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HTS by NMR guided identification of novel agents targeting the protein docking domain of YopH

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Abstract

Recently we described a novel approach, named HTS by NMR that allows the identification, from large combinatorial peptide libraries, of potent and selective peptide mimetics against a given target. Here we deployed the HTS by NMR approach for the design of novel peptoid sequences targeting the amino terminal domain of the *Yersinia* outer protein H (YopH-NT). We aimed at disrupting the protein-protein interactions between YopH-NT and its cellular substrates, with the goal of inhibiting indirectly YopH enzymatic function. These studies resulted in a novel agent of sequence Ac-F-pY-cPG-D-P-NH_2 (pY = phosphotyrosine; cPG = cyclopentyl glycine) with a Kd value against YopH-NT of 310 nM. We demonstrated that such pharmacological inhibitor of YopH-NT resulted in the inhibition of the dephosphorylation of a cellular substrate by full length YopH. Hence, potentially this agent represents a valuable stepping stone for the development of novel therapeutics against *Yersinia* infections. The data reported further demonstrate the utility of the HTS by NMR approach in deriving novel peptide-mimetics targeting protein-protein interactions.

Keywords

YopH; YopH amino-terminal domain; YopH-NT; combinatorial peptide library; protein-protein interactions inhibitor; HTS by NMR; positional scanning

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Conflict of interest

The authors declare no financial /commercial conflicts of interest.

Introduction

Contrary to conventional biochemical high throughput screening campaigns (HTS), which often result in the identification of several and deceiving false positive compounds,[1] fragment library screens have become popular in ligand design as an alternative and perhaps more effective method in drug discovery. [2] Nuclear Magnetic Resonance Spectroscopy (NMR) in solution provides a variety of techniques that collectively are, based on our experience, the most powerful, sensitive and robust approaches to detect and characterize ligand binding. [2k-p] However, ligand screening using NMR spectroscopy has remained of a relatively low throughput. To tackle this problem, we have recently developed a technique called HTS by NMR,[2k, 2o, 3] where combinatorial chemistry principles were applied to screen large compound collections.[4] In essence, in this approach a peptide library is arranged into mixtures where each position of a common backbone is fixed while the other positions contain all building blocks (natural and non-natural amino acids in this case); this array is called “positional scanning”.[5] In this study, we selected 46 natural and non-natural amino acids which were arranged into tripeptoids. The positional scanning mixtures were arranged by fixing one amino acid at one position while all possible combinations of the 46 building blocks sat on the remaining 2 positions. For example, a mixture Ac-Ala-XX-NH_2 represents all tri-peptoids that started with Alanine at position 1 into one mixture, where X stands for all 46 amino acids. Hence, we obtained systematically mixtures for each of the 46 amino acids at position 1, position 2, and position 3. This grouping method allowed the synthesis and subsequent screening of a large number of tri-peptoids by NMR with a manageable effort, given that only 46+46+46=138 mixtures (each containing in theory 2,116 compounds) were synthesized and tested, representing nearly 100,000 (46×46×46=97,336) possible tri-peptoids. Previously we reported on the successful application of the HTS by NMR in the *de novo* discovery of ligands to the EphA4 ligand binding domain.[3] Here we deployed the HTS by NMR to target a bacterial toxin essential for the virulence of *Yersinia pestis,* namely the phosphatase YopH.

The plague-causing pathogen, *Yersinia pestis*, is a Gram-negative bacterium that utilizes a type III secretion system (TTSS) to translocate effectors into the infected cells which help evading the host immune response.[6] Current treatments include aminoglycoside antibiotics such as streptomycin and gentamicin (http://www.cdc.org), however these are only effective when administered early in the course of the disease. Moreover, concerns about the emergence of antibiotic resistant strains for *Yersinia pestis* have prompted the search for alternative *druggable* targets to fight this pathogen.

One of the *Yersinia outer proteins* (Yop) effectors, called *Yersinia* outer protein H (YopH), has long been thought as a potential drug target to combat *Yersinia* infections because bacteria carrying deletions of YopH are avirulent.[7] YopH is a potent protein tyrosine phosphatase (PTPase), which dephosphorylates components of key signal transduction pathways in the host immune cells, resulting in suppression of innate immunity [8] and later rendering the adaptive immunity null.[9]

YopH consists of 468 amino acids, comprising structurally distinct N-terminal and C-terminal domains (here called NT and CT respectively). The structure of YopH-NT (residues

1 to 129) has been solved by both X-ray crystallography and solution NMR and was determined to be monomeric at physiological pH.[10] The first 70 amino acid of YopH-NT are essential for its secretion and translocation into the infected cells. [11] Once intracellular, YopH-NT also mediates docking to protein targets by recognizing the consensus sequence pYxxP, where pY represents a phosphorylated tyrosine. [10] It has been proposed that this protein-protein interaction of YopH-NT helps determine the enzyme substrate specificity and hence it is essential for its enzymatic activity. [12] The YopH-CT (residues 206 to 468) contains the phosphatase catalytic site, including the catalytic residue Cysteine 403. A Cys403Ala YopH mutant could not hydrolyze phosphotyrosine but retained its ability to bind to substrates, which has been useful in the identification of intracellular substrates of YopH.[12]

Several intracellular substrates of YopH have been identified in different cell types. In T cells, YopH dephosphorylates the kinase Lck (Lymphocyte-specific protein tyrosine kinase) at the positive regulatory site tyrosine 394 and blocks T cell antigen receptor signaling. [13] Lck belongs to Src-family of kinases and is responsible for the initiation of the T cell receptor activation pathway. [14] In macrophages YopH disrupts the activation of focal adhesion complexes, which are crucial for phagocytosis. In activated macrophages, two adhesion-associated scaffold proteins, Fyb (Fyn-binding protein) and SKAP-HOM (Src kinase-associated phosphoprotein of 55 kD- homologue) have been identified as YopH substrates.[15] SKAP-HOM is a Fyn-associated adaptor protein and only becomes phosphorylated upon T cell activation and/or macrophage adhesion.[15-16] From sequence analysis, YopH likely dephosphorylates SKAP-HOM at tyrosine 251, belonging to a consensus sequence for Src family kinase phosphorylation Y^{251}EEIP.[15] In solution, the structure of YopH-NT in complex with a SKAP-HOM derived peptide Ac-DEpYDDPF-NH_2 (compound **1, Table 1**; Kd = 180 nM) was reported, in which the negatively charged peptide interacted with YopH-NT at a largely positive charged surface near the first α helix and β-sheet. [10]

Hence, on these premises, we sought to explore the possibility of targeting the N-terminal docking domain of YopH, given that it has been proposed to be an essential domain to recruit Lck and other YopH substrates. Because targeting YopH-NT consists of targeting a protein-protein interface, we decided to test whether our recently reported HTS by NMR approach could lead to novel and potent antagonists. We report that the HTS by NMR primary screen resulted in compound **2** of micromolar affinity for YopH-NT. Subsequent synthesis of additional derivatives of compound **2** identified a compound (compound **14**) with nanomolar affinity and improved drug-like character (smaller molecular weight, only two negative charges, composed by non-natural amino-acid) compared to the known consensus binding sequence for YopH-NT (compound **1**). This example illustrates the usefulness of the HTS by NMR as a rapid and robust approach to derive novel and effective peptide-mimetics as antagonists to protein-protein interactions. Moreover, the identified compound **14** could be potentially translated into possible therapeutics targeting *Yersiniae*.

Methods and Materials

Protein expression and purification

A plasmid encoding the first 129 amino acids of YopH (YopH-NT) and with a carboxy terminal His tag was used for these studies. ^{15}N labeled YopH-NT was expressed as previously described.[17] In short, transformed BL21(DE3)pLysS competent cells (Promega catalog # L1195) were grown in 10 ml LB culture with appropriate antibiotic overnight at 37 °C shaking. The cells were transferred to 1 L M9 minimal media with $^{15}NH_4Cl$ (1g / 1L) supplement as sole nitrogen source and were allowed to grow to OD_{600} ~ 0.6 at 30 °C under shaking conditions. The culture was then induced with 1 mM of IPTG and left overnight shaking at 20 °C. Bacteria were collected and lysed at 4 °C. YopH-NT was purified with a nickel column using and dialyzed in a buffer consisting of 30 mM Tris, 150 mM NaCl, pH=8.

A plasmid encoding the full length YopH with an amino terminal GST tag was used for the enzyme activity and inhibition studies. The protein was expressed as previously described.[17] In short, transformed BL21(DE3)pLysS competent cells (Promega catalog # L1195) were grown in 10 ml LB culture with appropriate antibiotic overnight at 37 °C shaking. The cells were transferred to 1 L LB media and allowed to grow to OD_{600} ~ 0.6 at 30 °C shaking. The culture was then induced with 1 mM of IPTG and left overnight at 20 °C under shaking conditions. Bacteria were collected and lysed at 4°C. Full lenght GST-YopH was subsequently purified and dialyzed in the final buffer consisting of 30 mM Tris, 150 mM NaCl, 1 mM DTT, pH = 6.7.

HTS by NMR

A tri-peptide library was assembled using forty-six natural and non-natural amino acids as building blocks with all possible combinations (Pepscan Presto BV, Lelystad, The Netherlands). The forty-six amino acids presented a total of 97,336 combinations (46 × 46 × 46) and the peptide library was arranged into mixtures. Each mixture contained in theory 2,116 (1 × 46 × 46) tri-peptoids with one amino acid fixed at one certain position. Stock solutions where prepared for each mixture to reach a theoretical final concentration of 100 mM in d_6-DMSO, for the fixed position element. NMR spectra were acquired on 600 MHz Bruker Avance spectrometer equipped with a TCI cryoprobe and z-shielded gradient coils. All NMR data were processed and analyzed using TOPSPIN 2.1 (Bruker Biospin Corp., Billerica, MA, USA). In general, 2D [^{15}N, ^{1}H] HSQC experiments were acquired using 32 scans with 2048 and 128 complex data points in the ^{1}H and ^{15}N dimensions at 300 K. Compound binding was detected at 27 °C by comparing the 2D [^{15}N, ^{1}H] HSQC spectra of 10 μM YopH-NT in the absence and presence of compounds at molar ratio 50/1 (total compound mixture / YopH-NT). Chemical shift perturbations of the backbone amide cross-peak relative to F55 was used to calculate chemical shift perturbations to rank order the mixtures as reported in **Figure 1**. For the screen, a concentration of 10 μM is used to minimize proein consumption and to increase the sensitivity of the assay (higher ligand/ protein ratio). For subsequent Kd determinations and chemical shift mapping studies with individual hit compounds, a protein concentration of 50 μM was used (see below).

K_d values determinations by NMR

Dissociation constants (K_d) determinations were performed by comparing the 2D [^{15}N, ^{1}H] HSQC spectra of YopH-NT (50 μM) collected in the absence and in presence of different concentrations of test compounds (20, 40, 60, 100, 150, 200, and 250 μM) and by monitoring the backbone amide cross-peak of residue A32. The weight averaged chemical shift changes in both ^{1}H and ^{15}N dimensions were calculated using the following equation as previously reported: [2m, 18]

$$\Delta\delta = \sqrt{(\Delta^1 H)^2 + (0.17 \quad * \Delta^{15} N)^2}$$

Dissociation equilibrium constants (K_d) of compounds against YopH-NT were determined by monitoring the averaged chemical shift perturbations as function of compound concentration. Titration analysis was done by fitting chemical shift data into the following equation:[19]

$$\Delta\delta_{obs} = \Delta\delta_{max} \frac{(K_d + [L]_0 + [P]_0) - \sqrt{(K_d + [L]_0 + [P]_0)^2 - 4[P]_0[L]_0}}{2[P]_0}$$

where $\Delta\delta_{obs}$ represents the observed chemical shift perturbation value at each titration point; $\Delta\delta^{max}$ is the maximum chemical shift perturbation value of the fully complexed protein; $[L]_0$ and $[P]_0$ are the total concentrations of compound and protein.

Solid phase peptide synthesis

All reported peptides and peptide-mimetics were acetylated at the N-terminus and amidated at C-terminus. Compounds **1**, **3**, **13**, **14** and **15** were purchased from Innopep (San Diego, CA, USA) while compounds **2**, **4** to **12** were synthesized in house. Unless otherwise indicated, all anhydrous solvents were stored in sure-seal bottles under nitrogen. All other reagents and solvents were purchased as the highest grade available and used without further purification. Thin-layer chromatography (TLC) analysis of reaction mixtures was performed using Merck silica gel 60 F254 TLC plates, and visualized using ultraviolet light. NMR spectra were recorded on Joel 400 MHz instrument. Chemical shifts (δ) were reported in parts per million (ppm) referenced to ^{1}H (Me_4Si at 0.00). Coupling constants (*J*) were reported in Hz throughout. Mass spectral data were acquired on Shimadzu LCMS-2010EV for low resolution, and on an Agilent ESI-TOF for either high or low resolution. Purity of all compounds was obtained in a HPLC Breeze from Waters Co. using an Atlantis T3 3μm 4.6×150 mm reverse phase column. The eluant was a linear gradient with a flow rate of 1 ml/min from 95% A and 5% B to 5% A and 95% B in 15 min followed by 5 min at 100% B (Solvent A: H_2O with 0.1% TFA; Solvent B: acetonitrile with 0.1% TFA). The compounds were detected at λ=254 nm or 220 nm.

Compounds **2**, **4** to 1**2** were synthesized using standard Fmoc peptide synthesis protocol with the Rink amide resin (**supplementary scheme 1**). For each coupling reaction (for 0.1 mmol scale), 4 equivalents of Fmoc-amino acid, 6 equivalents of coupling agents (Oxyma

Pure and DIC), 6 equiv of DIEA in DMF (5 mL) were used. The coupling reaction was allowed to proceed for 2 hours. Fmoc deprotection was performed by treating the resin-bound peptide with 20% piperidine in DMF (4 mL DMF, 1 mL piperidine) for 30 min at room temperature twice. After each coupling reaction or Fmoc deprotection, the resin bound peptide was washed with DMF (5 mL, 6 times) and DCM (5 mL, 6 times) respectively. For phosphotyrosine, mono benzyl protected phosphotyrosine (Chem-impex) was used and its protecting group was removed during cleavage reaction. For acetylation, acetic anhydride (2 equiv.) in the presence of DIEA (3 equiv.) with a catalytic amount of DMAP (5 mol%) in DMF was used and the reagents were allowed to carry out for 2 hours. The peptide was cleaved from the resin by treating the resin with a cleavage cocktail containing 94% TFA, 2 % water, 2% triisopropylsilane, 2% phenol for 3 hours. All protecting groups were also removed during this cleavage reaction. TFA was removed under reduced pressure and the peptide was precipitated in diethyl ether, centrifuged and washed with diethyl ether prior to drying in high vacuum. The crude peptide was purified by preparative reverse phase HPLC. The final compounds were characterized by NMR and MALDI-Mass spectrometry. All individual compounds were purified at >95% level of purity.

The positional scanning library was obtained by PepScan (Lelystad, The Netherlands) using solid phase synthesis as described above, using 46 natural and non-natural amino-acids arranged in tri-peptoids. After the final de-protection step, each mixture was freeze-dried 3 times to eliminate excess of TFA.

Western blot analyses

Human recombinant Lck was purchased from Life Technologies (Carlsbad, CA; Catalog number: P3043). DMSO or compound **14** (at the indicated concentrations in **Figure 4B**) were first pre-incubated with 15 nM full length GST-YopH at room temperature for 10 min. Then a solution containing recombinant Lck was added to the reaction to reach a final concentration of 300 nM of protein, and incubated for another 20 min at room temperature. To stop the reaction, 4X LDS (with 1% β-mercaptoethanol) was added. In a polycrylamide gel, 7 μl of each reaction was loaded in a single well. Gels were run and transferred in accordance with manufacture's protocol (NuPage). Membranes were blocked for 1 hour in TBST + 5% BSA at room temperature rocking and then incubated with primary antibody overnight at 4°C. Primary antibodies used were phospho-Src family (Y416) antibody 1:1000 (Cell Signaling #2101) for phospho-Lck, and LCK(3A5) antibody 1:1000 (Santa Cruz catalog # sc-433) for total Lck. The membranes were washed with TBST for 10 min at room temperature rocking for four times. Then secondary antibodies were used at 1:10,000 dilutions and incubated at room temperature for 1 hour rocking. Chemo-luminescence substrates (Thermo Fisher catalog #34079) were added and incubated for 15 min room temperature and then expose to film to develop.

Isothermal titration calorimetry (ITC)

ITC measurements were performed using ITC200 calorimeter from Microcal (Northampton, MA) at 23 °C. Aliquots of 2.0 μl of solutions containing about 0.4 mM test compounds were injected into the cell containing a solution of 30 μM YopH-NT. A total of 19 injections were

made. The experiments were performed in buffer containing 30 mM Tris, 150 mM NaCl at pH = 8.4. ITC data were analyzed using the Origin software provided by Microcal.

Results

A combinatorial library was assembled using 46 natural and non-natural amino acids, formatted in tri-peptoids. Each of the three positions had 46 possibilities, resulting in a total of 97,336 possible combinations (46 × 46 × 46 = 97,336). To make the synthesis and subsequent NMR manageable, the peptoids were grouped into mixtures where, for each mixture, position 1, 2, or 3 contained a fixed amino acid while the other two positions comprised any of the possible 46 amino acids (1 × 46 × 46 combinations for each mixture) (**Figure 1A**). This arrangement eventually shaped the combinatorial peptide library into 138 mixtures (46 amino acids × 3 positions), which rendered the syntheses and the subsequent screen by NMR of the nearly 100,000 peptide mimetics possible, as we recently reported.[2k, 2o, 3]

Therefore, we screened a total 138 mixtures of tri-peptoids (46 × 3 = 138) in 2D [^{15}N, ^{1}H] HSQC experiments monitoring chemical shift perturbations induced by each mixture. In particular we chose to rank order the mixtures by monitoring the chemical shifts of the backbone ^{15}N, $^{1}H^{N}$ of YopH-NT residue F55 (**Figure 1B**). We observed that, at all 3 positions, phosphotyrosine (pY) (corresponding to amino acid number 19 in **Figure 1C**) was identified to be the most potent binder, especially in mixture 1 (**Figure 1C**). For position 1, only the mixture containing tri-peptoids Ac-pYXX-NH_2 (where X represents all 46 building blocks) caused a chemical shift perturbation larger than 0.5 ppm (**Figure 1C**). Interestingly, for position 2, we identified cyclopentyl glycine (cPG) (mixture 11 of composition Ac-X(cPG)X-NH_2) as a possible hit, while at position 3, natural aspartic acid (D), tryptophan (W) and serine (S) were also discovered (mixture 39, Ac-XXD-NH_2; mixture 38, Ac-XXW-NH_2; mixture 46 Ac-XXSNH_2), each producing significant chemical shift perturbations (**Figure 1C**). Therefore, from these observations, we deduced an initial possible consensus of sequence Ac-pY-cPG-D-NH_2 (compound **2**; **Table 1**). Hence, compound **2** was synthesized and tested by means of NMR spectroscopy titrations and Isothermal Titration Calorimetry (**Table 1**). The Kd value of compound **2** was determined by NMR to be 17.3 μM monitoring chemical shifts of backbone amide of residue A32 (**Figure 2A and 2B**), in relatively good agreement with the ITC value of 6.6 μM (**Figure 2C**, **Table 1**). The backbone amide cross-peak for residue A32 was chosen for the NMR titrations as it had been previously used to calculate the Kd value for reference compound **1** to YopH-NT.[17] In agreement with previous observations, we found that compound **1** bound to YopH-NT in slow exchange in the NMR timescale, making a Kd determination by NMR not accurate. However, the Kd of compound **1** for YopH-NT could be determined by ITC and was found to be about 180 nM (**Figure 3A**; **Table 1**). Surprisingly, and in agreement with our HTS by NMR screening data, replacing cPG in place of D at position 2 of compound **1** (**Table 1**) resulted in an equally potent compound **3**, which also displayed slow exchange in the 2D-[^{15}N, ^{1}H]-HSQC spectra of YopH-NT and showed a Kd value of 180 nM as determined by ITC (**Figure 3B, Table 1**). Comparison of the chemical shift perturbations caused by binding of compounds **1** and **3** suggested that two molecules bound to YopH-NT in a similar

fashion, affecting mostly the same amino acids (**Figure 3C, D**). Only relatively small differential chemical shift perturbations are noted, again suggesting that both peptides bound to YopH-NT in a similar fashion.

To further optimize the binding of compound **2**, we designed additional tetra-peptoids introducing a fourth amino acid at either its C- or N- amino termini (position 0 and position 4, respectively, in **Table 1**). We chose isoleucine (I) to represent an aliphatic amino acid and phenylalanine (F) to represent aromatic amino acid respectively, while lysine (K) and aspartic acid (D) were used to represent positively and negatively charged amino acids (**Table 1**). Proline (P) was also included at position 4 considering that the YopH-NT reportedly bound sequences of consensus pYxxP.[12] To assess the binding potency of the synthesized compounds, we used both NMR (following the backbone amide cross-peak of residue A32) and ITC. However, as mentioned above, for compounds that bound to YopH-NT in slow exchange fashion, we could not use NMR to accurately determine their Kd values. Conversely, ITC is not very reliable in measuring dissociation constants of weakly interacting compounds (with Kd in double and triple digit micromolar or weaker), but it is very accurate in quantifying the binding properties of potent binders. Therefore, to enable the comparison of compound potency, the Kd values reported below were determined by ITC, NMR or both depending on the relative potency of the compounds.

Among the C-terminal extensions, compound **8** with P (Ac-pY-cPG-D-P-NH_2) displayed a significantly improved Kd value at 1.4 μM as determined by ITC and exhibited slow exchange behavior in the 2D NMR spectra with YopH-NT. Compound **4** (Ac-pY-cPG-D-I-NH_2) and compound **5** (Ac-pY-cPG-D-F-NH_2) were within close range of the original compound **2**, with Kd values of 4.4 μM and 5.5 μM, respectively. Moreover, compound **7** (Ac-pY-cPG-D-D-NH_2) had Kd value of 6.5 μM, while the compound **6** (Ac-pY-cPG-D-K-NH_2) was the least potent, with a Kd value of 14.2 μM.

Among the additional four compound **2** derivatives extended at the N-terminus, compound **10** (Ac-F-pY-cPG-D-NH_2) was the most potent with a Kd value of 9.3 μM, while compound **9** (Ac-I-pY-cPG-D-NH_2) had the second best Kd value of 10.9 μM. However, surprisingly, compound **12** (Ac-D-pY-cPG-D-NH_2) with an additional D, was less active with a Kd value of 16.2 μM. Lastly, as for the C-terminal, inclusion of a K at the N-terminal of compound **2**, resulted in compound **11** that did not appreciably bind to YopH-NT by ITC (**Table 1**). Hence, if any, F or I at position 0 would be preferred, while a P residue at position 4 is warranted (**Table 1**). Therefore, we synthesized penta-peptoids **13** (Ac-I-pY-cPG-D-P-NH_2) and **14** (Ac-F-pY-cPG-D-P-NH_2) and found that both bound potently to YopH-NT, with Kd values of 520 nM and 310 nM, respectively (**Figure 4A**). Finally, considering that W was also a hit amino acid at position 3, we also tried to further replace the D at position 3 with a residue W to create compound **15**, which bound, however, to YopH-NT with a Kd value of only 3.1 μM (**Table 1**). Hence, in conclusion, the HTS by NMR screen of approximately 100,000 peptide mimetics lead to the discovery of compound **2**, and subsequently to compound **14** with nanomolar affinity for YopH-NT and improved properties compared to the previously known sequence (compound **1, Table 1**).

We subsequently tested if the compound could inhibit dephosphorylation of Lck, a cellular substrate of YopH. Full length GST-YopH was pre-incubated for 10 min with compound **14** at 60 μM, 6 μM and 0.6 μM and then recombinant Lck was added an incubated 20 min at room temperature. The phosphorylation state of Lck on tyrosine 394 (Y394) was detected using a general antibody against phospho-Src family proteins (from Cell Signaling # 2101) as previously reported [20], while the total level of Lck was also detected using an appropriate antibody. From the positive and negative control data, we demonstrated that the recombinant Lck was phosphorylated and after treatment with 15 nM of YopH for 20 min the phosphorylation was dramatically decreased (**Figure 4B**). We also observed that, at 60 μM, compound **14** inhibited the dephosphorylation of Lck while treatment with compounds alone did not affect the phosphorylation state (**Figure 4B**). Finally, compound **14** did not affect the catalytic activity of full length YopH in an enzymatic assay, using a fluorescent DiFMUP, in absence and presence of compound **14** (up to 60 μM) (**Supplementary Figure 2**). However, we observed that compound **14** got slowly hydrolyzed by YopH (**Supplementary Figure 3**) using 1D proton NMR experiments where the ^{1}H resonances of phosphorylated tyrosine were monitored. The resulting non-phosphorylated version of compound **14** did not appreciably bind YopH-NT by NMR.

Discussion

Protein tyrosine phosphatases (PTPases) are important regulators of various signal transduction pathways.[21] Many PTPases are considered interesting therapeutic targets for human diseases. For example, PTP1B has long been targeted for obesity and type II diabetes and lymphoid tyrosine phosphatase (LYP) was implicated as therapeutic candidate for autoimmune diseases.[22] However, PTPases have proven to be very challenging drug targets perhaps due to two main reasons. First, the natural active site substrates bind to PTPases in a phosphotyrosine dependent matter, therefore compound library screening for catalytic site antagonists often yields hits carrying highly negatively charged compounds such as suramin and its derivatives.[23] These highly charged hits often pose problems in terms of cell permeability and other pharmacological properties. Second, in inhibitor design, it is difficult to achieve specificity for a given phosphatase because the active site is shallow and only carries a small portion of the structural determinants of enzyme selectivity for its substrates. In some cases, secondary pockets in vicinity to the catalytic sites have been identified which may be responsible for specificity.[21, 24] We indeed previously reported the design of bi-dentate YopH inhibitors that bound to the active site and at a secondary pocket for higher specificity.[25] Recently, several studies reported the identification of allosteric PTPase inhibitors which hopefully avoid the problems of targeting the catalytic site. So far, allosteric inhibitors have been discovered for PTP1B,[26] LYP[22] and Eya2. [27]

Here we targeted a unique protein-protein interaction feature which is necessary for YopH to recognize its intracellular substrate and fully function. Therefore we hypothesized that by interrupting this specific phosphotyrosine-dependent protein-protein interaction, we could achieve inhibition of YopH physiological functions. Our previous efforts in targeting this substrate binding site involved the design of cyclic-peptides based on known binding sequences, that resulted in low micromolar binding agents (Kd ~15 μM for cyclo-DE(pY)DDPfK where f = d-phenylalanine and ~23 μM for cyclo-DE(pY)LDPfK) as

determined by 2D [^{1}H,^{15}N] HSQC experiments). [17] Hence, we thought that YopH-NT was potentially a suitable candidate for the HTS by NMR approach for deriving new antagonists. We therefore screened a combinatorial peptide library in 2D [^{1}H,^{15}N] HSQC experiments and identified compound **2** (Ac-pY-cPG-DNH$_2$) as a novel YopH-NT binding agent with a Kd value of 17.3 μM determined in the same fashion as our previously reported cyclized peptides. To further optimize this binding sequence, we then designed tetra-peptoids with extensions at both N- and C-termini. This resulted in the rapid design of the penta-peptoids **13** (Ac-I-pY-cPG-D-P-NH$_2$) and **14** (Ac-F-pY-cPG-D-P-NH$_2$), both showing dramatic improvement in binding affinity for YopH-NT with Kd values of 520 nM and 310 nM, respectively.

Furthermore, we chose a well-characterized cellular substrate of YopH, namely the protein kinase Lck, to develop an *in vitro* system to monitor the ability of our molecules to antagonize the dephosphorylation of Lck-Y394 by full length YopH. For YopH to dephosphorylate Lck on Y394, proper protein-protein interactions are required. We detected visible dephosphorylation of Lck from 10 to 30 min after incubation of full length YopH with recombinant full length human Lck (**Supplementary Figure 1**). Here we observed that in presence of compound **14**, the dephosphorylation was inhibited (**Figure 4**). However, taking in consideration that the catalytic site of YopH dephosphorylates phosphotyrosine and our compounds all contains a phosphotyrosine residue, we tested if our agent could inhibit the catalytic activity of YopH. We used a biochemical enzymatic assay using 6,8-difluro-4-methylumbelliferyl phosphate (DiFMUP) as substrate: when DiFMUP was hydrolyzed by full length YopH it became fluorescent. As predicted, compound **14** did not affect the catalytic activity of YopH when using such a small molecule substrate since the amino terminal docking domain was not required to bind to DiFMUP. Indeed we demonstrated that compound **14** did not affect the catalytic activity in an enzymatic assay and DiFMUP fluorescence readings were consistently high in presence of compound **14** (up to 60 μM) (**Supplementary Figure 2**). On the contrary, using 1D proton NMR experiments where the ^{1}H resonances of phosphorylated tyrosine were monitored, we observed that compound **14** got slowly hydrolyzed by YopH (**Supplementary Figure 3**). This probably explains why only a relatively high concentration of compound **14** caused a significant inhibitory effect in the western blot (**Figure 4B**). In the assay system, full length YopH dephosphorylates compound **14**, reducing its actual concentration in the assay. Hence, for increased efficacy, mimetics of compound **14** could be attempted that present non-hydrolysable pY mimetics. Hence, substantial work is therefore still needed to fully exploit the potential of this agent as possible therapeutic, including the identification of nonhydrolysable phospho-isosters, addressing issues of cell-permeability, *in vivo* adsorption, distribution, toxicity, etc. Nonetheless, we are confident that the data presented suggest that compound **14** constitutes a suitable starting point for continued drug development studies.

Conclusions

While YopH continues to prove to be a difficult drug target we demonstrated that targeting the YopH-NT substrate docking site is potentially a viable route to inhibitor design. The HTS by NMR proved again to be a very powerful technique in deriving antagonists of

protein-protein interactions,[2k, 2o, 3] and it allowed the identification, out of ~100,000 possible agents, of a potent a binding sequence with a relatively small effort. These studies resulted in compound **14** with a Kd value of 310 nM. The inhibition of the catalytic activity of full length YopH by this agent suggests that further optimizations of this compound are warranted for the development of potentially new agents against *Yersiniae*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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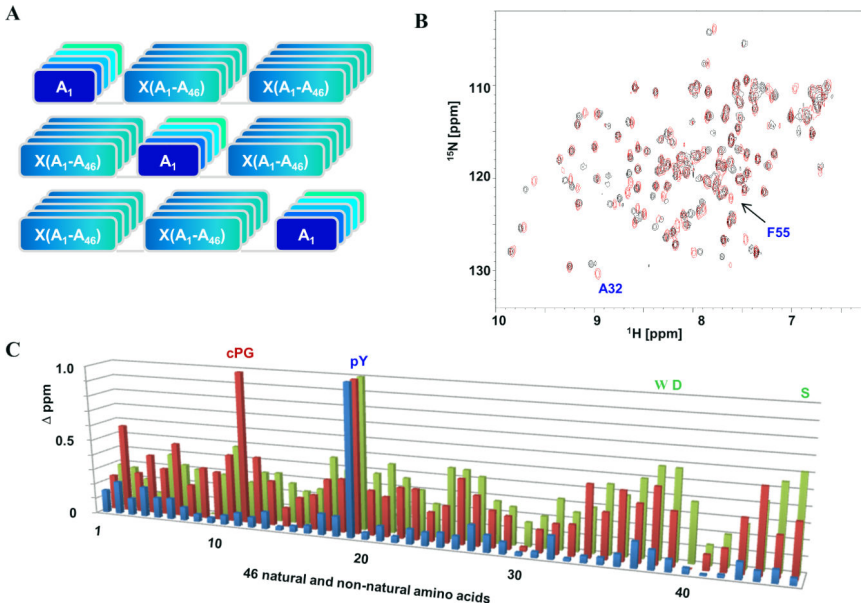

Figure 1. Schematic representation of the combinatorial peptide library and summary of the screening results

A) Schematic illustration of the mixtures used for the NMR screen. A_1 to A_{46} indicate each of the 46 natural and non-natural amino acids comprising the library. X(A_1-A_{46}) indicates a mixture comprised by all 46 amino acids. Hence a mixture A_1XX contains the amino acid A_1 at position P1 while the other 2 positions have all possible combinations. B) 2D [^{1}H, ^{15}N] HSQC spectra of ^{15}N labeled YopH-NT (at 50 μM concentration) acquired in presence (black) or absence (red) of compound **2** (200 μM). C) Summary of chemical shift perturbations induced by the peptide library, arranged as function of amino acid number at the fixed position: blue for position 1, red for position 2, and green for position 3.

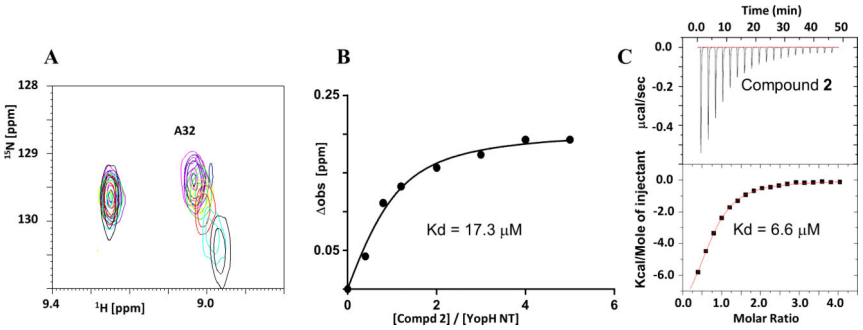

Figure 2. Binding data for compound 2

A) NMR titration of compound **2** binding to YopH-NT (50 μM) using 2D [^{1}H,^{15}N] HSQC spectra. The black contours represent the spectrum for the free protein, while the spectra in cyan, red, yellow, green, purple, blue and fuchsia, respectively, represent YopH-NT in presence of increasing amounts of compound **2** (20 μM, 40 μM, 60 μM, 100 μM, 150 μM, 200 μM and 250 μM, respectively). B) Kd determination for compound **2** using chemical shift perturbation titrations; Kd = 17.3 μM. C) Isothermal titration calorimetry data for the binding between compound **2** and YopH-NT (Kd = 6.6 μM, ΔH =−9.8 Kcal/mol, −TΔS = 2.7 Kcal/mole, n = 0.67).

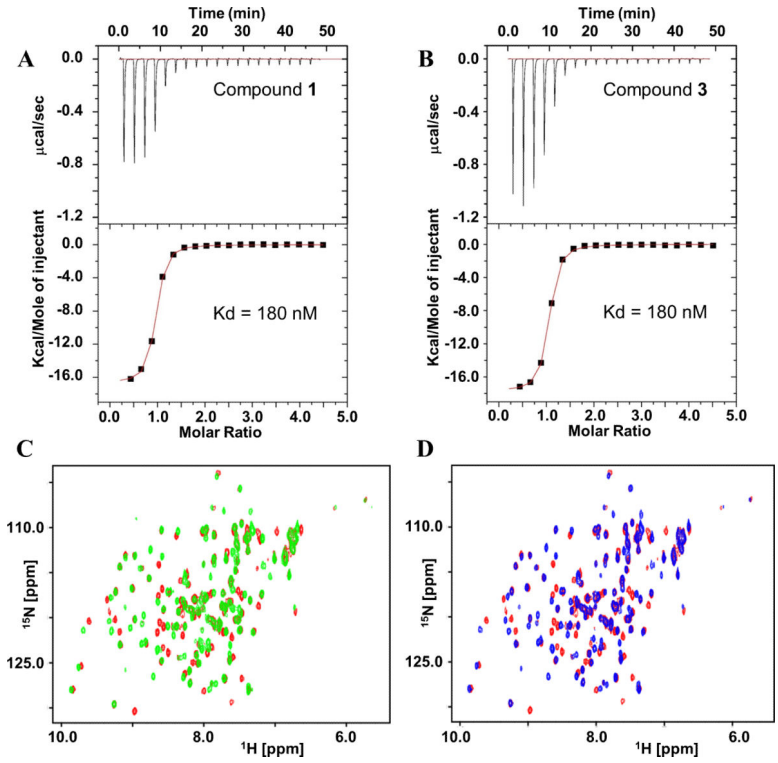

Figure 3. Comparative binding data of compounds 1 and 3

Isothermal titration calorimetry data of binding to YopH-NT for A) compound **1** (Kd = 180 nM, ΔH = −16.5 Kcal/mole, −TΔS= 7.4 Kcal/mole, n = 0.87) and B) compound **3** (Kd = 180 nM, ΔH = −17.6 Kcal/mole, −TΔS= 8.43 Kcal/mole, n =0.96). C) Overlay of [^{1}H,^{15}N] HSQC spectra of YopH-NT (50 μM) in free form (red) and in presence of compound **1** (green, 150 μM). D) Overlay of [^{1}H,^{15}N] HSQC spectra of YopH-NT (50 μM) in free form (red) and in presence of compound **3** (blue, 150 μM). Both compounds produce very similar perturbations to YopH-NT suggesting a similar binding mode.

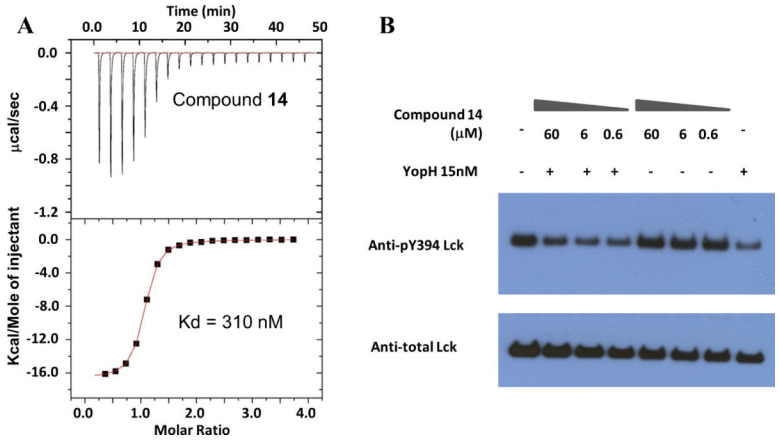

Figure 4. Compound 14 binds to YopH-NT and inhibits full length YopH catalytic activity

A) Isothermal titration calorimetry data for the binding between YopH-NT and compound **14** (Kd = 310 nM, ΔH = −16.6 Kcal/mol, −TΔS = 7.78 Kcal/mol; n =0.99). B) Western blot analysis for the inhibition by compound **14** of the dephosphorylation of Lck by YopH. Recombinant Lck phosphorylation state on pY394 and total Lck level were detected using selected antibodies, in the absence and presence of compound **14**. Full length GST-tagged YopH was pre-incubated with compound **14** for 10 min at room temperature and human recombinant Lck was added and incubated for 20 min.

Table 1

List of compound sequences and summary of Kd values against YopH-NT determined by 2D NMR titrations and/or ITC.

ID	Ac-Peptide sequence-NH_2							Kd value (μM)	
	P-1	P0	P1	P2	P3	P4	P5	YopH NT by NMR	YopH NT by ITC
1	D	E	pY	D	D	P	F	*nd*	0.18
2			pY	cPG	D			17.29	6.60
3	D	E	pY	cPG	D	P	F	*nd*	0.18
4			pY	cPG	D	I		1.47	4.40
5			pY	cPG	D	F		1.66	5.50
6			pY	cPG	D	K		62.69	14.20
7			pY	cPG	D	D		4.93	6.50
8			pY	cPG	D	P		*nd*	1.40
9		I	pY	cPG	D			10.47	10.90
10		F	pY	cPG	D			6.06	9.30
11		K	pY	cPG	D			127.60	*nb*
12		D	pY	cPG	D			21.67	16.20
13		I	pY	cPG	D	P		*nd*	0.52
14		F	pY	cPG	D	P		*nd*	0.31
15		F	pY	cPG	W	P		*nd*	3.10

The amino acid abbreviation pY stands for phosphorylated tyrosine, cPG stands for cyclopentyl glycine. *nd* indicates not determined; *nb* indicates no binding detected at the used experimental conditions. In each peptide, the N-terminus was acetylated and the C-terminus amidated.