Published in final edited form as:

Sci Transl Med. 2013 September 18; 5(203): 203ps12. doi:10.1126/scitranslmed.3006670.

All Signals Lost

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Abstract

Inhibitors of bacterial histidine kinases that globally deactivate bacterial signaling may offer a new offensive against antibiotic resistance.

New antibacterial targets are vital to combat the alarming rate of resistance development to available drugs. Histidine kinase (HK) proteins are the primary means by which microbes sense and respond to their environment. Thus, broad-spectrum HK inhibitors should render bacteria largely blinded to their surroundings, resulting in decreased viability, pathogenicity, and virulence.

WINDOW TO THE WORLD

HKs are the initiating proteins in bacterial two-component systems (TCSs) that translate environmental cues into an intracellular phosphorylation network, enabling bacteria to respond to the outside world. Each HK detects one or several of a diverse array of extracellular stimuli and catalyzes autophosphorylation of a conserved histidine residue. The phosphoryl group is subsequently transferred to a conserved aspartate on the HK's cognate response regulator (RR), the second protein constituent of the TCS. Phosphorylated RRs can perform many functions but often regulate gene transcription (Fig. 1) (1).

A well-studied example of TCS-regulated stimulus-and-response behavior is the PhoP/PhoQ pair (RR and HK, respectively) in *Salmonella typhimurium*. When *S. typhimurium* infects a host, PhoP/PhoQ activity is repressed in part by high extracytoplasmic concentrations of Mg²⁺ in serum. Once bacteria invade epithelial cells, PhoQ senses the decreased Mg²⁺ concentration within phagosomes, shifts to an activated state, and phosphorylates PhoP. This phosphotransfer event initiates the expression of more than 40 bacterial proteins, some of which facilitate host cell invasion, intramacrophage survival, and resistance to antimicrobial peptides (2).

In fact, TCSs are often crucial for bacterial survival, virulence, and promoting pathogenic responses. HKs have been implicated in acute and chronic infections caused by both Gram-negative and Gram-positive bacteria, and many of the phenotypes of infectious agents are consequences of TCS signaling (for example, quorum sensing in pneumonia and antibiotic

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resistance in *Staphylococcus* infections) (3). Experimental results from mouse infection models suggest that genetic inactivation of HK- or TCS-encoding genes attenuates virulence-associated signaling. For example, a *retS* mutant of *Pseudomonas aeruginosa* yields drastically decreased colonization of the lungs and dissemination to the liver and spleen relative to control strains (4), and mice infected with a *graRS* mutant of *Staphylococcus aureus* display fewer bacteria in their kidneys compared to those infected with control bacteria (5). Together, these findings suggest that therapeutic targeting of HKs may generate a new class of antibacterials with a mechanism of action that has not yet been exploited in the clinic.

WHY TRY AGAIN?

In the late 1990s, researchers began to identify HK inhibitors, but these compounds were ineffective as antibacterial drugs because of uncompetitive kinetics with adenosine 5'-triphosphate (ATP), inadequate selectivity, or nonspecific partitioning into both eukaryotic and bacterial cell membranes (6, 7). However, there are strong arguments for giving HK inhibitors a second look.

First, in the past decade, researchers have examined the causes of earlier failures and found that they likely stem from the fact that compounds populating pharmaceutical libraries are designed for eukaryotic protein targets. A recent analysis of the physicochemical properties of known antibacterial agents demonstrated that these molecules are higher in molecular weight and polarity than are most compounds found in the Comprehensive Medicinal Chemistry database (8, 9).

Furthermore, HK proteins are pervasive in all bacteria, each typically averaging ~20 TCSs (1), including the ESKAPE pathogens—organisms that pose the highest risk in nosocomial infections. Despite their decoration with individual sensing motifs, HKs share a highly conserved catalytic core, which presents an opportunity to discover and design HK inhibitors that disable numerous pathogens and simultaneously block many TCSs. Inhibitors that disarm multiple signal transduction networks in a single cell could conceivably cut off all detours that lead to retention of virulence or survival by the bacterium. Furthermore, multitargeted therapy is hypothesized to decelerate drug-resistance development because the mutation of several drug target–encoding genes concurrently in one bacterium is a low-probability event (10).

FORM AND FUNCTION

More appealing still, TCS-associated HKs are specific to bacteria with the exception of several lower eukaryotes (1). Comparison of HKs with eukaryotic serine/threonine and tyrosine kinases shows contrasting structural features and a lack of sequence similarity. Unlike Ser/Tr and Tyr kinases, HKs possess an ATP-binding domain with a Bergerat fold—a sandwich of α helices in one layer, several mixed β strands in a second, and a discrete and flexible ATP lid (11–13)—and the fold's hydrophobic pocket displays ATP-binding residues that differ from other kinases. Although a small number of mammalian proteins have been shown to perform His phosphorylation (such as histone H4 histidine kinase and nucleoside diphosphate kinase), these proteins do not share the Bergerat fold.

However, bacterial HKs do share the Bergerat fold with the eukaryotic GHF protein family [DNA gyrase, heat shock protein 90 (Hsp90), MutL] (11, 12) and with the mitochondrial proteins pyruvate dehydrogenase kinase and branched-chain α -ketoacid dehydrogenase kinase (Fig. 2). Among these proteins, the most conserved residues are those used to anchor ATP within the binding site: an Asp in the G1 box, which binds the exocyclic amine of adenine, and an Asn in the N box, which coordinates with the α -phosphate of ATP. This implies that ATP-competitive inhibitors of bacterial TCS HKs may also bind to these important eukaryotic proteins. Thus, it will be critical to develop HK inhibitors that discriminate between bacterial HKs and other Bergerat fold-containing proteins.

Although the Bergerat fold is a structural entity, many residues within this fold are not shared among eukaryotic and bacterial proteins (Fig. 2). In the N box, HKs bind the adenine of the ATP substrate with Tyr, Phe, His, or Ala residues, the first three of which are uniquely found in the HK active site. Also in the HK N box, an Asn facilitates coordination of Mg^{2+} and phosphates, whereas in GHF and mitochondrial proteins, this position is a Glu that serves as a general base for ATP hydrolysis (11), highlighting the functional differences between HKs and ATPases. Last, the greatest variability in the Bergerat folds of HKs and GHF proteins is found in their ATP lids; these long, flexible regions share little sequence similarity, differ in conformation, and interact distinctly with ATP. A part of the lid called the F box is specific to HKs (11, 12).

Drug design strategies that take advantage of such variations could yield selective HK inhibitors, and previous studies provide reasons for optimism. Guarnieri *et al.* compared binding of Hsp90 and the PhoQ HK to three GHF inhibitors: radicicol, geldanamycin, and novobiocin. Only radicicol bound to PhoQ, but with a reduced affinity ($K_{dPhoQ} = 715 \pm 78 \mu M$ versus $K_{dHsp90} = 19 \text{ nM}$) (12) that exceeds the 10-fold selectivity sought in drug screens (9). This selective affinity was attributed to the fact that radicicol bound in different orientations within the active sites of these two proteins, which was postulated to result from differences in their ATP-lid architecture (12). In a second study, a library of Hsp90 inhibitors and their analogs was screened for ATP-competitive inhibition of PhoQ. The top hit demonstrated only low PhoQ-binding affinity, with a K_d of $391 \pm 61 \mu M$ (14). These results illustrate that a given molecule does not necessarily affect all HK and GHF proteins to an equal extent, and thus differences within the Bergerat-fold family may be exploited for rational design of selective inhibitors.

HK DRUG DISCOVERY REDUX

The design of selective HK inhibitors will depend on the integration of multiple approaches and careful attention to the extensive biochemical and structural information that has been gathered in the past decade. Of the 179 structure hits for “histidine kinase” in the Protein Data Bank, 148 of them were released after 2005. These structural data will improve *in silico* screening results by providing a more accurate depiction of compound-protein interactions in the conserved ATP-binding domain. Furthermore, a more precise understanding of the three-dimensional form and molecular movements of the HK domains that facilitate signal transduction will help to establish which small-molecule leads have the greatest possibility of binding with high potency and selectivity. For example, a recent study reported the

identification of four compounds from an in silico high-throughput screen that were subsequently found to inhibit PhoQ in vitro (15). These inhibitors also prevented bacterial invasion of HeLa cells in culture and suppressed inflammation in a mouse model infected with *Shigella flexneri*.

A combination of in vitro screens and immediate assessment of lead compounds in vivo will be necessary to discover effective HK-directed drugs. In vitro biochemical screens are the best way to identify compounds that act via a desired mechanism and are the most amenable to targeting of the conserved ATP-binding domain because HK-ligand interactions can readily be observed. In contrast, in vivo, whole-cell, or high-content screens provide better models of the effectiveness of potential drugs under physiologically relevant conditions (16). Leads from in vitro biochemical screens can be difficult to translate to intact cells and organisms; however, confirming the mechanism of action for compounds discovered by using in vivo screens is often equally challenging. To indirectly analyze HK activity in vivo, assays have been designed to detect reporter gene expression under the control of the cognate RR, but the precise mode of inhibition must then be elucidated (17).

It is vital to integrate screens that examine HK activity in the context of infection rather than relying solely on in vitro studies. Innovative screening strategies developed after the “mini Gold Rush” of HK inhibitor discovery more than a decade ago should help promote lead-compound identification. For example, whole-cell screens now often include synergy testing and use bacteria that possess resistance, weakened cell walls, or hypersensitivity to inhibitors. In addition, by analyzing motility, adhesion, quorum sensing, or the presence of toxins, virulence-targeting mechanisms can now be readily evaluated (16).

Last, recent studies suggest that natural-product libraries should be tested for potential HK inhibitors. For example, walkmycin C produced by *Streptomyces sp.* inhibits purified HKs, and this activity translates into whole-cell activity. Minimum inhibitory concentration (MIC) values ranged from 0.063 to 16 µg/mL for 22 Gram-positive strains, including the ESKAPE pathogen *S. aureus* (18).

CARVING A NEW PATH

Given the current scarcity of diagnostic tools and the financial input required to develop narrow-spectrum antibacterial drugs against the myriad pathogens we encounter, the search for broad-spectrum agents that target multiple proteins represents a viable path for treatment of many microbial infections. However, as with all broad-spectrum agents, the host microbiota may also be affected. Reduction of the detrimental effects of antibacterial treatments on the microbiome has recently been accomplished through molecule selection and design or through coadministration with an absorbent to protect the large intestine (19, 20).

Characterization of HK activity throughout the course of infection also will be essential for the development of HK-targeting drugs (21–24). If HKs perform essential functions only before a patient experiences symptoms, then treatment with an HK inhibitor may not clear the infection. For an HK-directed drug to suppress or reverse an infection, HK activity must persist or become functional after symptoms appear. To determine the feasibility of medical

interventions that target HKs, we must be precisely informed about the chronology of HK functions throughout infection.

Because HKs have never been drugged, their appeal as an antimicrobial target is captivating. Microbes have found myriad mechanisms to overcome all known antibacterial agents, and a new approach to fight infections will require that we take the road less traveled.

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Fig. 1. Function

Bacterial HKs promote pathogenesis. Bacteria progress through microfold cells in the intestinal epithelium to macrophages, where they are encapsulated in phagosomes. Environmental signals inside these vesicles can be sensed by bacterial HKs, initiating a phosphorylation cascade (ATP to HK to RR) that results in the regulation of gene expression.

Fig. 2. Form

Represented as a cube, the ATP-binding pocket comprises various homology boxes (illustrated by shading). Structural differences between (A) GHL adenosine triphosphatases (ATPases) and (B) HKs are indicated: (1) HKs possess a conserved stacking interaction that positions adenine in the N box (yellow). (2) A conserved Glu in GHL ATPases, which acts as a general base for γ -phosphate hydrolysis, is replaced by an Asn in HKs that coordinates the Mg^{2+} ion and phosphates (N box). (3) GHL ATPases use more main-chain nitrogens to coordinate α - and γ -phosphates. (4) The F box is unique to HKs and forms a hydrophobic face in the ATP-binding pocket (green). (5) G2-box interactions with ATP are not conserved between GHL ATPases and HKs (teal). (6) HKs lack extra β strands seen in GHL ATPases (brown). (7) ATP-lid length, structure, and conformation upon ATP binding vary among GHL and HK proteins (13, 25, 26). Red circles symbolize residues in the pocket. (C) Conserved motifs are rendered in their corresponding colors onto the structure of PhoQ. AMP-PNP molecule abstracted from the PhoQ structure (1ID0) (25). Representative GHL proteins (PDB code): Hsp90-ATP (1AM1), Hsp90-AMPPNP (3H80), MutL-AMPPNP (1B63), GyrB-AMPPNP (1AJ6).