HHS Public Access

Author manuscript

Bioorg Med Chem. Author manuscript; available in PMC 2016 June 15.

Published in final edited form as:

Bioorg Med Chem. 2015 June 15; 23(12): 2781–2785. doi:10.1016/j.bmc.2015.02.040.

Strategies to make Protein Serine/Threonine (PP1, Calcineurin) and Tyrosine Phosphatases (PTP1B) druggable: achieving specificity by targeting substrate and regulatory protein interaction sites

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Abstract

The established dogma is that protein Serine/Threonine (PSPs) and Tyrosine (PTPs) Phosphatases are unattainable drug targets. This is because natural product inhibitors of PSP active sites are lethal, while the active sites of PTPs are exceptionally conserved and charged, making it nearly impossible to develop PTP inhibitors that are selective. However, due to a series of recent structural and functional studies, this view of phosphatases is about to undergo a radical change. Rather than target active sites, these studies have demonstrated that targeting PSP/PTP protein (substrate/regulatory) interaction sites, which are distal from the active sites, are highly viable and suitable drugs targets. This is especially true for Calcineurin (CN), in which the blockbuster immunosuppressant drugs FK506 and cyclosporine A were recently demonstrated to bind and block one of the key CN substrate interaction sites, the LxVP site. Additional studies show that this approach—targeting substrate and/or regulatory protein interaction sites—also holds incredible promise for protein phosphatase 1 (PP1)-related diseases. Finally, domains outside PTP catalytic domains have also recently been demonstrated to directly alter PTP activity. Collectively, these novel insights offer new, transformative perspectives for the therapeutic targeting of PSPs by interfering with the binding of PIPs or substrates and PTPs by targeting allosteric sites outside their catalytic domains.

Keywords

protein phosphatase 1; serine/threonine phosphatases; calcineurin; drug design; PTP1B

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Phosphorylation is one of the most ubiquitous, reversible posttranslational modifications in cells [1]. This is because the tightly-regulated phosphorylation of highly dynamic, interacting proteins is one of the key mechanisms used by cells to communicate external signals from the membrane to the nucleus. The enzymes responsible for controlling the phosphorylation state of the cell are *kinases*, which catalyze the transfer the γ-phosphate moiety of ATP to substrates, and *phosphatases*, which catalyze the reverse reaction, the removal of the phosphate moiety from phosphorylated substrates. Thus, in this way, phosphatases dynamically reverse the effects of kinases. Because phosphorylation is critical for biological processes from cell growth to differentiation to development, the location and duration of the reciprocal actions of kinases and phosphatases is exquisitely regulated both temporally and spatially within the cell. However, when this tight regulation is disrupted, dysregulation of phosphorylation signaling ensues and the consequence is almost always disease [2–7].

In the human genome, there is a near 1-to-1 ratio of tyrosine phosphatases (PTPs; 107) to kinases (90) [3, 8, 9]. In contrast, the serine/threonine phosphatases (PSPs) are woefully outnumbered by their abundant kinase counterparts (~40:418) [10–16]. Thus, while tyrosine phosphatases have been considered to be viable drug targets, serine/threonine phosphatases have been viewed as ‘house-keeping’ enzymes, with only a limited chance for drug selectivity. This assessment is due in large part because the active sites of the three most abundant and well-studied PSPs—protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 2B (PP2B; PP3; calcineurin [CN] [15, 16])—are 100% conserved and thus active site inhibitors would likely not be selective [14] (Figure 1). This has been confirmed by the discovery of natural product PSP inhibitors, including microcystin [17], nodularin [18], okadaic acid [19, 20] and tautomycin [18] among others, which inhibit all three phosphatases with only slight preferences of one versus the other. Furthermore, because PP1, PP2A and CN are responsible for the majority of the ser/thr dephosphorylation reactions in humans, inhibiting their active sites is also expected to disrupt many biological processes. This has also been demonstrated as these natural product inhibitors are potent and very lethal toxins.

PSP drugs: target PSP substrate and regulatory protein interaction sites

So is there any chance of successfully turning a serine/threonine phosphatase into a multi-billion dollar drug target? The answer is a resounding yes. In fact, such drugs already exist [21]. Cyclosporin A (CSA) [22] and FK-506 [23] are potent immunosuppressants that generate over a billion dollars per year in revenue. They function by potently inhibiting the activity of CN. However, they do not inhibit CN by binding and blocking its active site. Instead, as we have recently shown, these drugs bind to a critical substrate/regulatory protein recognition site on CN known as the LxVP site [24], and function by inhibiting substrates, especially the NFATs, from binding and, as a consequence, being dephosphorylated by CN [25, 26] (Figure 2). The second characterized substrate recognition site in CN is the PxIxIT site [27], which binds PxIxIT sequences in CN regulators and substrates [28]. However, the primary problem with targeting the PxIxIT and LxVP sites is that most CN substrates (as well as regulatory and targeting proteins, such as e.g. the AKAPs [29]) contain a PxIxIT and/or an LxVP site. This likely explains the severe side effects of CSA and FK-506: while

the targeted substrates - the NFATs - cannot be dephosphorylated and are result in the limited immune response that is critical for organ transplantations, other substrates with LxVP sites will also be unable to bind and be dephosphorylated by CN.

Thus, for CN, the identification of additional protein interactions sites that are used by very few substrates and/or targeting proteins ought to be seen as the key step for the development novel CN therapeutics, e.g. against neurological diseases. This is because novel drugs that target these sites will only disrupt the interaction of CN with a very small number of targeting and/or substrates. Once identified, these sites will provide a powerful avenue for highly specific modulation of CN activity.

Since PP1, PP2A and PP2B share a 100% conserved active site [14], the development of specific active site inhibitors/activators is impossible. Nature has confirmed this, as nearly all naturally-derived inhibitors show only minimal differences in potency against PP1, PP2A and CN [18, 30]. Thus alternative routes for creating PP1 specific drugs must be pursued. One innovative approach is to target the PP1 regulatory proteins. Although the specificity of the PP1 catalytic domains is low, PP1 dephosphorylates its substrates with high specificity. To achieve this, PP1 interacts with more than 100 distinct regulatory subunits [13]; namely, *inhibitory* proteins that potently inhibit phosphatase activity by binding and blocking the active site [5, 31–34], and *targeting* proteins, proteins the localize PP1 to distinct regions of the cell while also directly modulating PP1-substrate interactions (Figure 1,3). Many PP1 targeting subunits, such as NIPP1 [35], enhance the binding of specific substrates. For example, the FHA domain of NIPP1 enhances the PP1-mediated dephosphorylation of its substrates CDC5L and SAP155 [35, 36]. However, others, such as spinophilin and PNUTS [37–39], have been shown to bind PP1 substrate recognition sites, thereby inhibiting the dephosphorylation of a subset of substrates. Thus, they function identically to CSA and FK-506 with CN [40]; i.e., they inhibit substrates from binding the PSP and thereby selectively inhibit their dephosphorylation.

Therefore, the most promising approach for developing PP1 specific drugs is to identify protein interaction sites that are specific for only a limited number of substrates and/or regulatory proteins (Figure 3, 4). This strategy provides a powerful and specific way to *selectively* modulate PP1 activity against a small subset of substrates and, in turn, target distinct signaling cascades. However, this strategy will also only be successful if the PP1 regulatory code is fully understood [12, 13]. In recent years, much progress has been made in elucidating the PP1 regulatory code [37] (Figure 3, 4). This was mainly driven by the structural assessment of new, additional PP1 holoenzymes, which has allowed for novel primary sequence motifs to be identified that either are necessary for binding PP1 and/or for changing its substrate specificity.

The proof of principle of this approach has been already demonstrated in an exciting report from the Köhn laboratory [41–43]. Here, the authors developed a peptide based on the primary sequence of the PP1 regulator NIPP1 that includes the PP1 RVxF and φφ motifs [41], the two most prevalent PP1 binding motifs in all PP1 regulatory proteins [37]. In collaboration with the Bollen laboratory, they showed that the peptide binds to PP1 and displaces many weaker binding targeting and inhibitory proteins in vitro and in vivo,

abolishing the tight specificity of PP1 (by disrupting its interaction with targeting proteins) and increasing its activity (by disrupting its interaction with inhibitory proteins). Thus, this peptide based drug increases the overall general dephosphorylation in cells, something of potential use in diseases associated with global increases of phosphorylation, such as certain cancers. However, because the increase in dephosphorylation is now unregulated, drugs that target these interaction sites will likely not provide useful therapeutics for specific diseases. To further enhance this approach it will be necessary to target more unique sites that are share by only a very small number of PP1 regulatory proteins, similar as suggested before for CN.

There is now data that suggest it may be possible to selectively target a single PP1-specific pathway. Salubrinal [44, 45] and Guanabenz [46, 47] are small molecule drugs that have recently been shown to specifically inhibit translation by blocking the activity of eIF2α phosphatases, specifically CreP:PP1 and GADD34:PP1 [48–54]. Whether or not this is achieved by selectively disrupting the PP1-substrate (eIF2α) and/or the PP1-regulatory protein (CReP, GADD34) remains to be determined. Finally, small molecules might also be designed to bind specifically to the motifs/domains on substrates that mediate PSP binding, especially if substrate recruitment requires additional domains form the regulatory proteins that are distinct from the phosphatase binding domains. This approach can be applied to PP1 as well as to PP2A [55].

PTPs: developing drugs that bind to intrinsically disordered regions of the enzyme

As numerous other manuscripts in this issue of Bioorganic & Medicinal Chemistry focus on PTPs as drug targets this section focuses on PTP1B and its modulation via its intrinsically disordered C-terminus. PTPs have long been bona fide drug targets [56]. However, molecules that target PTP active sites are difficult to make selective against closely related PTPs. Unfortunately, all programs aimed at targeting the PTP1B active site have failed due to technical challenges arising from the chemical properties of the PTP active site, which leads to highly charged drugs and, as a consequence, limited drug development potential [57]. Recently, new approaches that aim beyond the active site have been introduced for PTPs [58]. Specifically, in a collaboration between the Tonks and Peti laboratories, the small molecule inhibitor MSI-1436 was recently discovered and shown that it binds to the disordered C-terminal domain of PTP1B, C-terminal to the catalytic domain [59]. Moreover, we also showed that MSI-1436 functions using an allosteric mechanism to direct the enzymatic activity of PTP1B. Thus, this work demonstrates that targeting binding sites in disordered domains [60] might also be a new strategy for PTP drug development. Intriguingly, most PP1 regulatory proteins are also intrinsically disordered proteins. Thus the success of identifying an inhibitor that binds to the intrinsically disordered region of PTP1B might also allow for the regulation of PSP regulatory proteins and PSPs.

Summary

Compared to their kinase counterparts, PTPs and especially PSPs were considered to be poor drug targets. However, advances during the few years have shown unequivocally that

PTPs, and especially PSPs, are potent and viable drug targets. In particular multiple structural studies has provided critical new avenues that will allow newly developed therapeutics to not only inhibit, but also be highly selective for only a small set of PSP and/or PTP substrates. These new efforts a sure to be augmented with additional efforts focused on developing biologics that can also modulate the PSPs and PTPs in specific manners. It will be exciting to see which approach will be successful, but the authors are confident that the next years will provide novel, successful route(s) for PSP and PTP drug design.

Acknowledgments

The authors thank the large numbers of highly skilled and ambitious coworkers for their strong support in the effort to better understand protein phosphatases. They are also inclined to many members of the phosphatase field – to many to name – for their support, for their collaborations and their input. This work was supported by NIH grant R01GM098482 to RP as well as an NIH grant R01NS091336 and an American Diabetes Association Pathway to Stop Diabetes Grant 1-14-ACN-31 to WP.

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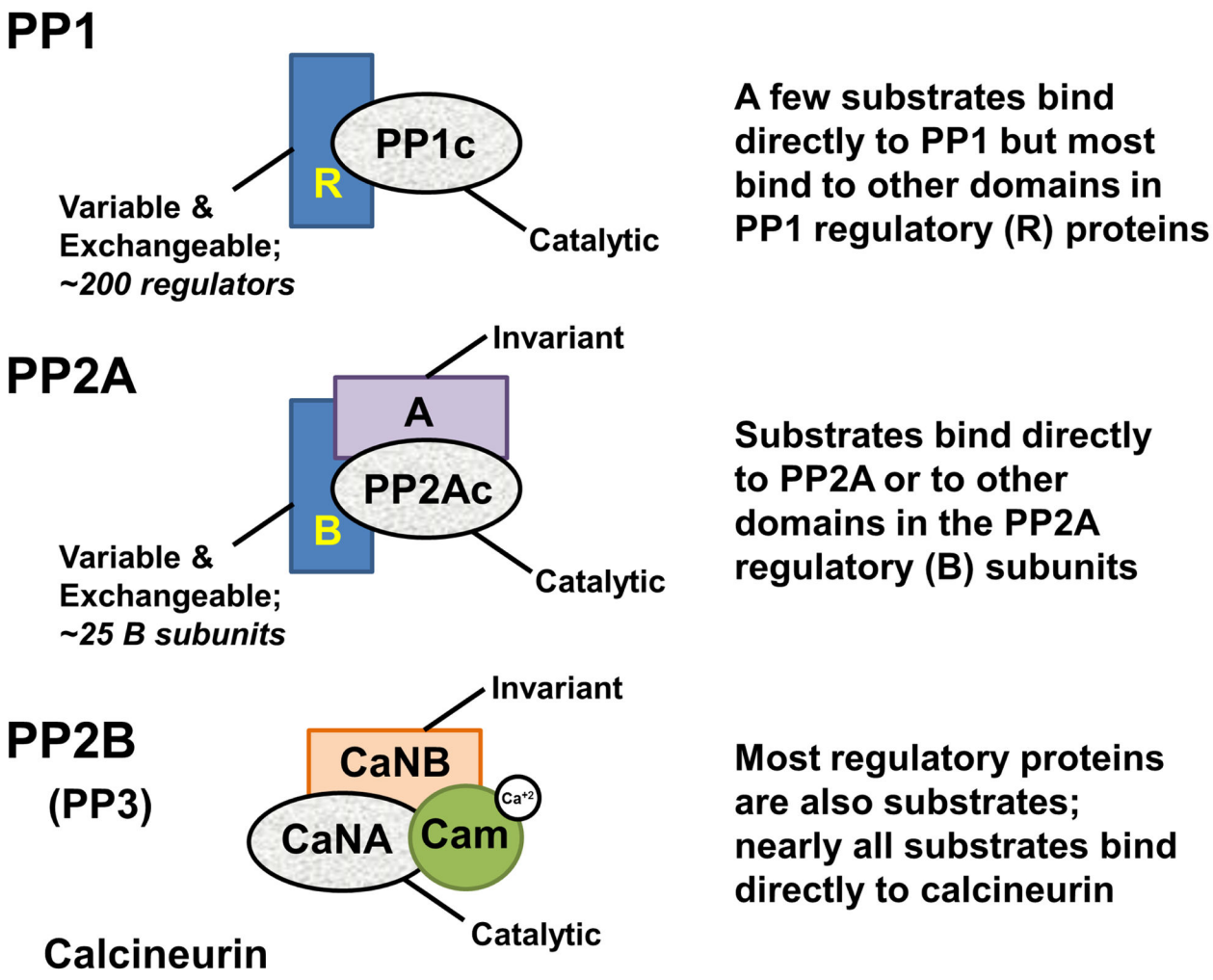

Figure 1. Conservation of PSP catalytic domains

PP1 (top), PP2A (middle) and PP2B/Calcineurin (bottom) have a highly conserved catalytic domain (grey). PP1 interacts with ~200 distinct regulatory proteins (R; blue), which function as inhibitory and targeting proteins. PP1 substrates bind directly to PP1, bind to other domains that are part of the PP1 regulatory proteins to enhance dephosphorylation or are dephosphorylated because PP1 is localized in proximity to the substrate via its targeting proteins. The catalytic domain PP2Ac interacts with an invariant A subunit and ~25 regulatory B subunits to achieve substrate specificity in a manner similar to that of PP1. Calcineurin (CN), on the other hand, is regulated by calcium, which is required for activation. CN binds directly to its substrates via protein interaction motifs that are also used by regulatory proteins.

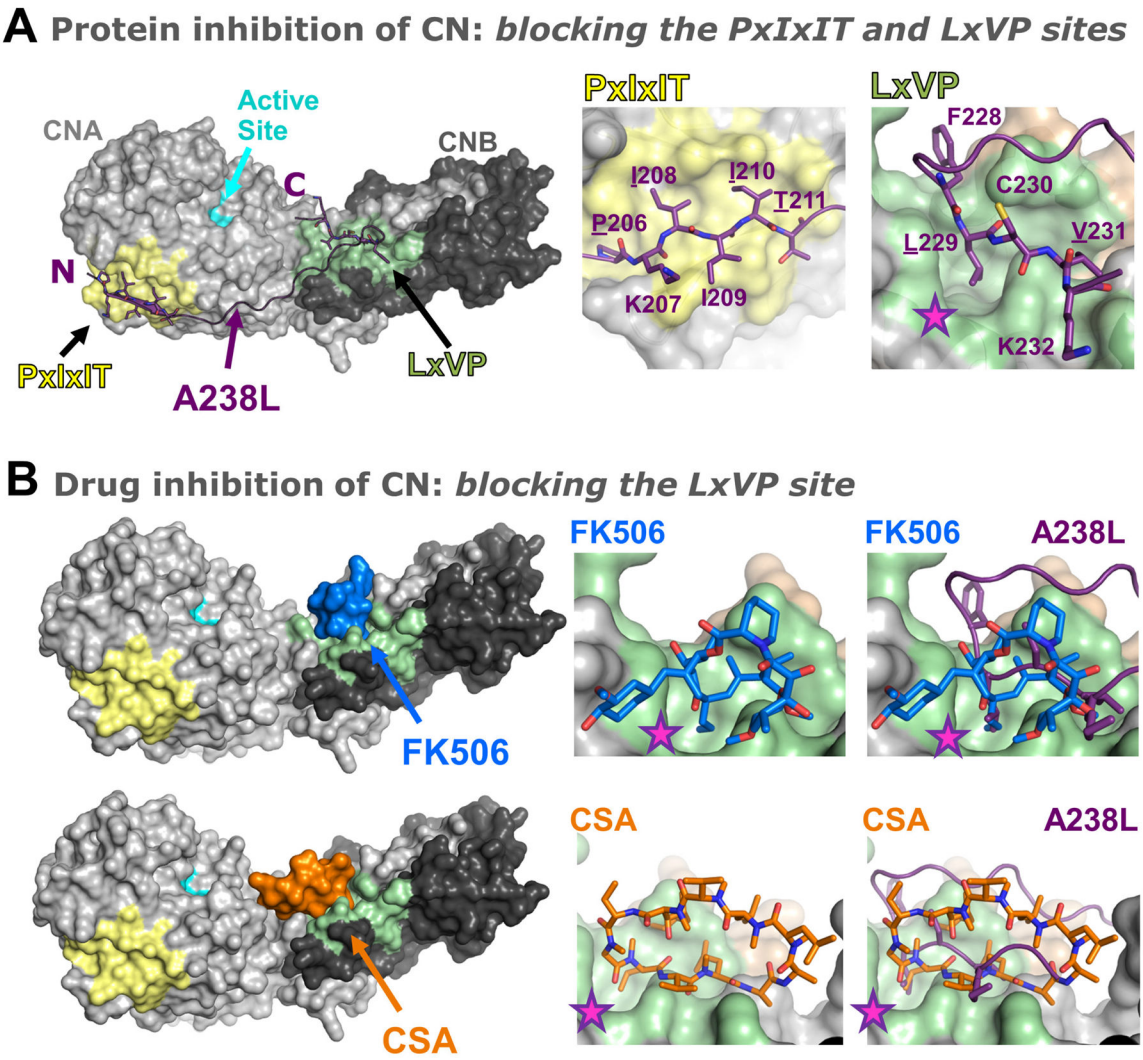

Figure 2. Inhibiting CN by blocking substrate binding

A. The CN protein inhibitor A238L inhibits CN activity not by blocking its active site, but instead by binding to CN substrate recognition grooves, which blocks CN from binding and dephosphorylating its substrates. *Left panel*, surface representation of CN (light/dark grey; left panel) bound to A238L (magenta; shown as a cartoon), a potent protein inhibitor of CN from the African Swine Fever Virus. The active site is shown in light blue. A238L binds CN via both a PxIxIT sequence (*middle panel*) and an LxVP sequence (*right panel*). The deep groove in CN engaged by the 'L' of the LxVP motif (Leu229 in A238L) is indicated by a star (pink). **B.** The immunosuppressant drugs FK506 and cyclosporine A (CSA) bind directly to the CN LxVP docking groove. *Left panels*, surface representations of CN (light and dark grey) bound to FK506 (blue) and CSA (orange). *Upper right panels*, close-up views of FK506 in the CN LxVP binding groove (left) and overlay with A238L (magenta, right). The deep groove in CN engaged by the 'L' of the LxVP motif is indicated by a star (pink) and is fully engaged by FK506. *Lower right panels*, close-up views of CSA in the CN LxVP binding groove (left) and overlay with A238L (magenta, right).

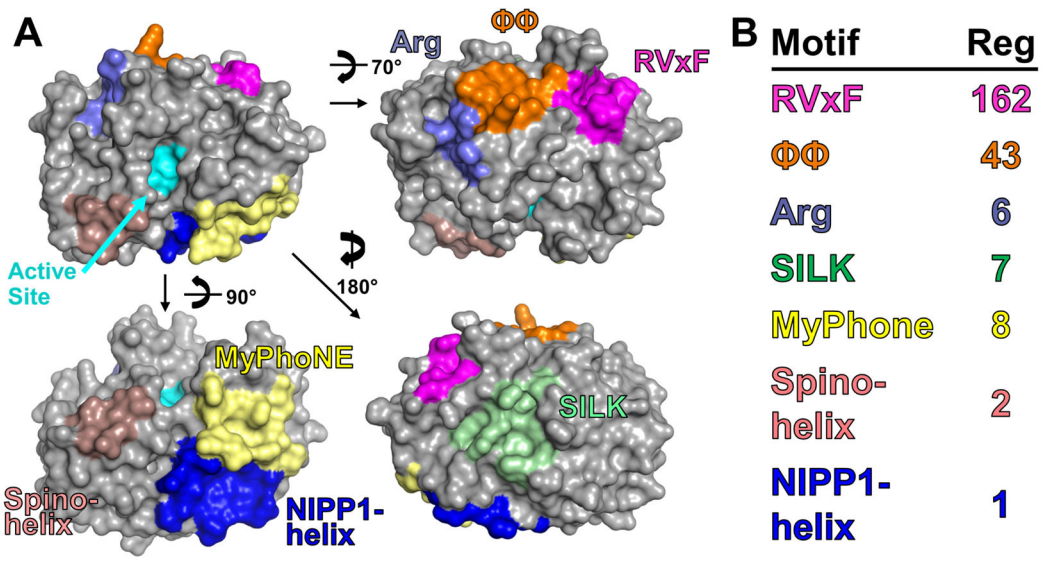

Figure 3. PP1 regulatory protein docking grooves

A. Surface representation of PP1 with its various regulatory protein docking grooves shown (RVxF, pink; φφ, orange; Arg, lavender; SILK, green; MyPhoNE, yellow; NIPP1-helix, dark blue; Spino-helix, light pink). The active site is in cyan. The Inhibitor-2 helix that lies over the active site to directly inhibit PP1 activity is not shown. **B.** The presence of various motifs in confirmed PP1 regulators (189).

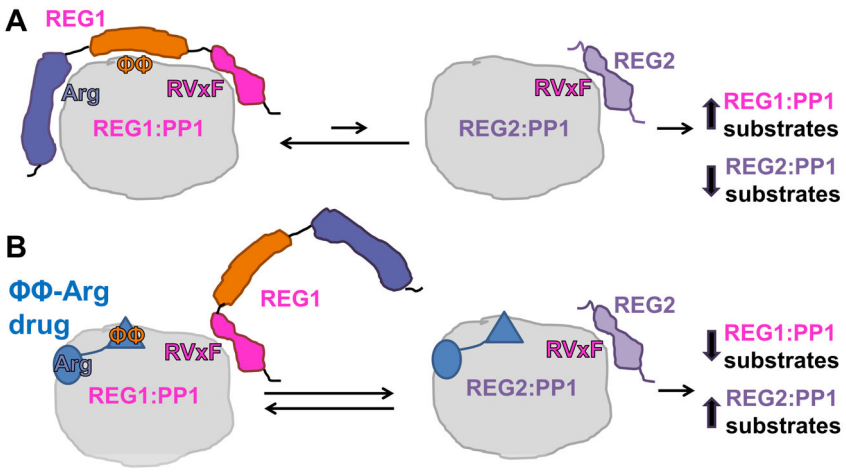

Figure 4. Developing drugs that inhibit only a small subset of PP1 substrates

Toxins that bind and block the PP1 active site are lethal. Thus, any potential drugs that target PP1 must interact *outside* the PP1 active site. One approach is to target the PP1 regulatory protein docking grooves. **A.** The REG1:PP1 holoenzyme (in this case, REG1 represents a PP1 regulatory protein that contains an RVxF-φφ-Arg motif, such as PNUTS) is preferentially populated compared to the REG2:PP1 holoenzyme (in this case, REG2 is a PP1 regulatory protein with only an RVxF motif), because the affinity of REG1 for PP1 is much higher than REG2 for PP1. **B.** A drug that targets only the φφ-Arg binding grooves will selectively displace the motifs in REG1 that bind at these sites, thereby reducing the affinity of REG1 for PP1 and, consequently, increasing the likelihood of forming REG2:PP1 holoenzymes. In this way, dephosphorylation of REG1:PP1 substrates will decrease while REG2:PP1 holoenzymes will increase.