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Review Article

Histidine Kinases as Targets for New Antimicrobial Agents

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Abstract—The emergence and spread of hospital acquired multi drug resistant bacteria present a need for new antibiotics with innovative mode of action. Advances in molecular microbiology and genomics have led to the identification of numerous bacterial genes coding for proteins that could potentially serve as targets for antibacterial compounds. Histidine kinase promoted two-component systems are extremely common in bacteria and play an important role in essential signal transduction for adapting to bacterial stress. Since signal transduction in mammals occurs by a different mechanism, inhibition of histidine kinases could be a potential target for antimicrobial agents. This review will summarize our current knowledge of the structure and function of histidine kinase and the development of antibiotics with a new mode of action: targeting histidine kinase promoted signal transduction and its subsequent regulation of gene expression system.

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Introduction

Antibiotics were one the great discoveries of the 20th century. However, the administration of this class of drug has been compromised by the emergence of new antimicrobial resistant bacterial strains. The first clinical antibiotic, penicillin, was introduced in the mid 1940s, while the existence of resistant mutants was recognized within 2 years of its introduction.[1] Currently, one of the most serious problems being encountered in the clinic is an increasing number of bacterial strains with resistance to vancomycin, which is often the antibiotic used as a last resort. Multi-antibiotic resistant Gram-positive strains, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant MRSA, and vancomycin-resistant *Enterococci* (VRE), are spreading at an alarming rate.[2] Consequently, novel approaches are urgently required to treat such bacterial infections. The complete genome sequences of several bacterial strains, including the clinically important MRSA,[3] vancomycin-resistant MRSA,[3] and *Escherichia coli* O157,[4,5] have been announced, and these genome projects have provided numerous bacterial genes coding for proteins that could potentially serve as targets for novel antibacterial compounds.[6] Indeed, the bioinformatics of those strains may provide a foundation for a structure- and mechanism-based design approach to develop innovative antimicrobial therapies.

Bacterial Two-Component Systems

Histidine kinase promoted two-component systems are extremely common in bacteria and play an important role in signal transduction that is essential for adaptation to bacterial stress.[7–11] Since signal transduction in mammals occurs by a different mechanism, inhibitors of histidine kinases could act as antimicrobial agents and, as such, two-component systems have attracted much attention from medicinal chemists in the past year.[12–17] This review will summarize our current knowledge of the structure and function of histidine kinase and the development of antibiotics with a new mode of action: targeting histidine kinase promoted signal transduction and its subsequent regulation of gene expression system.

Mechanism of two-component systems

During the past decade, it has become apparent that bacteria use sophisticated signal transduction mechanisms to control specific gene expression.[7–11, 13–16] Two-component systems comprising histidine kinases and response regulators allow bacteria to adapt themselves to physical and chemical extracellular environmental changes. In this system, histidine kinases function both as sensors and signal transducers.

Figure 1 shows a model for two-component system signal transduction. Histidine kinases usually function as dimeric proteins.[18] The signal transduction cascade starts with the sensing of a signal ligand in the divergent histidine kinase sensor domain. Binding of the ligand induces an autophosphorylation of the conserved histidine residue in domain A, using the γ-phosphate of ATP.[18] In this way, the information of the outer membrane stimuli is converted into the high-energy phosphoryl group as the signal transduction is mediated by the transphosphorylation. The phosphorylated histidine kinase interacts with its mated response regulator, and transfers the phosphate to a conserved aspartate residue in the receiver domain of the response regulator.[19,20] The transfer of this phosphoryl group tunes the DNA-binding property of the response regulator and regulates the initiation of developmental transcription.[7–11,13–16]

Structure of histidine kinase

NMR structures of the autophosphorylation and ATP binding domains of histidine kinase EnvZ have been determined (Fig. 2).[18,19] EnvZ is an *E. coli* transmembrane osmosensor containing 450 amino acid residues. The C-terminal cytoplasmic segment (residue 223–450) of EnvZ consists of two discrete sections, domain A (residue 223–289) and domain B (residue 290–450).[21,22] The NMR structure of histidine kinase CheA has also been

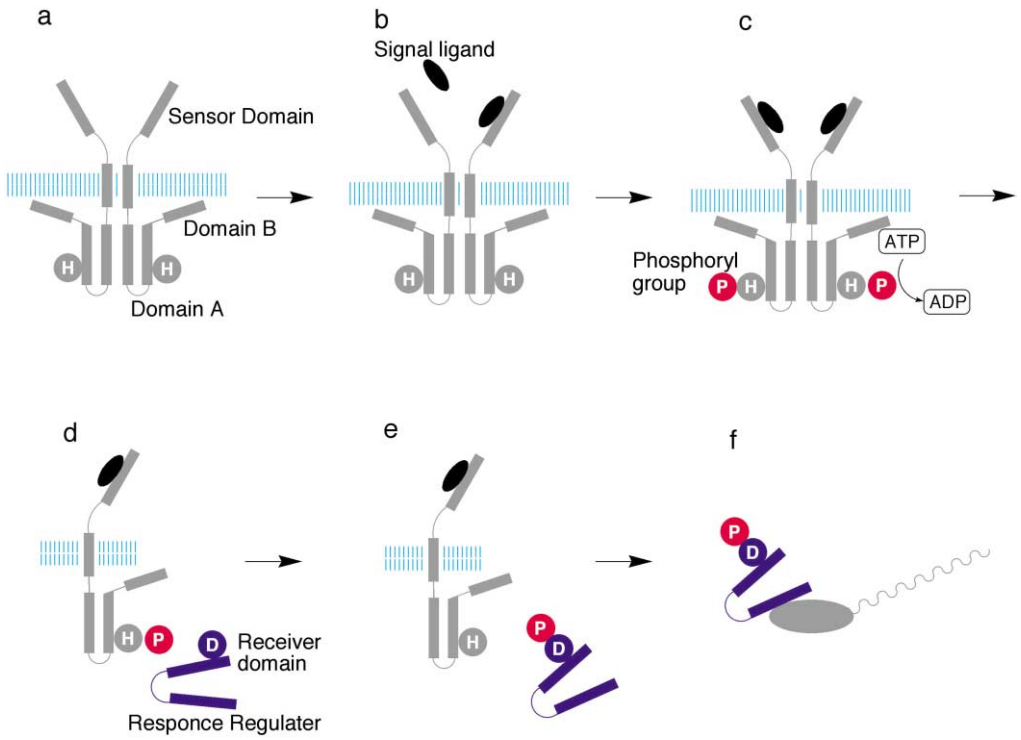


Figure 1. Signal transduction mechanism of the two-component system. (a) Sensor histidine kinase in its dimer form. (b) Signal ligand binds to the sensor domain. (c) ATP binds to domain B, then a conserved histidine residue is phosphorylated by ATP. (d) The response regulator binds to histidine kinase. (e) A Phosphoryl group is transferred to the asparagine residue in receiver domain of the response regulator. (f) The phosphorylated response regulator activates the particular DNA transcription.

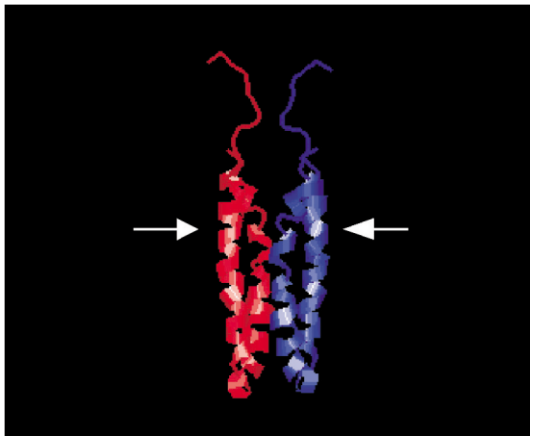


Figure 2. Ribbon diagram of EnvZ homodimeric core domain (domain A).[18] Two identical subunits are colored in blue and red, respectively. The conserved His 243 is indicated by the white arrow. Protein data bank accession number is 1joy.

reported.[23] Domain A exhibits autophosphorylation activity on His 243 in the H box, when in the presence of Domain B which contains the ATP binding site.[22]

Histidine kinases as targets for novel antimicrobial agents

Two-component systems are omnipresent in bacteria. With the advent of the microbial genomic sequencing project, the number of identified histidine kinases is expanding further.[6] For example, Gram-positive bacteria such as *Bacilus subtilis* contains 15 established histidine kinases as well as an additional 15 hypothetical histidine kinase genes among 4100 total genes (Table 1).[24] Also, clinically important bacteria, MRSA,[3] vancomycin-resistant MRSA,[3] and *E. coli* O157[4] each contain 10, six, and nine histidine kinase encoding genes, respectively.

Two-component systems regulate the transcription of various essential and non-essential gene products.[7–11,13–16] Regulation of osmolarity, nutrient uptake, redox potential, sporulation, and expression of virulence factors are under the control of two-component systems. Two-component signal transduction systems are used not only by bacteria, but also by procaryotes and higher plants. However, higher eucaryotes including mammals use a distinct signal transduction system that incorporate serine, threonine, and tyrosine phosphorylation. So far, several hundred histidine kinases have been identified. Recently, Kim and Forst have classified the histidine kinase family into five major categories based on genomic analysis (Table 2).[25] Despite the functional diversity between the two-component system of different subclasses, the H-box region sequence of each histidine kinase is highly conserved,[26,27] and thus this common locus in multiple two-component systems could be inhibited by a single molecule.

Two-Component Systems in Bacterial Resistance

One of the most attractive aspects of the two-component system is its regulation of antimicrobial resistance factors. Following the introduction of penicillin in the mid-1940s, bacterial resistance was reported within 2

Table 1. Histidine kinases (HK) in clinacally important bacteria[a]

HK[b]	*S. aureus* N315[c]	*S. aureus* Mu50[d]	*E. coli* O157 EDL933	*B. subtilis*
Identified HK genes	*vic*K, *lyt*S, *sae*S, *vra*S (4)	*vic*K, *lyt*S, *sae*S, *arl*S, *vra*S (5)	*rst*B, *rcs*C, *nar*Q, *arc*B, *env*Z, *uhp*B, *gln*L, *cpx*A (8)	*ybd*K, *ycb*A, *ycb*M, *ycl*K, *dct*S, *ydf*H, *yes*M, *cit*S, *yfi*J, *yko*H, *kin*E, *kin*D, *kin*A, *kin*C, *lyt*S (15)
Total HK genes	10	6	9	30
Total genes	2595	2697	5416	4100

[a]Genes were assembled using the completed genomes list of Kyoto Encyclopedia of Genes and Genomes (KEGG) on their web site:http://www.genome.ad.jp/kegg/
[b]Number of the total HK genes includes identified HK genes, predicted HK genes, and HK homolog genes.
[c]Methicillin-resistant *S. aureus* (MRSA).
[d]Vancomycin-resistant MRSA (VR-MRSA).

Table 2. Histidine kinase (HK) family[a]

HK subtype	HK	H-box[b]
Type IA	RstZ	GAA**HD**L**RTP**LVRLRYR
Type IB	BarA	NMS**HE**L**RTP**LNGVIGF
Type IC	AtoS	GVA**HE**V**RNP**LTAIRGY
Type II	DcuS	ERS**HE**FMNKLHVILGL
Type III	NarX	**RE**L**HD**SIAOSLSCMKM
Type IV	YehU	VN**PHF**LF**N**ALNTIKAV
CheA	CheA	RAA**HS**I**KG**GAGT----

[a]Based on a table by Kim and Forst.[25]
[b]Bold characters represent characteristic conserved residue of each subtype.

years, and by the late 1950s, up to 85% of clinically isolated staphylococci were found to be penicillin-resistant.[1,28] The action of one gene product and its time of propagation through the bacterial population results in drug resistance.[1] The resistance mechanisms of bacteria can be classified into three categories: (1) modification of the target site or enzyme, (2) prevention of access for the antibiotic, and (3) production of enzymes that destroy or inactivate the antibiotic.[29] These modifications are based on genetic changes: such as genetic mutation or gene introduction via transfer of a plasmid.[29]

Accounting for the large number of bacteria in an infection cycle, the rapid generation time, and the essential mutation of 1 in 10^7, then a pool of 10^{10} bacteria would have a diversity of thousands of mutants. If any member of this library was to acquire a resistance mutation to an applied antibiotic, its survival would allow it to grow and spread to become a dominant strain in the population.[1] The emergent antibiotic resistance gene would then spread rapidly between bacterial cells and strains by plasmid transfer. In this manner, as new antibiotics have been introduced, so the strains of resistant microbes have emerged also. Advances in molecular microbiology and genomics have led to the identification of a number of bacterial genes responsible for expression of antimicrobial resistance. It has been proven that two-component systems play an important role sensing cell disruption by drugs and regulate the expression of resistance factors. Table 3 shows the representative two-component systems that regulate the resistance to several antimicrobial agents. The function of these two-component systems will be discussed in the following section.

Two-component systems in vancomycin resistance

Vancomycin has been used as a 'last-resort' against hospital acquired infection by bacteria, such as methicillin-resistant *S. aureus* (MRSA). However, increased use of the drug has inevitably given rise to vancomycin resistant strains. Vancomycin possesses antimicrobial activity against Gram-positive bacteria by its strong binding to the cell wall peptidoglycan, L-Lys-D-Ala-D-Ala, so as to inhibit the cell wall synthesis. The vancomycin-resistant *Enterococci* bacteria (VRE) are characterized by the replacement of their cell wall peptidoglycan, D-Ala-D-Ala with D-Ala-D-Lac. This modification removes one hydrogen bond between vancomycin and its target, resulting in up to a 1000-fold loss of activity (Fig. 3).[30,49–51]

Table 3. Antibacterial resistance strains mediated by two-component systems (TCS)

TCS	Organism	Resistant drug	Refs
VanS/VanR	*Enterococcus faecium* BM4147 (VRE)	Vancomycin	30–33
$VanS_b/VanR_a$	*Escherichia coli*	Vancomycin	34
$VanS_c/VanR_c$	*Enterococcus gallinarum* BM4147	Vancomycin	35
VraR/VraS	*Staphylococcus aureus* Mu3 (VRSA)	Vancomycin	36
VncS/VncR	*Staphylococcus pneumoniae*	Vancomycin	37, 38
CiaH/CiaR	*Streptococcus pneumoniae*	Cefotaxime, penicillin	39–42
PhoQ/PhoP	*Salmonella typhimurium*	Polymixin B	43
PmrB/PmrA	*Salmonella typhimurium LT2*	Polymixin B	44–47
RprX/RprY	*Bacteroides fragilis*	Tetracycline	48

The genetic background of vancomycin resistance in *Enterococci* is now well understood. Five plasmid-born genes (*vanS*, *vanR, vanH, vanA,* and *vanX*) manage the reprogramming of the peptidoglycan (Fig. 4). Enzymes VanH, VanA, and VanX act together to reconstruct the peptidoglycan structure. The α-keto reductase, VanH, reduces pyruvate to D-lactate, while the dipeptidase, VanX, hydrolyses the D-Ala-D-Ala dipeptide. Then, the depsipeptide ligase, VanA couples the D-lactate (Lac) to D-Ala affording D-Ala-D-Lac. The expression of *vanH, vanA,* and *vanX* genes are regulated by a VanS-VanR two-component system. VanS, sensor histidine kinase, responds to the presence of the glycopeptide antibiotic and transfers the phosphoryl group to the response regulator, VanR. Phosphorylation of VanR enhances its binding capacity for the *vanH, vanA, and vanX* promoter regions to activate their expression. These antibiotic resistance genes can be carried either by plasmids or by mobile genetic elements between cells and strains.

Two-component systems in β-lactam resistance

The relevance of two-component signaling systems to antibacterials is not limited to VRE, as the resistance of *Streptococci pneumoniae* to ß-lactams is also under the control of this type of system. ß-Lactams exhibit their activity by inhibiting the penicillin-binding protein (PBP), constraining both its transpeptidase and transglycosylase capabilities which themselves are critical functions for cell wall synthesis.[29] Traditionally, the modification of PBP and production of β-lactamase have been identified as the major mechanisms responsible for bacterial β-lactam resistance. More recently, a higher level of mechanistic resistance has been discovered in several laboratories studying mutants of *S. pneumoniae*.[42]

The cefotaxime resistant strain of *S. pneumoniae* contains non-PBP genes that encode CiaH histidine kinase,

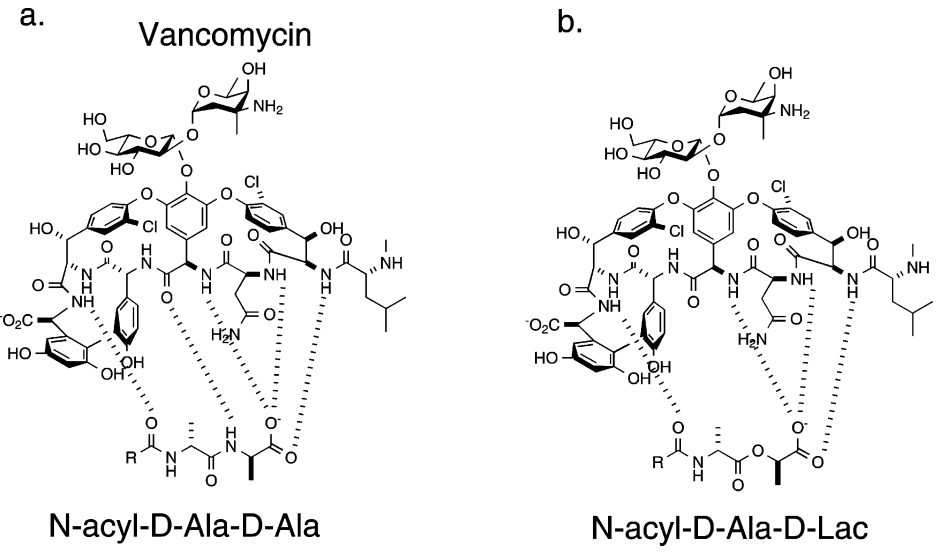


Figure 3. Mode of action of vancomycin. (a) Vancomycin binds to the cell wall peptidoglycan, *N*-acyl-D-Ala-D-Ala, to inhibit the cell wall synthesis. (b) The modified cell wall peptidoglycan, *N*-acyl-D-Ala-D-Lac, removes one hydrogen bond between vancomycin. Based on a figure by Walsh.[30]

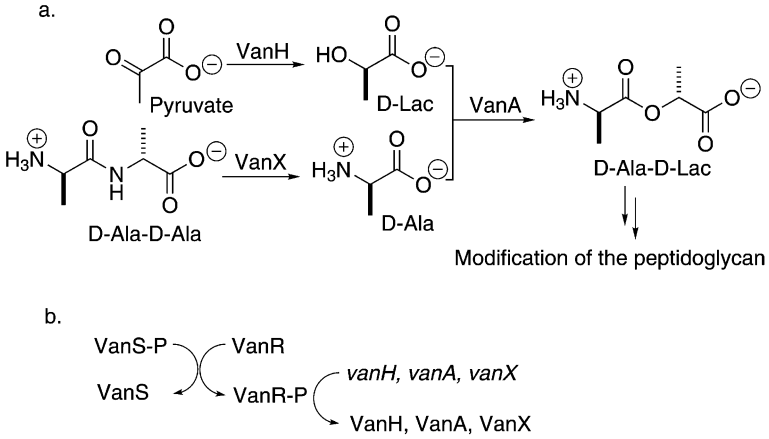


Figure 4. Modification of the peptidoglycan. (a) The biosysthesis of D-Ala-D-Lac. Based on a figure by Nicolaou.[49] (b) The VanR/VanS two-component system regulates the expression of *vanH*, *vanA*, and *vanX* genes.

and CiaR response regulator. The CiaH/CiaR two-component system senses alterations in the cell wall or PBP mediated by antibiotics and regulates the expression of several genes that reinforce the cell wall synthetic processes (Fig. 5).[39–41]

Two-component systems in polymixin resistance

Polymixin, a cationic antimicrobial peptide, kills a broad spectrum of Gram-negative bacteria by binding to their outer membranes through electrostatic interactions with the negatively charged groups of the lipopolysaccaride core and lipid A thereby effecting membrane disturbance. Several mutant strains of *Salmonella typhimurium* have survived antimicrobial attack through dynamic modification of their outer membrane lipid A moiety, using a two-component regulatory system.[44] Polymixin resistant *S. typhimurium* redecorate their lipid A with aminoarabinose and 2-hydroxymyristate (Fig. 6).[43] The decrease in affinity of polymixin to the modified lipid A membrane provides the basis of bacterial resistance. The plasmid encoded two-component system, PhoP/PhoQ, senses the membrane disturbance by polymixin and activates the lipid A modification mechanism.[43,46] Another two-component system, PmrB/PmrA, has also been reported to regulate similar lipid A modifications, by the activation of *pmrE* UDP-glucose dehydrogenation, and *pmrF* glycosyltransferase.[44,52,53]

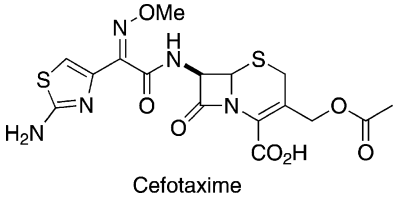


Figure 5. Structure of cefotaxime.

Two-component systems in tetracycline resistance

Tetracycline antibiotics are widely used against both Gram-positive and Gram-negative bacteria. The mode of action of the tetracycline is to inhibit protein biosynthesis in the bacterial cytoplasm. Porin proteins, OmpF and OmpC, regulate the cytoplasm level of tetracycline (Fig. 7). Passage of tetracycline across the outer membrane into the cell occurs via the porin OmpF; transport in the opposite direction occurs via porin OmpC. The tetracycline resistant *E. coli* does not contain an exogenous resistance gene, although it reduces the OpmF level to decrease drug delivery into the cell, thereby promoting survival.[48,54,55] The expressions of OmpF and OmpC are under the control of both EnvZ/OmpR and RprX/RprY two-component systems.[48]

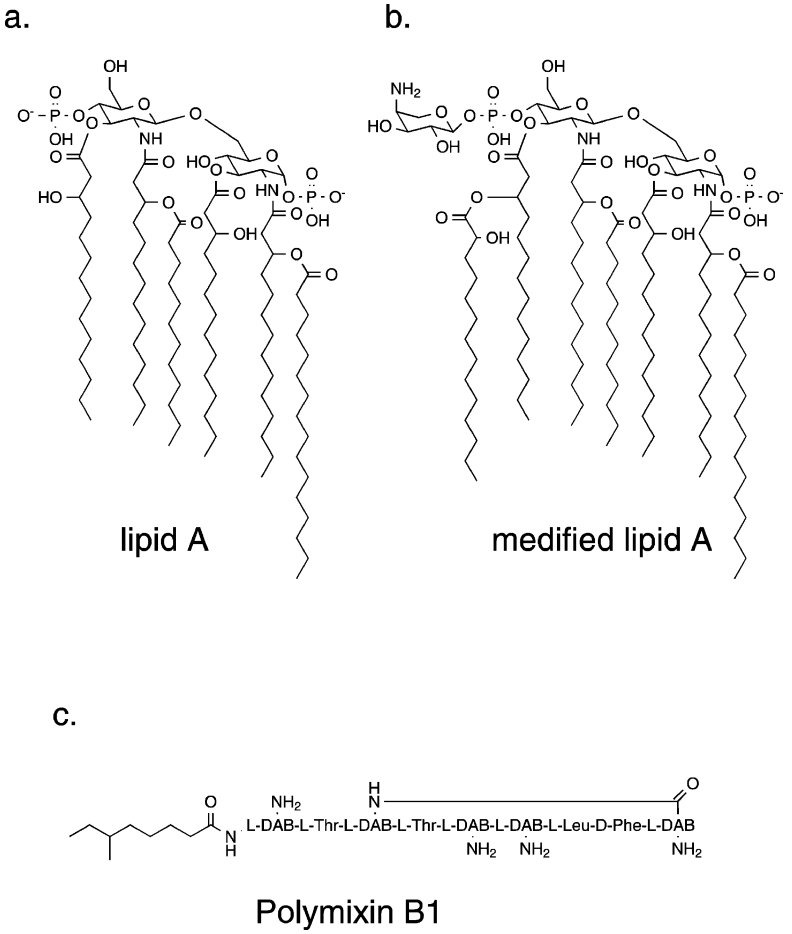


Figure 6. Modification of the outer membrane lipid A. (a) Structure of lipid A. (b) Structure of the modified lipid A in polymixin-resistant *S. typhimurium*. (c) Structure of cationic antimicrobial peptide, polymixin B1.

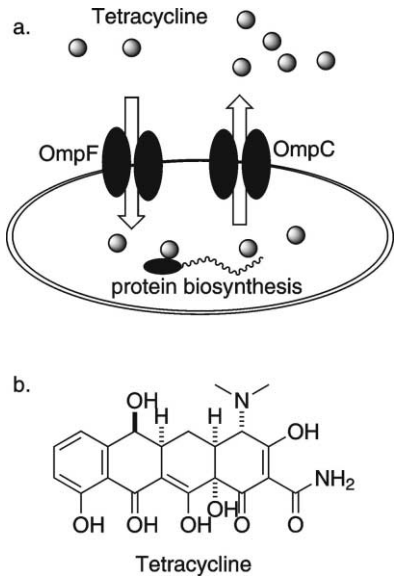


Figure 7. Mode of action of tetracycline. (a) Tetracycline (shown as gray circle) inhibits protein biosynthesis in the bacterial cytoplasm. Porin proteins OmpF and OmpC regulate the cytoplasm level of the tetracycline. (b) Structure of tetracycline.

Histidine Kinase Inhibitors

Aromatic structures

Inhibition of the gene expression regulated by two-component systems was first achieved by Roychoudhury.[56] Cystic fibrosis (CF) patients suffer from a major problem of pulmonary infection by mucoid strain *Pseudomonas aeruginosa*. In the CF lung, *P. aeruginosa* synthesize an exopolysaccaride coat, alginate, which inhibits access of a bactericidal drug to the site of infection. The AlgR2/AlgR1 two-component system regulates the transcription of alginate gene expression. Inhibitors **1** and **2** were shown to inhibit kinase activity of histidine kinase AlgR2 and inhibitor **3** interfered with DNA binding activity of response regulator AlgR1 (Fig. 8). Compounds **3** and **4** appear to be general two-component system inhibitors: they strongly inhibit the autophosphorylation activity of soluble histidine kinases, CheA, NRII, and KinA, and also inhibit transmembrane kinase, VirA. These bioactive compounds may be used in an adjunct therapy with a bactericidal agent to improve the therapeutic outcome.[56] Seebeck has demonstrated that **2** (LY266500) is also a potent inhibitor of histidine phosphorylation by succinyl CoA synthetase and diphosphate kinase in the important humanpathogenic parasite *Trypanosoma brucei*.[57,58]

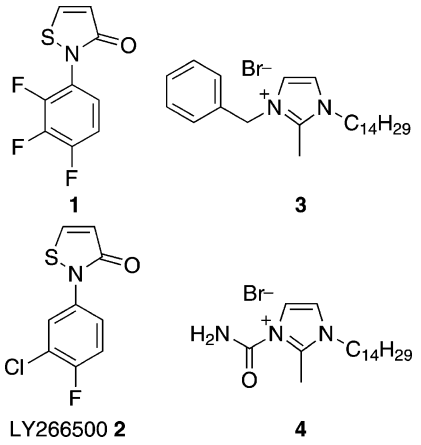


Figure 8. Structures of AlgR1/ArgR2 two-component system inhibitors. Compounds **1** and **2** inhibit the autophosphorylation of AlgR2, while compound **3** interferes with DNA binding activity of AlgR1.

A series of bacterial two-component system inhibitors has been described by R. W. Johnson Pharmaceutical researchers. The KinA/Spo0F two-component system consists of KinA histidine kinase and Spo0F response regulator, and serves to regulate the sporulation in *B. subtilis* (Fig. 9). RWJ-49815 **5**, a representative of a family of hydrophobic tyramines, inhibits the autophosphorylation of KinA with an IC_{50} of 1.6 μM.[59] Kinetic analyses revealed that **5** was competitive with ATP. Tyramine **5** has antibacterial activity against Gram-positive bacteria including MRSA, VRE, and penicillin-resistant *S. pneumoniae*. Furthermore, agent **5** exhibited a reduced resistance emergence in MRSA, compared with the potent quinolone, ciprofloxacin. These findings are important as they demonstrate the potential of two-component system inhibitors as leads for antibacterial therapy.[59]

The salicylanilides, closantel **6**, tetrachlorosalicylanilide **7**, and related derivatives **8** and **9** were all shown to be potent inhibitors of the autophosphorylation of KinA kinase (Fig. 10).[60] Anilide **7** inhibited the VanS/VanR two-component system that mediates vancomycin resistance in a genetically engineered *Enterococcus faecalis* cell line at concentrations sub-inhibitory for growth. Compound **7** could be an effective lead compound for adjunct therapy in the treatment of VRE. Cyano compounds **6**, **8**, and several related compounds have antibacterial activity against the drug-resistant organisms, MRSA and VRE. However, compound **8** affects the growth of *E. faecalis* only at concentrations >2 μM, whereas its effect on VanS/VanR-mediated protein expression could be detected at levels as low as 0.5 μM. Therefore, the antibacterial activities of compound **8** presumably result from not only inhibition of the two-component system, but also multiple mechanisms working in concert.

6-Oxa isosteres of anacardic acids were discovered to be potent inhibitors of bacterial two-component regulatory systems. SAR studies revealed that acid **10**, bearing a C14-alkyl substituent, was the most potent inhibitor (IC_{50} 2.2 μM) to date of the KinA/Spo0F two-component system (Fig. 11).[61] However, the antibacterial activity of this series of compounds against Gram-posi-

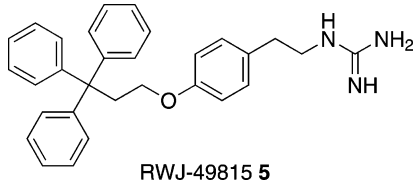


Figure 9. Structure of KinA/Spo0F two-component system inhibitor **5**.

tive bacteria was less potent than was expected based on the enzyme inhibitory potency (minimum inhibitory concentration (MIC) >64, >32, and 4 μg/mL against *S. aureus*, MRSA, and VRE).

While many hydrophobic compounds have been reported as two-component system inhibitors, their mechanism(s) of inhibition is largely unknown. Hilliard has reported studies into the mechanism of action for twenty four two-component system inhibitors including tyramine **5**, salicylanilides **6**, **7**, cyclohexene **11**, benzimidazole **12**, benzoxanine **13**, bis-phenol **14**, and their derivatives (Fig. 12).[62] These compounds were shown to inhibit the two-component signal transduction of KinA/Spo0F and NRII/NRI with IC_{50} ranging from 1.9 to >500 μM and MICs ranging from 0.5 to >1.6 μg/ml for Gram-positive bacteria. However, the majority of compounds did not exhibit a strong correlation between their IC_{50}s against two-component systems and MIC against bacteria. Twenty-three of the 24 two-component system inhibitors evaluated had an appreciable affect on the membrane integrity of *S. aureus* or caused hemolysis of equine erythrocytes. The membrane effect is very similar to those seen with some peptide antimicrobial agents, such as gramicidin S that disrupts cell membranes to inhibit macromolecular synthesis.[63] Therefore, the bactericidal properties exhibited by these HPK inhibitors has been attributed to multiple mechanisms, some of which are independent of HPK inhibition.[62]

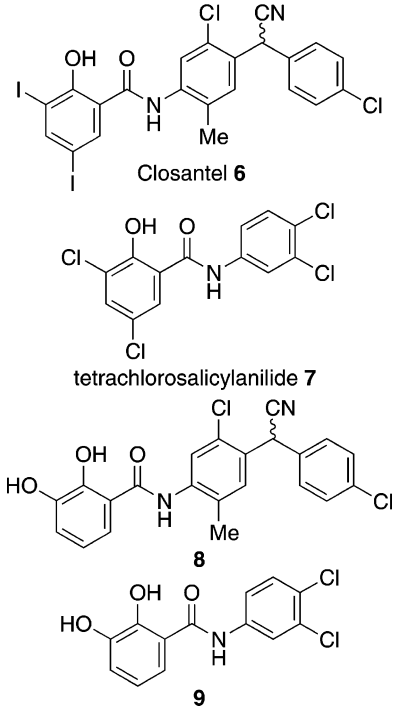


Figure 10. Structures of salicylanilide derivatives that inhibit the autophosphorylation of KinA. Compounds **6** and **8** have activity against MRSA and VRE.

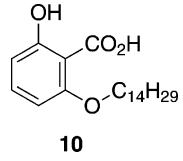


Figure 11. Structure of anacardic acid derivative **10** with potent KinA/Spo0F inhibitory activity.

Recently, Hoch has disclosed that **5** and **6** inhibit the KinA/Spo0F two-component system by interfering with autophosphorylation of histidine kinase KinA.[64] Inhibitors **5** and **6** target the carboxyl-terminal catalytic domain of the sensor kinase and exert their affect by causing structural alteration of the histidine kinase leading to aggregation. It was assumed that the inhibitors intercalate in the hydrophobic core of the four-helix bundle of KinA, driving structural denaturation nonspecifically. The phosphorylation of histidine kinase was also reported to be influenced by dimethylsulfoxide[65] and some detergents.[62]

TerraGen Discovery has reported several natural products that inhibit the NRII histidine kinase from *E. coli*. A screening assay of 64,000 microbial fermentation extracts revealed that a phenalenone derivative produced by *Penicillium cf. Herquei* 20421, XR770 **15**, inhibits histidine kinase NRIIc with an IC_{50} of 20 μM (Fig. 13).[66] Unfortunately, polyol **15** did not have any significant whole cell antibacterial activity against a panel of test organisms.

A series of halogenated pyrrolo [2,1][1,3] benzoxazines was isolated from fermentations of an actinomycete

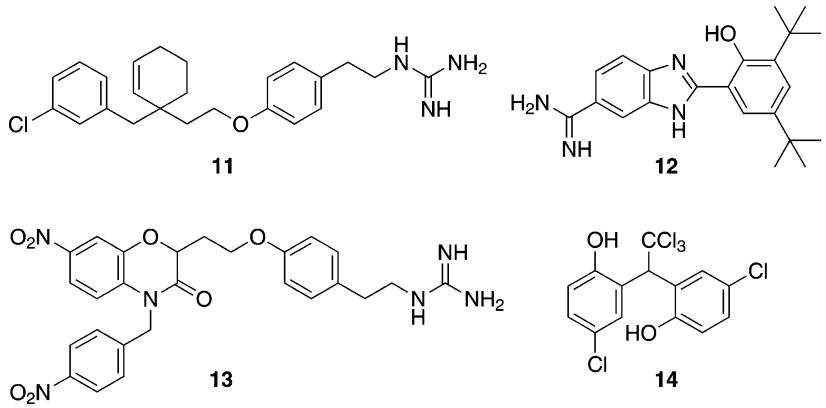
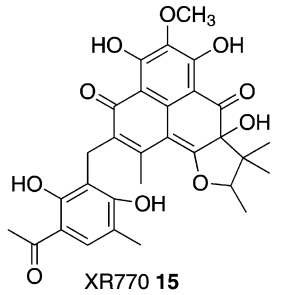


Figure 12. Structures of various chemical classes of two-component system inhibitors.



XR770 **15**

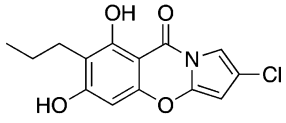


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Figure 13. Two-component system inhibitors isolated from fermentation extracts.

strain X10/78/978 (NCIMB40808), identified as *Streptomyces rimousus*. Streptopyrrole **16** inhibited NRIIc with an IC_{50} of 20 μM and exhibited antimicrobial activity against a range of Gram-positive bacteria including a drug resistant *S. aureus* strain (MIC as low as 1 μg/mL).[67]

Peptide inhibitors

Roychoudhury et al. have reported the identification of novel antibacterial compounds from a combinatorial library of *N*-acetylated, *C*-amidated D-amino acid hexapeptides.[68] A number of individual peptides were identified through a combination of deconvolution and positional scanning strategies. The most potent compounds, **17** and **18** not only inhibit CheA autophosphorylation (75 and 73% inhibition at 8 μM, respectively), but also show antibacterial activity against pathogenic Gram-positive bacteria (MIC 8–16 μM against *S. aureus*) (Fig. 14). Both compounds failed to show red blood cell lysis activity, though they inhibited mammalian protein kinase C with comparable potency to that exhibit against CheA.

Phage display approach

The phosphoenolpyruvate–sugar phosphotransferase system is another ubiquitous signal transduction system in bacteria. In this system, histidine kinase enzyme I transfers a phosphoryl group to the general phosphoryl carrier protein. Erni has reported a mechanism based peptide inhibitor of this phosphoryl transfer.[69,70] Phage libraries were amplified and affinity panned on enzyme I, then high-affinity clones that could be phosphorylated

Ac-D-Trp-D-Ile-D-Val-D-Ile-D-Leu-D-Arg-NH_2

17

Ac-D-Trp-D-Phe-D-Val-D-Ile-D-Leu-D-Arg-NH_2

18

Figure 14. Structures of two-component system inhibitors from a combinatorial library.

by enzyme I were identified by colony screening. Sixteen cationic oligopeptides including 15-mer **19** and 10-mer **20** were identified and evaluated, and the best peptide **19** inhibited enzyme I with an IC_{50} of 10 μM (Fig. 15).

Recently, Rognan has described the NMR docking study of Erni's oligopeptide and enzyme I.[71] The conformation of the decapeptide **20** bound to enzyme I was determined by ^1H NMR using a transferred nuclear Overhauser effect technique. Two possible phosphorylation models of the peptide inhibitor were presented. Interestingly, one model is very similar to that of the complex between enzyme I and its natural substrate HPr. NMR-restrained docking could prove to be a valuable tool for evaluating highly flexible ligands or protein-based lead identification of non-peptide inhibitors using virtual screening of a chemical database.

Phosphohistidine analogues

The extreme lability of phosphohistidines under acidic conditions has hampered the study of their biochemical function, in contrast to more stable congeners like phosphoserine, phosphothreonine or phosphotyrosine. The half-lives of 3-phosphohistidine **21** and 1-phosphohistidine **22** at pH 2.4 (46 °C) are 25, and 5 min respectively (Fig. 16).[15] Recently, some phosphohistidine analogues with improved stability have been reported. These analogues should be useful to study the phosphohistidine signal transduction pathway, for example: (1) Incorporating stable phosphohistidine analogues

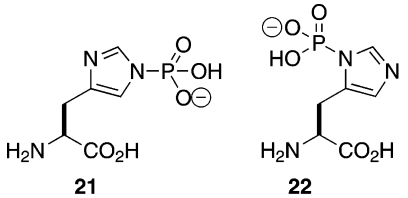
SGRLSSWVHVGWLVL

19

KISRHGRPTG

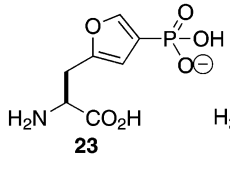
20

Figure 15. Inhibitors of histidine kinase enzyme I from a phage display library. (Amino acid sequences in one letter form.)

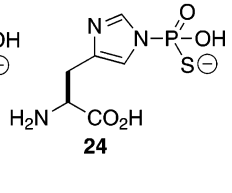


21

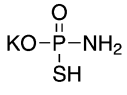
22



23



24



25

Figure 16. Structures of phosphohistidines (**21** and **22**) and their analogues (**23** and **24**) with agent thiophosphoramidate **25**.

into substrate proteins as probes or inhibitors and, (2) producing monoclonal antibodies that recognize the phosphohistidine residue. Reymond and co-workers have reported the design and enantioselective synthesis of (4′-phospho-2′-furyl)-alanine **23**, a non-hydrolyzable analogue of **21**.[72] In this compound, a stable phosphorus-carbon bond replaces the hydrolytically labile phosphorus nitrogen bond of **21**. The oxygen atom of the furan replaces the nitrogen atom in position 3 of phosphohistidine and similarly displays an in plane sp2 lone pair with hydrogen-bond acceptor properties.

Turck has reported the thiophosphorylation of a histidine residue in an oligopeptide.[73] A synthetic peptide with the sequence Ac-HGGGGAAAL-NH_2 was thiophosphorylated on the histidine employing $PSCl_3$ as the phosphorylation reagent. The thiophosphohistidine analogue preserves the overall structure of the phosphohistidine, though replacing one oxygen of the phosphate with sulfur leads to a more stable N-P bond due to the lower electronegativity of sulfur. More recently, Pirrung has reported the preparation of 3-thiophosphohistidine **24** using a novel phosphorus species, thiophosphoramidate **25** (Fig. 16).[74] Treatment of histidine with **25** has enabled specific thiophosphorylation to give the thermodynamically stable 3-thiophospho isomer **24**. The alkylation of **24** by phenacyl bromide is also reported, and the reaction serves as a model for the introduction of labeling or probe reagents into histidine phosphorothioate-containing proteins.

Negative regulators of two-component systems

Recently, several negative regulators that inhibit phospho-relay signal transduction systems have been discovered from genes of unknown function. Such inhibitors may play a role in the elucidation of new mechanisms of signal transduction.

Hoch has reported the first representative of a new class of signal transduction inhibitors, KipI, which is a protein encoded in an operon of genes whose role has yet to be established (Table 4).[75] KipI inhibits the autophosphorylation reaction of histidine kinase KinA potently, but does not inhibit phosphate transfer to the Spo0F response regulator. The effect of KipI was specific for KinA, and KipI did not inhibit histidine kinase NRII. Over expressing KipI in a wild-type strain inhibits sporulation to roughly the same extent as does deleting KinA. KipI is an inhibitor of the catalytic domain of KinA, affecting the autophosphorylation with ATP and not the phosphotransferase functions of this domain. Other protein negative regulators that inhibit specific histidine kinase have been identified, including PII,[76] SixA,[77] FixT,[78] and Sda.[79] It is noteworthy that these peptides display no amino acid sequence similarity to one another.

Global inhibitors

A large number of two-component system inhibitors have been reported, though very little is known about the extracellular signal ligands that presumably bind to receptor-histidine kinases to initiate signal transduction.

The synthesis of virulence factors in *S. aureus* is globally controlled by the AgrC/AgrA two-component system. Extracellular autoinducing peptides (AIPs), hepta- to nona-peptide thiolactones, trigger the signal transduction by acylating a signaling domain of the AgrC histidine kinase.[80] On the basis of this autoinducer-receptor specificity, *S. aureus* can be classified into four different groups. One unique aspect of the *agr* system is that AIP, which activates virulence expression in one group of *S. aureus* strains, also acts as an inhibitor within another group. For example, natural thiolactone **26** inhibits protein expression in group I *S. aureus*, while it activates protein expression in group II (Fig. 17).[80] Muir and Novick have demonstrated a structure–activity relationship for AIP.[81] AIP analogues lactone **27** and lactam **28** inhibit group I potently and no longer activate group II. Furthermore, the truncated thiolactone **29** is an inhibitor for all four specificity groups of *S. aureus*. Consequently, pentamer **29** could be the model for a global

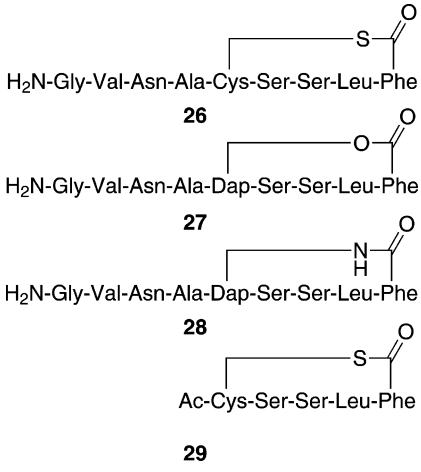
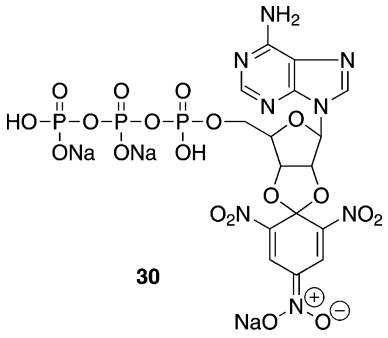


Figure 17. Structures of extracellular autoinducing peptides (AIPs).

Table 4. Negative regulators of two-component systems (TCS)

Negative regulators	TCS	Mode of action	Bacteria	Refs
KipI	KinA/Spo0F	Autophosphorylation inhibitor	*B. subtilus*	75
PII	NTRB/NTRC	Phosphatase stimulation	*S. typhimurium*	76
SixA	ArcB/OmpR	Phosphohistidinephosphatase	*E. coli*	77
FixT	FixL/FixJ	Autophosphorylation inhibitor	*S. meliloti*	78
Sda	KinA/Spo0F	Autophosphorylation inhibitor	*B. subtilus*	79



30

Figure 18. Structure of nucleotide binding domain inhibitor TNP-ATP **30**.

inhibitor of *S. aureus,* a quintessential feature in a lead compound for clinical use.[82]

Nucleotide binding domain inhibitors

The interaction of CheA bacterial histidine kinase with ATP has important consequences in the chemotaxis signal transduction pathway of *E. coli*. ATP binds to CheA thus promoting autophosphorylation. Stewart has reported TNP-ATP **30**, a fluorescent nucleotide analogue, binds CheA by competing with ATP (Fig. 18).[83] Experiments show that TNP-ATP binds CheA more tightly than it binds unmodified ATP. TNP-ATP is not a substrate for CheA and serves as a potent inhibitor of CheA autophosphorylation ($K_i < 1$ μM). Recently Bilwes has reported the crystal structure for the nucleotide binding domain of CheA with TNP-ATP.[84] The inhibitor TNP-ATP binds CheA with its phosphate moieties in a non-productive conformation and its adenine and trinitrophenyl groups in two adjacent binding pockets. Bilwes described that the trinitrophenyl interaction might be exploited for designing CheA-targeted drugs that would not interfere with host ATPases.

Conclusion

Since histidine kinase promoted two-component systems are extremely common in bacteria and play an important role in essential signal transduction for adapting bacterial stress, the inhibition of two-component systems have attracted considerable attention from medicinal chemists. A number of two-component system autophosphorylation inhibitors with inhibitory activity against multi-drug resistant bacteria have been developed. However, it should be noted that their bactericidal properties have been attributed to multiple mechanisms, some of which are independent of the two-component system inhibition. Alternative functional proteins, including extracellular sensor domains, ATP-binding sites, and negative regulators of two-component systems may also provide possible targets for new antibacterial drugs. Current bacterial genomics and proteomics will undoubtably uncover further targets for related structure-based rational drug design.

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