other phosphatases. Moreover, using a newly developed method to incorporate the unnatural amino acid 2-fluorine-tyrosine (F2Y) and 19F-NMR spectroscopy, we provide direct evidence that NC1 allosterically regulates LYP activity by restricting WPD loop movement. In conclusion, our approach has identified a new allosteric binding site in LYP useful for selective LYP inhibitor development; we propose that the 19F-NMR probe developed here may be useful also for characterizing allosteric inhibitors of other tyrosine phosphatases.

Selective inhibition of protein tyrosine phosphatases (PTPs) has potential to be developed as a new therapeutic strategy for the treatment of many human diseases, including cancer, inflammation, diabetes, Alzheimer's disease and autoimmune diseases(1-5). However, few selective PTP inhibitors have been developed because of their highly conserved active site, which includes a 9-Å-deep and 6-Å-wide phospho-tyrosine binding pocket surrounded by conserved catalytic residues. In the past, selective inhibitors of several classical PTPs, including PTP1B and LYP, were created by targeting both the phospho-tyrosine binding site and an adjacent site(6-12). Alternatively, non-competitive inhibitors have been recently identified for the treatment of several important disease-related PTPs, including PTP1B(2,13) and SHP2(1,14) as a way to achieve selectivity and circumvent the conserved nature of the PTP phospho-tyrosine catalytic site. These inhibitors regulate phosphatase activity through allosteric regulation and exhibit great potential for therapeutic development against cancer or diabetes.

A member of the PTP family, PTPN22, also called lymphoid tyrosine-specific phosphatase (LYP), is exclusively expressed in hematopoietic immune cells. Developing specific inhibitors toward LYP has raised tremendous interest in the autoimmunity therapeutics community, as a gain-of-function mutant of LYP, the R620W mutant (encoded by the C1858T single-nucleotide polymorphism), has been associated with many autoimmune diseases, including type1 diabetes(15,16), systemic lupus erythematosus(17,18), myasthenia gravis(19-21), rheumatoid arthritis(22,23) and Graves' disease(24). Conversely, impairing the phosphatase activity of LYP was found to reduce the risk of several autoimmune diseases, including systemic lupus erythematosus(25), ulcerative colitis(26) and rheumatoid arthritis(27). With remarkable efforts, several potent inhibitors that selectively target LYP without inhibiting other phosphatase members have been recently identified by our group and by others(3,9,11,12,28,29). However, most of these compounds inhibit LYP via a competitive mode, and an alternative allosteric inhibition mechanism for LYP that can fulfill therapeutic demands has not yet been discovered.

Here, we describe the identification of an allosteric LYP inhibitor (NC1) that was obtained via structural modifications of our previously reported competitive LYP inhibitor (i.e., A15 analogs). Importantly, NC1 displayed a non-competitive mode of LYP inhibition, showed selectivity in a panel of other phosphatases and inhibited LYP activity in T cells. Further mechanistic study revealed that NC1 concurrently bound to a “WPD” pocket adjacent to the classic phospho-tyrosine binding site and to a unique LYP-specific insert that accounted for its selectivity. Moreover, we used our newly developed unnatural amino acid F2Y incorporation technology and 19F-NMR spectroscopy to provide direct biophysical evidence for the allosteric mechanism underlying the non-competitive inhibition of LYP by NC1, in
which the compound restricts the closure of the catalytic WPD-loop.

Results

Identification of NC1 as a non-competitive LYP inhibitor with selectivity against a panel of phosphatases

Our recent efforts using target-ligand interaction-based virtual screening identified a series of competitive LYP inhibitors(28). To explore the diverse chemotypes underlying LYP inhibition, we performed hit-based similarity search of commercial database based on our previously published compound A15(28) and identified a new scaffold (2-iminothiazolidin-4-one) for LYP inhibition (Figure 1A). Subsequently, 10 compounds were purchased from the SPECS database and examined by NMR and mass spectrometry (Supplemental Figures 1-2). We then assessed their abilities to inhibit the LYP-catalyzed hydrolysis of p-nitrophenyl phosphate (pNPP) (Supplemental Table 1). The most active compound, NC1 (Figure 1B), showed LYP inhibitory activity ($K_i = 4.3 \mu M$) that was comparable to the original compound A15 ($K_i = 2.87 \mu M$). Interestingly, an analysis of the inhibition kinetics of NC1 unambiguously indicated a non-competitive inhibition mode toward LYP (Figure 1C), which was different from the competitive inhibition mode of the original compound A15. The dialysis analysis and reversible binding assays confirmed that the NC1 is a non-covalent reversible LYP inhibitor (Supplemental Figure 3-4). Consistently with the enzymology analysis, whereas the interactions between compound A15 and LYP active site is stable during 20ns MD simulation, the compound NC1 could not form stable interactions with LYP and run out from the active site (Supplemental Figure 5). Taken together, both biochemical and computational results suggested the non-competitive binding mode of compound NC1 to LYP.

Further enzyme inhibition tests indicated that NC1 displayed at least 1.9-fold higher selectivity against a panel of other protein phosphatases, including STEP, PTPN18, Glepp, VHR and the Ser/Thr phosphatases PPM1A and PP1, among others (Table 1). Unlike its inhibition of LYP, NC1 exhibited a competitive inhibition mode toward other tested phosphatases (Supplemental Figure 6).

NC1 enhances TCR signaling in lymphoid T cells

We next examined the ability and specificity of NC1 to inhibit LYP activity in cellular contexts. Downstream of T-cell receptor (TCR) activation in lymphoid T cells, LYP negatively regulates the phosphorylation levels of ERK at the pT202pY204 site and of LCK at the pY394 site. As shown in Figure 2, TCR activation in Jurkat T cells significantly increased the phosphorylation levels of ERK and LCK, which were substantially augmented by the application of 20 $\mu$M NC1. Importantly, knockdown of LYP by siRNA increased both the phosphorylation of ERK and LCK to a similar extent to solely administration of NC1 (Fig. 2A-2C and Supplemental Figure 7). Moreover, without endogenous LYP expression in T cells, the NC1 shows no effect on CD3 induced LCK and ERK phosphorylation in T cells (Fig. 2A-2C and Supplemental Figure 7). Taken together, these results suggest that compound NC1 effectively and specifically inhibited LYP-mediated TCR signaling in T cells.

Identification of a novel interaction mode of NC1 with LYP by mutagenesis and simulation analyses

To dissect the molecular mechanism underlying the inhibition of LYP by NC1, a panel of LYP mutants with mutations located on the LYP catalytic surface (Figure 3A and Supplemental Figure 8) was selected according to our previously published crystal structures of LYP(12,30). Six out of nine mutations were found to increase the $K_i$ values of NC1 toward LYP by more than 1.5-fold (Figure 3B). Despite the conserved nature of the PTP catalytic pocket, the combination of four of the mutated residues (H196, D197, F28 and T36) is unique among all PTPs, as indicated by sequence alignment (Figure 3C). This unique pattern contributes to the
selectivity of NC1 for LYP over other phosphatases. In particular, the LYP-specific insert is a unique PTPN22 sequence that is not shared by other phosphatases, as revealed by our previous crystallographic studies(12). More interestingly, two key residues, H196 and D197, are located in the WPD-loop, and one residue, T36, is located in the LYP-specific insert S\textsuperscript{35}TKYKADK\textsuperscript{42}. The WPD-loop harbors the essential catalytic residue D195, which moves more than 6 Å after substrate binding(12,31,32) to coordinate the stabilization of the leaving group after the phospho-ester bond is broken during catalysis. Notably, the LYP-specific insert is on the other side of the WPD-loop relative to the substrate phospho-tyrosine binding pocket. Therefore, the concurrent interaction of NC1 with both the LYP-specific insert and the WPD-loop suggests a unique binding mode of NC1 with LYP that is dissimilar to traditional inhibitor binding to the substrate-binding pocket(10,33).

To predict the allosteric binding mode of NC1 to LYP, we analyzed all possible binding pockets around the four key residues using AlphaSpace, a fragment-centric topographic mapping program. To deal with the protein flexibility of LYP, 8 available crystal structures of LYP were used in our pocket analysis(3,9,12,30,34). Three different WPD-loop conformations (closed, atypical-open and open) were found in the eight crystal structures, and the LYP-specific insert can exist in either α-helix or loop conformations (Supplemental Figure 9). The docking results suggested that NC1 is indeed able to concurrently target the “WPD pocket” and the LYP-specific insert and to interact with the four key residues in the crystal structure 3H2X, which possesses an “atypical-open” WPD-loop conformation (Supplemental Figure 9D)(34,35).

Eight representative docked poses of NC1 that bind the predicted allosteric pockets in opposite directions were selected and subjected to molecular dynamics (MD) simulations to evaluate their binding stabilities (Supplemental Figure 10A). One docked pose of NC1 remained in the predicted allosteric pockets during the 50-ns MD simulation; this pose is recognized as the allosteric binding mode of NC1 (Figure 4A and Supplemental Figure 10C), whereas others could not bind tightly to LYP, and NC1 moved out of the initial pocket (Supplemental Figure 10B-C). Individual residue contribution to the binding of compound NC1 with LYP was further calculated by the MM/GBSA binding free energy decomposition analysis (Figure 4B). Residue R266 and T36 were found to contribute substantially to the binding energy of NC1 to LYP. Consistently, the T36E/R266A double mutation showed a 8.2 folds decrease in its binding ability to NC1 (Figure 3B). According to the well-recognized general acid-base catalysis mechanism, residue D195, which is located in WPD-loop, works as the general acid. Therefore, the conformation of WPD-loop plays key roles in determination of the activity of LYP. To further examine the allosteric inhibition mechanism of NC1, we measured the distance between D195 and the small artificial substrate pNPP by MD simulation of LYP-pNPP system with or without NC1 bound. As shown in Figure 6, the MD analysis suggested that the binding of NC1 in the allosteric pocket, which is located between the substrate binding pocket and the WPD-loop (Figure 5C), may block the closure of the WPD-loop and thereby lock LYP in an inactive conformation.

To further understand why NC1 displayed a different inhibition mode for LYP compared to other protein phosphatases (Table 1), we performed a pocket analysis using the crystal structures of PTP1B, STEP, PTPN18 and Glepp, which all have crystal structures with an “open” WPD loop conformation (35,36). Interestingly, we detected a similar “WPD pocket” in the PTP crystal structures that possess “open” conformations for their WPD-loops (Figure 6). However, the “secondary pockets” were detected in PTP1B, PTPN18 and Glepp, and they were less connected to their “WPD pockets” than that of LYP. Thus, the binding of NC1 to both the “WPD pocket” and “secondary pocket” provides a potential structural basis for its different inhibition mechanism with LYP compared to its mechanisms with other protein phosphatases.

\textsuperscript{19}F-NMR spectroscopy reveals a non-competitive mechanism underlying LYP inhibition by NC1
We next examined the dynamic conformational changes of the WPD-loop using our recently developed unnatural amino acid F2Y incorporation together with 19F-NMR technology(37,38). The unnatural amino acid incorporation causes the fewest structural perturbations and maintains better protein structural integrity than traditional chemical labeling, whereas 19F-NMR is an excellent tool for examining the conformational rearrangement of proteins with higher molecular weights(39,40).

Residue L281 was selected as the F2Y incorporation site to generate a 19F-NMR probe to detect WPD-loop dynamics, as this residue is buried by the WPD-loop in the absence of substrate (2P6X)(35) but is substantially exposed after substrate binding (2QCJ)(12) (Figure 7A). We then mutated residue 281 to an amber stop codon and co-transfected the LYP mutant plasmid with the pEVOL-F2YRS plasmid, which encodes specific Methanocaldococcus jannaschii tyrosyl amber suppressor transfer RNA/tyrosyl-tRNA synthase mutants, into Escherichia coli strain BL21 and cultured it in medium containing F2Y (Figure 7B). After purification, we obtained approximately 95% pure L281F2Y-LYP, and mass spectrometry analysis unambiguously identified the incorporation of F2Y at position 281 (Figure 7C and Supplemental Figure 11 ). The L281F2Y incorporation did not perturb the overall LYP structure, as it showed similar activity toward a phospho-peptide substrate (Supplemental Figure 12). We then used 19F-NMR to monitor WPD-loop movement in response to the binding of the phosphate mimic Na3VO4 with or without compound NC1. A 0.56 ppm upfield shift was detected after incubation of Na3VO4 with the LYP-L281F2Y probe (Figure 7D). In contrast, the Na3VO4-induced upfield shift was reduced to 2/3 of its original value (±0.39 ppm) following pre-incubation with NC1, indicating suppressed movement of the WPD-loop after NC1 incubation (Figure 7D). The NMR results provided direct biophysical evidence that NC1 non-competitively inhibits LYP by restricting the movement of the WPD-loop, in agreement with the data obtained from the mutagenesis analysis and the MD simulations.

Discussion

The development of selective inhibitors of specific PTPs has been hampered by the fact that their conserved active site is shared by most PTP family members. In the past, potent and selective PTP inhibitors have been developed by targeting non-conserved second-layer residues close to the active site(33) or by simultaneously binding to both the active site and a second pocket in the vicinity(9,10,12). Alternatively, inhibitor selectivity can be achieved via allosteric regulation by targeting a pocket outside of the catalytic center. Such allosteric inhibitors have been identified for PTP1B(2,13) and CD45(41) and more recently for SHP2(1,14), and these compounds serve as promising new therapeutics to treat cancer and diabetes. Whereas PTP1B and SHP2 are important drug targets for cancer and diabetes treatment, modulation of LYP activity has the potential to treat autoimmune diseases. Several LYP inhibitors with both high potency and selectivity have been developed(3,9,11,12). Although one of these known LYP inhibitors has a mixed inhibition mode(3), an allosteric inhibitor for LYP was still lacking, and the mechanism of allosteric regulation of LYP by a small compound had not been revealed. Here, we identified NC1 as a non-competitive inhibitor of LYP using enzymology. Moreover, the results of site-directed mutagenesis, fragment-centric topographic mapping and MD simulations suggested that NC1 concurrently binds to a “WPD pocket” in WPD-loop and a “secondary pocket” in LYP-specific insert outside the active site of LYP. Two residues (H196 and D197) in the “WPD pocket” and two residues (F28 and T36) in the “secondary pocket” shared low sequence identity compared with other phosphatases, thus contributing to the selectivity of NC1 toward LYP.

Because the fragment-centric topographic mapping and all-atom MD simulations suggested that NC1 binds to LYP with the catalytically important WPD-loop assuming an open conformation, and because the efficient catalysis of substrate by PTPs requires the closed form of the WPD-loop(31,32,42), we reasoned that NC1 allosterically regulates WPD-loop movement and
thus inhibits enzyme activity. Traditionally, structural information can be acquired by co-crystallizing an inhibitor with a phosphatase, which captures a static image of how an inhibitor interacts with a phosphatase (1,13), but cannot provide further dynamic information. NMR spectroscopy can be used to characterize small phosphatases, such as VHR and PRL, but it is not easily applied to classic tyrosine phosphatases because of their large size. Here, using our newly developed unnatural amino acid F2Y incorporation technology (38), we were able to monitor WPD-loop dynamics with high resolution using 19F-NMR spectroscopy. The binding of LYP to the product mimic vanadate caused a significant upfield shift, which was restricted by approximately 1/3 after incubation with compound NC1. Therefore, the F2Y incorporation method together with 19F-NMR spectroscopy provided direct evidence that modulation of WPD-loop movement serves as an underlying mechanism for the non-competitive inhibition of LYP by NC1. Interestingly, both previously identified allosteric inhibitors of PTP1B also limited the movement of the WPD-loop. Therefore, limiting WPD-loop movement may be a common strategy to develop allosteric inhibitors for PTPs, and our newly developed unnatural amino acid F2Y incorporation method, together with 19F-NMR spectroscopy, may be used to characterize the inhibitory mechanisms of other classic PTPs.

Experimental Procedures

Materials

The selected compounds from hit-based screening were purchased from SPECS with purities confirmed by LC-MS and 1H NMR (data available at http://www.specs.net/). The p-nitrophenyl phosphate (pNPP) was purchased from Sangon Biotech Co., Ltd. Ni-NTA agarose was obtained from Amersham Pharmacia Biotech. The anti-Src/pY416 (catalog number 2101) and ERKpT202/pY204 (catalog number 9101) antibodies were obtained from Cell Signaling Technology. LYP-specific antibody was obtained from R&D (catalog number MAB3428). The anti-CD3 (OKT3) was purchased from eBioscience (catalog number 56-0037-42). The mouse anti-GAPDH monoclonal antibody was obtained from ZSGB-BIO Co (catalog number TA-08). LYP siRNAs were synthesized by China RiboBio Co., Ltd. (Guangzhou, China). All other chemicals and reagents were purchased from Sigma.

Plasmid construction

The constructs of His-LYP, His-PTP1B, His-VHR, His-STEP, His-PTPN18 (catalytic domain), His-Glepp, His-Slingshot2, His-PPM1A, His-PPM1G, His-PP1 have been described previously (28,36,43-45). The LYP mutants F28A, R33A, S35E, T36E, K61A, C129S, H196A, D197A and C231V were generated by PCR reactions with the QuickChange site-directed mutagenesis kit from Stratagene. The PAGE-purified oligonucleotide primers were from Beijing Genomics Institute (China). The LYP mutant L281TAG for F2Y-incorporated protein expression was constructed in a similar way. For F2Y-incorporated protein expression, the pEVOL-F2YRS plasmid used has been described previously (38). All mutations were verified by DNA sequencing from Beijing Genomics Institute.

Protein expression and purification

The expression of native proteins including the catalytic domain of LYP (residues 1-294) with an N-terminal His tag and other His-tagged proteins were described previously (46). Briefly, BL21 (DE3) cells were transformed with the expression plasmids and cultured in LB medium with shaking at 37 °C. The culture temperature was adjusted to 18 °C when the cultures reached an OD600 of 0.6, and expression was induced for 12 h with 0.3 mM IPTG at an OD600 of 0.8. For expression of C-terminal His-tagged LYP F2Y-incorporated protein, pEVOL-F2YRS was co-transformed with LYP L281TAG mutation into BL21 (DE3). The expression was induced with 0.3 mM IPTG and 0.02% L-arabinose at an OD600 of 1.0 in the presence of 0.5 mM F2Y. The cells were then harvested by centrifugation and resuspended in lysis buffer (20 mM Tris pH 8.0, 300 mM NaCl). After centrifugation, the supernatant was incubated with Ni-NTA resin with end-to-end mixing for 1 h at 4 °C. The beads were collected and washed with 20 ml of wash buffer (20 mM Tris, 300 mM NaCl, and 5 mM imidazole) and eluted with an imidazole gradient (20 mM Tris (pH 8.0), 300 mM NaCl and 20-200 mM.
imidazole. The protein was further purified through CM Sefinose85 with elution by a salt gradient. The low-salt solution contained 20 mM MES (pH 6.0), 100 mM NaCl, 1 mM EDTA and 2 mM DTT. The high-salt solution contained 20 mM MES (pH 6.0), 1 M NaCl, 1 mM EDTA and 2 mM DTT. After purification using CM Sefinose, the protein was further concentrated and stored at -80 °C.

**kcat and Km Measurements**

Initial rate measurements for the enzyme-catalyzed hydrolysis of pNPP were conducted as described previously (42). All assays were carried out at 25 °C in 50 mM 3,3-dimethylglutarate (pH 7.0) buffer, containing 2 mM DTT and 1 mM EDTA, with an ionic strength of 0.15 M adjusted by addition of NaCl. For the pNPP reaction, assay mixtures of 100 μl in total volume were set up in a 96-well polystyrene plate from Fisher. A substrate concentration range from 0.2 to 5 Km was used to determine the kcat and Km values. Reactions were started by the addition of an appropriate amount of enzymes. The reaction mixtures were quenched with 100 μl of 1 M sodium hydroxide, and the absorbance at 405 nm was read using a plate reader. All Michaelis-Menten parameters reported are based on non-linear curve fits of the raw data. The steady-state kinetic parameters were determined from a direct fit of the data to the Michaelis-Menten equation using GraphPad Prism 6.0 as follows:

$$v = \frac{V_{\text{max}} \times [S]}{K_m + [S]}$$

(1)

**IC50 Measurements**

Kinetics assay for LYP-catalyzed pNPP hydrolysis in the presence of small-molecular inhibitor were measured as described previously (28,46). The effect of each inhibitor on the LYP-catalyzed pNPP hydrolysis was determined at 25 °C in reaction buffer (50 mM 3,3-dimethylglutarate buffer with the ionic strength of 0.15 M adjusted by NaCl). The Km value of LYP toward pNPP hydrolysis (4mM for pNPP) were used to determine the IC50. The reaction was detected by monitoring the absorbance of pNP at 405 nm. The IC50 values were obtained by fitting the data to Equation 2 using GraphPad Prism 6.0 as follows:

$$A_t = A_0 \times \frac{IC_{50}}{IC_{50} + [I]}$$

(2)

**Ki measurements**

The phosphatases-catalyzed hydrolysis of pNPP in the presence of inhibitors were assayed at 25 °C. The reaction was initiated by addition of pNPP (ranging from 0.2 to 5 Km) to a reaction mixture containing different phosphatases and various fixed concentrations of inhibitors and stopped by addition of 1 M NaOH. All inhibition constant Ki were evaluated based on non-linear curve fits of the raw data using GraphPad Prism 6.0. Inhibition pattern were evaluated by fitting the data to the Michaelis-Menten equations (or Lineweaver-burk equation) for competitive inhibition (Equation 3, 4) and non-competitive inhibition (Equation 5, 6), using linear regression and the program GraphPad Prism 6.0 as follows:

$$\frac{1}{v} = \frac{K_{mobs}}{V_{\text{max}} \times [S]} + \frac{1}{V_{\text{max}}}$$

(3)

$$K_{mobs} = K_m/(1 + [I]/K_i)$$

(4)

$$\frac{1}{v} = \frac{K_m}{V_{\text{max inh}} \times [S]} + \frac{1}{V_{\text{max inh}}}$$

(5)

$$V_{\text{max inh}} = V_{\text{max}}/(1 + [I]/K_i)$$

(6)

**Cell culture, RNA interference and western blot analysis**

Cell culture and RNA interference were performed as previously described (28). Jurkat T cells were pre-incubated with 20 μM (final concentration) inhibitor (NC1) or DMSO for 45 minutes and then stimulated with 5μg/ml anti-CD3 antibody (OKT3) or medium for 5 min. The stimulation was terminated by transferring cells to ice and then lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM NaF, 2 mM EDTA, 10% Glycerol, 1% NP-40, 0.25% Sodium deoxycholate, 1 mM Na3VO4, 1 mM PMSF, 0.3 μM aprotinin, 130 μM bestatin, 1 μM leupeptin and 1 μM pepstatin) for 15 minutes. The lysates were then centrifuged at 12000 rpm for 15 minutes. The supernatants were collected and the protein concentrations were measured by the BCA Protein Quantitation Kit (Beyotime). Equal amounts of cell lysates were denatured in 2×SDS loading buffer and boiled for 10min. Protein samples were then subjected to western blot with specific anti-Src/pY416 or...
anti-ERK pT202/pY204 antibodies or GAPDH antibodies.

**Preparation of ligand and protein structures**

The initial structures of compound A15 and NC1 were constructed using Sybyl-x 1.1 (Tripos, Inc.) and the Tripos force field was employed in structure minimization. Eight crystal structures of LYP were retrieved from the Protein Data Bank (2P6X, 2QCT, 2QCJ, 3BRH, 3H2X, 3OLR, 3OMH, and 4J51) and prepared using the protein preparation workflow in Sybyl-x 1.1 (Tripos, Inc.). The mutated residues were changed back using Discovery Studio 2.5. The protonation states of specific residues were determined at constant pH 7 using the PDB2PQR server(47). Alignment of eight crystal structures were performed using PyMOL (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC).

**Pocket analysis**

Pocket analysis of LYP crystal structures were performed using AlphaSpace(48,49) which utilizes a geometric model based on Voronoi tessellation. Concave interaction space across the protein surface was identified and represented as a set of alpha-atom/alpha-space pairs, which are then clustered into discrete fragment-centric pockets. Details for the calculation of pocket score are described in previous study(48,49).

**Molecular docking**

All docking studies were carried out using the standard setting of Autodock Vina(50). Compound NC1 was docked into eight LYP crystal structures in the existence of substrate (pNPP) to generate the non-competitive binding model. The initial binding conformation of pNPP was judiciously determined based on the crystal structure of LYP complexed with a phospho-tyrosine peptide (PDB: 3OLR). The competitive binding models of compound A15 and NC1 were predicted by docking each ligand separately to the active site of LYP (PDB: 3H2X) in the absence of pNPP. A grid box with 30 Å units in x, y and z directions was used to cover the protein surface around five key interacting residues from mutational analysis. AutoDock Vina reports a series of lowest energy conformations and eight representative models were selected for NC1 to fully explored the potential non-competitive inhibitor binding mode. In addition, the competitive binding models for A15 and NC1 were selected according the docking scores. A total of 10 LYP-inhibitor complexes obtained from molecular docking were subjected to molecular dynamics simulations.

**Molecular dynamics simulation**

Molecular dynamics simulations were carried out using Amber14 package with Amber14SB forcefield(51). The LYP crystal structure (PDB: 3BRH) that possess a closed conformation of WPD loop was used for LYP_pNPP system without NC1 bound. Eight representative docked poses of compound NC1 with LYP crystal structure (PDB: 3HX2) were used for LYP_pNPP system with pNPP bound. Partial atomic charges for pNPP, A15 and NC1 were obtained from HF/6-31G (d) calculations using Gaussian 09 package(51-53). The RESP module in the Amber package was employed to fit the charges to each atomic center(54,55). Each system was neutralized with Na+ counterions and solvated with explicit TIP3P water in a rectangular periodic box with 10.0 Å buffer. After a series of minimizations and equilibrations, standard molecular dynamics simulations were performed with periodic boundary condition. Non-bonded interactions were treated using the Particle Mesh Ewald method(56,57) with 12.0 Å cutoff. The SHAKE algorithm(58) was utilized to constrain all bonds involving hydrogen atoms. The coordinates were stored every 2 ps, and the simulation time step was 2 fs. Berendsen thermostat method(59) was used to control the system temperature at 300 K. All other parameters were default values. MD trajectories were analyzed using cpptraj module in AmberTools 15. Protein-ligand interaction energies were calculated using molecular mechanics/generalized born solvent accessibility (MM/GBSA) method(60,61). MM-GBSA calculations were performed by MMPBSA.py module of Amber14. All figures and movies are produced using Pymol (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC), Chimera(62), and Microsoft Excel.

**Trypsin digestion and MS/MS analysis**
The LYP-F2Y protein was subjected to electrophoresis and the protein band was cut into small plugs and washed twice in 200 ml distilled water for 10 min. The gel bands were dehydrated in 100% acetonitrile for 10 min and dried in a Speedvac (Labconco) for 15 min. Disulfide bonds were reduced by adding 10 ml of 100 mM dithiothreitol (DTT) and subsequently alkylated by 40 mM IAA, 25 mM NH₄HCO₃ for 45 min at room temperature in the dark. The sample was then mixed with trypsin by a ratio of 100:1 in Tris buffer and digested at 37 °C for 12 h. Digestion was stopped by adding formic acid to 1 % final concentration. Digested samples were purified and desalted, and re-dissolved in 30 ml 50% CH₃CN/0.1% CF₃COOH buffer before MS/MS analysis.

LC-MS/MS analysis was performed using a Thermo Finnigan LTQ linear ion trap mass spectrometer in line with a Thermo Finnigan Surveyor MS Pump Plus HPLC system. The peptides generated by trypsin digestion were loaded onto a trap column (300SB-C18, 5×0.3 mm, 5 µm particle) (Agilent Technologies, Santa Clara, CA), which was connected through a zero dead volume union to the self-packed analytical column (C18, 100 µm i.d ×100 mm, 3 µm particle) (SunChrom, Germany). The peptides were then eluted over a gradient (0-45% B in 55 min, 45-100% B in 10 min, where B = 80% Acetonitrile, 0.1% formic acid) at a flow rate of 500 nl min⁻¹ and introduced online into the linear ion trap mass spectrometer (ThermoFisher Corporation, San Jose, CA) using nano electrospray ionization. MS data were analyzed by Bioworks 3.2 software.

NMR experiments

To detect Na₃VO₄-induced LYP WPD loop conformational changes, 100 µM LYP F2Y proteins were mixed with or without a 10-fold molar ratio of Na₃VO₄ and incubated in binding buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% D2O) with end-to-end rotation at room temperature for 30 min. The protein samples were then subjected to ¹⁹F-NMR experiments.

All NMR data were collected using an Agilent OD2 600 spectrometer fitted with a 5-mm broad band probe. The ¹⁹F 90° pulse lengths were 9.9 s and the spectra were typically obtained using 15,000 scans and a recovery delay of 1 s. Data were processed using 10-Hz Lorentzian line broadening and were referenced to the internal TFA standard ( -76.5 p.p.m.). All of the spectra were recorded at 25 °C.

Statistics

The data were analyzed using GraphPad Prism 6. All experiments were performed in at least triplicate, and the data were expressed as mean ± SD. Statistical comparisons between two groups were performed with Student t tests. Statistical comparisons between two factors were performed with two-way ANOVA analysis.

Data availability

The authors declare that data supporting the findings of this study are available in the article as well as its Supplementary Information file and from the authors on reasonable request.
Acknowledgements

We thank NYU-ITS and NYUAD for providing computational resources. This work was supported by grants from the National Key Basic Research Program of China (2013CB967700 to Dr. X.Y), the National Natural Science Foundation of China (31100580, 31470789 to Dr. J.P.S, 31471102 to Dr X.Y), the Shandong Natural Science Fund for Distinguished Young Scholars (JQ201320 to Dr X. Y; JQ201517 to Dr. J.P.S; JQ201319 to Dr. H.F), the Fundamental Research Funds of Shandong University (2016JC017 to J.P.S), the Shandong Province Science and Technology Development Program(2017GSF218023 to WX.B), the program for Changjiang Scholars and Innovative Research Team in University (IRT13028), and National Institute of Health (R01GM120736 to Y.Z.), Key research and development project of Shandong Province (No. 2017CXGC1401).

Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Chen, Y.-N. P., LaMarche, M. J., Chan, H. M., Fekkes, P., Garcia-Fortanet, J., Acker, M. G., Antonakos, B., Chen, C. H.-T., Chen, Z., Cooke, V. G., Dobson, J. R., Deng, Z., Fei, F., Firestone, B., Fodor, M., Fridrich, C., Gao, H., Grunenfelder, D., Hao, H.-X., Jacob, J., Ho, S., Hsiao, K., Kang, Z. B., Karki, R., Kato, M., and Larrow, J. a. (2016) Allosteric inhibition of SHP2 phosphatase inhibits cancers driven by receptor tyrosine kinases. Nature 535, 148--152

2. Krishnan, N., Koveal, D., Miller, D. H., Xue, B., Akshinthala, S. D., Kragelj, J., Jensen, M. R., Gauss, C.-M., Page, R., Blackledge, M., Muthuswamy, S. K., Peti, W., and Tonks, N. K. (2014) Targeting the disordered C terminus of PTP1B with an allosteric inhibitor. Nature chemical biology 10, 558--566

3. Stanford, S. M., Krishnamurthy, D., Falk, M. D., Messina, R., Debnath, B., Li, S., Liu, T., Kazemi, R., Dahl, R., He, Y., Xu, B., Chan, A. C., Zhang, Z.-Y., Barrios, A. M., Woods, V. L., Neamati, N., and Bottini, N. (2011) Discovery of a novel series of inhibitors of lymphoid tyrosine phosphatase with activity in human T cells. Journal of medicinal chemistry 54, 1640--1654

4. Xu, J., Chatterjee, M., Baguley, T. D., Brouillette, J., Kurup, P., Ghosh, D., Kanyo, J., Zhang, Y., Seyb, K., Ononenyi, C., Foscue, E., Anderson, G. M., Gresack, J., Cuny, G. D., Glicksman, M. A., Greengard, P., Lam, T. K. T., Tautz, L., Nairn, A. C., Ellman, J. A., and Lombroso, P. J. (2014) Inhibitor of the tyrosine phosphatase STEP reverses cognitive deficits in a mouse model of Alzheimer’s disease. PLoS biology 12, e1001923

5. Zhang, Z.-Y. (2017) Drugging the Undruggable: Therapeutic Potential of Targeting Protein Tyrosine Phosphatases. Accounts of chemical research 50, 122--129

6. Ala, P. J., Gonneville, L., Hillman, M., Becker-Pasha, M., Yue, E. W., Douty, B., Wayland, B., Polam, P., Crawley, M. L., McLaughlin, E., Sparks, R. B., Glass, B., Takvorian, A., Combs, A. P., Burn, T. C., Hollis, G. F., and Wynn, R. (2006) Structural insights into the design of nonpeptidic isothiazolidinone-containing inhibitors of protein-tyrosine phosphatase 1B. The Journal of biological
chemistry 281, 38013--38021

7. Barr, A. J. (2010) Protein tyrosine phosphatases as drug targets: strategies and challenges of inhibitor development. *Future medicinal chemistry*, 2, 1563--1576

8. Combs, A. P., Zhu, W., Crawley, M. L., Glass, B., Polam, P., Sparks, R. B., Modi, D., Takvorian, A., McLaughlin, E., Yue, E. W., Wasserman, Z., Bower, M., Wei, M., Rupar, M., Ala, P. J., Reid, B. M., Ellis, D., Gonneville, L., Emm, T., Taylor, N., Yeleswaram, S., Li, Y., Wynn, R., Burn, T. C., Hollis, G., Liu, P. C. C., and Metcalf, B. (2006) Potent benzimidazole sulfonamide protein tyrosine phosphatase 1B inhibitors containing the heterocyclic (S)-isothiazolidinone phosphotyrosine mimetic. *Journal of medicinal chemistry*, 49, 3774--3789

9. He, Y., Liu, S., Menon, A., Stanford, S., Oppong, E., Gunawan, A. M., Wu, L., Wu, D. J., Barrios, A. M., Bottini, N., Cato, A. C. B., and Zhang, Z.-Y. (2013) A potent and selective small-molecule inhibitor for the lymphoid-specific tyrosine phosphatase (LYP), a target associated with autoimmune diseases. *Journal of medicinal chemistry*, 56, 4990--5008

10. Sun, J.-P., Fedorov, A. A., Lee, S.-Y., Guo, X.-L., Shen, K., Lawrence, D. S., Almo, S. C., and Zhang, Z.-Y. (2003) Crystal structure of PTP1B complexed with a potent and selective bidentate inhibitor. *The Journal of biological chemistry*, 278, 12406--12414

11. Vang, T., Xie, Y., Liu, W. H., and Vidović (2011) Inhibition of lymphoid tyrosine phosphatase by benzofuran salicylic acids. *Journal of medicinal chemistry*, 54, 562--571

12. Yu, X., Sun, J.-P., He, Y., Guo, X., Liu, S., Zhou, B., Hudmon, A., and Zhang, Z.-Y. (2007) Structure, inhibitor, and regulatory mechanism of Lyp, a lymphoid-specific tyrosine phosphatase implicated in autoimmune diseases. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 19767--19772

13. Wiesmann, C., Barr, K. J., Kung, J., Zhu, J., Erlanson, D. A., Shen, W., Fahr, B. J., Zhong, M., Taylor, L., Randal, M., McDowell, R. S., and Hansen, S. K. (2004) Allosteric inhibition of protein tyrosine phosphatase 1B. *Nature Structural & Molecular Biology*, 11, 730--737

14. Garcia Fortanet, J., Chen, C. H., Chen, Y. N., Chen, Z., Deng, Z., Firestone, B., Fekkes, P., Fodor, M., Fortin, P. D., Fridrich, C., Grunenfelder, D., Ho, S., Kang, Z. B., Karki, R., Kato, M., Keen, N., LaBonte, L. R., Larrow, J., Lenoir, F., Liu, G., Liu, S., Lombardo, F., Majumdar, D., Meyer, M. J., Palermo, M., Perez, L., Pu, M., Ramsey, T., Sellers, W. R., Shultz, M. D., Stams, T., Towler, C., Wang, P., Williams, S. L., Zhang, J. H., and LaMarche, M. J. (2016) Allosteric Inhibition of SHP2: Identification of a Potent, Selective, and Orally Efficacious Phosphatase Inhibitor. *J Med Chem* 59, 7773-7782

15. Bottini, N., Musumeci, L., Alonso, A., Rahmouni, S., Nika, K., Rostamkhani, M., MacMurray, J., Meloni, G. F., Lucarelli, P., Pellechia, M., Eisenbarth, G. S., Comings, D., and Mustelin, T. (2004) A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nature Genetics*, 36, 337--338

16. Zheng, P., and Kissler, S. (2013) PTPN22 silencing in the NOD model indicates the type 1 diabetes-associated allele is not a loss-of-function variant. *Diabetes*, 62, 896--904

17. Elghzaly, A. A., Metwally, S. S., El-Chennawi, F. A., Elgayaar, M. A., Mosaad, Y. M.,
18. Kyogoku, C., Ortmann, W. A., Lee, A., Selby, S., Carlton, V. E. H., Chang, M., Ramos, P., Baechler, E. C., Batiwalla, F. M., Novitzke, J., Williams, A. H., Gillett, C., Rodine, P., Graham, R. R., Ardlie, K. G., Gaffney, P. M., Moser, K. L., Petri, M., Begovich, A. B., Gregersen, P. K., Langefeld, C. D., and Behrens, T. W. (2004) Genetic Association of the R620W Polymorphism of Protein Tyrosine Phosphatase PTPN22 with Human SLE. The American Journal of Human Genetics 75, 504–507

19. Seldin, M. F., Alkhairy, O. K., Lee, A. T., Lamb, J. A., Sussman, J., Pirskanen-Matell, R., Piehl, F., Verschuuren, J. J. G. M., Kostera-Pruszczczyk, A., Szczudlik, P., McKee, D., Maniaol, A. H., Harbo, H. F., Lie, B. A., Melms, A., Garchon, H.-J., Willcox, N., Gregersen, P. K., and Hammarstrom, L. (2015) Genome-wide Association Study of Late-Onset Myasthenia Gravis: Confirmation of TNFRSF11A, and Identification of ZBTB10 and Three Distinct HLA Associations. Molecular medicine (Cambridge, Mass.) 21, 1

20. Vandiedonck, C., Capdevielle, C., Giraud, M., Krumeich, S., Jais, J.-P., Eymard, B., Tranchant, C., Gajdos, P., and Garchon, H.-J. (2006) Association of the PTPN22*R620W polymorphism with autoimmune myasthenia gravis. Annals of Neurology 59, 404–407

21. Xiong, X., Xiang, M., Cheng, X., and Huang, Y. (2015) PTPN22 R620W Polymorphism is Associated with Myasthenia Gravis Risk: A Systematic Review and Meta-Analysis. Medical science monitor : international medical journal of experimental and clinical research 21, 2567–2571

22. Begovich, A. B., Carlton, V. E. H., Honigberg, L. A., Schrodi, S. J., Chokkalingam, A. P., Alexander, H. C., Ardlie, K. G., Huang, Q., Smith, A. M., Spoerke, J. M., Conn, M. T., Chang, M., Chang, S.-Y. P., Saiki, R. K., Catanese, J. J., Leong, D. U., Garcia, V. E., McAllister, L. B., Jeffery, D. A., Lee, A. T., Batiwalla, F., Remmers, E., Criswell, L. A., Seldin, M. F., Kastner, D. L., Amos, C. I., Sninsky, J. J., and Gregersen, P. K. (2004) A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. American journal of human genetics 75, 330–337

23. Ruiz-Noa, Y., Padilla-Gutirrez, J. R., Herrnandez-Bello, J., Palafoux-Sanchez, C. A., Valle, Y., Oregn-Romero, E., Pereira-Surez, A. L., Bernard-Medina, A. G., and Muoz-Valle, J. F. (2017) Association of PTPN22 Haplotypes (-1123G \textgreater C/+1858C \textgreater T) with Rheumatoid Arthritis in Western Mexican Population. International journal of genomics 2017, 8753498

24. Liu, J., Chen, M., Li, R., Yang, F., Shi, X., Zhu, L., Wang, H.-M., Yao, W., Liu, Q., Meng, F.-G., Sun, J.-P., Pang, Q., and Yu, X. (2012) Biochemical and functional studies of lymphoid-specific tyrosine phosphatase (Lyp) variants S201F and R266W. PloS one 7, e43631

25. Orr. (2009) A loss-of-function variant of PTPN22 is associated with reduced risk of systemic lupus erythematosus. Human molecular genetics 18, 569–579

26. Diaz-Gallo, L.-M., Espino-Paisn, L., Fransen, K., Gmez-Garca, M., van Sommeren, S., Cardea, C., Rodrigo, L., Mendoza, J. L., Taxonera, C., Nieto, A., Alcaín, G.,
Cueto, I., Lopez-Nevot, M. A., Bottini, N., Barclay, M. L., Crusius, J. B., van Bodegraven, A. A., Wijmenga, C., Ponsioen, C. Y., Geary, R. B., Roberts, R. L., Weersma, R. K., Urcelay, E., Merriman, T. R., Alizadeh, B. Z., and Martin, J. (2011) Differential association of two PTPN22 coding variants with Crohn’s disease and ulcerative colitis. Inflammatory bowel diseases 17, 2287--2294.

27. Rodriguez-Rodriguez, L., Taib, W. R. W., Topless, R., Steer, S., González-Escribano, M. F., Balsa, A., Pascual-Salcedo, D., González-Gay, M. A., Raya, E., Fernandez-Gutierrez, B., González-Ivars, I., Bottini, N., Witte, T., Viken, M. K., Coenen, M. J. H., van Riel, P. L. C. M., Franke, B., den Heijer, M., Radstake, T. R. D. J., Wordsworth, P., Lie, B. A., Merriman, T. R., and Martin, J. (2011) The PTPN22 R263Q polymorphism is a risk factor for rheumatoid arthritis in Caucasian case-control samples. Arthritis and rheumatism 63, 365--372.

28. Hou, X., Li, R., Li, K., Yu, X., Sun, J. P., and Fang, H. (2014) Fast identification of novel lymphoid tyrosine phosphatase inhibitors using target-ligand interaction-based virtual screening. Journal of Medicinal Chemistry 57, 9309--9322.

29. Vang, T., Liu, W. H., Delacroix, L., Wu, S., Vasile, S., Dahl, R., Yang, L., Musumeci, L., Francis, D., Landskn, J., Tasken, K., Tremblay, M. L., Lie, B. A., Page, R., Mustelin, T., Rahmouni, S., Rickert, R. C., and Tautz, L. (2012) LYP inhibits T-cell activation when dissociated from CSK. Nature chemical biology 8, 437--446.

30. Yu, X., Chen, M., Zhang, S., Yu, Z.-H., Sun, J.-P., Wang, L., Liu, S., Imasaki, T., Takagi, Y., and Zhang, Z.-Y. (2011) Substrate specificity of lymphoid-specific tyrosine phosphatase (Lyp) and identification of Src kinase-associated protein of 55 kDa homolog (SKAP-HOM) as a Lyp substrate. The Journal of biological chemistry 286, 30526--30534.

31. Jia, Z., Barford, D., Flint, A. J., and Tonks, N. K. (1995) Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B. Science (New York, N.Y.) 268, 1754--1758.

32. Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z.-Y., Dixon, J. E., and Saper, M. A. (1994) Crystal structure of Yersinia protein tyrosine phosphatase at 2.5 VAA and the complex with tungstate. Nature 370, 571--575.

33. Sun, J.-P., Wu, L., Fedorov, A. A., Almo, S. C., and Zhang, Z.-Y. (2003) Crystal structure of the Yersinia protein-tyrosine phosphatase YopH complexed with a specific small molecule inhibitor. The Journal of biological chemistry 278, 33392--33399.

34. Tsai, S. J., Sen, U., Zhao, L., Greenleaf, W. B., Dasgupta, J., Fiorillo, E., and Orr, G. (2009) Crystal structure of the human lymphoid tyrosine phosphatase catalytic domain: insights into redox regulation. Biochemistry 48, 4838--4845.

35. Barr, A. J., Ugochukwu, E., Lee, W. H., Mig, O. N. F., Filippakopoulos, P., Alfano, I., Savitsky, P., Burgess-Brown, N. A., Miller, S., and Knapp, S. (2009) Large-scale structural analysis of the classical human protein tyrosine phosphatome. Cell 136, 352--363.

36. Wang, H.-M., Xu, Y.-F., Ning, S.-L., Yang, D.-X., Li, Y., Du, Y.-J., Yang, F., Zhang, Y., Liang, N., Yao, W., Zhang, L.-L., Gu, L.-C., Gao, C.-J., Pang, Q., Chen, Y.-X., Xiao, K.-H., Ma, R., Yu, X., and Sun, J.-P. (2014) The catalytic region and PEST domain of PTPN18 distinctly regulate the HER2 phosphorylation and
ubiquitination barcodes. *Cell research* **2**, 1--24

37. Li, F., Shi, P., Li, J., Yang, F., Wang, T., Zhang, W., Gao, F., Ding, W., Li, D., Li, J., Xiong, Y., Sun, J., Gong, W., Tian, C., and Wang, J. (2013) A genetically encoded 19F NMR probe for tyrosine phosphorylation. *Angewandte Chemie (International ed. in English)* **52**, 3958--3962

38. Yang, F., Yu, X., Liu, C., Qu, C.-X., Gong, Z., Liu, H.-D., Li, F.-H., Wang, H.-M., He, D.-F., Yi, F., Song, C., Tian, C.-L., Xiao, K.-H., Wang, J.-Y., and Sun, J.-P. (2015) Phospho-selective mechanisms of arrestin conformations and functions revealed by unnatural amino acid incorporation and (19)F-NMR. *Nature communications* **6**, 8202

39. Latorraca, N. R., Venkatakriishnan, A. J., and Dror, R. O. (2017) GPCR Dynamics: Structures in Motion. *Chemical Reviews* **117**, 139--155

40. Zhang, H., and van Ingen, H. (2016) Isotope-labeling strategies for solution NMR studies of macromolecular assemblies. *Current Opinion in Structural Biology* **38**, 75--82

41. Perron, M. D., Chowdhury, S., Aubry, I., Purisima, E., Tremblay, M. L., and Saragovi, H. U. (2014) Allosteric noncompetitive small molecule selective inhibitors of CD45 tyrosine phosphatase suppress T-cell receptor signals and inflammation in vivo. *Molecular pharmacology* **85**, 553--563

42. Wang, H.-M., Xu, Y.-F., Ning, S.-L., Yang, D.-X., Li, Y., Du, Y.-J., Yang, F., Zhang, Y., Liang, N., Yao, W., Zhang, L.-L., Gu, L.-C., Gao, C.-J., Pang, Q., Chen, Y.-X., Xiao, K.-H., Ma, R., Yu, X., and Sun, J.-P. (2014) The catalytic region and PEST domain of PTPN18 distinctly regulate the HER2 phosphorylation and ubiquitination barcodes. *Cell research* **24**, 1067--1090

43. Li, R., Gong, Z., Pan, C., Xie, D.-D., Tang, J.-Y., Cui, M., Xu, Y.-F., Yao, W., Pang, Q., Xu, Z.-g., Li, M.-y., Yu, X., and Sun, J.-P. (2013) PPM1A functions as an ERK phosphatase. *FEBS Journal*, n/a--n/a

44. Li, R., Xie, D. D., Dong, J. H., Li, H., Li, K. S., Su, J., Chen, L. Z., Xu, Y. F., Wang, H. M., Gong, Z., Cui, G. Y., Yu, X., Wang, K., Yao, W., Xin, T., Li, M. Y., Xiao, K. H., An, X. F., Huo, Y., Xu, Z. G., Sun, J. P., and Pang, Q. (2014) Molecular mechanism of ERK dephosphorylation by striatal-enriched protein tyrosine phosphatase. *Journal of Neurochemistry* **128**, 315--329

45. Pan, C., Liu, H.-D., Gong, Z., Yu, X., Hou, X.-B., Xie, D.-D., Zhu, X.-B., Li, H.-W., Tang, J.-Y., Xu, Y.-F., Yu, J.-Q., Zhang, L.-Y., Fang, H., Xiao, K.-H., Chen, Y.-G., Wang, J.-Y., Pang, Q., Chen, W., and Sun, J.-P. (2013) Cadmium is a potent inhibitor of PPM phosphatases and targets the M1 binding site. *Scientific reports* **3**, 2333

46. Li, K.-S., Xiao, P., Zhang, D.-L., Hou, X.-B., Ge, L., Yang, D.-X., Liu, H.-d., He, D.-F., Chen, X., Han, K.-R., Song, X.-Y., Yu, X., Fang, H., and Sun, J.-P. (2015) Identification of para-Substituted Benzoic Acid Derivatives as Potent Inhibitors of the Protein Phosphatase Slingshot. *ChemMedChem* **230027**, 1980--1987

47. Dolinsky, T. J., Nielsen, J. E., McCammon, J. A., and Baker, N. A. (2004) PDB2PQR: An automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Research* **32**

48. Rooklin, D., Modell, A. E., Li, H., Berdan, V., Arora, P. S., and Zhang, Y. (2017) Targeting Unoccupied Surfaces on Protein-Protein Interfaces. *J Am Chem Soc* **139**, 15560-15563
49. Rooklin, D., Wang, C., Katigbak, J., Arora, P. S., and Zhang, Y. (2015) AlphaSpace: Fragment-Centric Topographical Mapping To Target Protein-Protein Interaction Interfaces. J Chem Inf Model 55, 1585-1599

50. Trott, O., and Olson, A. J. (2010) AutoDock Vina. J. Comput. Chem. 31, 445–461

51. Maier, J. A., Martinez, C., Kasavajhala, K., Wickstrom, L., Hauser, K. E., and Simmerling, C. (2015) ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. Journal of Chemical Theory and Computation 11, 3696–3713

52. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, G. E., Cheeseman, J. R., Scalmani, G., Barone, V., Mennucci, B., Petersson, G. A., Nakatsuji, H., Caricato, M., Li, X., Hratchian, H. P., Izmaylov, A. F., Bloino, J., Zheng, G., Sonnenberg, J. L., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Vreven, T., Montgomery, Jr., P. J., Ogliaro, F., Bearpark, M., Heyd, J. J., Brothers, E. K., K. N., Staroverov, V. N., Keith, T., Kobayashi, R., Normand, J. R., K., Rendell, A., Burant, J. C., Iyengar, S. S., Tomasi, J., C., M., Rega, N., Millam, J. M., Klene, M., Knox, J. E., Cross, J. B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R. E., Y., Austin, A. J., Cammi, R., Pomelli, C., Ochterski, J. W., M., R. L., Morokuma, K., Zakrzewski, V. G., Voth, G. A., Salvador, P., Dannenberg, J. J., Dauprich, S., Daniels, A. D., F., and O., Foresman, J. B., Ortiz, J. V., Cioslowski, J., Fox, D. J. (2009) Gaussian 09, Revision D01. Gaussian Inc. 10.1159/000348293

53. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Scalmani, G., Barone, V., Mennucci, B., Petersson, G. A., Nakatsuji, H., Caricato, M., Li, X., Hratchian, H. P., Izmaylov, A. F., Bloino, J., Zheng, G., Sonnenberg, J. L. (2009) Gaussian 09, Revision A02. Inc. Wallingford, CT

54. Bayly, C. C. I., Cieplak, P., Cornell, W. D., and Kollman, P. a. (1993) A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges: the RESP model. The Journal of Physical 10ds 97, 10269–10280

55. Cieplak, P., Cornell, W. D., Bayly, C., and Kollman, P. A. (1995) Application of the multimolecule and multiconformational RESP methodology to biopolymers: Charge derivation for DNA, RNA, and proteins. Journal of Computational Chemistry 16, 1357–1377

56. Darden, T., York, D., and Pedersen, L. (1993) Particle mesh Ewald: An N-log(N) method for Ewald sums in large systems. The Journal of Chemical Physics 98, 10089

57. Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., and Pedersen, L. G. (1995) A smooth particle mesh Ewald method. J Chem Phys 103, 8577--8593

58. Miyamoto, S., and Kollman, P. A. (1992) Settle: An analytical version of the SHAKE and RATTLE algorithm for rigid water models. Journal of Computational Chemistry 13, 952–962

59. Berendsen, H. J. C., Postma, J. P. M., van Gunsteren, W. F., DiNola, a., and Haak, J. R. (1984) Molecular dynamics with coupling to an external bath. The Journal
60. Hou, T., Wang, J., Li, Y., and Wang, W. (2011) Assessing the performance of the MM/PBSA and MM/GBSA methods. I. The accuracy of binding free energy calculations based on molecular dynamics simulations. *J Chem Inf Model* **51**, 69-82

61. Hou, T., Wang, J., Li, Y., and Wang, W. (2011) Assessing the performance of the molecular mechanics/Poisson Boltzmann surface area and molecular mechanics/generalized Born surface area methods. II. The accuracy of ranking poses generated from docking. *J Comput Chem* **32**, 866-877

62. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera—A Visualization System for Exploratory Research and Analysis. *J Comput Chem* **25**, 1605--1612

FOOTNOTES

The abbreviations used are: PTP, protein tyrosine phosphatases; LYP, lymphoid-specific tyrosine phosphatase; TCR, T-cell receptor; F2Y, 2-fluorine-tyrosine; pNPP, p-nitrophenyl phosphate
Table 1 The selectivity of NC1 against a panel of protein phosphatases

| Enzymes | Ki, µM   | Ratio of selectivity | Inhibition kinetics |
|---------|----------|----------------------|---------------------|
| LYP     | 4.3±0.3  | 1                    | Non-competitive     |
| PTP1B   | 8±0.6    | 1.9                  | Competitive         |
| VHR     | 9.1±0.9  | 2.1                  | Competitive         |
| STEP    | 10.1±1.2 | 2.3                  | Competitive         |
| N18     | 11.8±1.3 | 2.7                  | Competitive         |
| Glepp   | 15.2±1.2 | 3.5                  | Competitive         |
| Slingshot2 | >100   | >21.5                | -                   |
| PPM1A   | >100     | >21.5                | -                   |
| PPM1G   | >100     | >21.5                | -                   |
| PP1     | >100     | >21.5                | -                   |

All measurements were made by using pNPP as a substrate at pH 7.0, 25°C, and ionic strength of 0.15 M. For all statistical analyses, data from at least three independent experiments were quantified and presented as the mean ± SD.

Figure 1
The identification of a new non-competitive LYP inhibitor. (A) A “ring-opening” strategy based on our previously reported competitive LYP inhibitors (A15 analogs) was used to identify new LYP inhibitors. (B) The chemical structure of compound NC1. (C) A kinetic study of the inhibition mode of NC1 towards LYP. The pNPP concentrations used were 1.17, 1.75, 2.63, 3.95, 5.93, 8.89, 13.33, and 20 mM. Lineweaver-Burk plots displayed a characteristic pattern of intersecting lines, which indicates non-competitive inhibition.
Figure 2

The effect of NC1 on anti-CD3 antibody-stimulated TCR signaling in Jurkat T cells. (A) The effects of NC1 on the anti-CD3 (OKT3)-induced phosphorylation of ERK (pT202 and pY204) and LCK pY394 in control siRNA treated T cells or LYP-siRNA treated T cells. A representative Western blot selected from at least three independent experiments is shown. The GAPDH level was used as a control. (B-C) Statistical analysis of the phosphorylation of LCK Y394 (B) and of ERK (C) in T cells pre-incubated with NC1. Statistical comparisons between two groups were performed with Student t tests. *: p<0.05 when the anti-CD3 antibody-treated cells were compared with the untreated cells. Statistical comparisons among the anti-CD3 treated groups were performed with two-way ANOVA analysis. Difference between NC1 groups and con groups was significant (p< 0.001); Difference between siRNA treated groups and siRNA untreated groups was significant (p< 0.001); the interaction between these two factors was significant (p< 0.005). For all statistical analyses, data from at least three independent experiments were quantified and presented as the mean ± SD (error bars).
Figure 3

Mutagenesis and sequence alignment reveal a potential unique molecular mechanism underlying the non-competitive inhibition of LYP by NC1. (A) Structural representation of the locations of the selected mutations on the surface surrounding the active site of LYP, which may be involved in NC1-LYP interactions (pdb: 2QCJ). (B) The $K_i$ values of NC1 toward wild-type LYP and a panel of selected mutants. (C) Structure-based sequence alignment of LYP mutations with more than 1.5-fold $K_i$ values from different species together with other PTP members, including PTPN18, MEG1, MEG2, TCPTP, STEP and HePTP. Residues located in the yellow background indicate mutations with more than 1.5-fold $K_i$ values. Residues different from human LYP are colored in red. For all statistical analyses, data from at least three independent experiments were quantified and presented as the mean ± SD.
Figure 4
Molecular docking and MD simulation analyses reveal the molecular mechanism underlying the non-competitive inhibition of LYP by NC1. (A) Pocket analysis of predicted binding mode of NC1 to pNPP-bound LYP using representative MD snapshot. The WPD pocket (colored in blue) and Secondary pocket (colored in green) are represented as transparent surface and spheres. Compound NC1 was represented as orange stick and surrounding residues were represented as white sticks. (B) Individual residue contribution to the binding of compound NC1 with LYP. Data were calculated by the MM/GBSA binding free energy decomposition analysis. (C) Calculated occupied spaces of compound NC1 in WPD pocket and Secondary pocket during MD simulations.

Figure 5
MD simulations of LYP-pNPP systems with or without NC1 bound reveal the details of NC1 inhibition.
(A) Comparison of the distance between the catalytic residue D195 and the substrate pNPP during MD simulations of LYP-pNPP systems with or without NC1 bound. (B) Representative MD snapshot of LYP-pNPP system. (C) Representative MD snapshot of LYP- pNPP -NC1 system.

Figure 6

Comparison of the potential allosteric pockets in LYP with atypical-open WPD-loop (A, PDB: 3H2X), VHR with closed WPD-loop (B, PDB: 1J4X), PTP1B with open WPD-loop (C, PDB: 2HNP), STEP with open WPD-loop (D, PDB: 2CJT), PTPN18 with open WPD-loop (E, PDB: 2OC3), Glepp with open WPD-loop (F, PDB: 2GJT). The proteins were presented in transparent white surface with WPD-loop shown as red loop and substrate pNPP shown as yellow sticks. The predicted binding pose of NC1 was derived from representative MD simulation snapshot in Figure 5A and shown as green stick. Fragment-centric topographic mapping was performed using AlphaSpace. Good pockets (pocket score > 100) were presented with green sphere and auxiliary pockets (30 < pocket score < 100) were presented with blue sphere. Potential allosteric inhibitor binding pockets, which possess a series connected small pockets, were marked with yellow cycle.
Figure 7

$^{19}$F-NMR spectroscopy reveals suppression of WPD-loop conformational changes by NC1. (A) Crystal structures of LYP showing conformational changes in the WPD-loop and its adjacent residues with or without substrate. Left: Residue L281 is “buried” by the WPD-loop (PDB code: 2P6X). Right: Residue L281 is “exposed” after substrate binding (PDB code: 2QCJ). (B) Schematic flowchart of the incorporation of F2Y into LYP at position 281. (C) The purity of the protein was determined by electrophoresis (left panel). The purified protein was subjected to trypsin digestion and analyzed by MS/MS, which indicated the presence of the $y_{12}$± F2Y-V-Y-N-A-V-L-E-L-F-K-R fragment (MW 1550) and the $y_{13}$± E-F2Y-V-Y-N-A-V-L-E-L-F-K-R fragment (MW 1679). These results confirmed that F2Y...
was specifically incorporated into LYP at position 281, m/z, mass/charge ratio. (D) An upfield shift was observed in the $^{19}$F-NMR spectrum of the LYP L281F2Y $^{19}$F-NMR probe in response to Na$_3$VO$_4$ binding (upper panel). The $^{19}$F-NMR spectrum of the LYP-L281F2Y probe in response to Na$_3$VO$_4$ binding after pre-incubation with compound NC1 (lower panel).
