Protein Phosphatases Targeted Library

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Preamble

Phosphatases constitute a wide class of enzymes which remove a phosphate group from the target substrate by hydrolysing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group. This action is directly opposite to that of phosphorylases and kinases. While kinases phosphorylate cellular substrates by using energetic molecules like ATP, phosphatases, in turn, remove phosphate from the phosphorylated proteins (dephosphorylation). Among phosphatases, protein phosphatases (PPs) constitute the most promising biological targets in terms of modern medicinal chemistry. They are central players in many different cellular processes and their aberrant activity is associated with multiple human pathologies, including diabetes and obesity, osteoporosis and autoimmune diseases, chronic inflammation as well as cancer. It is generally believed that small molecule selective inhibitors of PPs activity could potentially be used for the treatment of a variety of serious diseases thereby providing promising clinical benefits.

1. A brief insight into the classification, structure and therapeutic significance of PPs

Reversible tyrosine phosphorylation is a crucial and vital mechanism to regulate protein-mediated processes that steer the proliferation and differentiation, functioning of cells and the development, contractility, homeostasis and metabolism, functioning of multicellular organisms, fertilization and memory (for reviews, see the combined reference [1]). This process includes two principal stages, phosphorylation and dephosphorylation, addressed directly to two different types of enzymes. The first one is the most common and essential form of reversible protein posttranslational modification. More than 30% of all proteins being phosphorylated during their intracellular activity, and it is absolutely necessary to say a few words about protein kinases (PKs) before the protein phosphatase description will follow. Protein kinases are the effectors of phosphorylation and catalyse the transfer of a γ-phosphate from ATP to specific amino acids on proteins. More than 500 different PKs have been identified to date. They are generally classified into several distinct super-families, including the key tyrosine and serine/threonine kinases. Considering the catalytic mechanism of action, target proteins are phosphorylated by kinases

predominantly on Ser, Thr and Tyr residues. Since PKs tend to have multiple functions *in vivo*, a vast number of endogenous proteins could be substrates for these enzymes.

Protein phosphatases constitute a divers group of enzymes having the opposite activity as against to PKs, thus they are the primary effectors of dephosphorylation (Fig. 1). In 2007, the New York SGX Research Center for Structural Genomics (NYSGXRC) had published the structures of 21 distinct protein phosphatases: 14 from human, 2 from mouse, 2 from the pathogen *Toxoplasma gondii*, 1 from *Trypanosoma brucei*, the parasite responsible for African sleeping sickness, and 2 from the principal mosquito vector of malaria in Africa, Anopheles gambiae. They can be classified in different ways. First, they can be divided into three main classes according to their substrate specificity: Ser/Thr phosphatases (PSPases), tyrosine phosphatases (PTPase) and dual-specificity phosphatases (DSPs). The two classes of enzymes that catalyze dephosphorylation are protein tyrosine phosphatases and protein serine/threonine phosphatases. Structural studies of these enzymes have revealed that although the two families of Ser/Thr protein phosphatases are unrelated in sequence, the architecture of their catalytic domains is remarkably similar, and distinct from that of the protein tyrosine phosphatases. Tyrosine-specific phosphatases and dual-specificity phosphatases are the members of a broader cysteine-dependent phosphatases (CDPs) family. DSPs are largely involved in the activation of cyclin-dependent kinases [2] and activation of MAP kinases [3]. It should be noted, that the cysteine phosphatases utilize a conserved 'C[X]5R' sequence motif to hydrolyze phosphoester bonds in proteins and in non-protein substrates [4].

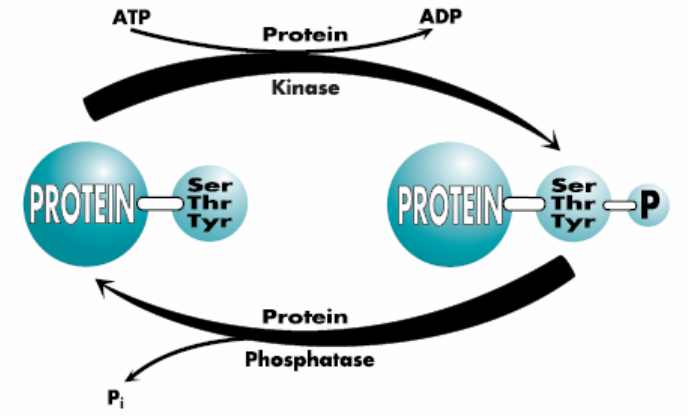


Fig. 1. Schematic view of kinase and phosphatase activity.

In turns, according to sequence alignment, structure and catalytic mechanisms, PPs can also be separated into the PPP (phosphoprotein phosphatase) family, PPM (metallo-dependent protein phosphatase, including the Mg^{2+}- or Mn^{2+}-dependent PPs) family [5], PTP (protein tyrosine phosphatase) family, and the more recently discovered phosphatases that utilize an aspartate-based catalytic mechanism. Both PPPs and PPMs possess unique signature sequence

motifs that span a region of about 220 or 290 aa, respectively. These families are Ser/Thr protein phosphatases. While most members of the PTP family are tyrosine phosphatases, some are dual-specificity protein phosphatases. Based on biochemical parameters, Ser/Thr protein phosphatases were initially divided into two classes: type-I phosphatases (PP1), which are inhibited by two heat-stable proteins, termed inhibitor-1 (I-1) and inhibitor-2 (I-2), and preferentially dephosphorylate the β-subunit of phosphorylase kinase, and type-2 phosphatases (PP2) that are insensitive to the heat-stable inhibitors and preferentially dephosphorylate the β-subunit of phosphorylase kinase. Type-2 phosphatases comprise three enzymes (PP2A, PP2B, and PP2C) that can be distinguished by their cation requirements. PP1, PP2A and PP2B exhibit broad specificities against a number of protein substrates, while PP2B relatively narrow.

PP1, PP2A and PP2B all belong to the PPP family and are important eukaryotic Ser/Thr protein phosphatases. Related novel protein phosphatases, such as PP4, PP5, PP6 and PP7, have recently been characterized, and occur at low abundance and in a tissue-and development-specific manner. The activities of PP1 and PP2A are independent of metal ions [6]. The catalytic subunit of PP1 is bound to the regulatory subunits that determine the subcellular localization and activity of the enzyme [7]. PP2A is inactivated by transient phosphorylation of tyrosine residues on the molecule [8]. PP2B, also known as calcineurin, consists of a catalytic subunit (A-subunit, 61 kDa) and a regulatory subunit (B-subunit, 19 kDa). It is dependent on the Ca^{2+}-calmodulin complex for complete activation [9]. PPP family protein phosphatases are involved in many cellular functions, including glycogen metabolism, muscle contraction, protein synthesis, etc [10]. Although there are differences between PP1, PP2A and PP2B in their bivalent metal ion requirements, inhibitor sensitivity and substrate specificity, their sequences are similar.

Metallo-phosphatases, e.g. PP2C, co-ordinate 2 catalytically essential metal ions within their active site. There is currently some confusion of the identity of these metal ions, as successive attempts to identify them yield different answers. There is currently evidence that these metals could be Magnesium, Manganese, Iron, Zinc, or any combination thereof. It is thought that a hydroxyl ion bridging the two metal ions takes part in nucleophilic attack on the phosphorus ion.

Kinases have achieved specificity by using very specific catalytic domains that recognize particular sequence motifs in target substrates. Phosphatases, on the other hand, have highly conserved catalytic domains that gain specificity through the formation of heteromultimeric holoenzymes [11], which localize phosphatases to specific areas of the cell where their activity is required [12]. This localization is facilitated through specific protein-protein interactions. For example, the tyrosine phosphatases, Shp-1 and Shp-2, contain SH2 (srchomology) domains, which target the phosphatases to sites of tyrosyl phosphorylation, such as receptor tyrosine

kinases [13]. PP1 is localized to different cellular compartments depending on the cell type. In liver cells, a "glycogen-targeting subunit" anchors PP1 to glycogen particles. In skeletal muscle, a different subunit anchors PP1 to the sarcoplasmic reticulum [14]. PP2A is a heterotrimer with a catalytic subunit, a structural subunit and a regulatory subunit. The regulatory subunit interacts with various subcellular structures (e.g., centrosomes, endoplasmic reticulum, golgi and nucleus) to compartmentalize the enzyme. PP2B also interacts with subcellular structures and associates with PKA and PKC on the A-kinase-anchoring protein, AKAP79 [15]. From the therapeutic point of view, two key groups of phosphatases are of the most prominent: those that dephosphorylate phosphotyrosine residues and those that dephosphorylate phosphoserine residues and/or phosphothreonine residues.

As briefly mentioned above, malfunction in PP activity has been associated with human diseases, including diabetes, obesity, cancer, and neurodegenerative and autoimmune disorders, and makes this class of enzymes attractive targets for chemical biology and medicinal chemistry research. Interestingly, although PKs represent reliable molecular targets for an increasing number of anticancer agents, there is a notable absence of drugs targeting specifically against PPs. This absence is particularly striking considering the fact that many cancer-relevant pathways are also deeply controlled by PPs; in cancer cells their functions are also grossly abnormal. Therefore, several research groups are currently searching for drug-like inhibitors of phosphatase-based cancer targets [16].

While the importance of PKs for the spatial and temporal control of mitotic events has long been recognized, mitotic phosphatases have only recently come into the limelight [17]. It is now well established that PPs counteract mitotic kinases, so contributing to the generation of switch-like responses at mitotic stage transitions. In addition, the timely dephosphorylation of mitotic phosphoproteins by tightly regulated phosphatases is required for the assembly and stability of the mitotic spindle, the initiation of anaphase, and exit from mitosis. Mitotic PPs also emerge as effectors of the DNA damage and spindle assembly checkpoints. These new findings show that PPs regulate every step of mitosis and provide novel insights into the dynamic and versatile nature of mitotic phosphoregulation.

It has also been suggested that PPs play a role in Alzheimer's disease. The underlying mechanism of action is associated with tau phosphorylation [18]. Therefore, PP inhibitors could provide clinical benefits in this area.

A number of strategies are currently explored for the identification and development of various classes of PP modulators and have resulted in a plethora of chemically distinct inhibitors. However, limited selectivity and adverse pharmacological properties of PP inhibitors are still major bottlenecks for further clinical development and resulted in only a few molecular entities

currently in clinical trials. Therefore there is a growing demand in novel PP inhibitors of different structural classes particularly targeted against Tyrosine and Ser/Thr protein phosphatases [19].

2. Protein tyrosine phosphatases

Tyrosine phosphorylation is a fundamental mechanism for numerous important aspects of eukaryote physiology, as well as human health and disease [20]. Compared to protein phosphorylation in general, phosphorylation on tyrosine is extensively utilized only in multicellular eukaryotes. Tyrosine phosphorylation is used for communication between and within cells, the shape and motility of cells, decisions to proliferate versus differentiate, cellular processes like regulation of gene transcription, mRNA processing, and transport of molecules in or out of cells. Tyrosine phosphorylation also plays an important role in the coordination of these processes among neighboring cells in embryogenesis, organ development, tissue ho-meostasis, and the immune system. Abnormalities in tyrosine phosphorylation play a role in the pathogenesis of numerous inherited or acquired human diseases from cancer to immune deficiencies. Although it is generally agreed that tyrosine phosphorylation is regulated by the equal and balanced action of PTKs and PTPs, proportionately much more research has focused on PTKs. This is partly for historical reasons: the first PTP was purified [21] and cloned [22] ten years later the first PTK [23]. Recent findings have now led to the emerging recognition that PTPs play specific and active, even dominant, roles in setting the levels of tyrosine phosphorylation in cells and in the regulation of many physiological processes.

Considering these findings, PTPs and PTKs are the enzyme classes that are key instrumental in controlling the spatial and temporal ratio of tyrosine-phosphorylated and non-phosphorylated targets and thus coordinately regulate cellular responses to intra- and extracellular cues. PTPs can be roughly divided into two distinct groups: the truly phosphotyrosine-specific PTPs on the one hand and the dual-specificity phosphatases (DSPs) on the other [24]. Disturbance of the normal balance between PTK and PTP activity has been linked to various disease states ranging from cancer to neurological disorders and diabetes. For example, a number of PTPs have been implicated in oncogenesis and tumor progression and therefore are potential drug targets for cancer chemotherapy [25]. These include PTP1B, which may augment signaling downstream of HER2/Neu; SHP2, which is the first oncogene in the PTP superfamily and is essential for growth factor-mediated signaling; the Cdc25 phosphatases, which are positive regulators of cell cycle progression; and the phosphatase of regenerating liver (PRL) phosphatases, which promote tumor metastases. As PTPs have emerged as drug targets for cancer, a number of strategies are currently been explored for the identification of various

classes of PTP inhibitors. These efforts have resulted many potent, and in some cases selective, inhibitors for PTP1B, SHP2, Cdc25 and PRL phosphatases. Structural information derived from these compounds serves as a solid foundation upon which novel anti-cancer agents targeted to these PTPs can be developed. In addition, PTP1B was also shown to be a negative regulator of the insulin signaling pathway [26] suggesting that PTP1B inhibitors (such as DMHV) may be beneficial in the treatment of type 2 diabetes. Consequently, PTPs have emerged as promising targets for therapeutic intervention in recent years [27]. Different strategies have been successfully applied to identify potent and selective PP inhibitors, including 3D-molecular docking and pharmacophore modeling as well as various *in silico* data mining techniques. These studies constitute the basis for the future development of PTP inhibitors as drugs.

In fact, PTPs constitute a large family of enzymes that parallel PTKs in their structural diversity and complexity. Structural analysis of protein tyrosine phosphatases has expanded considerably in the last several years, producing more than 200 structures in this class of enzymes (from 35 different proteins and their complexes with ligands). The classical, tyrosine-specific PTPs are encoded by 38 genes in humans. They belong to a larger family of cysteine-dependent phosphatases that comprises 106 genes in humans and numerous pseudogenes. Over 40 protein tyrosine phosphatases (PTPs) have been comprehensively characterized thus far. They possess a 230-amino acid catalytic domain and contain a number of regulatory subunits that appear to be essential for subcellular localization and regulation of enzymatic activity [28]. Developments in mammalian expression technology recently led to the first crystal structure of a receptor-like PTP extracellular region. Altogether, the PTP structural work significantly advanced our knowledge regarding the architecture, regulation and substrate specificity of these enzymes. Schematic depiction of the domain composition for all subfamilies of classical phosphotyrosine-specific PTPs is shown in figure 2.

As shown in figure 2, each of the 38 classical mammalian PTP genes is represented by a single protein isoform. PTP subtypes, according to Andersen et al. [29], are listed. It should be noted that because of, for example alternative splicing, a single PTP gene may encode multiple isoforms, sometimes including receptor-like and non-transmembrane enzymes (hence the R7 subtype classification for cytosolic KIM-containing PTPs). In addition, specific isoforms within subtype families may contain additional protein domains and/or targeting sequences (e.g. the ER anchoring tail in PTP1B and the nuclear localization signal in TCPTP) [30].

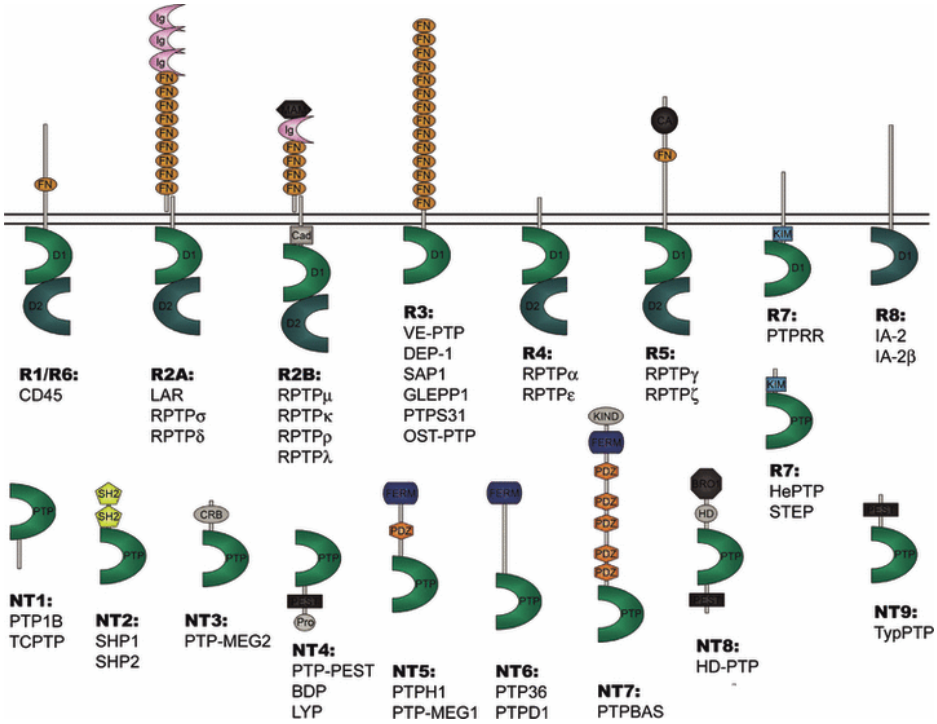


Fig. 2. Subfamilies of classical phosphotyrosine-specific PTPs. (Domain abbreviations: BRO1, baculovirus BRO homology 1; CA, carbonic anhydrase-like; Cad, cadherin-like; CRB, cellular retinaldehyde-binding protein-like; D1 and D2, membrane-proximal and membrane distal PTP domains, respectively (enzymatically active domains are in green, PTP domains with reduced or even no activity are in bluish green); FERM, band 4.1/ezrin/radixin/moesin homology (in blue); FN, fibronectin type-III repeat-like (orange ovals); HD, His domain; Ig, immunoglobulin-like; KIM, kinase interaction motif (light blue); KIND, kinase N-lobe-like domain; MAM, meprin/A2/RPTPμ homology; PDZ, postsynaptic density-95/discs large/ZO1 homology; Pro, proline-rich sequence; SH2, src homology 2 (in yellow))

As briefly mentioned above, the most significant trait of the PTP superfamily is conservation of the signature motif CX5R, which forms the phosphate-binding loop in the active site (known as the P-loop or PTP-loop). Despite relatively large sequence variations in the X5 segment, the conformation of the P-loop is strictly conserved and can be easily superimposed from different PTP structures, with minor deviations in the Cα tracing (< 1 Å). This structurally conserved arrangement ensures that the catalytic Cys, the nucleophile in catalysis, and the Arg, involved in phosphate binding, remain in close proximity and form a cradle to hold the phosphate group of the substrate in place for nucleophilic attack. The cysteine Sγ-atom is the nucleophile that attacks the substrate phosphorus atom leading to the cysteinyl-phosphate reaction intermediate. The arginine is involved both in substrate binding and in stabilization of the reaction intermediate [[31]]. Further to this, the amide groups in the P-loop point towards the interior of the cradle and form a network of hydrogen bonds to the phosphate oxygens (Fig. 3A). A conserved Ser/Thr residue in the P-loop has been proposed to play an important role in the

stabilization of the thiolate group in the transition state facilitating the breakdown of the phosphoenzyme intermediate [32] (Scheme 1).

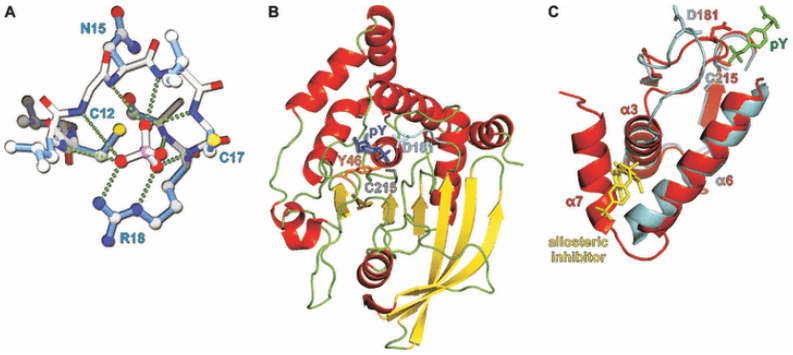
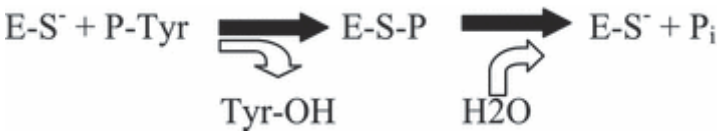


Fig. 3. (**A**) Structure of the phosphate-binding loop (P-loop). Stick representation of the consensus signature motif (CX5R) that forms the P-loop present in the active site of PTPs. (**B**) Structure of PTP1B (C215S mutant) in complex with phosphotyrosine (PDB entry 1PTV). Position of the substrate in the active site is illustrated by the phosphotyrosine ligand (blue). (**C**) Binding of an allosteric inhibitor of PTP1B keeps the catalytic WPD-loop in the open conformation.

As shown in figure 3(A), the P-loop from bovine LMW-PTP (1PNT) [33] is represented and the catalytic Cys12 and Arg18 are labelled. The amide nitrogens form hydrogen-bond interactions (dotted green lines) with the phosphatase bound showing network of interactions that involve the catalytic Arg. The cradle-like conformation of the P-loop is conserved in the structures of all PTPs. In figure 3(B), Tyr46 within the 'KNRY' conserved motif contributes the substrate recognition. Active-site nucleophile Cys215 (grey) (here mutated to Ser) attacks the substrate phosphorus leading to the formation of the cysteinyl-phosphate intermediate. Asp181 within the WPD-loop (cyan), here in the closed conformation, acts as a general acid donating a proton to the phenolate leaving group. And, figure 3(C) presents the structure of PTP1B (cyan) in complex with the allosteric inhibitor 3-(3,5-dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonic acid 4-sulfamoyl-phenyl)-amide (termed 'compound-2' in [34]) (PDB entry 1T49) overlain on the PTP1B (C215S mutant) structure (red) in complex with a p-Tyr substrate (PDB entry 1PTV). Only the main structural elements involved in allosteric inhibition are represented. In the presence of the allosteric inhibitor (yellow), the C-terminus of PTP1B is disordered while in presence of the phosphotyrosine (green) it adopts the α-helical structure α7. Binding of allosteric inhibitor impedes the interaction between helices α3, α6 and α7, thus preventing the closure of the WPD-loop.



Scheme 1. General mechanism of the PTPs-related catalysis.

The catalytic mechanism of PTP reaction requires the participation of a general acid and a general base. This is provided by a unique aspartic residue situated on the WPD-loop. During formation of the transition state intermediate, the catalytic Asp acts as a general acid protonating the oxygen of the leaving group in the tyrosine residue. In the second catalysis step, the same Asp functions as a general base during hydrolysis of the phospho-enzyme by accepting a proton from the attacking water and assisting in the conversion of the phospho-Cys enzyme to its resting Cys-SH state, thus regenerating the free enzyme [35]. Upon substrate binding, the WPD-loop closes over the active site bringing the catalytic Asp near the leaving group. An analogous Asp residue is found in the DPYY-loop of the low molecular weight protein tyrosine phosphatases (LMW-PTPs), although in this enzyme it appears to be less mobile than the WPD-loop and it adopts a fixed position near the active site.

Table 2 illustrates that the construction of knockout mouse models, via homologous recombination in embryonic stem cells, for the different PTP genes is rapidly nearing completion. The phenotypes obtained all advocate the importance of PTP signalling. PTP loss has lethal consequences during early embryonic development or results in no or only mild effects, presumably reflecting redundancy as a safeguard for the organism.

How PTP activity is regulated is still subject of intense investigation, but mechanisms include alternative mRNA splicing, modulation of steady state levels, posttranslational modification (including phosphorylation), dimerization and/or subcellular confinement [36]. With regard to the physiological functions of individual PTPs, also much more work needs to be done. Many studies at the cellular level are hampered by the very low endogenous expression levels of PTPs and their firm catalytic activity that is some log orders of magnitude over that of kinases. In combination, this may cause overexpression artifacts in transfected cell-based readouts, and thus, it should come as no surprise that most information on PTP functioning resulted from studies of mammalian pathologies or via exploitation of genetically modified animal models [37]. The scientists have correlated each PTP locus with genetic disease markers and identified 4 PTPs that map to known susceptibility loci for type 2 diabetes and 19 PTPs that map to regions frequently deleted in human cancers.

Table 2. Phosphotyrosine-specific class I PTP-related phenotypes in mouse and human.

Gene symbol	Protein name	PTP type[a]	Mouse model	Human/mouse/rat phenotype description (functional evidence from other sources)
PTPN1	PTP1B	NT1	Yes	M: NOP[b] – Increased insulin sensitivity, obesity resistance
PTPN2	TCPTP	NT1	Yes	M: Die 3–5 weeks postpartum; defective haematopoiesis and immune function
PTPN3	PTPH1	NT5	Yes	M: Enhanced growth due to augmented GH signalling, normal haematopoietic functions
PTPN4	PTP-MEG1	NT5	–	M: Involved in motor learning and cerebellar synaptic plasticity
PTPN5	STEP	R7	–	(duration of ERK signalling in the brain, neuronal plasticity)
PTPN6	SHP1	NT2	Yes	M: Die within first month; haematopoietic defects, splenomegaly, autoimmune disease, osteoporosis, increased insulin sensitivity H: Candidate tumour suppressor in lymphomas
PTPN7	HePTP	R7	Yes	M: NOP (suppresses ERK activation)
PTPN9	PTP-MEG2	NT3	Yes	M: Embryonic lethal; defective secretory vesicle function
PTPN11	SHP2	NT2	Yes	M: Lethal at preimplantation stage; defective cell survival signalling H: Mutated in Noonan syndrome and Leopard syndrome
PTPN12	PTP-PEST	NT4	Yes	M: Embryonic lethal; regulator of cell motility H: CD2BP1, a PTP-PEST binding protein, is mutated in PAPA syndrome
PTPN13	PTPBAS	NT7	Yes	M: NOP – Impaired regenerative neurite outgrowth, negative regulator of STAT signalling (control of oocyte meiotic maturation)
PTPN14	PTP36	NT6	Yes	M: Androgenization of female mice (US Patent 20020152493) (negative regulator of cell motility)
PTPN18	BDP	NT4	–	(involved in HER2 signal attenuation)
PTPN20	TypPTP	NT9	–	(regulator of actin cytoskeleton dynamics)
PTPN21	PTPD1	NT6	–	(modulator of Tec family kinases and Stat3 activity)
PTPN22	LYP	NT4	Yes	M: Enhanced immune functions, splenomegaly, lymphadenopathy. H: Gain of function mutant causes autoimmune diseases
PTPN23	HD-PTP	NT8	–	(candidate tumour suppressor on 3p21.3; regulates endothelial migration via FAK)
PTPRA	RPTPα	R4	Yes	M: NOP – affected neuronal migration and synaptic plasticity, learning deficit, decreased anxiety, impaired NCAM-mediated neurite elongation
PTPRB	VE-PTP	R3	Yes	M: Embryonic lethal, reduced vascular development, heterozygotes are normal
PTPRC	CD45	R1	Yes	M: No T cells, immature B cells, impaired differentiation of oligodendrocyte precursor cells, dysmyelination
PTPRD	RPTPδ	R2A	Yes	M: Impaired learning and memory, retarded growth, early mortality, posture and motor defects
PTPRE	RPTPε	R4	Yes	M: NOP – Hypomyelination, defective osteoclast functioning, reduced src activity, aberrant macrophage function
PTPRF	LAR	R2A	Yes	M: NOP – Mammary gland defect, altered neuronal circuitry, learning deficits, enhanced IGF-1 signaling
PTPRG	RPTPγ	R5	Yes	M: NOP (tumor suppressor candidate on 3p14)
PTPRH	SAP1	R3	–	(negatively regulates cell motility)

a PTP types according to Andersen et al. [38]. Phenotypic consequences of mutations in human (H), mouse (M) or rat (R) are given. In absence of such information, the functional data derived from cell models are mentioned between brackets and aligned to the right. *b* NOP (no obvious phenotype): normal and healthy appearance, normal breeding and behaviour. *c* The apparently conflicting phenotypes reflect different mouse mutants.

3. Serine/Threonine phosphatases

The most abundant protein phosphatases in mammalian systems are the serine/threonine protein phosphatases, which catalyse the dephosphorylation of serine and threonine residues in proteins. More than 98% of all protein-bound phosphate is attached to two amino acids, serine and threonine, whereas the remaining less than 2 % affects tyrosine. In eukaryotic organisms, serine/threonine protein phosphatases (PPases) have been grouped into two major families, designated as PPM (metal-dependent protein phosphatases) and PPP (phosphoprotein phosphatases). The phosphatases of the PPP family are among the most highly conserved proteins on earth, with homology across taxa greater than that of histones (2A and 2B). Serine/threonine protein phosphatases have been traditionally classified into type 1 (PP1) and type 2 (PP2), depending on their substrate specificity and sensitivity to inhibitors. Type 2 protein

phosphatases are subdivided into three major groups: ion-independent PP2A, calciumdependent calcineurin PP2B and magnesium-dependent PP2C. Other types of serine/threonine PPs designed PP4, PP5, PP6, PP7 and FCP/SCP have been described, but their role, substrate affinity and pharmacology are not well known. Most of the PPP-subfamilies contain isoforms, with mammals expressing two or more isoforms of PP1, PP2A, PP2B, and PP7 that share >80% identity. The sequence homology between them is relatively high, and tertiary structure is conserved [39]. Because of the complexity of the structure of PP2A and the diversity of its regulatory subunits, its structure is less well known than those of PP1 and PP2B. The PP2A holoenzyme consists of a heterodimeric core enzyme, comprising a scaffolding subunit and a catalytic subunit, as well as a variable regulatory subunit.

PP1, PP2A and PP2B participate in regulating many important physiological processes, such as cell cycle control, regulation of cell growth and division regulation, etc [40]. For example, recent studies in mammalian cells have highlighted a major role of serine/threonine protein phosphorylation in apoptosis [41]. Thus, it was suggested that the interaction of the serine/threonine protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) with certain regulators of the Bcl-2 family is critically involved in the control of apoptosis. Considering these findings, small molecule modulators of PP-related apoptosis can be regarded as promising anticancer agents.

Accumulating evidence indicates that serine/threonine protein phosphatases, such as PP1, PP2A and PP2B, participate in the neurodegenerative progress in Alzheimer's disease [42]. The general characteristics and pathologic changes of PP1, PP2A and PP2B in AD, and their relations with microtubule-associated proteins, focusing mainly on tau protein, neurofilament (NF), amyloid precursor protein (APP) processing and synaptic plasticity have been discussed.

6. Dual-specificity phosphatases

DSPs are deeply involved in the regulation of both developmental and postnatal essential processes, such as early embryogenesis, placental development and immune responses. DSPs can dephosphorylate phosphoserine, phosphothreonine and nonproteinaceous substrates including signaling lipids and complex carbohydrates in addition to phosphotyrosine residues, and some are also active against phospholipids [43]. Several DSP genes are implicated in familial and sporadic human diseases, including tumor-related, neurological and muscle disorders, and cardiovascular and inflammatory diseases. This association ranges from disease-causative mutations to disease-risk-prone single-nucleotide polymorphisms, promoter methylation or gene duplication (most often in cancer). Deconvolution of the role of DSPs in disease is challenging.

The enzymes' activities are regulated at many levels and they form part of extensive, intricate networks with other signaling components.

Mammalian class I cysteine-based dual-specificity phosphatases constitute a broad family of protein tyrosine phosphatases, both in number and diversity. PTP domain class I DSPs differ from classical PTPs in their native substrates. DSPs are nontransmembrane proteins (with the exception of TPIP and TPTE) that contain a single catalytic domain, and their modular structure ranges from the 'minimal PTP core' of the small atypical DSPs to the multimodular structure of some large myotubularins. DSPs can be clustered in several groups that include MAP-kinase phosphatases (MKPs), myotubularins (MTMs), PTEN-related phosphatases, slingshots (SSHs), PRLs, CDC14s and a heterogenous group named atypical DSPs [44]. The involvement of some of these DSPs in human disease is well established, because their genes (PTEN, MTMs, laforin) are targeted by mutations in sporadic tumors or in the germline of patients with hereditary disorders. In addition, recent findings indicate that many other DSPs may be associated with human disease because of pathological alterations in their expression patterns and/or functional activities. The growing information on DSP structure, specific inhibitory compounds and physiological function in mammalian animal models may be exploited to target-specific DSPs in particular diseases and/or subsets of patients. Several putative disease-relevant DSPs, as inferred from knockout mice models, are presented in Table 1 and Fig. 4. Detailed information on the biochemical and biological properties of the distinct DSP families has been provided in recent reviews [45].

Table 3. Functional properties and relation with human disease of DSPs*.

Gene name	Phosphatase name	Other names	Substrate	Associated with disease/ target for disease	Phenotype of KO mouse	Chromosomal location	Accesion
DUSP1	MKP-1	3CH134, PTPN10, erp, CL100, hVH1	p38, JNK, ERK	Overexpressed in many cancers	Increased innate immune response; resistant to diet-induced obesity	5q35.1	NM_004417
DUSP6	MKP-3	PYST1, rVH6	ERK,p38	Often suppressed in cancers	Excess mortality in homozygotes; skeletal deformations linked to excess FGFR signaling	12q22-q23	NM_001946
DUSP10	MKP-5		JNK, p38	KO animals protected from EAE	Protected from experimental autoimmune encephalitis	1q41	NM_007207
DUSP2	Pac-1		ERK, p38	KO animals protected from RA	Partial immunodeficiency; protected from rheumatoid arthritis	2q11	L11329
DUSP26	DUSP26	MKP-8	p38, JNK, ERK	Cancer	Not available (N/A)	8p12	NM_024025
PTP4A1	PRL-1	Phosphatase of regenerating liver, PTPCAAX1	Unknown	Cancer	N/A	6q12	U48296
PTP4A2	PRL-2	PTPCAAX2	Unknown	Cancer	N/A	1p35	NM_003479
PTP4A3	PRL-3	PRL-R	Unknown	Cancer; metastasis	N/A	8q24.3	NM_032611
MTM1	MTM1	Myotubularin	PI(3)P, PI(3,5)P2	X-linked myotubular myopathy (XLMTM)	Muscle myopathy; XLMTM-like	Xq27.3-q28	NM_000252
MTMR1	MTMR1		PI(3)P, PI(3,5)P2	Congenital myotonic dystrophy (cDM1)	N/A	Xq28	NM_003828
MTMR2	MTMR2		PI(3)P, PI(3,5)P2	Charcot–Marie–Tooth disease type 4B1 (CMT4B1); male infertility	Abnormal myelination; CMT4B-like; impaired spermatogenesis	11q22	NM_016156
MTMR13	MTMR13	SBF2, Set-binding factor 2	Enzyme inactive; required for MTMR2 activity	Charcot–Marie–Tooth disease type 4B2 (CMT4B2)	Abnormal myelination; CMT4B-like	11p15.4	NM_030962
MTMR5	MTMR5	SBF1, Set-binding factor 1	Enzyme inactive	Male infertility; cancer	Impaired spermatogenesis	22q13.33	U93181
EPM2A	Laforin		Complex carbohydrates; GSK3β	Lafora disease (progressive myoclonus epilepsy)	Neurodegeneration; Lafora disease-like	6q24	AF284580
PTEN	PTEN	MMAC-1, TEP1	PI(3,4,5)P3	Cancer	Early embryonic lethality in homozygotes; high incidence of cancer and autoimmunity in heterozygotes	10q23.3	U92436

* EAE, experimental autoimmune encephalitis; RA, rheumatoid arthritis; XLMTM, X-linked myotubular myopathy.

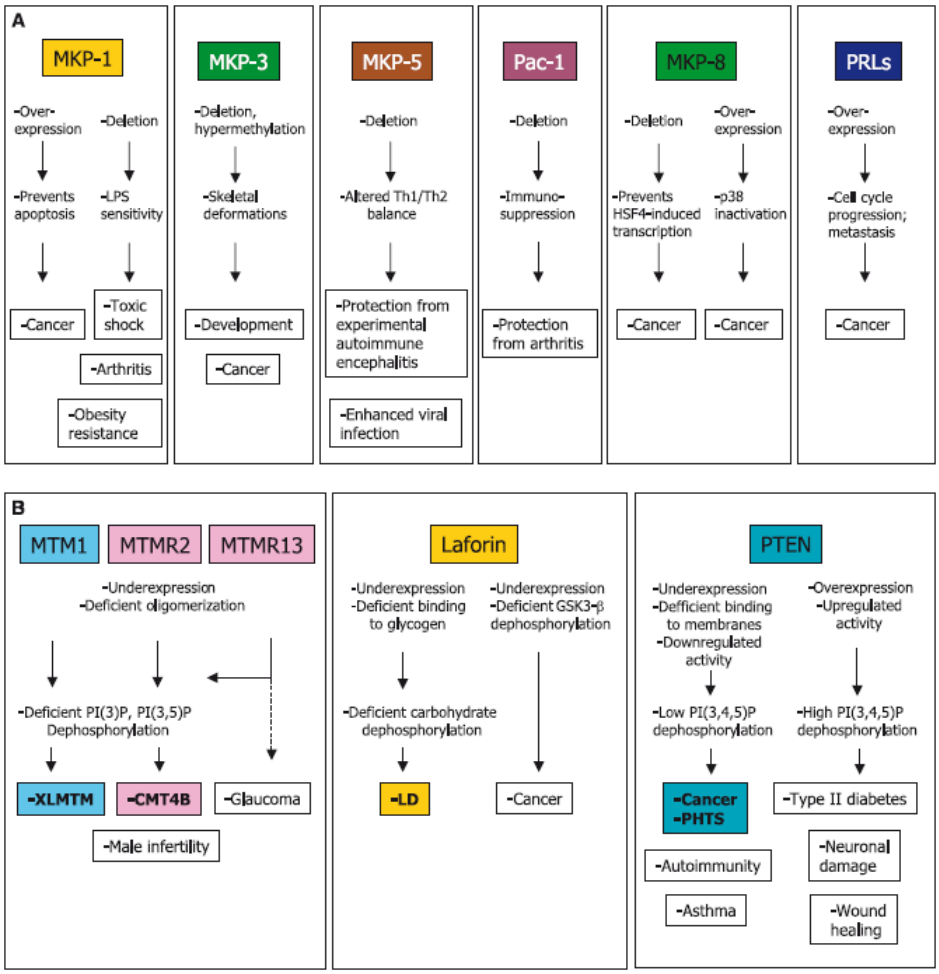


Fig. 4. DSPs in human disease. (**A**) Putative role of class I cysteine-based DSPs in human disease. The involvement of MKP-1/DUSP1, MKP-3/DUSP6, MKP-5/DUSP10 and Pac-1/DUSP2 in human disease is mainly based on the phenotype of knockout mice. The involvement of MKP-8/DUSP26 and PRLs in cancer is based on the high expression and function of these DSPs in tumor samples and/or tumor cell lines. (**B**) Class I cysteine-based DPS mutated in human disease. The functional properties altered in disease of MTMs, laforin, and PTEN are indicated. In bold are shown the major diseases caused by mutations in the DSP genes.

Dual-specificity phosphatases are implicated in a variety of processes that play a role in disease, but a number of complications precludes their simple validation as drug targets. Their activity is under stringent control and they may affect multiple signal transduction partners and feedback control mechanisms. While DSPs are clearly druggable (many reports describe small-molecule inhibitors), as a rule such compounds are not totally selective and will affect subsets of enzymes with consequences that are extremely difficult to predict from mechanistic studies alone. The path forward to DSP target validation appears therefore to attempt to come to a better understanding of DSPs using genetic and genomic approaches as combined with empirical studies using inhibitors as pharmacological tools.

Taking together, these members of PPs constitute a very attractive group of biological targets deeply implicated in various actual diseases. The following section provides our *in silico* approach to design of novel promising PP inhibitors under the cover concept of target diversity.

7. Concept and Applications

PTPs-targeted library design at CDL involves:

• *A combined profiling methodology that provides a consensus score and decision based on various advanced computational tools*:

1. Targeted diversity concept
2. Unique bioisosteric morphing and funneling procedures in designing novel potential PTP inhibitors with high IP value. We apply CDL's proprietary Chemosoft™ software and commercially available solutions from Accelrys, MOE, Daylight and other platforms.
3. Neural Network tools for target-library profiling, in particular Self-organizing Kohonen maps, performed in SmartMining Software. We have also used the Sammon mapping and Support vector machine (SVM) methodology as more accurate computational tools to create our PTP-focused library.
4. A molecular docking approach to the focused library design.
5. Computational-based `*in silico*` ADME/Tox assessment for novel compounds includes prediction of human CYP P450-mediated metabolism and toxicity as well as many pharmacokinetic parameters, such as Brain-Blood Barrier (BBB) permeability, Human Intestinal Absorption (HIA), Plasma Protein binding (PPB), Plasma half-life time ($T_{1/2}$), Volume of distribution in human plasma (V_d), etc. The fundamentals for these applications are described in a series of our recent articles on the design of exploratory small molecule chemistry for bioscreening [for related data visit ChemDiv. Inc. online source: www.chemdiv.com].

• *Synthesis, biological evaluation and SAR study for the selected structures*:

1. High-throughput synthesis with multiple parallel library validation. Synthetic protocols, building blocks and chemical strategies are available.
2. Library activity validation via bioscreening; SAR is implemented in the next library generation.

7.1. The concept of targeted diversity: a new algorithm for building screening libraries

ChemDiv introduces the concept of Targeted Diversity which is intended for the design of high quality libraries of drug-like compounds that have been focused against various biological targets. Targeted diversity signifies the superposition of highly diverse chemical space

on the assortment of divergent families or sub-families of targets and unique biomolecules. These targets may be congener or "orthogonal" (non-overlapping) and include:

(a) Different classes of targets.

(b) Distinct, structurally unrelated branches of the same target class.

(c) Independent targets.

The different classes of biomolecules are represented by G-protein coupled receptors (GPCR), nuclear hormone receptors (NHR), ligand- and voltage-gated ion channels (LGICh and VGICh), transporters (TR), various enzymes (kinases, proteases, phosphodiesterases, etc.), effector proteins and others. Examples of the branches of related proteins include serine/threonine protein kinases (STPK) and tyrosine kinases (TK) as sub-families of the kinome. An example of independent targets is GPCR-like Smo receptors. The current edition of the Targeted Diversity Library (TDL) is based on approximately 100 small molecule sets. Each of these sets is focused against distinct biological targets belonging to the different classes and sub-families of targets (list of targets selected is shown below) and includes about 5000 individual drug-like molecules. The selection process for these sets involves identifying active ligands/inhibitors as prototypes existing in the patent and research literature or databases and performing bioisosteric replacement strategies, e.g. a known peptide ligand may be substituted with a small non-peptide peptidomimetic. Then a similarity search based on these strategies is conducted within ChemDiv's collection for possible augmentation of the rational set. Other techniques include computer-assisted 3-D pharmacophore matching and when possible, in silico docking experiments. The directed synthesis of new chemotypes with functionality mimicking recognition elements (shapes, "warheads") of known active ligands/inhibitors has also been performed. In some cases, proof of concept has been established with in-house biological data. A special effort has been made to select respective compounds and synthetic templates with good IP potential, as deduced from Beilstein, SciFinder and Markush sub-structure searches. The special rules of ChemDiv's medchem filters (MCF) ensure the high quality and drug-like properties of selected molecules. The first edition of the TDL includes the most diverse compounds (250-750 members) from each of 100 target-specific sets. The current TDL is built around 1,000 diverse chemical templates to yield a library of about 40,000 individual drug-like molecules. Embellishment of the library is an ongoing effort at ChemDiv. Regular updates are being made as newly synthesized compounds become available and pass our QA specifications (>90% purity as established by LC/MS with UV and ELSD). Additionally, new proposals for target-specific sets are being evaluated, tested and made available.

Thus, the TDL may provide high-quality hits in screening against "difficult" targets with limited or no structure/ligand information, as well as "eclectic" biological targets, including

cellular processes (e.g. apoptosis and cell cycle), signaling pathways (e.g. WNT, Hh, RTK and Ras) or protein-protein interactions (e.g. XIAP, pGPCRs).

7.2. Virtual screening on PP-specific activity

Our multi-step *in silico* approach to PP-focused library design is schematically illustrated in Fig. 5.

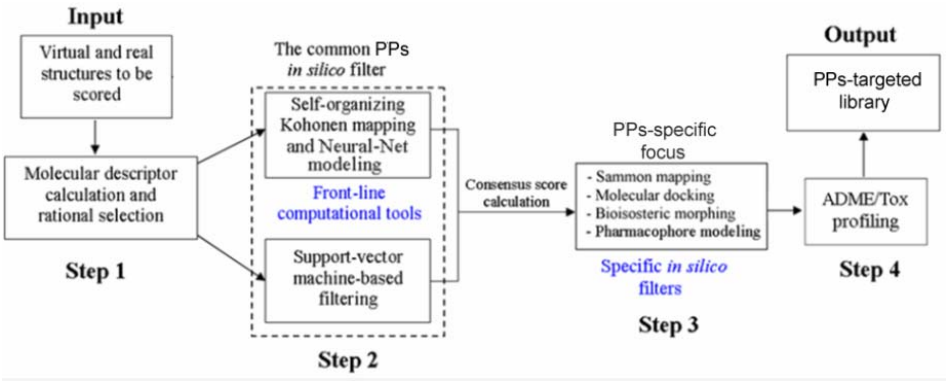


Fig. 5. Multi-step computational approach to PP-targeted library design.

At the initial stage of our PP-targeted library *in silico* design, we have collected a 22,110-compound database of known drugs and compounds entered into preclinical or clinical trials; their structures and assignments were obtained from Prous Science Integrity [46]. Each compound in this database is characterized by a defined profile of target-specific activity, focused against 1 of more than 100 different protein targets, including compounds acting against PPs (several already launched drugs, hundreds active compounds in advanced clinical evaluation and more than 1.2K compounds in early biological trials). The database was filtered based on MW (not more than 800). Molecular features encoding the relevant physicochemical and topological properties of compounds were calculated from 2D molecular representations and selected by PCA (Step 1, Fig. 5). These molecular descriptors encode the most significant molecular features, such as molecular size, lipophilicity, H-binding capacity, flexibility, and molecular topology. Taken in combination, they define both pharmacokinetic and pharmacodynamic behavior of compounds and are effective for property-based classification of target-specific groups of active agents. However, it should be noted that for each particular target-specific activity group, another, more optimal set of descriptors can be found, which provides better classification ability. As shown in Fig. 5, 'front-line' computational tools include Kohonen-based SOM generation as well as Neural-Net- and SVM-based modeling; these algorithms have been effectively used across the Step 2, decoded in Fig. 3. As an example, Kohonen-based method is described below in more detail.

Self-organizing Kohonen mapping

A Kohonen SOM of 22,110 pharmaceutical leads and drugs generated as a result of the unsupervised learning procedure is depicted in Fig. 6. It shows that the studied compounds occupy a wide area on the map, which can be characterized as the area of druglikeness. Distribution of various target-specific groups of ligands in the Kohonen map demonstrates that most of these groups have distinct locations in specific regions of the map (Fig. 7a-f), including PTP inhibitors (Fig. 7f). A possible explanation of these differences is in that, as a rule, receptors of one type share a structurally conserved ligand-binding site. The structure of this site determines molecular properties that a receptor-selective ligand should possess to properly bind the site. These properties include specific spatial, lipophilic, and H-binding parameters, as well as other features influencing the pharmacodynamic characteristics. Therefore, every group of active ligand molecules can be characterized by a unique combination of physicochemical parameters differentiating it from other target-specific groups of ligands. Another explanation of the observed phenomenon can be related to different pharmacokinetic requirements to drugs acting on different biotargets.

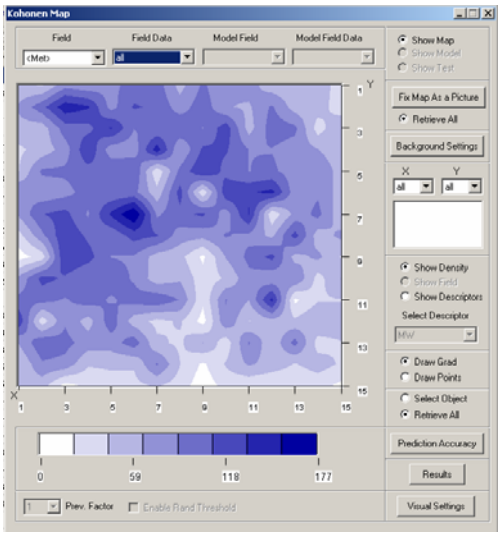


Fig. 6. Property space of 22,110 pharmaceutical leads and drugs visualized using the Kohonen map (the data have been smoothed).

The described algorithm represents an effective procedure for selection of target-focused compound subsets compatible with high throughput *in silico* evaluation of large virtual chemical space. Whenever a large enough set of active ligands is available for a particular receptor, the quantitative discrimination function can be generated allowing selection of a series of compounds to be assayed against the target. It is important to note that focusing on physicochemical rather than structural features makes this approach complementary to any available ligand structure similarity technique.

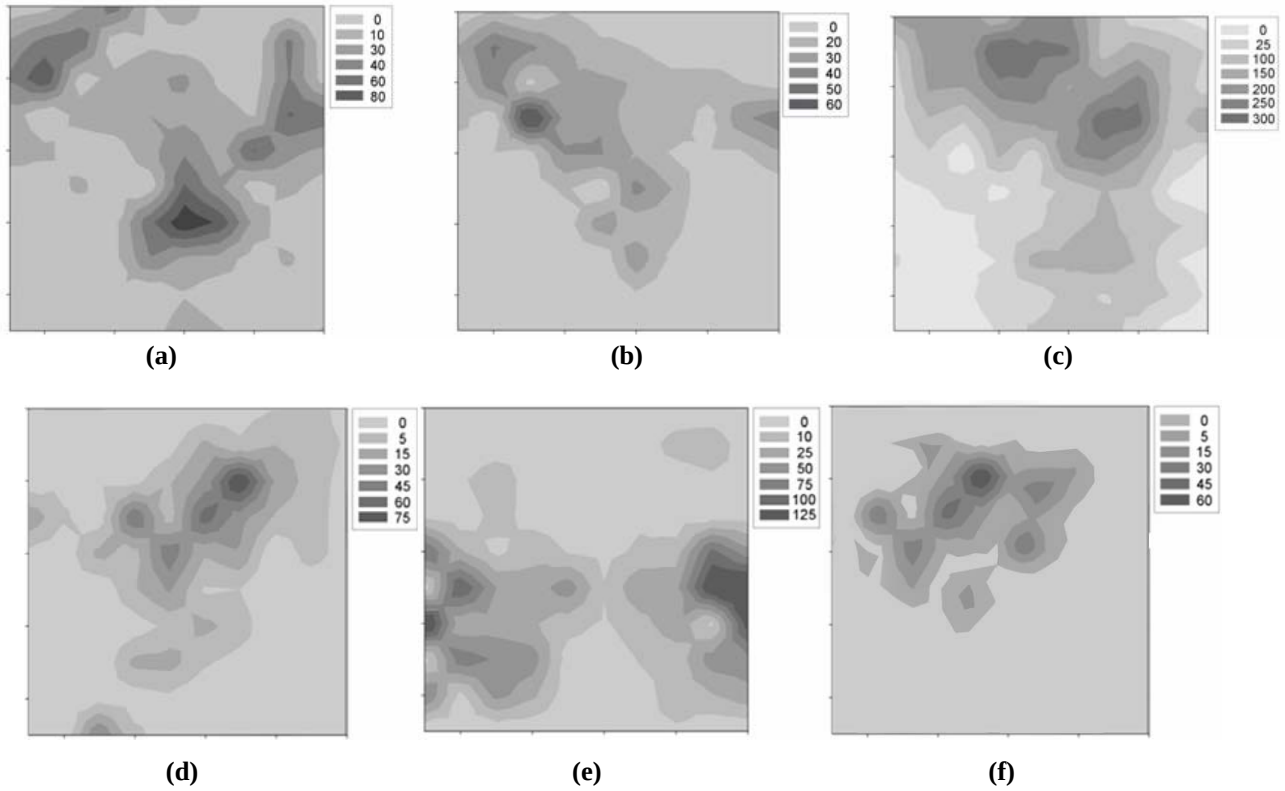


Fig. 7. Distribution of 5 large target-specific groups of pharmaceutical agents on the Kohonen map: (**a**) tyrosine kinase inhibitors (1423 cmpds); (**b**) ion channel agonists/antagonists (1080 cmpds); (**c**) GPCR agonists/antagonists (12,711 cmpds)); (**d**) dopamine receptors agonists/antagonists (664 cmpds); (**e**) protease inhibitors (2833 cmpds); (**f**) PPs inhibitors (1685 cmpds).

The predictive ability of the constructed model towards PPs inhibitors was approx. 80%. Therefore, this model can be satisfactorily used for targeted-library design and rational compound selection. Thus, we have evaluated our collection in stock as well as structures from the generated virtual library using this model. In the output, we have selected more than 25K individual compounds with high scores. We have also applied additional computational algorithms to generate a consensus scoring, see figure 5, and to reduce the related chemical space. As a result, we have selected 15 small molecule compounds with high *in silico* scores.

Molecular docking and pharmacophore-constrained screening

Key aspects of 3D-composition of PPs as well as interesting structural features of the PTP-active binding sites have been described briefly above. Within the following subsection we have focused particularly on our results obtained by 3D-molecular docking study for PTP-targeted compounds. Recent X-ray crystallographic data has demonstrated that the potency of the `template` lead compound (for the structure see Figure 8) is due to extensive hydrophobic interactions with the side chains of Tyr46, Phe182, and Ile219, and electrostatic interactions

between the positively charged Arg 221 and Lys 120 with the negatively charged sites of the molecule.

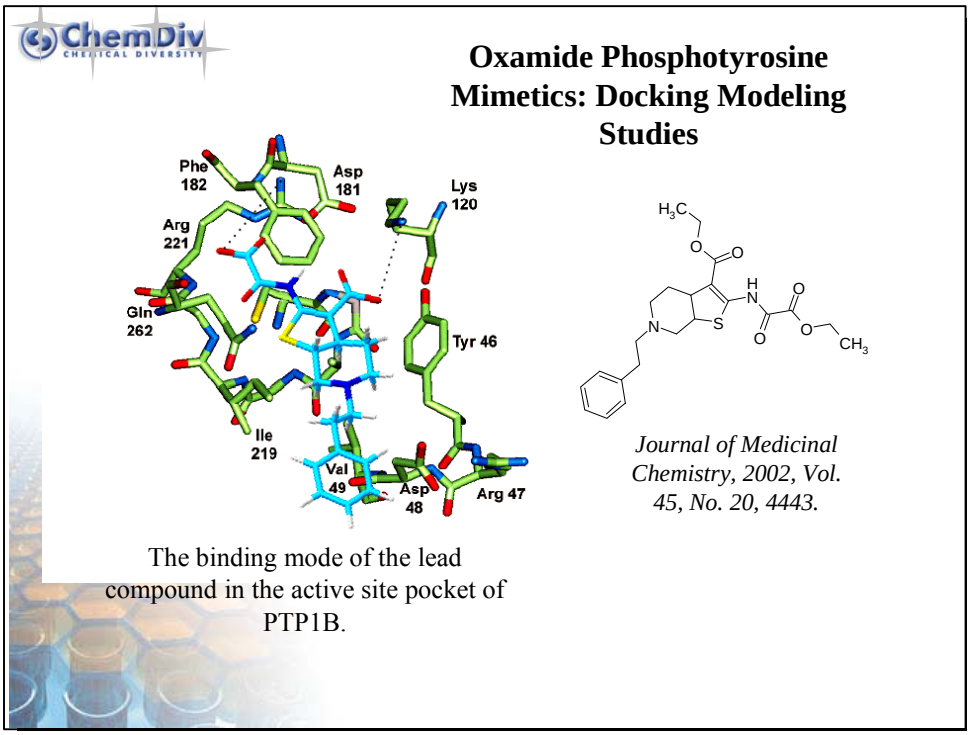
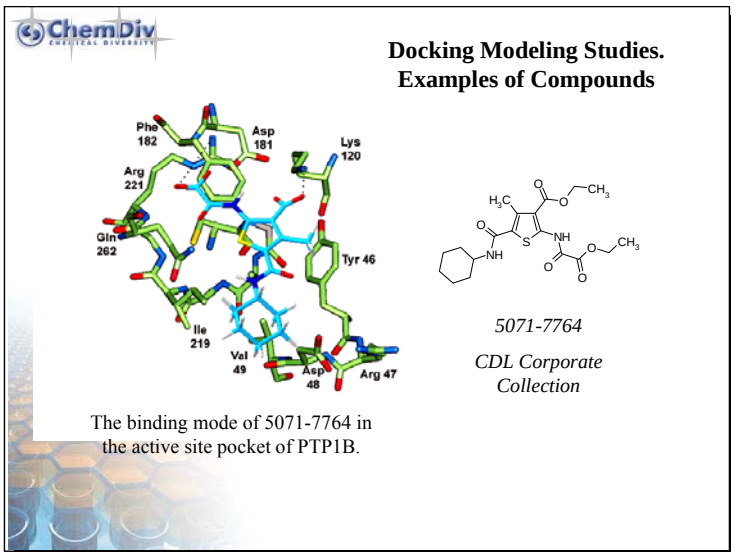
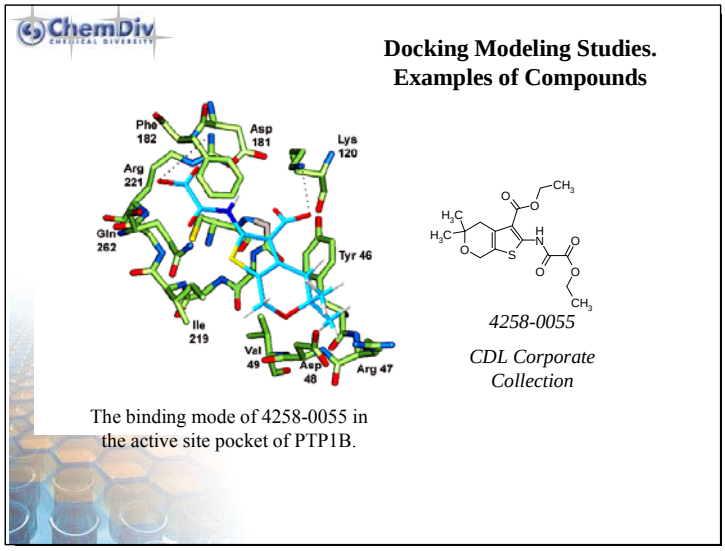


Fig. 8. Reference compound in the active binding site of PTP1B.

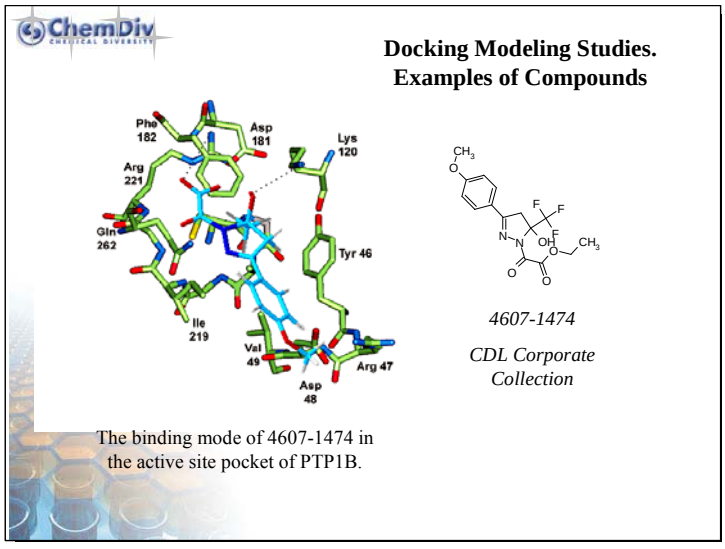
We have successfully used this data for the construction of our PTP1B docking model (Fig. 9). Thus, molecular docking of the previously selected compounds (15K cmpds) was performed in SurFlex docking computational program Version 1.24 (BioPharmics LLC). After the generation of `protomol`, a 3D-template for docking study, all structures have been directly docked into the active binding site of PTP. Ten different conformations generated for each structure have been used. There are two scores for each docked conformation: an affinity (-log(K_d)) (named as "polar") and a `pen score` (arbitrary units named as "penetration"). The pen score is a relative degree of geometric embedding (penetration) of a ligand into the active site of protein. Penetration scores that are close to 0.0 are favorable, however visual analysis of each conformer seems to be a more adequate. After the 3D-model was complete, the tested compounds from our pre-selected library were accurately docked into the enzyme's active site (for representative examples see next three figures). Please take it into account that all the shown compounds are obtained as the ethyl esters. However, their carboxilic acid metabolites (as they exist in human organism) are shown within the active site of the enzyme.



(**A**)



(**B**)



(**C**)

Fig. 9. Representative examples of our docking study towards PTP1B.

It should also be noted that based on the constructed model we have built a unique 3D PTP-pharmacophore model which has also been effectively used for our targeted library design (the corresponding results are not shown here). A similar approach has been effectively used for selecting the most promising compounds targeted against other PPs. At the final output, both the 3D-pharmacophore searching and 3D-molecular docking have provided more than 10K high-scored compounds with promising activity against PPs, and it is the main content of our targeted library.

Several structure-based modifications applied for our targeted library design are presented below.

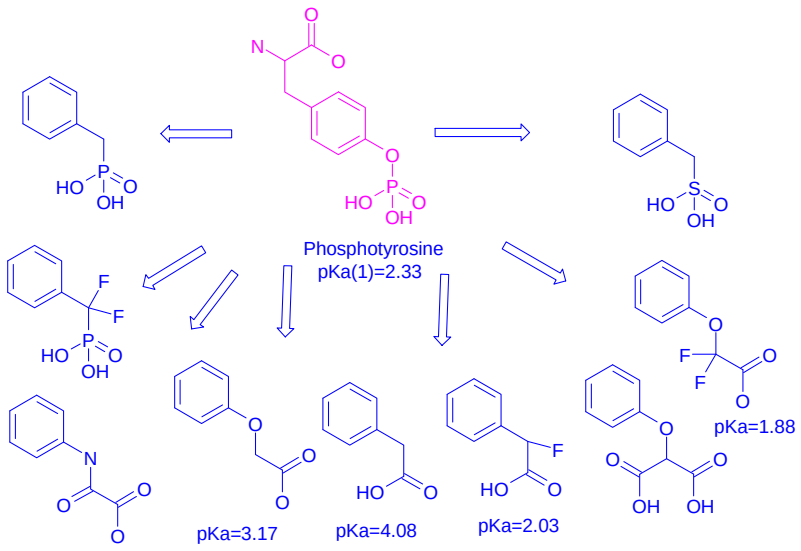


Fig. 10. Design of phosphotyrosine mimetics.

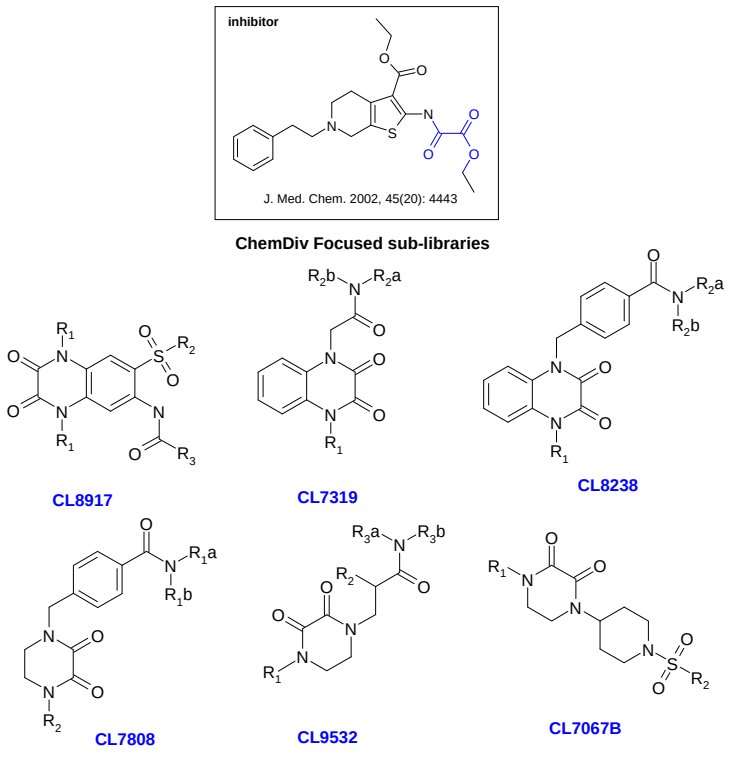


Fig. 11. Oxamide Phosphotyrosine mimetics.

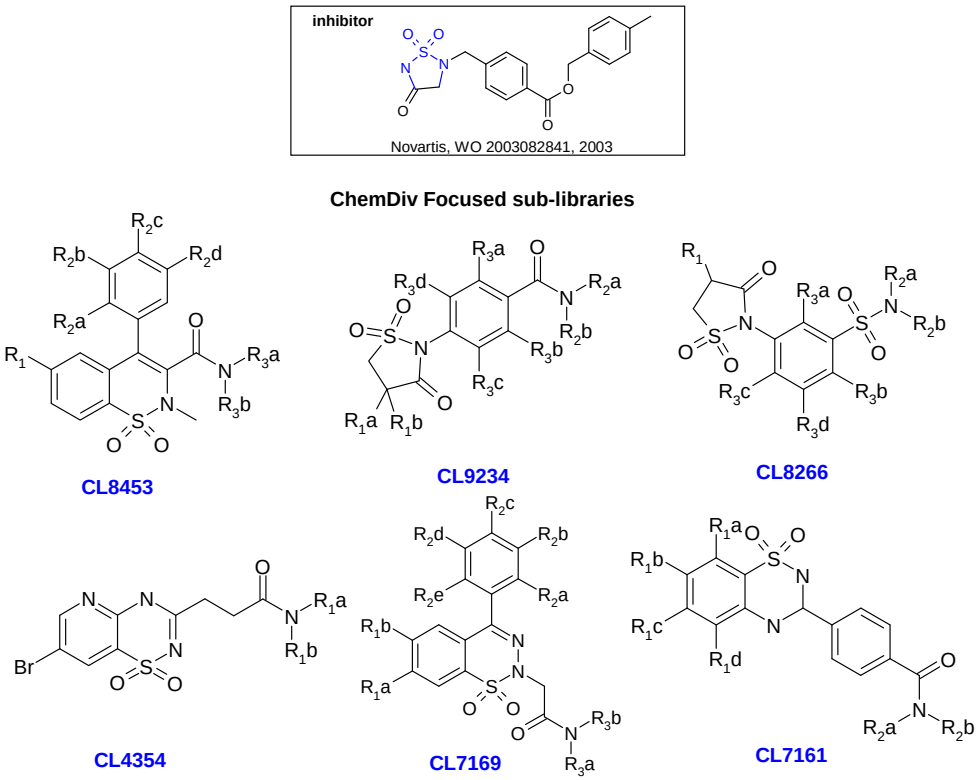


Fig. 12. Morphing of cyclic Phosphotyrosine mimetics.

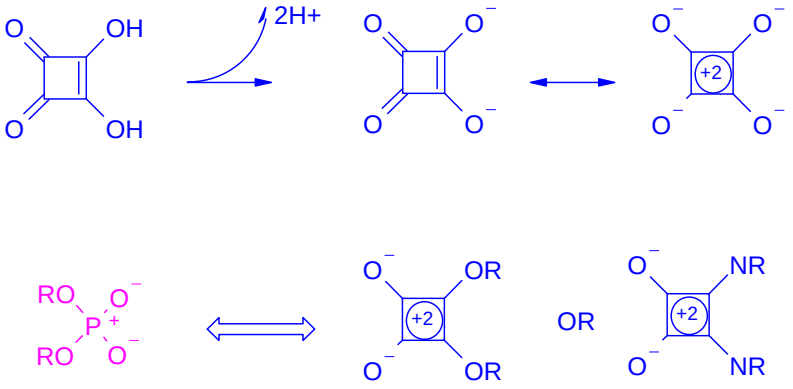


Fig. 13. Squaryl group as a new mimic of phosphate group.

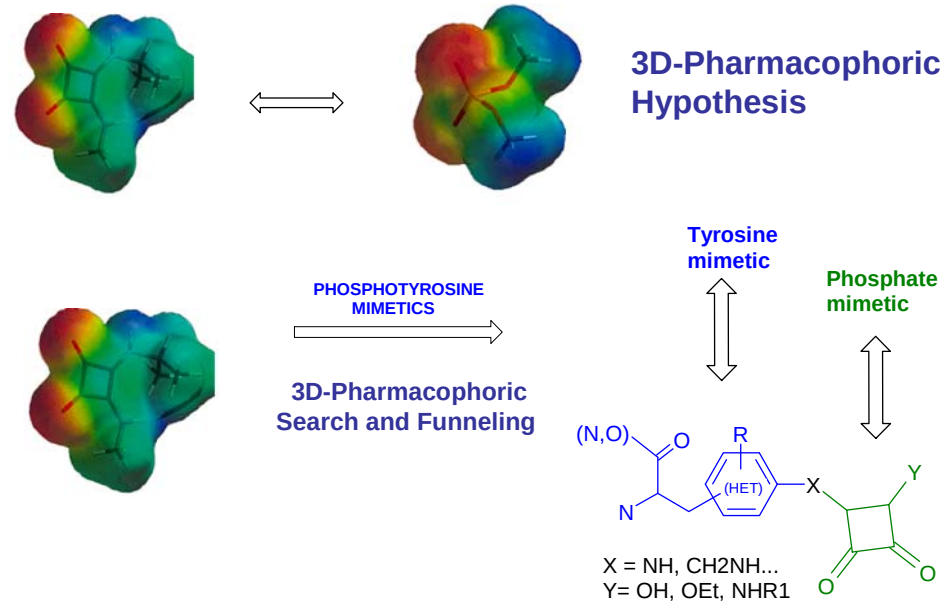


Fig. 14. Pharmacophoric Design of PTP focused library.

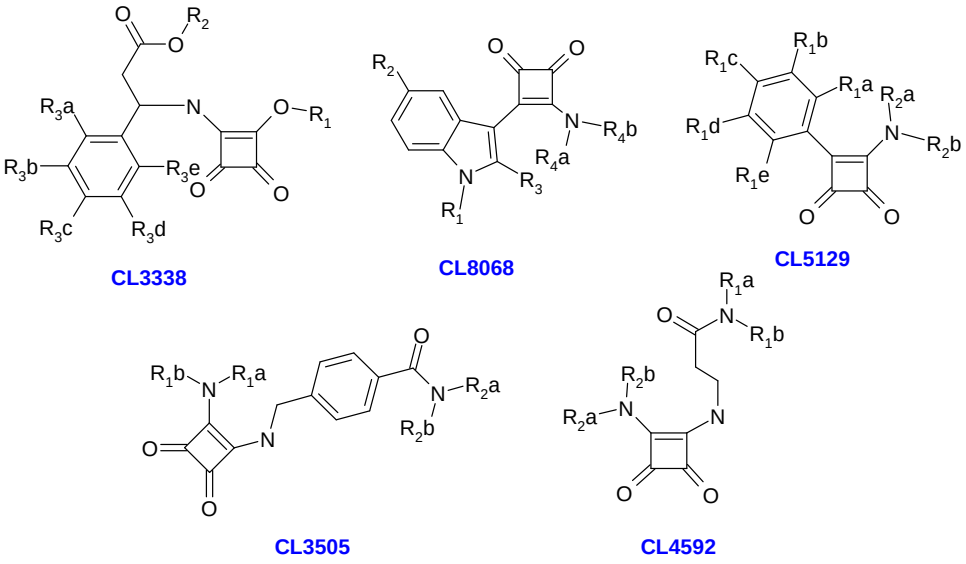


Fig. 15. Examples of ChemDiv probe PTP focused libraries.

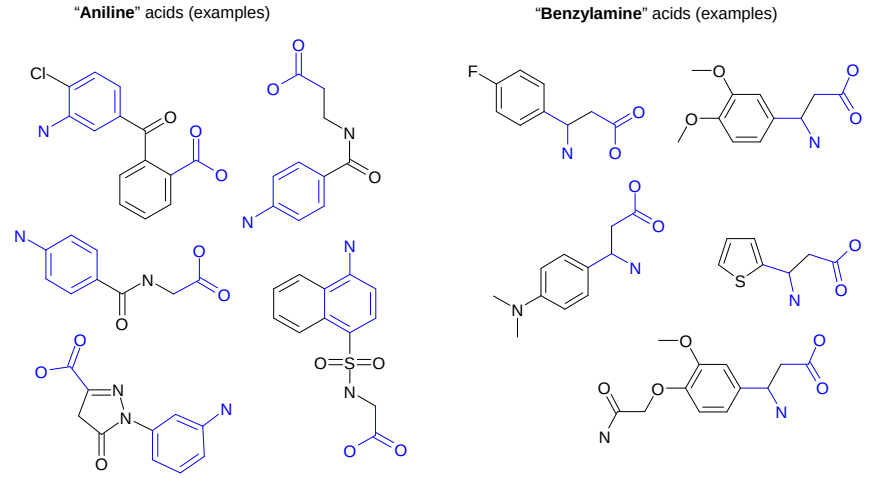


Fig. 16. Special set of Building Blocks for PTPs-focused library (tyrosine bioisosters).

The main statistical features are listed below:

PPs-targeted library profile:

• More than 1.5K known inhibitors of PPs comprising the knowledge base;

• Purity: all the compounds analyzed; > 90% average purity;

• The library contains > 130 unique heterocycles, > 150 unique chemotypes, > 2,500 "screen" subfragments;

• The library can be expanded in the feasible space of 50K structures.

Property range:

• 181 < MW < 554; **404** on average;

• 0 <H-bond acceptors < 10; **5** on average;

• 0 < H-bond donors < 5; **1** on average;

• 0 < rotatable bonds < 14; **6** on average;

• -1.1 < clogP < 5.5; **2.5** on average;

• -7.9 < log of solubility in water (pH 7.4) < 4.6; **-3.0** on average.

In summary, under the top concept of targeted diversity we have developed and effectively applied a multi-step *in silico* approach to design the PPs-targeted library of small molecule compounds therein. The related biological trials have revealed several highly potent inhibitors, and we can confidently conclude that described *in silico* pathway represents an effective method for PP-focused library design. Moreover, we provide rapid and efficient tools for follow-up chemistry on discovered hits, including single isomer chemistry, stereoselective synthesis and racemic mixture separation. The developed library is updated quarterly based on a "cache" principle. Older scaffolds/compounds are replaced by templates resulting from our in-house development (unique chemistry, literature data, computational approaches) while the overall size of the library remains the same (ca. 10-14K compounds). As a result, the library is renewed each year, proprietary compounds comprising 50-75% of the entire set. Clients are invited to participate in the template selection process prior to launch of our synthetic effort.

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