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Selective Detection of Allosteric Phosphatase Inhibitors

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Supporting Information

ABSTRACT: Normal cellular function, such as signal transduction, is largely controlled by the reversible phosphorylation of cellular proteins catalyzed by two major classes of enzymes, kinases and phosphatases. A misbalance in this complex and dynamic interplay leads to a variety of severe diseases, such as cancer, inflammation, or autoimmune diseases. This makes kinases as well as phosphatases equally attractive targets for therapeutic manipulation by small molecules. While the development of kinase inhibitors has resulted in several blockbuster drugs, such as imatinib, with remarkable success in the clinic and sales of many billions of U.S. dollars per year, not a single phosphatase inhibitor has yet been approved for clinical use. Similar to the kinase world, substrate-competitive phosphatase inhibitors have been developed but were not suitable for further development into clinical candidates due to their charge and limited selectivity. Research efforts, therefore, have shifted to the exploitation of allosteric sites that can regulate phosphatase activity and may enable the discovery of novel modulators of phosphatase activity with much improved pharmacological properties. However, assay systems, which enable the straightforward discovery of these inhibitor types, are missing. Here, we present a novel binding assay capable of detecting ligands of an allosteric pocket of the protein tyrosine phosphatase 1B. This assay is suitable for high-throughput screening and selectively detects ligands which bind to this unique site with a clear discrimination from substrate-competitive ligands.

Reversible phosphorylation of proteins is *the* critical post-translational modification by which cellular pathways are regulated. Enzymatic transfer of the γ-phosphate group of ATP onto substrate proteins triggers various biological responses by introducing recognition sites for interacting proteins or by directly regulating the activity of the substrate protein. In living cells, a fine-tuned and dynamic balance between phosphorylation (catalyzed by kinases) and de-phosphorylation (catalyzed by phosphatases) exists. A disruption of this balance can lead to severe diseases, including many forms of cancer and auto-immune, neurodegenerative, or inflammation disorders, making both enzyme classes highly attractive targets for chemical biology and medicinal chemistry research in the development of novel drugs.[1,2]

A huge effort has been undertaken by academia and the pharmaceutical industry over the past decades to target kinases as well as phosphatases with small molecules that modulate their activity.[1,2] Although still challenging, this effort has been remarkably more successful for kinases, resulting in 26 kinase inhibitors being approved for clinical use to date[3] and many more in clinical trials. On the other hand, not a single phosphatase inhibitor has made it to the clinic.[2] At first glance, this is surprising, since both enzymes are equally important for the regulation of cellular functions. An explanation for this difference lies at the molecular level. First, there are significantly more kinases (>500) than phosphatases (>130) encoded in the human genome, implying that the substrate specificity of phosphatases is much broader. Additionally, much like kinases, the active center of phosphatases are structurally very well conserved, contributing to poor inhibitor selectivity.[4,5] Second, the substrate pocket of phosphatases is positively charged to complement the high negative charge of phosphorylated substrates. In screening campaigns, this leads to the predominant discovery of negatively charged substrate mimetics which suffer from poor bioavailability and cell permeability, disqualifying them as lead candidates. One way to circumvent these issues is the identification and exploitation of allosteric sites that are less conserved. Using high-throughput screening (HTS) assays which enable the detection of ligands for such sites would likely identify novel chemical scaffolds for further development into much more potent phosphatase noncompetitive activators or inhibitors. However, for a selective discovery of allosteric ligands, novel assay systems must first be developed.

One prime example of a phosphatase drug target is the protein tyrosine phosphatase 1B (PTP1B). PTP1B has been identified as a highly interesting drug target for type II diabetes and for the treatment of obese patients, since mice lacking the PTP1B gene showed resistance and decreased incidence of obesity and diabetes.[6−9] However, inhibitors that target PTP1B must have a high selectivity, since inactivation of TC-PTP, a close homologue of PTP1B, can cause dramatic negative side effects, as shown with mice knockout studies.[10,11] The discovery of a druggable allosteric pocket ∼20 Å away from the catalytic site[12] (Figure 1) offers a promising new opportunity for the development of selective PTP1B modulators which lock the phosphatase in its inactive conformation. The allosteric inhibitor **1** described in this study[12] displaces helix α7 from the core of the phosphatase upon binding. This translates, over a cascade of amino acid side-chain interactions, into a conformational change of the catalytically important WPD loop, thereby disabling substrate

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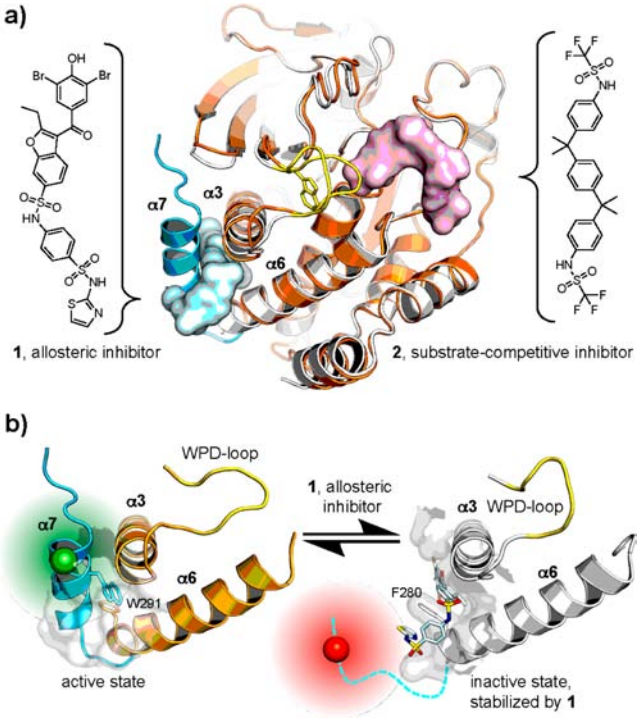

Figure 1. Principle of the FLiP assay. (a) Overlay of the active (orange; PDB entry 1PTY) and inactive (white; PDB entry 1T4J) forms of PTP1B (catalytic domain). Active site inhibitor **2** (pink) and allosteric inhibitor **1** (blue) are shown in a surface representation. (b) Left: In the active form, helix α7 (blue) docks onto helix α3. This interaction is stabilized by hydrophobic packing of W291 at the interface of helix α6 (orange) helix α3. The WPD loop (yellow) closes over the substrate in the active site. Right: Upon binding of allosteric inhibitor **1**, a conformational rearrangement occurs, disabling substrate recognition. The disordering of helix α7 (dashed line) upon ligand binding is reported by the fluorophore (colored sphere).

recognition.[12] The inhibitor is bound to the protein and stabilized by hydrogen bonds with residues from helices α3 and α6 and through the formation of $\pi-\pi$ stacking interactions between the benzofuran and the thiazole of the compound with the side chain of F280 sandwiched in between (Figure 1b). Since TC-PTP has a cysteine at this position in helix α6, a ~40-fold reduced affinity was reported for **1** as compared to PTP1B.[12]

Here we report the development of a novel detection method for ligands of this allosteric pocket in phosphatases. This assay is solely based on the conformational change of helix α7 introduced by binding of an allosteric ligand, rather than by the displacement of a phosphorylated substrate (as would be the case in classic activity-based assays). It therefore allows for an easy discrimination between allosteric and substrate-competitive inhibitors. We show that this "Fluorescent Labels in Phosphatases" (FLiP) assay is suitable for measuring the affinity and binding kinetics of allosteric ligands, has a robust performance in 384-well microtiter plates, and is suitable for HTS.

To enable specific labeling of the allosteric pocket, we first introduced a cysteine at the third turn of helix α7 (L294C) by site-directed mutagenesis. From available crystal structures it would be expected that a fluorophore attached to this cysteine would be close to the protein surface in the active state but fully solvent-exposed in the inactive state (Figure 1b). From the crystal structures it would not be expected that this substitution and subsequent labeling would disrupt the structure of helix α7 or disturb interactions between helices α3 and α7. To achieve monolabeling of the protein with fluorophore, solvent-exposed native cysteines (Figure S1) were conservatively replaced by serines (C32S, C92S, C121S, and C215S, the catalytic cysteine). We recorded CD spectra of wild-type (WT) and mutated FLiP variant PTP1B, calculated the content of secondary structure elements over a wide range of temperatures, and verified that the substitutions did not have a significant impact on protein structure and stability (Figure S2).

Prior to batch labeling, we performed initial FLiP experiments in which small quantities of protein were labeled with a variety of different fluorophores in order to identify a fluorophore that can report the change in its microenvironment caused by the conformational change of helix α7 upon ligand binding. From eight different fluorophores tested, Texas Red was ultimately chosen to further develop our binding assay (data not shown). Successful labeling of PTP1B with Texas Red was verified by ESI-MS (Figure S3).

We then recorded the emission spectra of Texas Red-labeled PTP1B (TR-PTP1B) after stepwise addition of inhibitors **1** and **2**. Upon binding of **1**, a bathochromic shift in the emission maximum from 614 to 616 nm and an intensity decrease were observed (Figure 2a), as would be expected from an increased

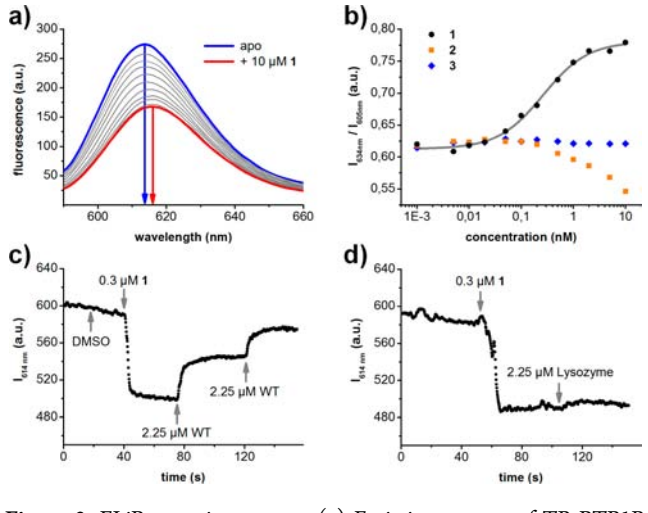

Figure 2. FLiP assay in cuvettes. (a) Emission spectra of TR-PTP1B supplemented with increasing concentrations of **1**. A bathochromic shift in the emission maximum (indicated by arrows) can be observed upon ligand binding. (b) Plotting the ratiometric fluorescence against the inhibitor concentration on a logarithmic scale results in an upward sigmoidal regression curve for **1**, while a decrease in the ratio was observed for **2** (for spectra, see Figure S4). **3** showed no effect. (c) **1** binds very rapidly and can be extracted from the allosteric site by addition of excess unlabeled PTP1b (WT). (d) Addition of lysozyme as negative control did not release the inhibitor.

solvent exposure of the fluorophore.[13] As reported elsewhere (Schneider et al., submitted), this shift can be translated into a robust assay readout by calculating the ratio of intensities measured at longer (634 nm) and shorter (605 nm) wavelengths ($R = I_{634\ nm}/I_{605\ nm}$). Ratiometric fluorescence changes are dose-dependent, and increasing values are obtained with allosteric ligand binding. Plotting these ratios against increasing concentrations of **1** on a logarithmic scale resulted in a sigmoidal regression curve from which the dissociation constant (K_d) of **1** can be directly determined as described

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earlier[14,15] (Figure 2b). The substrate-competitive inhibitor **2** showed an opposite effect with a *decrease* in the ratio, demonstrating that this assay is a strong tool for the selective detection of allosteric PTP1B inhibitors. Miconazole (**3**), a non-phosphatase inhibitor which forms aggregates at >3 μM onto which proteins adsorb,[16] is not detected, suggesting that the FLiP assay would be insensitive for promiscuous hits.

From five measurements using protein from two independent preparations (i.e., expression, purification and labeling), an average K_d = 0.22 ± 0.02 μM was determined, with a small standard deviation suggesting high reproducibility. The FLiP assay seems to be more sensible toward allosteric ligands as compared to activity-based assays, since the measured K_d is significantly lower than the published IC_{50} for **1** (8 μM).[12] This finding also suggests that the FLiP assay as a very simple detection system is preferable as a first-line screening method since molecules with low affinity may be more reliably detected.

We facilitated the FLiP assay to obtain kinetic parameters for the association and dissociation of the ligand in real time without relying on alternative instrumentation such as surface plasmon resonance. For this, the fluorescence intensity was measured at the emission maximum ($I_{614\ nm}$) of TR-PTP1B in its apo form. Addition of **1** to a stirring suspension of labeled PTP1B resulted in a very rapid signal decrease (Figure 2c) to a stable level, suggesting a very fast association of the inhibitor to the allosteric site. This effect was not observed when the vehicle alone (DMSO) was added and could be reversed by addition of unmodified PTP1B, which shifts the binding equilibrium in favor of the unlabeled PTP1B. This reversal suggests that helix α7 is rearranged after complete dissociation of the allosteric ligand.

We then optimized the assay to be used in a 384-well microtiter plate format suitable for HTS. The emission spectra of TR-PTP1B with and without addition of **1** measured in plates (Figure 3a) were very similar to those measured in

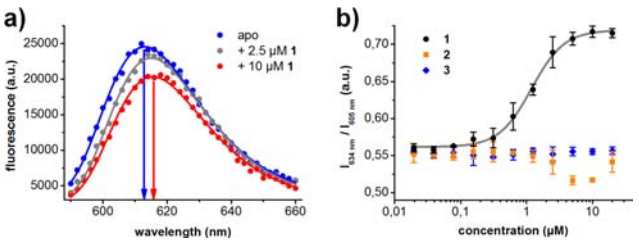

Figure 3. FLiP assay in 384-well plates. (a) Emission spectra. (b) Plot of the ratiometric fluorescence against the logarithmic inhibitor concentration.

cuvettes (Figure 2a), suggesting successful downscaling. Like in cuvettes, a sigmoidal regression curve could be obtained from the titration of TR-PTP1B with **1** (Figure 3b). The calculated K_d measured in the microtiter plate format was slightly higher (1.1 ± 0.3 μM; n = 5) than that measured from the cuvette format, an effect described elsewhere.[14] Finally, we calculated Z', a parameter traditionally used to assess the robustness of a HTS assay.[17] For this, the ratiometric fluorescence of TR-PTP1B incubated with vehicle alone (DMSO) or with a saturating amount of reference inhibitor (10 μM **1**) was measured, each in eight replicates. Traditionally, a $Z' > 0.5$ is regarded as suitable for HTS.[17] With a calculated Z' = 0.70 ± 0.07 (n = 5), the FLiP assay qualifies as a robust tool for HTS.

In summary, we have established a novel assay, FLiP, for the selective detection of allosteric phosphatase inhibitors. As proof of principle, we attached the fluorophore Texas Red to helix α7 of PTP1B so that the conformational change induced by binding of the allosteric ligand was reported by a bathochromic shift in its emission spectrum. We showed that this assay has a robust performance in 384-well plates. With an excitation maximum of ~590 nm, Texas Red is an ideal fluorophore, since small molecules typically identified in high-throughput screening do not display intrinsic fluorescence at this excitation wavelength (Schneider et al., submitted), reducing false-positive hits in a screening scenario to a minimum. This assay principle has already been applied to a variety of enzymes undergoing distinct conformational changes.[15,18−21] We speculate that FLiP can be easily transferred to other phosphatases such as SHIP2 and protein phosphatase 1D where allosteric modes of action are known.[4,5] The FLiP assay is a strong tool in the search for novel selective and potent phosphatase modulators with drug-like chemical composition.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of protein production (construct design, expression, purification), FLiP measurements, CD spectroscopic analysis, and supplementary Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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