Inhibition of Yersinia Tyrosine Phosphatase by Furanyl Salicylate Compounds*

Received for publication, November 19, 2004, and in revised form, December 15, 2004
Published, JBC Papers in Press, December 22, 2004, DOI 10.1074/jbc.M413122200

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To avoid detection and targeting by the immune system, the plague-causing bacterium Yersinia pestis uses a type III secretion system to deliver a set of inhibitory proteins into the cytoplasm of infected cells. One of these proteins is an exceptionally active tyrosine phosphatase termed YopH, which paralyzes lymphocytes and macrophages by dephosphorylating critical tyrosine kinases and signal transduction molecules. Because Y. pestis strains lacking YopH are avirulent, we set out to develop small molecule inhibitors for YopH. We used a novel and cost-effective approach, in which leads from a chemical library were analyzed and computationally docked into the crystal structure of YopH. This resulted in the identification of a series of novel YopH inhibitors with nanomolar Ki values, as well as the structural basis for inhibition. Our inhibitors lack the polar phosphate-mimicking moiety of rationally designed tyrosine phosphatase inhibitors, and they readily entered live cells and rescued them from YopH-induced tyrosine dephosphorylation, signaling paralysis, and cell death. These inhibitors may become useful for treating the lethal infection by Y. pestis.

To survive in humans, pathogenic bacteria have evolved numerous mechanisms to evade the immune response in the host (1, 2). One of the most successful strategies was adopted by Yersinia pestis, namely a type III secretion system that injects a set of paralyzing proteins directly into the cytoplasm of macrophages and lymphocytes that the bacterium encounters in the lymph nodes of infected individuals (3, 4). As a result, the targeted cells become unable to respond, and the bacteria can multiply unopposed by the normal mechanisms of host defense.

The natural route of Y. pestis infection is by transmission from infected rats or other animals by blood-sucking fleas, which are weakened by the bacteria in their gut and therefore expel bacterial mass into the epidermis of their next victim when trying to feed (5, 6). From these flea bites, the bacteria travel to local lymph nodes (7–9), where they multiply and cause a massive lymphadenitis within 2–6 days (5). These enlarged and painful lymph nodes, or “bubos,” give the disease its common name Bubonic Plague. Unless treated with high dose streptomycin- or tetracycline-type antibiotics during the first few days, the infection develops into a toxemic sepsis, which is often fatal (5, 6). A normally very rare, but much more rapidly lethal, form of the infection is caused by inhaled bacteria and is referred to as pneumonic plague or plague pneumonia (10). By this route of infection, the number of bacteria entering the body can be much larger than from microscopic flea bites, and the bacteria are efficiently disseminated to the peritracheal, mediastinal, and other central lymph nodes, from which they gain access to the bloodstream much earlier. Although several vaccines exist (11, 12), and Yersinia usually is sensitive to antibiotics, the pneumonic form of the disease is difficult to diagnose and still often results in death (10).

Despite efforts to eradicate the disease, natural reservoirs of Y. pestis still exist in wild rats and other rodent populations in parts of Africa, southeast Asia, and southwestern United States (13), and sporadic human cases of plague still occur every year. Although these cases pale by comparison to the devastating pandemics that killed an estimated 200 million people, mostly in Europe, during historical times (5, 6), the World Health Organization now recognizes plague as a re-emerging public health concern. There are also increasing fears that Y. pestis may be used for biological warfare or bioterrorism (14–16). The potential threat is heightened by the existence of multidrug-resistant strains of Y. pestis (17, 18) and the rapidly lethal course of the pneumonic form of the disease caused by aerosolized Yersinia. Clearly, new approaches to combat plague are urgently needed.

The molecular mechanisms employed by all virulent strains of Y. pestis and the two related species, Yersinia pseudotuberculosis and Yersinia enterocolitica, are based on an extrachromosomal virulence plasmid (19), which encodes a type III secretion system and several effector proteins called Yops (Yersinia outer membrane proteins) (20). The type III secretion system is a highly conserved macromolecular machinery found in many pathogenic Gram-negative bacteria and is induced by contact with a eukaryotic cell to inject effector Yops into the cytoplasm of the target cells (21). In the host cell, the Yops disrupt signaling cascades responsible for initiating key immune functions, such as phagocytosis (22–24), respiratory burst (25, 26), cytokine production, and lymphocyte activation (27). As a consequence, both the innate and adaptive immune responses are seriously impaired (28). However, a protective immunity can be acquired by vaccination (11, 12).

A key Yop protein is YopH, a 468-amino acid, exceptionally active protein-tyrosine phosphatase (PTP) (29, 30) with a C-terminal catalytic domain and a multifunctional N-terminal domain, which binds tyrosine-phosphorylated target proteins.

* This work was supported by Grants AI53114 and AI55789 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: PTP, protein-tyrosine phosphatase; BisTris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxyethyl)propane-1,3-diol; pNPP, p-nitrophenyl phosphate; mAb, monoclonal antibody.
of the substrate was corrected by measuring the control without addition of enzyme. To quantitate the inhibitory efficacy of the library compounds, we determined the ratio of inhibition in comparison to 200 µM orthovanadate, a PTP inhibitor. Every compound with a ratio of >1 was considered as a hit. ClogP for each compound was calculated with ChemDraw8.

**K** _Determination—_The YopH PTP-catalyzed hydrolysis of pNPP in the presence of inhibitors was assayed at 30 °C in 0.1 M BisTris, pH 6.0, assay buffer containing 1 mM dithiothreitol and 5% Me2SO. The ionic strength was adjusted to 150 mM with NaCl. The enzyme was preincubated with various fixed concentrations of inhibitors for 10 min. The reaction was initiated by the addition of various concentrations of pNPP (ranging from 0.2 to 10 _K_m) to the reaction mixtures to a final volume of 100 µL. The reaction was quenched by addition of 100 µL of 1 M NaOH. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without addition of enzyme. The amount of product formation was determined spectrophotometrically at 405 nm detected by a PowerWaveX340 microplate spectrophotometer (Bio-Tek Instruments, Inc.) using a molar extinction coefficient of 18,000 M⁻¹ cm⁻¹. The inhibition constant and inhibition pattern were evaluated by fitting the data to the Michaelis-Menten equations for either competitive (Equation 1), uncompetitive (Equation 2), or mixed (Equation 3) inhibition, using nonlinear regression and the program GraphPad Prism® (version 4.0).

\[
K_v = V_{max}[S]/(K_{app} + [S]) \quad \text{with} \quad K_{app} = K_v(1 + [I]/K_i) \quad \text{(Eq. 1)}
\]

\[
v_o = V_{max}[S]/(1 + [I]/K_i) \quad \text{with} \quad K_{app} = K_v(1 + [I]/K_i) \quad \text{(Eq. 2)}
\]

In the case of the mixed inhibition model, _K_v_ is the inhibition constant for the competitive participation, and _K_i_ is the inhibition constant for the uncompetitive participation. For a comparison of the fitting results, the second-order AIC's Information Criterion (AICc) was calculated with Equation 4, where _N_ is the number of data points, _SS_ the absolute sum of squares, and _K_ the number of parameters fit by nonlinear regression plus 1.

\[
AIC_c = Nln(SS/N) + 2K + (2K(K + 1))/N - K - 1 \quad \text{(Eq. 4)}
\]

The probability to have chosen the right model can be computed by Equation 5, where _Δ_ is the difference between AICc's Information Criterion (AICc) scores.

\[
\text{probability} = \exp(-0.5Δ)/(1 + \exp(-0.5Δ)) \quad \text{(Eq. 5)}
\]

**IC** _50_ **Measurements**—The PTP-catalyzed hydrolysis of pNPP in the presence of inhibitor was assayed at 30 °C in a 100-µL reaction system in the same assay buffer described above. At various concentrations of the inhibitor, the initial rate at fixed pNPP concentrations (equal to the corresponding _K_v_ values for each PTP) was measured by determining the free phosphate with the BIOMOL GREEN™ reagent, as described above. The _IC_{50_} was determined by plotting the relative pNPP activity versus inhibitor concentration and fitting to Equation 6 using GraphPad Prism®.

\[
V/V_0 = IC_{50}/IC_{50} + [I] \quad \text{(Eq. 6)}
\]

In this case, _V_ is the reaction velocity when the inhibitor concentration is [I], _V_ 0 in the reaction velocity with no inhibitor, and _IC_{50_} = _K_v + K[S]/K_{app_}.

**Molecular Modeling**—Molecular modeling studies were conducted on several R12000 SGI Octane workstations with the software package Sybyl version 6.9 (TRIPOS). Energy-minimized molecular models of the compounds were generated by the Sybyl/MAXIMIN2 routine. Flexible ligand docking calculations were performed with FlexX as implemented in Sybyl. For each compound, 20 solutions were generated and ranked-ordered via FlexX score and CSCORE. In all cases, there was a high degree of convergence for the salicylic acid-furanyl moiety and more variability in the position of the remaining molecules. The coordinates of three-dimensional structure of catalytic domain of YopH (Protein Data Bank codes 1YTS and 1QZ0) were used in the docking studies, and the binding pocket was defined as composed of the following amino acid residues: Arg-205, Arg-228, Phe-229, Ile-232, Asn-245, Ala-258, Cys-259, Gin-260, Tyr-261, Val-264, Leu-268, Ala-286, Ser-287, Gln-289, Ile-291, Phe-296, Met-296, Val-351, Trp-354, Pro-355, Asp-356, Gin-

**TABLE I**

| Compound  | Compound 2 | Compound 1 | Compound 2 |
|-----------|------------|------------|------------|
| IC_{50}   | IC_{50}    | IC_{50}    | IC_{50}    |
| µM        | µM         | fold IC_{50} | fold IC_{50} |
| YopH      | 0.39       | 3.9        | 1          | 1          |
| CD45      | 8.6        | 39         | 22         | 10         |
| PTP 1B    | 12         | 39         | 31         | 10         |
| VHX       | 10         | 20         | 26         | 5.1        |
| VHR       | 28         | 49         | 72         | 13         |
| HePTP     | >100       | >100       | >256       | >26        |
| LMPPT B   | >100       | >100       | >256       | >26        |
| VHI       | >100       | >100       | >256       | >26        |
| LAR       | >100       | >100       | >256       | >26        |

(31, 32). The catalytic domain of YopH is structurally similar to that of eukaryotic PTPs (33). A marked dephosphorylation of proteins in human epithelial cells and murine macrophages has been observed during infection with live bacteria (24, 30, 34, 35). In macrophages and neutrophils, the targets include the focal adhesion proteins Cas, focal adhesion kinase, and paxillin (22, 23), providing a molecular mechanism for inhibition of migration and phagocytosis by these cells (22, 23, 37, 38).

YopH also inhibits the activation of T and B lymphocytes (27, 39). We recently reported (39) that YopH in T cells directly dephosphorylated the Src family tyrosine kinase Lck at its positive regulatory site, Tyr-394, resulting in a complete loss of Lck activity. Because this kinase is the first upstream signal-generating molecule for the T cell antigen receptor, signaling from this receptor was completely abrogated. As a consequence, all tyrosine phosphorylation of downstream signaling proteins was inhibited; the T cells failed to form immune synapases with antigen-presenting cells, and they were unable to secrete any interleukin-2 into the medium (39). Similarly, T cells exposed to _Y. enterocolitica_ became unable to focus calcium and produce cytokines (40).

Because _Yersinia_ strains that carry a _pYV_ plasmid with a nonfunctional _yopH_ gene are avirulent (41-44) and even a point mutation that changes the catalytic _Cys-403_ to an ala nine eliminates the virulence of _Y. pseudotuberculosis_ in a murine infection model (30, 34), it is clear that the catalytic activity of YopH is critical for the lethality of _Yersinia_ infection. We therefore set out to develop small molecule inhibitors of YopH by a combination of chemical library screening, structure-activity analysis, and _in silico_ docking of lead compounds.

**MATERIALS AND METHODS**

*Reagents—p-Nitrophenyl phosphate (pNPP)* was purchased from Sigma. BIOMOL GREEN™ reagent was from BIOMOL Research Laboratories (Plymouth Meeting, PA). All other chemicals and reagents were of the highest grade available commercially. Anti-photophototin mAb 4G10 was from Upstate Biotechnology, Inc. (Lake Saranac, NY), and mAb PY20 was from BD Biosciences.

*Plasmids and Protein Purification*—The eukaryotic and prokaryotic expression plasmids for YopH were as described previously (39). YopH was expressed and purified as before (39). The PTPs VHX (51), VHR, VH1, LMPTP, and HePTP were expressed in _Escherichia coli_ and purified as described previously (45-49). Recombinant CD45, PTP1B, and LAR were expressed and purified as before (39). The PTPs VHX, VH1, LMPTP, and HePTP were expressed in _Escherichia coli_ and purified as described previously (45-49). Recombinant CD45, PTP1B, and LAR were purchased from BIOMOL Research Laboratories.

*Chemical Library Screening for YopH Inhibitors*—A subset of 10,000 compounds from the DIVERSet™ library of 50,000 drug-like molecules (ChemBridge, Inc.) was screened in a 96-well format in _vitro_ assay. Each reaction contained 50 nM YopH, 1 mM pNPP, and 0.03 mg/ml compound in 0.1 M BisTris, pH 6.0, reaction buffer. The final volume amounted to 50 µl and contained 2% Me2SO. The reaction was initiated by addition of pNPP after a preincubation with the enzyme with the compounds for 10 min at room temperature. After 7 min, the reaction was quenched by addition of 100 µl of BIOMOL GREEN™ reagent, and the pNPP hydrolysis was determined by measuring the absorbance of the complexed free phosphate at 620 nm. The nonenzymatic hydrolysis...
### TABLE II
Kinetic data of 2 initial hits and 21 representative analogs

| ID #     | Structure | MW    | CLogP | Ki(c) [μM] |
|----------|-----------|-------|-------|------------|
| Compound 1 |           | 331.30 | 3.03  | 0.311      |
| Compound 2 |           | 464.54 | 2.90  | 1.87       |
| Compound 3 |           | 432.45 | 5.60  | 0.143      |
| Compound 4 |           | 358.32 | 2.98  | 0.208      |
| Compound 5 |           | 432.39 | 4.01  | 0.432      |
| Compound 6 |           | 362.38 | 2.76  | 0.487      |
| Compound 7 |           | 432.45 | 5.65  | 1.27       |
| Compound 8 |           | 298.25 | 0.87  | 1.45       |
| Compound 9 |           | 359.42 | 3.68  | 1.71       |
| Compound 10 |          | 390.41 | 3.21  | 1.84       |
| Compound 11 |          | 421.49 | 4.92  | 1.94       |
| Compound 12 |          | 385.37 | 4.70  | 2.05       |
| Compound 13 |          | 232.19 | 2.88  | 2.08       |
| Compound 14 |          | 331.37 | 2.82  | 3.25       |
| Compound 15 |          | 365.34 | 4.74  | 3.67       |
| Compound 16 |          | 365.81 | 3.03  | 4.52       |
| Compound 17 |          | 331.37 | 2.82  | 5.34       |
| Compound 18 |          | 342.26 | 2.11  | 6.60       |
| Compound 19 |          | 421.49 | 4.92  | 12.7       |
| Compound 20 |          | 371.35 | 4.20  | 14.3       |
| Compound 21 |          | 331.37 | 1.94  | >100       |
| Compound 22 |          | 322.74 | 3.09  | >100       |
| Compound 23 |          | 195.20 | 0.89  | -          |
Thr-358, Ala-359, Val-360, Ile-401, His-402, Ser-403 (Cys-403 in wild-type YopH), Arg-404, Ala-405, Gly-406, Val-407, Gly-408, Arg-409, Thr-410, Ala-411, Gln-412, Leu-413, Ile-443, Met-444, Val-445, Gln-446, Lys-447, and Gln-450. Molecular surfaces were generated with MOLCAD as implemented in Sybyl. Comparisons with other PTPs were made by using the x-ray coordinates for PTP1B (Protein Data Bank code 1PA1), VHR (Protein Data Bank code 1VHR), and bovine LMPTPB (1DG9) and the computer models of the membrane-proximal domains of CD45, VHX, and HePTP. These were generated as described (50).

**Cells and Cell Treatments**—Normal T lymphocytes were isolated from venous blood of healthy volunteers by Ficoll gradient centrifugation. Monocytes/macrophages were eliminated by adherence to plastic for 1 h at 37 °C. Jurkat T leukemia cells were kept at logarithmic growth in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids, and 100 units/ml each of penicillin G and streptomycin. For T cell receptor- and CD28-induced tyrosine phosphorylation responses, normal T lymphocytes were incubated in ice for 15 min with 10 μg/ml OKT3 and anti-CD28 mAbs, washed, and incubated with a cross-linking rabbit anti-mouse Ig for 15 min, washed, and transferred to 37 °C for 5 min. Cells were pelleted and lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, protease inhibitors, and 250 μg/ml of phosphatase inhibitors (1 mM sodium fluoride and 100 μg/ml soybean trypsin inhibitor). Cell pellets were then lysed in 0.5% Nonidet-P40, 250 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 1% Triton X-100, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A. Lysates were sonicated on ice and cleared by centrifugation.

**Fig. 1.** Lineweaver-Burk plots for the four best YopH inhibitors. The structure and $K_i$ (in μM) are given for each compound.

![Lineweaver-Burk plot compound 3](image1)

**Compound 3**

Ki = 0.143 ± 0.029

![Lineweaver-Burk plot compound 4](image2)

**Compound 4**

Ki = 0.208 ± 0.047

![Lineweaver-Burk plot compound 1](image3)

**Compound 1**

Ki = 0.311 ± 0.053

![Lineweaver-Burk plot compound 5](image4)

**Compound 5**

Ki = 0.432 ± 0.093
mm NaCl, 5 mm EDTA containing 1% Nonidet P-40, 1 mm Na₂VO₄, 10 μg/ml aprotinin and leupeptin, 100 μg/ml soybean trypsin inhibitor, and 1 mm phenylmethylsulfonyl fluoride and clarified by centrifugation at 15,000 rpm for 20 min. Lysate was mixed with an equal volume of twice concentrated SDS sample buffer, boiled for 1 min, and resolved by SDS-PAGE.

**SDS-PAGE and Immunoblotting**—These procedures were done as before (39).

**Interleukin-2 Secretion Assay**—A 96-well format in vitro assay was used to screen the first 10,000 compounds of the DIVERSet™ library (ChemBridge, Inc.) of drug-like compounds. A total of 10 compounds inhibited YopH to a higher extent than 200 μM orthovanadate, a general PTP inhibitor. After determination of the kinetic parameters of these 10 first hits, we selected four compounds, which were showing a competitive or mixed inhibition pattern with a Kᵢ value <10 μM, for closer inspection. Two of these, a quinone and a charged thio-imidazole, were subsequently discarded, and we focused on a 5-methylenethiazolidin-4-one (additionally substituted at the nitrogen atom) similarly linked via a furanyl ring to a salicylic acid (compound 1) and a 5-methylene-2-thioxothiazolidin-4-one—2 linked via a furanyl ring to a nitrophenol (compound 2). These two inhibited YopH with competitive Kᵢ values of 0.311 ± 0.053 and 1.87 ± 0.924 μM, respectively, and were relatively selective for YopH (Table I).

The structures of compounds 1 and 2 are shown in Table II. These two represent our initial hits from the chemical library screen.

**Structure-Activity Relationship Analysis of Compound 1 and 2**—Encouraged by these findings, we investigated the structure-activity relationships for a total of 61 analogs that all contained a substituted phenyl ring linked via a furanyl moiety to a more diverse entity at the other end of the molecule, preferentially a 5-methylenethiazolidine ring. A total of 44 compounds inhibited YopH in a competitive manner with Kᵢ values of <100 μM. The structures and kinetic data for a representative set of 21 analogs are given in Table II. Significantly, 13 of the 16 salicylic acid analogs were among the 26 best compounds (competitive inhibition with Kᵢ values <10 μM). Elimination of the salicylic acid moiety (compound 23) led to a complete loss of YopH inhibition, whereas salicyl furaldehyde (compound 13) shows good competitive inhibition (Kᵢ = 2.08 μM). However, salicylic acid alone was a poor inhibitor (Kᵢ = 3.82 μM) compared with the most potent inhibitor, compound 3, which has a 6,211-fold lower inhibitory constant (Kᵢ = 0.143 μM). The very similar compound 7, which only differs in the location of a single methyl group, shows almost 10 times less activity, suggesting some steric constraints for the putative binding site or some very specific interactions of that methyl group. A comparison of the Kᵢ values for compounds 12 and 20, which differ by a methyl group in a very similar position, supports the latter.

Another example of a unique binding mode is presented by the second best inhibitor, compound 4 (Kᵢ = 0.208 μM). In this case, the structurally very similar compound 18 (Kᵢ = 6.60 μM), in which the positions of the carboxylic and the hydroxyl groups have been switched, has a 32 times higher Kᵢ value, supporting the notion of specific binding.

Substitution of the hydroxyl group in the original hit compound 1 by a chlorine in compound 16 led to a 14.5 times less inhibitory activity. Eliminating this hydroxyl group and shifting the carboxylic group to an ortho position (compound 21) results in a >300 times higher Kᵢ value. It is also worth mentioning that inhibitory activity is almost completely lost if the carboxylic and hydroxyl group are replaced by halogens, like fluorine and chlorine (compound 22), perhaps because these atoms are not able to mimic the phosphate group of a natural substrate.

Our structure-activity relationship analysis resulted in the identification of two compounds, which inhibited YopH with lower Kᵢ values than the first furanyl salicylate hit, compound 1. A comparison of the Lineweaver-Burk plots of the four best inhibitors is shown in Fig. 1.

**Virtual Docking Studies**—To provide further insights into specific interactions of our inhibitors with the enzyme, we performed flexible ligand docking with the best four inhibitors and the x-ray coordinates of the catalytic domain of YopH. In all cases, there was a high degree of convergence for the salicylic-furanyl moiety, which occupied the deep hydrophilic phosphate binding cavity (catalytic pocket) on the surface of YopH (Fig. 2). The salicylic group was found to be involved in a complex network of hydrogen bonding interactions (Fig. 2 and Table III) that correlate very well with the less potent inhibition by compounds analogs that lack either the carboxylic or...
the hydroxyl group (Table II). In addition, the oxygen atom of the furanyl ring is also invariably involved in hydrogen bonding interactions with the side chains of Gln-357 or Arg-404 (Table III and Fig. 2, A, C, E, and G), both of which are unique to YopH among PTPs. From these docking studies, it is evident that most of the binding energy of the four inhibitors resides in the interactions with the furanyl and salicylate moieties. In fact, docking studies performed with a virtual compound containing only these two moieties gave similar binding energies, in agreement with experimental data with compound 13 (Ki 2.08 \u03bcM; Table II). In contrast, the in silico elimination of the carboxylic and/or the hydroxyl groups in the salicylate produced compounds that failed to dock in the catalytic pocket of the protein. For example, the binding energy of compound 4 dropped from −46 kJ/mol (Table III) to −21 kJ/mol after removal of the carboxylic and hydroxyl groups. The docking studies also showed that a denser network of hydrogen binding interactions forms within the YopH catalytic pocket compounds when the carboxylic acid in the salicylate is in para position with respect to the furanyl ring (Table III and Fig. 2, A, C, E, and G).

In all four inhibitors, the positioning of the end of the molecule opposite from the salicylate was less defined among the 20 solutions generated with FlexX, correlating well with the high variability of tolerated substitutions at this position. However, a few important conclusions could be made. In compound 4, this moiety is involved in an additional hydrogen bonding interaction with the side chains of Gln-357 and Gln-446, whereas similar interactions occur with compound 5 and Arg-205 that could confer further affinity for YopH. The methyl groups in ring C of compound 3 make favorable steric contacts with YopH, in very close proximity to an additional groove (termed P2 in Fig. 2) on the surface of the protein. Based on the latter model, one could also predict that even small substitutions could result in unfavorable steric hindrance and decrease the binding affinity, as observed for compound 7 (Table II).

In silico docking was also used to evaluate if there is a structural basis for YopH selectivity compared with other PTPs. Although all PTPs have very similar catalytic cores, they differ dramatically in surface topology and charge distribution in the terrain that surrounds the catalytic pocket (50). Fig. 3 shows a comparison of the surface topology, surface electrostatic potential, and surface lipophilic potential of YopH with a set of six other PTPs also used in our selectivity assays. Each enzyme has a unique surface surrounding the catalytic pocket (indicated with a white circle in Fig. 3), with a different distribution, size, and shape of surface depressions and protrusions. In addition, each enzyme has a distinct surface charge and

### Table III

| ID   | Structure | Kᵢ [µM] | E FlexX [kJ/mol] | H-Bond   |
|------|-----------|---------|-----------------|----------|
| Compound 3 | ![Image](image3.png) | 0.143   | -35.8           | Arg 409 H (α) Arg 409 H (α) Ala 405 H (β) Arg 409 H (β) Val 407 H (β) Ser 403 H (β) Gln 357 H (γ) Arg 404 H (γ) Gly 408 H (α) Arg 409 H (α) Val 407 H (α) Ser 403 H (α) Thr 410 H (α) Arg 409 H (β) Ala 405 H (β) Arg 404 H (β) Gln 357 H (γ) Gln 446 H (γ) Gln 446 H (β) Gln 357 H (β) |
| Compound 4 | ![Image](image4.png) | 0.208   | -46.5           | Val 407 H (α) Arg 409 H (α) Arg 409 H (α) Gly 406 H (α) Ser 403 H (α) Ala 405 H (β) Arg 404 H (β) Arg 409 H (β) Arg 409 H (β) Gln 357 H (γ) Gln 446 H (γ) |
| Compound 1 | ![Image](image1.png) | 0.311   | -34.4           | Gln 450 H (α) Arg 409 H (α) Gln 357 H (γ) Gln 446 H (γ) Gln 357 H (γ) Arg 205 H (β) Gln 357 H (β) |
| Compound 5 | ![Image](image5.png) | 0.432   | -44.3           | Gln 446 H (γ) Arg 205 H (β) Gln 357 H (β) |
lipophilicity profile. These striking differences probably reflect preferences in substrate selection. It should also be possible to utilize these features for the development of small molecule inhibitors with a high degree of specificity. Indeed, attempts to dock our compounds into the crystal structures of PTP1B and VHR clearly demonstrated that none of our furanyl salicylates were relatively selective for YopH. (Table IV). Thus, as predicted by the docking studies, the IC\textsubscript{50} values of our inhibitors for a set of PTPs were 1–4 orders of magnitude higher than for YopH, as we have reported before (39). Compound 4 also restored Lck phosphorylation to normal levels (Fig. 5A, lower panel). Compounds 3 (Fig. 5B), 4, and 5 (not shown) also largely neutralized the effect of YopH on tyrosine phosphorylation in a dose-dependent manner.

To extend these observations to normal human T lymphocytes, we incubated freshly isolated peripheral blood T cells with 2 \mu M of membrane-permeable YopH (ANT-YopH) for 2 h at 37 °C, followed by stimulation with anti-CD3ε and anti-CD28 mAbs plus a secondary cross-linking antibody for 18 h at 37 °C in the presence of YopH inhibitors. Although YopH completely inhibited the secretion of interleukin-2, as before (39), cells stimulated in the presence of the YopH inhibitors produced almost as much interleukin-2 as cells without ANT-YopH (Fig. 5C). The four inhibitors varied somewhat in efficacy, but none of them affected the response of cells without ANT-YopH. Together, all these experiments demonstrate that our YopH inhibitors can reverse the strong inhibitory effects of YopH on T cell activation.

**DISCUSSION**

In this study we use an approach to inhibitor design, which can be characterized as a hybrid between traditional high throughput screening and rational design based on the structure of the substrate. Instead of starting with a nonhydrolysable phosphotyrosine analog, we used high throughput library screening to identify useful lead structures, which then were taken into in silico docking studies as the main platform on which the inhibitory properties of inhibitors were examined at the atomic level. This approach identified a novel pharmacophore, furanyl salicylate, a substrate mimic with better properties for drug design than the highly charged phosphotyrosine. In silico docking gave detailed insight into the complex network of hydrogen bonds between the enzyme and the inhibitors and thus allowed us to understand the experimental results with analogs of the first hits. This in turn will make it possible to rationally design even better inhibitors in the future.

Structurally, our inhibitors resemble a recently reported YopH inhibitor, aurintricarboxylic acid (51), that represents a symmetric joining of three salicylate moieties. This compound inhibited YopH with a \( K\) of 5 nM and an \( I_{50}\) of 10 nM, but did not penetrate into cells. Our inhibitors contain only one salicylate group and therefore have only one carboxylic group. For this reason, they penetrated into lymphocytes and were able to

**FIG. 3.** Surface representation of YopH in comparison with other PTPs used in selectivity determinations. The color code of MOLCAD represents topology (left-hand panels, blue, protrusion, and yellow, deepest cavity), the electrostatic potential (middle panels, red, most positive; purple, most negative), and lipophilic nature of the surface (right-hand panels, brown, more lipophilic; blue, more hydrophilic).

**FIG. 4.** Close up of surface surrounding the catalytic pocket of YopH in comparison with PTP1B and VHR. The pockets termed \( P1 \) (catalytic), \( P2 \), and \( P3 \) are indicated, and the arrow points to the large protrusion in PTP1B discussed in the text. Color codes are as in Fig. 3.

normal (Fig. 5A, upper panel). Similarly, the phosphorylation of the Lck kinase at its positive regulatory tyrosine residue, Tyr-394, was severely reduced by YopH, as we have reported before (39). Compound 4 also restored Lck phosphorylation to normal levels (Fig. 5A, lower panel). Compounds 3 (Fig. 5B), 4, and 5 (not shown) also largely neutralized the effect of YopH on tyrosine phosphorylation in a dose-dependent manner.
reverse the inhibitory effects of YopH on T cell antigen receptor signaling.

The docking studies showed that the salicylate moiety (“ring A”) mimics the phosphotyrosine residue of a substrate for YopH and fits into the catalytic pocket (P1). The furanyl ring (“ring B”) interacts specifically with Gln-357 on the rim of the catalytic pocket and with the side chain of Arg-404. Because both these residues are unique to YopH compared with other PTPs, the furanyl ring apparently provides selectivity to the inhibitors. The positioning and the interactions involving the other end of the compounds (“ring(s) C”) were more variable, as also seen experimentally by a higher tolerance for substitutions at this position. However, ring C may give us the opportunity to bridge the core salicylate-furanyl structure occupying the catalytic pocket to two other unique pockets present on the surface of YopH (Figs. 3 and 4). Thus, future compounds that reach into pockets may have further increased affinity and selectivity for YopH.

It should be pointed out that our best inhibitors showed a mixed type of inhibition (which is common), indicating that they not only fit into the catalytic cleft in a substrate-competitive manner, as demonstrated by the in silico docking, but also affect the substrate-enzyme complex. Because our inhibitors interact with a considerably larger surface than the pNPP substrate, it is possible that they bind to YopH with pNPP in its catalytic cleft and interfere with product release.

Our studies support the notion that small molecule inhibitors that are specific for individual members of the PTP family can be generated by taking advantage of unique surface features outside of the catalytic pocket. A comprehensive comparison of the crystal structures and computer models of 103 of the 107 human PTPs (data not shown) clearly shows that PTPs vary widely in topology and charge distribution surrounding the catalytic pocket. This variability probably reflects substrate preferences and the interactions with surface features of substrates other than the phosphotyrosine residue (36). As an example, Fig. 4 shows a close up of the surface that surrounds the catalytic pocket in YopH, PTP1B, and VHR. In YopH there is a large semicircular valley bordered by the three pockets and with a low ridge between P2 and P3 giving the valley a V-shaped floor. In PTP1B there are also two additional depressions, of which the equivalent of P3 is a pocket involved in substrate binding (36). In contrast to YopH, the access to P3 from P1 elongated and constricted into a very narrow passage, and access to the equivalent of P2 is completely blocked in PTP1B. In VHR, there are no well defined depressions other than the catalytic pocket, which is surrounded by more hydrophilic and acidic surfaces than in YopH or PTP1B. These striking differences in surface topology inspire confidence in a more rational design of selective inhibitors for PTPs.

Our results demonstrate that selective and potent YopH inhibitors can be developed and could be used as a starting point for the design of drugs to combat the virulence of Y. pestis. Particularly in the case of multidrug-resistant strains or following exposure to aerosolized Y. pestis, such an inhibitor may prove very useful. YopH is also a good target for drug design because it differs in several residues from endogenous PTPs in humans. In addition, an inhibitor with some effects on endogenous PTPs in immune cells may act to further strengthen the immune response against Y. pestis.

Acknowledgment—We are grateful to Dr. Zhong-Yin Zhang for discussions and advice.
REFERENCES

1. Ernst, J. D. (2000) Cell. Microbiol. 2, 379–386
2. DeVinney, I., Steele-Mortimer, I., and Finlay, B. B. (2000) Trends Microbiol. 8, 29–33
3. Persson, C., Nordfält, R., Holmström, A., Hakansson, S., Rosqvist, R., and Wolf-Watz, H. (1995) Mol. Microbiol. 18, 135–150
4. Cheng, L. W., and Schneewind, O. (2000) J. Bacteriol. 182, 3183–3190
5. Titball, R. W., and Leary, S. E. (1998) Br. Med. Bull. 54, 625–633
6. Hinnebusch, B. J. (1997) J. Mol. Med. 75, 645–652
7. Autenrieth, I. B., Vogel, U., Preger, S., Heymer, B., and Heesemann, J. (1993) Infect. Immun. 61, 2585–2595
8. Autenrieth, I. B., Haaschmann, P., Heymer, B., and Heesemann, J. (1993) Immunobiology 187, 1–16
9. Christie, A. B. (1982)
10. Friedlander, A. M., Welkos, S. L., Worsham, P. L., Andrews, G. P., Heath, J. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8778–8783
11. Titball, R. W., and Leary, S. E. (1998) Science 283, 571–575
12. Hawley, R. J., and Eitzen, E. M., Jr. (2001) J. Mol. Med. 79, 1187–1191
13. Inglesby, T. V., Dennis, D. T., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Rogers, D. S., and Fauci, A. S. (1999) Nat. Med. 5, 699–711
14. Galimand, M., Guiyoule, A., Gerbaud, G., Rasoamanana, B., Chanteau, S., Courvalin, P., and Carniel, E. (1997) J. Biol. Chem. 272, 3531–3538
15. Black, D. S., Mustelin, T., and Zhang, Z.-Y. (2003) J. Biol. Chem. 278, 4445–4453
16. Black, D. S., Montagna, L. G., Zitsmann, M., and Bliska, J. B. (1998) Mol. Microbiol. 29, 1263–1274
17. Galimand, M., Guiyoule, A., Gerbaud, G., Rasoamanana, B., Chanteau, S., Carniel, E., and Courvalin, P. (1997) N. Engl. J. Med. 337, 677–680
18. Guvieux, A., Gerbaud, G., Buchrieser, C., Galimand, M., Kehalissen, L., Chanteau, S., Courvalin, P., and Carniel, E. (2001) Emerg. Infect. Dis. 7, 43–48
19. Corneil, G. R., and Wolf-Watz, H. (1997) Mol. Microbiol. 23, 861–867
20. Corneil, G. R., Bolland, A., Boyd, A. P., Geurjes, C., Iriarte, M., Neyt, C., Sary, M. P., and Stainier, I. (1998) Microbiol. Mol. Biol. Rev. 62, 1315–1352
21. Juris, S. J., Shao, F., and Dixon, J. E. (2002) Cell. Microbiol. 4, 201–211
22. Black, D. S., and Bliska, J. B. (1997) EMBO J. 16, 2730–2744
23. Persson, C., Carballera, N., Wolf-Watz, H., and Fallman, M. (1997) EMBO J. 16, 2307–2318
24. Anderson, K., Carballera, N., Magnusson, K. E., Persson, C., Stendahl, O., Wolf-Watz, H., and Fallman, M. (1996) Mol. Microbiol. 20, 1057–1069
25. Aepfelbacher, M., Zumbihl, R., Rückdeschel, K., Jacob, C. A., Barz, C., and Heesemann, J. (1999) Biochim. Biophys. Acta 1401–1412
26. Green, P. S., Hartland, E. L., Roberts-Browne, R. M., and Phillips, W. A. (1995) J. Leukocyte Biol. 57, 972–977
27. Yao, T., Mecas, J., Healy, J. I., Falkow, S., and Chien, Y. (1999) J. Exp. Med. 190, 1343–1350
28. Cornelis, G. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8778–8783
29. Guan, K., and Dixon, J. E. (1999) Science 285, 531–536
30. Bliska, J. B., Guan, K., Dixon, J. E., and Falkow, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1167–1191
31. Montagna, L. G., Ivanov, M. I., and Bliska, J. B. (2001) J. Biol. Chem. 276, 5005–5011
32. Evdokimov, A. G., Tropea, J. E., Rutzahn, K. M., Copeland, T. D., and Waugh, D. S. (2001) Acta Crystallogr. 57, 793–799
33. Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z. Y., and Dixon, J. E. (1994) Nucleic Acids Res. 22, 711–717
34. Bliska, J. B., Clemens, J. C., Dixon, J. E., and Falkow, S. (1992) J. Exp. Med. 176, 1625–1630
35. Hartland, E. L., Green, S. P., Phillips, W. A., and Robins-Browne, R. M. (1994) Infect. Immun. 62, 4445–4453
36. Salmeen, A., Andersen, J. N., Myers, M. P., Tonks, N. K., and Barford, D. (2000) Mol. Cell 6, 1401–1412
37. Black, D. S., Montagna, L. G., Zitsmann, M., and Bliska, J. B. (1998) Mol. Microbiol. 29, 1263–1274
38. Rückdeschel, K., Roggenkamp, A., Schubert, S., and Heesemann, J. (1996) Infect. Immun. 64, 724–733
39. Alonso, A., Bettini, N., Bruckner, S., Rahmouni, S., Williams, S., Schoenberger, S. P., and Mustelin, T. (2004) J. Biol. Chem. 279, 4922–4928
40. Sauvonnnet, N., Lambermont, I., van der Bruggen, P., and Cornelis, G. R. (2002) Mol. Microbiol. 45, 805–815
41. Bolin, I., and Wolf-Watz, H. (1988) Mol. Microbiol. 2, 237–247
42. Rosqvist, R., Bolin, I., and Wolf-Watz, H. (1988) Infect. Immun. 56, 2139–2143
43. Botella, J., and Bowmer, W. S. (1986) Infect. Immun. 51, 445–454
44. Alonso, A., Merlo, J. J., Na, S., Khodol, N., Jaroszewski, L., Kharitonov, A., Williams, S., Godzik, A., and Mustelin, T. (2002) J. Biol. Chem. 277, 5524–5531
45. Alonso, A., Rahmou, S., Williams, S., van Stipdonk, M., Jaroszewski, L., Godzik, A., Abraham, R. T., Schoenberger, S. P., and Mustelin, T. (2003) Nat. Immun. 4, 44–48
46. Alonso, A., Rahmou, S., Williams, S., van Stipdonk, M., Jaroszewski, L., Godzik, A., Abraham, R. T., Schoenberger, S. P., and Mustelin, T. (2003) Nat. Immun. 4, 44–48
47. Khodol, N., and Mustelin, T. (2001) BioTechniques 31, 322–328
48. Saxena, M., Williams, S., Taskén, K., and Mustelin, T. (1999) Nat. Cell Biol. 1, 305–311
49. Slama, M., Williams, S., Brockdorff, J., Gilman, J., and Mustelin, T. (1999) J. Biol. Chem. 274, 11693–11700
50. Alonso, A., Sasin, J., Betini, N., Friedman, I., Osterman, A., Godzik, A., Hunter, D., Dixon, J. E., and Mustelin, T. (2004) Cell 117, 999–1011
51. Liang, F., Huang, Z., Lee, S.-Y., Liang, J., Alonso, A., Bliska, J. B., Lawrence, D. S., Mustelin, T., and Zhang, Z.-Y. (2003) J. Biol. Chem. 278, 41734–41741
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J. Biol. Chem. 2005, 280:9400-9408.
doi: 10.1074/jbc.M413122200 originally published online December 22, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M413122200

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