Histidine Kinases as Antimicrobial Targets: Prospects and Pitfalls

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Abstract: Histidine kinases are ubiquitous molecular sensors that are used by bacteria to detect and respond to a myriad of environmental signals. They are attractive antimicrobial targets because of their roles in mediating the virulence of pathogenic organisms, as well as the ability of bacteria to resist host defenses and develop resistance to antibiotics. In this review, we discuss the challenges involved in developing specific inhibitors of this highly diverse group of kinases.

Key Words: Histidine kinase, sensor kinase, two-component system, infectious disease, antimicrobial, antibiotic resistance.

1. INTRODUCTION

For an organism to grow and prosper, it must be able to detect and adapt to environmental changes. Phosphotransfer-mediated signaling pathways are vital tools used by both prokaryotic and eukaryotic cells to sense and respond to environmental stimuli, both from inside and outside the cell. In bacteria, the almost omnipresent device for phosphotransfer-mediated signal sensing is known as a two-component system (TCS). TCSs comprise a pair of proteins: a histidine kinase (HK) and a response regulator (RR). The HK serves as a molecular sensor that is responsible for detecting the environmental cue and autophosphorylating in response to the stimulus. As its name suggests, the RR is a molecular switch that accepts phosphate from the phosphorylated HK and subsequently mediates the bacterium's response to the initial stimulus. The RR is usually, but not always, a transcription factor [1], and hence the output response is typically a change in the pattern of gene expression.

Bacteria use HKs to respond to a myriad of environmental signals. However, it is their involvement in mediation of antibiotic resistance and virulence that makes HKs particularly interesting from a medical perspective. Since these kinases are so important to bacteria, but are not present in higher eukaryotes, they have become seductive targets for the development of a new class of antimicrobial drugs. Unfortunately, attempts to develop such drugs have proved unsuccessful, largely due to our limited understanding of the way that HKs function, and the mechanisms by which their activity is regulated. In this review, we describe recent advances in our understanding of HK structure, function, and regulation, and discuss how these advances might impact on current and future attempts to develop therapeutically useful HK inhibitors.

2. HK MEDIATION OF BACTERIAL VIRULENCE AND ANTIBIOTIC RESISTANCE

Bacterial HKs regulate a diverse array of cellular processes including osmoregulation, chemotaxis, photosensitivity, virulence, and antibiotic resistance [2]. Some HKs are essential for bacterial viability, an attribute that makes them attractive antimicrobial targets. A prototypic example is the YycG sensor kinase found in all low G+C Gram-positive bacteria, including pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, and *Enterococcus faecalis* [3-7]. The underlying reasons for the essentiality of YycG remain to be determined, but it presumably stems from an ability to mediate key cellular housekeeping functions. Recent studies have suggested that YycG plays a critical role in regulating the expression of genes involved in fatty acid and cell wall biosynthesis [8-10].

Many HKs have been identified that are not essential for cell survival but which are critically important for virulence. There are numerous examples of bacterial strains that have attenuated virulence due to the inactivation of one or more TCSs [11,12], and specific virulence-associated roles have been established for some HKs. In *Strept. pneumoniae*, for example, eight HK/RR pairs, and one orphan RR, are required for virulence in a mouse respiratory tract model [5]. In *Brucella abortis*, the BvrR/BvrS TCS is essential for cell invasion and intracellular survival by the bacterium [13,14]. Other examples of HKs that are critical for bacterial virulence include the BvgA/BvgS system of *Bordetella pertussis*, which controls biofilm formation and the expression of virulence genes [15], the *Chlamydia trachomatis* CtcB/CtcC pair, which is predicted to regulate the parasite's obligatory intracellular development [16], and the PhoQ/PhoP system used by *Pseudomonas aeruginosa* to regulate the modification of lipid A and other virulence factors, including those necessary for resistance to antimicrobial peptides produced by host cells [17-20].

HKs also mediate the resistance of several clinically important pathogens to key antibiotics such as penicillin and vancomycin. Resistance to vancomycin, one of the last lines of defense in antibiotic therapy, has ballooned in the bacterial pathogen population over the past 15 years. Vancomycin resistance in *E. faecalis*, which is mediated by the VanS/VanR TCS [21], spread from one clinical isolate in 1988 to the point where 52% of isolates were vancomycin resistant in 1999 [22]. In the United States, the frequency of penicillin resistance in populations of *Strept. pneumoniae*, which is mediated by the CiaH/CiaR TCS, increased from ~14% in 1993 to ~25% in 1997 [23] Clinical isolates of daptomycin-

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resistant *Staph. aureus* possess mutations in HK genes [24], while other HKs mediate resistance to antibiotics such as bacitracin [25].

TCSs constitute ~1% of all encoded proteins in eubacterial genomes [26]; as of May 2007, over 16,000 bacterial HKs had been catalogued by InterPro (Accession No. IPR005467). Many of these HKs are likely to be involved in mediating virulence, antibiotic resistance, or key cellular functions, and hence these kinases might be potential therapeutic targets.

3. THE MODULAR THREE-DIMENSIONAL ARCHITECTURE OF HISTIDINE KINASES

HKs and RRs have a modular architecture with versatile conserved domains that are readily adapted to the specific needs of individual systems [26,27] (Fig. **1A**). All HKs contain a conserved ATP-binding catalytic domain (the Cat or CA domain) that encodes the protein's kinase activity [28]. The Cat domain forms an α/β sandwich comprising a relatively flat five-stranded β sheet flanked on one side only by three α helices [29]. This unconventional Bergerat fold is unrelated to the catalytic domains of mammalian Ser/Thr and Tyr kinases. The core of the HK is composed of a four-helix bundle that comprises two antiparallel α-helices from each monomer (Fig. **1D**). This domain, termed the dimerization/histidine phosphotransfer (DHp) domain [30], mediates dimerization of the HK, which is essential for its biochemical functions, and it usually contains the histidine residue that is capable of receiving and donating a phosphate moiety. The Cat and DHp domains together are termed the autokinase (AK) domain.

Upon activation, the HK performs a *trans*-autophosphorylation reaction in which the ATP bound at the active site of one of the Cat domains is used to phosphorylate the His sidechain on the DHp domain *of the other monomer* (Fig. **2**). The resultant high-energy phosphoimidazole group is capable of donating the phosphoryl group to a conserved Asp sidechain on the downstream RR, thereby altering its activity and inducing an output response to the initial stimulus [34].

HKs are typically transmembrane sensors, with the components just described lying inside the cell at the C-terminal end of the molecule. The N-terminus of the molecule is devoted to signal sensing and signal transmission modules, which are highly variable in sequence and structure. In some instances the entire kinase is cytoplasmic, but in most cases part of the N-terminus lies outside the membrane, often flanked by one or more transmembrane (TM) domains (Fig. **1A**). The N-terminal sensor region of HKs is highly modular, and can become quite elaborate, often comprising multiple protein modules such as GAF, HAMP, and PAS domains (Fig. **1A–C**). These can either be part of the external sensor, or they can lie inside the membrane, between the external sensor module and the catalytic core, apparently regulating transmission of the input signal to the autokinase domain. In

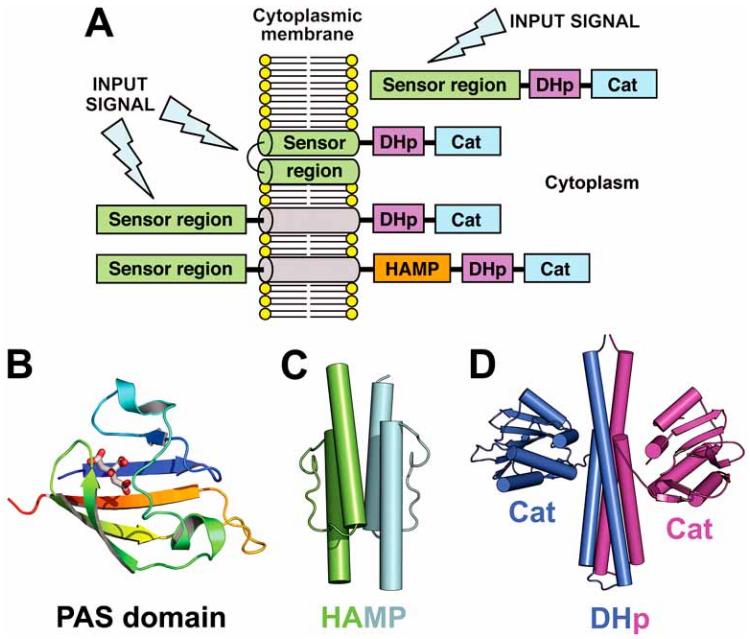


Fig. (1). (A) Cartoon of the domain architecture and topological arrangement of histidine kinases. The N-terminal sensor region (sage) can be intracellular, extracytoplasmic, or embedded in the cytoplasmic membrane. In membrane-tethered histidine kinases, an intracellular HAMP domain is often interposed between the transmembrane region and the cytoplasmic autokinase domain. The C-terminal autokinase region contains structurally autonomous catalytic (Cat) and dimerization/histidine phosphotransfer (DHp) domains (shown in cyan and magenta, respectively). **(B)** Richardson representation of the structure of the periplasmic PAS domain of the CitA sensor kinase; the bound stimulatory ligand (citrate) is depicted in red and gray tubes [31]. Color is ramped from blue at the N-terminus to red at the C-terminus. The sensor region of bacterial HKs commonly comprises one or more PAS domains. **(C)** Structure of the HAMP domain from *Archaeoglobus fulgidus* Af1503 [32]. This domain forms an unusual parallel coiled coil comprised of four helices, with two helices being contributed by each monomer (shown in green and cyan). **(D)** Structure of the entire autokinase region from the *Thermotoga maritima* histidine kinase TM0853 [33]. The autokinase region dimerizes by virtue of the four-helix bundle formed from two monomers of the DHp domain (shown in blue and magenta). In this view, the N-terminal sensor region would project from the top of the molecule.

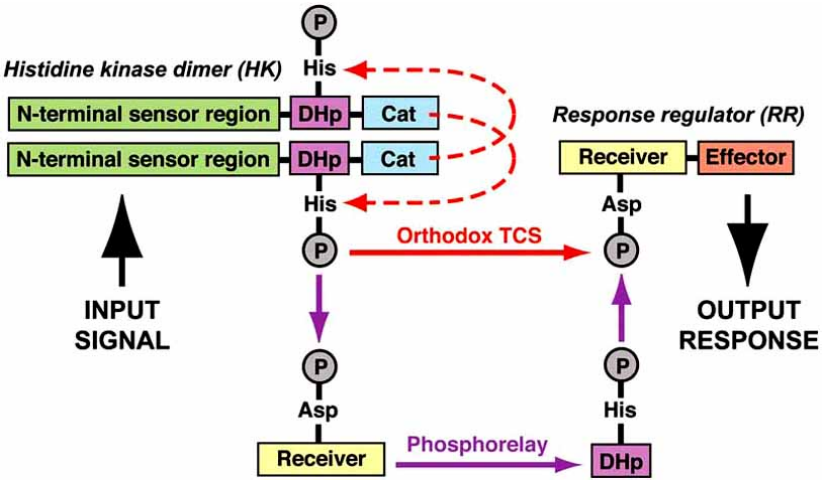


Fig. (2). Cartoon of phosphate flow in histidine kinase signaling pathways. In an orthodox two-component system (TCS), the input signal induces the catalytic domain (Cat) of the HK to phosphorylate a His residue on the DHp domain *of the other monomer* in the HK dimer. The phosphate is then passed directly to an Asp residue on the receiver domain of the cognate RR, leading to altered activity of the effector domain. In a His→Asp→His→Asp phosphorelay, the phosphate is first passed from the DHp domain of the HK to the receiver domain of an intermediate protein and then *via* an autonomous DHp domain to the downstream RR. Phosphorelays provide additional nodes for regulation of the HK signaling pathway.

other cases the sensor can have multiple TM helices that appear to act in concert to detect and transmit signals (e.g., the MASE1 and MASE2 domains [35]) (Fig. **1A**). The elaborations of the N-terminal sensor region of HKs appear almost limitless, and the reader is referred a recent review for further details [36].

The HKs just described are classed as orthodox, meaning that after autophosphorylation they pass the phosphate group directly to the RR. Some orthodox HKs use the conserved Asp and His groups of accessory transfer proteins to pass the phosphate to the response regulator, resulting in a multi-step His→Asp→His→Asp phosphorelay (Fig. **2**). To further complicate matters, there is a second class of "hybrid" kinases that utilize multiple additional internal phosphotransfer domains. These modules, which are located C-terminal to the autokinase domain, carry conserved Asp and His residues that mediate passage of the phosphate group to its final destination [37]. Some hybrid kinases even incorporate one (or more) response regulator/s, thus becoming an all-in-one multipurpose sensor/effector.

4. GENERIC HK INHIBITORS: REALITY OR PIPE DREAM?

Clearly, developing a universal inhibitor for such a disparate group of enzymes is likely to be a daunting task, but there are several avenues of approach that can be considered. An inhibitor could block signal sensing, either by blocking the ligand binding site, or by impeding the conformational changes in the sensor region that transmit information about the presence or absence of stimuli. Such an inhibitor is likely to be specific to subgroups of HKs that contain similar types and arrangements of sensor domains, since the sensor domains and the signals they recognize vary greatly across the HK family. An inhibitor could also block ATP binding by the Cat domain, or prevent phosphate transfer from the Cat domain to the His residue on the DHp domain by occluding the ATP binding site. Such inhibitors might be active against most HKs, as the ATP-binding domain is highly conserved.

HKs could also be inhibited by blocking or reversing the autophosphorylation event. An effective way to inhibit autophosphorylation is to block HK dimerization, since the autophosphorylation reaction occurs in *trans* and is therefore dependent upon dimer formation. Finally, an inhibitor could block activation of the RR, either by blocking access of the RR to the phosphohistidine on the DHp domain, by occluding the Asp residue on the RR that usually receives the phosphate group, or by dephosphorylating the RR.

In a concerted attempt to unmask new classes of widely useful antimicrobials, many academic and pharmaceutical labs have screened small-molecule libraries to isolate compounds that inhibit model HKs, primarily using *in vitro* assay systems. This search has yielded numerous potential inhibitor molecules, many of which share common features. They are generally large, aromatic, planar, hydrophobic molecules, and their chemistry has been reviewed [21,38]. Many of these compounds are proprietary, and consequently the details of their mechanism of action are not always available. Where details have been published, however, the results are less than encouraging. They tend to exhibit poor specificity for HKs, and if they do kill bacteria, they often do so by non-specific mechanisms that are independent of their ability to inhibit HK activity [21,39]. In addition, they often cytotoxic due to undesirable surfactant and membrane disrupting properties [39]. By 2002 there were already nine families of such compounds—salicylanilides, imidazoliums, bis-phenols, isothiazolones, trityls, benzoxazines, cyclohexenes, benzimidazoles and diaryltriazoles [21]—but none contained a broad-spectrum, HK-specific inhibitor that had antibacterial activity without generalized cytotoxicity.

This failure of large-scale library screening to yield desirable results has fostered more rational approaches towards

the development of HK inhibitors. These include structure-based discovery approaches, the development of chemical mimetics of natural inhibitors, refinement of initial lead molecules based on SAR data, and a concerted search for endogenous HK ligands and inhibitors that might act as drug leads or provide new paradigms for HK inhibition. In addition, there have been significant recent advances in understanding the three-dimensional architecture of HKs, as well as the molecular mechanism of signal transduction, which should facilitate the rational development of anti-HK chemistry. In the following sections, we review recent progress in each of these areas.

5. SIGNAL SENSING AS A TARGET FOR CHEMICAL INHIBITION OF HISTIDINE KINASES

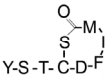
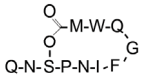
In the majority of orthodox HKs the sensor region is situated towards the N-terminus of the protein, and it is extracytoplasmic, although, as noted above, this is not always the case. Since HK sensors are so structurally, functionally, and chemically disparate, if one is to develop an inhibitor to the sensor of an HK, one has to consider the mode of sensing, the event that is sensed, the location of the sensor, and the way in which the sensing event is relayed to the catalytic core of the protein. Unfortunately, our understanding of all of these events is still in its infancy [25,36]. With a few notable exceptions, the ligands bound by HKs during sensing are unknown, and it is not always clear whether it is the presence or the absence of the ligand that activates the HK. In some cases, the putative sensor domain is not even necessary for the HK's response to the putative activating ligand or condition, indicating that the "sensor" is not always a *bona fide* target for inhibitor development [40].

Perhaps the "best bet" for developing inhibitors of HK signal sensing and/or intramolecular signal transduction lies in the development of compounds that mimic known sensor-domain ligands, and which can irreversibly bind to and desensitize the HK. Preferably, the targeted sensor domain would be external to the bacterial cytoplasmic membrane, which would obviate the complication of developing an inhibitor that had to traverse the bacterial inner membrane (the outer membrane of Gram negative bacteria and the thick peptidoglycan layer of Gram positive bacteria are both typically more permeable to solutes than the cytoplasmic membrane).

5.1. AIP Derivatives as Global Inhibitors of *Staphylococcus* virulence

One group of relatively well characterized sensor-domain ligands are endogenous peptides involved in the induction of competence (i.e., the ability to take up DNA), virulence, and other functions such as bacteriocin synthesis (Table **1**). Signal peptides are often more amenable to identification than other HK ligands since the genetic loci responsible for their production are usually adjacent to the gene encoding their receptor HK. Gram-positive bacteria use peptides for quorum sensing, which is often involved in the onset or control of virulence processes [41-43]. These peptides can be readily synthesized, making it possible to determine structure-activity relationships to aid the design of receptor antagonists that could be used therapeutically [44,45].

Table 1. Examples of Histidine Kinases that are Activated by Endogenous Peptides

Histidine Kinase	Signal Peptide	Sequence	Organism	References
Competence induction				
ComP	ComX	G-I-F-W*-E-Q	*Bacillus subtilis*	[50-53]
ComD	CSP/ComC	E-M-R-L-S-K-F-F-R-D-F-I-L-Q-R-K-K	*Streptococcus pneumoniae; Streptococcus mutans; Streptococcus gordonii*	[54-57]
Virulence induction				
AgrC	AIP[#]		*Staphylococcus aureus; Staphylococcus epidermis; Staphylococcus intermedius*	[43]
FsrC/ FsrA	GBAP		*Enterococcus faecalis*	[58]
Induction of bacteriocin synthesis				
PlnB	PlnA	K-S-S-A-Y-S-L-Q-M-G-A-T-A-I-K-Q-V-K-K-L-F-K-K-W-G-W	*Lactobacillus plantarum*	[59]
BlpH	BlpC-1, -2, -3	G-L-W-E-D-L-L-Y-N-R-Y-A-H-Y-I-T	*Streptococcus pneumoniae*	[60]

*The Trp sidechain is modified by covalent attachment of a geranyl group

[#]The AIP shown is AIP-1 from *Staph. aureus*. This staphylococcus utlizes four AIPs, all of which are short, thiolactone macrocyclic peptides that are recognized by a cognate HK. Other staphylococci utilize different AIPs, which are also peptidic macrocycles, but which sometimes contain lactone or lactam linkages instead of the thiolactone shown here.

One of the best studied bacterial peptide signaling systems is encoded by the *agr* locus of *Staphylococcus. Staph. aureus* unleashes a barrage of weapons during the first three hours of host infection [46]. Secreted toxins that attack host cells or interfere with the immune system, tissue-degrading enzymes, and cell-wall-associated proteins that may be involved in adhesion and protection against host defenses, all contribute to its successful virulence. The global regulatory locus, *agr,* which encodes the AgrC/AgrA TCS, is largely in control of this process. The AgrC HK recognizes, and autophosphorylates in response to, a processed autoinducing peptide (AIP) that is encoded by another gene in the *agr* locus. The AIP/AgrC pair shows significant interstrain variation, and there are at least four *Staph. aureus agr* specificity groups (Groups I–IV) [47,48]. Remarkably, however, the AIP of each group exhibits inter-group *inhibition* of AgrC-mediated virulence with an IC_{50} in the same range as the EC_{50} for *activation* of its cognate intra-group AgrC.

AIPs are typically 7–9 residues long, and they contain an unusual 16-membered thiolactone ring structure in which the α-carboxyl group of the C-terminal amino acid is linked to the sulfhydryl group of a cysteine, which is always the fifth amino acid residue from the C-terminus of the peptide (e.g., *Staph. aureus* AIP-1, compound **1**, Fig. **3**) [49]. In addition to the five residues that form the thiolactone ring, there is usually an exocyclic "tail" of 3–4 amino acids N-terminal to the cysteine. The thiolactone macrocycle appears to be necessary for activation of the virulence response, as the corresponding lactone and lactam analogues (i.e., those with oxygen or nitrogen in place of sulfur in the ring structure), as well as linearized versions of the AIP, are inactive against their cognate HKs [49]. However, as for the native AIPs, the macrocyclic lactam and lactone analogues (but not the linear mutants) are potent *intergroup inhibitors*. These findings indicate that the AIP ring structure is critical for activity, and that the chemical nature of the ring determines the *type* of activity conferred (i.e., agonist or antagonist).

The observation that cross-inhibition is more tolerant of sequence and structural diversity in the AIP than activation of intragroup HKs has led to facile development of AIP-based HK inhibitors. A simple AIP-1 derivative in which the native Asp5 residue is replaced with Ala (compound **2**, Fig. **3**) potently inhibits both its cognate HK (AgrC-1, Group I) and ArgC-2 (Group II) with IC_{50} values of 21 and 4 nM, respectively [61]. Replacement of Asp5 with 2-aminobutyric acid (Abu) yielded a derivative (compound **3**, Fig. **3**) with increased inhibition of ArgC-2 (IC_{50} = 2.8 nM) but reduced antagonism of ArgC-1 (IC_{50} = 137 nM) [61]. Remarkably, with the exception of the final two residues in the macrocyclic ring, the replacement of *any residue* in *Staph. aureus* AIP-II with alanine leads to a derivative that inhibits the heterologous *agr* response in Group I cells with greater potency than native AIP-II (IC_{50} < 1 nM, compared with 3 nM for native AIP-II) [49]. A tail-free derivative of AIP-II is a global inhibitor of the *agr* response in all *Staph. aureus* specificity groups [45], but it is not as potent as the Ala derivatives described above (IC_{50} = 10–270 nM).

Clearly, there is much scope for further exploration of this mechanism of inhibiting *Staphylococcus* virulence. In addition to the four *agr* specificity groups in *Staph. aureus*, there are at least 20 more in other staphylococci [62], and the discovery that both *Staph. epidermis* and *Staph. intermedius* synthesize macrocyclic AIPs [63,64] indicates that AIP-like HK inhibitors might become important therapeutic weapons against this clinically important group of bacteria. It should be noted, however, that the therapeutic utility of AIP derivatives might be limited by the poor bioavailability that is often inherent to peptide-based drugs because of their proteolytic sensitivity *in vivo* and their limited penetration of human intestinal mucosa [65]. Although cyclic peptides are typically more stable *in vivo* than their linear counterparts [66], the thiolactone macrocycles are likely to have limited *in vivo* half-lives in humans since, at physiological pH, the thioester linkage will be slowly hydrolyzed, resulting in an inactive and protease-sensitive linear peptide. Lactone and lactam macrocycles should be more stable *in vivo*, and consequently they might represent better starting points for the development of global inhibitors of *Staphylococcus* virulence.

5.2. Hijacking Human Hormones to Develop Novel Antimicrobials

Another potential antimicrobial target is the sensor domain of the HK used by enterohemorrhagic *Escherichia coli*

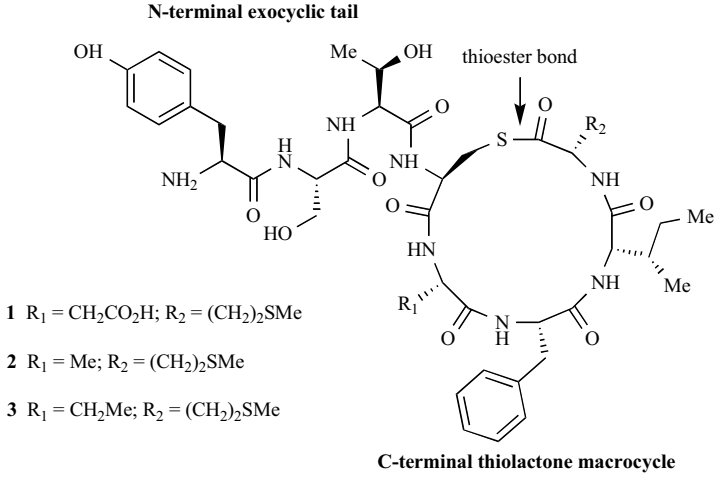


Fig. (3). *Staph. aureus* AIP-1 (**1**) and derivatives (**2**, **3**) that antagonize its cognate histidine kinase AgrC.

(EHEC) O157:H7 to activate transcription of virulence genes. EHEC O157:H7 is the etiological agent of hemorrhagic colitis and hemolytic uremic syndrome [67]. Upon entering the human colon, EHEC produces Shiga toxins that are both potently cytotoxic and able to promote colonization of epithelial cells [68]. EHEC O157:H7 directs production of Shiga toxins and other virulence-associated proteins through their synthesis and release of AI-3, a quorum-sensing molecule. The structure of AI-3 is unknown, but the mammalian hormones epinephrine (compound **4**, Fig. **4**) and norepinephrine (compound **5**, Fig. **4**) cross-talk with the AI-3 quorum sensing system [69], suggesting that they have structural homology with AI-3. Indeed, EHEC O157:H7 takes advantage of the mammalian hormones to activate its virulence activity upon infection of the host gut [70].

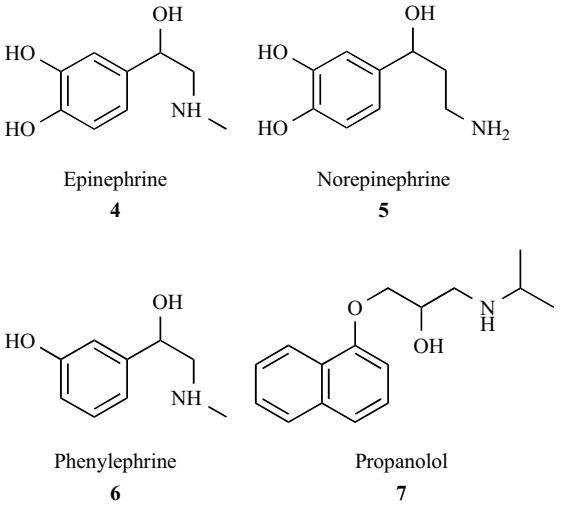


Fig. (4). The human hormones epinephrine (**4**) and norepinephrine (**5**) and analogues (**6**,**7**).

It was recently shown that the bacterial receptor for AI-3, epinephrine, and norepinephrine is the histidine kinase QseC, whose cognate RR is QseB [71]. In silico analysis indicates that the sensor domain of QseC is widely distributed, being present in numerous Gram-negative pathogens such as *Salmonella*, *Shigella*, *Yersinia*, *Haemophilus*, and *Pasteurella*. Thus, there may be a whole class of bacterial HKs that parasitize the human hormone signaling system and use it as a weapon against the host. It is possible, therefore, that derivatives of the human hormones could be used to inhibit these HKs. Reasoning that an α-adrenergic agonist might be active against a molecule that recognizes catecholamines, Clarke and coworkers [71] tested the ability of propanolol (compound **7**, Fig. **4**) and phenylephrine (compound **6**, Fig. **4**) to inhibit QseC activity in an *in vitro* model. Although propanolol was not effective, phenylephrine reduced the response of QseC to epinephrine, and thus it provides a lead in the search for inhibitors of EHEC virulence.

6. INHIBITING INTRAMOLECULAR SIGNAL TRANSDUCTION

An alternative to inhibiting signal recognition by the HK is to block intramolecular transmission of the signal recognition event to the catalytic core of the kinase. Unfortunately, however, the method of intramolecular signal transmission in HKs is still poorly understood. It is clear that some sensor domains can undergo structural perturbations upon ligand binding or signal recognition. For example, Per-Arnt-Sim (PAS) domains, which are commonly found in the sensor region of HKs, undergo subtle, but well documented, structural rearrangements upon signal sensing [72-74]. It is not clear, however, how this structural rearrangement is reported to the catalytic core, and hence, how this event could be inhibited.

Recent studies on the HAMP domain, which is present in over 10,500 bacterial proteins including HKs, adenylyl cyclases, chemotaxis receptors, and phosphatases [75] (see InterPro entry IPR00360), have begun to reveal details of how this mysterious event might occur in the subclass of HKs containing cytoplasmic HAMP domains. About one fifth of all HKs contain an intracellular HAMP domain, a 50-residue sequence located immediately C-terminal to, and continuous with, the last helix of the TM domain [32]. The HAMP domain is thought to play a crucial role in transducing the signal from the sensor to the catalytic core, which generally lies immediately C-terminal to it. Experiments in which the HAMP domains were switched between HKs, or between HKs and chemotaxis receptors, yielded chimeras that had altered activities, but which retained function [76-78]. This suggests that there must be some universality to the mode of HAMP action.

Recent NMR structural analyses of a prototypic HAMP domain [32] indicate that it forms a homodimeric four-helical, parallel coiled coil, with two helices contributed by each monomer (Fig. 1C). The interhelical packing is unusual in that it relies on knobs-into-knobs packing rather than the knobs-into-holes interactions typically associated with non-engineered coiled coils [79,80]. On the basis of this structure and studies of a mutant that oscillates between two different coiled-coil conformations, it was proposed that the HAMP domain relays the signal recognition event to the catalytic core of the kinase *via* a concerted 26° rotation of all four helices in the coiled coil, thus converting it from knobs-into-knobs to canonical knobs-into-holes packing [32]. This proposed rotary mechanism, along with the high-resolution structure of the HAMP domain, opens the door to modeling other HAMP domains, and to determining which residues lie on the surface regions that are buried by the helical rotations. It may be possible to design drugs that bind these surfaces, thus blocking helix-rotation and HAMP-mediated switching. Unfortunately, since HAMP domains display very limited sequence conservation, it is likely that specific inhibitors would have to be developed for each target HK.

7. MASTERS OF THEIR OWN DOMAIN(S): HISTIDINE KINASES AS SELF-INHIBITORS

Recent studies on *Bacillus anthracis* have raised the possibility that fragments or domains of HKs could be self-inhibitory (i.e., act as dominant negatives) at the level of ligand sensing [81]. Like other *Bacillus* species, *B. anthracis* is capable of forming metabolically dormant endopores when deprived of nutrients. However, during infection, this process (sporulation) is likely antithetical to pathogenesis. The decision to sporulate is controlled by a HK phoshophorelay

that determines the level of phosphorylation of the master transcriptional regulator SpoOA [2]. It appears that *B. anthracis* has developed a novel method of limiting the activity of this phosphorelay during pathogenesis.

B. anthracis produce two proteins (pXO2-61 and pXO1-118) that are encoded by the virulence plasmids pXO2 and pXO1, respectively. The pXO proteins are ~30% identical to the sensor domain of BA2291, the major sporulation HK of *B. anthracis* [82]. BA2291 induces sporulation when expressed in *B. subtilis* or *B. anthracis*, but when co-expressed with pXO2-61 or pXO1-118, it becomes an effective *inhibitor* of *Bacillus* sporulation [81]. Taken together with other evidence derived from both overexpression studies of BA2291 in *B. subtilis* and *in vitro* studies of the purified HK, this finding suggests that BA2291 acts as a kinase when activated by bound ligand, but adopts the role of a regulatory phosphatase when ligand-free. Since the pXO proteins appear to regulate the activity of BA2291, they presumably either bind the activating ligand for BA2291, or otherwise disrupt the HK's ability to autophosphorylate. Based on several lines of evidence, competition for the BA2291 ligand appears most likely [81]. The ligand itself, which must be present in both *B. subtilis* and *B. anthracis*, has not been identified.

With the exception of a virulent strain of *B. cereus* associated with inhalation disease, which carries a similar system of HK paralogs on its virulence plamids [83], this is the only known bacterial system in which a partial homologue to a HK is encoded elsewhere on the chromosome. Nevertheless, it does raise the possibility that a "ligand mop" paradigm could be used as the basis for development of HK inhibitors (with all the attendant problems associated with protein drugs). In cases where ligand binding influences the state of HK activation, it may be possible to exert control over the HK *via* a homologue, or direct copy, of the HK's sensor domain. This could be an effective means by which to attempt inhibition of an HK when the ligand itself has not been identified.

8. INHIBITION OF ATP BINDING TO THE HK CATALYTIC DOMAIN

The unconventional Bergerat fold found in the HK Cat domain places it in the GHKL ATPase/kinase superfamily [84]. Because the Cat domain has multiple structural homologues in both eukaryotes and prokaryotes (e.g., DNA gyrase, the Hsp90 molecular chaperone, and the MutL mismatch repair protein), it has been considered a difficult target for the development of HK inhibitors with sufficient selectivity for therapeutic use. However, there has been considerable recent progress in the development of specific inhibitors of ATP binding to human Hsp90 [85,86], another member of the GHKL superfamily, and this has prompted re-examination of the Cat domain ATP-binding site as a potential target for HK inhibitors [87].

Several inhibitors of *Staph. epidermis* YycG were recently obtained by high-throughout virtual screening of 85,000 drug-like compounds based on their probability of interaction with the ATP-binding site in a model of the protein's Cat domain [88]. *Staph. epidermis* is a common cause of infections associated with implanted medical devices, and there has been a rapid increase in the incidence of vancomycin- and multidrug-resistant strains [89]. In silico screening led to the selection of 76 compounds for biological assay, five of which proved to be effective inhibitors of the growth of both biofilm-forming and non-biofilm-forming strains of *Staph. epidermis* with minimum inhibitory concentrations (MICs) $\leq$ 50 μM. These compounds were chemically diverse and included benzamides (compounds **8** and **9**, Fig. **5**), thiazolidinones (compounds **10** and **11**, Fig. **5**), and a furan derivative (compound **12**, Fig. **5**). The most potent inhibitor (compound **10**) had a MIC of ~6 μM against both motile and biofilim-forming strains of *Staph. epidermis*. Several compounds proved inhibitory to other pathogens that contain YycG (*Staph. aureus*, *Strept. pyogenes*, and *Strept. mutans*) but not bacteria that lack this HK (*E. coli* and *P. aeruginosa*). These compounds were shown to bind the YycG autokinase domain (with K_d values of 7–48 μM) and block the autophosphorylation reaction [88], consistent with (but not proof of) an inhibitory effect on ATP binding to the Cat domain. Importantly, in contrast with many other reported HK inhibitors, these compounds showed little cytotoxicity against mammalian cells.

TEP (compound **13**, Fig. **5**) was recently isolated as a competitive inhibitor of Cat-domain ATP binding from a high-throughput screen of Eli Lilly compound libraries [87]. TEP inhibits the autokinase activity of a range of HKs, but with only moderate affinity; the lowest reported IC_{50} was 5.5 μM against *Thermotoga maritima* HpKA, the HK used in the initial screen. TEP had IC_{50} values >20 μM against a set of 10 common mammalian Ser/Thr kinases, indicating that it does not strongly inhibit any of these enzymes. At the current stage of development, however, TEP specificity appears to be determined largely on a kinase-to-kinase basis, since its IC_{50} against the VanS HK from *E. faecium* is only 104 μM. TCP is not toxic to rat myoblasts, but it is also benign to a range of bacteria. Moreover, the effects of TEP on bacterial cell growth may be unrelated to HK inhibition, since it accumulates in the cell membrane and at incipient division sites in *Staph. aureus* cells [87].

Although TEP is not a highly effective HK inhibitor, it does provide a starting point for the development of higher affinity blockers of ATP binding by the Cat domain. However, a major concern that needs to be addressed in future studies is whether competitive inhibitors of ATP binding to bacterial HK Cat domains will be promiscuous inhibitors of structurally related GHKL family members, such as human Hsp90. Conversely, it should prove interesting to examine whether any of the recently developed Hsp90 inhibitors are active against HKs. Interestingly, it was recently shown that radicicol, a macrolide from *Monosporium bonorden* that potently inhibits Hsp90, also inhibits the Snl sensor kinase from *Saccharomyces cerevesiae* [90].

9. TARGETING THE HK DIMERIZATION DOMAIN

Because the autophosphorylation event occurs in *trans*, inhibition of dimerization is a potential mechanism for HK inhibition. An elegant high-throughput genetic assay was recently developed to screen for specific inhibitors of HK dimerization, which led to the discovery of I-8-15 (compound **14**, Fig. **6**). Although I-8-15 only weakly inhibits YycG auto-

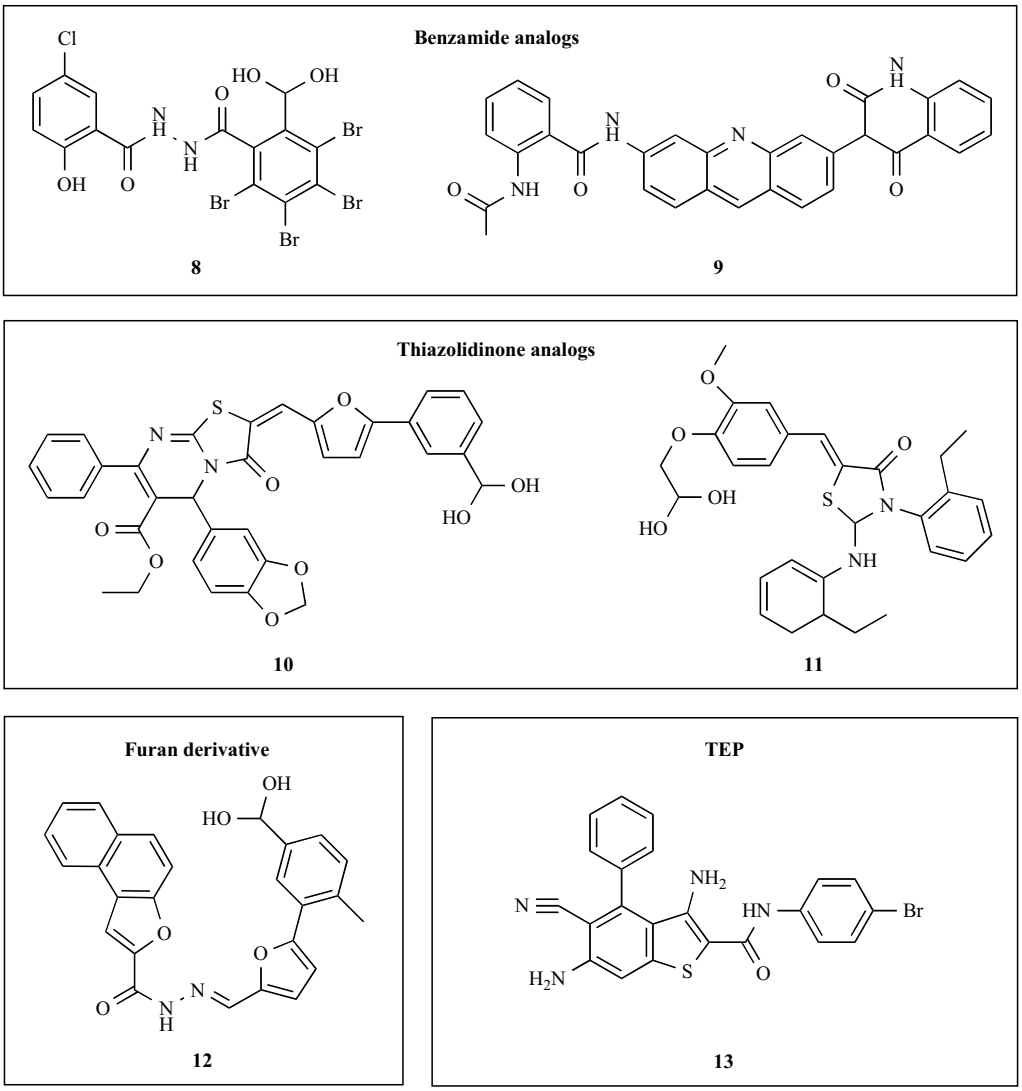
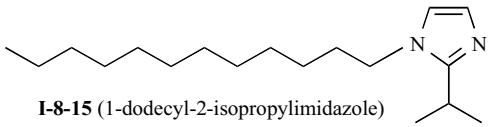


Fig. (5). Competitive inhibitors of ATP binding to histidine kinase Cat domains.

phosphorylation, with an IC_{50} of 77 μM, it exhibits antibacterial activity against methicillin-resistant *Staph. aureus* and vancomycin-resistant *E. faecalis* with MICs of 25 and 50 μg/ml, respectively. The mechanism by which I-8-15 blocks YycG dimerization, and whether it is toxic to mammalian cells, remain to be determined. Nevertheless, inhibition of dimerization appears to be a promising paradigm for the development of novel HK inhibitors.



I-8-15 (1-dodecyl-2-isopropylimidazole)

14

Fig. (6). Inhibitor of the dimerization of the YycG histidine kinase.

Are there any other features of the HK dimerization domain that make it amenable to therapeutic intervention? A 3D cluster analysis of the DHp domain of 35 HK/RR pairs revealed that the only conserved part of the DHp domain is the 3–4 residues immediately surrounding the active site His residue, with the remainder of the α helices showing high sequence diversity [91]. Thus, it appears that as long as the appropriate inter- and intra-molecular contacts are made to maintain the helical bundling, the niceties of sequence conservation can be dispensed with. In fact, the high sequence diversity in this part of the protein is presumably an advantage to bacteria, allowing them to develop highly specific endogenous inhibitors of individual HKs. *Bacillus subtilis*, for example, has developed specific antikinases, Sda and KipI, for regulating the autophosphorylation activity of KinA, its major sporulation HK [2,92-94]. Unfortunately, however, this lack of sequence conservation means that it will probably be difficult to develop broad-spectrum HK inhibitors that target the DHp domain.

10. TO BE OR NOT TO BE: WHEN IS A HISTIDINE KINASE A VALID TARGET?

Whenever one is dealing with a developmental process, the issue of timing is important. The ArgC-mediated virulence process in *Staph. aureus* is strictly temporally regu-

lated, and thus there is only a small window of opportunity during which it can be blocked. In a mouse model system, administration of synthetic AIP-derived inhibitory peptides at the same time as virulent *Staph. aureus* virtually eliminated abscess formation by the virulent strain. This was only the case, however, if the AIP was administered at the same time as the infective bacteria, since all of the HK-mediated activity necessary for abscess formation occurs in the first three hours of infection [46].

This highlights a potential limitation of antimicrobials developed against specific HKs, as any plan for targeting an HK must take into account the *time* or *stage of infection* at which the HK is essential for pathogenesis. Unlike conventional antimicrobials, which usually act at the level of cell wall synthesis, nucleic acid metabolism, or protein production (all housekeeping processes for most bacteria), drugs developed against HKs that regulate developmental processes have the potential drawback that they may only work during certain phases of infection, and in some cases, these phases may occur before the patient becomes symptomatic. For example, a drug which inhibited a HK that is critical for the establishment of infection, but not long-term survival in the host, might be useful as a prophylactic but not as a treatment for chronic infections.

One way to obviate this problem is to determine the precise stage of infection during which an HK is *essential* for pathogenesis. The idea that different proteins are essential at different times during infection was ably demonstrated by an elegant study that followed the genetic changes in *P. aeruginosa* that occurred during long-term infection of a cystic fibrosis patient [95]. Numerous genetic changes were detected over a period of eight years during the progression from acute to chronic infection. Two of the accumulated mutations were in the gene encoding the HK LadS, a major regulator of *P. aeruginosa* virulence. The other 62 HK genes were unchanged, suggesting that they are either essential for housekeeping, or are not detrimental to the bacterium's capacity to maintain a chronic infection. Combining this type of temporal genetic information with knockout data should provide a clearer picture of which HKs are valid targets for the development of therapeutically useful antimicrobials.

11. ARE THERE ARE OTHER TARGET SITES IN TWO-COMPONENT SYSTEMS?

The fact remains that no inhibitors have been developed that are active against a broad range of HKs, but which are not cytotoxic and do not display activity against eukaryotic kinases. Why is this the case? The answer probably lies in the structure (and variability) of the HK itself. As discussed above, the inherent variability in the sensor and DHp domains does not favor the development of a generic inhibitor. The presence of the Cat-domain fold in other prokaryotic and eukaryotic ATPases and kinases also makes it a challenging target that might be difficult to specifically inhibit, although efforts are still underway to develop such drugs.

How else could the activity of a target TCS be modulated? An obvious possibility is to target the cognate response regulators that receive phosphate from the HK, rather than the HK itself. Typical RRs comprise two domains—a receiver domain which contains the Asp residue that receives phosphate from the HK, and an effector domain, usually a DNA-binding domain, that mediates the output response (usually a change in pattern of gene expression). In contrast to the DHp domain of HKs, the RRs possess a large, conserved surface-exposed patch around the active-site aspartate that is used for interaction with both the HK and, in some cases, regulatory phosphatases. In addition, RRs contain a conserved set of residues that mediate the interaction between the receiver and effector domains [91]. Both of these regions are potential targets for TCS inhibition. Although there are many examples of naturally occurring regulators of RR function [96,97], to date there have been few attempts to develop drug-like inhibitors of RR function (although see Ref. [98]).

Other potential targets for TCS inhibition include the various inter-domain interactions that occur during HK sensing, autophosphorylation, and phosphotranfser to the RR. As already discussed, the intramolecular transfer of a phosphate group within an HK depends on (and is controlled by) a highly modular series of domains (Fig. **2**). It was previously thought that these domains behaved like beads on a string and orchestrated a linear relay of the input signal from the most N-terminal sensor domain to the C-terminal autokinase domain. However, the recently published crystal structure of the *T. maritima* ThkA HK [99] suggests this interpretation is naïve. In this structure, the N-terminal PAS sensor domain makes intra-monomer interactions with both the Cat domain *and* the bound RR (TrrA). This congested molecular organization suggests that the subtle conformational changes purported to occur in a PAS domain upon ligand binding could conceivably regulate both autokinase activity and subsequent phosphate transfer to the RR. This new picture of HK regulation suggests that the interaction between the accessory domains of an HK and the catalytic core of the molecule could be a critical intervention point for drugs, and these interdomain interactions should prove a fruitful avenue for further investigation.

12. CONCLUSIONS AND FUTURE PROSPECTS

Histidine kinases were first discovered in 1988 [100,101] and with the advent of large-scale genome sequencing their importance in pathogenesis has become increasingly apparent for a large number of bacteria. They are intimately involved in both virulence and antibiotic resistance, and in some cases they are essential for bacterial survival. Since HKs are not present in mammals, their potential utility as an antimicrobial target appears obvious. To date, however, it has proven very difficult to develop generic HK inhibitors that are specific, potent, and not detrimental to mammalian cells. This does not imply that the search for therapeutically useful HK inhibitors should be abandoned, but it does indicate that more rational approaches to the development of such drugs need to be implemented. In this regard, it will be critical to develop a better understanding of the way in which HKs sense input signals as well as the mechanisms by which this signal is transduced to the autokinase domain.

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