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Challenges and Opportunities in the Development of Protein Phosphatase-Directed Therapeutics

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ABSTRACT: Protein phosphatases have both protective and promoting roles in the etiology of diseases. A prominent example is the existence of oncogenic as well as tumor-suppressing protein phosphatases. A few protein phosphatase activity modulators are already applied in therapies. These were however not developed in target-directed approaches, and the recent discovery of phosphatase involvement followed their application in therapy. Nevertheless, these examples demonstrate that small molecules can be generated that modulate the activity of protein phosphatases and are beneficial for the treatment of protein phosphorylation diseases. We describe here strategies for the development of activators and inhibitors of protein phosphatases and clarify some long-standing misconceptions concerning the druggability of these enzymes. Recent developments suggest that it is feasible to design potent and selective protein phosphatase modulators with a therapeutic potential.

PROTEIN KINASE *VERSUS* PHOSPHATASE-DIRECTED THERAPEUTICS

Nearly every cellular process is controlled by phosphorylation of key regulatory proteins on specific serine (Ser), threonine (Thr), or tyrosine (Tyr) residues. The covalent attachment of a bulky and negatively charged phosphate group by a kinase usually induces a conformational change that affects protein function. Mammalian genomes harbor more than 500 genes that encode protein kinases.[1] Almost all of these belong to the same protein kinase superfamily and have a highly similar catalytic core and mechanism, implying that they originate from a common ancestor. In recent years protein kinases have become one of the most popular drug targets. Kinase inhibitors are used as a therapy for diverse pathologies but are most successful for the treatment of cancers that are driven by a single oncogenic kinase, such as chronic myeloid leukemia or gastrointestinal stromal tumors.[2,3] At present, 17 small-molecule kinase inhibitors have been approved by the USA-FDA for therapeutic use and many more are undergoing clinical trials. Kinase inhibitors, including those that cannot be used as drugs for reasons of toxicity or poor bioavailability, are also extremely useful tools for studying protein kinase functions since they act within minutes, before compensatory effects can be developed as often encountered with knockdown or knockout approaches.

Small-molecule inhibitors of protein kinases are classified into four groups.[4] Type-1 inhibitors compete with ATP for the nucleotide binding site and are usually rather aspecific due to the high conservation of the catalytic site residues. However, some type-1 inhibitors show a considerable specificity due to contacts with nonconserved residues in the hinge region between the two kinase lobes. Type-2 inhibitors are generally more selective because they not only bind to the nucleotide binding site but also penetrate into a flanking allosteric pocket that is specific for a subset of protein kinases. Type-3 inhibitors target exclusively this allosteric pocket. Finally, type-4 inhibitors bind to sites that are remote from the catalytic site. Despite some spectacular successes, the therapeutic use of kinase inhibitors has its limitations. An unanticipated side effect of some inhibitors is that they stabilize a specific protein conformation that promotes kinase-activity-independent scaffolding functions.[5] Another recurring problem is the development of drug resistance, which can mostly be explained by (1) mutation of the targeted kinase, (2) activation of other kinases that substitute for the inhibited kinase, (3) inactivation of phosphatases that oppose the targeted kinase, or (4) bypassing of the inhibited kinase by activation of downstream pathway components.[3] Drug resistance can be delayed or prevented by using polypharmacological approaches such as applying multi-kinase inhibitors, cocktails of specific kinase inhibitors, or combinations of kinase inhibitors and drugs that target other key proteins. An effective strategy to overcome drug resistance has been to use "next generation" inhibitors once the targeted kinase is mutated and has become resistant to the "first-line" inhibitor.

Most protein phosphorylations have a high turnover rate because the involved kinase and counteracting phosphatase are simultaneously active. Hence, a change in the phosphorylation status often just reflects an altered balance between both enzyme activities. From this viewpoint protein kinases and phosphatases are equally attractive targets to interfere with protein phosphorylation. Yet, compared to protein kinases, much less effort has been put into the development of small-molecule effectors of protein phosphatases. There are a number of obvious explanations for this limited interest in the

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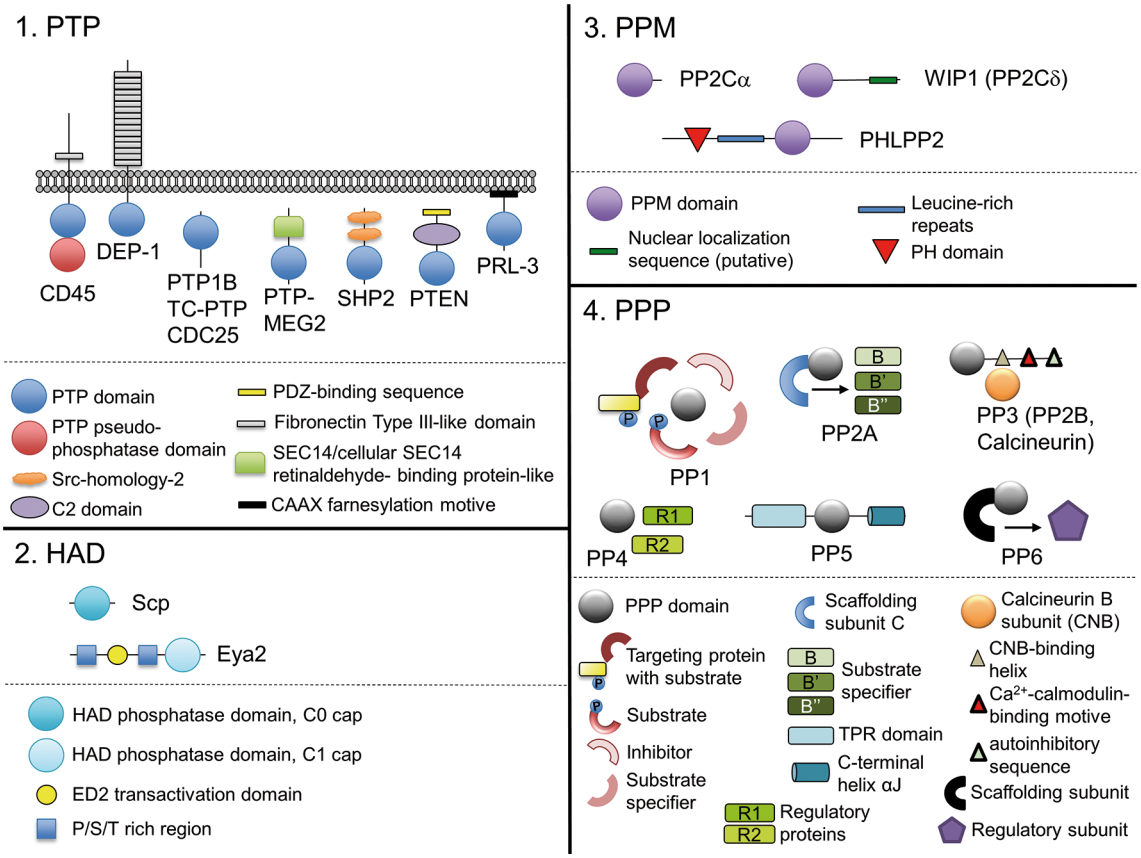

Figure 1. Subunit structure of protein phosphatases. Phosphatases of the superfamilies PTP,[9,100] PPP,[101,102] HAD,[8] and PPM[103,104] discussed in this article are shown.

development of protein phosphatase-directed therapeutics. First, the catalytic site of protein phosphatases lacks a pocket for a small molecule, such as ATP for kinases, which can be used as a starting point for the screening or design of analogues. Second, the catalytic site of protein phosphatases is positively charged, and the screening of compound libraries usually yields negatively charged inhibitors with a poor cell permeability and bioavailability. Third, the persistent misapprehension that protein phosphatases are less specific and less tightly regulated than protein kinases renders them less appealing as therapeutic targets. Fourth, the equivalent of a kinase inhibitor is a phosphatase activator, and enzyme activators are generally considered to be difficult to make.[6]

In this Review we will discuss recent data that have brought protein phosphatases into the limelight of therapeutics. There is now compelling evidence for a therapeutic benefit of both phosphatase activators and inhibitors, depending on the targeted pathology and phosphatase. Moreover, novel structural insights suggest avenues for the design of potent bidentate or allosteric phosphatase inhibitors as well as for the generation of activators of some protein phosphatases. Finally, initial data show synergisms between small-molecule kinase inhibitors and phosphatase activators, which pave the way for the development of combination therapies with reduced problems of toxicity and drug resistance.

PROTEIN PHOSPHATASES: DIVERSITY AND SELECTION OF SUITABLE DRUG TARGETS

Vertebrate genomes contain ~150 genes that encode protein phosphatase catalytic subunits.[7,8] They can be classified into four structurally unrelated superfamilies (Figure 1). The PTP (protein tyrosine phosphatase) superfamily comprises ~105 enzymes, which is similar to the diversity of protein tyrosine kinases. PTPs include the classical receptor-like and non-receptor-like protein tyrosine phosphatases, the rhodanese-derived Cdc25 phosphatases and the "dual-specificity" protein phosphatases, named for their ability to dephosphorylate Ser, Thr, and Tyr residues. Some members of the PTP superfamily are also able to dephosphorylate phospholipids, RNA, or glycogen.[9] The diverse superfamily of HAD (haloacid dehalogenase) hydrolases includes a group of ~10 Ser/Thr/Tyr protein phosphatases. Among the best characterized HAD members are the RNA polymerase-II C-terminal domain phosphatases. The ~20 members of the PPM (protein phosphatase metal) superfamily are Mg^{2+}- or Mn^{2+}-dependent monomeric phosphatases that specifically dephosphorylate Ser and Thr residues. Finally, the PPP (phosphoprotein phosphatase) superfamily comprises only ~15 protein phosphatases. However, together PPPs catalyze over 90% of all Ser and Thr dephosphorylations in eukaryotic cells. This broad activity spectrum can be explained by the ability of some PPP members, in particular protein phosphatases PP1 and PP2A, to form stable complexes with a wide array of regulatory subunits to generate hundreds of distinct dimeric or trimeric holoenzymes, each with a unique set of substrates and regulation.[10,11] Thus, although there are far fewer Ser/Thr protein phosphatases (~45) than Ser/Thr protein kinases (~400), they have a similar diversity and substrate specificity at the holoenzyme level. This shows that the targeting of Ser/Thr

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Table 1. Some Small-Molecule Effectors of Validated Protein Phosphatase Drug Targets

phosphatase	effector	modulator[a]	targeted pathology	ref
PTP Superfamily				
TC-PTP	mitoxantrone	Type 2′	cancer	41
	phosphonodifluoromethyl phenylalanine derivatives	Type 2	diabetes, Crohn's disease and reumatoid arthritis	71
CDC25	vitamin K3-like quinone derivatives	Type 1	cancer	73
DEP-1 (R-PTPη)	dimerizing peptide	Type 7′	cancer	62
SHP2 (PTP-1D)	sodium stibogluconate	Type 1	cancer	72, 76
	II-B08	Type 2	cancer	77
PTP1B	ISIS-PTP1BRx	antisense oligonucleotide	diabetes and obesity	
	dibenzo[*b*,*d*]furan monocarboxylic acid derivatives	Type 2	diabetes and obesity	15, 66, 69
PRL-3	thienopyridone and anthraquinones	?	cancer	74, 75
LYP	LTV-1	Type 4	autoimmune diseases	83
CD45	L158429, R164259, S349631, and purpurin	?	immune and inflammatory diseases	81, 82
RPTPα		Type 9	cancer	
PTP-MEG2	phosphonodifluoromethyl phenylalanine derivatives	Type 2	diabetes	70
PTEN	bisperoxovanadate derivatives	Type 1	ischemia and reperfusion injury	84, 85
HAD Superfamily				
Eya2	NCGC00241225	?	cancer	91
Scp	rabeprazole	Type 3	neurodegenerative disorders	90
PPM Superfamily				
PP2Cα	NPLC 0393	Type 1′	liver fibrosis	33
WIP1 (PP2Cδ)	thioether peptide	Type 1	cancer	93
PHLPP2	NCS 134149	Type 1	diabetes and heart disease	92
PPP Superfamily				
PP1/GADD34	guanabenz	Type 6	viral infection and protein folding diseases	29
PP1/RVxF-PIP	RVxF-mimics	Type 1′	cancer	37
	PDP3	Types 4′ and 5′	cancer	61
PP1/Tat	1H4	Type 5	HIV	38
PP1/G_L	CP-320626	Type 3′	diabetes	44, 45
PP2A and PP4	fostriecin	Type 1	cancer	87
PP2A/CIP2A	bortezomib and rabdocoetsin B	Type 6′	cancer	51, 57−60
PP2A/SET	FTY720	Type 5′	cancer	55, 56
PP3 (calcineurin, PP2B)	cyclosporin A and FK506	Type 8	organ transplant rejection	28
PP5	chaulmoogric acid	Type 2′	Alzheimer's disease	40
PP6			cancer	19

[a]The modulators refer to phosphatase activators or inhibitors, as defined in Figures 2 and 3, respectively. The activator types are indicated with a prime (′) to distinguish them from inhibitors.

protein kinases or phosphatases has a similar selectivity when directed against holoenzymes.

Several strategies can be employed to identify or validate protein phosphatases with a therapeutic potential (Table 1). One approach is to focus on protein phosphatases that act antagonistically or synergistically with protein kinases that are already validated drug targets. A large proportion of the current efforts in kinase inhibitor development for the treatment of cancer targets receptor tyrosine kinases and components of the MAP-kinase or PI3-kinase pathways.[3,4] Not surprisingly, the protein phosphatases that function in these pathways also emerge as attractive targets for novel anticancer therapies.[12−16] These include both tumor-suppressing (*e.g.*, DEP-1, PTEN, PHLPP, PP2A) and oncogenic phosphatases (*e.g.*, SHP2, RPTPα, PRL-3, WIP1 (PP2Cδ)). Some phosphatases (*e.g.*, TC-PTP) act as either oncogenes or tumor suppressors, depending on the cellular context. Hence, it is a misconception that phosphatase-directed therapeutics necessarily involves enzyme activators. A contribution of specific protein phosphatases to the etiology of diseases can also be indirectly inferred from their altered activity or expression level. Thus, oncogenic protein phosphatases are often activated in specific cancers by gain-of-function mutations or gene amplification, whereas the tumor suppressive functions of protein phosphatases are countered by inactivating mutations, loss of heterozygosity, a decreased expression of the phosphatase due to promoter hypermethylation, or an increased expression of inhibitory proteins. For example, inactivating mutations of a series of PTPs and noncatalytic subunits of PP2A in cancer provide strong evidence for their tumor-suppressive function.[17,18] Large scale whole-exome sequencing recently identified mutations in the catalytic subunit of the PPP phosphatase PP6 as a key driver for the development of melanoma.[19] This unbiased approach can also be adopted for the identification of other protein phosphatases that are implicated in specific pathologies. Knockdown techniques are also helpful for the mapping of phosphatases or their regulators as candidate therapeutic targets. For example, the down-regulation of the PPM phosphatase WIP1 (PP2Cδ) sensitizes ovarian carinoma cells to the DNA-damaging agent cisplatin,[20]

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suggesting that WIP1 inhibition can be implemented to treat chemoresistant tumors. siRNA screens have been used to map "survival" phosphatases, which cause cell death when suppressed by RNAi and hence are possible targets for novel cancer therapies.[21] Similarly, siRNA screens can be employed to map protein phosphatases that rescue or aggravate a disease phenotype or a kinase-inhibitor induced phenotype.[22−24] For some protein phosphatases (*e.g.*, PP1, PP2A, CDC25) knockdown approaches are not suited to delineate a link with diseases because their loss is either lethal or compensated by isozymes. In view of well-established activity-independent scaffold functions of some protein phosphatases,[25−27] it should be kept in mind that the knockdown or inhibition of a phosphatase does not necessarily have the same phenotypic outcome.

Serendipity has also played an important role in protein phosphatase target selection and validation. Cyclosporin A and FK506 were approved for clinical use as potent immunosuppressants long before they were known to act *via* inhibition of the PPP member PP3, also known as PP2B or calcineurin.[28] Guanabenz, a clinically approved α2-adrenergic agonist used to treat hypertension, was recently found to specifically disrupt one of the PP1 holoenzymes that dephosphorylates eIF2α.[29] Phosphatase inhibitors that keep eIF2α phosphorylated at Ser51, and thereby inactive, hold great promises for the treatment of protein misfolding disorders and viral infections.[29,30] The sphingosine-like molecule FTY720, an immunomodulator that downregulates the shingosine-1-phosphate receptor 1 and is approved for the treatment of multiple sclerosis,[31] was recently found to activate PP2A and kill leukemia cancer cells that have become resistant to the protein kinase inhibitor imatinib.[32] These and other (see below) serendipitous findings represent excellent evidence that small molecules can be generated that modulate protein phosphatase activity and are beneficial for the treatment of protein phosphorylation diseases. These data also indicate that it may be worthwhile to systematically examine the effects of all clinically approved drugs on protein phosphatases.

SMALL-MOLECULE ACTIVATORS OF PROTEIN PHOSPHATASES

Allosteric Activators of the Catalytic Domain. Very little is known about physiological allosteric activators that directly interact with the catalytic domain, making it is difficult to rationally design small-molecule activators of this kind (Figure 2, type 1′). Screening of a natural product library identified NPLC0393 as an activator of the PPM phosphatase PP2Cα in the low micromolar range.[33] Since PP2Cα is monomeric and consists of little more than a catalytic domain, this compound presumably acts as an allosteric activator of the catalytic domain. NPLC0393 did not affect the activity of PTP and PPP superfamily members and prevented the development of liver fibrosis in various mouse models, making it a therapeutically attractive lead compound. Other structurally unrelated activators of PP2Cα have been identified but they were active only at much higher concentrations.[34] The bioactive sphingolipid ceramide activates not only PP2C but, surprisingly, also the structurally unrelated catalytic subunits of the PPP phosphatases PP1 and PP2A.[35,36]

The catalytic subunit of PP1 has a hydrophobic surface groove that is remote from the catalytic site and mediates the binding of regulatory subunits *via* the so-called RVxF-type (one-letter residue code, x is any residue) docking motif.

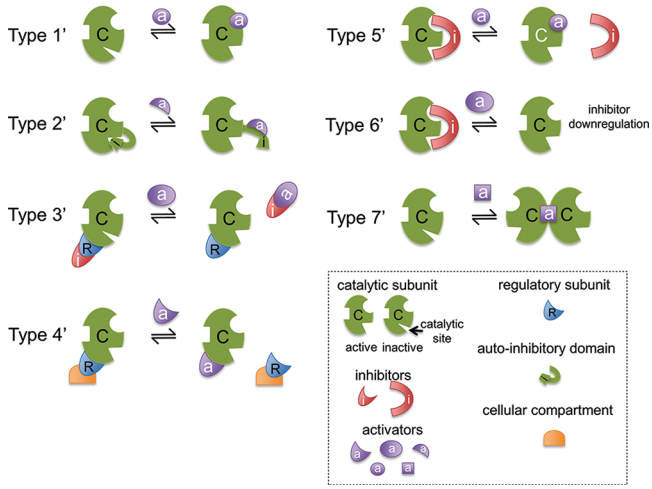

Figure 2. Small-molecule activators of protein phosphatases.

Intriguingly, short synthetic RVxF-containing peptides activate the purified catalytic subunit of PP1 up to 2-fold, hinting at an allosteric effect.[37] This activation is recapitulated with a small molecule that shows some structural resemblance with RVxF-peptides,[37] but not with another RVxF-mimick that was identified by a virtual screening.[38]

Allosteric Activators of Regulatory Domains or Subunits. Many protein phosphatases are kept in a dormant state by an allosterically regulated noncatalytic domain or regulatory subunit. For example, the activity of the PPP phosphatase PP5 is suppressed by its tetratricopeptide repeat (TPR) domain, but this TPR-mediated inhibition is alleviated by the binding of fatty acids or heat shock protein 90.[39] A biochemical screening identified chaulmoogric acid as an allosteric activator of PP5 (Figure 2, type 2′), which was subsequently shown to affect the tertiary structure of the TPR domain.[40] Fatty acids and chaulmoogric acid acted synergistically, suggesting that they have distinct TPR binding sites. A similar regulation applies to TC-PTP, which is activated by the α-cytoplasmic tail of $\alpha1\beta1$ integrin through alleviation of autoinhibition of the phosphatase by its C-terminal tail. Mattilla *et al.*[41] identified small molecules that mimick the TC-PTP/α1 interaction and activate the phosphatase. One of these compounds is the topoisomerase-II inhibitor mitoxantrone, which is already clinically used for chemotherapy.

A complex of PP1 and the hepatic glycogen-targeting subunit G_L dephosphorylates and activates glycogen synthase, the rate-limiting enzyme of glycogen synthesis. This PP1 holoenzyme is allosterically inhibited by the active *a* form of the glycogen-degrading enzyme phosphorylase.[42] Since disruption of phosphorylase *a* binding, *e.g.*, by mutation of its docking site, improves glucose tolerance in mice,[43] inhibitors that block the interaction of G_L with phosphorylase *a* are being sought as a new type of antidiabetic drug. One such small-molecule, CP-320626 (Figure 2, type 3′), has already been shown to cause the activation of the PP1/G_L complex as a glycogen synthase phosphatase.[44,45]

Activators That Disrupt PPP Holoenzymes. Some regulatory subunits of PPP members anchor the phosphatase at a specific cellular location and thereby limit the enzyme's action radius to local substrates. In addition, some PPP-interacting proteins sterically occlude specific substrate binding sites[46,47] or act as true inhibitors by blocking access to the active site.[48,49] Hence, these phosphatases can be activated with

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molecules that decrease the concentration or binding of inhibitory subunits.

PP2A is a well-established tumor suppressor, and the activity of some PP2A holoenzymes is inhibited in cancer cells by upregulation of the oncogenic inhibitors CIP2A and SET.[18,49,50] These inhibitors have been advanced as molecular targets to induce PP2A-mediated apoptosis in cancer cells.[51,52] A short peptide derived from apolipoprotein E activates PP2A by displacing SET.[52,53] The sphingolipid ceramide[54] and the sphingosine-like molecule FTY720 appear to do the same[55,56] and therefore represent attractive lead compounds for the development of more potent small-molecule activators of PP2A (Figure 2, type 5′). The natural compound rabdocoetsin B activates PP2A by inhibiting the transcription of CIP2A (Figure 2, type 6′).[51] Likewise, the *N*-protected dipeptide bortezomib induces apoptosis in cancer cell lines by a mechanism that is unrelated to its action as a proteasome inhibitor and involves the activation of PP2A through downregulation of the CIP2A transcript.[57−59] Interestingly, bortezomib acts synergistically with the kinase inhibitor sorafenib and the agonistic death-receptor antibody CS-2008 to induce apoptosis, highlighting its potential for a combination therapy.[57,58,60]

The recruitment of many targeting or inhibitory subunits of PP1 is mediated by the earlier described RVxF motif.[11,48] Recently, a cell-permeable and modified RVxF-based peptide (PDP3) was described that competitively disrupts a subset of PP1 holoenzymes and generates PDP3-associated PP1 that is fully active and dephosphorylates established PP1 substrates.[61] This shows that small-molecule competitors can be used to generate a pool of free PP1 that is no longer restrained by targeting (Figure 2, type 4′) or inhibitory subunits (Figure 2, type 5′). PDP3 or small molecules with the same effect may have therapeutic benefits, for example, to sensitize tumor cells to protein kinase inhibitors.

An Activator That Causes PTP Dimerization. The receptor-like PTP phosphatase DEP-1 opposes various oncogenic receptor tyrosine kinases. Recently, DEP-1 agonists were isolated from a random peptide phage-display library.[62] Intriguingly, these agonists circularize *in vitro via* an intramolecular disulfide bridge and form stable dimers that induce the dimerization-mediated activation of DEP-1 (Figure 2, type 7′). Consistent with the activation of DEP-1 as a tumor suppressor, these peptides reduced proliferation and triggered apoptosis of cancer cells.

SMALL-MOLECULE INHIBITORS OF PROTEIN PHOSPHATASES

Inhibitors of the Catalytic Domain. By far most efforts from academia and industry in the rational development of protein phosphatase-directed therapies have gone into the search for specific inhibitors of protein tyrosine phosphatases.[15,63−67] This has turned out to be a great challenge since the screening for active-site inhibitors of PTPs (Figure 3, type 1) often yields negatively charged phosphotyrosine mimetics with a limited cell membrane permeability. Another recurrent problem is the recovery of nonspecific oxidative compounds that form covalent adducts with the active-site cysteine of PTPs. The low bioavailability of lead compounds has been partially addressed by charge reduction, the introduction of hydrophobic groups, and their cellular delivery as prodrugs.[63,64] However, even the best phosphotyrosine mimetics only bind with a rather low affinity and also exhibit a poor specificity due to the conserved nature of the PTP catalytic site. This has inspired the development of bidentate inhibitors, which bind to both the catalytic site and a unique adjacent peripheral site (Figure 3, type 2). An alternative strategy aims at the development of type-4 inhibitors, which bind to an allosteric site that is remote from the active site and stabilize an inactive conformation. One set of type-4 inhibitors prevents the covering of bound substrates at the active site by a phosphate-capping "WPD" loop. Using conformation-specific antibodies it was recently demonstrated that it may also be possible to target PTPs with compounds that stabilize a naturally occurring oxidized inactive form.[68]

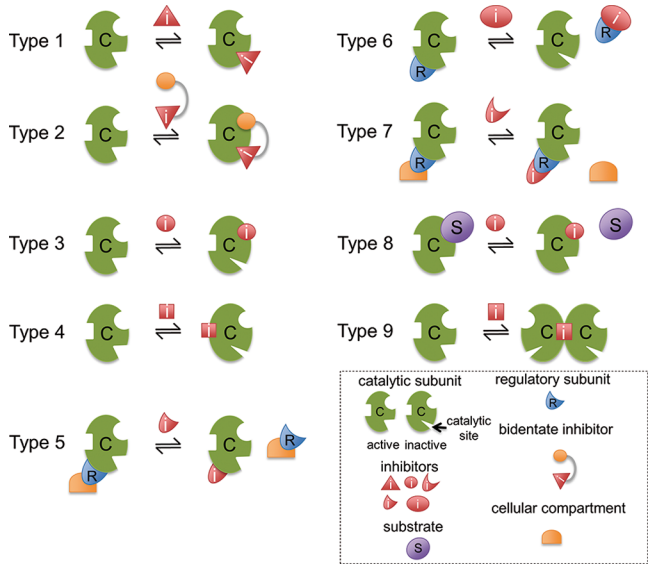

Figure 3. Small-molecule inhibitors of protein phosphatases.

The "hottest" PTP target is PTP1B, which opposes insulin and leptin signaling and is a validated target for the treatment of type-2 diabetes and obesity.[63,64,66,68] A PTP1B-directed antisense-based oligonucleotide (ISIS-PTP1BRx) has entered phase 1 clinical trials. In parallel, numerous fairly selective inhibitors of PTP1B have been developed that act in the low nanomolar range. Some of these can be administered orally and have reasonable pharmacokinetic properties, including dibenzo-[*b*,*d*]furan monocarboxylic acid derivates.[15,66,69] Although there are still many hurdles to be overcome, PTP1B inhibitors are likely to become the first rationally designed protein phosphatase inhibitors that make it to the clinic. PTP1B-directed drug design has also inspired the development of inhibitors for other PTPs. For example, Zhang *et al.*[70] used an elegant stepwise focused library approach to transform a weak and aspecific phosphotyrosine mimetic into a highly potent and specific bidentate inhibitor of PTP-MEG2, another antagonist of insulin signaling. A similar approach enabled the development of a potent inhibitor of TC-PTP,[71] a modulator of cytokine signaling through the Jak/Stat pathways. Other PTPs, including SHP2,[72] CDC25,[73] and PRL-3,[74,75] are being developed as targets for anticancer therapies. SHP2 and some related phosphatases are inhibited by sodium stibogluconate, a known agent against leishmaniasis.[76] Sodium stibogluconate is currently clinically tested as an anticancer agent in combination with interferon alpha. The structurally unrelated SHP2 inhibitor II-B08 was identified from a focused library of indol-based salicylic acid derivatives and shown to act synergistically with a PI3 kinase inhibitor to prolong the survival of leukemic mice.[77] The cell-cycle regulator Cdc25 is

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irreversibly inhibited by vitamine-K3-like quinone derivatives, but toxicity concerns have prompted a search for other small-molecule inhibitors.[73] PRL-3 is inhibited by different compounds such as thienopyridone[78] and anthraquinones,[79] which could serve as starting points for the development of more potent inhibitors. To date, however, these inhibitors are not selective, and their mode of action is often not known. Recently, the application of PRL-3-specific chimeric antibodies was shown to reduce the formation of PRL-3-expressing tumors in mice, suggesting the possibility of antibody therapy against intracellular targets in cancer.[80] The receptor-like tyrosine phosphatase CD45 is an attractive target for the treatment of immune and inflammatory diseases. However, only a few nonselective inhibitors have been described so far.[81,82] A high-throughput screening led to the identification of LTV-1, a type-4 inhibitor that rather selectively interferes with the phosphate-capping "WPD" loop of the lymphoid tyrosine phosphatase Lyp.[83] LTV-1 was active in intact T-cells and has potential as a lead compound for the treatment of autoimmune diseases. Finally, the inhibition of PTEN by bisperoxovanadate molecules has been reported to enhance PKB/Akt signaling and to protect the myocardium and liver from ischemia-reperfusion injury.[84,85]

A considerable number of natural cytotoxins are potent type-1 inhibitors of PPP phosphatases.[86,87] These include compounds as diverse as polyether fatty acids (*e.g.*, okadaic acid), terpenoids (*e.g.*, cantharidin), phosphate esters (*e.g.*, fostriecin), and cyclic peptides (*e.g.*, microcystin). Most of these toxins do not show a high selectivity for specific PPP members. An exception is fostriecin, which inhibits PP2A and PP4 with an IC_{50} of ∼1−5 nM but inhibits PP1 only at ∼10,000-fold higher concentrations. This shows that it is possible to generate specific inhibitors for PPP phosphatases based upon minor differences in catalytic site residues. Cantharidin is used as a topical medication to remove warts but, like most PPP-directed toxins, is too toxic for internal use. However, cantharidin derivatives entrapped in nanoparticles have recently been shown to display a lower cytotoxicity and higher tumor-targeting efficacy,[88] which represents a promising avenue to exploit PPP-directed toxins therapeutically. Fostriecin is much less toxic than most PPP inhibitors, probably because it is more selective. This inhibitor forces cancer cells prematurely into mitosis, ultimately resulting in apoptosis. At first glance, this antitumor action of fostriecin seems contradictory with the well-established function of PP2A as a tumor suppressor. However, the tumor-suppressor function only involves a subset of PP2A holoenzymes, whereas fostriecin indiscriminately inhibits all PP2A and PP4 holoenzymes, some of which fulfill essential functions in cell-cycle progression. Despite its potent antitumor activity, clinical trials with fostriecin have been suspended, mainly because of its low stability. The cardiac expression of specific inhibitory peptides of PP1 result in a hyperphosphorylation of phospholamban, a critical regulator of Ca^{2+} uptake in the sarcoplasmic reticulum.[89] Hence, type-1 inhibitors of PP1 can potentially be used as a novel therapeutic strategy to restore Ca^{2+} homeostasis and contractility of the failing heart.

Zhang *et al.*[90] identified rabeprazole as a specific inhibitor of the HAD phosphatase Scp, which can serve as a lead compound for the development of drugs that induce neuronal stem cell differentiation. Rabeprazole binds to a hydrophobic pocket adjacent to the active site and thus qualifies as a type-3 inhibitor (Figure 3). The inhibition of Scp does not explain the clinical use of rabeprazole as an antiulcer drug, which stems from its ability to bind covalently to the active-site cysteine of the H^+/K^+ ATPase. Recently, a set of mixed-type inhibitors of the HAD phosphatase Eya2 were described.[91] Eya2 is a metastasis-enhancing tyrosine protein phosphatase that dephosphorylates histone H2AX at Ser142. Eya2 inhibitors could be useful as anticancer drugs, possibly in combination with DNA-damaging agents.

PHLPP phosphatases, which belong to the PPM superfamily, inactivate the survival protein kinases Akt and PKC. Sierecki *et al.*[92] identified specific type-1 and type-2 inhibitors of PHLPP2, which could be useful where survival pathways are relevant, notably diabetes (islet transplant and survival) and heart disease. However, PHLPP is also a tumor suppressor,[16] indicating that the long-term use of PHLPP inhibitors may be detrimental. The oncogenic action of the PPM phosphatase PP2Cδ (encoded by WIP1) is related to its ability to inactivate several proteins that are important for stress responses, including p53 and a number of protein kinases. Hayashi *et al.*[93] described a potent and selective cyclic thioether peptide inhibitor of PP2Cδ. However, this inhibitor carries two phosphoric acid groups that limit its cellular bioavailability.

Inhibitors That Disrupt Holoenzymes. Most receptor-like PTPs are inactivated by homo- or heterodimerization.[15,94] Hence, bivalent antibodies that bind to the extracellular domain of these phosphatases and induce their dimerization could be used to block downstream signaling (Figure 3, type 9). Possible targets include oncogenic receptor-like PTPs like RPTPα. A major challenge for this antibody approach is the expected lack of specificity due to the absence of unique motifs in the extracellular domains. An alternative approach would be to screen a peptide library for dimerizing cyclic peptides. Such peptides have already been identified for DEP-1, which is, however, activated by dimerization.[62]

PP1 holoenzymes are created by the binding of PP1-interacting proteins (PIPs) *via* short docking motifs to surface grooves of the catalytic subunit.[11,48] About 10 distinct PP1-docking motifs have already been characterized in some detail, including the earlier described RVxF motif. PIPs differ in the number and combination of binding motifs, which forms the basis for a PP1-binding code that accounts for the existence of hundreds of functionally distinct holoenzymes. The relative importance of specific PP1-docking sites is holoenzyme-dependent. Thus, synthetic RVxF peptides can only be used as competitive disruptors of PP1 holoenzymes with an engaged RVxF-binding groove if there are no other high-affinity interaction sites between the subunits. Consistent with this notion, the RVxF-mimicking molecule 1H4 disrupts the interaction between PP1 and the HIV-transactivator protein Tat but has no effect on the association of PP1 with any of the other tested RVxF interactors.[38] The PP1-binding code can be exploited for the rational design of (modified) peptides that disrupt subsets of PP1 holoenzymes (Figure 3, types 5−7) and prevent the dephosphorylation of their substrates. The specificity of these disruptors can be enhanced by targeting less common PP1 docking sites. Even if such peptides or small-molecule mimics would disrupt multiple PP1 holoenzymes, this could still be therapeutically beneficial, as demonstrated by the clinical use of compounds such as sunitinib that inhibit dozens of distinct protein kinases. If the targeted PP1 holoenzymes are quantitatively important, off-target effects of the disrupting peptides could arise from uncontrolled dephosphorylation by the released catalytic subunit. This problem could be addressed

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by the design of bidentate inhibitors that not only disrupt the targeted holoenzyme(s) but also inhibit the released catalytic subunit.[61] An alternative and more selective strategy to disrupt PP1 holoenzymes is to screen for molecules that bind to PIPs. This is nicely illustrated by the specific binding of guanabenz to GADD34 (Figure 3, type 6), which prevents the recruitment of PP1α and hampers the dephosphorylation of eIF2α.[29] PP1-docking motifs often reside in intrinsically disordered domains that only become structured upon binding to PP1.[48] These interactions are characterized by a high specificity but low affinity and are excellent targets for drugs that prevent PP1 recruitment by promoting folding of the disordered region.[95]

Inhibitors of Substrate Recruitment or Subcellular Targeting. Many PIPs are themselves substrates for associated PP1.[11] Hence, the PP1-binding code can also be exploited to design compounds that interfere with the recruitment of subsets of substrates (Figure 3, type 8). Likewise, PP3 has hydrophobic pockets for the high-affinity binding of substrates *via* so-called PxIxIT- or LxVP-type docking motifs.[28] In fact, the clinically used PP3 inhibitors cyclosporine A and FK506 act by blocking access to these substrate-docking sites. These inhibitors have considerable long-term toxic effects that are, however, largely unrelated to the inhibition of PP3 *per se* and stem from the sequestration of immunophilin proteins that form a PP3-inhibitory complex with cyclosporine A or FK506. Hence, the substrate-docking sites of PP3 remain attractive targets for the development of novel immunosuppressants.

Signaling by many protein phosphatases is critically dependent on their targeting to substrate-containing compartments, which is often mediated by short docking motifs of scaffolding proteins.[96−98] Interference with subcellular targeting is another attractive but hitherto unexplored opportunity to generate specific inhibitors of protein phosphatases (Figure 3, type 7).

THE PROMISE OF PROTEIN PHOSPHATASE-TARGETING THERAPIES

Members of all four superfamilies of protein phosphatases have now been validated as therapeutic targets for various diseases (Table 1). These enzymes either promote or prevent human disease, explaining the quest for activatory as well as inhibitory drugs. Many natural and man-made compounds are already known to directly interfere with the activity of specific (subsets of) protein phosphatases *in vivo*, demonstrating that these enzymes are druggable. Also, some FDA-approved drugs have only been found to target protein phosphatases after their introduction into the clinic, justifying a systematic screening of drugs as potential modulators of protein phosphatases. A more rational approach for the development of phosphatase-directed therapeutics is also coming of age. The design of protein phosphatase inhibitors has mainly been driven by work on PTPs and has, unwillingly, largely followed the path of kinase-inhibitor drug discovery. Indeed, while in both fields the initial focus was largely on the search for active-site inhibitors, the attention has later shifted toward the design of bidentate and allosteric inhibitors. For PTPs this has already resulted in the development of some highly specific and potent inhibitors with a proven therapeutic value in mouse models. However, these inhibitors are still limited in their oral bioavailability, which obviates their testing in clinical trials. Recently acquired insights into the structure and regulation of PPP phosphatases, in particular PP1, PP2A, and PP3, have also ignited an interest into the rational development of therapeutically useful small-molecule modulators that interfere with the quaternary structure or substrate recruitment of these enzymes. The discovery of oncogenic PP2A inhibitors and a binding code for interactors of PP1 offer particularly attractive perspectives for the design of new types of activity modulators. Although the development of protein−protein interaction inhibitors is challenging, it certainly is an avenue worth pursuing.[99] We predict that rationally designed small-molecule modulators of protein phosphatases will reach the clinic in the coming decade, to be used as single therapies or as components of combination therapies, for the treatment of major human diseases.

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Notes

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KEYWORDS

Allosteric phosphatase modulator: A molecule that modulates the activity of a phosphatase through binding to a site that is distinct from the active site; **Bidentate phosphatase inhibitor**: An inhibitor that binds to both the catalytic site and an adjacent site; **HAD phosphatases**: Protein Ser/Thr/Tyr phosphatases belonging to the superfamily of haloacid dehalogenase hydrolases; **Phosphotyrosine mimetic inhibitor**: An active-site inhibitor of tyrosine phosphatases that structurally resembles phosphotyrosine; **PPM**: The superfamily of monomeric Mg^{2+} or Mn^{2+}-stimulated protein Ser/Thr phosphatases; **PPP phosphatases**: The superfamily of phosphoprotein Ser/Thr phosphatases; **Protein kinase**: An enzyme that catalyzes phosphorylation reactions, i.e., the transfer of the γ-phosphate of ATP to a side chain of an amino acid. Usually the phosphate is transferred to the hydroxyl side chain of a serine, threonine or tyrosine residue, resulting in the formation of a phospho-mono-ester bond; **Protein phosphatase**: An enzyme that removes covalently bound phosphate groups from proteins. Usually protein phosphatases hydrolyze phospho-mono-ester bonds; **Protein phosphorylation diseases**: Diseases caused by an excessive or deficient action of protein kinases or phosphatases, resulting in an altered substrate phosphorylation level; **PTP phosphatases**: The superfamily of protein tyrosine phosphatases and dual-specificity protein phosphatases.

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