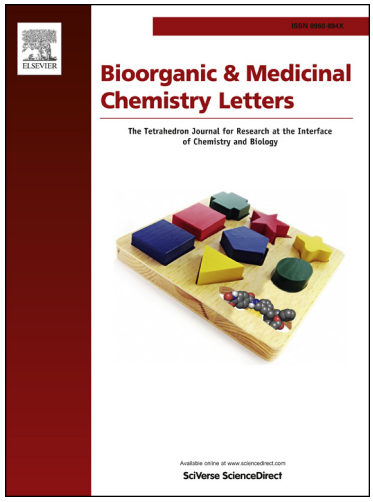
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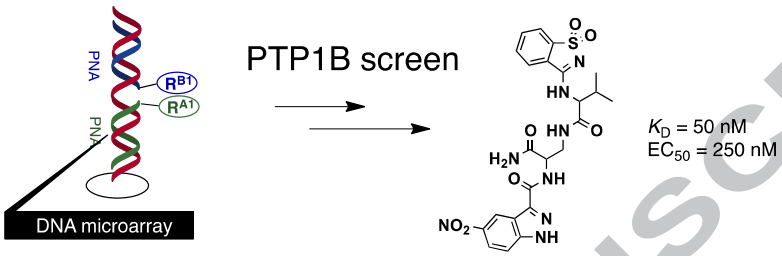
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Novel PTP1B inhibitors identified by DNA display of fragment pairs

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ABSTRACT

DNA display of PNA-encoded libraries was used to pair fragments containing different phosphotyrosine surrogates with diverse triazoles. Microarray-based screening of the combinatorially paired fragment sets (62 500 combinations) against a prototypical phosphatase, PTP1B, was used to identify the fittest fragments. A focused library (10 000 members) covalently pairing identified fragments with linkers of different length and geometry was synthesized. Screening of the focused library against PTP1B and closely related TCPTP revealed orthogonal inhibitors. The selectivity of the identified inhibitor for PTP1B *vs* TCPT was confirmed by enzymatic inhibition assay

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DNA encoding technologies for small molecule libraries are increasingly used in the discovery of novel drug leads or probes for chemical biology.[1] Several technologies have been developed to access encoded libraries including DNA-templated synthesis, split and mix synthesis using DNA-tag ligation (enzymatic or chemical), or hybridization to route the DNA tags.[2] In parallel, an encoding strategy leveraged on peptide nucleic acid (PNA) has been pursued on the basis that its encoding chemistry utilizes SPPS that it is compatible with co-synthesis of peptides and small molecule libraries.[3] Several studies have established that hybridization of DNA or PNA-tagged molecules can be used to display multiple ligands in order to interrogate the synergy of their interaction with a given target.[3g,h,m,4]

Protein phosphatases are an important class of enzymes for their role in post-translational regulation of protein function, acting as the counter-part of kinases.[5] Aberration of protein phosphatase activity has been implicated in numerous disease states including metabolic disorders, cancer, neurodegenerative and autoimmune pathologies.[6] Despite the interest in inhibitors for various protein phosphatases, developing selective and cell-permeable compounds has proved challenging, owing to the requirement of a negatively charged phosphotyrosine mimetic and a fairly conserved binding pocket. PTP1B, a prototypical phosphatase that epitomizes inhibitor development challenges, has received significant attention based on the discovery that mice lacking this phosphatase had increased insulin sensitivity and were not prone to obesity.[7] More recently, it has been shown that PTP1B ablation in mice delayed HER2/Neu-induced breast cancer whereas its overexpression resulted in spontaneous breast cancer.[8] Additionally, PTP1B has been shown to participate in oncogenic RAS-mediated gene silencing by de-phosphorylation of Argonaute AGO2.[9] Inspired by the development of novel phosphotyrosine mimetic based on aryl sulfonamide or salicylic acid that afford cell permeable inhibitors,[2m,10] coupled to the presence of an allosteric pocket in the vicinity of PTP1B's active

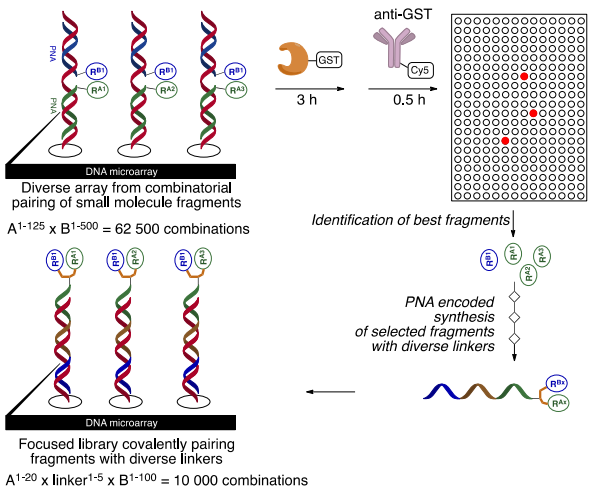


Figure 1. Schematic representation of microarray-based DNA display of fragment pairs screen and resynthesis of focused library based on identified fragments

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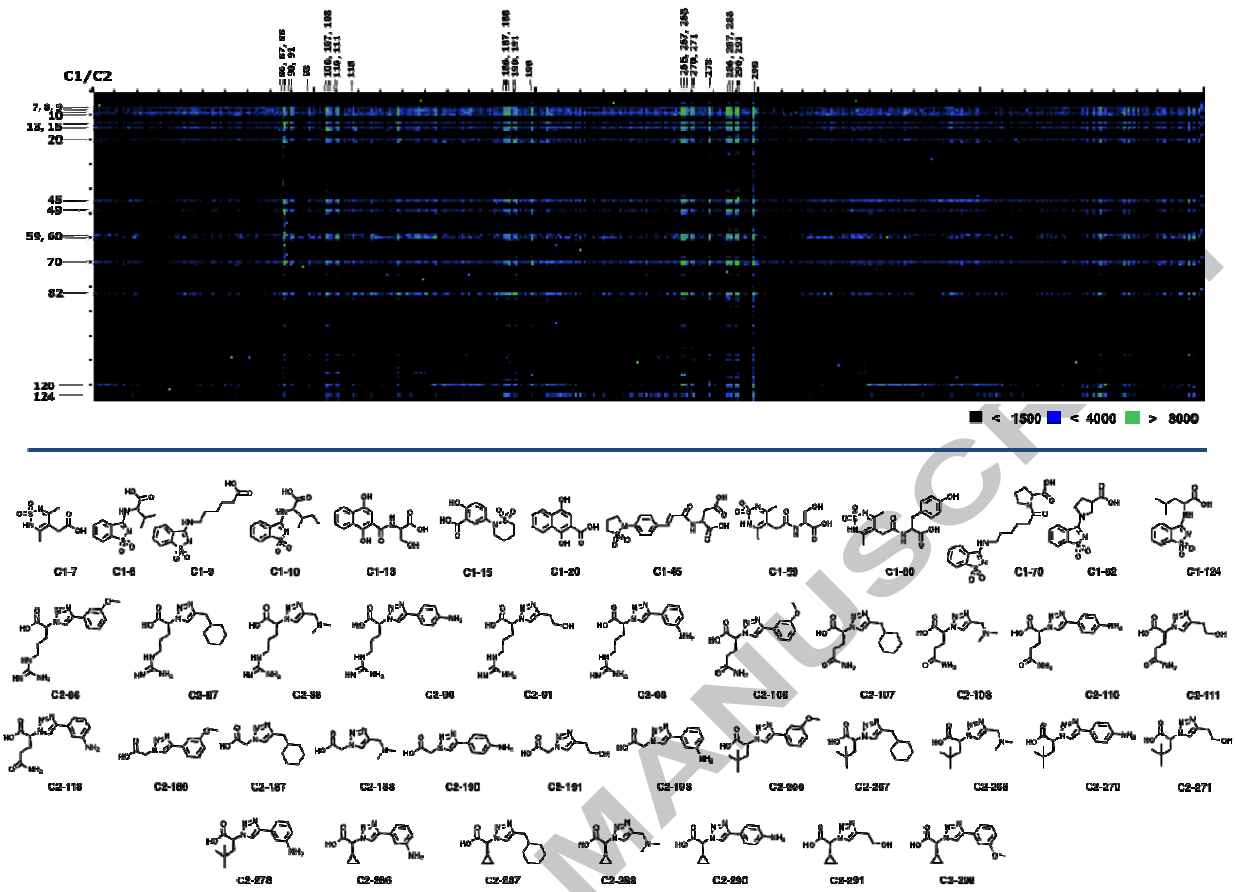


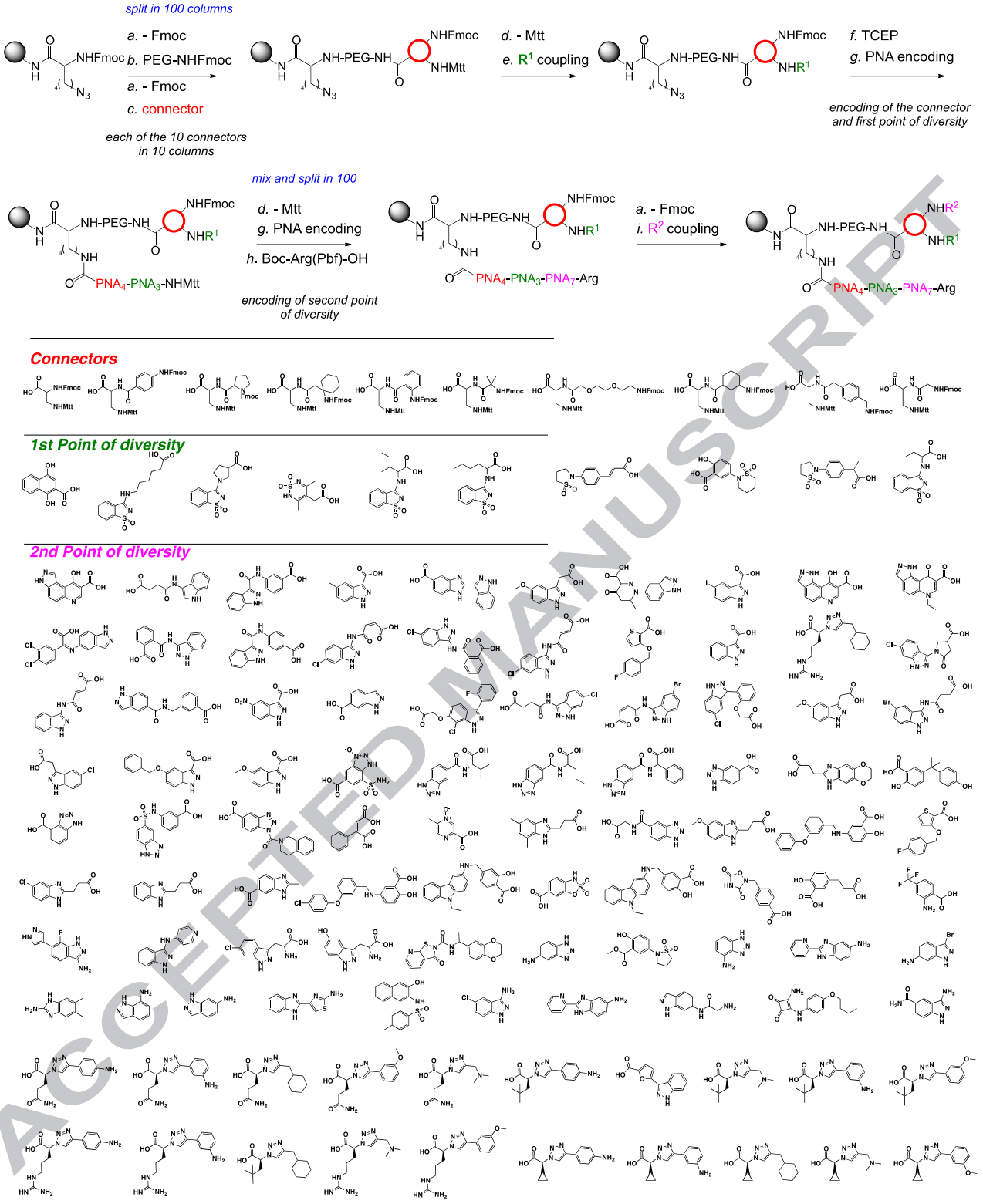
Figure 2. Top: Microarray/based screen of combinatorial-paired fragments against PTP1B (125 fragments C1 horizontally x 500 fragments C2 vertically = 62000 combinations); Bottom: Structure of identified fragments.

site,[11] we sought to use DNA-display of fragments to pair a phosphotyrosine mimetic with a library of triazoles (Figure 1).[12]

A set of 125 putative phosphotyrosine mimetics were individually coupled to an appropriate PNA tag on solid phase according to previously established protocols[3h] (see SI for complete list of fragments and PNA sequences). It is noteworthy that a coupling efficiency > 90% was obtained for 106 of the 125 fragments, the remaining fragments were coupled with 50-80% efficiency.[13] A microarray containg 62 500 unique DNA sequence was used to combinatorially pair this fragment library to a library of 500 heterocyles including triazoles.[3h,m] The screening was performed by incubating a microarray slide with GST-tagged hPTP1B (Figure 1) for 3h, washing the slide to remove unbound PTP1B and revealing PTP1B binders using an anti-GST antibody labelled with a Cy5 fluorophore. The result of this screen is shown in Figure 2, arranged for the 125 fragment set and 500 fragment set on horizontal and vertical lines respectively. The recurrence of binding across a line reflects the fitness of a given fragment. The fittest fragments from this screen were used to prepare a focus library covalently pairing the fragments (Scheme 1). This allows supplementing the selected set with new fragments (near neighbors) and enriching the local diversity space around these initial hits while optimizing the linker by systematically evaluating different linker lengths and geometries for each combination. To this end a focused library composed of the 10 highest intensity phosphotyrosine mimetics (See Figure 3 for structures) coupled via 10 different linkers to 100 fragments (20 of the highest intensity fragments from the primary screen supplemented by 80 novel fragments) was prepared by split and mix combinatorial synthesis (Scheme 1). The synthesis makes use of three orthogonal protecting groups (Mtt, N_3 and Fmoc) to mask the amines used in the coupling of the linker between the two fragments and the PNA oligomerization. After derivatization and encoding, an analytical sample of each pool was analyzed by MALDI to assess the quality of the chemistry (90% of the pools has >85% purity; the remaining 10% had different levels of truncation with the desired molecular weight being the most intense peak).

This focused library was screened according to the same protocol as the fragment library against (i) PTP1B; (ii) PTP1B in the presence of phosphotyrosine (0.5 mM) in order to verify that hits obtained in (i) are a product of an interaction with the phosphotyrosine binding site; (iii) against a closely related phosphatase TCPTP, a phosphatase presenting a high homology to PTP1B[14] but for which inhibition is undesirable due to unspecific lethality in cell-based assays (Figure 3). The screens were performed on arrays containing four-fold redundancy allowing statistical analysis of the hits. Analysis of the best binders yielded a coefficient of variation of 5% or less from the median intensity. As shown in Figure 3, the tightest PTP1B binders were all outcompeted by the addition of phosphotyrosine concurring the fact that compounds interact directly or allosterically with the phosphatase binding pocket. Comparison between PTP1B and TCPTP binders showed that while >50% of the tightest PTP1B binders were also TCPTP binders; several fragment combinations afforded preferential PTP1B binding

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Scheme 1. PNA-encoded synthesis of a focused PTP1B library and structure of the fragments used.

suggesting that selectivity in the inhibition can be achieved. Based on these results, 10 compounds were resynthesized without their PNA tags and with a biotin *in lieu* of the PNA. The biotin-tagged compounds provide a simple immobilization method for affinity measurements with streptavidin-coated surface using bio-layer interferometry (BLI). Gratifyingly, all the 10 compounds were reconfirmed for their binding affinity to PTP1B using BLI (Table 1) with affinities ranging from 50 to 290 nM. The compounds lacking the biotin were then assayed for PTP1B and TCPT inhibition *in vitro* by monitoring the

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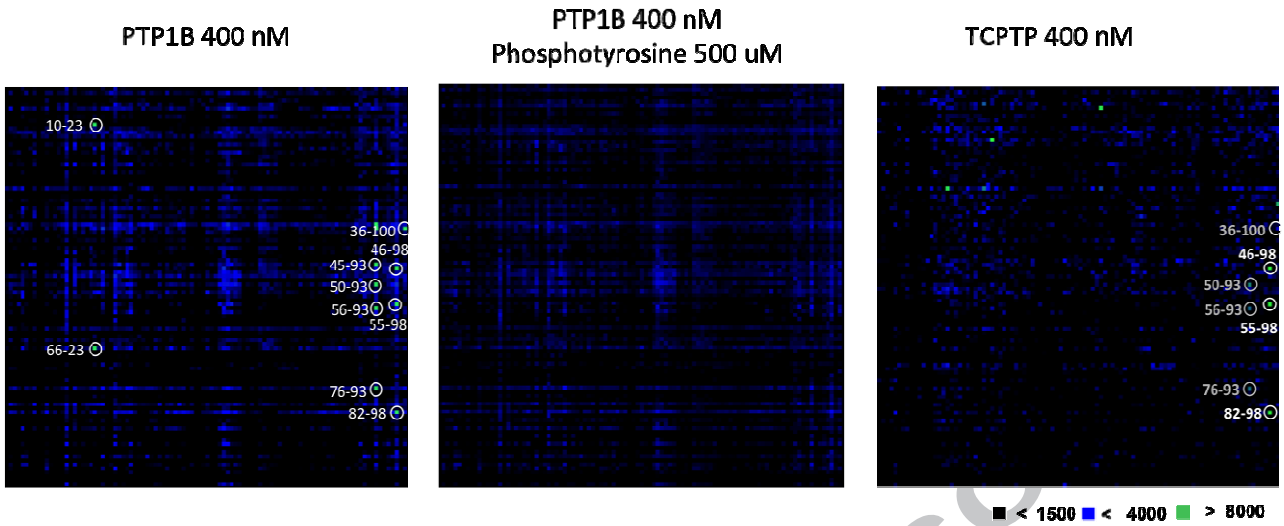


Figure 3: PTP1B focus 10K library incubated against PTP1B 400 nM (left), PTP1B 400 nM in the presence of 500 uM phosphotyrosine (middle) and TCPTP 400 nM (right). The intensities were calculated as a median of 4 redundant spots.

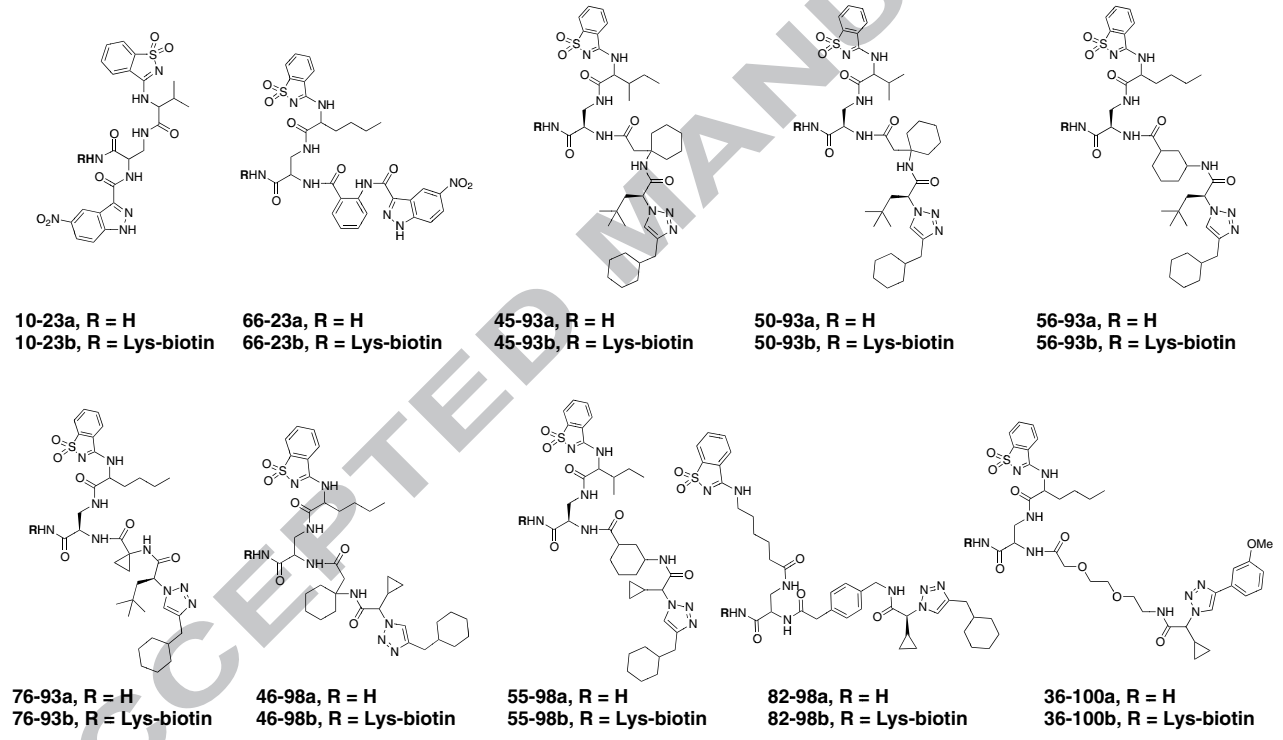


Figure 4: Structure of compounds selected and synthesized for hit validation.

hydrolysis of a fluorogenic substrate (DifMup). The 10 compounds selected on the basis on PTP1B binding were confirmed to inhibit PTP1B with Ki ranging from 0.250 uM to 16.8 uM. Importantly, three compounds were found to be selective for PTP1B inhibition relative to TCPTP (10-23a, 66-23a, 45-93a) concurring the differences observed in the microarray screen. While the microarray screen also revealed compounds that binding selectively to TCPTP vs PTB1B, their selectivity was not assayed in the present study. Based on the recent demonstration that PTP1B inhibitors induce growth inhibition in BT474,[15] a breast cancer cell line, we assayed the growth inhibition potential of the selective PTP1B compounds. To enhance water solubility, the compounds were resynthesized with an arginine at the C-terminus. While 10-23- and 66-23-adduct had no impact on cellular growth, 45-93-adduct showed a GI_{50} of 0.9 uM.

In conclusion, the work reported herein demonstrates the potential of using DNA-display of fragment libraries to guide the design of a focus library covalently paring selected fragments with different linkers. While fragment based drug discovery has gained a lot of momentum in the drug discovery process, identifying the optimal linker strategy to pair fragments remains a trial and error process generally approached by an iterative

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process. The ability to combinatorially address this problem with library size of 10^4 compounds greatly facilitates this process. The fact that PNA-encoded libraries can be prepared using standard SPPS protocols also facilitates library synthesis. The present study demonstrates the robustness of this process for a target that has proven challenging to drug and illustrates the scope of the chemistry. The identification of a compound with cellular efficacy that is selective for PTP1B relatively to a closely related phosphatase (TCPTP) demonstrates its potential.

Table 1. Efficacy of selected inhibitors: binding affinity measured by BLI and Ki measured by inhibition of enzymatic hydrolysis of fluorescent substrate.

Compound	Binding affinity uM	Ki PTP1B uM	Ki TCPTP uM
10-23	0.05	0.25	>20
66-23	0.27	1.2	>100
45-93	0.29	1.7	>100
50-93	0.10	3.5	6.2
56-93	0.09	3.1	12.2
76-93	0.21	2.2	7.6
46-98	0.12	16.8	2.7
55-98	0.24	3.4	0.8
82-98	0.11	6.8	3.01
36-100	0.22	2.9	7.01

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Supplementary Material

Experimental procedures and MALDI analysis of library synthesis are shown in the Supplementary material.

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