Methods 65 (2014) 219–228

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Integrating virtual and biochemical screening for protein tyrosine phosphatase inhibitor discovery

Katie R. Martin [a], Pooja Narang [b], José L. Medina-Franco [b], Nathalie Meurice [b], Jeffrey P. MacKeigan [a,*]

[a] Laboratory of Systems Biology, Van Andel Research Institute, Grand Rapids, MI, United States
[b] Mayo Clinic, Scottsdale, AZ, United States

ARTICLE INFO

Article history:
Available online 20 August 2013

Keywords:
Protein tyrosine phosphatase
PTP
PTPσ
Inhibitor
Virtual screen
Small molecule

ABSTRACT

Protein tyrosine phosphatases (PTPs) represent an important class of enzymes that mediate signal transduction and control diverse aspects of cell behavior. The importance of their activity is exemplified by their significant contribution to disease etiology with over half of all human PTP genes implicated in at least one disease. Small molecule inhibitors targeting individual PTPs are important biological tools, and are needed to fully characterize the function of these enzymes. Moreover, potent and selective PTP inhibitors hold the promise to transform the treatment of many diseases. While numerous methods exist to develop PTP-directed small molecules, we have found that complimentary use of both virtual (*in silico*) and biochemical (*in vitro*) screening approaches expedite compound identification and drug development. Here, we summarize methods pertinent to our work and others. Focusing on specific challenges and successes we have experienced, we discuss the considerable caution that must be taken to avoid enrichment of inhibitors that function by non-selective oxidation. We also discuss the utility of using "open" PTP structures to identify active-site directed compounds, a rather unconventional choice for virtual screening. When integrated closely, virtual and biochemical screening can be used in a productive workflow to identify small molecules targeting PTPs.

1. Introduction

1.1. The PTP family

Estimates predict that 30% of the human proteome is subject to phosphorylation [1]. While tyrosine phosphorylation constitutes <0.1% of total phosphorylation in mammalian cells, it represents a critical regulatory mechanism in signal transduction. Balanced signaling is achieved through the exquisite coordination of protein tyrosine kinases (PTKs) and phosphatases (PTPs), which catalyze the phosphorylation and dephosphorylation of diverse substrates, respectively [2,3]. While the active role of PTKs in signaling has long been accepted, PTPs were originally associated with housekeeping functions and their active and direct role in signaling was initially underappreciated [4]. This connotation has since been dismissed by decades of research revealing dynamic regulation, substrate specificity, and activity of the large family of PTPs.

The PTP superfamily consists of over 100 enzymes that can be classified by catalytic mechanism, substrate specificity, and sequence similarity. There are 38 members of the classic PTP family, which harbor strict specificity for tyrosine residues [5]. These enzymes are typified by deep and narrow catalytic grooves that accommodate large aromatic phosphotyrosine rings, while occluding shorter phosphoserine or threonine residues from the base of the active site [6]. Catalysis is initiated when the phosphate group of a substrate extends to the base of the active site and is attacked by a nucleophilic cysteine within a conserved phosphate-binding loop, or P-loop (hallmarked by a H/V-CX_5R-S/T motif). Binding is accompanied by closure of a flexible WPD loop (named for conserved tryptophan, proline, and aspartic acid residues) around the substrate, positioning the invariant aspartate to protonate the oxygen leaving group of the tyrosyl substrate. Finally, a conserved Q-loop coordinates a water molecule and the aspartate of the WPD loop, which catalyze the scission of the phospho-enzyme intermediate complex. This restores the enzyme to its initial state, while producing a free phosphate group and a dephosphorylated

Abbreviations: PTP, protein tyrosine phosphatase; RCC, redox cycling compounds; ROS, reactive oxygen species; HTS, high-throughput screen; PDB, protein data bank; pNPP, para-nitrophenyl phosphate; VS, virtual screening; DTT, dithiothreitol; pTyr, phosphotyrosine.

* Corresponding author.
E-mail address: jeff.mackeigan@vai.org (J.P. MacKeigan).

1046-2023
http://dx.doi.org/10.1016/j.ymeth.2013.08.013

substrate [7]. Despite sharing this highly conserved mechanism, PTPs show specificity for a diverse set of substrates that regulate a wide variety of cellular and molecular pathways.

When the normal function of PTPs are dysregulated (by altered expression or activity), they contribute to the aberrant signaling that drives pathological phenotypes of many human diseases [2–4]. In fact, roughly half of all PTP genes have been implicated in at least one human disease to date [1]. The most widely studied PTP in the context of human disease is PTP1B (*PTPN1*), the first member of this class to be purified and characterized [8,9]. Loss of PTP1B promotes insulin and leptin signaling, and has been shown to combat diabetes and obesity in animal models [10–13]. In addition, mutations and SNPs involving PTP1B have been linked to type 2 diabetes [14–18]. In total, over 20 PTPs have been associated with hereditary human diseases, notably SHP2 (*PTPN11*) which is mutated in both Noonan and Leopard syndromes [19,20]. Variants in Lyp phosphatase (*PTPN22*) have been strongly linked to autoimmune diseases, including type I diabetes, rheumatoid arthritis, Graves' disease, and systemic lupus erythematosus [21,22]. Furthermore, PTPs have been linked extensively to cancer, including the identification of 22 PTPs in chromosomal regions frequently amplified or deleted in cancer [23]. Point mutations and epigenetic silencing have also been found to alter PTP expression and activity in numerous cancer types (reviewed in [24]). While many alterations are consistent with tumor suppressive functions, for example those associated with PTEN and PTPδ (*PTPRD*), PTPs that promote cancer initiation and progression have also been discovered [24]. Of note, oncogenic mutations drive SHP2 activation in many hematological cancers, as well as breast cancer and neuroblastoma [25,26]. PRL3 (*PTP4A3*) has been found overexpressed in colorectal cancer metastases, an observation attributed to the positive regulation of cell motility and invasion by the PRL phosphatases [24,27–29]. Collectively, PTPs with positive roles in cancer and other diseases have garnered particular interest as drug targets, with inhibitors to PTP1B, SHP2, Lyp, and others being actively pursued [30–32].

In addition to the dysregulation of PTPs in disease, their physiological functions can also make them attractive as therapeutic targets. An important example of interest to us is PTPσ (*PTPRS*) [33,34]. PTPσ is enriched in the brain where it controls axon guidance and neurite outgrowth [35–38]. Recently, PTPσ was shown to interact with chondroitin sulfate proteoglycans (CSPGs), released at the site of spinal cord injury, and profoundly suppress neural regeneration [39,40]. Thus, the ability to therapeutically block this PTPσ-mediated activity has the potential to improve recovery following spinal cord, and other nervous system injuries.

Taken together, PTPs serve critical functions in normal physiology and actively drive disease phenotypes when their activity or expression is altered. As such, they represent important molecular targets for basic research and drug development. Selectively targeted small molecules are essential tools to interrogate the function of individual PTPs, offering unique advantages of reversibility (in the case of competitive inhibitors), as well as temporal and dose control. In addition to their use as biological tools, small molecules directed to PTPs hold considerable promise as potential therapeutics and innovative approaches chemically targeting phosphatases would certainly unlock a critical class of enzymes for disease modification.

1.2. Challenges in chemical targeting of PTPs

Confounding the fact that PTPs have not been always been accepted as drivers of cell signaling and disease, molecular targeting of these enzymes has been slowed by their perceived "undruggability" [41,42]. First, the functional role of PTPs poses a challenge for drug targeting. Within a single pathway, multiple PTPs may serve important roles, so targeting just one may not elicit the desired effect. Conversely, a single PTP may serve several distinct functions in complex signaling networks. In this case, selectively targeting a single enzyme may elicit several off target and possibly undesired cellular consequences.

Second, and more problematic, there are several chemical properties of PTPs that render drug targeting extremely challenging. The PTP family is characterized by an exceptionally high degree of sequence conservation across their active sites. This sequence similarity accompanies several highly conserved physical domains in and surrounding the active site [5]. This common sequence and structure makes building selectivity into small molecules quite challenging. In addition, the chemical environment of the active site, which so elegantly permits phosphatase activity, has impaired drug discovery efforts. The PTP active site is positively charged, which facilitates its interaction with phosphotyrosine substrates. Unfortunately, this environment also attracts negatively charged molecules with high affinity in drug screening initiatives. Generally, such polar compounds represent undesirable drugs owing to their poor membrane permeability and limited oral bioavailability [31]. In addition, PTP active sites must be maintained in a reduced state to preserve the catalytic activity of the nucleophilic cysteine residue. Consequently, they are extremely susceptible to oxidation. Molecules that support oxidation, such as redox cycling compounds (RCCs), are commonly identified in drug screening initiatives [43]. Because these oxidizing agents will elicit pleiotropic effects on many targets and cellular processes, they do not represent promising selective PTP-directed compounds.

Despite these issues, the important biological and disease roles that PTPs play provides rationale to pursue drug discovery initiatives. In this report, we discuss the integration of both biochemical and *in silico*, or virtual, screening approaches to develop PTP-directed inhibitors. We focus on our recent identification of small molecule inhibitors of PTPσ, highlighting challenges and considerations that arose from that work, while reviewing related efforts for other PTPs. While diverse targeting approaches exist, we specifically discuss methods to find active-site directed small molecules predicted to function as competitive inhibitors.

2. Virtual and biochemical approaches to identify PTP inhibitors

A number of useful methods are available to develop small molecules directed to PTPs (Fig. 1) [41,44]. Most strategies can be classified into one of the following: (1) rational design of inhibitors from substrate-like molecules or molecules with known activity against PTPs; and (2) broad screen of chemical libraries to identify scaffolds that bind and inhibit a PTP of interest. For the former, a substrate-like template mimicking phosphotyrosine [45–49], or a molecule previously shown to bind and inhibit the PTP active site [50–55], is used as a non-selective template while potency and selectivity are improved through chemical modification. A useful resource for this type of initiative is the human phosphatase-substrate network recently developed by Li et al. [56]. In this work, phosphatases have been classified according to their structures and information about known substrates and functions collated. This can aid in the identification of substrate-based templates for chemical development, as well as identify closely and distantly related phosphatases for selectivity evaluation. In this type of approach, small collections of chemicals sharing similarities with these templates can be designed and screened *in silico* or *in vitro* [45,48–51,53,54]. Additionally, when target structures are available, molecular docking studies can be used to rationally design lead molecules with desired properties [46,47,55,57–59]. This type of methodology has been effective in the iterative improvement of inhibitors of PTP1B, YopH, Cdc25, and others.

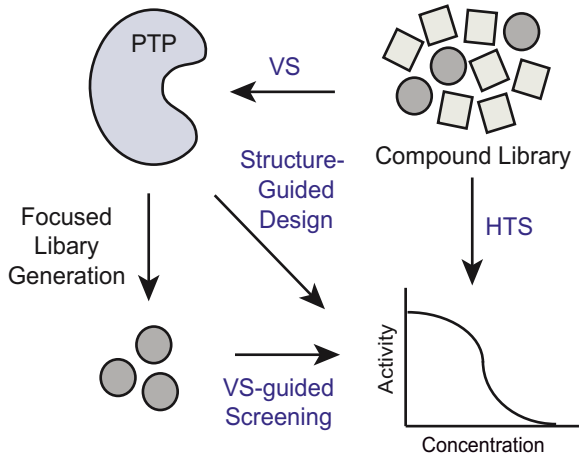

Fig. 1. Common approaches to identify PTP-directed small molecules. A traditional high-throughput screen can be performed using a large chemical library and*in vitro* phosphatase assays. To create a target-focused library of smaller size prior to screening *in vitro*, a virtual screen (VS) can be completed by molecular docking chemicals of a large library into the active site of a PTP. As an alternative or complement to screening, structure-guided design can be used to analyze the structure of a PTP and engineer compounds to form molecular interactions of interest.

The second approach, which we discuss here, includes screening platforms used to identify molecules that are predicted to bind the PTP active site *in silico* (virtual screen) or that inhibit PTP activity *in vitro* (biochemical screen). Importantly, both of these screening methods can be utilized together via an iterative screening approach to most efficiently identify, refine and filter lead molecules. A general discussion of the integration of *in silico* screen with other experimental techniques for accelerated drug discovery has been discussed elsewhere [60].

2.1. Virtual screening (VS)

In silico or virtual screening (VS) is an innovative approach to guide the identification of novel bioactive molecules, usually from combining large chemical libraries with computational methods [61]. VS methods can be divided into two major groups, namely ligand-based and structure-based. Ligand-based approaches utilize structure–activity data from a set of known actives in order to identify candidate compounds for experimental evaluation, while structure-based approaches leverage the three-dimensional structure of a target. Although VS is under constant development and improvement, successful cases of VS are frequently reported in the literature. Comprehensive reviews of VS including methods, successful applications, pitfalls, and workarounds are published elsewhere [61–63].

One of the most common structure-based strategies used today is (high-throughput) molecular docking [64]. Docking compound libraries to select hit compounds for experimental evaluation involves several preparation steps that can be grouped in two broad categories: (a) preparation of the target receptor, and (b) preparation of the structures in the compound library. Both steps are detailed elsewhere [65,66], and briefly outlined below:

(a) *The target receptor structure* (experimental structure or computational model) is prepared by adding any missing atoms, including hydrogen, and optimizing the structure [67]. If a structure of receptor-ligand complex is available, the ligand binding site may be used for definition of the docking binding pocket. In cases where no information is available for the binding site, structural knowledge of related proteins and/or available biological data can be used to locate the binding site. Several computer programs like SiteFinder (MOE) [68], SiteMap (Schrödinger) [69] and Q-SiteFinder [70] can also be used to predict the binding site topology.

(b) *The compound library* is prepared by assigning proper tautomeric, stereoisomeric and protonation states, and assigning proper charges to each ligand in the library [67]. Typically, compounds are considered flexible and several plausible conformations of each are sampled before or during the docking process. Filtering of compounds with potential problems of toxicity or with undesirable absorption, distribution, metabolism and excretion (ADME) properties can be applied to reduce the number of compounds with utility for docking purposes in therapeutic applications.

After the preparation steps are completed, the prepared computational compound library is molecularly oriented into the binding site usually with automated docking algorithms that predict the preferred orientation of the ligand in the binding site by modeling ligand–receptor interactions. Examples of widely used docking programs include ICM, DOCK, GLIDE, and FlexX [69,71,72]. A mathematical equation, also known as scoring function [73], is then used to evaluate the strength of interactions of the docked molecules and reported as a numerical binding score. The compounds are ranked on the basis of these scores and top scoring compounds are selected as virtual hits for subsequent and extensive experimental validation. In-depth reviews of the theory and practice of the docking and scoring are published elsewhere [65,66].

2.1.1. Validating and benchmarking a PTP template structure

Since three-dimensional structures of PTPs are available, structure-based approaches such as docking are an attractive avenue to conduct VS of chemical libraries to select compounds for biochemical screening. Arguably one of the most critical determinants of VS success is the quality of the receptor template. Fortunately, increased research attention to PTPs over recent years has led to the generation of many high-quality X-ray crystal structures of active sites. Notably, a resource has compiled and analyzed crystal structures from 22 members of the PTP superfamily, including 16 new structures [74]. Crystal structures are exquisite receptor templates for VS as they represent direct physical models of PTPs. However, further receptor sampling may be needed as X-ray structures only capture one state of the protein geometry. An alternative and commonly used approach is to construct a homology model using the PTP sequence of interest and the known structure of a closely related PTP utilized as the template.

For any structure, it is important to establish its quality as a receptor for docking purposes. In addition to general crystal structure criteria that should be met (e.g., sufficient resolution and completeness), the structure should prove its ability to dock known substrates or ligands. To prepare for a PTPσ-targeted VS, we extracted a phosphotyrosine peptide from a CD45-phosphotyrosine co-crystal and used ICM to dock it into the PTPσ active site [75]. We found that the phosphotyrosine peptide was structurally amenable, and thus efficiently docked into the active site, in a manner similar to that of the co-crystal and observed in a parallel docking with PTP1B. Specifically, we confirmed that the tyrosyl phosphate group extended to the base of the active site and made predicted interactions with conserved residues of the P-loop [75]. To further validate the receptor selected for VS, an analysis known as benchmarking can be completed. Here, a known ligand (in the case of PTPs, a phosphotyrosine peptide or established active site compound) is recovered from a decoy set of drug-like molecules by virtual screening [76]. A quality receptor will bind the known ligand efficiently, producing this molecule as a top hit in this exercise. In addition to further authenticating the template, benchmarking

allows the validation of the docking workflow, algorithm choice, and the optimization of docking parameters for the actual VS.

2.1.2. Using "Open" PTP structures in VS

Two primary conformations of PTP active sites can be found in crystal structures – open or closed. Open conformations are typically associated with apo enzymes that are not engaged in substrate interaction or catalysis. In contrast, closed receptors are characterized by a shift in the flexible WPD loop over a substrate or ligand as part of the catalytic mechanism. It is generally recommended, and typically followed, that the closed conformation of enzymes be used for VS [41]. This is because the closed conformation is considered a representation of the active pocket and permits interactions with all residues, including those of the closed WPD loop. However, challenging this straightforward interpretation of open and closed receptors has been the crystallization of apo PTPs in closed conformation, and open PTPs with molecules bound in the active site [74,77,78]. Accordingly, while the use of closed conformations is valid, and has indeed produced inhibitors to several PTPs, we rationalize that the open conformation could represent the unoccupied enzyme as encountered by an active-site directed inhibitor. Thus, to identify small molecule inhibitors of PTPσ, we concluded that it would be more advantageous to target this inactive receptor configuration.

To test this, we performed a virtual screen using the available apo crystal structure of the PTPσ active site (PDB 2FH7), specifically, the D1 domain that harbors phosphatase activity. The successful docking of a phosphotyrosine peptide provided further indication that this receptor configuration would be useful for library design. Indeed, we found molecules that bind with high affinity into the active site pocket and confer inhibition *in vitro* [75]. As further support for this open receptor approach, Park et al. performed a VS and biochemical screen using the same open PTPσ crystal structure and identified a series of potent inhibitory small molecules as well [79].

Of interest to our study, Wu and colleagues explored docking of both an open and closed conformation of the Lyp phosphatase (*PTPN22*) [80]. In this inhibitor study, they identified virtual hits with both templates that were largely non-overlapping, suggesting unique accommodation by structurally distinct molecules into each conformation. Hits from both VS were experimentally confirmed to represent active inhibitors of Lyp. Intriguingly, they discovered that the lead actives identified using the open template are predicted to form interactions with the WPD loop in the open position. The authors hypothesized that such compounds function by stabilizing the PTP active site in the open conformation, inhibiting WPD loop closure and consequently catalysis [80].

In light of this hypothesis, we performed a careful binding analysis of our lead molecule, compound 36, with the open conformation of the PTPσ active site. Indeed, we discovered that compound 36 forms interactions with the P-loop at the base of the active site as well as two residues of the open WPD loop, consistent with the open Lyp hits (Fig. 2A). Also important, the model predicts several interactions with the Q-loop, a conserved motif in the active site pocket. The Q-loop contains a glutamine residue (Gln1637 in PTPσ) that positions an essential water molecule to catalyze cleavage of the phosphoenzyme intermediate, completing the dephosphorylation reaction [7]. Compound 36 is predicted to form a hydrogen bond with Gln1637, suggesting this interaction may be disruptive to catalysis and partially responsible for inhibition (Fig. 2B). Taken together, these and other studies demonstrate the potential of open PTP structure conformations in the development of inhibitors. In fact, such compounds may offer unique properties of inhibition compared to those found from closed receptor docking.

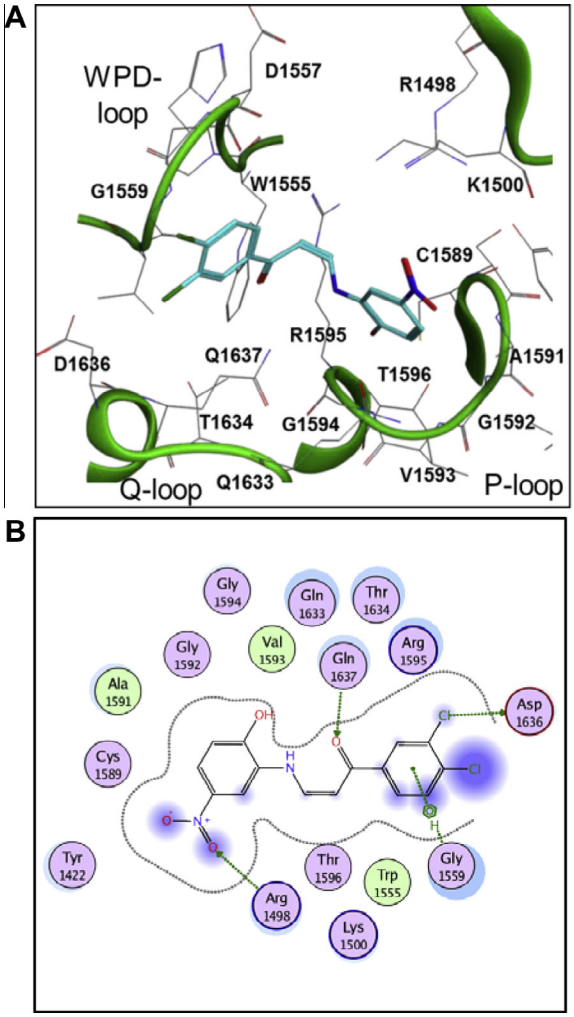

Fig. 2. A lead compound interacts with the open conformation of the PTPσ D1 active site. (A) Three important motifs and the interacting residues around the binding site are highlighted. PTPσ is represented by ribbon diagram in green, and the side chain of interacting residues are represented in grey. (B) Ligand interaction diagram of compound 36 docked into the active site of PTPσ, as generated by MOE v2011. Light green circles, hydrophobic residues; purple circles, polar residues; purple/blue circles, basic residues; purple/red circles, acidic residues; green arrows, side-chain donor H bonds; blue spheres, ligand exposure; cyan spheres, receptor exposure.

2.1.3. Promoting diversity of virtual hits

In general, small molecule hits are selected from a docking-based virtual screen as those compounds with the lowest predicted binding free energies, as defined by the docking algorithm scoring function. While the specific threshold for hit selection can vary depending on experimental bandwidth, it is critical to consider chemical diversity in this selection process. For example, we screened over 1 million compounds *in silico* and chose the top 200 compounds with the lowest binding scores as candidate inhibitors [75]. We further filtered these molecules for structural diversity using clustering analyses in MolSoft (ICM) and found that 66 unique scaffold families were represented. While all top ranked compound hits could be screened, choosing representative and structurally diverse structures from major chemical classes reduces unnecessary redundancy and promotes a diverse subset library for screening *in vitro*.

Selection of diverse compounds strongly depends on the structural representation of the molecules. Usually, there are three major types of different yet complementary representations, namely (a) physicochemical properties including drug-like descriptors,

(b) molecular scaffolds, and (c) fingerprints. Each of these methods has its own advantages and disadvantages as outlined below [81]:

(a) *Whole molecule properties* have the advantage of being intuitive and straightforward to interpret. Indeed, the drug-like and lead-like criteria, and "rule of 3" for fragment-based lead-discovery have been formulated using only physicochemical properties. A disadvantage of this representation is that physicochemical properties do not provide information regarding the sub-structural patterns and molecules with different chemical structures can have the same or similar physicochemical properties.
(b) *Molecular scaffolds or chemotypes*, similar to physicochemical descriptors, are straightforward to interpret and facilitate communication with experimental groups. For example, scaffold analysis has led to concepts that are widely used in medicinal chemistry and drug discovery, such as "scaffold hopping" and "privileged structures". One of the disadvantages of the scaffold analysis is the lack of information regarding structural similarity contributed by the side chains and the inherent similarity or dissimilarity of the scaffolds themselves. A straightforward solution is the analysis not only of the molecular frameworks but also the side chains, functional groups and other sub-structural analysis strategies.
(c) *Molecular fingerprints* are continuously applied to a number of chemoinformatic and computer-aided drug design applications. A disadvantage of some fingerprints is that they are more difficult to interpret. Also, it is well known that chemical space will depend on the types of fingerprints used. To meliorate these problems, the use of multiple structural fingerprints for structure–activity relationship studies [82] and combination of structural representations for structural diversity analysis have been implemented [81,83].

A number of compound databases from different sources can be used in VS [84]. The type of screening library utilized should be closely related with the objective of the specific screening campaign [85]. Chemically diverse libraries, such as those generated with diversity-oriented synthesis [86], are particular attractive for identifying novel scaffolds for relatively unexplored targets. If the goal of the screening is directed to a specific target family, one may use target-oriented synthesis [87], focused, or targeted libraries [88]. If the goal is lead optimization, chemical libraries with high intermolecular similarity are an attractive source.

Screening a diverse library improves the success rates of high-throughput screening, especially when it is performed against new targets. The goal is to improve the chemical content without significantly increasing the size of compound libraries [89]. Several cheminformatics approaches utilizing molecular fingerprints, scaffold-based, and graph-based representations have been used to represent the diversity of chemical space [90]. Large databases are pre-filtered to create smaller databases that capture the diversity of the entire set. Comprehensive description of molecules often leads to multidimensional data describing molecular spaces, which requires advanced data mining tools and visualization techniques for quantitative evaluation [91]. Unexplored regions of chemical space that have biological relevance can be visualized with techniques such as principal component analysis (PCA), self-organizing maps, multi-fusion similarity maps, and scaffold trees [89]. Selection of diverse subsets is usually performed by selecting a smaller, representative ensemble of compounds by using methods such as cluster-based [92] or dissimilarity-based selection [93]. Overall, molecular diversity provides a rational framework for removing redundant molecules and increasing the chance of identifying new leads.

2.2. Biochemical screening

2.2.1. In Vitro phosphatase assays

A high-quality virtual screen will generate a focused, yet diverse library of molecules covering a target-oriented chemical space. These hits must then be experimentally tested and ranked using a biochemical screening platform [41,44]. The basic chemical reaction catalyzed by a phosphatase converts a phosphosubstrate into a dephosphorylated product and free phosphate. The amount of free phosphate released or the generation of dephosphorylated product can be measured as a surrogate for phosphatase activity *in vitro*. Physiological substrates of most PTPs are not known, and thus a relevant phosphorylated peptide is difficult to predict. Therefore, many universal PTP substrates with colorimetric or fluorescent readouts are available and frequently used to assess PTP activity, especially in the context of screening.

One of the most widely used phosphatase substrates is pNPP (*para*-nitrophenyl phosphate), a chromogenic substrate analog suitable for most phosphatases. The phosphatase reaction yields free phosphate as well as *para*-nitrophenol, which becomes deprotonated to *para*-nitrophenolate (pNP) under alkaline conditions (i.e., with addition of sodium hydroxide stop solution). pNP is intense yellow in color and can be measured at 405 nm using a spectrophotometer. The pNPP assay is robust, has incredibly low background signal, and shows a large dynamic range (5–500 μM), making it useful for a variety of enzymes [41]. Importantly for screening initiatives, pNPP is an economical reagent and amenable to high-throughput screening (HTS) applications. However, pNPP is a small and very generic pTyr-like substrate, containing only a single phosphorylated nitrophenyl ring. Some enzymes may prefer a more natural peptide with residues flanking the phosphoryl group, which have been shown to be important for phosphatase activity [94]. Also, it is important to note that many compounds appear yellow in solution and will interfere with the pNP absorbance. To avoid yellow compounds being identified as false negative inhibitors (since their yellow solution will mimic the phosphatase activity readout), compound-only samples (all reaction components minus the PTP enzyme) should be measured [41]. In the case of yellow compounds, and as a secondary validation to a primary pNPP assay, alternative phosphatase assays based on fluorescence or malachite green absorbance could be incorporated [41,42].

As an alternative to the measurement of dephosphorylated product, malachite green assays measure the concentration of free phosphates produced as a byproduct of the phosphatase reaction. Free orthophosphates are sequestered by malachite green molybdate, producing a colorimetric emission between 620 and 640 nm that directly correlates with free phosphate concentration, and serves as a proxy for phosphatase activity [41]. The versatile compatibility of this assay with any phosphosubstrate (e.g., pNPP, pTyr-peptide) makes it an attractive option. However, the ease of phosphate contamination represents a considerable shortcoming of this assay, creating the potential for high and variable background signal. We have found that common culprits for free phosphate contamination are bacterial protein preparations and synthetic phosphosubstrate reagents. Care must be taken to ensure all reagents are phosphate-free in order to minimize background signal and preserve the dynamic range of the assay. Finally, certain buffer components are incompatible with malachite green and should be avoided [41]. When these considerations are taken into account, this assay still provides a versatile and relatively economical HTS platform.

Finally, fluorescent PTP assays have been developed using phospho-substrates that do not fluoresce until phosphates are removed, including fluorescein diphosphate (FDP), 3-*O*-methyl fluorescein phosphate (OMFP), and 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP). These substrates can be tested with a PTP

of interest and similar to pNPP considerations, caution should be taken to avoid misinterpretation of results from autofluorescent compounds [42]. In addition, dynamic range and background signal for these systems should be assessed prior to screening [42,44].

2.2.2. Biochemical screening recommendations

2.2.2.1. Preparing ecombinant PTP protein. We have had considerable success expressing and purifying GST-fusions of PTPs from bacteria using pGEX-KG, an inducible bacterial expression plasmid containing an N-terminal glutathione S-transferase (GST) tag [95]. The PTP of interest – either an isolated PTP domain or full-length enzyme – is cloned into pGEX-KG and transformed into BL21 *E.coli*. Protein expression is induced in using IPTG, bacteria are lysed, and recombinant proteins are purified by affinity-based techniques. While purity, yield, and activity will be highly dependent on the PTP of interest, we have purified several PTPs using this approach and routinely obtain milligram quantities of recombinant protein from several liters of bacterial cultures. In our hands, PTPs have retained activity, and preparations are enriched for full-length expression constructs, with few partially translated fragments and small amounts of free GST. This is just one example of many expression vectors and systems that exist for producing recombinant proteins (using GST or other epitope tags) in the laboratory, which we find to be an economical approach. In addition, many commercial sources provide pure and active recombinant PTPs, or offer custom production services.

2.2.2.2. Optimizing the phosphatase reaction for inhibitor screening. To identify competitive inhibitors of phosphatases, reactions should be carried out under initial velocity conditions, that is, an early point in the reaction when less than 10% of substrate is consumed [96,97]. This allows several important assumptions to be met during the reaction, including (a) the substrate concentration does not change significantly over time, (b) the reverse reaction does not occur, (c) the detection system does not become saturated, (d) product inhibition does not occur, and (e) the enzyme does not lose stability [96]. Initial velocity conditions can be found by optimizing the amount of substrate, enzyme, and reaction time. This is comprehensively described in [97], and briefly summarized below:

(a) *Step 1: Determine optimal buffer composition, temperature, and pH*- Phosphatase activity will differ in buffers of various compositions, including those that are Tris or HEPES-based and contain additional factors (e.g., EGTA, EDTA, BSA, NaCl, sodium acetate). Base buffers are created and titrated to a physiological range of pHs (pH 5.5–7.5). To preserve the reduced state of the catalytic cysteine, a reducing agent like dithiothreitol DTT (1–5 mM) must be included fresh in the reaction buffer. Phosphatase reactions are then compared across these buffers while reaction temperature (23–37 °C) and duration (10–30 min) are tested. PTPs will likely show activity in several conditions and practicality should be considered when choosing the best (for instance, a cooler temperature and longer reaction might be more feasible for HTS than short reaction at higher temperature, despite both producing similar activity).

(b) *Step 2: Determine an enzyme concentration that yields linear product formation*-Using optimized buffer and assay conditions, a range of enzyme concentrations are titrated into reactions [41]. Saturating substrate concentrations are used to ensure substrate is not limiting for this analysis. Depending on purity and specific activity, phosphatases are typically required at nanograms to low microgram quantities per reaction. Enzyme titration experiments are executed in a kinetic experiment, for example, by stopping the reactions at 5-min intervals for 30 min. An enzyme amount should be chosen that yields linear product formation through the reaction time chosen, while displaying a sufficient signal to background ratio ($\geqslant 5$) (Fig. 3A) [97].

(c) *Step 3: Titrate substrate concentration to measure initial velocities*- Using the chosen enzyme concentration, substrate concentrations are next titrated into reactions captured over the same kinetic conditions [97]. Substrate concentration will vary by assay but are generally on a micromolar scale. Under these conditions, phosphatase activity should be linear and concentration-dependent (Fig. 3B).

(d) *Step 4: Plot initial velocities to determine K_m*-The slopes of the lines from the substrate titration experiment are derived by linear regression to yield initial reaction velocities (in units of activity per minute). These values are used to generate a Michaelis–Menten reaction plot with velocities on the *Y*-axis and corresponding substrate concentrations on the *X*-axis. Statistical software (e.g., GraphPad PRISM) can be used to derive the V_{max} and K_m, the substrate concentration at which the reaction velocity is half the V_{max} (Fig. 3C) [41,97].

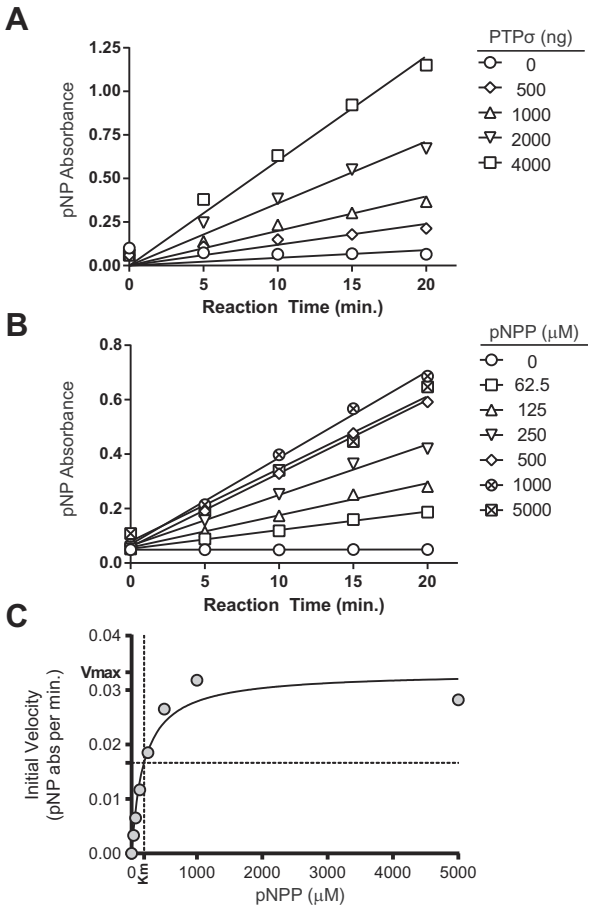

Fig. 3. Optimizing an *in vitro* phosphatase assay to screen for competitive inhibitors. (A) With saturating (1 mM) pNPP substrate, increasing amounts of recombinant PTPσ (GST-tagged; all residues C-terminal to the transmembrane domain) were titrated into reactions. The linear formation of product was observed through time-course reactions. Background-corrected absorbance of dephosphorylated product are plotted by time of reaction. Each plot stems from the quantities of PTPσ indicated in the legend. (B) 2 μg enzyme was chosen from (A) for analysis of activity with varying concentrations of pNPP substrate. Each plot represents a unique concentration of pNPP (indicated in the legend). Background-corrected absorbance of dephosphorylated product are plotted by time of reaction. (C) Initial velocities of PTPs phosphatase activity (Y-axis; in pNP product formed per minute) were derived from the slopes of the plots in (B) at each of the indicated pNPP substrate concentrations (*X*-axis). From this, V_{max} (labeled with tick mark) and km (denoted by dashed lines) can be derived. Figure modified from [75].

2.2.2.3. Designing the inhibitor screen. Screening phosphatase reactions under initial velocity conditions is critical to the successful identification of competitive inhibitors. The optimized buffer, assay duration, and enzyme concentration should be used, along with a substrate concentration at or below the calculated K_m [96]. This allows detectable competition between the substrate and compound under the reaction conditions. In addition, several important screening considerations should be used. Important negative controls include a vehicle control (all reaction components with the compound solvent in place of test compounds), and compound-only controls that contain compound, substrate, and buffer, without phosphatase. Compound-only controls are important to determine the baseline colorimetric properties of compounds, which as mentioned, can often be yellow in color. In addition, an established PTP inhibitor, such as sodium ortho-vanadate, should be included on each plate as a positive control for chemical inhibition. With positive and negative controls in place, Z' scores can be determined to ensure an appropriate dynamic range and precision of the screening platform prior to large-scale screening [41].

An important decision for HTS is the concentration(s) of the test compound to be used. Typically, compounds are screened at a concentration for which 50% inhibition is desired as a minimum hit criterion. For example, if compounds are sought that have IC_{50} values less than 10 μM, compounds can be incubated at 10 μM and hits selected as those compounds that confer at least 50% inhibition at such a concentration. Depending on library size, several concentrations of each compound can be used as part of the primary screen. Primary hits should be subjected to secondary screening that establishes concentration–response curves (IC_{50} values) and time-dependent inhibition. It is advised to confirm hits using complementary phosphatase assays to eliminate false positives resulting from a nuance of a specific reagent. Notably, if pNPP is used a primary screening platform, a malachite green-based assay using a pTyr peptide, or fluorescent phosphatase assay, should be queried in follow-up assays. Finally, inhibition by lead molecules can be characterized in detail to determine the precise mode of action (competitive, non-competitive, or mixed) [98].

2.2.2.4. Overcoming oxidation artifacts. The pKa of the catalytic cysteine in the active site is unusually low, which renders it ionic at a neutral pHs and permits strong nucleophilic activity [99]. This also makes the cysteine incredibly vulnerable to oxidation-mediated inhibition [100,101]. Upon oxidation, the cysteine thiol is converted to sulphenic acid, which is incapable of performing the nucleophilic attack on its substrates. Elegantly documented for PTP1B, sulphenic acid can quickly be converted to a covalent, cyclic sulphenamide that alters the conformation of the active site, prevents further detrimental oxidation, and primes the cysteine for reduction and reactivation [3,102]. A significant body of research has shown that this reversible oxidation of PTP active sites is mediated by reactive oxygen species (ROS) in cells and represents an important physiological mode of regulation in cell signaling [99,103,104]. However, excessive oxidation (converting sulphenic acid into further oxygenated sulphinic or sulphonic acid species) renders the cysteine irreversibly transformed (Fig. 4A).

The inherent susceptibility of PTPs to oxidation poses a serious concern when screening compounds *in vitro*. As described, the catalytic cysteine must be maintained in a reduced state for full activity. To ensure this, strong reducing agents, namely dithiothreitol (DTT), are included as an essential component of phosphatase assay buffers. Redox cycling compounds (RCCs) are molecules that can utilize DTT in oxygenated aqueous environments to create ROS, such as hydrogen peroxide (H_2O_2), by a mechanism that involves cyclic oxidation and reduction of the RCC itself [43]. This perpetual cycling creates a time-dependent production of H_2O_2 in the buffer that can reach micromolar, or even millimolar, concentrations in a short period of time [43,105]. H_2O_2 then oxidizes the catalytic cysteine, potentially to the irreversible sulphinic or sulphonic derivatives. Thus, RCCs confer indirect, and non-selective inhibition of PTPs *in vitro*.

Such agents have a significant history of false identification as direct PTP inhibitors. One example is BVT.948, originally identified as a PTP1B inhibitor through high throughput screening [106]. After the pattern of inhibition with BVT.948 suggested it was non-competitive and dependent on DTT concentration, it was confirmed to catalyze peroxide formation and irreversibly oxidize PTP1B [42,106]. A similar conclusion was made for pyridazine compounds that were initially pursued as potentially selective PTP1B inhibitors, but later found to function by oxidation [107,108]. Oxidizing compounds are not unique to PTP1B inhibition. *Ortho*-quinone inhibitors were found to catalyze redox cycling, concentrating reaction buffers with high levels of H_2O_2, and triggering PTPα inhibition [105]. In addition to ROS production, quinones are Michael acceptors that can directly alkylate cysteines and other residues [109]. Accordingly, quinone-containing compounds have frequently been identified as PTP inhibitors [42].

To gauge the extent of the problem that RCCs pose in chemical screening, Soares and colleagues profiled compound libraries for the presence of RCCs [110]. The study confirmed that majority of *ortho*-quinones and pyrimidotriazinediones function as RCCs in solution. In total, while RCCs represented only a small fraction of two libraries queried – 2 of 1280 molecules (0.16%) in the Library of Pharmacologically Active Compounds (LOPAC) and 37 of 195,826 compounds (0.02%) in the NIH Small Molecule Repository (SMR) library – their presence could easily enrich screen hit lists as only a small percentage of active molecules are pursued [110]. As evidence of this, over 85% of active compounds identified in an HTS for inhibitors of Caspase 8 (a protease which is sensitive to oxidation similar to PTPs) were found to be artifacts of RCCs [111].

While the identification of oxidizing compounds has facilitated the characterization of oxidation-mediated PTP regulation, which holds important physiological implications, RCCs are not ideal PTP inhibitors. Their ROS-mediated cysteine modification renders the PTP inactive via a promiscuous, non-selective mode of inhibition, that can elicit a number of unintended effects *in vivo* [109]. Instead, potent, direct, and selective inhibitors of PTPs are needed. To promote their identification, oxidation must be considered and accounted for in screening initiatives.

A simple step to reduce the contribution of RCCs in chemical screening is to minimize reaction time, as RCCs catalyze the accumulation of oxidative species over time [41]. Reducing the reaction time will favor the identification of direct PTP modulators, while minimizing the effects of oxidation. In our study, we executed a primary screen using compounds pre-incubated with PTPσ for 30 min, followed by 30-min assays with substrate. Follow-up investigation revealed that several lead molecules identified by this screen mediated inhibition through the production of H_2O_2. To address this, we revisited our experimental design, reducing the pre-incubation period to 10 min and the phosphatase assay to 15 min in a refined biochemical screen. This method led to the identification of alternative lead molecules, including at least one whose inhibition appeared to be largely oxidation-independent [75].

In conjunction with minimized reaction time, several direct methods can be employed to reduce the identification of false positive RCCs. While RCCs catalyze ROS generation in the presence of strong reducing agents, like DTT, they fail to do so with weaker agents like glutathione (GSH) or β-mercaptoethanol (βME) [43]. These agents can be substituted to reduce the activity of RCCs, assuming conditions are optimized to retain high PTP activity. Second, a colorimetric assay based on the HRP-catalyzed oxidation of

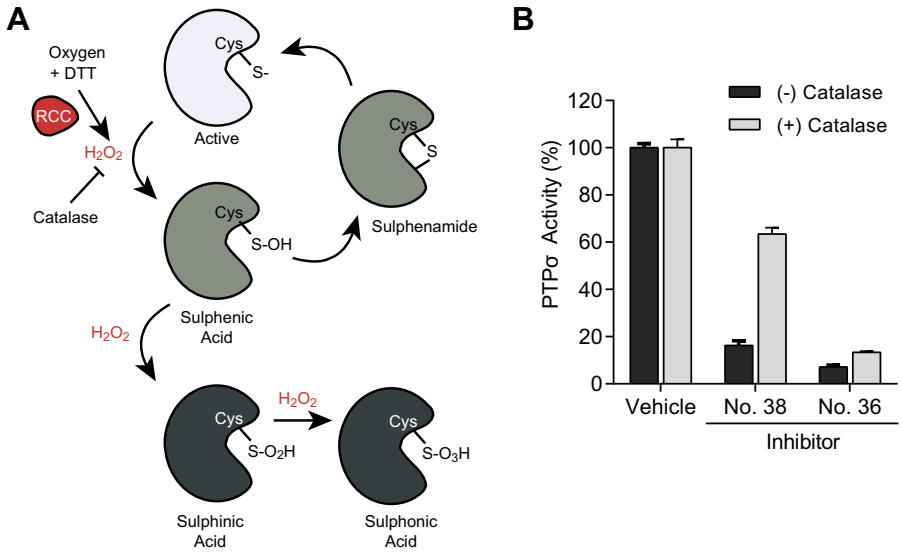

Fig. 4. PTP inhibition by oxidation is a serious concern in compound screening. (A) The conserved cysteine in the PTP active site functions as a nucleophile at neutral pH (light gray). Upon oxidation, the cysteine is converted to sulphenic acid (medium gray) which his incapable of participating in catalysis. This oxidation can be mediated by peroxide (H_2O_2) that is generated in buffers by the activity of redox cycling compounds (RCCs) in the presence of DTT and oxygen. Sulphenic acid can be converted to a cycling sulphenamide (medium gray) that alters the conformation of the active site and prevents further oxidation while priming the cysteine for reactivation. Conversely, the sulphenic acid can be subject to one or two more oxidation events, generating sulphinic and sulphonic acid species, respectively (dark gray). These oxidation events are irreversible. (B) H_2O_2 can be converted into water and oxygen by catalase. To determine the contribution of oxidation to PTPσ inhibition mediated by two lead compounds (36 and 38), we performed pNPP phosphatase assays in the presence or absence of 1 mM catalase. Relative PTPσ activity (expressed as percent vehicle control) is plotted on the *Y*-axis. While compound 38-mediated inhibition was largely negated by co-incubation with catalase, compound 36 activity is primarily independent of oxidation. Figure modified from [75].

phenol red can be used to counter-screen an entire library, or test active molecules, for those that produce H_2O_2 [112]. Third, compound inhibition can be tested in the presence and absence of an agent that removes peroxide species, such as catalase (which converts H_2O_2 into water and oxygen). We found this to be an incredibly simple, economical, and robust filter for oxidative compounds. We repeated phosphatase assays with lead molecules in the presence of 1 mM catalase (from bovine liver; Sigma–Aldrich), or a vehicle control. These experiments led us to conclude that several primary hits from our first PTP inhibitor screen were functioning as RCCs owing to their complete inactivity in the presence of 1 mM catalase. However, we were able to identify at least one lead compound which inhibits PTPσ with minimal oxidative artifacts (Fig. 4B) [75]. Many chemical moieties that confer RCC potential have been identified and are described [43], so lead molecules can be carefully analyzed for such properties if oxidation is suspected.

3. Integrating biochemical and virtual approaches

As discussed, both biochemical and virtual screening offer unique advantages for the identification of PTP inhibitors; however, neither method can completely satisfy a drug discovery effort in isolation. Molecular docking analyses completed *in silico* can be used to create focused libraries for evaluation *in vitro*, can be used to explain results obtained biochemically, and used to develop new hypotheses to improve inhibitors. Complementarily, *in vitro* screening can be used to prioritize the most promising scaffolds and inform structural observations. When leveraged together, virtual and biochemical screening improve the efficiency and success of one another.

We utilized both *in silico* and *in vitro* screening platforms in tight coordination to identify small molecules targeted to PTPσ. After molecular docking was employed to identify primary virtual hits, we evaluated these agents as a focused library *in vitro*. A series of phosphatase assays led us to conclude that many lead molecules displayed oxidative properties, prompting the retrieval of additional unique scaffolds virtually. These new compounds were again screened *in vitro* using refined assay conditions, and a promising lead molecule was identified. Binding analyses *in silico* have now generated predictions about the precise mechanism of inhibition by this molecule, which we can test *in vitro* by mutating key residues predicted to mediate activity. Importantly, we can improve the potency and selectivity of our lead molecule by modifying additional molecular interactions through structure-guided design.

4. Summary and conclusions

In summary, PTPs represent an important class of enzymes critical for the maintenance of normal cell function, and are increasingly implicated in the pathology of many diseases. While the development of small molecules to PTPs is essential to their characterization and therapeutic targeting, several features of these enzymes – including high sequence conservation, charged active sites, and susceptibility to oxidation – have plagued such efforts. Despite this, approaches and tools are being developed to overcome such concerns.

Here, we have described how a rational integration of both biochemical and virtual methods can provide an effective workflow for chemical targeting of PTPs. Notably, structure-based virtual screening can be used to identify compounds that bind the PTP active site *in silico*. While PTP crystal structures with closed WPD loops, which reflect conformations associated with substrates interaction and catalysis, are often used as templates in VS, we and others have found utility in open PTP structures as models of enzymes accessible to active site targeting. Regardless of the template, a VS can be used to generate a focused library to evaluate *in vitro*, reducing the scope and scale of biochemical phosphatase assays. Moreover, when biochemical screening conditions are carefully optimized – including reaction time, use of reducing agents,

and inclusion of peroxide scavengers – they can minimize the influence of oxidation in chemical libraries, common culprits for non-selective PTP inhibition.

Our recent work provides one example of an effective workflow where the transition between virtual and biochemical methods is iterative and informative. The continued integration of these and other approaches will be instrumental in developing our lead molecules into selective and active inhibitors of PTPσ. Importantly, the methods discussed here can be applied to drug discovery initiatives for other members of the PTP family, facilitating their characterization and fostering the therapeutic potential of this class of enzyme the future.

Funding

This work was supported by Award Number R01CA138651 from the National Cancer Institute and the Department of Defense Prostate Cancer Research Program of the Office of Congressionally Directed Medical Research Programs PC081089 to JPM.. Award Number RC2MH090878 from the National Institute of Mental Health awarded to NM provided partial support for the work described in this paper. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank H. Eric Xu and Yong Xu for contributions to original PTPσ virtual screen. We thank Julianna Sacoman and Jennifer Kordich for careful review of this manuscript and helpful feedback.

References

[1] L. Tautz, M. Pellecchia, T. Mustelin, Expert Opin. Ther. Targets 10 (1) (2006) 157–177.
[2] P. Lahiry et al., Nat. Rev. Genet. 11 (1) (2010) 60–74.
[3] N.K. Tonks, Nat. Rev. Mol. Cell Biol. 7 (11) (2006) 833–846.
[4] N.K. Tonks, FEBS J. 280 (2) (2013) 346–378.
[5] J.N. Andersen et al., Mol. Cell Biol. 21 (21) (2001) 7117–7136.
[6] J.O. Lee et al., Cell 99 (3) (1999) 323–334.
[7] K. Kolmodin, J. Aqvist, FEBS Lett. 498 (2–3) (2001) 208–213.
[8] N.K. Tonks, C.D. Diltz, E.H. Fischer, J. Biol. Chem. 263 (14) (1988) 6731–6737.
[9] N.K. Tonks, C.D. Diltz, E.H. Fischer, J. Biol. Chem. 263 (14) (1988) 6722–6730.
[10] M. Elchebly et al., Science 283 (5407) (1999) 1544–1548.
[11] L.D. Klaman et al., Mol. Cell Biol. 20 (15) (2000) 5479–5489.
[12] J.M. Zabolotny et al., Dev. Cell 2 (4) (2002) 489–495.
[13] A. Cheng et al., Dev. Cell 2 (4) (2002) 497–503.
[14] S.M. Echwald et al., Diabetes 51 (1) (2002) 1–6.
[15] A. Mok et al., J. Clin. Endocrinol. Metab. 87 (2) (2002) 724–727.
[16] R. Di Paola et al., Am. J. Hum. Genet. 70 (3) (2002) 806–812.
[17] J.L. Bento et al., Diabetes 53 (11) (2004) 3007–3012.
[18] N.D. Palmer et al., Diabetes 53 (11) (2004) 3013–3019.
[19] M. Tartaglia et al., Nat. Genet. 29 (4) (2001) 465–468.
[20] W.J. Hendriks et al., FEBS J. 280 (2) (2013) 708–730.
[21] P.K. Gregersen, Nat. Genet. 37 (12) (2005) 1300–1302.
[22] G.L. Burn et al., FEBS Lett. 585 (23) (2011) 3689–3698.
[23] J.N. Andersen et al., FASEB J. 18 (1) (2004) 8–30.
[24] S.G. Julien et al., Nat. Rev. Cancer 11 (1) (2011) 35–49.
[25] N. Aceto et al., Nat. Med. 18 (4) (2012) 529–537.
[26] K.S. Grossmann et al., Adv. Cancer Res. 106 (2010) 53–89.
[27] P. Rios, X. Li, M. Kohn, FEBS J. 280 (2) (2013) 505–524.
[28] B.J. Stephens et al., Mol. Cancer Ther. 4 (11) (2005) 1653–1661.
[29] S. Saha et al., Science 294 (5545) (2001) 1343–1346.
[30] R. He et al., FEBS J. 280 (2) (2013) 731–750.
[31] A.J. Barr, Future Med. Chem. 2 (10) (2010) 1563–1576.
[32] L.M. Scott et al., Curr. Pharm. Des. 16 (16) (2010) 1843–1862.
[33] K.R. Martin et al., J. Cell Sci. 124 (Pt 5) (2011) 812–819.
[34] J.P. MacKeigan, L.O. Murphy, J. Blenis, Nat. Cell Biol. 7 (6) (2005) 591–600.
[35] J. McLean et al., J. Neurosci. 22 (13) (2002) 5481–5491.
[36] K.M. Thompson et al., Mol. Cell Neurosci. 23 (4) (2003) 681–692.
[37] P.S. Sapieha et al., Mol. Cell Neurosci. 28 (4) (2005) 625–635.
[38] R. Siu, C. Fladd, D. Rotin, Mol. Cell Biol. 27 (1) (2007) 208–219.
[39] E.J. Fry et al., Glia 58 (4) (2010) 423–433.
[40] Y. Shen et al., Science 326 (5952) (2009) 592–596.
[41] L. Tautz, T. Mustelin, Methods 42 (3) (2007) 250–260.
[42] M.A. Blaskovich, Curr. Med. Chem. 16 (17) (2009) 2095–2176.
[43] P.A. Johnston, Curr. Opin. Chem. Biol. 15 (1) (2011) 174–182.
[44] E. Mattila, J. Ivaska, Anticancer Agents Med. Chem. 11 (1) (2011) 141–150.
[45] Y.S. Heo et al., Exp. Mol. Med. 34 (3) (2002) 211–223.
[46] M. Bahta et al., J. Med. Chem. 54 (8) (2011) 2933–2943.
[47] K. Shen et al., J. Biol. Chem. 276 (50) (2001) 47311–47319.
[48] C.E. Hubbard, A.M. Barrios, Bioorg Med Chem Lett 18 (2) (2008) 679–681.
[49] M. Sodeoka et al., J. Med. Chem. 44 (20) (2001) 3216–3222.
[50] D.F. McCain et al., J. Biol. Chem. 279 (15) (2004) 14713–14725.
[51] F. Liang et al., J. Biol. Chem. 278 (43) (2003) 41734–41741.
[52] K. Umezawa, M. Kawakami, T. Watanabe, Pharmacol. Ther. 99 (1) (2003) 15–24.
[53] J.H. Ahn et al., Bioorg. Med. Chem. Lett. 12 (15) (2002) 1941–1946.
[54] M.R. Karver et al., J. Med. Chem. 52 (21) (2009) 6912–6918.
[55] L.F. Iversen et al., Biochemistry 40 (49) (2001) 14812–14820.
[56] X. Li et al., Sci. Signal 6 (275) (2013) rs10.
[57] Q. Wang et al., Eur. J. Med. Chem. 49 (2012) 354–364.
[58] M. Bahta, T.R. Burke Jr., ChemMedChem 6 (8) (2011) 1363–1370.
[59] G. Navarrete-Vazquez et al., Eur. J. Med. Chem. 53 (2012) 346–355.
[60] F. Lopez-Vallejo et al., Comb. Chem. High Throughput Screen 14 (6) (2011) 475–487.
[61] T. Scior et al., J. Chem. Inf. Model 52 (4) (2012) 867–881.
[62] B.K. Shoichet, Nature 432 (7019) (2004) 862–865.
[63] P. Ripphausen, B. Nisius, J. Bajorath, Drug Discov. Today 16 (9–10) (2011) 372–376.
[64] M. Congreve, C.W. Murray, T.L. Blundell, Drug Discov. Today 10 (13) (2005) 895–907.
[65] D.B. Kitchen et al., Nat. Rev. Drug Discov. 3 (11) (2004) 935–949.
[66] S. Kalyaanamoorthy, Y.P. Chen, Drug Discov. Today 16 (17–18) (2011) 831–839.
[67] G.M. Sastry et al., J. Comput. Aided Mol. Des. 27 (3) (2013) 221–234.
[68] Molecular Operating Environment (MOE), Chemical Computing Group, Inc.: 1010 Sherbooke St. West, Suite 910, Montreal, QC, Canada, H3A 2R7.
[69] R.A. Friesner et al., J. Med. Chem. 47 (7) (2004) 1739–1749.
[70] A.T. Laurie, R.M. Jackson, Bioinformatics 21 (9) (2005) 1908–1916.
[71] M. Rarey et al., J. Mol. Biol. 261 (3) (1996) 470–489.
[72] X.Y. Meng et al., Curr. Comput. Aided Drug Des. 7 (2) (2011) 146–157.
[73] S.Y. Huang, S.Z. Grinter, X. Zou, Phys. Chem. Chem. Phys. 12 (40) (2010) 12899–12908.
[74] A.J. Barr et al., Cell 136 (2) (2009) 352–363.
[75] K.R. Martin et al., PLoS One 7 (11) (2012) e50217.
[76] N. Huang, B.K. Shoichet, J.J. Irwin, J. Med. Chem. 49 (23) (2006) 6789–6801.
[77] S. Liu et al., J. Am. Chem. Soc. 130 (50) (2008) 17075–17084.
[78] A.K. Pedersen et al., Acta Crystallogr. D Biol. Crystallogr. 60 (Pt 9) (2004) 1527–1534.
[79] H. Park, P.N. Chien, S.E. Ryu, Bioorg. Med. Chem. Lett. 22 (20) (2012) 6333–6337.
[80] S. Wu et al., ChemMedChem 4 (3) (2009) 440–444.
[81] N. Singh et al., J. Chem. Inf. Model 49 (4) (2009) 1010–1024.
[82] J.L. Medina-Franco et al., J. Chem. Inf. Model 49 (2) (2009) 477–491.
[83] J.L. Medina-Franco et al., PLoS One 7 (11) (2012) e50798.
[84] Moura. Barbosa, Curr. Top. Med. Chem. 12 (8) (2012) 866–877.
[85] A.A. Shelat, R.K. Guy, Curr. Opin. Chem. Biol. 11 (3) (2007) 244–251.
[86] K.M.G. O'Connell, W.R.J.D. Galloway, D.R. Spring, The basics of diversity-oriented synthesis, in: A. Trabbocchi (Ed.), Diversity Oriented Synthesis: Basics and Applications in Organic Synthesis, Drug Discovery, and Chemical Biology, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2013, pp. 1–28.
[87] S.L. Schreiber, Science 287 (5460) (2000) 1964–1969.
[88] R. Gozalbes et al., J. Med. Chem. 51 (11) (2008) 3124–3132.
[89] L.B. Akella, D. DeCaprio, Curr. Opin. Chem. Biol. 14 (3) (2010) 325–330.
[90] A.D. Gorse, Curr. Top. Med. Chem. 6 (1) (2006) 3–18.
[91] J.L. Medina-Franco, Chemoinformatic characterization of the chemical space and molecular diversity of compound libraries, in: A. Trabocchi (Ed.), Diversity-Oriented Synthesis: Basics and Applications in Organic Synthesis, Drug Discovery, and Chemical Biology, John Wiley & Sons, Inc., 2013, pp. 325–352.
[92] G.M. Downs, Clustering methods and their uses in computational chemistry, in: K.B. Lipkowitz (Ed.), Reviews in Computational Chemistry, Wiley-VCH, New York, 2002, pp. 1–40.
[93] M.S. Lajiness, Perspec. Drug Discov. Des. 7–8 (20) (1997) 65–84.
[94] Z.Y. Zhang, Annu. Rev. Pharmacol. Toxicol. 42 (2002) 209–234.
[95] K.L. Guan, J.E. Dixon, Anal. Biochem. 192 (2) (1991) 262–267.
[96] H.B. Brooks, S. Geeganage, S.D. Kahl, C. Montrose, S. Sittampalam, M.C. Smith, J.R. Weidner, Basics of enzymatic assays for HTS, in: S. Sittampalam, J.R. Weidner (Eds.), Editor Assay Guidance Manual, Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda, MD, 2004.
[97] M.B. Tierno et al., Nat. Protoc. 2 (5) (2007) 1134–1144.
[98] T.O. Johnson, J. Ermolieff, M.R. Jirousek, Nat. Rev. Drug Discov. 1 (9) (2002) 696–709.
[99] N.K. Tonks, Cell 121 (5) (2005) 667–670.
[100] T.C. Meng, T. Fukada, N.K. Tonks, Mol. Cell 9 (2) (2002) 387–399.
[101] J.M. Denu, K.G. Tanner, Biochemistry 37 (16) (1998) 5633–5642.
[102] A. Salmeen et al., Nature 423 (6941) (2003) 769–773.

[103] J. den Hertog, A. Groen, T. van der Wijk, Arch. Biochem. Biophys. 434 (1) (2005) 11–15.
[104] A. Salmeen, D. Barford, Antioxid. Redox. Signal 7 (5–6) (2005) 560–577.
[105] M.P. Bova et al., Arch. Biochem. Biophys. 429 (1) (2004) 30–41.
[106] C. Liljebris et al., J. Pharmacol. Exp. Ther. 309 (2) (2004) 711–719.
[107] C. Liljebris et al., Bioorg. Med. Chem. 10 (10) (2002) 3197–3212.
[108] A. Tjernberg et al., Bioorg. Med. Chem. Lett. 14 (4) (2004) 891–895.
[109] J.L. Bolton et al., Chem. Res. Toxicol. 13 (3) (2000) 135–160.
[110] K.M. Soares et al., Assay Drug Dev. Technol. 8 (2) (2010) 152–174.
[111] G.K. Smith et al., Arch. Biochem. Biophys. 399 (2) (2002) 195–205.
[112] P.A. Johnston et al., Assay Drug Dev. Technol. 6 (4) (2008) 505–518.