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ARTICLE *in* NATURE PROTOCOL · FEBRUARY 2007

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Methods of using click chemistry in the discovery of enzyme inhibitors

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Published online 25 October 2007; doi:10.1038/nprot.2007.323

This protocol describes the step-by-step procedures for the efficient assembly of bidentate inhibitor libraries of a target enzyme, using the so-called 'click chemistry' between an alkyne-bearing core group and an azide-modified peripheral group, followed by direct biological screening for the identification of potential 'hits'. The reaction is highlighted by its modularity, high efficiency (~100% yield in most cases) and tolerance toward many functional groups present in the fragments, as well as biocompatibility (typically carried out in aqueous conditions with small amounts of biocompatible catalysts). The approach consists of three steps: (i) chemical synthesis of alkyne-bearing protein tyrosine phosphatase or matrix metalloprotease core groups and diverse azide-modified peripheral groups; (ii) click chemistry to assemble the bidentate inhibitor libraries; and (iii) direct screening of the libraries with target enzymes using 384-well microplate assays. Following the chemical synthesis of the core and peripheral groups and optimization of the click chemistry conditions (~1 week), steps (ii) and (iii) take 3 d to complete (~1–2 d for library assembly and 1 d for inhibitor screening).

INTRODUCTION

Catalomics—an emerging branch in chemical biology that aims at the high-throughput studies of enzymes at the organism-wide scale—relies heavily on robust chemical reactions compatible with high-throughput instruments[1]. One such reaction that we have intensively explored in recent years is the Cu(I)-catalyzed 1,3-dipolar cycloadditon between an azide and an alkyne[2–4], a prime example of a class of reactions known as 'click chemistry'—a term coined by Barry Sharpless[4]. It has been consistently proven to be one of the most effective reactions to date that 'stitch' two orthogonal building blocks together under extremely mild reaction conditions. The reaction is characterized by its high chemoselectivity, modularity, near-perfect yield and biocompatibility in that aqueous conditions are typically employed in the reaction, which renders the products 'ready-to-use' without further purifications. As a result, click chemistry has become an attractive tool in many research fields ranging from materials sciences, biology to medicinal chemistry/chemical biology[5]. It is for the same reasons that click chemistry has emerged as an integral part of the drug discovery pipeline and of the field of catalomics by providing a high-throughput amenable chemical reaction platform for compound synthesis. Unlike other fragment-based approaches, such as the NMR/X-ray-based structure-activity-relationship (SAR) strategy[6] or the mass spectrometry-based tethering strategy[7], click chemistry-based strategies require neither sophisticated instruments nor mutations in the target proteins, and at the same time enable the exploration of N^2 possibilities with $N + N$ chemical combinations. This is particularly useful for small-molecule lead discovery against enzymes (and other proteins) that possess an extended active site (as in the case of most proteases) or multiple binding pockets (as in the case of most kinases and phosphatases). We have thus far successfully explored this approach for the rapid synthesis and discovery of small-molecule inhibitors against two important classes of enzymes, protein tyrosine phosphatases (PTPs) and matrix metalloproteases (MMPs; **Fig. 1**). We expect that this method will find widespread applications in the emerging field of catalomics and drug discovery.

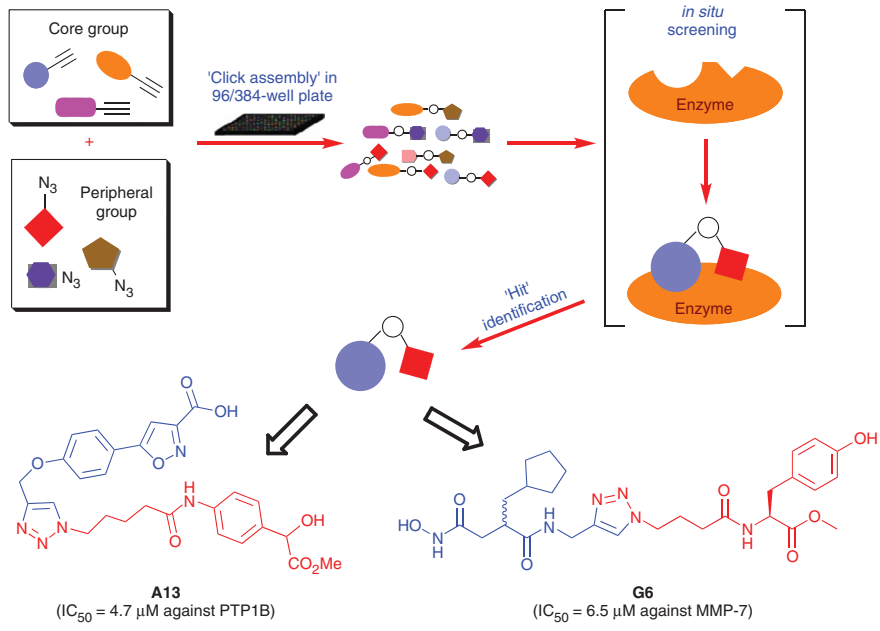

Figure 1 | Inhibitor discovery by click chemistry, followed by direct screening against two major classes of enzymes—PTPs and MMPs. Most enzymes possessing extended active sites or multiple binding pockets are suitable for this strategy.

PTPs, together with protein tyrosine kinases, regulate the intracellular signal-transduction pathways in the biological system[8]. There are more than 100 PTPs encoded in the human genome, many of which are well-documented therapeutic targets of major human diseases. Among them, PTP1B is the major regulator of both the insulin and leptin signaling pathways. Malfunctioning of PTP1B has been shown to lead to a variety of human diseases, including cancer, diabetes, obesity and inflammation[9]. Hence, PTPs are sometimes referred to as an attractive 'druggable genome'. The active site of most PTPs, however, is highly conserved, rendering it extremely difficult to develop specific inhibitors against a particular PTP while maintaining a negligible cross-reactivity against other PTPs.

The active site of PTP1B, as in the case of other PTPs, possesses critical residues that form an extensive hydrogen-bond network with the phosphotyrosine residue of its bound substrate (**Fig. 2**). Recently, a unique secondary binding site was discovered near the active site of PTP1B, prompting the call for the development of the so-called 'bidentate inhibitors', which, by targeting both the core and peripheral sites concomitantly (**Fig. 1**), promises to offer a novel strategy that could potentially yield highly potent and selective small-molecule inhibitors against PTP1B and even other PTPs[10]. We recently combined click chemistry with the direct screening method pioneered by Wong *et al.*[11–13] and developed for the first time a 66-member PTP1B inhibitor library[14]. The core group in our bidentate library, as shown in **Figure 2a** (right), was an *N*-phenyloxamic acid or its analogs, recently identified by Abbott Laboratories (from NMR-based fragment approaches) as a new class of cell-permeable, potent bioisosteric mimic of phosphotyrosine[15]. An alkyne handle was strategically introduced into the core group such that it could be easily assembled into bidentate inhibitors by 'clicking' with an azide-containing secondary binder (**Fig. 3**).

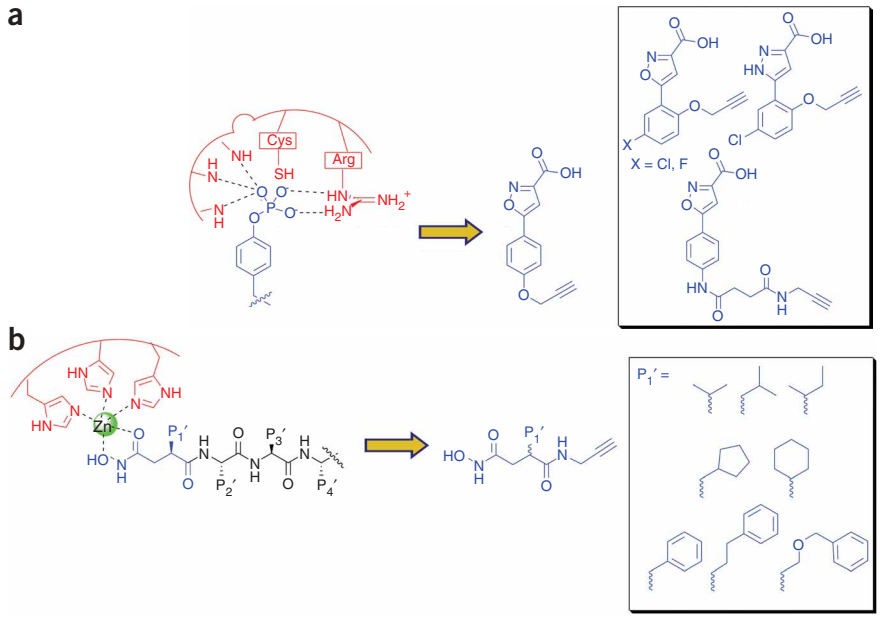

Figure 2 | Core groups used in the 'click' assembly of bidentate inhibitors against PTP1B and MMPs. (**a**) Phosphotyrosine-bound active site of PTP1B (left) and the cell-permeable *N*-phenyloxamic acid and analogs (inset) that mimic pTyr (right). (**b**) Succinyl hydroxamate-based peptide inhibitors bound to the active site of MMPa (left) and the alkyne-containing core groups used in the 'click-based' MMP inhibitors (right). Drawings in red denote the active site of the enzyme, whereas those in blue are from substrate/inhibitor.

MMPs are another class of important enzymes we have thus far explored successfully using the above-described strategy (**Fig. 1**). MMPs are a family of zinc-dependent endopeptidases that represent a family of at least 23 members in human alone[16]. They are highly homologous both in their structure and enzymatic activities. Dysregulation of MMP activities contributes to tumor metastasis, tumor angiogenesis, cancer, neurodegenerative and cardiovascular diseases, osteoporosis and arthritis. A recent survey of MMPs has divided MMPs into two distinct classes in terms of their therapeutic potential. One class is the so-called 'target MMPs', which have been shown unequivocally to contribute to tumor progression. The other class is the anti-target MMPs, which are essential for normal cell and tissue functions[17]. The central challenge, therefore, in MMP research is to develop potent MMP inhibitors that work against only the target MMPs but spare the fate of anti-target MMPs. The fact that many MMPs contain an extended substrate-binding pocket in their active site (a property shared by many other

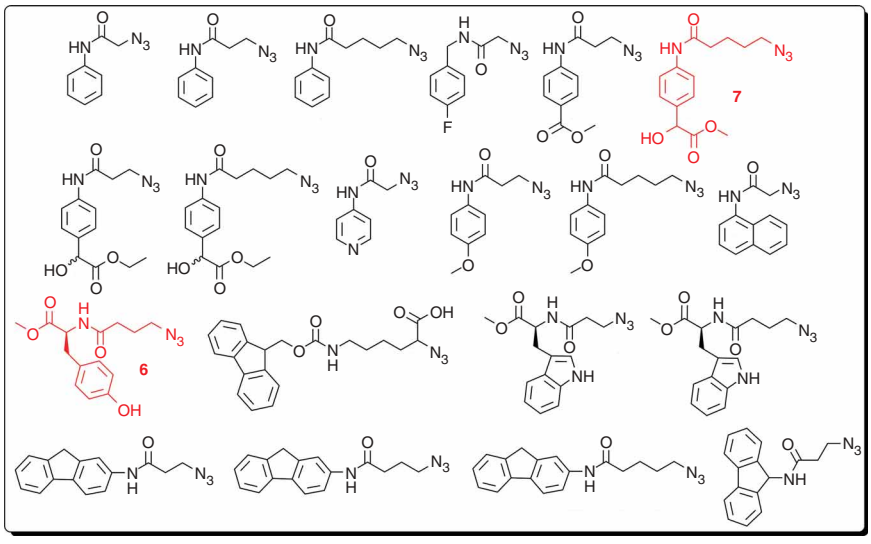

Figure 3 | Representative examples of azide-containing secondary-site binders used in our studies[14,18]. Highlighted in red are the azide constituents identified from our screening results against PTP1B and MMP-7.

proteases) makes them ideal candidates to develop suitable small-molecule inhibitors using click chemistry.

We have explored click chemistry to identify a novel MMP inhibitor (**Fig. 1**, bottom right) from a small-molecule library assembled with a hydroxamate-containing core group (**Fig. 2b**, right) and an azide-containing peripheral group (**Fig. 3**)[18]. The succinyl hydroxamate core group is the most potent zinc-binding group known. It works by chelating to the three histidine residues present in the active site of MMPs (**Fig. 2b**, left). By chemical modification of the core group with an alkyne handle (**Fig. 2b**, right), the inhibitor could be easily assembled via click chemistry, using the same azide-containing generic secondary-site binders, which, in the case of MMPs, would be expected to occupy the P′ substrate-binding sites of the enzymes (**Fig. 3**).

In this protocol, we describe the synthesis of a representative core group for the PTP1B and MMP libraries, the 'click' assembly of the inhibitor libraries in microplates, as well as the general procedure for biological screening of the putative inhibitors (**Fig. 4**). We found it essential to find the optimal click chemistry conditions to obtain quantitative ligation products with good purity. Both solvents and catalysts used in click chemistry have a pronounced effect on the yield and quality of the product generated. As such, we also detail the procedure we adopted for the optimization process. Representative examples of the synthesis of warheads for PTP1B and MMPs, as well as the synthesis of the azide library, are described in this protocol (**Figs. 5, 6** and **7**, respectively). The reader is referred to refs. 14 and 18 for further details regarding the synthesis of other core groups, their key intermediates and other related protocols. The screening procedure described herein is general and can be applied to the studies of other PTPs and MMPs by using the corresponding enzymes, substrates and buffers with conditions optimized for each enzyme. The IC_{50} values are obtained using dose-dependent reactions by varying the concentration of the inhibitor, under the same enzyme concentration. For further details, see refs. 14 and 18.

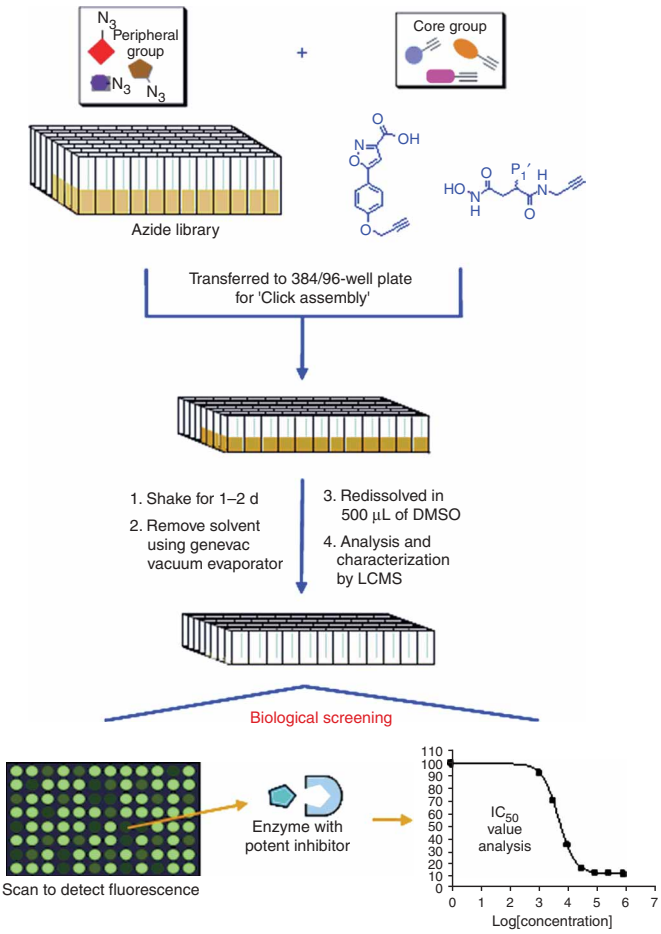

Figure 4 | Schematic of the whole 'click' synthesis and biological screening processes.

MATERIALS

REAGENTS

- 4-Hydroxyacetophenone (Fluka, cat. no. 54180)
- Propargyl alcohol (Aldrich, cat. no. P5080-3)
- *p*-Toluenesulfonic acid (*p*-TsOH; Sigma-Aldrich, cat. no. t3751)
- Dibenzo-18-crown-6 (Aldrich, cat. no. 15839-9)
- Potassium carbonate (K_2CO_3; GCE Laboratory Chemicals, cat. no. E4820)
- Dimethyl oxalate (Aldrich, cat. no. 13562-3)
- Sodium metal (Comak, cat. no. L537) ! CAUTION Flammable.
- Hydroxylamine hydrochloride ($NH_2OH \cdot HCl$; Aldrich, cat. no. 25558-0)
- Sodium hydroxide (NaOH; Chemicon, cat. no. 5013)
- Sodium ascorbate (Aldrich, cat. no. A7631)
- Sodium azide (NaN_3; Aldrich, cat. no. S2002) ! CAUTION Toxic.
- Sodium sulfate (Na_2SO_4; GCE Laboratory Chemicals, cat. no. E0476)
- 4-Bromobutanoyl chloride (Fluka, cat. no. 16515)
- *O*-(7-Azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU; GL Biochem, cat. no. 00703)
- Propargylamine (Aldrich, cat. no. P5090-0)
- Trifluoroacetic acid (TFA; Sigma-Aldrich, cat. no. T6508) ! CAUTION Corrosive.
- Triisopropylsilane (Sigma-Aldrich, cat. no. 233781)
- Copper iodide (CuI; Merck, cat. no. B392648)
- Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$; Merck, cat. no. A840490)
- Pyridine (Fluka, cat. no. 82703) ! CAUTION Toxic and corrosive.
- *N*,*N*-diisopropylethylamine (DIEA; Sigma, cat. no. D3887)
- Acetonitrile (CH_3CN; Tedia, cat. no. AS1122-001)
- Methanol (MeOH; Tedia, cat. no. MS1922-001) ▲ CRITICAL When specified, MeOH is dried by distilling over magnesium shavings.
- *tert*-Butyl alcohol (*t*-BuOH; Mallinckrodt, cat. no. 1998)
- Dichloromethane (DCM; Tedia, cat. no. CS-1334)
- 1,2-Dichloroethane (Aldrich, cat. no. D61563)
- Dimethylformamide (DMF; Fischer Scientific, cat. no. D/3846/17)
- Dimethyl sulfoxide (DMSO; Fischer Scientific, cat. no. D/4121/PB17)

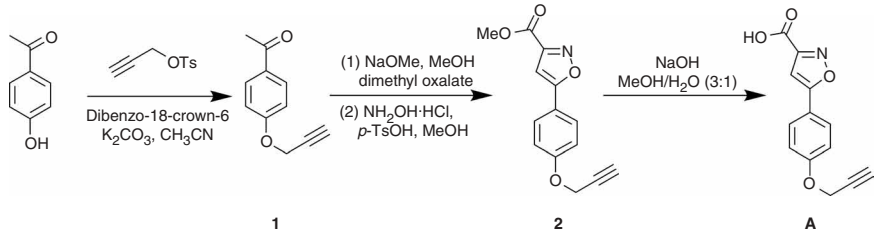

Figure 5 | Synthesis of the alkyne-functionalized isoxazole warhead for PTP1B[14]. **1**=1-(4-(prop-2-ynyloxy)phenyl)ethanone; **2**=methyl 5-(4-(prop-2-ynyloxy)phenyl)isoxazole-3-carboxylate.

- Deionized water
- Ethyl acetate (EA)
- Brij-35 (Amresco Inc., cat. no. M106)
- MMP-7 (Calbiochem, cat. no. 444270)
- MMP-7 fluorogenic substrate (MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2; Calbiochem, cat. no. 03-32-5032)
- MMP-7 assay buffer
- PTP1B (Calbiochem, cat. no. 539735)

EQUIPMENT

- Common synthetic organic chemistry glassware
- pH paper (Universalindikator, Merck)
- Rotary evaporator (Buchi)
- Vacuum evaporator (Genevac HT-4X)
- Centrifuge (model Rotina 35, Hettich-Zentrifugen)
- Liquid chromatography-mass spectrometry (LCMS) column (analytical reverse phase): C18 (250 mm × 4.6 mm) (Phenomenex, cat. no. 00G-4041-E0)
- Shimadzu liquid chromatography-mass spectrometry-ion trap-time of flight (LCMS-IT-TOF) system equipped with auto-sampler (cat. no. LCMS-2010EV)
- Sciclone ALH 3000 liquid handler workstation (Caliper Life Sciences)
- Multidrop Combi dispenser (Thermo Scientific)
- 5–50 µl multichannel pipette (Brandtech Scientific)
- Black flat-bottomed polypropylene 384-well plates (Nunc)
- 96-well 1-ml polypropylene stock plates (Greiner Bio-One, cat. no. 780215)
- 96-well polypropylene stock plates (Greiner Bio-One, cat. no. 655201)
- 384-well polypropylene plate (Genetix, cat. no. X7020)
- 384-well cap-mat (Greiner Bio-One)
- Adhesive film (ABgene, cat. no. AB-0558)
- SpectraMax Gemini XS fluorescence plate reader (Molecular Devices)
- Microplate shaker (GFL)
- Dessicator with dry storage box
- Ice box
- Powder-free gloves

REAGENT SETUP

0.5 M sodium methoxide in methanol (NaOMe solution) Add dry MeOH (38 ml) into a flame-dried round-bottomed flask (rbf). Weigh 0.45 g of sodium pieces in mineral oil into a beaker containing hexane. Dry the sodium with an adsorbent paper and quickly add it into the rbf. Stir the solution using a magnetic stirrer under a nitrogen atmosphere until the sodium completely dissolves.

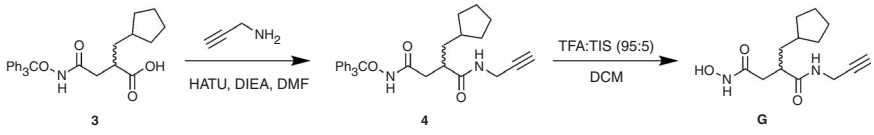

Figure 6 | Synthesis of the alkyne-functionalized hydroxamic acid warhead for MMPs[18]. **3**=2-(cyclopentylmethyl)-4-oxo-4-(trityloxyamino)butanoic acid; **4**=2-(cyclopentylmethyl)-N1-methyl-N4-(trityloxy)succinamide.

1.25 mM $CuSO_4 \cdot 5H_2O$ 16 mg of 1.25 mM $CuSO_4 \cdot 5H_2O$ in 50 ml of deionized water.

2.5 mM sodium ascorbate 25 mg of 2.5 mM sodium ascorbate in 50 ml of deionized water.

1.25 mM CuI 12 mg of 1.25 mM CuI in 50 ml of DMSO.

25 mM pyridine in CH_3CN 0.01 g of 25 mM pyridine in 5 ml of CH_3CN.

Catalyst mix A (for 100 reactions) 200 µl of 1.25 mM $CuSO_4 \cdot 5H_2O$, 500 µl of 2.5 mM sodium ascorbate and 4.3 ml H_2O.

Catalyst mix B (for 100 reactions) 200 µl of 1.25 mM CuI and 500 µl of 25 mM pyridine in CH_3CN ▲ **CRITICAL** Always use freshly prepared CuI solution for the catalyst mixes in the click reactions.

PTP1B assay buffer 50 mM HEPES, 300 mM NaCl and 0.2 mg ml^{-1} of BSA, pH 7.5.

T-cell protein tyrosine phosphatase (TCPTP), protein phosphatase 1 (PP1), leukocyte antigen-related (LAR), YOP, λPPase, PP1 assay buffer 25 mM Tris, pH 7.5.

MMP-7 assay buffer 0.1 M Tris-HCl, 0.1 M NaCl, 10 mM $CaCl_2$ and 0.05% (vol/vol) Brij-35, pH 7.5.

EQUIPMENT SETUP

LCMS-IT-TOF setup Configure the LCMS-IT-TOF system with an analytical C18 reverse phase column. Use eluents A (0.1% TFA in acetonitrile) and B (0.1% TFA in water) as mobile phases and C (100% acetonitrile) as the washing solvent. Configure the system with a photodiode array (PDA) detector and IT-TOF mass spectrometer. Set up the system for automatic batch injections from the 384-well plate. The injection volume is 2 µl. Before each injection, equilibrate the column with 30% A (with 70% B) for 10 min. After each injection, run a solvent gradient of 30% A to 100% A over 10 min followed by 100% A over 5 min at a flow rate of 0.6 ml min^{-1}. After each run is completed, flush the column with C for 10 min.

Spectrofluorometer setup Set the spectrofluorometer to an excitation wavelength of 325 nM and an emission wavelength of 393 nM.

PROCEDURE

Synthesis of alkyne-functionalized PTP1B core group A ● TIMING 5 d

1| Equip a two-necked rbf with a Teflon-coated magnetic stir bar. Fit a reflux condenser to one neck and cap a glass stopper to the other neck.

2| Charge the rbf with propargyl tosylate (4.63 g, 22 mmol), dibenzo-18-crown-6 (0.4 g, 1.1 mmol), K_2CO_3 (4.6 g, 33 mmol) and 4-hydroxyacetophenone (3 g, 22 mmol). Add ~75 ml of acetonitrile. Reflux with stirring at 80 °C for 6 h.

3| Cool the reaction mixture to room temperature (RT; 25 °C). Remove the acetonitrile with a rotary evaporator equipped with a water aspirator at 45 °C.

4| Add DCM (100 ml) to the slurry and transfer the solution into a 250 ml separating funnel. Wash the solution with deionized water (2 ml × 50 ml) and finally once with brine (50 ml). Dry the DCM layer over anhydrous sodium sulfate (~3 g) for 2 min.

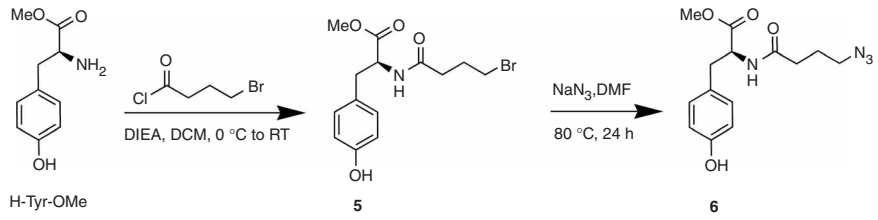

Figure 7 | Representative synthesis of the azide-functionalized secondary-site binder 6. **5**=methyl 2-(4-bromobutanamido)-3-(4-hydroxyphenyl)propanoate; **6**=methyl 2-(4-azidobutanamido)-3-(4-hydroxyphenyl)propanoate.

5| Carefully decant the DCM solution into an rbf and concentrate *in vacuo* with a rotary evaporator equipped with a water aspirator.

6| Purify the resulting oil residue by column chromatography according to Still's method[19] (20–30% EA/hexane; Rf = 0.3) over silica gel (~200 g) packed in a glass column (60 mm internal diameter × 150 mm height).

7| Equip an oven-dried two-necked rbf with a Teflon-coated magnetic stir bar. Fit a pressure-equalizing funnel to one neck and cap the other neck with a glass stopper.

8| Fit an oven-dried 250-ml two-necked rbf containing a Teflon-coated magnetic stir bar with an adapter connected to a nitrogen inlet. Loosely cap the other neck with a glass stopper. Flush the rbf with nitrogen gas for ~1 min. Charge the rbf with **1** (3.37 g, 19.36 mmol) and dimethyl oxalate (3.19 g, 19.36 mmol).

9| Add 0.5 N of a NaOMe solution (38 ml, 19.36 mmol) to the rbf. Reflux the reaction mixture at 80 °C for 12 h. A thick suspension forms.
▲ **CRITICAL STEP** Use freshly prepared sodium methoxide and add the solution dropwise over 10 min to the rbf using a pressure-equalizing funnel under a nitrogen or argon atmosphere.

10| Stop the heating and cool the reaction to RT.

11| Add a catalytic amount of *p*-TsOH (~50 mg), followed by hydroxylamine hydrochloride (1.35 g, 19.36 mmol). Reflux the reaction mixture at 80 °C for over 3 d.

12| Cool the reaction mixture in an ice bath for about 15 min. Filter the suspension through a sintered funnel. Wash the solid repeatedly with ice-cold methanol. Dry the solid *in vacuo* for 3 h to obtain **2**.
? TROUBLESHOOTING

13| Suspend the methyl ester **2** (0.972 g, 4 mmol) in 90 ml of methanol in an rbf.

14| Dissolve NaOH (1.6 g, 40 mmol) in 30 ml of water and add to the rbf slowly at RT. Stir the suspension at RT for 4 h.

15| Remove the methanol at 45 °C with a rotary evaporator equipped with a water aspirator. Place the flask in an ice bath. Add 4 N HCl solution to the suspension until the pH of the solution is ~2. The pH may be approximated using pH papers.

16| Filter the suspension through a sintered funnel and repeatedly wash the precipitate with ice-cold water (~150 ml in total). Dry the precipitate under vacuum in a dessicator connected to an oil pump for 12 h to obtain **A**.
■ **PAUSE POINT** The solid may be stored indefinitely at −20 °C.

Synthesis of alkyne-containing MMP core group G ● TIMING 12 h

17| Fit an oven-dried 250-ml two-necked rbf containing a Teflon-coated magnetic stir bar with an adapter connected to a nitrogen inlet. Loosely cap the other neck with a glass stopper. Flush the rbf with nitrogen gas for 2 min.

18| Load the rbf with **3** (0.46 g, 1.5 mmol) and add 5 ml of DMF. Stir the mixture with a magnetic stirrer. Add DIEA (0.26 ml, 1.8 mmol), HATU (0.685 g, 1.8 mmol) and propargylamine (0.123 ml, 1.8 mmol) sequentially into the rbf. Stir the reaction mixture at RT for 3 h.

19| Remove the DMF with a rotary evaporator equipped with a vacuum pump. Dissolve the oil residue in DCM (10 ml) and extract with deionized H_2O (2 ml × 8 ml). Wash the DCM layer with brine (8 ml). Dry over sodium sulfate (~1 g) for 5 min. Carefully decant the DCM solution into an rbf and concentrate *in vacuo* at 45 °C with a rotary evaporator equipped with a water aspirator.

20| Purify the resulting oil residue by column chromatography (60–100% DCM/hexane–5% MeOH/DCM; Rf = 0.3) over silica gel (~15 g) packed in a glass column (30 mm internal diameter × 150 mm height).

21| Pool the fractions containing the pure product and remove the solvent *in vacuo*. Remove the residual traces of solvent at 45 °C with a rotary evaporator equipped with an oil pump to give alkyne **4**.

22| Dissolve alkyne **4** (0.49 g, 1.2 mmol) in a clean 10 ml rbf with DCM (3 ml). Prepare the cleavage cocktail (95% vol/vol TFA/triisopropylsilane (TFA: 0.92 ml)) and add the solution into the rbf. Stir for 15 min.
! **CAUTION** TFA is corrosive. Wear lab coat, gloves and goggles.

23| Evaporate the TFA and DCM at 45 °C with a rotary evaporator equipped with a water aspirator. Purify the crude residue by column chromatography as outlined in Steps 22 and 23. A reddish-brown solid is obtained.

Synthesis of azide-containing peripheral group 6 ● TIMING 27 h

24| Equip an oven-dried two-necked rbf with a Teflon-coated magnetic stir bar. Connect one of the necks to a nitrogen line and cap the other neck using a rubber septum.

25| Maintain a positive nitrogen atmosphere in the rbf. Place the rbf in an ice bath.

26| Charge the rbf with the amine (H-Tyr-OMe) (0.36 g, 2 mmol) and dissolve it in distilled DCM (30 ml).
▲ CRITICAL STEP Use freshly distilled DCM for better yields.

27| Add DIEA (0.28 ml, 2.2 mmol) and then 4-bromobutanoyl chloride (0.37 g, 2 mmol) slowly using a syringe. Remove the ice bath and stir for 2 h.

28| Transfer the contents of the flask into a 100 ml separating funnel. Extract twice with 1 N HCl (30 ml), followed by deionized water (30 ml) and brine (30 ml). Dry the DCM solution over anhydrous sodium sulfate (~1 g). Remove the solvent by rotary evaporation.
? TROUBLESHOOTING

29| Cap a single-necked rbf containing a Teflon-coated stir bar with rubber septum. Pierce a needle into the septum.

30| Charge the rbf with the alkyl bromide **5** (0.34 g, 1 mmol), NaN_3 (0.1 g, 1.5 mmol) and 30 ml DMF. Stir the suspension vigorously at 80 °C for 24 h.
! CAUTION NaN_3 is a highly toxic and explosive compound. Avoid shock and heating to very high temperatures.

31| Remove DMF at 55 °C using a rotary evaporator equipped with an oil pump.

32| Dissolve the crude viscous mass in EA (30 ml) and transfer the solution into a 100 ml separating funnel.

33| Wash the organic solution with deionized water (2 ml × 30 ml) followed by brine (30 ml). Dry the organic layer using anhydrous sodium sulfate (~3 g).

34| Remove the solvent by rotary evaporation.
■ PAUSE POINT The compound **6** may be stored for a long period at temperatures below 4 °C.

Optimization of the click reaction ● TIMING **2 d**

35| Refer to **Table 1** for the conditions and volumes of each reagent and catalyst needed for the optimization of the click reaction. Each reaction is carried out in a 384-well microplate with a total volume of 100 µl in each well.

36| Prepare the following alkyne and azide stock solutions in DMSO:
25 mM alkyne **A**: 30 mg of **A** in 5 ml of DMSO
50 mM azide **7**=methyl 2-(4-(5-azidopentanamido)phenyl)-2-hydroxyacetate: 77 mg of **7** in 5 ml of DMSO

37| Add alkyne **A** (25 mM, 10 µl), azide **7** (50 mM, 7 µl), catalyst mix and organic solvent sequentially into the 384-well plate. The amounts of the catalyst mix and the organic solvent are varied based on **Table 1**.
▲ CRITICAL STEP As DCM and dichloroethane are highly volatile, the addition should be fast and the reaction vessels immediately sealed.
? TROUBLESHOOTING

38| Seal the 384-well plate tightly with a silicon-based cap-mat.
■ PAUSE POINT Shake the plate thoroughly for 36 h using a microplate shaker.

39| Remove the solvents in the 384-well plate to dryness using a vacuum evaporator and redissolve in 125 µl of DMSO. The concentration is assumed to be 2 mM.

40| Carry out LCMS analysis of the inhibitors in each well in the plate (see **Table 2** for representative results). (Optional) If LCMS is not available, carry out parallel thin-layer chromatography to determine whether the reaction is complete.

TABLE 1 | Detailed reaction contents for the click optimization reaction.

	Catalyst mix		Organic solvent		
Entry	**Mix**	**Volume (µl)**	**Solvent**	**Volume (µl)**	**Vol. of H_2O (µl)**
1	A	50	*t*-BuOH	33	—
2	A	50	DMSO	33	—
3	A	50	DCM	33	—
4	A	50	DCE	33	—
5	B	50	*t*-BuOH	33	—
6	B	50	DMSO	33	—

Library synthesis of bidentate inhibitors in 384-well plates ● TIMING 2 d

41| Prepare 25 mM stock solutions of each of the alkynes and 50 mM stock solutions of each of the azides.

42| From the LCMS profiles obtained in Step 44, pick the entry that gives the highest product and yield. Using the set of conditions, assemble the library by reacting a different alkyne and azide in individual wells in a 384-well plate. Refer to refs. 14 and 18, respectively, for further details of the synthesis of a 66-member PTP1B (5 alkynes × 13/14 azides) and a 96-member MMP-7 library (8 alkynes × 12 azides).

43| Dispense the solutions, solvents and the catalyst mix using an automatic liquid handler, a bulk dispenser and a multichannel pipette for quick and robust transfer into the 384-well plate.

44| Repeat Steps 38–40.

■ **PAUSE POINT** The 384-well plate is the master plate used directly for enzymatic screening and can be stored in a −20 °C freezer for an extended period of time.

Microplate-based screening of MMP inhibitors against MMP-7 ● TIMING 1 d

45| Assume the concentration of the inhibitors in each well to be 2 mM. Prepare a diluted stock plate of the inhibitors with an approximate concentration of 20 μM. Transfer 10 μl of each stock solution from the master plate prepared in Step 43 to individual wells of a 96-well polypropylene microplate containing 90 μl of water and mix by shaking on a microplate shaker.

46| Transfer 10 μl of each subsequently diluted sample into another 96-well polypropylene microplate containing 90 μl of water, to result in a 100 times dilution. Steps 45 and 46 may be performed using a multichannel pipette or a liquid transfer robot.

■ **PAUSE POINT** The diluted stock plate can be sealed with an adhesive film and stored at −20 °C for several months. For long-term storage, storage at −80 °C is recommended.

47| In a 384-well, black, flat-bottomed polypropylene microplate, aliquot 25 μl of MMP-7 assay buffer (2×) into each well using a multichannel pipette or a liquid transfer robot. A typical reaction volume is set at 50 μl buffered with substrate, inhibitor and enzyme components.

▲ **CRITICAL STEP** Constituents of the reaction buffer change with the use of different enzymes, catered to their requirements for activity.

48| Aliquot MMP-7 fluorogenic substrate into each well of the reaction plate using a multichannel pipettor or a liquid transfer robot to 80 pmol per assay.

▲ **CRITICAL STEP** It is essential that equal amounts of liquid be transferred from each well. Perform calibration checks on liquid handler if necessary.

49| Thaw the diluted stock plate at RT and mix on a microplate shaker. Spin in a centrifuge with plate carrier briefly (1 min) at 1,000*g* at 4 °C before the seal is removed.

50| Transfer 10 μl of each diluted stock solution from Step 46 into individual wells, using a multichannel pipette or a liquid transfer robot. Keep three wells as positive controls (e.g., no inhibitor or replacing the inhibitor with an equal volume of water/DMSO). These positive controls will provide benchmark readouts of enzyme assayed without inhibitor.

▲ **CRITICAL STEP** It is essential that all wells receive equal amounts of liquid and thus equivalent concentrations of inhibitors. The final concentration of inhibitors in the reaction mix may be optimized when working with different enzymes or inhibitor libraries, which is influenced by the expected potency of inhibitors. Replicate experiments should be performed to minimize errors. Screening at a range of inhibitor concentrations could also improve the quality of the results obtained.

51| Cover the reaction microplate with an adhesive film, mix on a microplate shaker and spin in a centrifuge with plate carrier briefly (1 min) at 1,000*g* at 4 °C to remove bubbles that might interfere with fluorescence readouts.

52| Remove the adhesive film and scan the microplate using a fluorescence plate reader and take the fluorescence readout as a background value to be subtracted from the final reaction readouts.

53| Aliquot the enzyme to initiate the reaction, at a final amount of 0.6 pmol per assay. Spin in a centrifuge with plate carrier briefly (1 min) at 1,000*g* at 4 °C if the solution sticks to the side or bubbles are observed.

▲ **CRITICAL STEP** It is essential that the enzyme is simultaneously added to individual wells and that an equal amount of the enzyme is added. The final concentration of the enzyme used in the reaction mix may be optimized, as it is influenced by the level of activity of the enzyme.

54| Incubate plates at 37 °C for 30 min, before scanning using the fluorescence plate reader for end-point fluorescence.
? TROUBLESHOOTING

55| Export plate readings from the scanner into Microsoft Excel.

56| Normalize plate readings obtained by subtracting readings from the initial uncleaved substrate and inhibitor backgrounds (obtained from Step 52) from the end-point fluorescence (obtained from Step 54). Calculate the relative potencies of each inhibitor from the normalized data using the following relationship:

$$\text{inhibition potency of } x = \left(1 - \frac{\text{measured intensity } x}{\text{uninhibited intensity}}\right) \times 100\%$$

Uninhibited intensity is the normalized readings from the positive controls (the three wells containing no inhibitor, as stated in Step 50).
? TROUBLESHOOTING

TABLE 2 | Results of the optimization of the click reactions based on Table 1.

Entry	Yield[a] (%)	Purity[a] (%)
1	~100	>95
2	>95	>95
3	~100	~100
4	~100	~100
5	>90	>80
6	>95	>90

[a]Estimation based on the LCMS profile.

Calculation of IC_{50} values for selected inhibitors

57| Prepare a twofold dilution series of an inhibitor, from approximately 745 to 1 μM (final concentrations) in the case of MMP-7. For other enzymes, a different concentration range may be determined experimentally.

58| Incubate the enzymatic reactions at RT for 0–60 min. Scan for end-point fluorescence using the fluorescence plate reader.

59| Calculate the IC_{50} values by fitting the fluorescent outputs obtained using the Graphpad Prism software v.4.03 (GraphPad). Use 7–8 data points to generate each of the IC_{50} plots.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
12	Low yield of the isoxazole product	NaOMe used has degraded Insufficient acid catalyst added in the cyclization step	Use freshly prepared NaOMe Add another portion of *p*-toluenesulfonic acid
28	Low yield of the N-acylation product	Solvent Hydrolysis of acid chloride	Use distilled DCM Use freshly prepared acid chloride. Always store the acid chloride below 0 °C under argon
37	Click reaction is incomplete	Evaporation of DCM and DCE	Seal the plate tightly with silicon-based cap-mat. Top up the DCM and DCE if necessary
54 and 56	No substrate activity is detected for the entire library, including positive control	Enzyme is inactive	Repeat experiment with active enzyme, perform small-scale substrate assay to confirm the activity of enzyme
54 and 56	No substrate activity is detected for the library except for positive control	Inhibitor concentrations are too high	Lower inhibitor concentrations. The optimal concentration would result in a good distribution of inhibition levels between 0% and 100% among the different library members
	No or little inhibition is observed	Inhibitor concentrations are too low	Increase inhibitor concentrations. Optimize the amount of inhibitor to be used (see above)

ANTICIPATED RESULTS

Analytical data

Compound A. Yield = 87%. ^{1}H-NMR (300 MHz, DMSO-d6) δ 7.91–7.85 (d, J = 9.6 Hz, 2H), 7.26 (s, 1H), 7.16–7.14 (d, J = 8.7 Hz, 2H), 4.90 (d, J = 1.98 Hz, 2H), 2.50 (m, 1H); ESI $[M + 1]^+$ = 244.1, ESI-MS: m/z $[M + Na]^+$ = 266.0.

Azide 6. Yield = 75%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.96–6.93 (m, 2H), 6.76–6.71 (m, 2H), 6.06 (br s, 1H), 4.90–4.83 (m, 1H), 3.75 (s, 2H), 3.30–3.25 (m, 2H), 3.13–2.94 (m, 2H), 2.27 (t, J = 7.41 Hz, 2H), 1.87 (quintet, J = 6.86 Hz, 2H); ESI-MS: m/z $[M + 23]^+$ = 329.1.

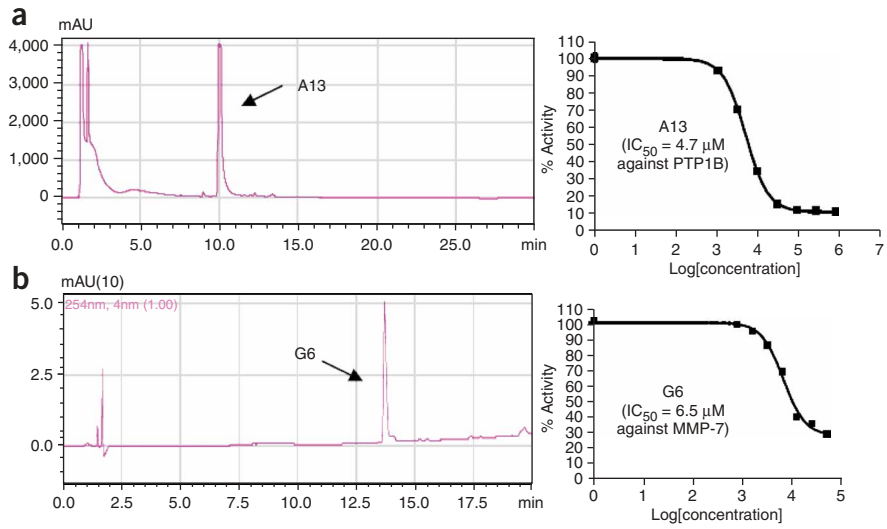

Figure 8 | LCMS (left) and IC_{50} curves (right) of two 'hits' identified against PTP1B and MMP-7, respectively: (**a**) PTP1B; (**b**) MMP-7.

Compound G. Yield = 43%. ^{1}H-NMR (800 MHz, MeOD) δ 4.06–3.96 (m, 1H), 3.93–3.87 (m, 1H), 2.86–2.76 (m, 1H), 2.64–2.53 (m, 1H), 2.39–2.29 (m, 1H), 2.23–2.13 (m, 1H), 1.61–1.56 (m, 2H), 1.26–1.16 (m, 1H), 0.98–0.89 (m, 6H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 177.9, 171.5, 81.3, 73.0, 43.4, 40.3, 30.3, 27.8, 24.4, 23.2; ESI-MS: m/z $[M + 1]^+$ = 559.2.

Click chemistry of PTP1B inhibitors

A 66-member PTP1B library was constructed on the basis of the above protocols, from five different alkyne-bearing isoxazole core groups as shown in **Figure 2a** and more than a dozen azide-containing peripheral groups. Upon optimization of click chemistry conditions, we found that a mixed solvent system containing t-BuOH, water and DMSO (1:1:0.1), with $CuSO_4$ and sodium ascorbate as catalysts, enabled the formation of the triazole products in nearly quantitative yield with few by-products in most cases, as indicated by LCMS analysis (**Table 2** and **Fig. 8a**, left). Equally important, the products could be taken directly, without purifications, and screened for inhibition against PTP1B using standard microplate-based enzyme assays. We routinely screen for possible cross-reactivity with other enzymes. Most library members in our studies showed good inhibition toward PTP1B and a good degree of inhibition toward other PTPs (e.g., TCPTP, YOP and LAR)[14]. Significantly, they did not show any appreciable inhibition against other non-PTP phosphatases (e.g., λPPase and PP1). From this bidentate 'click' library, we have successfully identified a specific PTB1B inhibitor, A13 (**Fig. 1**, bottom left), which showed good inhibition (IC_{50} = 4.7 μM; **Fig. 8a**, right) and selectivity (5 and 25 times more selective toward PTP1B over TCPTP and other PTPs, respectively)[14].

Click chemistry of MMP inhibitors

Our MMP library design was based on the general structure of hydroxamate-based MMP inhibitors[18]. It was previously shown that hydroxamate inhibitors with (a) hydrophobic P_1' residues are generally preferred, (b) a variety of substitutions are tolerated at P_2' and P_3' positions and (c) hydrophobic P_4' residues are preferred and sometimes could confer a good degree of specificity among different MMPs[16]. There we used 8 different alkyne-containing succinyl hydroxamates as the core groups of our 'click' MMP library (**Fig. 2b**, inset), and 12 different aromatic compounds tethered with an azide linker of a varied chain length (**Fig. 3**). By changing the linker length of the azides, our design facilitates the projection of the hydrophobic moiety into the P_4' binding pocket of the target MMP, thereby aiming to improve both the potency and specificity of the inhibitors. Subsequent click chemistry followed by LCMS analysis indicated that the triazole products were formed with quantitative yield and excellent purity in most cases (**Fig. 8b**, left). The products were screened directly against MMP-7; the results indicated that the most potent inhibitor was G6 (**Fig. 1**, bottom right), which showed moderate inhibition against MMP-7 (IC_{50} = 6.5 μM) and good selectivity (>10 times more potent toward MMP-7 over other metalloproteases such as thermolysin and collagenase).

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