Published in final edited form as:

Bioorg Med Chem. 2018 October 15; 26(19): 5322–5326. doi:10.1016/j.bmc.2018.04.047.

Screening serine/threonine and tyrosine kinase inhibitors for histidine kinase inhibition

Kaelyn E. Wilke^a, Conrad A. Fihn^b, and Erin E. Carlson^{a,b,c,d,e,*}

^aDepartment of Chemistry, Indiana University, 800 East Kirkwood Avenue, Bloomington, IN 47405, United States

^bDepartment of Medicinal Chemistry, University of Minnesota, 308 Harvard Street SE, Minneapolis, MN 55455, United States

^cDepartment of Chemistry, University of Minnesota, 207 Pleasant Street SE, Minneapolis, MN 55454, United States

^dDepartment of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 321 Church Street SE, Minneapolis, MN 55455, United States

^eDepartment of Molecular and Cellular Biochemistry, Indiana University, 212 South Hawthorne Drive, Bloomington, IN 47405, United States

Abstract

Histidine kinases of bacterial two-component systems are promising antibacterial targets. Despite their varied, numerous roles, enzymes in the histidine kinase superfamily share a catalytic core that may be exploited to inhibit multiple histidine kinases simultaneously. Characterized by the Bergerat fold, the features of the histidine kinase ATP-binding domain are not found in serine/threonine and tyrosine kinases. However, because each kinase family binds the same ATP substrate, we sought to determine if published serine/threonine and tyrosine kinase inhibitors contained scaffolds that would also inhibit histidine kinases. Using select assays, 222 inhibitors from the Roche Published Kinase Set were screened for binding, deactivation, and aggregation of histidine kinases. Not only do the results of our screen support the distinctions between ATP-binding domains of different kinase families, but the lead molecule identified also presents inspiration for further histidine kinase inhibitor development.

Keywords

Histidine kinases; Two-component systems; Roche Published Kinase Set; Antibacterial targets; Inhibitors

*Corresponding author. carlsone@umn.edu (E.E. Carlson).

A. Supplementary data

Supplementary data (screening data for the entire compound library, activity assays, structurally-related compounds that were not hits, and descriptions of the materials and methods) associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.bmc.2018.04.047>.

The increase in bacterial resistance to all current antibiotics is a threat to global public health. As nearly all classes of antibiotics are based on initial developments from four or more decades ago, a new class of antibiotics is needed. Ubiquitous signaling processes in bacteria, two-component systems (TCSs) may provide a novel target. Key players in the response to changing environments, the prototypical bacterial TCS includes a cognate pair of proteins: a membrane-bound histidine kinase (HK) and a cytosolic response regulator (RR). Upon activation by a signal, the HK autophosphorylates with the γ -phosphate of adenosine triphosphate (ATP) on a conserved histidine residue. Subsequently, the phosphoryl group is transferred to the RR that usually acts as a transcription factor to bind DNA and alter gene expression (Fig. 1A).^{1,2}

TCS proteins are promising targets for new antibacterial agents. Myriad TCSs facilitate diverse and virulent microbial functions,^{3,4} have been identified only in low-level eukaryotes, such as yeast or fungi, and are absent in mammals.⁵ TCSs have also been implicated in resistance to antibiotics such as vancomycin resistance in *Staphylococcus aureus* (VanSR),⁶ carbapenem resistance in *Pseudomonas aeruginosa* (CzcRS),⁷ and multidrug resistance in *Mycobacterium tuberculosis* (MtrAB).⁸ Despite their varied roles, TCS proteins share a catalytic core that may be exploited by a multitargeted therapeutic agent to deactivate several TCSs simultaneously.⁹ We have focused our attention on HKs, the sensor proteins of TCSs that begin the phosphorylation cascade. HKs exhibit a high degree of conservation in the ATP-binding domain that is characterized by the Bergerat fold: a sandwich of α helices in one layer, mixed β strands in another, and a discrete and flexible ATP lid containing homology boxes G1-, G2, G3, F-, and N-, which are strings of conserved residues that play important roles in ATP binding.⁹⁻¹² A conserved Asp is housed in the G1-box that forms an important salt bridge with the N6 in adenine, the G1-, F-, and G3- boxes are partially responsible for positioning the adenine through interactions with many hydrophobic residues, and in the N-box, and Asn residue interacts with the phosphate groups through a bound Mg^{2+} ion.¹²⁻¹⁵ This architecture is absent in serine/threonine and tyrosine kinases that are abundant in human biology (Fig. S1 and 2).^{10,14} This distinction between the ATP-binding domains of the HKs and the eukaryotic kinases suggests that an inhibitor could be designed to selectively target HKs to treat bacterial infections in a human host.⁹ Initial attempts in the 1990s to identify bioactive molecules that targeted HKs were thwarted by problematic compounds that inhibited through aggregation of the protein.¹⁶ Although research has been progressing to identify molecules that inhibit HKs,¹⁷⁻²¹ none have progressed to a clinical stage.

We previously published the design and execution of a high-throughput screen to detect lead compounds for HK inhibition. The screen was comprised of a fluorescence polarization (FP) displacement assay using the model protein HK853 from *Thermotoga maritima*²² and an ADP-BODIPY ligand (Fig. 1B). As the fluorescent ligand was shown to bind HK853 (K_d of $6.79 \pm 0.11 \mu M$ with 10 nM ADP-BODIPY), its displacement is indicative of the presence of a potential inhibitor that affects substrate binding to the ATP-binding domain. Using this screen with ~53,000 diverse small molecules, we identified nine new lead compounds that deactivate HKs.¹⁸

In parallel to sourcing diversity libraries for the search of novel HK inhibitors, we questioned if we could find inspiration from established serine/threonine (Ser/Thr) or tyrosine (Tyr) kinases inhibitors. While Ser/Thr and Tyr kinases lack the Bergerat fold of HKs, we postulated that some scaffolds might exhibit HK inhibitory activity because all kinase families bind the same ATP substrate. Thus, we applied our FP assay in conjunction with 222 published kinase inhibitors (Hoffmann-La Roche, Inc.). Each compound was assessed for inhibition of ADP-BODIPY binding to HK853 in 96-well plates (Z' -factor 0.95; Fig. S3). A threshold of 65% inhibition (Fig. S4 and Table S1) was used to select nine Ser/Thr and Tyr kinase inhibitors to pursue in follow-up assays (Fig. 2; nine compounds).

Dose-response inhibition was determined for the nine hits using the FP assay in the presence and absence of the detergent Triton X-100 (Figs. 3; S5 and Table S2). Large differences in the two dose-response curves (DRCs) were suggestive of aggregation-induced inhibition. As a result, four compounds (**46**, **81**, **110**, **161**) were removed from further testing. Next, we performed secondary assays on two HK proteins, HK853 and VicK (*Streptococcus pneumoniae*),^{18,23} to further assess inhibition using a coupled competition-aggregation assay.²⁴ We used two proteins as we sought to identify pan-HK inhibitors and thus desired inhibition of more than one HK. Utilizing ATP[γ -³³P], we measured the degree of inhibition of enzymatic activity by the remaining nine lead compounds (Figs. S6 and S7). In parallel, the samples were subjected to glutaraldehyde cross-linking and native-PAGE gel analysis to investigate compound-induced aggregation (Fig. S8). Lead compounds **46**, **81**, **108**, **110**, **114**, and **161** aggregated HK853 and/or VicK at concentrations where significant inhibition was observed. These results indicate that their activity in our assays is likely due to their promiscuous, aggregatory effects rather than specific interactions with the ATP-binding domain of the HKs. As such, these compounds were eliminated from further testing. The remaining leads were subjected to a pan assay interference compound (PAINS) filter using the Schrodinger Canvas suite.²⁵ PAINS are false positive compounds that are likely to nonspecifically interact with numerous targets.²⁶ Compounds **86** and **58** were flagged in three separate PAINS filters, leading us to eliminate these two compounds from further testing.

These studies resulted in the validation of one lead for HK inhibition from the Roche collection, compound **32** (Figs. 2 and S9), which was first shown to displace ADP-BODIPY from the HK ATP-binding domain and subsequently demonstrated to inhibit HK and VicK activity at non-aggregating concentrations (Figs. S6, S7, S9). We next assessed **32** for efficacy in live cells. Our hope is that because most HKs are not genetically essential, but are instead required for virulence and pathogenicity, that this compound may operate as an anti-virulence agent as opposed to an antibacterial agent.²⁷ Indeed, **32** did not cause a significant decrease in cell viability as indicated by optical density measurements and resazurin indicator dye in both Gram-positive (*Methicillin-sensitive Staphylococcus aureus*) and Gram-negative strains (*Pseudomonas aeruginosa*) compared to a DMSO control (Fig. 4). It has been theorized that eliminating bacterial virulence would be a promising alternative to current antibiotic strategy.^{28,29} According to the US National Institute of Health, biofilms are involved in a large number of antibiotic-resistant infections (~80%) and are a common

mark of pathogenesis.³⁰ TCS are directly implicated in the formation of these biofilms in both *S. aureus* and *P. aeruginosa*; thus inhibiting HKs would likely have an effect on this virulence marker.^{31,32} As such, biofilm formation was analyzed, using a crystal violet staining assay.³³ Treatment of bacteria with **32** showed little effect on the biofilm production of *S. aureus* or *P. aeruginosa* compared to a DMSO control. This result led us to hypothesize the lead **32** may only poorly penetrate the bacterial envelope or that it lacks sufficient potency to yield measurable effects. Conversely, this compound is a potent inhibitor of several mammalian kinases, the cytokine biosynthesis regulation associated p38 MAP kinase (53 nM), and protein kinase D, a diacylglycerol-regulated serine/threonine protein kinase.^{34,35} Indeed, it is promising that there is limited crossover between inhibitors that target traditional mammalian kinases and the histidine kinase, providing further evidence that it will be possible to target the HKs selectively.

Inhibitor cross-reactivity and poor cell penetration are undesirable but do not preclude the potential of these scaffolds to be developed into HK-selective molecules with proper optimization. Although we found that most of the molecules in this library were ineffective against the HKs – either due to lack of binding or promotion of protein aggregation at inhibitory concentrations – we can take inspiration from **32** for the design of future inhibitors. Recently, we reported that purine is a promising scaffold for the development of selective HK inhibitors. Through docking studies, we postulated the necessity of a nitrogen for hydrogen bonding in the HK active site through a conserved aspartate residue.²¹ Compound **32** shares some structural similarities with this base, but it is composed of pyrazine fused to a pyrrole as opposed to a pyrimidine and an imidazole. We could also glean information from the examination of related compounds from the library that were not leads from the HTS (Fig. S10). Four additional molecules in the library contain a similar scaffold and substituents to **32** but instead possess a pyridine ring in place of the pyrazine. Indeed, the difference of a single nitrogen in this heterocycle correlates to inactive analogs (e.g., comparison of **32** to RO1153853–000). As we have seen in our previous studies, it is possible that this nitrogen is critical for interacting with key active site residues, such as the conserved aspartate. Docking studies with this compound show a binding mode that is similar to these previously reported molecules (Fig. 5).^{18,21} Asp 411 and Gly 415 participate in hydrogen bonding interactions to the nitrogen atoms in the bicyclic core in the same manner to that observed in the adenine core. In addition, the pyridine nitrogen is predicted to participate in a hydrogen bond with Asn 380, mimicking the same interaction we had previously reported, though this may be water-mediated given the distance (3.3 Å). These insights can be utilized for the incorporation of the structural features of **32** into other scaffolds that have demonstrated activity in live cells in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Hoffmann-La Roche Inc. for providing the kinase inhibitor library. We thank S. Francis for synthesis of ADP-BODIPY. We thank M. Goswami for helpful discussions. We thank A. Zlotnick and L. Li for use and help with a microplate reader for assay development. Indiana University's Physical Biochemistry

Instrumentation Facility and the Indiana Molecular Biology Institute provided essential instrumentation. We thank M. Winkler for an overexpression strain for the VicK construct. This work was supported by the National Institutes of Health (NIH) DP2OD008592, a Pew Biomedical Scholar Award (E.E.C.), an Indiana University Dean's Fellowship (E.E.C.), and an Indiana University Quantitative and Chemical Biology training fellowship (K.E.W.) and the University of Minnesota.

References

1. Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. *Annu Rev Biochem.* 2000;69:183–215. [PubMed: 10966457]
2. Stock JB, Stock AM, Mottonen JM. Signal transduction in bacteria. *Nature.* 1990;344:395–400. [PubMed: 2157156]
3. Gotoh Y, Eguchi Y, Watanabe T, Okamoto S, Doi A, Utsumi R. Two-component signal transduction as potential drug targets in pathogenic bacteria. *Curr Opin Microbiol.* 2010;13:232–239. [PubMed: 20138000]
4. Stephenson K, Hoch JA. Virulence- and antibiotic resistance-associated two-component signal transduction systems of gram-positive pathogenic bacteria as targets for antimicrobial therapy. *Pharmacol. Therapeut* 2002;93:293–305.
5. Thomason P, Kay R. Eukaryotic signal transduction via histidine-aspartate phosphorelay. *J Cell Sci.* 2000;113:3141–3150. [PubMed: 10954413]
6. Bisicchia P, Bui NK, Aldridge C, Vollmer W, Devine KM. Acquisition of VanB-type vancomycin resistance by *Bacillus subtilis*: the impact on gene expression, cell wall composition and morphology. *Mol Microbiol.* 2011;81:157–178. [PubMed: 21542863]
7. Dieppois G, Ducret V, Caille O, Perron K. The transcriptional regulator CzcR modulates antibiotic resistance and quorum sensing in *Pseudomonas aeruginosa*. *PLoS ONE.* 2012;7:e38148. [PubMed: 22666466]
8. Nguyen HT, Wolff KA, Cartabuke RH, Ogowang S, Nguyen L. A lipoprotein modulates activity of the MtrAB two-component system to provide intrinsic multidrug resistance, cytokinetic control and cell wall homeostasis in *Mycobacterium*. *Mol Microbiol.* 2010;76:348–364. [PubMed: 20233304]
9. Wilke KE, Carlson EE. All signals lost. *Sci Transl Med.* 2013;5:s12.
10. Dutta R, Inouye M. GHKL, an emergent ATPase/kinase superfamily. *Trends Biochem Sci.* 2000;25:24–28. [PubMed: 10637609]
11. Guarnieri MT, Zhang LD, Shen JP, Zhao R. The Hsp90 inhibitor radicicol interacts with the ATP-binding pocket of bacterial sensor kinase PhoQ. *J Mol Biol.* 2008;379:82–93. [PubMed: 18440021]
12. Tanaka T, Saha SK, Tomomori C, et al. NMR structure of the histidine kinase domain of the *E. coli* osmosensor EnvZ. *Nature.* 1998;396:88–92. [PubMed: 9817206]
13. Bem AE, Velikova N, Pellicer MT, Baarlen P, Marina A, Wells JM. Bacterial histidine kinases as novel antibacterial drug targets. *ACS Chem Biol.* 2015;10:213–224. [PubMed: 25436989]
14. Casino P, Rubio V, Marina A. The mechanism of signal transduction by two-component systems. *Curr Opin Struct Biol.* 2010;20:763–771. [PubMed: 20951027]
15. Marina A, Mott C, Auyzenberg A, Hendrickson WA, Waldburger CD. Structural and mutational analysis of the PhoQ histidine kinase catalytic domain. Insight into the reaction mechanism. *J Biol Chem.* 2001;276:41182–41190. [PubMed: 11493605]
16. Stephenson K, Yamaguchi Y, Hoch JA. The mechanism of action of inhibitors of bacterial two-component signal transduction systems. *J Biol Chem.* 2000;275:38900–38904. [PubMed: 10978341]
17. Worthington RJ, Blackledge MS, Melander C. Small-molecule inhibition of bacterial two-component systems to combat antibiotic resistance and virulence. *Future Med Chem.* 2013;5:1265–1284. [PubMed: 23859207]
18. Wilke KE, Francis S, Carlson EE. Inactivation of multiple bacterial histidine kinases by targeting the ATP-binding domain. *ACS Chem Biol.* 2015;10:328–335. [PubMed: 25531939]

19. Velikova N, Fulle S, Manso AS, et al. Putative histidine kinase inhibitors with antibacterial effect against multi-drug resistant clinical isolates identified by in vitro and in silico screens. *Sci Rep*. 2016;6:26085. [PubMed: 27173778]
20. Boibessot T, Zschiedrich CP, Lebeau A, et al. The Rational Design, Synthesis, and Antimicrobial Properties of Thiophene Derivatives That Inhibit Bacterial Histidine Kinases. *J Med Chem*. 2016;59:8830–8847. [PubMed: 27575438]
21. Goswami M, Wilke KE, Carlson EE. Rational Design of Selective Adenine-Based Scaffolds for Inactivation of Bacterial Histidine Kinases. *J Med Chem*. 2017;60:8170–8182. [PubMed: 28933546]
22. Marina A, Waldburger CD, Hendrickson WA. Structure of the entire cytoplasmic portion of a sensor histidine-kinase protein. *EMBO J*. 2005;24:4247–4259. [PubMed: 16319927]
23. Gutu AD, Wayne KJ, Sham LT, Winkler ME. Kinetic characterization of the WalRKSpn (VicRK) two-component system of *Streptococcus pneumoniae*: dependence of WalkSpn (VicK) phosphatase activity on its PAS domain. *J Bacteriol*. 2010;192:2346–2358. [PubMed: 20190050]
24. Foster JE, Sheng Q, McClain JR, et al. Kinetic and mechanistic analyses of new classes of inhibitors of two-component signal transduction systems using a coupled assay containing HpkA-DrrA from *Thermotoga maritima*. *Microbiology*. 2004;150:885–896. [PubMed: 15073298]
25. Canvas S Schrödinger Release 2018–1. New York, NY: LLC; 2018.
26. Baell JB, Nissink JWM. Seven Year Itch: Pan-Assay Interference Compounds (PAIS) in 2017-Utility and Limitations. *ACS Chem Biol*. 2018;13:36–44. [PubMed: 29202222]
27. Tiwari S, Jamal SB, Hassan SS, et al. Two-Component Signal Transduction Systems of Pathogenic Bacteria As Targets for Antimicrobial Therapy: An Overview. *Front Microbiol*. 1878;2017:8.
28. Beceiro A, Tomas M, Bou G. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clin Microbiol Rev*. 2013;26:185–230. [PubMed: 23554414]
29. Johnson BK, Abramovitch RB. Small Molecules That Sabotage Bacterial Virulence. *Trends Pharmacol Sci*. 2017;38:339–362. [PubMed: 28209403]
30. National Heart, L., Blood Institute, 2002 Research on microbial biofilms (PA-03–047). National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD.
31. Mikkelsen H, Sivaneson M, Filloux A. Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. *Environ Microbiol*. 2011;13:1666–1681. [PubMed: 21554516]
32. Sharma-Kuinkel BK, Mann EE, Ahn JS, Kuechenmeister LJ, Dunman PM, Bayles KW. The *Staphylococcus aureus* LytSR two-component regulatory system affects biofilm formation. *J Bacteriol*. 2009;191:4767–4775. [PubMed: 19502411]
33. Skogman ME, Vuorela PM, Fallarero A. A platform of anti-biofilm assays suited to the exploration of natural compound libraries. *J Vis Exp*. 2016.
34. Trejo A, Arzeno H, Browner M, et al. Design and synthesis of 4-azaindoles as inhibitors of p38 MAP kinase. *J Med Chem*. 2003;46:4702–4713. [PubMed: 14561090]
35. Tandon M, Wang L, Xu Q, Xie X, Wipf P, Wang QJ. A targeted library screen reveals a new inhibitor scaffold for protein kinase D. *PLoS ONE*. 2012;7:e44653. [PubMed: 23028574]

Fig. 1. HK853 and ADP-BODIPY. a) HK853 from *Thermotoga maritima*. In white, the dimerization and histidine phosphotransfer domain houses the conserved His residue. The catalytic and ATP-binding domain is shown in blue with bound ADP. Nucleotide and His are shown in yellow stick form. PDB accession code 3DGE. b) ADP-BODIPY containing the adenosine core and BODIPY fluorophore.

Fig. 2.

Lead compounds that inhibited 65% in the FP screen. Numbers are assigned based on the positioning of the compounds within the source plates, followed by the reference code for each molecule assigned by Roche.

Fig. 3.

Assessment of the IC_{50} values for the nine Roche Published Kinase set molecules. a. Representative dose-response curves with HK853. Solid line represents inhibition without detergent, and dotted line denotes the addition of 0.1% Triton X-100. FP data from eight concentrations of inhibitor were used to generate DRCs in GraphPad Prism. Numbers in the plots are IC_{50} values. Large differences in the two curves is suggestive of aggregation-induced inhibition. As a result, compounds **46** (red), **81**, **110**, and **161** were removed from further testing. Assays were performed in $n = 1$ due to the limited quantity of each compound that was available. b. IC_{50} values obtained with all nine lead compounds. Grey highlight indicates substantially different curve shape and/or IC_{50} values.

Fig. 4.

Assessment of the ability of **32** to impact biofilm formation in *S. aureus* and *P. aeruginosa*. Values are plotted as “percent response” in comparison to a DMSO control. Optical density and resazurin were measured (in MSSA) to illustrate the ability of this compound to act as a bactericidal agent. Biofilm formation was assessed using a crystal violet assay. Given the limited quantity of compound available, only one replicate could be performed.

Fig. 5. Docking of compound **32** into HK853 (PDB:3DGE). Illustrates hydrogen bonding between core nitrogen atoms and key, conserved active-site residues such as Gly 415, Asp 411, and Asn 380.