Bioorganic & Medicinal Chemistry 19 (2011) 2145–2155

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Using small molecules to target protein phosphatases

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ARTICLE INFO

Article history:
Received 7 January 2011
Revised 21 February 2011
Accepted 23 February 2011
Available online 2 March 2011

Keywords:
Inhibitor
Structure
Drug
Design

ABSTRACT

The site specific functionalization of phosphate groups with amino acid side chains of substrate proteins represents one of the most important regulatory mechanisms of biological systems. Phosphorylation and dephosphorylation are reversibly catalyzed by protein kinases and protein phosphatases, and the aberrant regulation of these enzymes is associated with the onset and progression of various disease states such as cancer, diabetes, neurodegenerative and autoimmune disorders, making these proteins attractive targets for drug discovery. Here we report on strategies currently explored for the identification and development of various inhibitors directed against clinically relevant phosphatases. While over the last years, inhibition of phosphorylation has evolved into a key strategy in targeted therapies, the development of clinically relevant phosphatase inhibitors still faces major bottlenecks and is often plagued by limited selectivity and unfavorable pharmacokinetics. The reader will gain a better understanding of the importance of the field and its current limitations.

1. Introduction

The dynamic nature of biological systems owes itself to a variety of carefully orchestrated posttranslational modifications. One of the most important mechanisms by which nature regulates gene product function in cellular systems is the reversible phosphorylation of proteins. The transfer of the γ-phosphate group from ATP to the side chains of serine, threonine or tyrosine residues of substrate proteins is catalyzed by protein kinases (PKs) and can lead to conformational changes, promote protein–protein interactions or switch proteins and/or substrates between their active and inactive states to allow cells to transduce information between distinct cellular sites. Protein phosphatases (PPs) are the physiological counterparts of protein kinases and catalyze the dephosphorylation of protein substrates to restore them to their native states. The dynamic interplay between PKs and PPs is tightly regulated to ensure the maintenance of physiological processes. This balance is crucial since aberrant regulation of phosphorylation events is known to lead to fatal disease such as diabetes, cancer, autoimmune and neurodegenerative disorders. The enormous wealth of knowledge on protein phosphorylation and dephosphorylation accumulated in the past years consequently spurred the initiation of discovery projects in both academia and the pharmaceutical industry to develop small organic molecules to perturb unwanted PK and PP function in disease states.[1–8] In particular, the discovery of such molecules combined with better fundamental understanding of kinase malfunction in cancer biology has culminated in an uprise in targeted cancer therapies.[9] One example is the pyrimidine derivative Imatinib (Gleevec®), an inhibitor of the mutant oncogene BCR-Abl kinase developed by Novartis, which was approved by the FDA in 2001. Since then, it has led to historically unprecedented improvements in the progression-free survival of leukemia patients and marked a new era in medicinal chemistry research to modulate unwanted phosphorylation events in diseases. While a dozen kinase inhibitors are on the market and several more are in clinical trials, the development of clinically relevant phosphatase inhibitors faces major bottlenecks and is often plagued by limited selectivity and unfavorable pharmacokinetics. Although phosphatases and their inhibitors are heavily investigated by researchers from academia and industry, until now no phosphatase inhibitor was approved by the FDA for the treatment of human diseases.

PPs are classified by their substrate specificities and the human genome encodes for more than 130 PPs. A total of 107 tyrosine phosphatases (PTPs) can be further subdivided into 38 tyrosine-specific PTPs and 61 dual-specificity phosphatases (DSPs) which catalyze the dephosphorylation of both serine/threonine and tyrosine residues.[10] In comparison to the 90 tyrosine kinases

Abbreviations: ASO, antisense-oligonucleotides; DSPs, dual-specific phosphatases; F_2Pmp, difluorophosphonomethylphenylalanine; PK, protein kinase; PP, protein phosphatase; FDA, Food and Drug Administration; CML, chronic myeloid leukemia; PTP, protein tyrosine phosphatase; RNAi, RNA interference; siRNA, short interfering RNA; STP, serine/threonine phosphatase; TB, tuberculosis; TCPTP, T-cell protein tyrosine phosphatase.

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doi:10.1016/j.bmc.2011.02.047

encoded in the human genome, this suggests an equal partnership of these enzymes in regulating protein function through tyrosine phosphorylation. However, the majority of protein phosphorylation occurs on serine and threonine residues and is catalyzed by the remaining 428 protein serine/threonine kinases, suggesting that the fewer serine/threonine phosphatases (STPs) must display much broader substrate specificities than the complementary kinases. The catalytic domains of PPs are highly conserved and differences in substrate specificity are largely determined by regulatory domains or subunits. To achieve the essential temporal and spatial specificity in modulating protein function in signaling events, nature uses a variety of mechanisms such as posttranslational modifications and protein–protein interactions to regulate PK and PP activities. In particular the catalytic domains of STPs interact with a large variety of regulatory subunits and the incorporation of catalytic subunits in multi domain complexes helps to explain why eukaryotes contain fewer genes that encode for PPs than genes that encode for PKs.[3,11–13] PPs are often kinase substrates themselves and vice versa and in-depth biochemical and structural biology studies allowed for detailed understanding of the different catalytic mechanisms of STPs and PTPs as well as substrate specificities. While many classical phosphatase inhibitors are designed to target the catalytic site as phosphate mimics, insights into the regulatory mechanisms and conformational changes in phosphatase structure and the dynamic interplay with other regulators, might prove crucial for the design and development of next generation inhibitors with improved pharmacological properties. Here we report on strategies currently explored for the identification and development of various inhibitors directed against clinically relevant phosphatases (Table 1).

2. Clinically relevant phosphatases

2.1. PTP1B

The impact on society and the costs associated with lifestyle related diseases such as Type 2 diabetes and obesity are overwhelming and estimated to increase further in the near future. Type 2 diabetes and obesity are often linked to acquired insulin resistance and the loss of proper glucose homeostasis. The tyrosine phosphatase PTP1B was discovered to be a negative regulator of the insulin and leptin receptor pathways.[14–16] The peptide hormone insulin induces autophosphorylation of its receptor and thereby triggers a kinase signaling cascade that ultimately induces the synthesis of glycogen, fatty acids and proteins. Dephosphorylation of the receptor by PTP1B leads to its inactivation and shuts-down additional downstream processes. The notion that pharmacological blockage of PTP1B should therefore counteract insulin resistance made this phosphatase an attractive drug target.[17,18] About a decade ago animal studies demonstrated that PTP1B-deficient mice are insulin sensitivity and the use of antisense oligonucleotides (ASOs) designed to down-regulate expression of PTP1B resulted in normalized blood glucose levels and improved insulin sensitivity without changing the regular diet of mice.[19] These findings inspired nearly every major pharmaceutical company to launch a program for the identification of potent PTP1B inhibitors. Interestingly, PTP1B function is not only associated with the insulin cascade but is also found to be overexpressed or up-regulated in human breast, colon and ovarian cancers.[20–22] However, the discovery and development of clinically useful PTP1B inhibitors has proven to be extremely difficult since it is often challenged by limited inhibitor selectivity and low bioavailability.[4,5,23,24]

2.1.1. Structural biology and structure-based design

The relative ease of recombinantly producing catalytic domains of protein phosphatases made structural biology and structure-based design approaches an important means for the discovery and development of phosphatase inhibitors. The plethora of over 100 protein crystal structures of PTP1B deposited in the Protein Data Bank (PDB) best exemplifies the structure-based design approaches widely applied in phosphatase inhibitor research. In a recent work, Barr et al. used large-scale structural comparison to identify shared as well as target-specific structural features of members of the classical PTP family.[25] As a result, the authors report on four diverse conformations of the WPD-loop and propose a mechanism for loop closure of this catalytically important structural element which also regulates the recognition of active site directed inhibitors. In addition, the authors determined 22 catalytic domain structures of the human PTP family, including 16 not published previously. The generated structures include at least one member of each PTP subgroup and provide a comprehensive coverage of the structural features of the classical phosphotyrosine-specific protein tyrosine phosphatome. More recently Vidović and Schurer reviewed the entire human PTP family and presented a systematic knowledge-based characterization of global and local similarity relationships, which might prove relevant for the development of small-molecule inhibitors.[26] The authors used extensive parallel homology modeling to expand the current structure space of PTPs and demonstrate the importance of binding site similarities in understanding cross-reactivity and inhibitor selectivity in the design of small-molecule inhibitors.

2.1.2. Active site inhibitors

The design of active site directed inhibitors was stimulated by one of the first structures of PTP1B (pdb code: 1pty). This complex crystal structure featured a phosphotyrosine in the active site of the catalytic domain of the phosphatase and in addition revealed a second pTyr molecule in an adjacent pocket and immediately stimulated ideas to chemically fuse the two moieties using a suitable linker region. This hybrid approach resulted in bidentate inhibitors with dramatically increased affinities and the second substrate pocket has since then been highly important in lead structure optimization for PTP1B inhibitors. Fragment based design approaches both by protein X-ray crystallography and NMR techniques were widely employed to identify, for example, small organic acids as binders to this second site and resulted in an

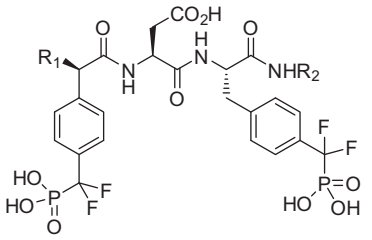
Table 1
Selected phosphatases as target proteins

Family	Phosphatases	Disease, therapeutic approach
Serine/threonine	PP1	Tumor suppression, malignancy
	PP2B, PP2C	Cystic fibrosis, immune suppression, asthma, cardiovascular
Tyrosine phosphatase	PTP1B	Diabetes, obesity
	MptpA/B	Tuberculosis
	CD45	Alzheimer's disease, autoimmune disease, inflammation, organ transplantation
	SHP-1/2	Neuron protection, obesity, Noonan syndrome, leukemia, regulation of RAS/MAP-kinases
Dual-specific phosphatases	VHR	Regulation of MAP-kinases
	Cdc25	Cell-cycle progression, tumor therapy (various human cancers)
	PRL-1/2/3	Promoting tumor cell, leukemia, Hodgkin's disease, prostate cancer

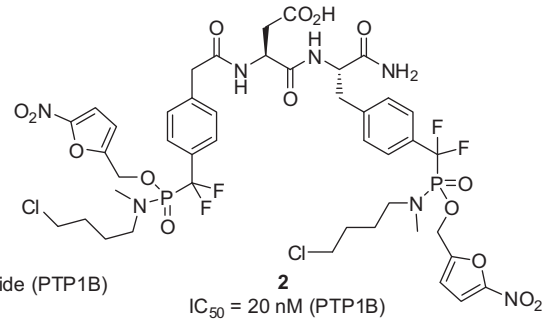
impressive array of potent nM inhibitors when fused to phospho mimics (Fig. 1). Most active-site-directed PTP inhibitors reported to date are non-hydrolysable pTyr mimics that take advantage of the positively charged active site. However, most of these pharmacophores are characterized by a high charge density which does not favor cell penetration. In addition, the conserved mode of action of these mimics reduces selectivity and results in cross-reactivity with multiple phosphatases. The most widely used and potent pTyr surrogate devised for PTP1B thus far contains the non-hydrolyzable difluorophosphonomethylphenylalanine (F_2Pmp) group.[27] Compound **1a**, identified from a combinatorial chemistry approach, displays a K_i value in the low nM range for PTP1B and exhibits notable selectivity in favor of PTP1B when tested against a panel of PTPs including Cdc25A, SHP-2 and VHR.[28] However, as mentioned above, compound **1a** is not cell permeable due to its high polarity. To enhance penetration across membranes, derivatives of **1a** were prepared either by coupling to a cell permeable (D)-Arg_8-peptide tag (**1b**),[29] which showed a significant improvement in leptin-dependent suppression of food intake in leptin resistant rats,[30] or by coupling to a highly lipophilic fatty acid (**1c**).[31] Furthermore, a prodrug concept was utilized in the development of **2**.[32] Recently, Han et al. reported on the discovery of orally active F_2Pmp-containing PTP1B inhibitors,[33] of which the nitrile analogue **3** was shown to have the best overall profile in terms of potency and oral bioavailability and was found to be efficacious in animal models of diabetes and cancer. Several strategies have been applied to identify pTyr mimics with more favorable pharmacological properties. One such approach is referred to 'Breakaway Tethering' and involves the use of disulfide-containing small molecule fragments in combination with a Cys residue introduced on the surface of the target enzyme to act as a tethering point which captures corresponding ligands under reducing conditions.[34] Subsequent mass spectrometry analysis allows for the detection of the modified mutant target protein and the identification of the captured small molecule fragment. Using this powerful approach, Erlanson et al. identified PTP1B inhibitors with enhanced bioavailability. In a more recent analysis, 1,2,5-thiadiazolidin-3-one-1,1-dioxide (IZD) was identified as a novel phosphomimic and the isothiazolidinone group was utilized in the synthesis of the peptide-based inhibitor **5** which shows an IC_{50} in the mid nM range.[35] In addition, using the same phosphomimic, the non-peptide-based compound **6** was identified, which displays nM potency in biochemical assays and increases the level of insulin receptor phosphorylation (2.8-fold at 80 μM) in cellular systems.[36] A closely related five-membered heterocyclic pTyr mimic, 1,2,5-thiadiazolidin-3-one 1,1-dioxide (TDZ), was independently discovered by AstraZeneca **7**,[37] Incyte **8**,[38] Novartis **9**,[39] and Vertex.[40] In 2005, Black et al. reported the discovery of TDZ **7a** (IC_{50} = 1.6 mM) by using a fragment-based NMR screening approach for the identification of pTyr mimics initially derived from PTP1B crystallographic data.[37] Follow-up structure-based ligand design led to the identification of the *o*-methoxybiphenyl analogue **7b**. The *o*-methoxy and 5-aryl substituents both contribute to the observed 100-fold increase in potency (IC_{50} = 2.5 μM). Later in 2005, Incyte published a series of unique five-membered heterocyclic pTyr mimics derived from structure-based design which included the TDZ- and the previously mentioned IZD-group (**5**, **6**).[38] Remarkably, the TDZ-based analogue **8** was found to be four times less potent when compared to the corresponding IZD-containing derivative **6**. Novartis filed several patent applications covering a variety of scaffolds bearing an *o*-hydroxyaryl TDZ.[39] The most active derivative, compound **9**, has a reported IC_{50} value of 80 nM and a ligand efficiency of 0.36. In addition, the 'Institutes for Pharmaceutical Discovery' (Branford, CT, USA) disclosed new scaffolds bearing the TDZ-moiety as exemplified by the biarylmethylene bridged aryl-TDZ **10**.[41] Recently, Taylor and co-workers designed and synthesized the analogous sulfonic acid, difluoromethylenesulfonic acid (DFMS), to the widely used pTyr mimic F_2Pmp.[42] Unfortunately, compounds possessing the DFMS group, such as **11**, are several orders of magnitude less potent when compared to the corresponding F_2Pmp containing derivatives. More recently, Zhang and co-workes identified aryl diketoacids **12** as novel pTyr surrogates and showed that neutral amide-linked aryl diketoacid dimers **13** also exhibit PTP inhibitory activity. Detailed enzyme kinetics studies and protein X-ray crystallography revealed that these derivatives stabilize PTP1B in its inactive, WPD-loop open conformation and act as noncompetitive inhibitors.[43] Using a high-throughput screening approach, a series of monocyclic thiophenes were identified as PTP1B inhibitors.[44] Further optimization resulted in the development of the potent inhibitor **14**. Introduction of a tetrazole ring or 1,2,5-thiadiazolidine-3-one-1,1-dioxide as a carboxylate mimic led to the discovery of two unique starting points that improved cell permeability and increased potency up to 300 nM.[45] Recently, thiazolidinedione derivatives were identified as a novel class of PTP1B inhibitors.[46] The most active derivative from this class, compound **15** shows an IC_{50} in the low μM range. Evaluation of **15** in mouse models as a potential anti-obesity and hypoglycemic drug revealed that it indeed significantly improves glucose tolerance and suppresses weight gain. Maccari et al. reported the closely related thiazolidinedione derivative **16** as an inhibitor of both PTP1B and LMW-PTP.[47] Additional examples for active-site-directed PTP1B inhibitors include arylbenzonaphthothiophenes and arylbenzonaphthofurans. Both scaffolds were shown to improve insulin sensitivity in rodents. One compound from these efforts, ertiprotafib (**17**), progressed to clinical trials for the treatment of Type-2 diabetes. Development was discontinued in phase II due to insufficient efficacy and strong unwanted side effects.[48] Trodusquemine (MSI-1436) (**18**) is another clinical candidate, which is being developed by Genaera Corp for the potential treatment of Type 2 diabetes and obesity and has proceeded to Phase Ib.[49] Recently, trodusquemine was also reported to reduce food intake, induce significant weight loss via selective reduction of fat and to reduce insulin and leptin levels in a mouse model for diet-induced obesity.[50] Researches at Japan Tobacco, Inc. reported the development and the in vitro and in vivo evaluation of new PTP1B inhibitor, JTT-551 (**19**). In diabetic mice **19** substantially reduced blood glucose level and showed antidiabetic effects without change in body weight.[51] Recently, a series of novel thiophene derivatives was identified and their activities as substrate competitive, active site directed inhibitors of PTP1B were evaluated in in vitro and in vivo experiments.[52] The reported compounds were shown to have significant membrane permeability and exerted extensive cellular effects on the activation of the PI3K/AKT signaling pathway in CHO-K1 cells. More recently 2,4-disubstituted polyhydroquinolines were identified as a novel class of promising antihyperglycemic and lipid modulating agents.[53] Although great progress has been made to increase potency of PTP1B inhibitors, these initial successes were compromised by unwanted cross reactivity to the structurally-related T-cell tyrosine phosphatase TCPTP (77% sequence identity with PTP1B) and the discovery that TCPTP knockout mice are born healthy but die after about 4 weeks. Even more disturbing was the finding that double knock-out of TCPTP and PTP1B turned out to be lethal. The design and synthesis of selective PTP1B inhibitors which are less potent against TCPTP still remains a particular challenge in current PTP1B medicinal chemistry research.

2.1.3. Allosteric inhibitors

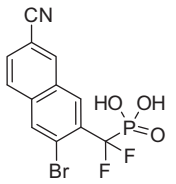
The work on phosphatase inhibitors that target pockets located remotely from the active site in order to overcome the current limitations in selectivity and bioavailability of active site directed phosphatase modulators was pioneered by researchers at Sunesis.



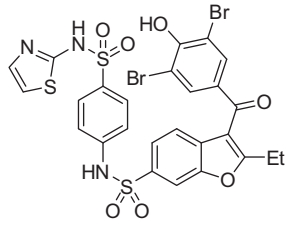
1a $R_1 = R_2 = H$; K_i = 2.4 nM (PTP1B)
1b $R_1 = H$, $R_2 = (CH_2)_2SS(CH_2)_2NH$-(D)$Arg_8$-amide (PTP1B)
1c $R_1 = NH(CO)C_{14}H_{29}$; K_i = 26 nM (PTP1B)



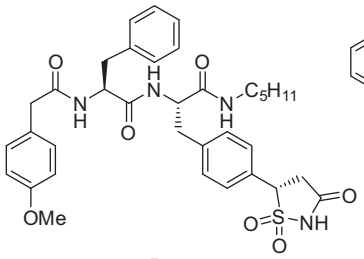
2
IC_{50} = 20 nM (PTP1B)



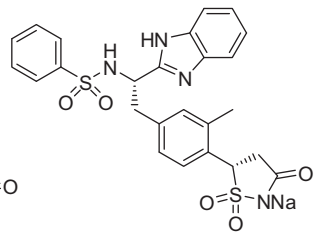
3
IC_{50} = 120 nM (PTP1B)



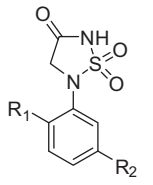
4
IC_{50} = 8 μM (PTP1B)



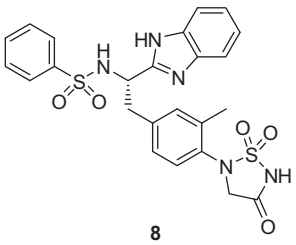
5
IC_{50} = 40 nM (PTP1B)



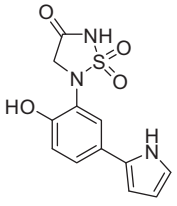
6
IC_{50} = 35 nM (PTP1B)



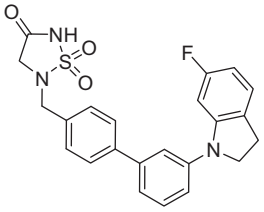
7a $R_1 = R_2 = H$
IC_{50} = 1.6 mM (PTP1B);
7b R_1 = OMe; R_2 = Ph
IC_{50} = 2.5 μM (PTP1B)



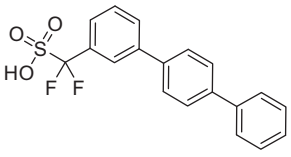
8
IC_{50} = 140 nM (PTP1B)



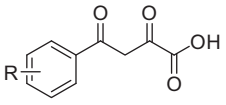
9
IC_{50} = 80 nM (PTP1B)



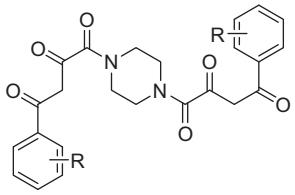
10
IC_{50} = 130 nM (PTP1B)



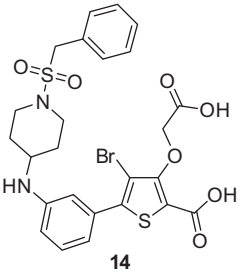
11
IC_{50} = 8 μM (PTP1B)



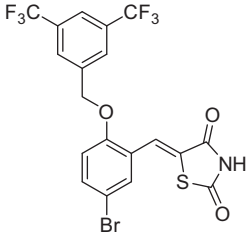
12 R = H; *o*-F; *p*-CF_3; *m*-OBn; *m*-NBn_2, IC_{50} = 20 μM (PTP1B)
m-(2'-fluorobenzyl)
IC_{50} = 40 μM (PTP1B)



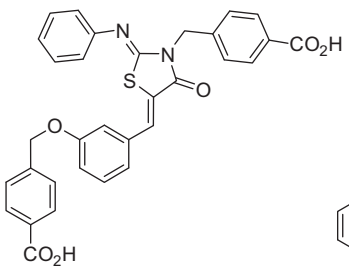
13 a R = *m*-(2'-fluorobenzyl),
IC_{50} = 3.7 μM (PTP1B)
13 b *m*-NBn_2, IC_{50} = 2.8 μM (PTP1B)



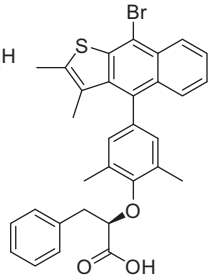
14
IC_{50} = 5 nM (PTP1B)



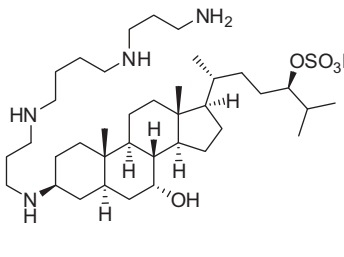
15
IC_{50} = 5 μM (PTP1B)



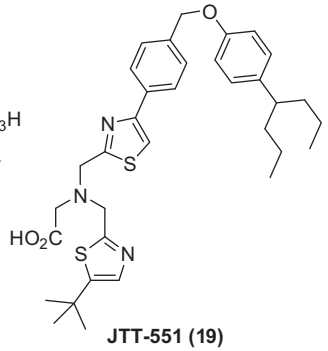
16
IC_{50} = 220 nM (PTP1B)



Ertiprotafib (17)
IC_{50} = 1.6 μM (PTP1B)



Trodusquemine (18)
IC_{50} = 1.3 μM (PTP1B)



JTT-551 (19)
IC_{50} = 0.22 μM (PTP1B)

Figure 1. Structures of selected PTP1B inhibitors, IC_{50} or K_i values are given.

In 2004, they reported an allosteric site located at the back of the catalytic domain of PTP1B about 20 Å away from the catalytic center. The crystal structure of PTP1B in complex with the allosteric inhibitor **4** (pdb code: 1t4j) proved that the ligand stabilizes the inactive phosphatase conformation by preventing the WPD-loop from adopting a catalytically competent conformation and revealed a novel but general mechanism to inhibit tyrosine phosphatases.[54] In the allosteric pocket, the inhibitor forms π–π interactions with the side chain of a central Phe residue. Notably, the structural homolog TCPTP holds a Cys residue at this position and **4** binds only with reduced affinity.

2.1.4. Antisense oligonucleotides directed against PTP1B

Meanwhile, a new clinical focus is on antisense oligonucleotides (ASO) which are directed against PTP1B. The main advantage of oligonucleotides compared to classical small molecular weight inhibitors is their *genetic selectivity* for PTP1B without perturbing the function of related proteins although off-target effects cannot be ruled out. PTP1B ASO have been developed by ISIS Pharmaceuticals Inc. and the second generation ASO, ISIS-113715, was reported to have positive top-line phase 2 clinical data for the treatment of patients with Type 2 diabetes in the fall of 2009.

2.2. Cancer related PTPs

2.2.1. Cdc25

Several PTPs have been identified as critical oncogenes in human malignancy and are now considered to be potential drug targets.[5,6,55,56] Phosphatases of the Cdc25 subfamily are dual-specific and regulate cyclin-dependent kinases (Cdks) which are key players in the regulation of the cell cycle, transcription and mRNA processing. The three human isoforms, Cdc25A, Cdc25B and Cdc25C, seem to have overlapping substrate specificities for the different Cdk-cyclin. Cdc25 phosphatases are also key participants of the checkpoint pathway and are negatively regulated to induce cell cycle arrest in response to DNA damage, thereby initiating DNA repair or apoptosis. Thus, the disregulation of these processes and Cdc25 overexpression can contribute to genomic instability and can often lead to more aggressive tumor growth and poorer clinical outcome.[57,58] The inhibition of Cdc25 phosphatase activity may represent a novel approach for the development of anticancer therapeutics. Reviews by Garuti et al.[59] and Contour-Galcera et al.[60] provide a comprehensive overview of current Cdc25 inhibitor development. The isoform unspecific naphthoquinone NSC95397 (**20**) shows IC_{50} values in the mid nM range and is the most potent Cdc25 inhibitor reported to date[61] (Fig. 2). This compound displays significant inhibition when tested against human and murine carcinoma cells and blocks the G2/M phase transition of the cell cycle. NSC663284 (**21**) is an isoform unspecific irreversible Cdc25 inhibitor and arrests cells in the G1 and G2/M phases and induces significant growth inhibition of human breast cancer cell lines.[62] Contour-Galcera and co-workers recently reported the identification of a new class of thiazoloquinones and showed that BN82685 (**22**) inhibits Cdc25 in the high nM range in biochemical assays and retains activity in human xenografts.[63] Low concentrations of BN82685 in combination with the micro tubulin stabilizer paclitaxel (Taxol®) inhibit proliferation of colon cancer cells. It should be noted that most quinine-based Cdc25 inhibitors reported to date are irreversible binders and act via arylation of the nucleophilic catalytic Cys of the phosphatase. In addition, the redox properties of quinones are prone to generate reactive oxygen species (ROS), which may cause toxicity to normal tissues and thus reduce therapeutic potential. In order to overcome this problem, Carr and co-workers recently synthesized a series of non-quinone sulfone analogues of vitamin K_3, including H32 (**23**),[64] which inhibits Cdc25 by reversibly binding to the catalytic cysteine and leads to G1 phase arrest during the cell cycle. As an alternative strategy, the above mentioned phosphate surrogates, which potentially anchor the ligand in the active site of the phosphatase, are also utilized for the design of Cdc25 inhibitors.

The sesterterpenoid Dysidiolide (**24a**) isolated from the Caribbean sponge *Dysidea etheria* was the first natural product reported to be active on Cdc25 and the γ-hydroxybutenolide moiety is thought to mimic the substrate phosphate. Facilitating **24a** as a biologically validated starting point, our group used solid-phase synthesis for the generation of a small focused library of dysidiolide analogues[65,66,132] and was able to identify inhibitors of Cdc25C that are more potent then the parent natural product **24a**. Analogues **24b** and **24c** displayed IC_{50} values in the low μM range. Moreover, these analogues showed considerable biological activity in cytotoxicity assays employing different cancer cell lines. Using the structures of dysidiolide and vitamin D_3 as starting points, Shimazawa et al. also designed several potent Cdc25 inhibitors, including **25** which inhibits Cdc25A and Cdc25B with IC_{50} values of 0.44 and 1.9 μM, respectively.[67]

Recently, 2-methoxyestradiol (2-ME) was reported as a potent, selective and relatively non-toxic inhibitor of hepatoma growth both in vitro and in vivo and it was suggested that 2-ME binds to the catalytic site Cys.[68] Another class of Cdc25 inhibitors are maleic anhydride derivatives bearing a fatty acid chain at the C-4 position.[69] One such compound, **26**, inhibits Cdc25s with IC_{50} values in the low μM range and induces G0/G1 phase accumulation with subsequent inhibition of Cdk2 activity. Moreover, **26** triggered apoptosis within a 48-h treatment without oxidative burst. *N*-Arylmaleimide derivatives are potent electrophiles and reagents for thiol-selective modifications and were introduced as a novel class of Cdc25 inhibitors.[70] An example is PM-20 (**27**), which is selective for Cdc25A with an IC_{50} value of 1 μM. Furthermore, PM-20 inhibits growth of several human tumor cells, especially Hep3B cells with an IC_{50} of 0.7 μM. More recently, several Cdc25 phosphatase inhibitors with micromolar activities were discovered from structure-based virtual screening.[71] The most active of them was compound **28**, which inhibits Cdc25A and B with IC_{50} values in the low μM range.

2.2.2. SHP-2

SHP-2 and its paralogue SHP-1 are non-receptor protein tyrosine phosphatases that mediate cell signaling via the RAS/MAP-kinase pathway and are functionally regulated by two N-terminal SH2 domains.[56,72] Mutations of the SHP-2 gene (*PTPN11*) can cause hyperactivation of its catalytic activity and have been identified as contributing to Noonan syndrome, a developmental disorder which is frequently associated with short stature[73] and childhood leukemia.[74] Recent reports indicate, that knockdown of SHP-1 inhibits G1/S progression in prostate cancer cells presumably through the interaction with PI3-kinases (PI3K), involved in PI3K-AKT signaling pathway.[75] Although SHP-2 represents an attractive target for the treatment of cancer, only a few SHP-2 inhibitors are known from the literature. The pentavalent antimony derivative sodium stibogluconate, a known agent against leishmaniasis, has recently been found to inhibit SHP-2 and SHP-1 activity[76] and is the first SHP inhibitor to enter clinical trials. A phase I clinical trial of sodium stibogluconate in combination with interferon alpha-2b followed by cisplatin, vinblastine, and temozolomide in treating patients with advanced melanoma or other cancer types was launched in 2007 (http://clinicaltrials.gov). Recently, the design and synthesis of a compound collection containing SHP-2 inhibitors inspired by furanodictines and the concept of biology-oriented synthesis was reported[77] in which inhibitor **29** (Fig. 3) exhibits an IC_{50} of 2.5 μM against SHP-2. A screening initiative at the National Cancer Institute resulted in the identification of NSC-87877 (**30**)[78] and NSC-117199 (**31**)[79] which inhibit SHP-2

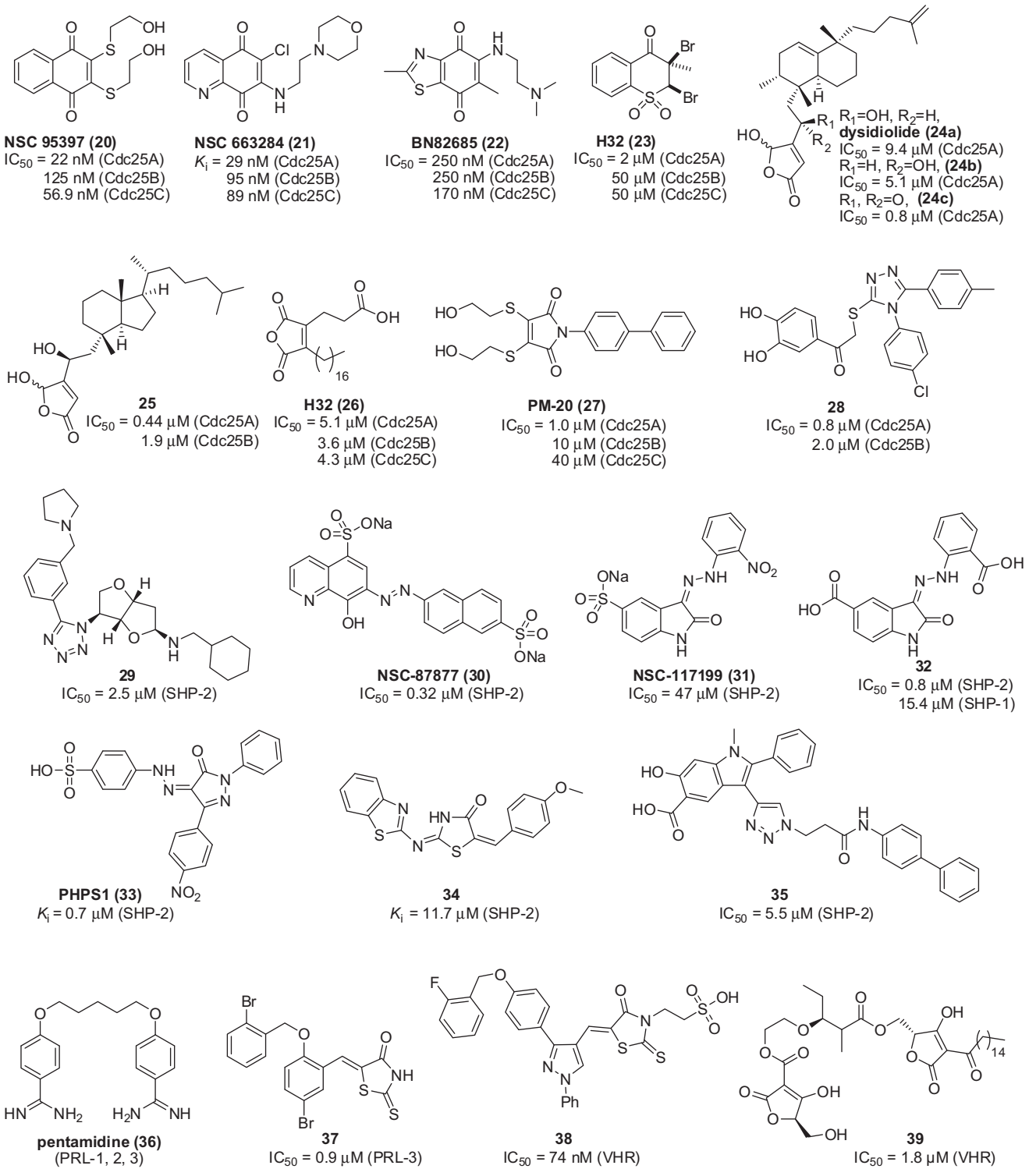


Figure 2. Structures of selected inhibitors of Cdc25, SHP-2, PRLs, and VHR. IC_{50} or K_i values are given.

with IC_{50} values of 0.32 and 47 μM, respectively. Based on the oxindole **30**, a focused library was designed and **32** was identified as a selective inhibitor of SHP-2 over SHP-1 and PTP1B with an IC_{50} value of 0.8 μM for SHP-2.[79] Recently, Birchmeier et al. performed an in silico screen of low-molecular-weight compounds that may bind to the catalytic site of SHP-2 and resulted in the discovery of PHPS1 (**33**) as a potent and cell-permeable inhibitor which is selective for SHP-2 over SHP-1 and PTP1B.[80] PHPS1 efficiently inhibits the activation of Erk1/2 by a leukemia-associated mutant variant of SHP-2 and blocks growth of a variety of human tumor cell lines. In addition, Geronikaki et al. reported the synthesis and biological evaluation of thiazolidin-4-one derivatives as a novel class of SHP-2 inhibitors.[81] Compound **34** exhibited the best inhibitory activity with a K_i of 11.7 μM. Recently, Zhang et al. reported a salicylic acid based combinatorial library design approach to target both the PTP active site and a unique nearby subpocket

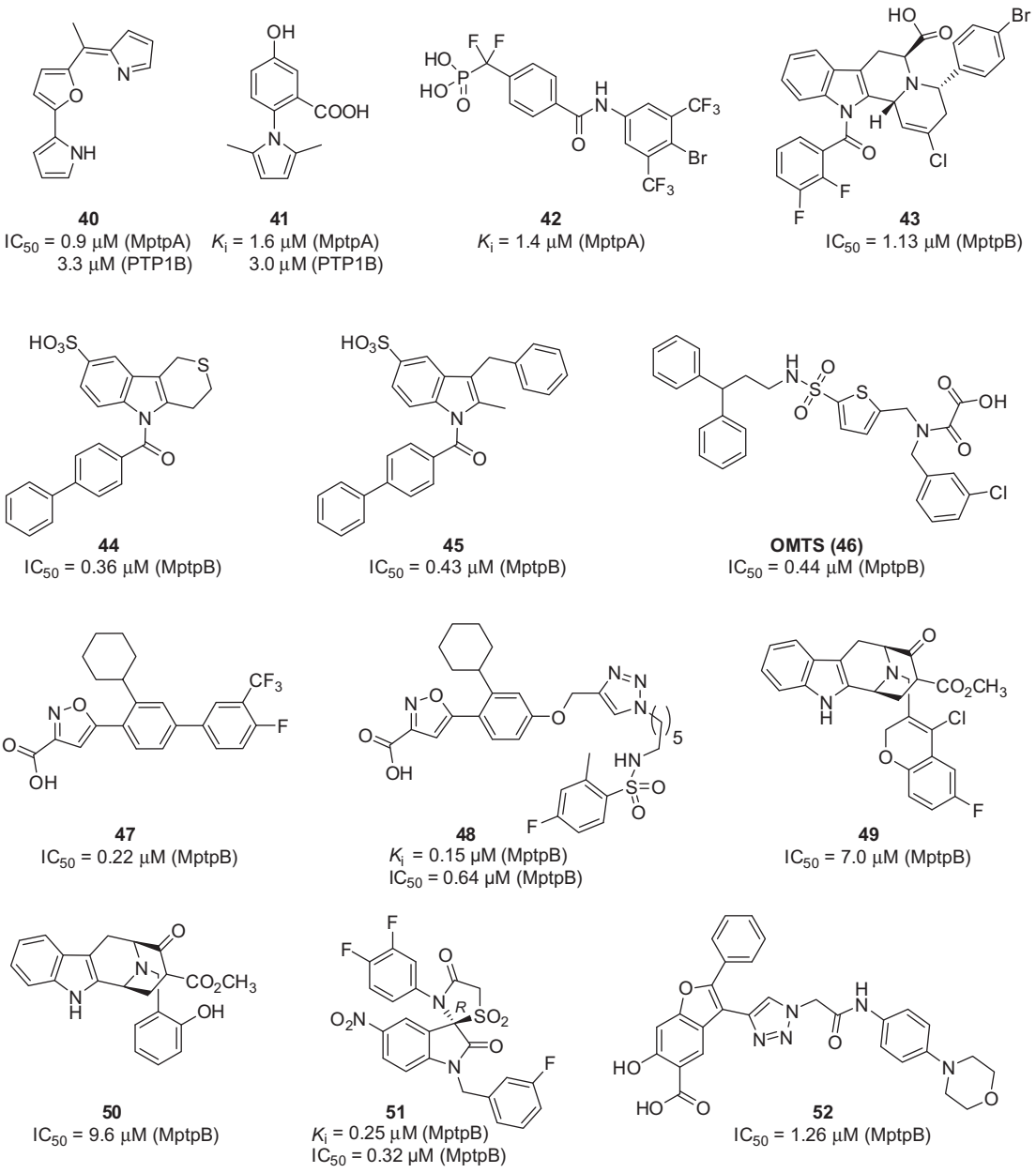


Figure 3. Structures of selected inhibitors of MptpA, MptpB and CD45. IC_{50} or K_i values are given.

for enhanced affinity and selectivity.[82] High-throughput screening of the library led to the identification of a SHP-2 inhibitor **35** with promising potency and selectivity. Remarkably, **35** blocks growth factor stimulated ERK1/2 activation and hematopoietic progenitor proliferation, providing supporting evidence that chemical inhibition of SHP-2 may be therapeutically useful for the treatment of cancer and leukemia.

2.2.3. PRL

PRL-1, PRL-2 and PRL-3 form the *phosphatases of regenerating liver* subfamily. They are dual-specific phosphatases and represent potential targets for the treatment of various cancers.[6,83] PRL-3 overexpression correlates with metastasis in many malignancies and several recent reports suggest that PRLs may play key causal roles in promoting tumor cell motility and invasion. The genetic knockdown of PRL-3 with interfering RNA in cancer cells can abrogate cell motility and the ability to metastasize in a mouse model[84] but the discovery of PRL phosphatase inhibitors has lagged behind the extensive pharmacological and structural studies on this target protein and only a few inhibitor classes have been reported so far. Pentamidine (**36**) is a known anti-leishmaniasis drug and was also reported to inhibit all three PRL isoforms in vitro and induced tumor shrinkage in a melanoma mouse xenograft model.[85] Additionally, recent studies by Lee et al. showed that combination of pentamidine with the phenothiazine antipsychotic agent chlorpromazine exerts synergistic anti-proliferative effects. Pentamidine treatment resulted in chromosomal segregation defects and delayed progression through mitosis, which is consistent with inhibition of PRL.[86] Benzylidene rhodanine derivatives were identified through the screening of chemical libraries and showed good inhibitory activity against PRL-3. Compound **37** was the most active with an in vitro IC_{50} of 0.9 μM and showed reduced cellular invasion in cell-based assays.[87] In addition, bioflavonoids isolated from young branches of *Taxus cuspidata* inhibit PRL-3 with IC_{50} values in the low μM range.[88] Park et al. identified inhibitors of PRL-3 by means of virtual screening and docking simulations and showed

that initial hits inhibit PRL-3 phosphatase activity with IC_{50} values in the mid μM range.[89]

2.2.4. VHR

VHR is a dual-specificity phosphatase, which dephosphorylates activated ERK1/2 and weakens the ERK signaling cascade in mammalian cells.[90] Moreover, VHR can regulate cell-cycle progression and is itself modulated during the cell cycle.[91] Cells that lack VHR can be arrested at the G1–S and G2–M transitions of the cell cycle and show the initial signs of senescence. VHR activity has been known to be promoted by the tyrosine kinase ZAP-70 which plays a critical role in the immune response of activated T cells,[92] or by the interaction with vaccinia-related kinase 3 (VRK3).[93] Recently, it was reported that VHR is upregulated in several cervical cancer cell lines as well as in carcinomas of the uterine cervix.[94] These results suggest that VHR may serve as a new marker for cancer progression in cervix carcinoma and represents a potential new target for targeted cancer therapies. Wu et al. recently reported on the development of multidentate small-molecule inhibitors of VHR that inhibit its enzymatic activity at nanomolar concentrations and exhibit anti-proliferative effects on cervix cancer cells.[95] The authors applied SAR analysis in the search for analogs with improved potency and selectivity, which resulted in the discovery of novel inhibitors that are able to interact with both the phosphate-binding pocket and several distinct hydrophobic regions within the active site of VHR. At a concentration of 20 μM, compound **38** (IC_{50} = 74 nM) significantly inhibited the spontaneous proliferation of HeLa and CaSki cells. Other types of VHR inhibitors have been identified by Usui et al. which designed and synthesized a novel dimeric derivative (**39**) of the natural product RK-682.[96] This compound showed increased potency against VHR in comparison to its parent natural product (IC_{50} = 1.8 μM). Additionally, Park et al. recently identified a novel classes of VHR inhibitors by means of a virtual screening and in vitro enzyme assays.[97]

2.3. MptpA and MptpB

Among infectious diseases, tuberculosis (TB) continues to be a major cause of morbidity and mortality throughout the world. The World Health Organization estimates that one-third of the world's population is infected with *Mycobacterium tuberculosis*[98] and about 35 million people are expected to die from TB in the first 20 years of this century. Although new combination therapies with known drugs might prove useful in the treatment of TB,[99] the increasing occurrence of drug-resistant mycobacteria, requires the identification of new targets and are in high demand for drugs for new therapeutic interventions. *M. tuberculosis* protein tyrosine phosphatase A (MptpA) and MptpB are two enzymes secreted by growing mycobacteria and are believed to mediate *M. tuberculosis* survival in host macrophages by dephosphorylation of proteins that are involved in interferon signaling, which represents a crucial pathway of the immune system.[100–102] Remarkably, MptpB shows resistance to the oxidative conditions that prevail within infected host macrophages. X-ray crystallographic studies revealed that a closed, two-helix lid structure blocks the active side and retards oxidative inactivation.[103] Recently, Flynn et al. used single-molecule Förster-type resonance energy transfer (FRET) experiments to explore the dynamics of two helices that constitute the lid. The authors obtained direct proof for large, spontaneous opening transitions of MptpB with the closed form of both helices favored (~3:1).[104]

The importance of MptpB to the intracellular survival of *M. tuberculosis* was recently confirmed by a study in which specific inhibitors directed against MptpB were shown to impair mycobacterial survival in murine macrophages.[105,106] In addition, inhibitors might also prove useful as probe molecules in chemical biology approaches to dissect the detailed functional role of MptpA/B phosphatases in host-pathogen invasion. Recently, our group reported the identification of MptpA inhibitors from the screening of natural-product inspired compound libraries.[107] Compound **40**, an analogue of roseophilin found in Streptomyces species, was found to be an inhibitor of MptpA with an IC_{50} value of 0.9 μM. Utilization of fragment-based library design resulted in the discovery of several novel classes of MptpA inhibitors, among which compound **41** was the most active one, exhibiting a K_i value of 1.6 μM. Recently Rawls et al. reported the application of fragment-based approaches to discover selective inhibitors of MptpA.[108] The most potent of these inhibitors, compound **42**, (K_i = 1.4 ± 0.3 μM) was found to be selective when tested against a panel of human tyrosine and dual-specificity phosphatases, including MptpB, PTP1B, TC-PTP, VHR, CD45, LAR and HCPtpA. Additional MptpA inhibitors are based on the indolizine-1-carbonitrile scaffold.[109] Using biology-oriented synthesis (BIOS) as an efficient means of discovering new compound classes for medicinal chemistry and chemical biology research,[77,110] we identified indole derivatives **43**, **44** and **45** which were at least 100-fold more selective for MptpB and displayed IC_{50} values in the low μM and high nM range. Additionally, Alber and co-workers reported the development of a potent and selective (oxalylamino-methylene)-thiophene sulfonamide inhibitor for MptpB (**46**) (OMTS).[111] OMTS (**46**) shows an IC_{50} value of 440 ± 50 nM and >60-fold specificity for MptpB over six other human PTPs. According to the X-ray crystal structure of MptpB in complex with OTMS, binding of the inhibitor induces significant structural rearrangements of the enzyme's active site, with some residues shifting >27 Å relative to the MptpB:PO_4 complex. More recently, Ellman and co-workers developed a substrate-based fragment based approach termed substrate activity screening (SAS) to identify novel PTP inhibitors with submicromolar inhibitory activities.[112] Application of this method to MptpB resulted in the discovery of the isoxazole based inhibitor **47**. With a K_i of 220 nM, compound **47** is one of the most potent MptpB inhibitors reported in literature so far and was selective for MptpB against a panel human PTPs (VHR, TCPTP, CD45, LAR). Building on the structure of isoxazole **47**, Yao and co-workers recently synthesized and screened a click chemistry-based library of MptpB inhibitors and identified **48** as the most active representative with a K_i value of 0.15 μM for MptpB, but with only moderate selectivity when screened against PTP1B, TCPTP, YopH and LMW-PTP.[113] Recently, the discovery of a new class of MptpB inhibitors was facilitated by the previously mentioned BIOS approach. A stereoselective solid-phase synthesis of macroline derivatives yielded 120 natural product analogues. Kinetic studies and extensive NMR spectroscopy suggest that inhibitors identified from these macrolines inhibit MptpB not via the substrate binding site, but rather via an allosteric mechanism yet to be identified.[114] Compounds **49** and **50** selectively inhibit MptpB with IC_{50} values of 7.0 and 9.6 μM, respectively, and did not show inhibition of other phosphatases (MptpA, VE-PTP, PTP1B, TC-PTPN2, Cdc25A) up to a tested concentration of 100 μM. In a recent study our group reported, spiro-fused indolin-2-one-thiazolidinones, exemplified by compound **51** in Figure 3, as a novel class of potent and selective substrate competitive inhibitors of MptpB.[115] Detailed SAR studies, revealed key structural elements that are essential for strong inhibitory activity against MptpB. Remarkably, the configuration of the spiro-centre was found to be crucial for the inhibitory activity and the separation of the racemate revealed the *R*-(−)-enantiomers as the biologically active component. Recent studies of Zhou et al. revealed that MptpB suppresses the innate immune responses by blocking the ERK1/2 and MAP kinase mediated IL-6 production and promoting host cell survival by activating the Akt pathway.[104] The authors identified a potent and selective inhibitor of cellular MptpB activity, **52**, from a combinatorial library of

bidentate benzofuran salicylic acid derivatives assembled by click chemistry. It was shown that inhibition of MptpB in macrophages with **52** reverses the perturbation of host immune responses induced by the bacterial phosphatase and prevents growth of the TB bacterium in host cells.

3. Conclusion

In the physiological setting of a cell, protein phosphatases function as antagonists of protein kinase activity and there is a high therapeutic potential in targeting these hydrolases. After more than a decade of extensive research and advances in early PTP1B inhibitor identification and development, the discovery of phosphatase modulating agents has progressed steadily in the recent years and has resulted in the generation of a variety of more potent inhibitors. However, since most of these newly developed chemical scaffolds which qualify as PP inhibitors embody phosphate mimics, these recent advances have been accompanied by problems of limited selectivity, limited cell permeability and inadequate pharmacological properties, thereby leaving the door open for the development of next generation drugs and therapeutics which circumvent these major roadblocks in phosphatase inhibitor research. The identification of hit molecules which can be developed into promising new lead compounds is mainly driven by the screening of large compound collections, where up to millions of chemical entities form a single compound library. These large screening campaigns very often focus on the perturbation of a single enzymatic activity and essentially examine the isolated function of the target protein without the added complexity of additional cellular processes associated with various disease states. Along these lines, the screening of PPs using classical phosphatase assays is dependent on the use of enzymatically active phosphatase preparations. As a result, screening initiatives often result in an enrichment of hits which are substrate competitive and block access to the active site of the phosphatase. This may be problematic since the majority of known active site directed phosphatase inhibitors are phosphomimetics which rely on interaction with the highly charged catalytic active site of the phosphatase. Frequently such ligands tend to be relatively unselective.

These limitations may be direct consequences of hit identification using standard enzymatic assays to screen compound libraries. Moreover, such purely activity-based assays make the identification and differentiation of binders which have an alternative mode(s) of inhibition, such as ligands which stabilize inactive phosphatase conformations via an allosteric mechanism, a challenging task. In our view, new directions for future phosphatase inhibitor research can be learned from the success stories of the kinase field and the current endeavors used to identify and develop inhibitors that target alternative binding sites and stabilize inactive kinase conformations.[116] In the kinase field, similar roadblocks are presented by the highly conserved ATP binding site shared by all kinases. The most recent successes in targeted cancer therapy are the newest approved kinase inhibitors sorafenib (Nexavar®; Bayer), nilotinib (Tasigna®; Novartis) and lapatinib (Tykerb®; GlaxoSmithKline) and the more experimental probe molecule GNF-2[117] which target specific types of cancer.[118] The key to their success is that many of these more 'modern' drugs adopt alternative binding modes and take advantage of inactive kinase conformations.[119] To allow for the identification and characterization of such desired molecules from compound libraries, we recently reported the development of FLiK (Fluorescent Labels in Kinases) as a novel direct binding assay for protein kinases.[120–122] This system takes advantage of conformational changes in the kinase domain triggered by ligand binding and can specifically identify and enrich for compounds which stabilize the inactive kinase conformation. By avoiding the requirement of using activated enzyme, which in the case of kinases, shifts conformational equilibrium away from the inactive conformation, the use of such systems may unlock the full potential of pre-existing inhibitor libraries. In combination with structure-based design approaches, this method even allowed the emerging clinical problem mutation-associated drug resistance in protein kinases to be addressed.[123] Although conformational changes in phosphatases are known to be triggered either by protein–protein interactions or ligand binding and the exploitation of less-conserved allosteric pockets available in PPs seems to be a promising strategy for the identification and further development of new marketable compounds to treat diseases of PP disregulation,[54] methods that can reliably identify such modulators in high-throughput are absent from current phosphatase research. As a direct consequence, only a few PP inhibitors have entered clinical trials and new inhibitor classes are in high demand. In addition, relatively few phosphatases are being explored chemically and new targets may be more promising although general limitations such as polarity of active site directed inhibitors prevail. Since PPs often function in multi-enzyme complexes and directing phosphatases to distinct cellular compartments to ensure proper spatial and temporal enzyme activity is a key requirement in the dynamic regulation of cellular processes,[124] targeting protein–protein interactions either using stabilizers[125] or disruptors[126] may also offer a suitable strategy to overcome the current limitations of small molecules directed against the active site of PPs. Up to this point, large scale investigations on the generality of such approaches are still missing from current phosphatase research. Given the difficulties in modern phosphatase inhibitor development, researchers are starting to find success using alternative methods such as the use of short interfering RNAs (siRNAs) in therapeutic approaches which target PPs.[127,128] Interfering RNAs lead to the degradation of messenger RNA and facilitate the use of nucleic acids as 'drugs' against otherwise challenging drug targets.[129] Recently Sirna Therapeutic Inc. claimed to have developed a RNAi directed against PTP1B that potently reduces cellular PTP1B expression levels.[130] However, at this point only time will tell whether any of the discussed therapeutic approaches will succeed.

As soon as the first phosphatase targeted drug reaches the market, a flood of new development programs will likely follow suit, just as it did for kinases at the end of the 1980s. About a decade ago, the successful sequencing of numerous genomes and the development of powerful 'omics'-techniques has sparked a new era in life sciences that aims to translate the genetic and proteomic information into a detailed understanding of cellular processes in order to foster the development of new and innovative medicines to tackle the healthcare challenges of the 21st century. Phosphatase inhibitor research has reached a critical juncture where we now realize that an exclusive understanding of either genomic or proteomic data alone is not sufficient for fully understanding PP function within the orchestrated complexity of cellular networks, living cells, whole organisms or their basic role in mediating diseases. A particularly suitable and powerful approach used to unravel how cellular processes are orchestrated by gene products is to perturb protein function and to compare observed differences between perturbed and unperturbed states. The analysis of disease states associated with different splice variants of phosphatases—which adds an additional layer of complexity to the regulation of PP activity—and monitoring the different states of the phosphoproteome using mass spectrometry[131] in combination with innovative chemical-biological approaches such as Systems Chemical Biology, will also help to unravel how the biological function of PPs and cellular processes emerge from the dynamic interplay of PPs and PKs in cellular systems. However, different yet compatible technologies embedded in an applied basic research-driven interdisciplinary environment will have to be combined in

order for such a multifaceted endeavor to be realized. The combination of such techniques will enable us to draw a more complete image of what, where, when and in which form a particular PP of interest functions. Additionally, these techniques will help to identify effector molecules which can modulate the function of such proteins, and thereby help to clarify the most relevant key interfaces that govern particular aspects of cell behavior. We are confident that the combination of innovative medicinal chemistry research with state-of-the-art chemistry, small molecule screening, cell/model organism and biochemistry-based assays, structural biology and proteomics approaches together with integrated translational research principles shall permit the investigation of aberrantly regulated PP processes that can lead to abnormal cell behavior such as neurodegenerative diseases, cancer and pathogen invasion and guide towards innovative medical applications.

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