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A Combinatorial Strategy for the Acquisition of Potent and Specific Protein Tyrosine Phosphatase Inhibitors

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Abstract

Protein tyrosine phosphatases (PTPs), a large family of signaling enzymes, regulate many cellular processes, such as proliferation, differentiation, migration, apoptosis, and immune responses. Small molecule inhibitors against PTPs are valuable both as powerful tools to study the functions of target PTPs and as lead compounds for pharmacological development. Here, we describe a novel combinatorial library approach to target simultaneously both the active site pocket and a peripheral secondary binding site for the acquisition of potent and specific PTP inhibitors. Fluorescence tagging during combinatorial library synthesis enables fluorescence polarization-based high-throughput screening of the resulting library, leading to identification of a TC-PTP inhibitor.

Keywords

Protein tyrosine phosphatases (PTPs); PTP inhibitor; Peripheral binding site; Fluorescence tagged; Fluorescence polarization; High-throughput screening

1. Introduction

Protein tyrosine phosphatases (PTPs), which catalyze the dephosphorylation of phosphotyrosine-containing proteins, play important roles in a broad range of cellular processes, such as proliferation, differentiation, migration, apoptosis, and immune responses (1, 2). Malfunction of PTP activity is known to be associated with cancer, metabolic syndromes, and autoimmune disorders (3). Small molecule inhibitors of the PTPs not only can be used as research tools to study the function of these enzymes in normal physiology and in diseases, but also can be potential leads for therapeutic development. However, achieving specificity for PTP inhibition is not trivial, particularly when the target is a member of a large protein family. Indeed, the common architecture of PTP active sites impedes the development of selective PTP inhibitors. Fortunately, PTP substrate specificity studies have shown that pTyr alone is not sufficient for high-affinity binding and residues flanking pTyr are important for PTP substrate recognition (4). These results suggest that unique PTP sub-pockets that border the active site (i.e., the pTyr-binding site) may be targeted to enhance inhibitor potency and selectivity. In principle, active site-directed, potent and selective PTP inhibitors can be devised by tethering a nonhydrolyzable pTyr mimetic to appropriately functionalized moieties to engage both the active site and unique nearby sub-pockets (Fig. 1) (5). In this bidentate inhibitor approach, the pTyr mimetic may interact in

the desired inhibitory fashion with all PTPs; the molecular scaffolds attached to it should render the inhibitors PTP isozyme-selective. Moreover, the peripheral site binder will also enhance inhibitor potency due to additivity of free energy of binding. Utilizing this strategy we have obtained several PTP inhibitors with desired potency as well as specificity in a relatively efficient way in terms of both time and cost (6–10). Here, we will describe the design of a bidentate PTP inhibitor library and a fluorescence-tagged combinatorial synthesis and fluorescence polarization-based high-throughput screening, which we used to obtain a potent and selective TC-PTP inhibitor (8).

1.1. Bidentate Inhibitor Library Design

The basic structure of the bidentate PTP inhibitors is shown in Fig. 2, with phosphonodifluoromethyl phenylalanine (F₂Pmp) targeting the PTP catalytic pocket. As shown in this figure, the bidentate inhibitor contains three components: (a) F₂Pmp as the active site-directed motif, (b) an R group as the peripheral site-binding motif, and (c) the linker that connects the F₂Pmp and the R group. Other bidentate inhibitor libraries can be designed similarly, with different active site targeting motifs and/or different linkers. The library of potential bidentate PTP inhibitors can be prepared by condensation reaction of the common core (compound 1 in Fig. 3) with different carboxylic acids. In addition to condensation reactions, the library can be constructed using a variety of assembly modalities (e.g., “Click Chemistry”) (7, 9, 10) either in solution or on solid support (6). Once prepared, the library can then be screened against the target PTP in order to identify lead inhibitory species. The latter typically need to be resynthesized on a larger scale for detailed studies to confirm inhibitory activity and evaluate intracellular behavior.

The fluorophore-tagged compound library compounds are designed in the same way as described above, except that a fluorescein tag is attached to each library member at a position separated from critical inhibitory functionality (Fig. 4). The fluorophore allows the binding affinity of the library compounds for the target PTP to be measured using a fluorescence polarization (FP)-based displacement assay (11). Since nonfluorescent impurities do not affect the assay, the library can be screened as is without the need for further purification, an otherwise time-consuming and laborious process. In addition, the FP value is an intensive property (as versus to extensive property), which means it is independent of the concentration of fluorophore-tagged compound. As a result, any variation in the concentration of the compound will not bias the screening result. Finally, we note that the fluorophore-tagged library can be prepared in a fashion analogous to that of the corresponding non-tagged library (Fig. 5).

2. Materials

Prepare all the aqueous solutions with Milli-Q water. Prepare all the organic solutions with certified ACS grade solvents. All the organic solvents and reagents should be handled in a fume hood. Avoid any contact or inhalation.

2.1. DMF Solution of the Coupling Reagents

1. HBTU solution (Advanced ChemTech): 35 mM HBTU in DMF (Fisher Scientific). Weigh 1.32 g HBTU and transfer to a dry Erlenmeyer flask. Add 100 mL DMF to the flask, and gently shake the flask until all the HBTU dissolves. Transfer the HBTU solution into a glass bottle. Use a cap with TFE liner to cap the bottle tightly, and seal it with Parafilm. Store the HBTU solution at -20°C (see Note 1).
2. HOBT solution (Advanced ChemTech): 50 mM HOBT in DMF. Weigh 0.76 g HOBT monohydrate and transfer to a dry Erlenmeyer flask. Add 100 mL DMF, and gently shake the flask until all the HOBT dissolves. Transfer the HOBT solution into a glass bottle and cap it tightly. Store the HOBT solution at -20°C .
3. NMM solution (Fisher Scientific): 200 mM NMM in DMF. Weigh 2.02 g NMM and transfer to a dry Erlenmeyer flask. Add 100 mL DMF, and gently shake the flask to mix the NMM with DMF. Transfer the NMM solution into a glass bottle and cap it tightly. Store the NMM solution at -20°C .
4. Cyclohexylamine solution (Aldrich): 87 mM in DMF. Weigh 0.86 g cyclohexylamine and transfer to a dry Erlenmeyer flask. Add 100 mL DMF and gently shake the flask to mix the cyclo-hexylamine with DMF. Transfer the cyclohexylamine solution into a glass bottle and cap it tightly. Store the cyclohexylamine solution at -20°C .
5. The library core **2**: Synthesize compound **2** using standard solid-phase peptide synthesis (SPPS) protocol (8). Following the synthesis, purify it by HPLC. Make a 2 mM DMF solution of the core **2** and store the solution at -20°C .
6. Carboxylic acid library: Make a 40 mM DMF solution for each acid, and transfer the acid solution into a 96-well polypropylene DeepWell plate (NUNC). Seal the plates with aluminum sealing tape (NUNC) and store the plates at 4°C (see Note 2).
7. DMG buffer: 50 mM DMG, pH 7.0. Weigh 4.01 g 3,3-dimethyl glutaric acid, 0.52 g sodium chloride, and 0.186 g ethylenediaminetetraacetic acid (EDTA) and transfer to a 1-L graduated cylinder. Add 400 mL Milli-Q water into the

¹All glassware should be dried in an oven ($>100^{\circ}\text{C}$) overnight and cooled to room temperature in a desiccator before use. The HBTU solution can be stored for months if it is free of water and sealed properly with Parafilm.

²The plate should be sealed carefully with the sealing tape to ensure that every well is tightly sealed, so that the acid solution will not absorb moisture. For better protection, the sealed plate can be wrapped with three layers of plastic food wrap.

cylinder. Gently shake the graduated cylinder until all the solid dissolve. Adjust pH to 7.0 using NaOH. Make up the volume to 500 mL with Milli-Q water. Store the DMG buffer at room temperature.

8.

The target PTP protein with GST tag can be over-expressed in *Escherichia coli* and purified using GST-tag purification resins. Add 30% glycerol to the purified protein and store the protein at -20°C .

3. Methods

Carry out all procedures on a Freedom EVO workstation (Tecan) with a 96-channel MCA tip block using disposable tips. The layout of the workstation with the plates and reagent troughs is shown in Fig. 6. The complete EVO program is listed in Note 3.

³EVOware program for the automatic synthesis of library.

```

Comment Begin of the library synthesis
1
Comment Transfer acid to the Reaction Plate
2
Get DiTis Grid 1; Site: 1 (200ul RaininTips
TecanBox)
Fetch 8 rows and 12 columns
3
Aspirate 10 µl SZ-DMF
"Acid Plate" (Col. 1, Rows 1-8)
4
Dispense 10 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
5
Drop DiTis Grid 1; Site: 1 (200ul RaininTips
TecanBox)
6
Comment Add HBTU to the Reaction Plate
7
Get DiTis Grid 1; Site: 2 (200ul RaininTips
TecanBox)
Fetch 8 rows and 12 columns
8
Aspirate 10 µl SZ-DMF
"HBTU Trough" (Col. 1, Rows 1-8)
9
Dispense 10 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
10
Begin Loop 3 times "Mixing"
11
Aspirate 15 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
12
Dispense 15 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
13
End Loop "Mixing"
14
Drop DiTis Grid 1; Site: 2 (200ul RaininTips
TecanBox)
15
Start Timer 1

```

3.1. Combinatorial Synthesis of Fluorescence-Tagged Library

1.

Put four polypropylene troughs on the Freedom Evo workstation. Label them as “HBTU,” “HOBt,” “NMM,”

```
16
Comment Add HOBt to the Reaction Plate
17
Get DiTis Grid 1; Site: 3 (200ul RaininTips
TecanBox)
Fetch 8 rows and 12 columns
18
Aspirate 10 µl SZ-DMF
"HOBt Trough" (Col. 1, Rows 1-8)
19
Dispense 10 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
20
Begin Loop 3 times "Mixing"
21
Aspirate 25 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
22
Dispense 25 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
23
End Loop "Mixing"
24
Drop DiTis Grid 1; Site: 3 (200ul RaininTips
TecanBox)
25
Wait for Timer Timer 1: 300 sec
26
Comment Add NMM to the Reaction Plate
27
Get DiTis Grid 7; Site: 1 (200ul RaininTips
TecanBox)
Fetch 8 rows and 12 columns
28
Aspirate 10 µl SZ-DMF
"NMM Trough" (Col. 1, Rows 1-8)
29
Dispense 10 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
30
Begin Loop 3 times "Mixing"
31
Aspirate 35 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
32
Dispense 35 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
33
End Loop "Mixing"
34
Comment Add reaction solution to the Library
Plate
35
Aspirate 40 µl SZ-DMF
"Reaction Plate" (Col. 1, Rows 1-8)
36
Dispense 40 µl SZ-DMF
"Library Plate" (Col. 1, Rows 1-8)
37
Begin Loop 3 times "Mixing"
38
Aspirate 40 µl SZ-DMF
```

and “Cyclohexylamine.” Fill them with 30 mL HBTU, HOBt, NMM, and cyclohexylamine solution accordingly (see Note 4).

2. Take a 96-well polypropylene plate, and label it as “Library.” Manually add 10 μ L of library core **2** solution to each well. Put the “Library” plate on the Freedom Evo workstation.
3. Put another empty 96-well polypropylene plate on the Freedom Evo workstation, and label it as “Reaction.”
4. Put the 96-well DeepWell plate of carboxylic acids on the Freedom Evo workstation.

```
"Library Plate" (Col. 1, Rows 1-8)
39
Dispense 40  $\mu$ L SZ-DMF
"Library Plate" (Col. 1, Rows 1-8)
40
End Loop "Mixing"
41
Drop DiTis Grid 7; Site: 1 (200ul RaininTips
TecanBox)
42
Start Timer 2
43
Wait for Timer Timer 2: 3600 sec
44
Comment Add cyclohexylamine to the Library
Plate
45
Get DiTis Grid 7; Site: 2 (200ul RaininTips
TecanBox)
Fetch 8 rows and 12 columns
46
Aspirate 10  $\mu$ L SZ-DMF
"Cyclohexylamine Trough" (Col. 1, Rows 1-8)
47
Dispense 10  $\mu$ L SZ-DMF
"Library Plate" (Col. 1, Rows 1-8)
48
Begin Loop 3 times "Mixing"
49
Aspirate 50  $\mu$ L SZ-DMF
"Library Plate" (Col. 1, Rows 1-8)
50
Dispense 50  $\mu$ L SZ-DMF
"Library Plate" (Col. 1, Rows 1-8)
51
End Loop "Mixing"
52
Drop DiTis Grid 7; Site: 2 (200ul RaininTips
TecanBox)
53
Comment End of the library synthesis
54
```

⁴If the DMF solution is stored in freezer, allow it to warm up to room temperature before pouring into trough, because the cold solution will absorb moisture while warming up.

5. Load the 96-channel MCA tip block with new disposable tips. Transfer 10 μ L of carboxylic acid solution from the 96-well DeepWell plate of the carboxylic acid library to the empty “Reaction” plate. Dispose the tips.
6. Load the tip block with new tips. Transfer 10 μ L of HBTU solution from the “HBTU” trough to the “Reaction” plate. Mix the solution by pipetting up and down three times. Dispose the tips. Start a timer with 5 min countdown time.
7. Load the tip block with new tips. Transfer 10 μ L of HOBt solution from the “HOBt” trough to the “Reaction” plate. Mix the solution by pipetting up and down three times. Dispose the tips.
8. After the 5 min countdown time is over, load the tip block with new tips. Transfer 10 μ L of NMM solution from the “NMM” trough to the “Reaction” plate. Mix the solution by pipetting up and down three times. Transfer all 40 μ L solution from the “Reaction” plate to the “Library” plate. Mix the solution by pipetting up and down three times. Dispose the tips.
9. After 1 h, load the tip block with new tips. Transfer 10 μ L of cyclohexylamine solution from the “Cyclohexylamine” trough to the “Library” plate. Mix the solution by pipetting up and down three times. Dispose the tips.
10. After 30 min, manually add 190 μ L DMSO to each well of the “Library” plate using a multichannel pipette. Mix the solution by pipetting up and down three times. Dispose the tips. Seal the “Library” plate with aluminum sealing tape and store the plate at -20°C . The final concentration of the fluorescence-tagged library compound is about 80 μM .
11. Repeat steps 2–10 for all the plates of the carboxylic acid library.

3.2. Fluorescence Polarization-Based High-Throughput Screening

1. The fluorescence-tagged library compound from the DMSO solution should be diluted with DMG buffer, until the concentration of the library compound is about 75 nM. Store the diluted library (75 nM) in a 96-well plate.
2. Dilute the PTP protein stock to 2 μM with DMG buffer. About 20 mL of the 2 μM PTP solution is enough for four 96-well plates of library compounds (384 compounds).

3. Take a black 384-well polystyrene plate, and label it as “assay” plate. Add 50 μ L 2 μ M PTP solution to each well of the “assay” plate using Multidrop. Use Freedom EVO workstation to transfer 2 μ L library compounds (75 nM stock) to the “assay” plate. Mix the solution by pipetting up and down three times (see Note 5).
4. Use EnVision to measure the fluorescence anisotropy value (A_1) of each well of the assay plate (see Note 6). The anisotropy value of each library compound can be exported to an Excel file.
5. Prepare another 2 μ M protein solution with 0.5 mM Fmoc-F₂Pmp-OH, a known active center binding PTP inhibitor. Repeat steps 3 and 4. The result fluorescence anisotropy value (A_2) of each compound can be exported to another Excel file.
6. For each compound, check the A_1 value: If the A_1 is larger than 50 mA, calculate the displacement percentage using the equation: displacement percentage (%) = $(A_1 - A_2) / (A_1 - 30) \times 100\%$; if the A_1 is smaller than 50 mA, set the displacement percentage (%) as 100% (see Note 7). Sort the compound based on the displacement percentage. The compounds with the smallest displacement percentage value are the hits from the screening.
7. The hit compounds need to be resynthesized without the fluorescence tag and their inhibitory activity needs to be confirmed in the activity-based enzymatic assay.

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⁵The library compounds are in 96-well plates and will be reformatted to 384-well assay plates for screening. The relationship of the two types of plates is as follows: for 96-well plate no. 1, the well “A1” goes to “A1” of 384-well plate; for 96-well plate no. 2, the well “A1” goes to “A2” of 384-well plate; for 96-well plate no. 3, the well “A1” goes to “B1” of 384-well plate; and for 96-well plate no. 4, the well “A1” goes to “B2” of 384-well plate. After screening, the result (associated with 384-well format) needs to be assigned to each of the library compound (associated with 96-well format) according to this reformatting relationship.

⁶Most plate readers can give both fluorescence polarization (P) value and anisotropy (A) value as result. For this screening, the fluorescence anisotropy value should be used instead of fluorescence polarization value, because only the fluorescence anisotropy, but not fluorescence polarization, holds the linear superposition principle with each fluorophore component in solution (12). Therefore, the displacement percentage can only be calculated using the anisotropy values.

⁷Occasionally, there may be some library compounds that do not bind to the target protein. These compounds will give small A_1 and A_2 value (both close to 30 mA). So we set their displacement percentage as 100% to prevent these compounds from being selected as hits.

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Fig. 1.
“Peripheral Site” strategy for acquiring potent and selective PTP inhibitors.

Fig. 2.

Structure of the bidentate PTP inhibitor. The bidentate PTP inhibitor contains F₂Pmp as the active site-directed motif (shown in *oval*), an R group as the peripheral site-binding motif (shown in *circle*), and a linker.

Fig. 3.
Synthesize the bidentate ligand library by condensing core **1** with different carboxylic acids.

Fig. 4. Structure of bidentate PTP inhibitor library with a fluorescein tag. Each library member contains F2Pmp as the active site-directed motif (shown in *oval*), an R group as the peripheral site-binding motif (shown in *circle*), and a fluorescein tag (shown in *rectangle*).

Fig. 5.
Synthesize the fluorescein-tagged library by condensing core **2** with different carboxylic acids.

Fig. 6.
Suggested layout for the troughs and plates for the library synthesis.