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Development of molecular probes for second site screening and design of protein tyrosine phosphatase inhibitors

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Abstract

We report on the design, synthesis and evaluation of series of furanyl-salicyl-nitroxide derivatives, as effective chemical probes for second site screening against phosphotyrosine phosphatases (PTPs) using NMR-based techniques. The compounds have been tested against a panel of *PTPs* to assess their ability to inhibit a broad spectrum of these phosphatases. The utility of the derived compounds is illustrated with the phosphatase YopH, a bacterial toxin from *Yersinia pestis*. Novel chemical fragments were identified during a NMR-based screen for compounds that are capable to bind on the surface of YopH in regions adjacent the catalytic site in presence of the spin-labeled compounds. Our data demonstrate the value of the derived chemical probes for NMR-based second-site screening in PTPs.

Introduction

Protein tyrosine phosphatases (PTPs) have been the focus of considerable drug discovery efforts in recent years owing to evidence of their role in regulating cell-signaling pathways [1,2,3]. However, many inhibitors identified so far are peptide-based, and because of the conserved nature of the active site among phosphotyrosine binding pockets, obtaining selectivity is often difficult. Only very recently, several examples point to fragment-based approaches as possible route for the identification of potent and selective protein phosphatase inhibitors [4,5]. A first example in designing selective PTP1B inhibitors was recently reported by Szczepankiewicz *et al.* [5]. In this work, the authors screened a library of 10,000 compounds by using heteronuclear [¹⁵N,¹H] NMR correlations spectra to assess binding. This approach has the advantages that even small fragments that bind fairly weakly can be identified, and that information on the binding mode can also be obtained [6,7]. This screen resulted in a compound that mimics the natural phosphotyrosine substrate and inhibits PTP1B in the micromolar range. Subsequently this hit was optimized with the aim of fully occupying adjacent sites by modular addition of scaffolds [5]. In fact, an additional compound that bound in a second adjacent site was identified by exploiting another NMR screen. The subsequent chemical linkage of both compounds lead to a bi-dentate molecule that inhibited PTP1B in the nanomolar range [5].

Another example on the use of fragment-based approaches to obtain inhibitors of a PTP is the application of the “click chemistry” [4]. In this case, Srinivasan *et al.* employed alkyne-containing compounds targeting the PTP active site of PTP1B and azide-containing compounds targeting an already known secondary aryl-phosphate binding site near the catalytic site. With these two types of fragments a library of 66 compounds was synthesized and screened against

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different PTPs obtaining one compound with an IC_{50} of 5 μ M for PTP1B and a selectivity of 5 to 25 times with respect to the rest of PTPs tested [4].

Recent studies from our laboratories focused on the inhibition of the bacterial protein tyrosine phosphatase YopH [8]. Aided by a combination of chemical library screening, structure-activity relationships analysis and *in silico* docking of lead compounds, we developed small-molecule inhibitors of YopH [8]. Our inhibitors contain a single salicylate linked to a furanyl moiety as phosphotyrosine mimic and a more variable group that could be exploited to achieve selectivity and higher affinity [8]. In fact, while very small differences can be seen in the phosphotyrosine binding pockets of tyrosine phosphatases, unique sub-pockets can be found in adjacent regions [3,8]. Therefore, it appears evident that by tailoring a second-site ligand, it should be possible to develop potent and selective inhibitors of therapeutically relevant protein tyrosine phosphatases. An interesting approach to screen for second site binders was recently reported by Jahnke and co-workers [9,10,11]. This method utilizes initial binders chemically labeled with organic nitroxide radicals (“spin labels” such as the 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO)) to perform second-site NMR spectroscopic screens of fragment libraries. The binding of a second-site ligand can be simply detected by measuring the relaxation enhancement induced by the spin-labeled first ligand (Figure 1) [9,10,11]. Other common approaches for second-site screen via NMR are the well known SAR by NMR method [12], where the second ligand is screened via protein [^{15}N , 1H] correlation spectra, and the SAR by ILOEs method, where the second site ligand is detected via protein-mediated ligand-ligand NOEs [13,14]. Compared with these methods, a major drawback of the spin-labeling approach consists in the necessity to produce an organic nitroxide radical derivative of a first ligand, which may be different for each target (Figure 1). A more practical approach consists in preparing first-site spin-labeled compounds that are cross-reactive within different members of a protein family, such as protein kinases, as recently reported [15], or phosphatases as we report in this work. Such chemical tools are then useful for the design and synthesis of bidentate compounds with increased affinity but also specificity for a given target. In fact, if specificity is a major issue, second site ligands that are specific for a given protein may be selected by performing the NMR screening against counter targets. Based on these premises, we report herein the synthesis and characterization of novel furanyl-salicyl-nitroxide derivatives (Table 1) as versatile probes for NMR-based second-site screening in protein tyrosine phosphatases.

Results and discussion

Chemical Synthesis

The synthesis of all the nitroxide compounds reported in Table 1 was accomplished employing easy and straightforward synthetic schemes that included as last step the attachment of the 4-amino 2,2,6,6-tetramethylpiperidine 1-oxyl (4-amino TEMPO) through an amide bond to the phosphotyrosine mimic (Figures 2 and 3).

The compounds **1a–b** were obtained through the synthesis of the intermediate carboxylic acids **6a–b** by the reaction between the anilines **5a–b** and glutaric anhydride. A subsequent amide formation with 4-amino TEMPO, employing water soluble carbodiimide (WSC) as activating agent, led to the stable radical compounds **1a–b** (Figure 2).

Compounds **4a–b** were obtained from commercial aldehydes **10a–b**. A Horner-Wadsworth-Emmons reaction between these aldehydes and methyl diethyl phosphonoacetate afforded the corresponding methyl esters **11a–b** in 89% and 83% yields respectively. After saponification and amide formation with 4-amino TEMPO in presence of WSC, compounds **4a–b** were isolated (Figure 2).

The first synthetic step towards compounds **2a–b** and compound **3** is the same in both cases. A Suzuki coupling between the methyl 5-bromo-2-hydroxybenzoate and the 5-formylfuran-2-ylboronic acid allowed us to obtain aldehyde **7** with a 62% yield. A reductive amination of this aldehyde with 4-amino TEMPO afforded compound **2a** with an 87% yield. Then, compound **2b** was obtained after the saponification of **2a**. In order to obtain compound **3**, a Horner-Wadsworth-Emmons reaction was done between aldehyde **7** and the methyl diethyl phosphonoacetate obtaining the methyl diester **8** with 84% yield. This compound was saponificated obtaining the dicarboxylic acid **9** with 88% yield. Knowing by previous experience in the laboratory that the carboxylic acid of the salicylic acid moiety is not particularly reactive, we could obtain compound **3** after an amide bond formation between the compound **9** and 4-amino TEMPO with a 46% yield (Figure 3).

Biochemical Evaluation

In order to characterize affinity of the compounds, we tested their inhibitory activity against a diverse panel of protein tyrosine phosphatases (Table 1). As it can be seen, compound **3** is the most cross-reactive showing inhibition constants in the range from 3.0 μM (YopH) to 24 μM (HePTP), representing an ideal compound candidate for second site screen in the proteins tested, and presumably to many others that have similar phosphotyrosine binding pocket characteristics.

To further characterize the binding of compound **3** to protein tyrosine phosphate binding pockets, we isolated the C-terminal domain (340 residues) of the PTP YopH (468 residues) from *Yersinia pestis* and monitored the effect of the ligand on resonance intensities in [^{15}N , ^1H] correlation spectra (Figure 4). As it can be seen, the binding of compound **3** to the C-terminal domain of YopH cause selective broadening of several resonance lines, presumably those located in the proximity of the binding pocket of the protein due to their closeness to the relaxation enhancer spin-labeled compound. In fact, the main nuclear spin relaxation mechanism in proteins and small molecule binders derives from the dipole-dipole interaction between a given nucleus and surrounding spins. The magnitude of this effect is proportional to the distance between the nucleus of the spins and to the gyromagnetic ratio of the spins. The unpaired electron possesses a gyromagnetic ratio which is 657.4 times that of a hydrogen nucleus, thus producing the most efficient relaxation effect even up to 10–15 Å from a given nucleus. As recently pointed out by Jahnke and co-workers [11], these data also further prove the utility of the chemical probes in the identification of binding site residues in isotopically labeled protein samples, particularly when combined with selective labeling of the target [11]. Finally, we have used compound **3** to screen for fragments that are capable to bind in adjacent pockets on the surface of the C-terminal domain of YopH. This was accomplished by exposing a mixture of potential second-site binders (1 mM) to compound **3** (500 μM) in presence and absence of a substoichiometric amount of YopH (10 μM). 1D ^1H - $T_{1\rho}$ NMR experiments are subsequently recorded and the differential loss of signal intensity is used to detect second-site binders. In fact, close proximity of any given second-site binder to the spin-labeled compound will result in rapid nuclear spin relaxation of hydrogen nuclei of the compound with concomitant line broadening in a simple 1D ^1H -NMR experiment. In order to amplify this effect, the transverse ^1H magnetization of the compounds is “locked” on the xy plane for 50 to 200 ms prior the acquisition of the 1D ^1H -NMR experiment. Such technique is usually referred as a $T_{1\rho}$ measurement [16]. Figure 5A shows a typical example of second site binders that can be easily identified with this approach. While binding of a given compound to the protein target would also result in decreased transverse relaxation times and thus reduce signal intensity (Figures 5B,C), the consequence of close proximity with a spin-labeled compound has a much more dramatic effect (Figure 5D). Thus, it is possible to discriminate protein binders in the proximity of the spin-labeled compound among non-binders or even compounds that bind in different locations. Intriguingly, some of the identified compounds,

for example the triazole derivative shown in red in Figure 5A, largely resemble scaffolds that were used to obtain potent bi-dentate PTP inhibitors discovered by the SAR by NMR approach [1,5].

In conclusion, we report here on the synthesis and characterization of a series of furanyl-salicylnitroxide derivatives that can be used as probes for NMR-based second-site screening in protein tyrosine phosphatases and to map PTP binding site residues. Although it is clearly emerging that the misregulation of protein tyrosine phosphatase activity is associated with the onset, development and progression of several human malignancies, the discovery of novel, safe and selective inhibitors has not been as successful as for other enzymes, mainly due to the high degree of conservation of the phosphotyrosine binding pocket [17]. We believe that the reported spin-labeled probes and the second-site screening approach proposed here may result very useful in developing novel, potent and selective lead compounds for further target validation and continued drug development.

Experimental Procedures

Chemistry

General methods and materials—Every chemical was used as received from the specified supplier. Anhydrous dimethylformamide (DMF) and anhydrous dichloromethane (DCM) were purchased from AKROS Chemical. Ethyl acetate, hexane and methanol were purchased from Fisher Scientific. Pyridine, toluene, diethylether, tetrahydrofuran (THF), glutaric anhydride, methyl 5-amino salicylate, 5-aminosalicylic acid, 5-bromo-2-hydroxybenzoate, 5-formylfuran-2-ylboronic acid, tetrabutylammonium chloride, palladium acetate, potassium carbonate, 4-amino TEMPO, sodium cyanoborohydride, methyl diethyl phosphonate, lithium hydroxide, 5-(4-nitrophenyl)-furfural and 5-(3-nitrophenyl)-furfural were all purchased from SIGMA-ALDRICH. The water soluble carbodiimide (WSC) was purchased from Novabiochem and the silica gel from Silicycle. ^1H and ^{13}C NMR data was collected using a 300 MHz Varian apparatus.

5-(4-hydroxy-3-(methoxycarbonyl)phenylamino)-5-oxopentanoic acid 6a—To a suspension of the glutaric anhydride (1.053 g, 9.2 mmol) in 50 mL of pyridine-dichloromethane (1:1) under nitrogen it was added the aniline **5a** (4.630 g, 27.7 mmol) as powder. The mixture was stirred at r.t. for 16 h under N_2 . After that time, the solvent was removed under reduce pressure and diluted with EtOAc. The organic phase was washed with 2N HCl, water and brine and it was dried over Na_2SO_4 . The solvent was removed under reduce pressure and the resultant oil was treated with diethyl ether to give a light purple solid that it was washed with a small amount of cold ether (1.281 g, 52%).

^1H -NMR (300 MHz, $\text{DMSO}-d_6$) δ 1.70–1.85 (m, 2H, CH_2), 2.15–2.35 (m, 4H, $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 3.34 (bs, 1H, CONH), 6.89 (d, $J = 8.8$ Hz, 1H, aromatic), 7.63 (dd, $J_1 = 8.8$ Hz $J_2 = 2.5$ Hz, 1H, aromatic), 8.10 (d, $J = 2.5$, 1H, aromatic), 9.85 (s, 1H, PhOH), 11.01 (bs, 1H, CO_2H); ^{13}C -NMR (75 MHz, $\text{DMSO}-d_6$) δ 21.1, 33.6, 35.9, 53.0, 53.4, 113.0, 117.9, 118.4, 120.3, 120.9, 127.8, 128.1, 131.9, 156.5, 171.2; MS(ESI) m/z : 268.1 $[\text{M}+\text{H}]^+$; R_f (EtOAc) = 0.58.

5-(4-carboxybutanamido)-2-hydroxybenzoic acid 6b—To a suspension of glutaric anhydride (1.053 g, 9.2 mmol) in 50 mL of pyridine-dichloromethane (1:1) under nitrogen it was added the aniline **5b** (4.245 g, 27.7 mmol) as powder. The mixture was stirred at r.t. for 16 h under N_2 . After that time, the solvent was removed under reduce pressure and diluted with EtOAc. The organic phase was washed with 2N HCl, water and brine and it was dried over Na_2SO_4 . The solvent was removed under reduce pressure and the resultant oil was treated with

diethyl ether to give a white solid that it was washed with a small amount of cold ether (1.165 g, 45%).

$^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ 1.70–1.85 (m, 2H, CH_2), 2.15–2.35 (m, 4H, $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{Me}$), 3.88 (s, 3H, CO_2Me), 6.92 (d, $J = 8.8$ Hz, 1H, aromatic), 7.61 (dd, $J_1 = 8.8$ Hz $J_2 = 2.5$ Hz, 1H, aromatic), 8.13 (d, $J = 2.5$, 1H, aromatic), 10.24 (s, 1H, PhOH); $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ 21.1, 33.6, 35.9, 113.0, 117.6, 118.0, 120.7, 121.4, 127.8, 128.3, 131.7, 157.6, 171.1, 172.4; MS(ESI) m/z : 282.1 $[\text{M}+\text{H}]^+$; R_f (EtOAc) = 0.03.

Compound 1a—To a solution of the carboxylic acid **6a** (0.200 g, 0.75 mmol) in a mixture of DCM:DMF (4:1) anhydrous (5 mL) in an ice bath, WSC (0.158 g, 0.82 mmol) was added already dissolved in the same previous solution (5 mL). After 30 min of agitation the 4-amino TEMPO (0.256 g, 1.5 mmol) was added and the reaction was allowed to reach room temperature and to continue for 16h. After this time the solution was diluted with DCM (20 mL) and washed with 0.1 N HCl (3×20 mL), H_2O (3×20 mL) and brine. The organic phase was dried over Na_2SO_4 and filtered. The solvent was then removed under reduced pressure to afford 0.146 g of an orange solid (45%). HRMS (ESI-TOF) m/z : calcd for $[\text{M}+\text{H}]^+$ 434.2291, found 434.2285; Anal. ($\text{C}_{22}\text{H}_{32}\text{N}_3\text{O}_6$) H, calc 7.42, found 7.22; C, calc 60.81, found 59.95.

Compound 1b—The same procedure as before was employed with the carboxylic acid **6b** obtaining a yellow solid (20%). HRMS (ESI-TOF) m/z : calcd for $[\text{M}+\text{H}]^+$ 421.2207, found 421.2194; Anal. ($\text{C}_{21}\text{H}_{30}\text{N}_3\text{O}_6$) H, calc 7.19, found 7.09; C, calc 59.99, found 59.24.

Methyl 5-(5-formylfuran-2-yl)-2-hydroxybenzoate 7—5-bromo-2-hydroxybenzoate (0.344 g, 1.4 mmol), 5-formylfuran-2-ylboronic acid (0.25 g, 1.7 mmol), tetrabutylammonium chloride (0.405 g, 1.4 mmol), palladium acetate (0.006 g, 2 mol %), and potassium carbonate (0.489 g, 3.5 mmol) were added to a 25 mL round-bottom flask. Then ethanol (4 mL) and of deionized water (4 mL) were added in this sequence and the reaction was stirred vigorously for 3h. After this time the ethanol from the mixture was removed under reduced pressure and diluted with water (15 mL). The product was extracted with EtOAc (3×50 mL). The organic phase was then stirred over charcoal (~ 1 g) for 30 min and Na_2SO_4 was added. The solution was filtered and concentrated to give a yellow oil. The residue was purified by silica gel column chromatography eluting with hexanes/EtOAc (4:1) to give 0.216 g of an off white solid (62%).

$^1\text{H-NMR}$ (300 MHz, CDCl_3) δ 4.09 (s, 3H, CO_2Me), 6.76 (d, $J = 3.7$ Hz, 1H, CH furanyl), 7.07 (d, $J = 8.8$ Hz, 1H, aromatic), 7.33 (d, $J = 3.7$ Hz, 1H, CH furanyl), 7.89 (dd, $J_1 = 8.7$ Hz $J_2 = 2.3$ Hz, 1H, aromatic), 8.33 (d, $J = 2.3$, 1H, aromatic), 9.63 (s, 1H, PhOH), 11.02 (s, 1H, CHO); $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ 53.2, 53.5, 108.4, 108.6, 115.1, 119.2, 119.5, 120.9, 126.9, 127.6, 132.5, 133.1, 161.3, 178.0, 178.4; MS(ESI) m/z : 247.1 $[\text{M}+\text{H}]^+$; R_f (Hexanes/EtOAc (4:1)) = 0.27.

Compound 2a—Compound **7** (0.173 mg, 0.7 mmol) was dissolved in toluene (6 mL) with 5% of acetic acid. Then the 4-amino TEMPO (0.124 g, 0.7 mmol) was added and the reaction was placed at 65 °C under N_2 for 16h. After this time the solvent was removed under reduced pressure until dryness. The residue was dissolved in MeOH (10 mL) and NaBH_3CN (0.050 g, 0.8 mmol) was added to the solution at 0 °C. After this another 3ml of 0.4 M HCl in MeOH were added and the reaction was allowed for 30 min at 0 °C. The reaction was warmed up to r.t. for 30 min and the solvent was removed under reduced pressure until almost dryness. The residue was diluted with water (20 mL) and 3M NaOH was added until pH 7. The product was extracted with chloroform (3×30 mL). The organic phase was dried over Na_2SO_4 , filtered and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography eluting with a gradient from hexanes/EtOAc (4:1) to hexanes/EtOAc (1:4) to give 0.244 g of an orange oil (87%).

HRMS (ESI-TOF) m/z : calcd for $[M+H]^+$ 402.2149, found 402.2141; Anal. (C₂₂H₂₉N₂O₅) H, calc 7.28, found 7.21; C, calc 65.82, found 66.04; R_f (EtOAc/MeOH (4:1)) = 0.48.

Compound 2b—Compound **2a** (0.1 g, 0.25 mmol) was dissolved in MeOH (3 mL) and cooled to 0 °C. 2N NaOH (3 mL) was added dropwise during 10 min and the reaction was placed at r.t. for 16h. After this time MeOH was removed under reduced pressure and water (15 mL) was added. Extractions with EtOAc (2 × 15mL) were done and the pH of the water solution was acidified to 6. Then, the product was extracted with EtOAc (3 × 15 mL). The organic solution was dried over Na₂SO₄ and filtered. The solvent was removed under reduced pressure to give a yellow solid (0.051 g) in 52% yield. HRMS (ESI-TOF) m/z : calcd for $[M+H]^+$ 388.1993, found 388.2002; Anal. (C₂₁H₂₇N₂O₅) H, calc 7.02, found 7.35; C, calc 65.10, found 63.85; R_f (EtOAc/MeOH (4:1)) = 0.07.

(E)-methyl 2-hydroxy-5-(5-(3-methoxy-3-oxoprop-1-enyl)furan-2-yl)benzoate 8

—A suspension of aldehyde **7** (0.125 g, 0.51 mmol), methyl diethyl phosphonoacetate (0.105 mL, 0.56 mmol) and LiOH (0.014 g, 0.56 mmol) in THF (10 mL) was stirred at r.t under Ar for 4h. After the usual work-up, purification by silica gel column chromatography with hexanes/EtOAc (6:1) as eluent was done to give 0.128 g of a pale yellow solid (84%).

¹H-NMR (300 MHz, DMSO-*d*₆) δ 3.72 (s, 3H, CO₂Me), 3.93(s, 3H, CO₂Me), 6.34 (d, J = 15.7 Hz, 1H, CH=CHCO₂Me), 7.05–7.06 (m, 2H, furanyl), 7.08 (d, J = 8.7 Hz, 1H, aromatic), 7.47 (d, J = 15.7 Hz, 1H, CH=CHCO₂Me), 7.97 (dd, J_1 = 8.7 Hz J_2 = 2.3 Hz, 1H, aromatic), 8.13 (d, J = 2.3 Hz, 1H, aromatic), 10.69 (bs, 1H, PhOH); ¹³C-NMR (75 MHz, DMSO-*d*₆) δ 52.0, 52.3, 53.1, 53.5, 108.3, 108.7, 114.3, 114.6, 114.8, 118.9, 119.3, 119.7, 121.8, 125.9, 126.6, 131.6, 132.4, 150.2, 155.3, 160.5, 167.3, 169.2; MS(ESI) m/z : 303.1 $[M+H]^+$; R_f (Hexanes/EtOAc (4:1)) = 0.49.

(E)-5-(5-(2-carboxyvinyl)furan-2-yl)-2-hydroxybenzoic acid 9—To suspension of compound **8** (0.1 g, 0.33 mmol) in MeOH (5 mL) at 0 °C was added dropwise 2N NaOH (5 mL). After the addition, the solution was warmed up to r.t. and then it was heated to 80 °C for 2h. After this time the reaction was cooled down and the methanol was removed under reduced pressure. The solution was diluted with water (20 mL) and extractions with EtOAc (2 × 15 mL) were done. The water solution was then acidified to pH 5 and the product was extracted with EtOAc (3 × 15 mL). The organic phase was dried over Na₂SO₄, filtered and the solvent was removed under reduced pressure affording 0.080 g of a yellow solid (88%).

¹H-NMR (300 MHz, DMSO-*d*₆) δ 6.27 (d, J = 15.7 Hz, 1H, CH=CHCO₂H), 6.95–7.10 (m, 3H), 7.40 (d, J = 15.7 Hz, 1H, CH=CHCO₂H), 7.97 (dd, J_1 = 8.7 Hz, J_2 = 2.3 Hz, 1H, aromatic), 8.16 (d, J = 2.3 Hz, 1H, aromatic); ¹³C-NMR (75 MHz, DMSO-*d*₆) δ 108.1, 108.5, 114.3, 115.9, 116.2, 118.6, 119.0, 121.7, 126.0, 126.7, 131.1, 150.3, 155.1, 161.8, 168.1, 172.1; MS (ESI) m/z : 275.0 $[M+H]^+$; R_f (EtOAc) = 0.1.

Compound 3—To a solution of the dicarboxylic acid **9** (0.070 g, 0.25 mmol) in a mixture of DCM:DMF (4:1) anhydrous (3 mL) in an ice bath, WSC (0.054 g, 0.28 mmol) was added already dissolved in the same previous solution (2 mL). After 30 min of agitation the 4-amino TEMPO (0.087 g, 0.51 mmol) was added and the reaction was allowed to reach room temperature and to continue for 16h. After this time the solution was diluted with DCM (20 mL) and washed with 0.1 N HCl (3 × 20 mL), H₂O (3 × 20 mL) and brine. The organic phase was dried over Na₂SO₄ and filtered. The solvent was then removed under reduced pressure to afford 0.049 g of a yellow solid (46%). HRMS (ESI-TOF) m/z : calcd for $[M+H]^+$ 428.1942, found 428.1938; Anal. (C₂₃H₂₇N₂O₆) H, calc 6.37, found 6.56; C, calc 64.62, found 64.23.

(E)-methyl 3-(5-(4-nitrophenyl)furan-2-yl)acrylate 11a—A suspension of aldehyde **10a** (0.5 g, 2.3 mmol), methyl diethyl phosphonoacetate (0.485 mL, 2.5 mmol) and LiOH (0.062 g, 2.5 mmol) in THF (10 mL) was stirred at r.t under Ar for 4h. After the usual work-up, purification by silica gel column chromatography with hexanes/EtOAc (4:1) as eluent was done to give 0.56 g of a yellow solid (89%).

¹H-NMR (300 MHz, DMSO-*d*₆) δ 3.75 (s, 3H, 3H, CH=CHCO₂Me), 6.55 (d, *J* = 15.8 Hz, 1H, CH=CHCO₂Me), 7.16 (d, *J* = 3.6 Hz, 1H, CH furanyl), 7.47 (d, *J* = 6.3 Hz, 1H, CH furanyl), 7.53 (d, *J* = 15.8 Hz, 1H, CH=CHCO₂Me), 8.11 (d, *J* = 8.9 Hz, 1H, aromatic), 8.30 (d, *J* = 8.8 Hz, 1H, aromatic); ¹³C-NMR (75 MHz, DMSO- 52.2, *d*₆) δ 52.5, 113.5, 116.7, 117.0, 119.2, 119.5, 124.8, 125.3, 125.4, 126.0, 131.2, 131.4, 135.6, 152.3, 167.1; MS(ESI) *m/z*: 274.1 [M+H]⁺; R_f (Hexanes/EtOAc (4:1)) = 0.83.

(E)-methyl 3-(5-(3-nitrophenyl)furan-2-yl)acrylate 11b—The same procedure as before was employed with the aldehyde **10b** obtaining a yellow solid (83%).

¹H-NMR (300 MHz, DMSO-*d*₆) δ 3.74 (s, 3H, CH=CHCO₂Me), 6.54 (d, *J* = 15.8 Hz, 1H, CH=CHCO₂Me), 7.14 (d, *J* = 3.5 Hz, 1H, CH furanyl), 7.45 (d, *J* = 3.6 Hz, 1H, CH=CHCO₂Me), 7.52 (d, *J* = 15.8 Hz, 1H, CH furanyl), 7.76 (t, *J* = 8.0 Hz, 1H, aromatic), 8.19 (dd, *J*₁ = 8.1 Hz *J*₂ = 1.5 Hz, 1H, aromatic), 8.30 (dd, *J*₁ = 8.1 Hz *J*₂ = 1.6 Hz, 1H, aromatic), 8.60 (t, *J* = 1.6 Hz, 1H, aromatic); ¹³C-NMR (75 MHz, DMSO-*d*₆) δ 52.1, 52.4, 111.8, 116.2, 119.0, 119.3, 123.2, 123.8, 130.6, 131.1, 131.2, 131.4, 131.6, 149.2, 151.5, 164.6, 167.2; MS (ESI) *m/z*: 274.2 [M+H]⁺; R_f (Hexanes/EtOAc (4:1)) = 0.83.

(E)-3-(5-(4-nitrophenyl)furan-2-yl)acrylic acid 12a—To solution of compound **11a** (0.4 g, 1.5 mmol) in MeOH (10 mL) at 0 °C was added dropwise 2N NaOH (10 mL). After the addition the solution was warmed up to r.t. and then it was heated to 80 °C for 2h. After this time the reaction was cooled down it was acidified to pH 6 after dilution with water (20 mL). The methanol was removed under reduced pressure and the product was extracted with EtOAc (3 × 20 mL). The organic phase was dried over Na₂SO₄, filtered and the solvent was removed under reduced pressure. Purification by silica gel column chromatography, with hexanes/EtOAc (4:1) as eluent, afforded 0.345 g of a pale orange solid (91%).

¹H-NMR (300 MHz, DMSO-*d*₆) δ 6.46 (d, *J* = 15.7 Hz, 1H, CH=CHCO₂H), 7.13 (d, *J* = 3.6 Hz, 1H, CH furanyl), 7.44 (d, *J* = 15.6 Hz, 1H, CH=CHCO₂H), 7.46 (d, *J* = 3.6 Hz, 1H, CH furanyl), 8.10 (d, *J* = 8.9 Hz, 1H, aromatic), 8.30 (d, *J* = 9.0 Hz, 1H, aromatic); ¹³C-NMR (75 MHz, DMSO-*d*₆) δ 113.5, 118.3, 118.5, 118.8, 124.8, 125.2, 125.4, 125.5, 130.8, 131.7, 152.5, 168.0; MS(ESI) *m/z*: 260.1 [M+H]⁺; R_f (Hexanes/EtOAc (1:1)) = 0.46.

(E)-3-(5-(3-nitrophenyl)furan-2-yl)acrylic acid 12b—The same procedure as before was employed with the compound **11b** obtaining a yellow solid (93%).

¹H-NMR (300 MHz, DMSO-*d*₆) δ 6.45 (d, *J* = 15.8 Hz, 1H, CH=CHCO₂H), 7.10 (d, *J* = 3.6 Hz, 1H, CH furanyl), 7.43 (d, *J* = 3.7 Hz, 1H, CH furanyl), 7.45 (d, *J* = 15.7 Hz, 1H, CH=CHCO₂H), 7.76 (t, *J* = 8.0 Hz, 1H, Arom), 8.19 (dd, *J*₁ = 1.3 Hz, *J*₂ = 8.4 Hz, 1H, Arom), 8.30 (dd, *J*₁ = 1.3 Hz, *J*₂ = 8.3 Hz, 1H, Arom), 8.58 (t, *J* = 1.4 Hz, 1H, Arom); ¹³C-NMR (75 MHz, DMSO-*d*₆) δ 111.8, 117.8, 118.1, 118.3, 118.8, 119.3, 123.1, 123.7, 130.5, 131.0, 131.2, 131.5, 151.7, 153.3, 168.0; MS(ESI) *m/z*: 260.1 [M+H]⁺; R_f (Hexanes/EtOAc (1:1)) = 0.45.

Compound 4a—To a solution of the carboxylic acid **12a** (0.133 g, 0.51 mmol) in a mixture of DCM:DMF (4:1) anhydrous (5 mL) in an ice bath, WSC (0.108 g, 0.56 mmol) was added already dissolved in the same previous solution (5 mL). After 30 min of agitation the 4-amino TEMPO (0.205 g, 1.02 mmol) was added and the reaction was allowed to reach room

temperature and to continue for 16h. After this time the solution was diluted with DCM (20 mL) and washed with 0.1 N HCl (3 × 20 mL), H₂O (3 × 20 mL) and brine. The organic phase was dried over Na₂SO₄ and filtered. The solvent was then removed under reduced pressure to afford 0.068 g of an orange solid. HRMS (ESI-TOF) *m/z*: calcd for [M+H]⁺ 413.1945, found 413.1941; Anal. (C₂₂H₂₆N₃O₅) H, calc 6.35, found 6.61; C, calc 64.06, found 63.67.

Compound 4b—The same procedure as before was employed with the carboxylic acid **12b** obtaining a yellow solid (27%). HRMS (ESI-TOF) *m/z*: calcd for [M+H]⁺ 413.1945, found 413.1943; Anal. (C₂₂H₂₆N₃O₅) H, calc 6.35, found 6.41; N, calc 10.19, found 9.94.

Expression and purification of recombinant YopH N- and C-terminal domains

Soluble recombinant uniformly ¹⁵N-labeled YopH is expressed in *E. coli* (BL21-DE3) as GST-fusion in minimal-media (with 0.5g of ¹⁵NH₄Cl per liter as sole source of nitrogen). The GST-YopH was purified from clarified lysates using glutathione-Sepharose beads (GE Healthcare) according to manufacturer's recommendations. The eluted GST-YopH was dialyzed against 5L of dialysis buffer (30 mM TRIS, 150 mM NaCl) and a second time in dialysis buffer with 1mM DTT. In the case of the C-terminal domain of YopH, after the first dialysis the protein was subject to thrombin cleavage (Thrombin Cleancleave KIT, Sigma-Aldrich, St Louis, MO) for 16h at 4°C following manufacturer's recommendations. In addition to the thrombin cleavage site between GST and YopH, a second cleavage site is found between the N- and the C-terminal domains (**sequence 126GARGHV131**). The three components (GST, YopH(1–128) and YopH(129–468)) were separated by a second glutathione-Sepharose purification (to eliminate GST). Pure YopH N- and C-terminal domains were further isolated by passing the elutes through a Sephacryl S-100 gel-filtration column pre-equilibrated with the dialysis buffer. The protein sequences of both domains were further confirmed by tryptic digestion followed by MALDI-TOF analysis.

Enzyme inhibition assay

The phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate (*p*NPP; Sigma-Aldrich, St Louis, MO) in the presence of compounds in DMSO (5% final concentration) was assayed at 30°C in 0.15 M Bis-Tris buffer, pH 6.0, having an ionic strength of 150 mM (adjusted with NaCl) and containing 1.0 mM dithiothreitol in a 100-μl 96-well plate format. YopH (expressed and purified as reported before) [8] was at 10 nM, PTP1B (BIOMOL International, L.P., Plymouth Meeting, PA) at 25 nM, HePTP (gift of Dr. Rebecca Page, Brown University) at 75 nM, TCPTP (BIOMOL International, L.P., Plymouth Meeting, PA) at 12 nM, CD45 (BIOMOL International, L.P., Plymouth Meeting, PA) at 5 nM, and VHR (expressed and purified as reported before) [18] at 50 nM. Compound concentrations were 0.0128, 0.064, 0.32, 1.6, 8, 40, 200, and 1000 μM. After preincubation of enzyme and compound for 10 min at room temperature, *p*NPP was added and the reaction mixture was incubated for 20 min at 30°C. The initial reaction rate at a fixed *p*NPP concentration, which was equal to the corresponding *K_m* value of 1 mM for YopH, PTP1B, TCPTP, and CD45, 2.5 mM for HePTP, and 1.5 mM for VHR was determined using a ELx808 micro plate reader (Bio-Tek Instruments, Inc., Winooski, VT), measuring absorption of the cleavage product *p*-nitrophenol at 405 nm after quenching the reaction with 100 μl 1 M NaOH. Nonenzymatic hydrolysis of the substrate was corrected by measuring the control without enzyme. IC₅₀ values were determined by plotting the relative activity versus inhibitor concentration using Prism® (GraphPad Software, San Diego, CA) and fitting to the equation $V_i/V_0 = IC_{50}/(IC_{50} + [I])$, where *V_i* was reaction velocity at inhibitor concentration [I], *V₀* is the reaction velocity without inhibitor and $IC_{50} = K_i + K_i[S]/K_m$, where *K_i* is the dissociation constant for binding of inhibitor to enzyme, [S] is the substrate concentration and *K_m* is the Michaelis-Menten constant.

NMR spectroscopy

NMR spectra were acquired on a 500 MHz or 600 MHz Bruker Avance spectrometer equipped with a Bruker TXI probe (500) or a TCI-Cryoprobe (600). All 1D ^1H experiments were carried out with samples containing unlabeled GST-YopH at a concentration of 10 μM . 1D $T_{1\rho}$ experiments were measured with a spin-lock time of 100 ms, a recycle delay of 1.5 sec, and water suppression based on the Watergate sequence [19]. A library of 200 compounds (1 mM) was tested in presence of compound **3** (500 μM). Compounds were tested in mixtures of 10 compounds and hits further deconvoluted aided by the 1D NMR spectra of the individual compounds of the library (Maybridge). For the 2D [^1H - ^{15}N]-HSQC experiments in Figure 4, samples of 280 μM of YopH-C were used to acquire the spectra in the absence and in presence of compound **3** (500 μM).

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Abbreviations

PTP	phosphotyrosine phosphatase
SAR	structure-activity relationship
ILOE	inter-ligand Overhouser effect
TEMPO	2,2,6,6-tetramethylpiperidine 1-oxyl
WSC	water soluble carbodiimide

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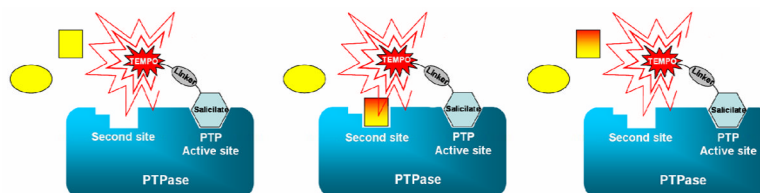
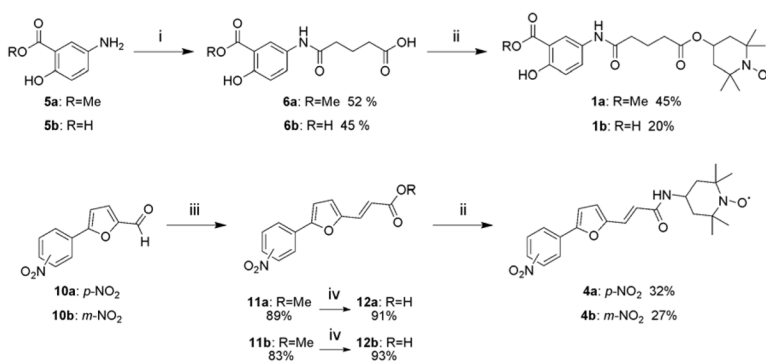
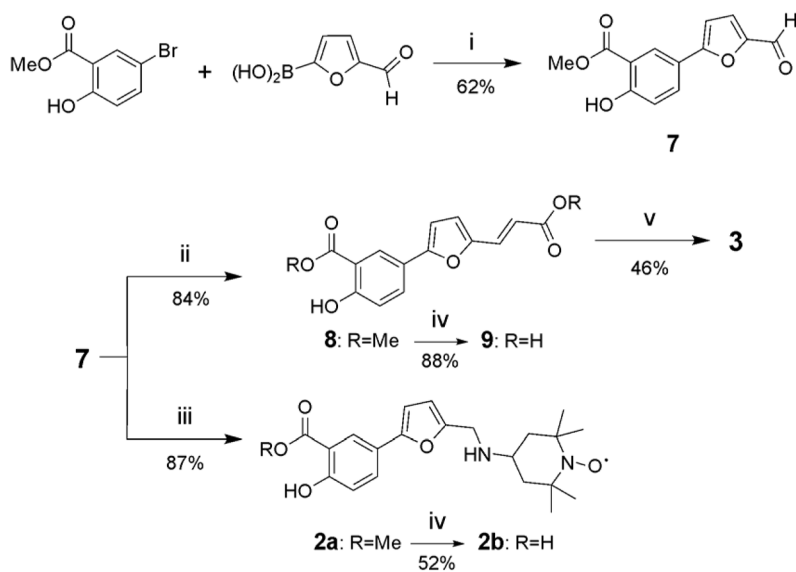


Figure 1. Schematic representation of the second-site screening approach proposed to design potent and selective PTPase inhibitors. The approach is based on the hypothesis that a pharmacophore for potent and selective PTP inhibitors would consist of a salicyl-furanyl moiety, as phospho-Tyrosine mimicking scaffold, connected to a second site binder whose nature would be different for various PTP targets.

**Figure 2.**

Synthetic scheme for compounds **1a–b** and **4a–b**: i) Glutaric anhydride (1 eq.), **5a–b** (2 eq.) in Pyridine/DCM (1:1) under N₂, r.t. 16h; ii) 4-amino-TEMPO (2 eq.), WSC (1.1 eq.) in DCM/DMF (9:1); iii) **10a–b** (1 eq.), methyl diethyl phosphonoacetate (1.1 eq.), LiOH (1 eq.) in THF under Ar; iv) MeOH/NaOH 2M (1:1) at 80 °C, 2h.

**Figure 3.**

Synthetic scheme for compounds **2a–b** and **3**: i) 5-Bromo-2-hydroxy-benzoic acid methyl ester (1 eq.), 5-formylfuran-2-ylboronic acid (1.2 eq.), Pd(OAc)₂ (3 mol%), K₂CO₃ (2.5 eq.), Bu₄NCl (1 eq.) in H₂O/MeOH (1:1); ii) methyl diethyl phosphonoacetate (1.1 eq.), LiOH (1 eq.) in THF under Ar; iii) 4-amino-TEMPO (1 eq.) in toluene with 5% AcOH o.n., then NaBH₃CN (1.1 eq.) in MeOH with 0.5% HCl; iv) MeOH/NaOH 2M (1:1) at 80 °C (**9**) for 2h or r.t. (**2a**) for 16h; v) 4-amino-TEMPO (2 eq.), WSC (1.1 eq.) in DCM/DMF (9:1).

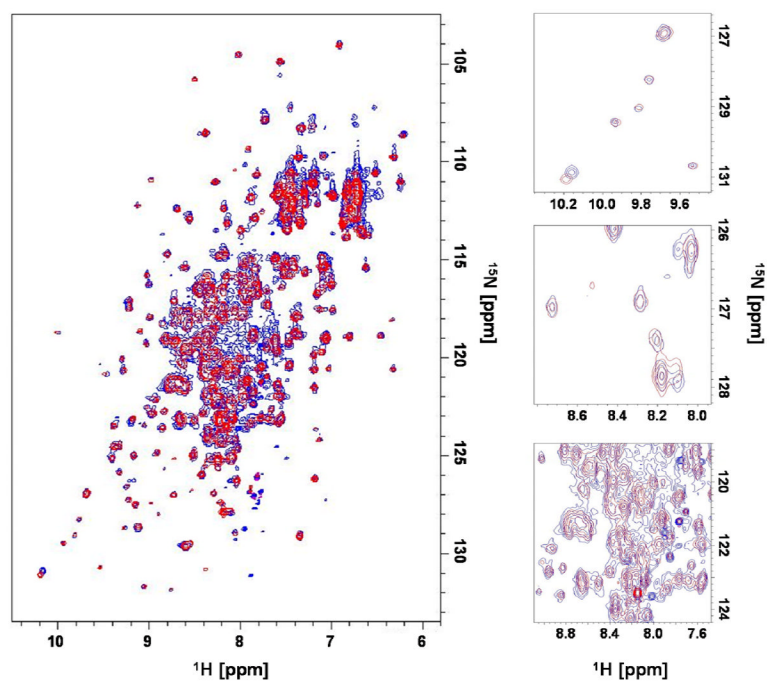


Figure 4. Effect of compound **3** on the [^{15}N , ^1H] correlation spectra of the C-terminal domain of YopH. The spectra were recorded in absence (**blue**) or in presence (**red**) of such compound. The expanded 2D regions (**right**) show disappearance or movement of some protein signals as consequence of ligand binding.

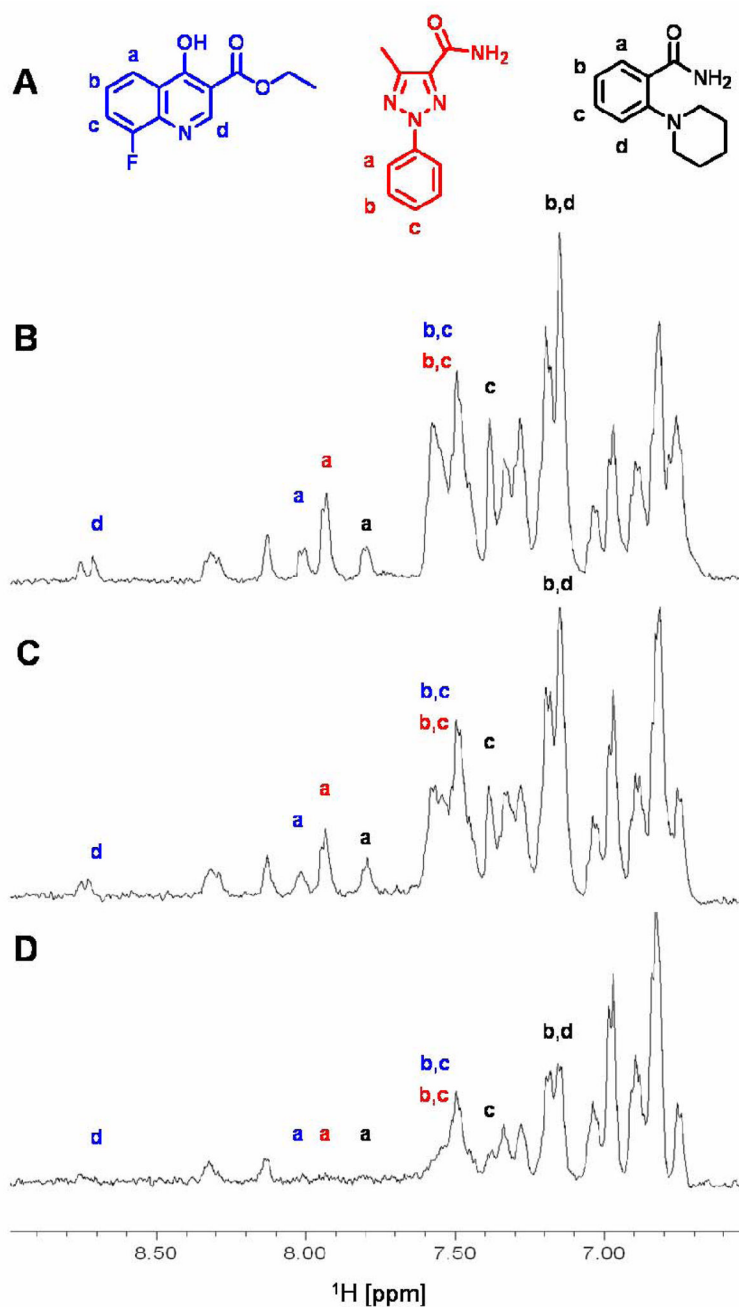


Figure 5. Example of second site binders (A) discovered by using compound **3** as spin-labeled probe. The aromatic region of 1D $T_{1\rho}$ spectra of a mixture of 10 compounds (1 mM each) are reported: B) in the presence of 500 μM of compound **3**; C) in the presence of 10 μM of GST-YopH; and, D) in the presence of both 500 μM of compound **3** and 10 μM of GST-YopH. Letters indicate resonances from the compounds shown in panel A in corresponding colors. The additional peak shown around 8.7 ppm comes from an analogue of the quinoline compound (blue in panel A) present in the mixture.

Table 1
Chemical structures and measured IC₅₀ values (μM) for the spin-labeled probes against different PTPs.

Compound	YopH	PTPIB	HePTP	TCPTP	CD45	VHR
	336	>1,000	>1,000	>1,000	>1,000	~1,000
	>1,000	>1,000	>1,000	>1,000	>1,000	~850
	50	>1,000	>1,000	685	>1,000	241
	3.0	7.5	24	14	14	12
	47	>1,000	>1,000	>1,000	>1,000	~1,000
	46	211	~1,000	260	>1,000	~200