Discovery of Inhibitors of Bacterial Histidine Kinases

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To my family and all the wholeheartedly devoted teachers

На моето семейство и на всички всеотдайни учители
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CHAPTER 1

GENERAL INTRODUCTION

Nadya Velikova
THE GROWING PROBLEM OF BACTERIAL RESISTANCE TO ANTIBIOTICS

Bacterial multi-drug resistance (MDR) is defined as acquisition of non-susceptibility to at least one agent in three categories of antibacterials by pathogenic bacteria. MDR is a growing problem worldwide. The World Health Organisation (WHO) has identified antibacterial resistance and the antibiotics crisis as ‘bigger than AIDS’; The so-called “ESKAPE” pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.) are the main cause of hospital infections and are resistant to virtually all the currently marketed antibiotics. Infections due to resistant bacteria have 1.3 – 2 fold higher associated healthcare costs than susceptible bacteria due to increased mortality, morbidity and treatment costs. The aging population and growing number of immunocompromised patients due to HIV, cancer therapy or transplantation are making the need for novel antibacterials even more acute.

CLASSICAL AND POST-GENOMIC ANTIBACTERIAL DRUG DISCOVERY

All currently used antibiotic classes have been discovered by screening natural products from various sources or synthetic compounds for antibacterial activities against a spectrum of different bacteria. Over the past decade the large number of available sequenced genomes from multiple strains of pathogenic bacterial stimulated a change in the screening approaches for antibacterials. Rather than screening for whole-cell antibacterial activity, efforts shifted to screening for inhibitors of rationally preselected targets, otherwise known as target-based drug discovery.

CLASSICAL PHENOTYPIC SCREENING

Phenotypic screening for inhibition of bacterial growth with libraries of chemical compounds, including natural products from soil or marine ecosystems, fungi or plants, and bacterial secondary metabolites, has to date been the most successful way of discovering novel antibacterials. Typically, inhibition of bacterial growth in the pre-clinical screening stage is followed up by isolation and purification of the active compound and evaluation of its toxicity. Promising hits are then optimized by synthesizing a series of similar compounds to investigate their structure-activity relationship and identify a lead compound. Ultimately, the mode of action of the lead and its antibacterial spectrum are determined. Nearly all antibacterials on the market are based on antibiotics produced by Streptomyces and their chemically modified derivatives. However, since the 1950s the number of novel classes of antibiotics discovered by phenotypic screenings has dwindled and novel strategies have been investigated to overcome the raising problem of bacterial multi-drug resistance and the lack of novel antibacterials.
In the genomic era, more potential antibacterial targets were identified and rational target-based antibacterial drug design gained popularity as an approach. This approach is based on the assumption that some targets, proteins or whole biological pathways, are essential or conditionally essential for bacterial survival. Therefore, blocking them will lead to bacterial death. In target-based drug discovery, selected targets are purified and used in in vitro screenings of compound libraries for inhibitors of the target activity. Typically, screenings are performed in a high-throughput manner (i.e. high-throughput screening, HTS) and libraries of hundreds of thousands of compounds are screened.

Recent advances in computational biology and chemistry made it became possible to screen compound libraries for putative target ligands in silico commonly referred to as virtual screening. This reduced the number of hits that have to be evaluated in vitro for the desired biochemical activity. Target-based screening in silico (or structure-based virtual screening) utilizes a three-dimensional structure of the target protein obtained experimentally by X-ray crystallography or NMR or a homology model based on the structure of homologous proteins. Then, large virtual libraries of chemical compounds are screened in silico to identify ligands predicted to bind to the target site on the protein. The most promising hits with biochemical activity against the target are optimized by iterative rounds of structure-based drug design.

Another approach combining the principles behind high-throughput in vitro screening and structure-based drug design is fragment-based drug discovery (FBDD). In FBDD a library of few thousands fragment-like compounds is screened for ligands of a selected drug target in vitro. Ideally, hits are confirmed by elucidating the target-fragment structure by X-ray crystallography or NMR. The obtained structural information is used to guide structure-based design of more complex inhibitors with enhanced selectivity and potency by chemically linking fragment hits binding to different regions of the target site.

Signaling by two-component systems (TCS) is one of the promising antibacterial targets to be proposed since the genomics revolution. Prototypical TCS consist of two proteins, a sensor histidine kinase (HK) and an effector response regulator (RR).
Figure 1. Two-component systems signalling and inhibition. A prototypical two-component system (TCSs) consists of a membrane-bound histidine kinase (HK) and a cognate response regulator (RR). HKs sense environmental stimuli via an extracellular sensor domain (pink) and this triggers HK autophosphorylation at conserved His residues in the dimerization and phosphotransfer (DHp) domain (orange). ATP accommodated in the ATP-binding and catalytic (CA) domain (blue) serves as a phosphodonor. Phosphoryl group is transferred from the bound ATP in the CA domain to the His residue of the DHp domain (autophosphorylation) and then from the DHp domain to Asp residue of the RR (phosphotransfer). RR phosphorylation induces changes of the expression of target genes. RR phosphorylation levels are regulated via dephosphorylation mediated by the phosphatase RR intrinsic or HK-induced phosphatase activity or by ancillary proteins. TCS inhibition can be targeted at different steps of the TCS signalling (indicated A to G) and the most promising and exploited so far is autophosphorylation inhibition.
phosphate and is implicated to the regulation of virulence and pathogenesis of Gram-negative bacteria.

The sensor HK is membrane-bound and signal recognition alters the phosphorylation state of a cognate response regulator (RR). HK sensing of specific signals occurs via a variable domain, which is commonly exposed to the extracellular milieu, whereas the remaining protein domains are generally conserved, cytoplasmic and required for signal transduction. RRs are usually transcription factors of which the DNA-binding capacity and consequently, gene transcription is determined by their phosphorylation state. TCS may directly or indirectly control numerous genes, including those involved in the regulation of metabolism, cell physiology, virulence and resistance to antibiotics or antimicrobial peptides. TCS represent an attractive target for novel antibacterial agents as they are pervasive in microorganisms but absent in mammals. Furthermore, some TCS are essential or conditionally essential for viability in several important bacterial pathogens. Many TCS play
Figure 3. Structural information on TCS has facilitated the discovery of TCS inhibitors using structure-based discovery and rational structure-based design. A) Structure of *S. mutans* WalK (PDB:4I5S). *S. mutans* WalK is a long-rod dimer anchoring a HAMP signal-transducer domain (light green) and a PAS sensor domain (dark green) directly connected to the catalytic DHp (orange) and CA (blue) domains. High-resolution structures of full-length HKs will shed light on how the different domains interact with each other and how this can be used for inhibitor design B) Structure of the CA domain of *B. subtilis* WalK (PDB:3SL2). CA is the most exploited domain in structure-based TCS inhibitors discovery so far because of its well defined and generally conserved ATP-binding site (shown as yellow surface) C) Structure of the *T. maritima* HK853-RR468 (PDB:3DGE) reveals the interaction between the catalytic DHp (orange) and CA (blue) domains of the HK with the response receiver (REC) domain of the cognate RR (magenta). TCS catalytic domains, especially the CA domain, are highly conserved and suitable for the development of broad-spectrum inhibitors. Narrow –spectrum inhibitors can be designed/ discovered by targeting the interaction of a HK and its cognate RR. For this high-resolution structures of TCS complexes are needed Putative ligand binding sites were calculated with PyMol and are shown as yellow surface (detection radius and detection cut-off is 5 solvent radii). Ligands are shown as sticks.

role in bacterial adaption to environmental stresses and the regulation of virulence factor production. Thus drugs inhibiting TCS function are expected to attenuate pathogen virulence and/ or compromise pathogen growth under a range ofphysiological stresses. Moreover, HKs and RRs possess a high degree of homology in their active sites, suggesting that it might be possible to identify inhibitors targeting multiple TCS. Developing resistance to a drug with multiple targets is expected to be slower than to a drug with a single target.
**TWO-COMPONENT SYSTEM SIGNALLING**

Upon sensing of a ligand or physiological stimulus TCS signal transduction is initiated by autophosphorylation of a conserved histidine (His) residue in the dimerization and phosphotransfer (DHp) domain. Autophosphorylation involves the helical DHp domain hosting the conserved His and the C-terminal catalytic and ATP-binding (CA) domain that binds ATP and phosphorylates the His (Figure 1 and 3). The phosphoryl group from the phosphorylated His in the DHp domain is then transferred to an exposed Asp that belongs to the response receiver (REC) domain of the cognate RR. Phosphotransfer, i.e. phosphorylation of the RR, triggers changes in the conformation of the RR, modulating the affinity of the effector domain for its targets, typically DNA binding motifs in specific gene promoters. In some cases the RR lacks the effector domain and the REC domain takes over the effector role. The phosphorylation levels of the RR are tightly regulated by the phosphatase activity of the HK; the RR itself or a partner protein. The DHp, CA, and REC domains are always present in all TCS and relatively well conserved in amino-acid sequence (Figure 4), compared to the sensor domains of HKs (the periplasmic domain, HAMP, PAS and GAF domains) or the effector domains of RRs. The presence or absence and the variability of the sensor and effector domains is determined by the wide range of specific signals or targets to be recognized by the different TCS. Therefore, inhibitors of the catalytic activity of the highly conserved DHp, CA and REC domain are expected to possess broad-spectrum antibacterial activity.

**DISCOVERY OF TWO-COMPONENT SYSTEM INHIBITORS**

There are several publications describing the discovery of TCS inhibitors which have been reviewed elsewhere and some aspects are discussed in more detail in the following sections. The target TCS include among others, *S. pneumoniae* and *S. epidermidis* WalKR, *E. coli* QseCB, *S. flexnerii* and *S. enterica* PhoQP. The identified TCS inhibitors were from libraries of natural products, small molecules and antibacterial peptides (Figure 5). Different approaches have been employed to discover TCS inhibitors, including high-throughput target-based screening and virtual structure-based screening.
Figure 4. Conservation of the different domains of TCS A) *S. mutans* WalK (PDB:4I5S) the catalytic domains (DHp and CA) are relatively more conserved than the sensor domains (HAMP and PAS). Furthermore DHp and CA domains are always present in all HK, whereas the sensor domains vary. B) REC domain of *B. subtilis* WalR is relatively more conserved than C) the DNA-binding effector domain of *B. subtilis* WalR. The variability of the HK sensor domains and the RR effector domains are determined by the variety of input signals and adaptation responses TCS are involved in. Targeting the relatively more conserved DHp, CA and REC domain might facilitate the development of broad-spectrum antibacterials with low potential of resistance development.
Figure 4 Published two-component systems inhibitors can be used as starting point for the design of more potent inhibitors following structure-based drug design approaches A) Led209 inhibits the binding of adrenergic signals to the periplasmic domain of *E. coli QseC*, preventing its autophosphorylation and consequently inhibiting QseC-mediated activation of virulence gene expression. B) Compound 5 was identified in a structure-based screening for ligands of the CA domain of *S. epidermidis* WalK. Compound 5 inhibited *S. epidermidis* WalK autophosphorylation with IC$_{50}$ = 14 µM and showed antibacterial effect against Gram-positive bacteria with minimal inhibitory concentrations (MICs) in the range of 2 – 6 µM. C) NSC48630 inhibited the formation of *S. enterica* PhoP-DNA complex with IC$_{50}$ = 3.6 µM. NSC4836 was identified as putative PhoP ligand in a structure-based screening for ligands of the activated response regulator PhoP (PDB: 2PL1). Compound 5 and NSC48630 and other TCS inhibitors identified by structure-based virtual screenings indicate the potential of structure-based approaches to discover promising TCS inhibitors with antibacterial and therapeutic effect.
PROMISING TWO-COMPONENT SYSTEM TARGETS

WalKR

WalKR (aka YycF/G, MicA/B, VicK/R) is highly conserved and has been identified in the genomes of the major group of Gram-positive bacteria with a low genomic GC-content (Figure 2A)\(^\text{52-55,32,30}\). This includes pathogenic species belonging to the genus *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Clostridium*, and *Listeria*. In the important pathogens *S. aureus*, *S. pneumoniae*, and *Enterococcus faecalis* WalKR has been shown to be essential for viability in rich laboratory growth medium\(^\text{52,30,56}\). WalKR regulates genes responsible for cell wall metabolism and cell wall homeostasis\(^\text{55,57,53}\) but the specific genes and pathways regulated by WalKR may differ from species to species. Additionally, *walKR* regulates genes involved in metabolism, stress response, virulence, host-microbe interactions, transport and regulatory pathways depending on the species\(^\text{27,58}\).

Of all the TCS WalKR is considered one of the most attractive targets for antibacterial drugs due to its essentiality, links to virulence and antibiotic resistance. Imidazole and zerumbone derivatives were the first inhibitors of WalK to be identified\(^\text{59,46}\). Following this pioneering discovery, Utsumi’s group developed biochemical and genetic high-throughput screening methods to search for WalK and WalR inhibitors. This led to the discovery of WalK inhibitors such as aranorosinol, walkmycin B, waldiomycin, signermycin B and the WalR inhibitors, walrycin A and B\(^\text{48,49}\). Later, some of these inhibitors were not proven to be selective to WalKR and other mechanisms such as membrane damage and adverse effects on macromolecules biosynthesis most likely account for the observed antibacterial effects.

Elucidation of the structure of HK catalytic domains opened up the possibility for structure-based virtual screening (SBVS) for WalK inhibitors\(^\text{60,61,43,43}\). The catalytic ATP-binding domain (CA domain) appeared to be the most promising target for this approach as this domain contains a well-defined and partially conserved pocket where ATP is accommodated (Figure 3B). Before the elucidation of WalK structure (PDB: 3SL2 and PDB:4I5S), homology models of the CA domains of *S. pneumoniae* and *S. epidermidis* WalK based on the three-dimensional structures of *Thermotoga maritima* HK853 (PDB: 2C2A) and *E. coli* EnvZ (PDB: 1BXD) HKs were used for SBVS of drug-lead compound libraries. These screenings yielded WalK autophosphorylation inhibitors belonging to different classes of chemical structures, such as imidazole analogues and derivatives of furan, thiophene, thiazolidinone, benzamide and pyrimidinone (Figure 4). The SBVS hits inhibited the growth of *S. pneumoniae* and *S. epidermidis* and showed bactericidal effects towards both planktonic and biofilm cells\(^\text{62,63}\). Furthermore, some of these inhibitors decreased the mortality of mice infected with *S. pneumoniae* in an *in vivo* sepsis model, demonstrating that SBVS is a valuable tool for the identification of WalK inhibitors with therapeutic effect\(^\text{43}\). Nevertheless,
the design of potent ATP-competitive inhibitors specific for WalK is challenging, even more so when structural homology models are used instead of experimental (e.g. X-ray) data. This issue is crucial when attempting to generate a WalK inhibitor with higher specificity and affinity in the hit-to-lead optimization phase. Although the overall fold of the CA domain as well as the catalytic residues is generally conserved, there are large variations in size and sequence in the ATP-lid, a HK distinctive flexible loop that covers the ATP-binding pocket and is crucially involved in ATP binding and in HK catalytic reactions $^{26,64,65}$. The recently published WalK structures of the entire intracellular portion from *Streptococcus mutans* (PDB: 4I5S) (Figure 3A) and the CA domain from *Bacillus subtilis* (PDB: 3SL2, Figure 3B) might provide the key to design of improved WalK inhibitors $^{66,67}$. These structures have not only revealed specific characteristics of the WalK CA domain but also insights into the molecular mechanism of WalK autokinase activation and clues how it can be effectively inhibited by exploring HK domains for structure-based drug design $^{25}$.

**QseCB**

Targeting microbial virulence without inhibiting growth has been proposed as a promising strategy in antibacterial drug discovery as it was proposed that antivirulence drugs present less selective pressure for resistance development $^{33,24,51}$. QseCB is a TCS involved in recognizing the host-derived adrenergic signals and the bacterial aromatic signal autoinducer-3 AI-3 (epinephrine, norepinephrine) to trigger expression of virulence genes (Figure 2B) $^{68}$. Homologues of QseC are present in at least 25 important human and plant pathogens, therefore, a QseC inhibitor is expected to be a promising drug for antivirulence therapy against a wide range of pathogenic bacteria $^{33}$.

Adrenergic signals, as well as, AI-3 are recognized by QseC periplasmic domain $^{68}$. Drugs targeting QseC periplasmic domain have an advantage compared to drugs with intracellular targets of Gram-negative bacteria as they only need to pass the outer membrane to reach the target.

High-throughput screening to search for a compound that inhibits the activation of QseC by AI-3 has been reported $^{33}$. LED209 (Figure 4) was identified as inhibitor of the binding of the signaling molecules to QseC from different Gram-negative pathogenic bacteria. LED209 inhibited QseC autophosphorylation, bacterial pathogenicity *in vitro* and *in vivo*, but did not inhibit cell growth. The discovery of LED209 demonstrates that targeting microbial virulence without inhibiting growth is a promising strategy to identify antibacterials with therapeutic effect $^{33}$.
**PhoPQ**

The PhoQ/PhoP two-component regulatory system is a major regulator of virulence in the enteric pathogen *Salmonella enterica* serovar Typhimurium and is present in a range of Gram-negative bacteria. It also controls the adaptation to low Mg\(^{2+}\) environments by governing the expression and/or activity of Mg\(^{2+}\) transporters and of enzymes modifying the Mg\(^{2+}\)-binding sites on the bacterial cell surface (Figure 2C). The RR PhoP modifies expression of about 3\% of the Salmonella genes in response to the periplasmic Mg\(^{2+}\) concentration detected by the HK PhoQ\(^{71}\). Genes that are directly controlled by PhoP often differ in their promoter structures, resulting in distinct expression levels and kinetics in response to the low Mg\(^{2+}\) inducing signal. PhoP regulates a large number of genes indirectly via other transcription factors and TCS that form a panoply of regulatory networks. These regulatory networks include transcriptional cascades, feed-forward loops and the use of connector proteins to modify the activity of RRs\(^{72}\). These networks confer distinct expression properties that may be important contributors to the lifestyle of Salmonella.

The structure of the catalytic domain of PhoQ has been well studied\(^ {73}\). PhoQ has also been co-crystallized with the inhibitor radicicol\(^ {45}\). The available structural information has been exploited in structure-based screening for PhoQ inhibitors that attenuate the virulence of *S. flexneri* in vitro and in vivo\(^ {44}\). PhoP inhibitors have also been discovered via structure-based screenings and shown to inhibit PhoP-DNA complex formation (Figure 4)\(^ {51}\). Taken together these findings highlight good potential to develop novel antibacterials against PhoPQ using structure-based screening and design.

**PhoRB**

PhoRB is a two-component system sensing the extracellular concentration of phosphate (Figure 2D)\(^ {74}\). It consists of the HK PhoR and its cognate RR PhoB. PhoRB responds to phosphate limitation, when the extracellular concentration of phosphate falls below 4 \(\mu\)M. In phosphate-limiting conditions, PhoR phosphorylates PhoB, which then binds to specific DNA sequences, known as Pho boxes. PhoB binding either induces or represses the genes belonging to the Pho (phosphate) regulon, which includes genes involved in acquisition and metabolism of different phosphates.

The Pho regulon in *E. coli* K12 comprises of 31 genes, and it is not only involved in phosphate homeostasis, but it is also related to bacterial virulence as the induction of Pho regulon results in attenuated pathogenesis\(^ {75, 76}\). Examples of virulence attributes altered by induction of the Pho regulon are a significant reduction in the amount of capsular antigen at the cell surface, resistance to the bactericidal effect of serum, to cationic antimicrobial peptides, and to acid and oxidative stress, as well as the production of type 1 fimbriae\(^ {75, 76}\). Therefore, PhoRB presents a promising target for antibacterials that inhibit bacterial virulence.
ANTIMICROBIAL PEPTIDES INHIBITING TCS SIGNALING

Antimicrobial peptides (AMPs) have been proposed as promising strategy to address the growing problem of MDR\textsuperscript{77, 78}. Eukaryotic cationic AMPs are produced at sites of infection or inflammation in many different organisms\textsuperscript{79, 80}. Typically they are peptides of 12 to 45 amino acids with a net positive charge and a high proportion of hydrophobic amino acids\textsuperscript{81}. Bovine lactoferricin (LfcinB; Figure 6) is a typical cationic antibacterial peptide generated by proteolytic cleavage of lactoferrin that has a broad-spectrum antibacterial activity\textsuperscript{82, 83, 84, 82}.

**Figure 5.** LactoferricinB is an antimicrobial peptide identified as a TCS inhibitor. LactoferricinB was shown to inhibit the phosphorylation activity of *E. coli* BasR-BasS and the ability of CreC to recognize CreB\textsuperscript{47}.

It has been shown that LfcinB derived peptides (LfcinB\textsubscript{17-41}, LfcinB\textsubscript{17-31} and D-LfcinB\textsubscript{17-31}) enter the cytoplasm of *E. coli* and *S. aureus*\textsuperscript{85}. In a recent study aimed at elucidation of the intracellular targets of LfcinB, the TCS BasSR and CreCB were among the 16 proteins shown to specifically interact with LfcinB\textsuperscript{47}. This discovery suggested that LfcinB-derived peptides were a promising starting point for the development of AMP-based TCS inhibitors.
Discovery of Inhibitors of Bacterial Histidine Kinases

THESIS OUTLINE AND AIMS

The main aims of the current thesis were:

1. To discover hits that can be further developed to broad-spectrum TCS inhibitors following a multidisciplinary approach.
2. To provide structural-knowledge on TCS inhibition to facilitate the optimization of the identified TCS inhibitors using structure-based drug design.
3. To evaluate the identified hits for their biochemical, antibacterial and cytotoxic effects in vitro.
4. To explore ways to improve hit compound permeability of the Gram-negative bacteria outer membrane using nanoparticles and AMPs.

In Chapter 2, we describe the discovery of histidine kinase (HK) autophosphorylation inhibitors targeted at the ATP-binding and catalytic (CA) domain of multiple HKs using combined structure-based and ligand-based virtual screening approaches.

In Chapter 3, we describe the application of fragment-based screenings by differential scanning fluorimetry and ligand-based similarity searches to discover fragment-like HK autophosphorylation inhibitors targeted at the CA domain of multiple HKs.

Due to the weak or absent antibacterial effect of identified HK inhibitors (Chapter 2 and 3) against Gram-negative bacteria, nanoparticles were investigated as potential drug delivery vehicles. Silica-based mesoporous nanoparticles capped with ε-poly-L-lysine were loaded with HK autophosphorylation inhibitors and their antibacterial effect, cytotoxicity and immunotoxicity were studied and described in Chapter 4.

In Chapter 5 we used computer-aided approaches in an attempt to discover improved HK inhibitors.

As lactoferricinB-derived peptides seemed to present a promising starting point for the development of antimicrobial-peptides-based TCS inhibitors, in Chapter 6 we investigated the possibility to use T. maritima HK853-RR468 and E. coli PhoRB for structural studies of TCS inhibition by LactoferricinB – derived peptides.

In Chapter 7 the (co-)crystallization and preliminary X-ray diffraction analysis of the T. maritima CheA-inhibitors co-crystals and CopM periplasmic domain are described.

In Chapter 8 the potential of targeting WalK for developing antibacterial drugs with low potential of resistance development is discussed in the light of current literature and results.

Chapter 9 is a concluding discussion on the results described in the thesis and their implications for antibacterial drug discovery and development.
REFERENCES

Discovery of Inhibitors of Bacterial Histidine Kinases

34. Worthington, R. J.; Blackledge, M. S.; Melander, C., Small-molecule inhibition of bacterial two-component systems to combat antibiotic resistance and virulence. Future medicinal chemistry 2013, 5 (11), 1265-84.
controls major staphylococcal virulence genes and is involved in triggering the host inflammatory response. Infection and immunity 2012, 80 (10), 3438-53.


CHAPTER 2

DISCOVERY OF BACTERIAL HISTIDINE KINASE INHIBITORS WITH ANTIBACTERIAL ACTIVITY AGAINST CLINICAL ISOLATES OF MRSA AND STAPHYLOCOCCUS EPIDERmidIS

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TO BE SUBMITTED TO ASM ANTIMICROBIAL AGENTS AND CHEMOTHERAPY
ABSTRACT

The emergence of multi-drug resistant bacteria is an overwhelming world-wide public health problem. Two-component systems, consisting of a sensor histidine kinase (HK) and an effector response regulator, are the main signaling devices in bacteria and have been proposed as promising targets for the development of novel broad-spectrum antibacterials. Rational design of competitive inhibitors for the nucleotide-binding site of HKs that inhibit the autophosphorylation activity is considered a promising strategy to generate a novel class of antibacterials. To discover HK inhibitors we employed a virtual screening approach, including structure-based virtual screening (SBVS) and a ligand-based similarity search (LBSS). Two of the hits identified using SBVS inhibited the autophosphorylation activity of multiple HKs in vitro and had a weak antibacterial activity against several Gram-positive bacteria species. Using the two hits as query molecules for LBSS, three compounds were identified with greater autophosphorylation inhibitory capacity (IC₅₀ ≥16 µg/m for the best hit, B13) and stronger antibacterial activity against different Gram-positive laboratory strains and clinical isolates of Staphylococcus epidermidis and methicillin-resistant Staphylococcus aureus (MRSA). One of these compounds, B14, inhibits the growth of multi-drug resistant clinical isolates of both Gram-positive and Gram-negative bacteria. Analysis of the predicted binding modes of the reported inhibitors suggests different modes of interaction with the ATP-binding site of HKs and thus can be used as a starting point for designing inhibitors with higher affinity and selectivity for HKs.
INTRODUCTION

There is an urgent need to discover new antibacterials to combat the growing problem of antibiotic resistance world-wide. Two-component systems (TCS) have been proposed for almost two decades as promising antibacterial drug-targets. TCS are highly pervasive in bacteria and used for signal transduction but are not present in mammals, making them attractive antibacterial drug targets. Moreover, TCS are involved in the regulation of a variety of processes related to bacterial pathogenicity, including virulence, biofilm formation, antibiotic resistance, and bacterial persistence. Some TCS are essential or conditionally essential for bacterial growth. Furthermore, the high degree of conservation among TCS active sites and the existence of multiple TCS in each bacterium suggest that it should be possible to identify an inhibitor of multiple TCS regulatory networks with a broad-spectrum activity. Overall, targeting TCS is expected to effectively incapacitate the ability of bacteria to adapt to environmental and physiological changes.

A prototypical TCS consists of a membrane-bound histidine kinase (HK) and a cognate response regulator (RR) with transcription activity. Upon sensing environmental stimuli, the HK is autophosphorylated at conserved histidine residues. Subsequently, the phosphoryl groups attached to His are transferred to conserved aspartic acid residues in the receiver domains (RECs) of the cognate RRs. For some RRs, phosphorylation has been shown to alter affinity of binding to the operator sites and alter gene transcription due to conformational changes and protein dimerization. HK autophosphorylation involves two well-conserved domains, a dimerization and histidine phosphotransfer domain (DHp) containing the phospho-accepting His and a C-terminal catalytic ATP-binding (CA) domain that phosphorylates His through ATP hydrolysis. The structure and amino acid sequence of the ATP-binding domain is well conserved so inhibitors targeted at ATP-binding would be expected to inhibit multiple TCS. Here we describe the identification of HK autophosphorylation inhibitors (HKAs) by combined virtual screening approach including structure-based virtual screening (SBVS), followed by ligand-based similarity search (LBSS) for homologues of the initial hits. The compounds were demonstrated to inhibit Escherichia coli and Staphylococcus aureus PhoR autophosphorylation and are expected to inhibit the autophosphorylation of other HKs. The HKAs were also tested for their antibacterial activity against reference and multi-drug resistant bacterial strains, including MRSA.
MATERIALS AND METHODS

STRUCTURE-BASED VIRTUAL SCREENING

Target preparation
The chosen molecular targets for molecular docking were the CA domains of *Thermotoga maritima* HK853 (PDB: 3DGE) \(^{15}\), *Geobacillus stearothermophilus* KinB (PDB: 3D36) \(^{16}\) and *T. maritima* CheA (PDB: I58B) \(^{17}\). Residues corresponding to the CA domain of each A chain (320-480 for 3DGE, 270-415 for 3D36 and 354-540 for I58B) were selected for each structure and additional atoms corresponding to water molecules, ions or ligands were removed. Hydrogen atoms were added in the absence of the cognate ligand using the GOLD program \(^{18}\).

Docking parameters
All docking calculations were performed with the GOLD docking software (version 5.2) using ChemPLP as a scoring function \(^{19}\). Binding sites were defined as being 10 Å around the geometric center of the cognate ligand.

Library
For the initial screening, a diversity set (600,000) of the Scopius – CSpace database (over 6 million commercially available drug-like compounds) \(^{20, 21}\) was docked into each of the three HK structures. The search efficiency parameter was set to 30 % and 10 solutions were generated for each compound of which only the highest-scoring poses were saved.

Post-processing of docking results
Compounds with unwanted functional groups (in-house rules used by InhibOx) were removed and the resulting set of compounds was ranked in two lists: i) by the ChemPLP GOLD docking score (ChemPLP) and ii) by a ligand efficiency score (i.e.) which is ChemPLP divided by the number of non-hydrogen atoms in the compound \(^{22}\). The top 3500 compounds in each list were used to extract the top 100 compounds docking to all three HK CA domain structures. This resulted in two final lists of compounds: one with respect to ChemPLP and one with respect to ligand efficiency. The top 100 compounds of each list were finally visually inspected and ten compounds were purchased for experimental testing.

LIGAND-BASED SIMILARITY SEARCH
The database from the Developmental Therapeutics program of the National Cancer Institute and the National Institute of Health (DTP) was searched for analogue structures of the initial hits A5 and A6. The similarity search with A5 or A6 as query molecules was performed using the Morgan fingerprint as implemented in RDKit \(^{23}\), which is a variation of the “extended connectivity fingerprints” (ECFP) \(^{24}\). The top 100 hits of each similarity search were visually inspected of which in total 25 compounds were ordered and experimentally tested.
BINDING AND INTERACTION MODE PREDICTION

Docking calculations to predict the binding mode of selected inhibitors were performed with the initial hits, A5 and A6, and the LBSS hits showing antibacterial effect, B7, B13 and B14, using the CA domain of *T. maritima* HK853 (PDB: 3DGE, chain A) and GOLD docking software. For each ligand 100 solutions were generated, of which the top 20 were visually inspected.

CHEMICAL REAGENTS

Compounds A1 to A10 from the initial SBVS screening were purchased from Ukrainian Organic Synthesis (Kiev, Ukraine). Compounds B1 to B25 from the ligand-based similarity search (LBSS) were obtained from DTP. Compounds were dissolved in 100% DMSO and stored at 4ºC protected from direct light. \([\gamma^{32} \text{P}] \text{ATP}\) was purchased from Perkin Elmer.

CLONING

*Streptococcus pneumoniae* walK encoding the catalytic portion (DHp and CA domain) of WalK (amino acids from 208 to 449) was amplified by PCR from *S. pneumoniae* CDC3059-06 genomic DNA using the following primers: forward 5´-aagttctgttccaggccagttgagcaggagaagaacgc-3´ and reverse 5´-atggtctagaaagctctagtcttcatccac-3´. The PCR product was purified by PCR product purification kit (Macherey-Nagel) and cloned into a gel-purified pOpinF vector (kindly provided by Nick Berow, IRB, Spain) linearized with KpnI and HindIII (Fischer Scientific). The insert was cloned into the pOpinF vector with In-Fusion HD cloning system (Clontech). Positive clones were confirmed by colony PCR and DNA sequencing.

PROTEIN EXPRESSION AND PURIFICATION

The catalytic portions (DHp and CA domain) of *T. maritima* HK853 (HK853), *E. coli* PhoR (PhoR^E^) and *S. aureus* PhoR (PhoR^S^) were expressed and purified as previously described \(^{15, 25}\). In brief, the proteins were expressed in *E. coli* RIL and purified by Ni-affinity (PhoR^E^ and PhoR^S^) or anion-exchange (HK853) and size-exclusion chromatography. *S. pneumoniae* WalK (WalK) was expressed in *E. coli* RIL. Luria Broth (LB) media supplemented with 100 µg/ml ampicillin and 33 µg/ml chloramphenicol was inoculated with an overnight pre-culture (1/50 of the culture volume). At exponential phase (OD\(_{600}\) 0.2 – 0.4) protein expression was induced by addition of 1 mM IPTG for 3 to 5 h at 37°C. The cells were harvested by centrifugation at 4000 g, 4 °C for 25 min and the pellets were stored at -80°C until use. The cell pellets were resuspended in lysis buffer (100 mM Tris pH 8.0, 150 mM NaCl, 0.1 mM PMSF) and sonicated at 4°C for 5 min with pulses of 15 sec at intervals
of 1 minute. The cell debris and the supernatant were separated by centrifugation at 11 000 g, 4°C for 60 min. The cell debris was resuspended in equilibration buffer (100 mM Tris pH 8.0, 150 mM NaCl) containing 2M urea and incubated overnight at 4°C with rotation. After centrifugation at 11 000 g, the supernatant was injected into a Ni-affinity chromatography column (GE Healthcare) equilibrated with equilibration buffer, washed with 5 volumes of equilibration buffer and eluted with equilibration buffer containing 0.5 M imidazole. WalK was concentrated with AmiconUltra (Millipore) centrifugal filters, aliquoted and stored at −80°C until use. The yield was ≤ 0.5 mg/L culture.

**KINASE ASSAY**

Kinase assay was performed as previously described 
26. When comparing the inhibitory capacity of ligands and measuring IC$_{50}$, the final DMSO concentration in the assay was 10% (v/v). Controls lacking ligands and containing an equal concentration of DMSO were carried out in parallel. Inhibition of HK autophosphorylation was determined by incubating 0.12 mg/ml (≈4 µM HK) and up to 20 mM compound in kinase buffer (50 mM Tris HCl, pH 8.5, 50 mM KCl, 5 mM MgCl$_2$, 0.5 mM EDTA and 0.1 mM DTT). Autophosphorylation reactions were initiated by addition of 0.1 µCi/µl [$\gamma^{32}$P] ATP containing from 0.03 to 0.06 µM ATP (final concentrations). Autophosphorylation was quenched with 2x SDS-PAGE sample buffer supplemented with 50 mM EDTA. Samples were applied without heating to 15% (w/v) Tris-glycine SDS-polyacrylamide gels. After electrophoresis, the bottoms of the gels were removed to lower the background signal from the unincorporated radiolabeled ATP. Gels were dried without staining on a Bio-Rad Gel Air drying system and the phosphorylated protein was quantified by phosphor-imaging using a Fluoro Image Analyzer FLA-5000 (Fujifilm) and evaluated with the MultiGauge software (Fujifilm). IC$_{50}$ is the concentration at which 50% residual enzyme activity was observed compared to the negative control, DMSO. Prism GraphPad v.4 was used for curve fitting and statistical analysis 
27.

**AGGREGATION ANALYSIS BY NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS**

PhoRE and PhoRS (0.12 µg/ml, final concentration) were prepared in kinase buffer. Compounds were added to a final concentration of 5 mM or 2 mM for the initial SBVS or the secondary LBSS hits, respectively. DMSO in the assays was maintained to a final concentration 10% (v/v). After 30 min of incubation at room temperature Native polyacrylamide gel electrophoresis (Native-PAGE) loading buffer was added and samples loaded. Coomassie blue staining was used for protein visualization.
BACTERIAL STRAINS

Bacterial strains used in this study for antibacterial susceptibility testing are listed in Table S3. The strains (Table S3) were propagated using standard microbiological procedures.

Uropathogenic *Escherichia coli* CFT 073, *S. aureus* DSM 20231 and *S. epidermidis* DSM 20044 were obtained from the German Collection of Microorganisms and Cell culture (DSMZ). *S. aureus* ATCC 25293, *S. epidermidis* RP62A and RP62A/1, *E. coli* ATCC 25276, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, and *S. pneumoniae* ATCC 49619 were obtained from the American Type Culture Collection (ATCC). *Streptococcus suis* 3881/S10 was provided by the Central Veterinary Institute of Wageningen University and Research Centre (CVI, Lelystad, The Netherlands).

Six clinical sporadic isolates of MRSA (127/08, 145/08, 274/08, V4180, S908, and T4/6) were obtained from wounds of patients admitted to Tawam hospital (Al Ain, UAE). Characterization of the MRSA strains by multilocus sequence typing (MLST), staphylococcal cassette chromosome (SCCmec) typing, accessory gene regulator (agr) typing, *Staphylococcus* protein A (spa) typing, and toxin gene carriage has been described previously [36]. The MRSA strains were resistant to all β-lactam antibiotics tested and to a range of non-β-lactam antibiotics.

Three isolates of *S. epidermidis* (T7/3, T6/19, and T37/8) were obtained from wounds of patients admitted to Tawam hospital (Al Ain, UAE). The biofilm-producing *Staphylococcus epidermidis* RP62A strain produces polysaccharide intercellular adhesin that protects the bacteria against the components of the human innate immune system. Its full genome sequence is in the GenBank: NC002976. *S. epidermidis* RP62A/1 is a stable biofilm non-producer phase variant of RP62A.

Five independent well-characterized multidrug-resistant *Acinetobacter baumanii* strains (NM8, NM35, NM75, NM109, and NM124) and three *Stenotrophomonas maltophilia* strains (B32/1, B5/5, and B6/2) were included in the study. These strains were isolated at four different hospitals in Abu Dhabi Emirate, UAE and their clonal lineages and antibiotic susceptibilities have been previously described.

ANTIBACTERIAL SUSCEPTIBILITY TESTING

For all microorganisms tested except *S. pneumoniae*, the minimal inhibitory concentrations (MICs) were determined as previously described following a standard double-dilution method. MICs were recorded as the lowest concentration of the compound where no visible growth was observed. After plating the dilutions around the MIC, MBC was recorded as the lowest concentration of the compound at which no colonies were formed after 24 h of incubation at 37°C. For *S. pneumoniae* MICs were determined by adapting the standard double-dilution method of this microorganism (use of Todd Hewitt Yeast extract (THY) with 200U/mL of catalase and continuous monitoring of growth) to anaerobic conditions. MBCs for *S. pneumoniae* were determined by inoculation of 10 µl from each well that did not show visible bacterial growth on THY 0.5% 3% blood agar plates. After 24 h of incubation at 37°C 5% CO2,
the first dilution yielding three colonies or fewer was scored as the MBC, as described by the CLSI for starting inoculate of $1 \times 10^5$ CFU/ml.

**HEMOLYSIS ASSAY**

Hemolytic activity against human erythrocytes taken from a healthy donor was measured as previously described. Erythrocytes were incubated with up to 500 µg/ml compounds and the LC$_{50}$ value was recorded as the mean concentration of compound producing 50% hemolysis in three independent incubations.

**CELL LINES AND MEDIUM**

Caco-2 BBE cells (CRL 2102) were purchased from the American Type Culture Center (Manassas, VA) and grown in DMEM (Invitrogen, Paisley, UK) containing Glutamax and supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Colbe, Germany) and 100 U/ml penicillin/100 µg streptomycin (Sigma, St. Louis, MO) in an atmosphere of 5% CO$_2$-95% O$_2$ at 37°C. Cells were trypsinized weekly.

**NEUTRAL RED UPTAKE ASSAY OF CELL VIABILITY**

Neutral red uptake assay of cell viability with Caco-2 cells was performed as previously described. Briefly, after overnight incubation (16 to 24 h) with concentration ranges of B7, B13 or B14, 10 µl of neutral red solution (33 µg/ml) was added to the wells. After 3 h of incubation at 37°C the medium was removed and cells were washed rapidly with PBS. Neutral red was extracted from the cells with 150 µl 1% acetic acid-50% ethanol, shaken for 10 min at RT. The neutral red content was measured on a SpectraMax M5 microplate reader (Molecular Devices) at 540 nm. The readings were expressed as neutral-red uptake relative to the neutral-red uptake of the cells exposed to DMSO. IC$_{50}$ is the concentration causing 50% reduction in neutral-red uptake. Prism GraphPad v.4 was used for curve fitting and statistical analysis.

**HUMAN PBMCs ISOLATION AND FLOW CYTOMETRY**

This study was approved by Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Buffy coats from healthy blood donors were obtained from the Sanquin Blood bank in Nijmegen (The Netherlands). A written informed consent was obtained from each volunteer before sample collection.

PBMCs were isolated from buffy coats of healthy donors using Ficoll Paque Plus density gradient (GE Healthcare, Diegem Belgium) according to the manufacturer’s protocol. After centrifugation, the mononuclear cells were collected, washed in IMDM + glutamax (Invitrogen, Breda, The Netherlands) and resuspended in IMDM + glutamax supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin and
100 µg/ml streptomycin (Sigma, St. Louis, MO). PBMCs were seeded at 1 x 10⁶ cells/well in 48-well plates and, treated with B7 and B13. After 24 hours, the cells were stained with annexin V and PI (BD Biosciences, Breda, The Netherlands) and analysed on a flow cytometer (FACS Canto II, BD).
RESULTS

VIRTUAL SCREENING STRATEGY FOR POTENTIAL HISTIDINE-KINASE INHIBITORS

The ATP-binding pocket of the CA domains of HKs is considered to be the most promising target for inhibition of TCS HKs and has been used previously in structure-based virtual screenings. To identify drug-like ligands of the ATP-binding sites of the CA domains of three different HKs: *T. maritima* HK853 (PDB: 3DGE), *T. maritima* CheA (PDB: 1I58), and *G. stearothermophilus* KinB (PDB: 3D36). These three CA domains were selected because the structures were solved in the presence of a nucleotide and re-docking of the cognate ligand was successful (RMSD 1Å for 3DGE and 3D36, and 3 Å for 1I58). Virtual screening with the three structures that have some sequence variability at the ATP-binding site was expected to facilitate the identification of broad-spectrum histidine kinase autophosphorylation inhibitors (HKAI). The screened compounds were ranked based on the raw docking score, ChemPLP, as well as ligand efficiency. The top 100 docked compounds in common for the three HKs were ranked on both scoring schemes (i.e. ChemPLP and ligand efficiency) and then visually inspected to select 10 compounds, A1-A10 (Figure 1, Tables 1, S1 and S2), for experimental testing.

INHIBITION OF HK AUTOPHOSPHORYLATION IN VITRO AND ANTIBACTERIAL SUSCEPTIBILITY TESTING REVEALED TWO HIT COMPOUNDS

The inhibitory activity of the 10 selected compounds from the SBVS on HKs autophosphorylation was tested in vitro using four different HKs: *T. maritima* HK853 (HK853), as a representative of the structures used in the docking assays, the highly extended HK PhoR from a Gram-negative (*E. coli*; PhoR\textsuperscript{E}), and Gram-positive (*S. aureus*; PhoR\textsuperscript{S}) representative and *S. pneumoniae* WalK (WalK) as a representative of the essential WalKR TCS ubiquitous among Gram-positive bacteria. As a fast way to compare the autophosphorylation inhibitory capacity of the 10 selected compounds the kinase assays were performed at a single and high (5 mM) compound concentration and at one time point (30 sec). Autophosphorylation activity of HK853 was not or weakly (up to 30%) inhibited by the 10 compounds compared to the...
Figure 1. Identification of antibacterial compounds form virtual screening. (A) Schematic representation of the screening workflow including structure-based virtual screening (SBVS; blue) followed by ligand-based similarity search (LBSS; green). In the SBVS a diversity set of the Scopius CSpace database was screened for putative ligands of the ATP-binding site of the CA domain of three HK structures using GOLD. Out of the top 3500 hits for each HK the top 100 in common for the three HKs based on ChemPLP or ligand efficiency were visually inspected and 10 compounds (B) were selected for experimental testing. The results of the in vitro evaluation by kinase assay and antibacterial susceptibility testing of these 10 compounds are provided in Table 1.

negative control (Fig S1A) and it was not possible to identify (a) more potent inhibitor(s) based on HK853 autophosphorylation inhibition. The kinase assays with WalK, PhoRE and PhoRS revealed that compounds A5 and A6 had a higher autophosphorylation inhibitory activity than the other selected compounds identified by SBVS and were general inhibitors of HK autophosphorylation (Figure 1B, Table 1). A5 and A6 inhibit HK autophosphorylation activity in a dose-dependent manner, with IC50 in the high micromolar / milimolar range (Table 1, Figure S2). A5 inhibits PhoRE, PhoRS and WalK with IC50 ≈ 1000 µM and it seems it is not soluble in kinase buffer in the presence of 10% DMSO at concentrations higher than 1.3 mM.
**Table 1.** Selected compounds from the SBVS and their corresponding IC$_{50}$ and MICs

<table>
<thead>
<tr>
<th>Initial hits</th>
<th>IC$_{50}$ [mM]</th>
<th>MIC µg/ml</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Pho$^S$</td>
<td>Pho$^E$</td>
<td>HK853</td>
</tr>
<tr>
<td>A1</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>A2</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>A3</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>A4</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
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<td>A7</td>
<td>&lt; 5*</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>A8</td>
<td>&lt; 5*</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>A9</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>A10</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
<td>&gt; 5</td>
</tr>
</tbody>
</table>

* % Inhibition at 5 mM ≥ 70%, i.e. IC$_{50}$ < 5 mM

# MBC > 500 µg/ml

A6 inhibits Pho$^E$, Pho$^S$ and WalK autophosphorylation with IC$_{50}$ of 372 µM, 1141 µM and > 1000 µM, respectively (Table 1, Figure S2). The inhibitory activity on HK autophosphorylation was most likely due to inhibition of ATP binding in the CA domain as neither inhibitor caused Pho$^E$ or Pho$^S$ protein aggregation when added in high concentrations (Figure S3).

To evaluate the antibacterial effect of the 10 selected compounds from SBVS, their minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined for Gram-positive (S. aureus DSM 20231 and S. epidermidis DSM 20044) and Gram-negative (E. coli UCF 073) bacteria. Only A5 and A6 were able to inhibit bacterial growth at high compound concentrations, which is in agreement with their inhibitory activity on HKs (Table 1). S. epidermidis DSM 20044 growth was inhibited by A5 and A6 while E. coli UCF 073 growth was only inhibited by A6, in all the cases with a modest MICs of 500 µg/ml that are in line with the low HK affinity suggested by the measured IC$_{50}$ (Table 1 and Figure S1). Both compounds were bacteriostatic against the three bacterial strains in the tested concentration range (MBC > 500 µg/ml). On the basis of these results, A5 and A6 were used for a ligand-based similarity search (LBSS) in order to identify more potent inhibitors with stronger antibacterial effect.
LIGAND-BASED SIMILARITY SEARCH

It is a well-accepted assumption that similar compounds have similar activity; however, small structural changes in a compound can result in significant difference in potency (so called ‘activity cliffs’) \(^4\). The latter is exemplified by A5 and A6 since the two compounds are structurally similar but they show different inhibitory capacity toward the HKs assayed (Figures 1B and S1, Table 1). Thus, to explore the chemical space around the scaffolds represented by the initial hits from the SBVS and to identify more potent HK inhibitors with stronger antibacterial effect, analogue compounds of A5 and A6 in the database from the Developmental Therapeutics Program of the National Cancer Institute and the National Institute of Health (DTP) were identified using LBSS (i.e. by circular topological fingerprints). The top 100 hits for the LBSS with A5 or A6 were visually investigated and 25 representative compounds, B1-B25 (Figure 2 and Table S1), were experimentally evaluated for their HK autophosphorylation inhibitory capacity and antibacterial effect in vitro.

LBSS IDENTIFIED MORE POTENT HKs INHIBITORS WITH STRONGER ANTIMICROBIAL ACTIVITY AGAINST GRAM-POSITIVE BACTERIA

To evaluate the 25 selected hits from LBSS, inhibition of autophosphorylation was measured at a single time point (30 sec) using one concentration of each putative inhibitor. PhoR\(^S\) and PhoR\(^E\) were used as targets as these HKs were more strongly inhibited by A5 and A6 than WalK and HK853. The concentration of putative inhibitors was reduced from 5 mM to 2 mM in order to identify inhibitors with higher potency than A5 and A6. B2, B11, B13 inhibit autophosphorylation activity of both PhoR\(^S\) and PhoR\(^E\) by more than 75%. B7, B14 and B15 inhibit PhoR\(^S\) and PhoR\(^E\) autophosphorylation by more than 75% and more than 40 % compared to the negative control, respectively (Figure S2).

The antibacterial effect evaluation of the 25 compounds identified using LBSS showed that B7 was bacteriostatic for \textit{S. aureus} DSM 20231 with a MIC of 250 \(\mu\)g/ml (Table 2). B13 was bactericidal for \textit{S. aureus} DSM 20231 and \textit{S. epidermidis} DSM 20044 with MIC of 8 and 1 \(\mu\)g/ml, respectively, and MBCs of 33 and 8 \(\mu\)g/ml, respectively (Table 2). Additionally, B14 was bactericidal for \textit{S. aureus} DSM 20231 and \textit{S. epidermidis} DSM 20044 at a MIC and MBC of 500 \(\mu\)g/ml (Table 2). The rest of the tested compounds did not inhibit the growth of \textit{S. aureus} DSM 20231 or \textit{S. epidermidis} DSM 20044 at the highest tested concentration of 500 \(\mu\)g/ml (MICs > 500 \(\mu\)g/ml). None of the 25 tested LBSS hits inhibited the growth of \textit{E. coli} CFT 073 at the highest tested concentration of 500 \(\mu\)g/ml (MICs > 500 \(\mu\)g/ml; Table 2). Compounds B7, B13 and B14, which had the highest inhibitory activity in the biochemical assays
Figure 2. Chemical structures of LBSS selected hits. The results of the in vitro evaluation by kinase assay and antibacterial susceptibility testing are presented in Table 2.
Table 2. LBSS hits and their corresponding IC$_{50}$ and MICs

<table>
<thead>
<tr>
<th>Secondary hits</th>
<th>IC$_{50}$ [mM]</th>
<th>MIC [µg/ml]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PhoR$^S$</td>
<td>PhoR$^E$</td>
</tr>
<tr>
<td>B1</td>
<td>&gt; 2</td>
<td>&gt; 2</td>
</tr>
<tr>
<td>B2</td>
<td>&lt;2</td>
<td>&lt;2</td>
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<td>B25</td>
<td>&gt; 2</td>
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*A7 S. aureus MBC > 500 µg/ml

*B13 MBC S. aureus DSM20231 31 µg/ml; B13 MBC S. epidermidis DSM20044 8 µg/ml

†B14 MBC S. aureus DSM20231 and B14 MBC S. epidermidis DSM20044 500 µg/ml
and antibacterial activity were selected for further evaluation *in vitro*. First, their IC\(_{50}\) against PhoR\(_E\) and PhoR\(_S\) was measured (Figure S2, Table 2). B7 inhibits PhoR\(_E\) autophosphorylation with IC\(_{50}\) in the lower micromolar range (≥ 100 µM) and PhoR\(_S\) in the higher micromolar/millimolar range (IC\(_{50}\) PhoR\(_S\) ≥ 1000 µM). The IC\(_{50}\) curves indicated that B7 is not soluble in concentrations higher than 1000 µM in kinase buffer in the presence of 10% DMSO. B13 inhibits PhoR\(_E\) and PhoR\(_S\) with IC\(_{50}\) PhoR\(_E\) < 100 µM and IC\(_{50}\) PhoR\(_S\) = 212 µM and possess good solubility in kinase buffer in the presence of 10% DMSO. B14 IC\(_{50}\) against PhoR\(_E\) and PhoR\(_S\) is higher than 2000 µM and higher than 1000 µM, respectively. The possibility of non-specific inhibition by aggregation was ruled out since none of the compounds caused protein aggregation of PhoR\(_S\) or PhoR\(_E\) at 2 mM in native PAGE (Figure S3). Another reported side effect of previously published TCS inhibitors is membrane damage. To exclude that the antibacterial activity of these compounds could be mediated by this mechanism, the hemolytic activity of B7, B13 and B14 was tested. B7 and B14 did not cause hemolysis at 500 µg/ml (i.e. LC\(_{50}\) > 500 µg/ml). Hemolysis was observed with compound B13 at concentrations higher than the observed MICs (LC\(_{50}\) 277 µg/ml).

**Clinical isolates of multidrug resistant bacteria are susceptible to selected HKAI s in vitro**

The most promising LBSS hits B7, B13 and B14 (Figure 2) were tested for their ability to inhibit the growth of well-characterized methicillin resistant clinical isolates of *S. aureus* (MRSA) \(^{28}\) and clinical isolates of *S. epidermidis*, as well as reference strains of *S. pneumoniae* and *S. suis* and additional reference strains of *S. aureus* and *S. epidermidis*. Since B7, B13 and B14 are expected to inhibit the autophosphorylation of multiple HKs, including HKs from Gram-negative bacteria, their antibacterial effect against multi-drug resistant clinical isolates of the important pathogens *A. baumannii* \(^{45, 46}\) and *S. maltophilia* \(^{31}\) and against reference strains from *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were also tested (Table 3, Table S3). B7 inhibited the growth of two of the 6 MRSA strains *in vitro* with MICs ≥250 µg/ml, which was comparable to the MICs for the *S. aureus* reference strains 25293 and DSM 202231. The other four MRSA strains were not susceptible to B7. As expected from the results of the antibacterial susceptibility testing with *S. epidermidis* DSM 20044, compound B7 had no antibacterial effect on the clinical isolates of *S. epidermidis*. B7 inhibits the growth of all tested *S. pneumoniae* strains with MIC of 16 µg/ml, which is similar to the MICs for the biofilm forming *S. epidermidis* RP62A and non-biofilm forming *S. epidermidis* RP62A/1. Like B7, B13 inhibited the growth of the *S. pneumoniae* strains with MIC of 16 µg/ml.

B13 inhibited the growth of all tested MRSA strains with MICs between 8 and 16 µg/ml, which was comparable to the MICs for the reference strains of *S. aureus*. B13 also inhibited the growth of clinical isolates of *S. epidermidis* with MICs between 8 and 16 µg/ml, which is similar to the MICs for, the biofilm forming *S. epidermidis* RP62A and non-biofilm forming *S. epidermidis* RP62A/1. Like B7, B13 inhibited the growth of the *S. pneumoniae* strains with MIC of 16 µg/ml.

B14 inhibits growth of MRSA with MICs of 250 µg/ml, which was comparable to the MICs for the reference strains of *S. aureus*. B14 inhibited growth of all *S.
epidermidis strains except *S. epidermidis* strain RP62A, with MICs in the range of 250 to 500 µg/ml. B14 was not active against *S. pneumoniae* strains but inhibited growth of the *S. suis* S10 with MIC of 125 µg/ml.

Even though the three selected HKAI s, B7, B13 and B14, inhibit PhoR, homologues of which are present in many Gram-negative bacteria, only B14 inhibits the growth of the Gram-negative *A. baumanii* and *S. maltohilia* strains with MICs from 250 µg/ml to 500 µg/ml. None of the 3 selected inhibitors inhibit the growth of *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 at the highest tested concentration (MIC > 500 µg/ml).

INCREASED ANTIBACTERIAL ACTIVITY IS NOT RELATED TO INCREASED CYTOTOXICITY TO MAMMALIAN CELLS

To check the effect of the most promising inhibitors (B7, B13 and B14) on mammalian cell viability, cytotoxicity was evaluated i) with neutral-red uptake assays with Caco-2 cells (Figure 3) and ii) measurement of dead (propidium iodide positive) and apoptotic (annexin V positive) freshly isolated human peripheral blood mononuclear cells (PBMCs) by flow cytometry (Figure 4). After overnight incubation (16 to 20 h), B7, B13 and B14 inhibited neutral-red uptake with IC$_{50}$ values of 105, 29 and 3 µg/ml, respectively. B7 and B14 IC$_{50}$ are lower than the observed MICs. However, B13 IC$_{50}$ is from 1.8 to 29 times higher than the observed MICs for the panel of Gram-positive strains tested. PBMCs were incubated with 0.2, 1, 3, 13 and 50 µg/ml B7 and B13 for 24 h. There was no significant difference in the number of dead cells in the control (DMSO treated) and B13 treated samples. However, the number of cells binding annexin V, a marker of early apoptosis, was significantly higher in cells incubated with 50 µg/ml of B13 than the control cells (DMSO). This is reflected in a reduction of viable (PI and annexin V negative) cells (Figure 4). Similar results were obtained for compound B7 (Figure 4C) suggesting that a concentration between 13 and 50 µg/ml induces apoptosis in a proportion of the PBMCs after 24 h incubation.
Table 3. Antibacterial effect of selected HK inhibitors against reference strains (marked with *) and (multi-drug resistant) clinical isolates

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<td>145/08</td>
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<tr>
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Figure 4. Mammalian cell viability assessed by neutral-red uptake assays with Caco-2 cell line. The error bars represent the SEM of at least two independent experiments in duplicate. IC\textsubscript{50} is the concentration at which 50\% reduction of neutral-red uptake is observed compared to the negative control (DMSO).

Figure 5. Dose effect of HKAI\textsubscripts{s} on PBMCs viability (A) and apoptosis evaluated by propidium iodide and annexin V (AV) staining. The highest tested concentration (50 µg/ml) of both B7 (C) and B13 (B) induces expression of the early apoptotic marker AV which is reflected in reduction of the live cells with more than 50\% compared to the control (DMSO-treated PBMCs). The error bars represent the SEM of three independent donors.
The HKAI5s are predicted to interact with conserved residues in the ATP-binding site of the HK CA domain

To gain insights into the mechanism of inhibition at the atomic level, the binding modes of A5, A6, B7, B13 and B14 to *T. maritima* HK853 (PDB: 3DGE) were predicted with GOLD and visually investigated. All selected inhibitors were predicted to occupy the ATP-binding pocket and interact with key residues involved in binding of the natural substrate. A common feature for all the compounds was the presence of an aromatic ring that accommodates into the hydrophobic cavity occupied by the pyrimidine ring of adenine. (Figure 6) The aromatic ring forms π–π stacking interactions with Y384 on one side and van der Waals contacts with I416 on the other side of the ring. Similar hydrophobic interactions have been observed for the adenine in the structures of *T. maritima* HK853 and other HKs in complex with nucleotides. Located at the bottom of the hydrophobic pocket is the conserved Asp (D411) in the G1 box that gives specificity for recognition of the N6 amino group in the pyrimidine ring of adenine (Figure 3). For B13, the most potent inhibitor, and B14, the hydroxyl group of the phenolic ring stacked in the adenine pocket is predicted to be hydrogen bonded to the conserved D411 residue (Figure 5). Similar mode of interaction was observed for the binding of the Hsp90 inhibitor radicicol to the ATP-binding domain of the HK PhoQ. In contrast, A5, A6 and B7 are predicted to exploit their common amide moieties to mediate polar interaction on the other part of the active site, which in HK structures with the native ligand is occupied by the nucleotide phosphates and the Mg²⁺ cation (Figure 5). In all the three compounds, A5, A6, and B7, the nitrogen of the amide moiety is predicted to be hydrogen bonded to the conserved N-box Mg²⁺ chelating residue N380 whereas the oxygen is predicted to bind to the main-chain of the G2-Box residues G443, L444 and G445, mimicking in this way the interactions of the ATP γ-phosphate.
Figure 6. Predicted binding modes of selected HK autophosphorylation inhibitors (HKAI). All HKAI (shown in blue as sticks) dock in the ATP-binding site of HK853 with a predicted binding mode resembling the experimental data (3DGE) for the natural substrate ADP (top left). They interact with key elements involved in ATP-binding and autophosphorylation, i.e. the N-, G1-, G2-boxes (shown in orange, blue and yellow, respectively) and the ATP-lid (shown in red).
DISCUSSION

The rapidly growing problem of multi-drug resistant bacteria requires urgent development of a pipeline of new classes of antibiotics. A new class of antibiotics ideally should be broad-spectrum, potent and the initial ‘hit’ scaffold should be amenable to structural changes to allow for optimization of the potency, specificity, efficacy and the ADMET properties. Inhibitors targeting virulence factors including the ability of bacteria to form biofilms have been proposed as a useful approach. As TCS are involved in the regulation of bacterial pathogenesis, including virulence, and biofilm formation, they are absent in mammals and some TCS are essential or conditionally essential for bacterial growth, TCS in general, and HKs in particular, are recognized as promising antibacterial drug targets. The first attempts to discover TCS inhibitors resulted in hydrophobic compounds with non-specific activity, which inhibited HK autophosphorylation by indirect mechanisms such as protein aggregation and showed antibacterial effect due to decreasing membrane integrity. After the elucidation of HK structures SBVS for ligands of the CA domain of HK were reported demonstrating that SBVS is a viable tool for discovery of HKAI with antibacterial, antivirulence and therapeutic effect.

HK CA domains present a characteristic fold with highly conserved residues involved in nucleotide-cation selection and binding. Therefore, autophosphorylation inhibitors discovered using structures of one HK CA are expected to have broad activity against orthologous kinases. However, using one protein structure for SBVS does not take into account the highly flexible and catalytically competent ATP-lid which is variable in length and sequence or the conformation and target plasticity. To address these and to increase the probability of identifying broad-spectrum HKAI, a SBVS by molecular docking using as receptors the ATP-binding site of three HK CA-domains: T. maritima HK853 (PDB: 3DGE), G. stearothermophilus KinB (PDB 3D36) and T. maritima CheA (PDB: 1I58) was performed. The use of multiple structures in docking experiments increases the chance of finding broad-spectrum ligands because it implicitly considers the receptors plasticity, sequence and conformation variability in the docking approach. This approach mimics an ensemble based docking which has been shown to improve the overall performance of virtual screening experiments. The methodology was validated by “re-docking” experiments where experimental protein-ligand complexes were reproduced with low RMSD values using the computational approach followed in the reported SBVS. This provides some confidence that novel ligands can be found using the same protocol. Encouragingly, two of the selected hits from the SBVS, A5 and A6, inhibited the autophosphorylation of HKs different from the HKs used in the SBVS and showed modest antibacterial effect. It seems that the observed HK autophosphorylation inhibition could be specifically mediated via the binding to the CA domain since A5 and A6 did not cause protein aggregation which is a common non-specific mechanism of action of previously reported HK inhibitors. Furthermore, there was a good correlation between the in vitro biochemical activity and the antibacterial effects of A5 and A6.
To identify other possible inhibitors with stronger antibacterial effect, A5 and A6 were successfully used as query molecules in a ligand-based similarity search (LBSS). Among the LBSS hits, the most potent inhibitor was B13 (IC$_{50}$ PhoR$^E$ = 16 µM, IC$_{50}$ PhoR$^S$ = 212 µM), which inhibited the growth of clinical isolates of MRSA and S. epidermidis with MICs $\leq$ 16 µg/ml. The MICs against multi-drug resistant clinical isolates are comparable with the MICs against reference strains. This suggests that the putative mechanism of action of B13 differs from the known antibiotics and/or the mechanisms of resistance of the tested strains to known antibiotics are not functional against B13. Furthermore, B13 is amenable for chemical modification and further optimization. Encouragingly, the stronger antibacterial effect of B13 was not related to higher cytotoxicity. B13 inhibited neutral-red uptake by Caco-2 cells with IC$_{50}$ of 29 µg/ml, which is higher than the observed MICs for Gram-positive strains. Furthermore, even 24 h incubation of PBMCs with up to 50 µg/ml of B13 and B7 did not increase proportion of dead cells. However, the highest B13 concentration tested (50 µg/ml) increased the proportion cells expressing an early marker of apoptosis. Together these results suggest that B13 is a good lead candidate for the development of antibacterials for Gram-positive bacteria. Even though B13 is the most potent PhoR$^E$ inhibitor among the once here reported it did not inhibit the growth of any of the tested Gram-negative strains at the highest tested concentration (500 µg/ml). The cell envelope of Gram-negative bacteria is well known to be more effective barrier to penetration of drugs than that of Gram-positive bacteria but ultimately the compound may be modified to promote uptake or penetrate prokaryotic membranes according to experience with other antibacterials entering into Gram-negatives or by applying nanotechnology approaches$^{59,60,49}$. The predicted binding mode of B14 exploits similar features to B13 as well as polar interaction with the conserved G1-box Asp residue, inhibits Gram-positive (S. aureus reference strains and MRSA, S. epidermidis strains and S. suis 3881/S10) and Gram-negative (A. baumannii and S. maltophilia) strains with MICs in the range of 125 to 500 µg/ml. The MICs range is higher that the observed for B13 in close agreement with B14 higher than B13 IC$_{50}$ for PhoR$^E$ and PhoR$^S$ autophosphorylation inhibition. Therefore, B13 and B14 could represent a promising scaffold to re-design new compounds that could be expected to inhibit a broad spectrum of HKs. Furthermore, B14 scaffold seems to possess good safety profile since the screening of B14 with 60-cell lines$^{61}$ showed that except for HL-60(TB) and UO-31, the compound was not cytotoxic (cell growth > 90%).

On the other hand, the relatively weaker than B13 HKAI B7 (IC$_{50}$ PhoR$^E$ $\approx$ 100µM, IC$_{50}$ PhoR$^S$ $\geq$ 1000 µM) inhibits the growth of the two reference S. aureus strains and 2 of 6 tested MRSA strains. B7 inhibited the growth of S. pneumoniae with MICs in the same range as the MICs for the S. aureus strains. However, B7 was not active against S. epidermidis or any of the Gram-negative bacteria tested. This strain specificity of B7 correlated with the quite different IC$_{50}$ observed for PhoR$^E$ and PhoR$^S$ HKs. A tentative explanation for this fact can arise from the predicted binding mode of B7 that suggests a main interaction mediated by its amide group with the variable part of the ATP-binding site, the ATP lid. All together these indicate that B7 would inhibit autophosphorylation of different HKs with affinity affected by sequence variability. B7 did not cause protein aggregation or hemolysis and seems to possess good toxicity properties as intraperitoneal injection on mice leukemia model L1210$^{62}$.
with up to 400 mg/kg of B7 led to from 93 to 98% survival of the treated animals, showing B7 was nontoxic \textit{in vivo}. All together these data \cite{62}, indicate B7 as a good hit candidate for development of an antibacterial drug. Furthermore, the effect of B7 on PBMCs was similar to that of B13 and B7 (Fig 5). As B7 has been shown to be nontoxic \textit{in vivo} it could be expected that B13 would also be nontoxic \textit{in vivo}.

Except of the number of rotatable bonds, the reported inhibitors B7, B13 and B14 are fragment-like, i.e. they follow the so called “rule of 3” (MW < 300 DA, hydrogen bond donors (HBD) < 3, hydrogen bond acceptors (HBA) < 3). Therefore, they present a promising starting point for the rational re-design of more potent and/ or specific HKAI\textsc{s}. The predicted binding modes of these candidates point to different ways of compound binding to the HK active site that could be exploited in the re-design process. In this way, the interaction with the conserved Asp in the G1 box mediated by the phenolic ring predicted for B13, the most potent inhibitor, and B14 could be combined with the amide group of B7 that could interact with the ATP-lid at the other side of the active center. This new generation of structure-based re-designed compounds could increases potency following the features of B13 and/or specificity exploiting the sequence and structural differences between HK\textsc{s}, mainly located at the ATP-lid, to generate general or strain-specific HK inhibitors.
REFERENCES
14. Casino, P.; Rubio, V.; Marina, A.
Discovery of Inhibitors of Bacterial Histidine Kinases

23. RDKit: Open-source cheminformatics.
2014

56. Li, Y.; Kim, D. J.; Ma, W.; Lubet, R. A.; Bode, A. M.; Dong, Z., Discovery of novel checkpoint kinase 1 inhibitors by virtual screening based on multiple crystal structures. Journal of chemical information and modeling 2011, 51 (11), 2904-14.
**Figure S1.** Autophosphorylation inhibitory activity of the initial hits from the SBVS. (A) One-time (30 sec) one-concentration (5 mM) kinase assay with the SBVS hits and PhoR<sup>S</sup>, PhoR<sup>E</sup>, WalK and HK853 distinguished A5 and A6 as relatively stronger multiple HKs autophosphorylation inhibitors. The IC<sub>50</sub> for PhoR<sup>S</sup>, PhoR<sup>E</sup> and WalK in presence of A5 (B) and A6 (C) were calculated from the autophosphorylation reaction assays at different concentrations of compounds. Error bars represent the standard errors of the mean (SEM) of two independent assays with two replicates.
Figure S2 Autophosphorylation inhibitory activity of the LBSS hits. (A) One-time point (30 sec), one-concentration (2 mM) kinase assay with the LBVS hits and PhoR<sup>S</sup> and PhoR<sup>E</sup> HKs. The IC<sub>50</sub> for PhoR<sup>S</sup> and PhoR<sup>E</sup> in presence of B7 (B), B13 (C) and B14 (D) were calculated from the autophosphorylation reaction assays at different concentrations of compounds. Error bars represent the standard errors of the mean (SEM) of two independent assays with two replicates.
Figure S3. Native PAGE-based aggregation analysis of five inhibitors. A5 and A6 (5 mM), and B7, B13 and B14 (2 mM) do not cause HK aggregation as demonstrated by native-PAGE with PhoR$^S$ and PhoR$^E$ HKs.
**Table S1.** Selected compounds for experimental testing from SBVS (A1 to A10) and LBSS (B1 to B17)

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<td>ClC1=CC=CC(=C1)CNC(=O)NC2=CC=CC=C2</td>
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<td>CCC(C(C(=O)C1=CC=C(=O)C)C=C1)C2=CC(=CC=C2)Br</td>
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<td>CC[N]1C2=C(C=CC=C2)C3=C1C=CC=C(C3)NC(=C)</td>
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Table S2. Compounds selected from SBVS and their corresponding ChemPLP and ligand efficiency.

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<th>Initial hits</th>
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<th>Ligand efficiency</th>
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<td>A10</td>
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<sup>¥</sup> The values for HK853 (PDB: 3DGE, chain A) are presented
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<th>Resistance</th>
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<tr>
<td><em>S. aureus</em> 25293</td>
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<td>Control strain</td>
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<tr>
<td><em>S. aureus</em> 274/08</td>
<td>Tawam Hospital, Al Ain, UAE</td>
<td>β-lactams, Ka, Ne, C, E</td>
</tr>
<tr>
<td><em>S. aureus</em> V4180</td>
<td>Tawam Hospital, Al Ain, UAE</td>
<td>β-lactams, A, G, Ka, Ne, S, Sxt, Tet, C, Rif, E, Cl</td>
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<tr>
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<td>β-lactams</td>
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<td>β-lactams, C, E, Cl</td>
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<td>Control strain</td>
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<td>Control strain</td>
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<td><em>S. epidermidis</em> RP62A/1 (non-biofilm producer)</td>
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</tr>
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<td><em>Streptococcus suis</em> 3881/S10</td>
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<td>All antibiotics commonly used to treat <em>Acinetobacter</em> infections</td>
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**Table S3 (continued). Bacterial strains**

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<td>meropenem</td>
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<td><em>S. maltophilia</em> B32/1 31</td>
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<td>meropenem</td>
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<tr>
<td><em>Escherichia coli</em> CFT 073</td>
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<td>Control strain</td>
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<tr>
<td><em>E. coli</em> 25276</td>
<td>ATCC</td>
<td>Control strain</td>
</tr>
<tr>
<td><em>Klebsiealla pneumoniae</em> 700603</td>
<td>ATCC</td>
<td>Control strain</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 27853</td>
<td>ATCC</td>
<td>Control strain</td>
</tr>
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</table>

*A, amikacin; C, ciprofloxacin; Cl, clindamycin; E, erythromycin; Fu, fusidic acid; G, gentamycin; Ka, kanamycin; Ne, neomycin; Rif, rifampicin; S, streptomycin; Sxt, sulfamethoxazole+trimethoprim; Tet, tetracyline.*
Discovering Inhibitors of Bacterial Histidine Kinases

Chapter 3

HISTIDINE-KINASE INHIBITORS WITH BROAD-SPECTRUM ANTIBACTERIAL EFFECT IDENTIFIED BY FRAGMENT-BASED SCREENS

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2014
ABSTRACT

Novel antibacterials are urgently needed to address the growing problem of bacterial resistance to conventional antibiotics. Two-component systems (TCS) have been previously proposed as promising antibacterial targets. TCS are widely used by bacteria to regulate gene expression in response to various environmental stimuli and physiological stress. They consist of a response regulator and a histidine kinase (HK) containing a highly conserved ATP-binding site that could be a good target for broad spectrum antibacterial drugs. Here we describe fragment-based screens using differential scanning fluorimetry and virtual ligand-based similarity searches that yielded HK autophosphorylation inhibitors with antibacterial effect against multi-drug resistant clinical isolates.

Keywords: antibacterials, histidine kinase autophosphorylation inhibitors, fragment-based screening, two-component systems

Abbreviations: TCS – two-component system/s; HK- histidine kinase; RR – response regulator; FBS – fragment-based screening; LBSS – ligand-based similarity search; DSF – differential scanning fluorimetry
INTRODUCTION

Bacterial two-component systems (TCS) are signal transduction systems used by nearly all bacteria. TCS regulate a variety of processes including bacterial growth, cell-wall metabolism, virulence, biofilm formation and resistance to antibiotics. A prototypical TCS consist of a membrane bound histidine kinase (HK) and its cognate response regulator (RR). Upon sensing an environmental stimulus the HK is autophosphorylated on conserved histidine residues in the dimerization and histidine phosphotransfer (DHp) domain. Subsequently, the phosphoryl group from the His is transferred to a conserved aspartic acid residue in the receiver (REC) domain of the RR. The phosphorylated state of the RR affects its binding affinity to a cognate DNA motif and/or other protein partners thereby modulating transcription of target genes. Inhibitors of HK autophosphorylation targeted at the nucleotide binding site of the catalytic and ATP-binding (CA) domains of HKs are expected to simultaneously inhibit multiple TCS due to the conservation between HKs CA domains (Figure S1). Here we describe the step-wise application of fragment-based screenings (FBS) by differential scanning fluorimetry (DSF) and ligand-based similarity searches (LBSS) to identify HKs autophosphorylation inhibitors with antibacterial activity against multi-drug resistant clinical isolates (Figure 1).

Figure 1. Histidine kinase autophosphorylation inhibitors with antibacterial effect for multi-drug resistant Gram-positive and Gram-negative bacteria were identified by the following workflow (A): First, a fragment library was screened for ligands of both the CA domain of Synechococcus sp. PCC 7942 NblS and CA and DHp domains of Streptococcus pneumoniae WalK. (B) The two hits in common (F1 and F2) were used as query molecules in a ligand-based similarity search of the National Cancer Institute Developmental Therapeutics Programme Database and 19 compounds (Figure S5) were selected for experimental testing. (C) 5 of the 19 tested fragments and F1 inhibited S. pneumoniae WalK and E. coli PhoR autophosphorylation and showed an antibacterial effect against Gram-positive or both Gram-positive and Gram-negative bacteria (Table 1)
### Table 1. Antibacterial activities of selected fragments for a panel of clinical isolates and reference strains.

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<th>F1.6</th>
<th>F1.8</th>
<th>F2.3</th>
<th>F2.4</th>
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</table>
**MATERIALS AND METHODS**

**REAGENTS**

A fragment library of 898 compounds (>95% purity) was purchased from Chem-X-Infinity (Romanville, France) and the individual compounds were stored at -80°C at a concentration of 20 mM. For screening purposes cocktails of 10 compounds at final concentrations of 2 mM were prepared in a 96-well-plate. Re-supply of compounds F2 and F2.5 to F2.9 (Table S2) were purchased from Sigma-Aldrich (Spain). Re-supply of F1 was purchased from Apolo Scientific (United Kingdom). Compounds F1.1 to F1.10 and F2.1 to F2.3 (Table S2) were obtained from Developmental Therapeutics program of the National Cancer Institute and the National Institute of Health (DTP NCI/NIH). γ-32P-ATP was purchased from Perkin Elmer.

**CLONING, EXPRESSION AND PURIFICATION**

*S. pneumoniae* walK encoding the catalytic portion (DHp and CA domain) of WalK (amino acids from 208 to 449) was amplified by PCR from *S. pneumoniae* CDC3059-06 genomic DNA using the following primers: forward 5´-aagttctgaggccgatggagcaggagaaggaacgc-3´ and reverse 5´-atggtctagaaagctctagtcttctacttcatccac-3´. The PCR product was purified by PCR product purification kit (Macherey-Nagel) and cloned into a gel-purified pOpiF vector (kindly provided by Nick Berow, IRB, Spain) linearized with KpnI and HindIII (Fermentas). The insert was cloned into the pOpiF vector with InFusion HD cloning system (Clontech). Positive clones were confirmed by colony PCR and DNA sequencing.

*S. pneumoniae* WalK (WalK) was expressed in *E. coli* RIL. Luria Broth (LB) media supplemented with 100 µg/mL ampicillin and 33 µg/mL chloramphenicol was inoculated with an overnight pre-culture (1/50 of the culture volume). At exponential phase (OD<sub>600</sub> 0.2 – 0.4) protein expression was induced by addition of 1 mM IPTG for 3 to 5 h at 37°C. The cells were harvested by centrifugation at 4000 g, 4°C for 25 min and the pellets were stored at -80°C until use. The cell pellets were resuspended in lysis buffer (100 mM Tris pH 8.0, 150 mM NaCl, 0.1 mM PMSF) and sonicated at 4°C for 5 min at pulses of 15 sec every 1 minute. The cell debris and the supernatant were separated by centrifugation at 11 000 g, 4°C for 60 min. The cell debris were resuspended in equilibration buffer (100 mM Tris pH 8.0, 150 mM NaCl) containing 2M urea and incubated overnight at 4°C with rotation. After centrifugation at 11 000 g, the supernatant was injected into a Ni-affinity chromatography column (GE Healthcare) equilibrated with equilibration buffer, washed with 5 volumes of equilibration buffer and eluted with equilibration buffer containing 0.5 M imidazole. WalK was concentrated with AmiconUltra (Millipore, USA) centrifugal filters, aliquoted and stored at -80°C until use. The yield was ≤ 0.5 mg/L culture.
The catalytic portion (DHp and CA domain) of *E. coli* PhoR (PhoRE), *E. coli* EnvZ, and *S. aureus* PhoR, and the CA domain of *Synechococcus sp.* PCC 7942 NblS were expressed and purified as previously described \(^5\), \(^6\), \(^7\), \(^8\). Shortly, proteins were expressed in *E. coli* RIL and purified by Ni-affinity and size-exclusion chromatography.

Purified proteins were stored in 20 – 50 µl aliquots at -80°C.

**DIFFERENTIAL SCANNING FLUORIMETRY**

To monitor protein unfolding, the fluorescent dye Sypro orange was used \(^9\). Differential scanning fluorimetry experiments (DSF) were conducted in the iCycleriQ Real Time Detection System (Bio-Rad, Hercules, CA). Solutions of 20 µl of 0.1 mg/mL protein (final concentration), 200 µM fragment cocktails or individual fragments (final concentration), 10X sypro orange (final concentration) and buffer (100 mM TrisCl pH 8, 150 mM NaCl) were added to the wells of the 96-well iCycler iQ PCR plate. The plate was heated from 20 to 85 or 99 ºC at a heating rate of 1 ºC/min. The fluorescent intensity was measured with Ex/Em: 490/530 nm. Prism GraphPad was used for curve fitting and statistical analysis \(^10\).

**KINASE ASSAY**

To evaluate the inhibitory capacity of selected hits from DSF and LBSS *in vitro* autophosphorylation kinase assays with γ-\(^32\)P-ATP was performed as previously described \(^11\). Ligands were dissolved in 100% DMSO. When comparing the inhibitory capacity of ligands in one concentration-one time point experiments or when measuring IC\(_{50}\) (the concentration at which 50% residual HK autophosphorylation activity is observed), the final DMSO concentration in the assay was 10% (v/v). Controls lacking ligands contained an equal concentration of DMSO. Inhibition of autophosphorylation was determined by incubating 0.12 mg/mL (≈ 4 µM) HK and up to 20 mM fragment in kinase buffer (50 mM Tris HCl, pH 8.5, 50 mM KCl, 5 mM MgCl\(_2\), 0.5 mM EDTA and 0.1 mM DTT). Autophosphorylation reactions were initiated by addition of 0.1 µCi/µl γ-\(^32\)P-ATP containing from 0.03 to 0.06 µM ATP (final concentrations). Autophosphorylation was quenched with 2xSDS-PAGE sample buffer supplemented with 50 mM EDTA. Samples were applied without heating to 15% (w/v) Tris-glycine SDS-polyacrylamide gels. After electrophoresis, the bottoms of the gels were removed to lower the background from the unincorporated radiolabeled ATP. Gels were dried without staining on a Bio-Rad Gel Air drying system and the phosphorylated protein was quantified by phosphor-imaging. Prism GraphPad was used for curve fitting and statistical analysis \(^10\).
AGGREGATION ANALYSIS BY NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

E. coli PhoR and EnvZ, and S. aureus PhoR (0.12 µg/mL, final concentration) were prepared in kinase buffer. F1 and F2 were added to a final concentration of 2 mM. DMSO in the assays was maintained to a final concentration 10% (v/v). After 30 min of incubation at room temperature native polyacrylamide gel electrophoresis (native-PAGE) loading buffer was added and samples loaded. Coomassie blue staining was used for protein visualization.

LIGAND-BASED SIMILARITY SEARCH

The database from the National Cancer Institute (NCI) was searched for analogous structures of the F1 and F2 as query molecules; the similarity search was performed using the Morgan fingerprint as implemented in RDKit, which is a variation of the ECFP “extended connectivity fingerprints”. The top 100 compounds of each similarity search were visually inspected of which in total 14 compounds were selected and experimentally tested.

ANTIBACTERIAL SUSCEPTIBILITY TESTING

Bacterial strains used in this study for antibacterial susceptibility testing (Table S3, Chapter 1) were propagated using standard microbiological procedures. Minimal inhibitory concentrations (MICs) were determined following a standard double-dilution method. MICs were recorded as the lowest concentration of the compound where no visible growth was observed. After plating the dilutions around the MIC or growing them in fresh MH media, minimal bactericidal concentration (MBC) was recorded as the lowest concentration of the compound at which no colonies were formed or no growth was observed, respectively. For S. pneumoniae MICs were determined by adapting the standard double-dilution method to anaerobic conditions and of this microorganism (use of Todd Hewitt Yeast extract with 200U/mL of catalase and continuous monitoring of growth). MBCs for S. pneumoniae were determined by inoculation of 10 µl from each well that did not show visible bacterial growth on THY 0.5% 3% blood agar plates. After 24 h of incubation at 37°C 5% CO2, the first dilution yielding three colonies or fewer was scored as the MBC, as described by the CLSI for starting inoculate of 1 x 10^5 CFU/mL.

HEMOLYSIS ASSAY

Hemolytic activity against human erythrocytes taken from a healthy donor was measured as previously described. Erythrocytes were incubated with (up to) 500 µg/mL compounds and the LC₅₀ value was recorded as the mean concentration of compound producing 50% hemolysis in three independent incubations.
BINDING MODE PREDICTION

Docking calculations to predict the binding modes of the reported fragments were performed using the ATP-binding domain of HK853CD (PDB: 3DGE, chain A, residues from 270 to 415) and the GOLD docking software \(^{18}\). For each ligand 100 solutions were generated of which the top 20 were visually inspected. In Figure 2 the dominant binding mode within the top 20 solutions is shown for the respective ligand.
RESULTS AND DISCUSSION

To identify fragment-like ligands (MW < 300, ClogP < 3, number of hydrogen bond donors and hydrogen bond acceptors < 3, number of rotatable bonds < 3) of the CA domains of HKs we used the Chem-X-Infinity library (Chem-X-Infinity, Romanville, France) comprising 898 fragments and differential scanning fluorimetry (DSF) as screening method. As targets, we selected the HKs of two essential TCS, WalK-WalR of *Streptococcus pneumoniae* and NblS-RapB of *Synechococcus sp.* PCC 7942. The presence of 4-(4-bromophenyl)-1,3-thiazol-2-amine (F1) and 2-hydroxycarbazole (F2) increased the temperature at which HK NblS (CA domain) unfolds (Tm) by 2.1 and 2.2 °C, respectively, suggesting both F1 and F2 are ligands for the CA domain of NblS. Encouragingly, the screening for ligands of HK WalK (DHp and CA domain) showed that F1 and F2 were also among the hits increasing WalK Tm. F1 and F2 increased WalK Tm by 4.5 and 3.9 °C, respectively (Figure S2). Furthermore, in vitro kinase assays with γ-32P-ATP showed that F1 and F2 inhibited the autophosphorylation of the screened catalytic portion of WalK. In addition, these compounds also inhibited the autophosphorylation of PhoR from the Gram-negative *Escherichia coli* (PhoRE, Figure S3), suggesting a general HK inhibitory activity for F1 and F2.

Compound library screens have previously identified non-drug like inhibitors of HK autophosphorylation with an unspecific mechanism of action due to protein aggregation. However, even at high concentrations (up to 2 mM) F1 and F2 did not cause protein aggregation of the catalytic portions (DHp and CA domains) of *E. coli* PhoR, *E. coli* EnvZ or *S. aureus* PhoR as determined by native polyacrylamide gel electrophoresis (Figure S4).

In an attempt to identify more potent HK autophosphorylation inhibitors than F1 and F2, LBSS were performed using the Morgan fingerprint as implemented in RDKit, the National Cancer Institute Developmental Therapeutics Programme database (NCI/DTP) and F1 and F2 as query molecules. From the LBSS 14 compounds were selected plus 5 additional substituted carbazoles similar to F2, to a final pool of 19 compounds (Figure S5) that was evaluated for WalK and PhoRE autophosphorylation inhibitory activity and antibacterial effect in vitro.

WalK and PhoRE kinase assays with γ-32P-ATP and the 19 compounds at one concentration (2 mM) and one time point (30 sec) showed that the fragments inhibited WalK and PhoRE autophosphorylation activity compared to the negative control from 11 to 62% and 17 to 80%, respectively (Figure S3A). Only F1.6 inhibited WalK autophosphorylation by more than 50% at a concentration of 2 mM meaning that the remaining 18 of the tested compounds are weak (Ki >> 2 mM) WalK autophosphorylation inhibitors. Inhibition of PhoRE autophosphorylation was greater than 50% for F1, F1.8, F2, F2.1, F2.2, F2.8 and F2.9 meaning that the remaining 12 of the tested compounds are weak PhoRE autophosphorylation inhibitors. The IC50 of the active compounds (F2.2. and F2.9 were excluded since they did not show antibacterial effect, see below) were measured in a multiple concentrations one time point (30 sec) PhoRE kinase assays (Table 1, Figure S3B). F1 and F1.8 inhibited PhoRE
autophosphorylation with $IC_{50} \approx 2$ mM and $\leq 1$ mM, respectively, and the two compounds were not soluble in kinase buffer in the presence of 10% DMSO at concentrations higher than 1 mM (Table 1, Figure S3B). F2 and F2.1 inhibited PhoR$^E$ autophosphorylation with $IC_{50}$ values of 0.3 and 0.24 mM, respectively, and F2.8 inhibited PhoR$^E$ autophosphorylation with an $IC_{50}$ value of 0.72 mM (Table 1, Figure S2B). F2, F2.1 and F2.8 showed good solubility in kinase buffer in the presence of 10% DMSO.

The antibacterial effect of the 19 selected compounds was first studied by determining their minimal inhibitory concentrations (MICs) by standard micro-dilution antibacterial susceptibility testing$^{21}$ for two Gram-positive (Staphylococcus aureus DSM 20231 and Staphylococcus epidermidis DSM 20044) and one Gram-negative (the uropathogenic E. coli CFT 073) strains (Table 1, Table S1, and Table S3, Chapter 1). The highest tested concentration was 500 µg/mL. F1, F1.6, F1.8, F2.3, F2.4 and F2.8 showed moderate antibacterial effects with MICs in the range of 31 to 250 µg/mL for S. aureus DSM 20231 and 4 to 500 µg/mL for S. epidermidis DSM 20044 (Table 1). Only F1.8 and F2.8 had antibacterial action on the Gram-negative E. coli CFT 073 with MICs < 250 µg/mL. With the exception of F2.2 and F2.9, those compounds showing HK autophosphorylation inhibitory activity also showed antibacterial activity, suggesting the possibility that the antibacterial activity might be mediated through the inhibition of HK autophosphorylation.

Next, we studied the antibacterial effect of F1, F1.6, F1.8, F2.3, F2.4 and F2.8 on a panel of clinical isolates and reference strains of pathogenic bacteria (Table S3, Chapter 1). The methicillin-resistant S. aureus (MRSA) strains are well characterized and are resistant to all β-lactam antibiotics and a range of non-β-lactam antibiotics$^{22}$. S. epidermidis clinical isolates were obtained from wounds of patients admitted to Tawam Hospital (Al Ain, United Arab Emirates). The clinical isolates of the Gram-negative Acinetobacter baumannii$^{23}$ and Stenotrophomonas maltophilia$^{24}$ are well characterized and show multi-drug resistance. Additional reference strains included the Gram-positive S. aureus ATCC 25293, S. epidermidis RP62A and RP62A, S. pneumoniae 49619 and the emerging zoonotic pathogen Streptococcus suis 3881/S10, and the Gram-negative of E. coli ATCC 25276, Klebsiella pneumoniae ATCC 700603 and Pseudomonas aeruginosa ATCC 27853.

F1 and F1.6 (halogen-substituted phenyl-thiazoleamines) showed similar antibacterial activities for the panel of clinical isolates and reference strains. Both F1 and F1.6 showed antibacterial effect for the MRSA strains (with the exception of F1.6 for V4180 MRSA strain; Table 2) and S. aureus 25293 with MICs in the range of 125 – 500 µg/mL (Table 2). The V4180 MRSA strain is resistant to a wider range of antibiotics compared to the other MRSA strains tested (Table S3, Chapter 1) including the small molecule antibiotics chloramphenicol and sulfamethoxazole. Given the broad range of antibiotic resistance of V4180 MRSA it is reasonable to propose that the presence of putative efflux pumps for small molecules could be responsible for the lack of susceptibility to F1.6. F1 and F1.6 showed antibacterial effect on one of the three tested S. epidermidis clinical isolates with MICs of 63 and 125 µg/mL, respectively. F1.6 MIC for S. pneumoniae 49619 was 256 µg/mL. F1 and F1.6 did not show antibacterial effect on S. suis 3881/S10 or on any of the Gram-negative strains tested.
F1.8 (bromophenyl-pyrimidinediamine) showed antibacterial effect on all the Gram-positive strains tested. F1.8 MICs for the *S. aureus* reference strains and the MRSA strains were in the range of 125 to 250 µg/mL. F1.8 MICs for the *S. epidermidis* strains were in the range of 31 to 500 µg/mL. F1.8 MICs for *S. suis* 3881/S10 and *S. pneumoniae* 49610 were 250 µg/mL and 128 µg/mL, respectively. F1.8 showed antibacterial effect for all the Gram-negative strains tested except for the *S. maltophilia* B32/1 strain. F1.8 MICs for *A. baumannii* strains, *E. coli* ATCC 25276 and *K. pneumoniae* ATCC 700603 were in the range of 125 – 250 µg/mL and the MIC for *P. aeruginosa* ATCC 27853 was 500 µg/mL. In a similar way to F1.6 with V4180 MRSA, the lack of susceptibility of *S. maltophilia* B32/1 to F1.8 could be explained by the presence of efflux pumps. *S. maltophilia* B32/1 is also resistant to the small-molecule β-lactam antibiotic, meropenem so that efflux pumps with broad substrate specificity may be involved.

The MICs of F2.3, F2.4 and F2.8 (substituted carbazoles) for the MRSA strains and *S. aureus* 25293 were in the range of 125 – 500 µg/mL. F2.3 and F2.8 MICs for the *S. epidermidis* strains were in the range of 31 – 250 µg/mL. F2.3 and F2.4 MICs for *S. pneumoniae* 49610 were 4 µg/mL and 64 µg/mL, respectively. F2.4 and F2.8 MICs for *S. suis* 3881/S10 were 125 and 250 µg/mL, respectively. F2.3 and F2.8 did not show antibacterial effect for the *A. baumannii* and *S. maltophilia* strains. F2.4 MICs for 3 of the 5 *A. baumannii* strains and 2 of the 3 *S. maltophilia* strains tested were 500 µg/mL. F2.4 and F2.8 MICs for *E. coli* ATCC 25276 were 500 µg/mL. F2.8 MIC for *K. pneumoniae* ATCC 700603 was 250 µg/mL. None of F2.3, F2.4 and F2.8 showed antibacterial effect for *P. aeruginosa* ATCC 27853.

One of the side effects of previously described TCS inhibitors is membrane damage. To exclude this mechanism of action we performed hemolysis experiments with erythrocytes from a healthy donor. None of the selected inhibitors (F1, F1.6, F1.8, F2.3, F2.4 and F2.8) caused erythrocyte hemolysis at 500 µg/mL (LC<sub>50</sub> > 500 µg/mL) indicating that the inhibitors do not cause loss of integrity of the erythrocyte plasma membrane.

To evaluate the cytotoxic effect of the selected fragments cell viability assays by neutral-red uptake by Caco-2 cells were performed. F1, F1.6, F1.8, F2.3, F2.4 or F2.8 inhibited neutral-red uptake by Caco-2 cells with IC50 values of 294, 121, 134, 30, 51 and 61 µg/ml, respectively, (Figure S6).

Additionally, cytotoxicity was evaluated by 24 h incubation of 0.2, 1, 3, 13 and 50 µg/ml of F1, F1.8, F2, F2.1, F2.3 and F2.8 with freshly isolated human peripheral blood mononuclear cells (PBMCs) and measurement of dead (propidium iodide (PI) positive) and apoptotic (annexin V (AV) positive) cells by flow cytometry. There was no significant difference in the number of dead cells in the control (DMSO treated) and inhibitors-treated samples. However, the number of cells binding annexin V, a marker of early apoptosis, was significantly higher in cells incubated with 50 µg/ml of F1.8, F2.3, and F2.8 (Figure S5) than the control cells (DMSO-treated). This is reflected in a reduction of viable (PI and annexin V negative) cells (Fig S5A). The results suggest that a concentration between 13 and 50 µg/ml of F1.8, F2.3 and F2.8 induces apoptosis in a proportion of the PBMCs after 24 h incubation.

The IC<sub>50</sub> values for neutral-red uptake by Caco-2 were mostly higher than the observed MICs and 24 h incubation with some of the hits (F1.8, F2.3 and F2.8)
induced apoptosis in human PBMCs suggesting off-target effects of the described HK autophosphorylation inhibitors. This requires that the further optimization of the hits should be multidirectional, including improving the affinity and specificity to the target bacterial HKs, enhancing the antibacterial effect and improving the toxicity profile of the hits.

To get insights into the putative interaction mode of the described HK autophosphorylation inhibitors with the CA domains of HKs, molecular docking experiments were performed. F1, F1.6, F1.8, F2.3, F2.4, F2.8 and ADP as an internal control were docked to the CA domain of *T. maritima* HK853 (PDB: 3DGE) using the GOLD docking software. The root-mean square deviation (RMSD) between the docked ADP and the cognate ADP structure in the KH853 was 1.0 Å. This corresponds to a successful redocking calculation and, therefore, validates the used docking parameters. Due to low molecular weight of the fragments, it is possible that the fragments possess more than one binding mode. Encouragingly, within the top 20 solutions for each fragment only one or two binding modes were predicted and the predicted binding modes of similar fragments were consistent with each other. This together with the low RMSD for the docked ADP gave us confidence about the predicted binding modes of the studied fragments.

The predicted binding modes of the studied fragments resemble the binding mode of the cognate ligand, ADP (Figure 2), by forming also stabilizing hydrogen bonds with the conserved Asp411 (located at the bottom of the hydrophobic ATP-binding pocket in the so called G1 box) as well as with the ATP-lid (i.e. Ile424, Tyr429 and Arg430): All six fragments form hydrogen bonds with Asp411 either by classical hydrogen bond donor groups such as NH2 or OH (F1.6, F2.8) or by other polar groups such as Br and CH (F1, F1.8, F2.3, F2.4). The ATP-lid interacts by hydrogen bonds with four of the six compounds (F1, F1.8, F2.3, F2.4). The ATP-lid is a HK distinctive flexible and variable loop that covers the ATP-binding pocket and is crucially involved in nucleotide binding and in autophosphorylation reactions. Overall, the predicted binding modes indicate that stabilizing interactions are formed by all six fragments with binding siteresidues that are conserved and crucial for autophosphorylation. This supports the *in vitro* results and suggests that the fragments might also inhibit further HKs not tested in this study.

**CONCLUSIONS**

In conclusion, by combining fragment-screenings by differential scanning fluorimetry and ligand-based similarity searches of a public repository database we identified multiple HK autophosphorylation inhibitors with antibacterial effect against Gram-positive and Gram-negative pathogens including multidrug resistant clinical isolates. The reported inhibitors present a promising starting point for the rational design of more potent HK general or specific inhibitors and the discovery of antibacterials with improved physicochemical and ADMET properties.
Figure 2. Predicted binding modes of selected fragments to the CA domain of *T. maritima* HK853 (PDB: 3DGE)\(^\text{15}\). The predicted binding modes indicate that these fragments form stabilizing hydrogen bonds with conserved elements of the CA domain of HKs represented by *T. maritima* HK853. The formed interactions include the D411 which also forms interactions with the adenine of the natural ligand ADP. Fragments interact as well with residues of the G1 box of the ATP-lid which are crucial for ligand binding and recognition. The binding modes are visualized with MOE \(^\text{28}\). The sequence alignment in the upper right corner demonstrates that the binding sites residues Ile424, Tyr429, and Asp411, are conserved in HKs suggesting that the compounds might also bind to other HKs not tested in this study.
REFERENCES
7. RDKit: Open-source cheminformatics.
Supplementary Material Chapter 3

Figure S1. The ATP-binding site of HK CA domain is a well-defined and highly conserved pocket and allows the discovery and design of HK autophosphorylation inhibitors with broad-spectrum antibacterial activity following structure-based approaches A) Structure of *B. subtilis* WalK CA domain (PDB: 3SL2). The conserved N-, G1- and G2-boxes are shown in red, blue and green, respectively. The variable ATP-lid is shown in pink. B) *B. subtilis* WalK CA domain (PDB: 3SL2) coloured by conservation. The conservation scores were calculated using ConSurf^30^. The ATP-binding pocket was calculated using PyMol^31^ and is shown as a semi-transparent yellow surface. ATP is shown in ball-and-stick representation with carbon, nitrogen, oxygen and phosphate atoms in yellow, blue, red and orange, respectively. C) Alignment of WalK CA domains from different organisms D) Alignment of the CA domains of different *E. coli* HKs
Figure S2. Identification of F1 (D4.1) and F2 (G5.9) as putative CA ligands by DSF. The fragment-library was divided in cocktails of ten fragments, each at a final concentration of 2 mM in a 96-well plates. Each plate included negative controls (DMSO) and positive-control (ADP, ATP, AMP-PNP). A) The individual compounds from the cocktails which increased the Tm of NblS (A) and the Tm of WalK (D) were tested. F1 (D4.1) and F2 (G5.9) were identified as the compounds present in the D4 and G5 cocktails that most likely caused the observed increase of NblS and WalK Tm (B, C, E, F).

Figure S3. Biochemical evaluation by in vitro kinase assay A) First, the autophosphorylation inhibitory activity was evaluated in a one concentration (2 mM) one time-point (30 sec) in vitro kinase assays with WalK and PhoRE. The fragments inhibited WalK and PhoRE autophosphorylation with 10 to 62% and 17 to 80%, respectively. B) IC50 of the more potent inhibitors (% inhibition at 2 mM > 50 %) with antibacterial effect were measured in a multiple-concentrations one time-point (30 sec) experiments.
Figure S4. F1 and F2 (2 mM) do not cause HKs aggregation as demonstrated by native-PAGE with E. coli PhoR and EnvZ, and S. aureus PhoR.

Figure S5. Selected fragments for experimental testing. F1 and F2 were identified as hits in a screening for ligands of both the CA domain of NblS and the CA and DHp domain of WalK. F1.1 to F1.10 and F2.1 to F2.4 were identified by ligand-based similarity searches of the National Cancer Institute Developmental Therapeutics Programme database using F1 and F2 as query molecules.
**Figure S6.** Cell viability assessed by neutral-red uptake assays with Caco-2 cell line. The error bars represent the SEM of at least two independent experiments in duplicate. IC$_{50}$ is the concentration corresponding to 50% reduction of neutral-red uptake by Caco-2 cells.
Figure S7. Dose effect of identified inhibitors on PBMCs viability (A) and apoptosis evaluated by propidium iodide and annexin V (AV) staining. 50 µg/ml of F1.8 (C) and F2.3 (E), F2.8 (F) induce stronger expression of the early apoptotic marker AV compared to the control (0 µg/ml, DMSO-treated PBMCs) which is reflected in reduction of the live cells (A) with around or more than 50% compared to the control (DMSO-treated PBMCs). Error bars represent SEM for three independent donors.
**Table S1.** Autophosphorylation inhibitory activities and antibacterial activities of the tested compounds.

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<th>S. epidermidis DSM 20044</th>
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Table S2. Compounds purchased for experimental testing.

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CHAPTER 4

BROADENING THE ANTIBACTERIAL SPECTRUM OF HISTIDINE KINASE AUTOPHOSPHORYLATION INHIBITORS VIA THE USE OF E-POLY-L-LYSINE CAPPED MESOPOROUS SILICA-BASED NANOPARTICLES

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TO BESubmitted TO NANOMEDICINE: NANOTECHNOLOGY, BIOLOGY AND MEDICINE AFTER PUBLISHING OF CHAPTER 2 AND 3
ABBREVIATIONS

Gr- Gram negative
Gr+ Gram positive
TCS Two-component systems
HK Histidine kinase
RR Response regulator
HKAI Histidine-kinase autophosphorylation inhibitor
f n-[(3-trimethoxysilyl)propyl]ethylenediamine triacetic acid trisodium salt
ePLL ε-poly-L-lysine
N.X MCM-41 funcionalized with n-[(3-trimethoxysilyl)propyl]ethylenediamine triacetic acid trisodium salt and capped ε-poly-L-lysine (ePLL) cationic polymer and loaded with:
N.Van – vancomycin
N.HKAIs – histidine kinase autophosphorylation inhibitors
N.Rho – rhodamine
F.HKAIs – free histidine kinase autophosphorylation inhibitors
INTRODUCTION

Infections caused by Gram negative (Gr-) multidrug-resistant (MDR) bacteria have become a growing challenge worldwide. The most important resistance problems are encountered in Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter* spp, which have developed resistance to last-line antibiotics such as expanded-spectrum cephalosporins and/or carbapenems. Urinary tract infections (UTIs) caused by Gr- uropathogenic *Escherichia coli* (UPEC) are among the most common bacterial infections in humans. Recently multi-drug resistant *E. coli* have been reported.

Most of the bacterial infections in fish are caused by Gr- bacteria, including *Aeromonas hydrophilia*, *Aeromonas salmonicida*, *Flavobacterium columnare*, *Vibrio*, and *Pseudomonas* spp. Currently antimicrobials are routinely used to directly control bacterial infections in pet (ornamental) fish and are added to the water these fish are shipped in to suppress the growth of potential pathogens during transport. This activity has led to high prevalence of multi-drug tolerant or resistant bacteria and associated antimicrobial resistance genes both in ornamental fish and their carriage water. This might present a potential health risk as studies have suggested that there is bi-directional transfer of resistance genes between aquatic and other Animalia as evidenced by the fact that antimicrobial resistance (AMR) genes of bacteria recovered from the aquatic environment can share very high sequence homology to clinically important AMR genes on plasmids and integrons found in clinical isolates of human pathogens. The transmission of zoonotic multidrug resistant Gr- bacteria between food-producing animals and humans and between companion animals and humans has also been reported.

Therefore, novel strategies to target infections caused by Gr- bacteria are urgently needed. Two-component systems (TCS) are signal transduction systems found in nearly all bacteria and have been proposed as promising antibacterial drugs targets for both Gram positive (Gr+) and Gr- infections. A prototypical TCS consist of membrane bound histidine kinase (HK) and its cognate response regulator (RR). Upon environmental stimuli the catalytic and ATP-binding (CA) domain of the HK autophosphorylates a conserved histidine found within the dimerization and histidine phosphotransfer (DHp) domain, which subsequently serves as the phosphodonor for a cognate RR. Many HKs are bi-functional and also dephosphorylate their cognate response regulator. The changes in the phosphorylation levels of the RR are usually related to changes of the expression of target genes involved in the regulation of variety of processes including growth, virulence, antibacterial resistance and adaptation to environmental changes. Following structure-based and fragment-based drug discovery approaches (Chapter 2 and 3), bacterial histidine kinase autophosphorylation inhibitors (HKAIs) were identified (Figure 1). The HKAIs inhibited the autophosphorylation of HKs from both Gr+ and Gr- bacteria, including *E. coli* PhoR. PhoR belongs to the PhoR-PhoB TCS, which is involved in the regulation of the *pho* regulon. The *pho* regulon is not only a regulatory circuit of phosphate homeostasis but also plays an important adaptive role in stress response and bacterial virulence. Despite the fact that the HKAIs inhibited
autophosphorylation of HKs from Gr- bacteria, they showed only moderate to strong antibacterial activity against Gr+ bacteria and not against Gr- bacteria. This was anticipated to be a consequence of the permeability barrier provided by the outer membrane (OM) of Gr- negative bacteria, which are inherently resistant to many hydrophobic antibiotics. This resistance limits the arsenal of antibiotics that are effective in treating Gr-negative bacterial infections. To overcome this problem, several compounds have been used to facilitate permeability of the OM and sensitize Gr- bacteria to hydrophobic antibiotics. These compounds are typically cationic, amphiphilic molecules that can be prepared from peptides or steroids. Nanotechnology has also been proposed and exploited in addressing the problem related to Gr-membrane permeability.

A nanodevice loaded with vancomycin was shown to possess antibacterial activity against a range of Gr- bacteria including *E. coli* (DH5α, 100, 405), *Salmonella typhimurium* and *Erwinia carotovora*. The device consisted of a nanometric mesoporous MCM-41 phase (ca. 100 nM; MCM-41) loaded with vancomycin, functionalized with n-[(3-trimethoxysilyl)propyl]ethylenediamine triacetic acid trisodium salt (f) and capped with a cationic polymer of ε-poly-L-lysine (ePLL), referred to here as N.Van. Vancomycin is glycopeptide antibiotic that selectively targets Gr+ bacteria. Most Gr- bacteria are intrinsically resistant to vancomycin due to its relatively high molecular weight and size and inability to pass through porins in the OM of Gr-. Capping the NPs with polycationic ePLL is anticipated to promote ionic bonding with the negatively charged OM and facilitate permeability or antibacterial effects of the inhibitor through its disruptive effects on the bacterial membrane. Indeed, N.Van broadened the spectrum of vancomycin and enhanced the efficacy of ePLL.

Here we employed a similar approach to N.Van to investigate the potential to deliver novel HKAIs to Gr- bacteria. Silica-based nanoparticles were synthesized, loaded with HKAIs and capped with ePLL, referred here as N.HKAIs. The antibacterial effect of N.HKAIs against Gr- bacteria *in vitro* was studied against a range of Gr- bacteria. To facilitate the potential clinical translation of these nanomedicines we tested their potential cytotoxicity using epithelial and immune cells, as well as their possible immunotoxicity to macrophages, which are crucial for immune defence against pathogens. Furthermore, we evaluated the uptake of the nanoparticles in zebrafish models.
MATERIALS AND METHODS

CHEMICALS

The chemicals tetraethylorthosilicate (TEOS) (98 %), n-cetyltrimethylammonium bromide (CTAB) (≥99 %), sodium hydroxide (≥98 %), and rhodamine B were provided by Aldrich. ε-poly-L-lysine (εPLL) was purchased from Chengdu Jinkai Biology Engineering Co. Ltd. N-[(3-Trimethoxysilyl)propyl]ethylenediamine triacetic acid trisodium salt was purchased from FluoroChem. Na₂HPO₄·7H₂O, KH₂PO₄, NaCl, NH₄Cl, MgSO₄, glucose and CaCl₂ were purchased from Scharlab (used for the preparation of M9 minimal medium). All reagents were used as received.

Histidine kinase autophosphorylation inhibitors (HKAs; Table S1, Figure 1) were obtained from the National Cancer Institute Developmental Therapeutics Programme (NCI/DTP).

GENERAL TECHNIQUES

Powder XRD, TG analysis, elemental analysis, TEM and N₂ adsorption-desorption techniques were employed to characterize the prepared materials as previously described. Powder X-ray diffraction measurements were performed on a Philips D8 Advance diffractometer using Cu Kα radiation. Thermo-gravimetric analysis were carried out on a TGA/SDTA 851e Mettler Toledo balance, using an oxidant atmosphere (air, 80 mL/min) with a heating program consisting of a heating ramp of 10°C per minute from 393 to 1273 K and an isothermal heating step at this temperature for 30 minutes. Elemental analysis was performed in a CE Instrument EA-1110 CHN Elemental Analyzer. TEM images were obtained with a 100 kV Jeol JEM-1010 microscope. N₂ adsorption-desorption isotherms were recorded on a Micromeritics ASAP2010 automated sorption analyser. The samples were degassed at 120°C in vacuum overnight. The specific surface areas were calculated from the adsorption data in the low pressures range using the BET model.

SYNTHESIS OF THE MESOPOROUS SILICA SUPPORT

The starting nanoparticulated MCM-41 mesoporous solid was prepared following well-known procedures using n-cetyltrimethylammonium bromide (CTAB) as template and tetraethylorthosilicate (TEOS) as hydrolytic inorganic precursor. N-cetyltrimethylammonium bromide (CTAB, 1.00 g, 2.74 mmol) was first dissolved in 480 mL of deionised water. Then 3.5 mL of NaOH 2.00 M in deionised water were added to the CTAB solution. The solution temperature was adjusted to 80°C. TEOS (5.00 mL, 2.57 x 10⁻² mol) was then added dropwise to the surfactant solution. The mixture was stirred for 2 h to give a white precipitate. Finally, the solid product was centrifuged, washed with deionised water and ethanol, and was dried at 60°C (MCM-41 as-synthesised). To prepare the final porous material (MCM-41), the as-synthesised
solid was calcined at 550°C using an oxidant atmosphere for 5 h in order to remove the template phase.

**SYNTHESIS OF MESOPOROUS SILICA-BASED NANOparticles LOADED WITH RHODAMINE AND CAPPED WITH E-POLy-L-lySINE (N.RHO)**

The material designed and synthesized in our previous work \(^3\)2, (named in the current paper as N.Rho) was used as control solid in some of the experiments. It consisted on MCM-41 silica nanoparticles loaded with the Rhodamine B dye and externally functionalized with N-[(3-Trimethoxysilyl)propyl]ethylenediamine triacetic acid trisodium salt (f) and e-poly-L-lysine (ePLL).

**SYNTHESIS OF E-POLy-L-lySINE CAPPED MESOPOROUS NANOparticles LOADED WITH HISTIDINE-KINASE AUTOPHOSPHORYLATION INHIBITORS (N.HKAI\(\text{S}\))**

\(\varepsilon\)-poly-L-lysine capped mesoporous nanoparticles loaded with histidine-kinase autophosphorylation inhibitors (N.HKAI\(\text{s}\); N.B2, N.B11, N.B13, N.B14, N.B15, N.F1.8, N.F2.3) were obtained essentially as previously reported \(^3\)2. The solids N.HKAI\(\text{s}\) consist of mesoporous nanoparticles loaded with the free HKAI\(\text{s}\) (F.HKAI\(\text{s}\); F.B2, F.B11, F.B13, F.B14, F.B15, F.F1.8 and F.F2.3; Table S1), externally functionalized with N-[(3-Trimethoxysilyl)propyl]ethylenediamine triacetic acid trisodium salt (f) and capped with \(\varepsilon\)-poly-L-lysine (ePLL). In a typical synthesis, 100 mg (except for NB.11 where 30 mg was used) of the template-free MCM-41 was suspended in solution containing the corresponding antibacterial derivative. F.B13, F.B14 and F.F1.8 (corresponding to N.B13, N.B14, N.F1.8 final solids loading) were dis-solved in DMSO. The rest of the F.HKAI\(\text{s}\) (F.B2, F.B7, F.B11, F.B15, F.F2.3) were dis-solved in \(\text{H}_2\text{O}:2\text{DMSO}\). After stirring at room temperature for 24 h, an excess (15 mmol/g MCM-41) of N-[(3-Trimethoxysilyl)propyl]ethylenediamine triacetic acid trisodium salt (f) was added and the mixture stirred for 12 h at room temperature. After that, the solid was centrifuged and washed with 1mL of water. Then, the solids were dried under vacuum. In a second step, the pre-functionalized solid was suspended in a solution containing \(\varepsilon\)-poly-L-lysine in \(\text{M9 minimal medium (1.8 mL/100mg of prefunctionalized solid of a 0.033M } \varepsilon\)-poly-L-lysine solution). This suspension was stirred for 1 h at room temperature. Finally, each solid was filtered by centrifugation with 1 mL of \(\text{M9 medium. The final solids were labelled as N.B2, N.B11, N.B13, N.B14, N.B15, N.F1.8 and N.F2.3 and were dried under vacuum for 12 h.}

**BACTERIAL STRAINS**

*Escherichia coli* CFT 073, *E. coli* DH5α and *S. marcescens* 21639 were obtained from Leibniz Institute DSMZ – German Collections of Microorganisms and Cell
Cultures (Braunschweig, Germany) and were propagated using standard microbiological procedures.

**ANTIMICROBIAL SUSCEPTIBILITY TESTING**

Minimal inhibitory concentrations (MICs) of MCM-41, N.Rho, ε-poly-L-lysine (ePLL), N.HKAIs (N.B2, N.B7, N.B11, N.B13, N.B14, N.B15, N.F1.8 and N.F2.3), and F.HKAIs (F.B2, F.B7, F.B11, F.B13, F.B14, F.B15, F.F1.8 and F.F2.3) were determined using a standard double dilution method. Antimicrobial susceptibility testing was performed with inoculum of 1.10^5 – 1.10^6 CFU/ml. N.HKAIs were suspended in water and Mueller Hinton (MH) broth (1:1) to concentration corresponding to 1.10^7 µg/ml (N.B2, N.B7, N.B11, N.B13, N.B14, N.B15) or 2.5x10^4 µg/ml (N.F1.8 and N.F2.3) F.HKAIs and sonicated until obtaining homogenous suspension. The suspensions were serially diluted in MH broth in 96-well plates. The MIC was recorded as the lowest concentration where no visible growth was observed. Minimal bactericidal concentrations (MBCs) were determined by plating 10 µl of the wells where no growth was observed on MH agar plates and incubating overnight at 37 °C or by inoculating 90 µl MH broth with 10 µl of the wells where no growth was observed. MBC was recorded as the lowest concentration where no colonies were formed or no visible growth was observed after overnight incubation at 37°C.

**SYNERGY ASSESSMENT**

Synergy between ε-poly-L-lysine (ePLL) and F.HKAIs (F.B2, F.B7, F.B11, F.B13, F.B14, F.B15, F.F1.8 and F.F2.3) was assessed by the check-board method in 96-well-plates. Fractional inhibitory concentrations (FICs) were calculated based on the content in the wells in the growth-no growth area. FIC > 0.5 indicates no synergy.

**CELL LINES AND MEDIUM**

Cells were grown in medium supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Colbe, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO) in an atmosphere of 5% CO₂-95% O₂ at 37°C and were passaged weekly.

Caco-2 BBE cells (CRL 2102), and Raw 264.7 (Mouse leukemic monocyte macrophage cell line) were purchased from the American Type Culture Center (Manassas, VA) and grown in DMEM (Invitrogen, Paisley, UK) containing Glutamax, in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 2mM Glutamine, in DMEM, respectively.
Neutral red uptake assay of cell viability

Neutral red uptake assay of cell viability with Caco-2 cells was performed as previously described. Briefly, after overnight incubation with the NPs (MCM-41, N.Rho and N.HKAIs), the F.HKAIs or ePLL, 10 µl of neutral red solution (33 µg/ml) was added to the wells. After 3 h of incubation at 37°C, the medium was removed and cells were washed rapidly with PBS. Neutral red was extracted from the cells with 150 µl 1% acetic acid-50% ethanol, shaken for 10 min at RT. The neutral red content was measured on a SpectraMax M5 microplate reader (Molecular Devices) at 540 nm. The readings were expressed as neutral-red uptake relative to the uptake of the cells exposed to the negative control (medium or DMSO).

Activation of macrophages and treatment with NPs and free histidine kinase autophosphorylation inhibitors

Raw cells were seeded in 96-well-plates (1.10^5 cells/well) and allowed to attach to the bottom of the wells at 37°C and 5% CO₂ for 1 h. Cells were treated with NPs (MCM-41, N.Rho, N.HKAIs) or F.HKAIs, in the presence or absence of 1 µg/ml LPS. Controls containing solvent (water or DMSO) were run in parallel. A NO assay and a viability assay (XTT) were run consecutively for every treatment group. The macrophages cultures were incubated at 37°C and 5% CO₂ for 24 h before performing the appropriate assays.

Nitric oxide assay

The NO production of the macrophages was indirectly determined using the Griess reaction, a colorimetric assay to measure the accumulation of the stable end product of NO degradation, nitrite, within the culture supernatant. Briefly, after overnight incubation with NPs or F.HKAIs, plates were centrifuged at 2000 rpm, at RT for 7 min. 75-µl volumes of the supernatants were transferred to a new 96-well plate. 100 µl of 1% sulfanilamide (Sigma) in 2.5% phosphoric acid was added to each well. This was followed by addition of 100 µl of 0.1% N-naphthyl-ethylenediamine (Sigma) in 2.5% phosphoric acid. The plate was gently tapped to mix the contents, incubated at RT for 5 min, and the optical density (OD) at 540 nm with 690 nm as a reference was determined using an automated spectrophotometer SpectraMax M5 microplate reader (Molecular Devices).

Cell respiration (XTT viability assay)

Changes in macrophage viability were assessed using the XTT viability assay. This assay measures the respiratory activity of mitochondria by determining the accumulation of a coloured formazan by-product within the supernatants of treated wells. Briefly, a 20% volume of 1 mg/ml sodium 3,3′-1[(phenylamino)carbonyl]-
3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulphonate acid hydrate (XTT) in 1× RPMI was added to each well of the treatment plate. The plates were gently tapped to mix the contents, incubated for up to 2 h at 37°C and 5% CO2, and the OD at 450 using 690 nm as a reference was determined using an automated spectrophotometer SpectraMax M5 microplate reader (Molecular Devices).

**TUMOR NECROSIS FACTOR ALPHA ELISA**

Tumor-necrosis factor alpha (TNFα) protein levels were measured by quantitative enzyme linked immunosorbent assays (ELISA) using the Mouse TNF alpha ELISA Ready-SET-Go!® reagent set (Affymetrix, eBioscience, Netherlands). Briefly, after overnight incubation of Raw cells with NPs or F.HKAIs before or after stimulation with 1 µg/ml LPS, plates were centrifuged at 2000 rpm, at RT for 7 min. 75-µl volumes of the supernatants were transferred to a new 96-well plate and ELISA was performed according to the manufacturer’s protocol.

**ZEBRAFISH MAINTENANCE**

Zebrafish (*Danio rerio*) were bred at the animal facility of Wageningen University and Research Centrum as previously described 42, 43. Experiments with zebrafish embryos were conducted in accordance with the guidelines of the Wageningen University Animal Experiments Committee. The breeding tank was cleaned twice a day until hatching by removing debris and unfertilized eggs. Embryos were collected as eggs on different days and maintained in oxygenated water at 28°C. For experiments zebrafish embryos between 1 and 4 days post-hatching were used. After 4 days post-hatching, embryos were killed by incubation on ice for at least 30 minutes.

**INCUBATION OF ZEBRAFISH WITH N.RHO**

Zebrafish were cultured to 96-well plates (1 embryo/ well) in a total volume of 100 µl oxygenated water at 28° C. Then 1, 2, 5, 10 µl of the nanoparticle stock solution (1 mg/ml) were added to the wells. Each treatment group contained 5 embryos 3 days post-hatching and 5 replicates were tested per group. To visualize the NPs two embryos per group were anesthetized using tricaine (MS-222) at standard concentration. Images were taken after 1.5, 6 and 24 hours post incubation using Leica M205 FA Fluorescence Stereo Microscope (Leica Microsystems).
RESULTS AND DISCUSSION

The growing number of reports about the spread of multi-drug resistant (MDR) Gr- pathogens demands urgent development of novel approaches to combat the health risks associated with infections caused by Gr- bacteria. Due to low number of new classes of antibacterials entering the market over the past decade and growing incidence of infections due to MDR bacteria, effort is also being directed towards the improving pharmacological properties, potency and delivery of known antibiotics. Here we describe a new approach using polycationic ε-poly-L-lysine capped nanoparticles loaded with bacterial histidine kinase autophosphorylation inhibitors (HKAs) as a mean to enhance permeability of the outer membrane (OM) of Gr-negative bacteria. Newly identified two-component systems (TCSs) inhibitors (Chapter 2 and 3, Figure 1, Table S1) were used to evaluate the nanodelivery approach as they were demonstrated to inhibit HKs from Gr- in vitro but lacked antibacterial activity against Gr- bacteria.

Figure 6.
Histidine-kinase autophosphorylation inhibitors (HKAs) Selected HKAs inhibited the autophosphorylation of E. coli PhoR with IC₅₀ ranging from 0.2 to > 2 mM. The HKAs showed antibacterial effect for a panel of Gram-positive bacteria (Chapter 2 and 3) with MICs in the range of 8 to > 500 µg/ml. Only B14 and F1.8 showed antibacterial effect for Gram-negative bacteria with MICs ≥ 250 or ≥ 125, respectively. The IC₅₀ (the concentration causing 50% reduction in autophosphorylation activity; mM) of each HKAs for E. coli PhoR (PhoR IC₅₀) is presented together with a consensus MIC (minimal inhibitory concentrations) in antimicrobial susceptibility testing assays with a panel of Gram-positive (Gr+) and Gram-negative (Gr-) bacteria.
SYNTHESIS AND CHARACTERIZATION OF MESOPOROUS SILICA-BASED NANOPARTICLES CAPPED WITH E-POLY-L-LYSINE AND LOADED WITH HK AUTOPHOSPHORYLATION-INHIBITORS

To evaluate the antibacterial effect of the newly identified HKAIIs to Gr- bacteria, mesoporous silica-based nanoparticles (MCM-41) were loaded with HKAIIs or rhodamine dye as a control (N.HKAIs or N.Rho) and capped with ε-poly-L-lysine (ePLL, Figure 2 and 3) as previously described 32. The organic content of N.HKAIs and N.Rho was verified and is shown in Table 1 and details are provided in the Supporting Information. The content of the N.HKAIs was between 5 and 138 mg HKAIr/g solid (0.02 to 0.45 mM HKAI/g solid). The ePLL and silica content for the N.HKAIs or N.Rho was in the same range, between 91 and 220 mg ePLL/g solid (0.022 – 0.054 mmol ePLL/g solid) and between 51 and 59% silica, respectively.

Figure 2. Schematic representation and putative mechanism of action of the nanoparticles. Nanometric mesoporous MCM-41 phase (ca. 100 nM; MCM-41) functionalized with n-[(3-trimethoxysilyl)propyl]ethylendiamine triacetic acid trisodium salt (blue cylinder) was capped with ε-poly-L-lysine cationic polymer (ePLL) and loaded with histidine kinase autophosphorylation inhibitors (HKAIIs, yellow balls). Putative mechanism of action of the nanoparticles (NPs) is attributed to interaction of the positively charged capped NPs with the negatively charged Gr- bacterial cell wall (blue lines) and displacement of the capping ePLL which results in release of the loaded HKAIIs and subsequently to inhibition of two-component systems (TCS) signalling.
Figure 3. TEM image of the inorganic MCM-41 calcined matrix (A) and some of the final solids (B, C, D and E, correspond to N.Rho, NB.2, NB.14 and NF1.8 final solids, respectively). The images clearly show the mesoporous structure of the matrix which was maintained after capping with ePLL and loading with HKAs.
Table 1. Content (α, mmol/g of solid) of the different HKAI (αHKAI), n-[(3-trimethoxysilyl)propyl]ethylenediamine triacetic acid trisodium salt (f, αf), ε-poly-L-lysine (ePLL, αePLL) and silica content (silica, %) in the prepared nanoparticles.

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Table 2. Antibacterial activities of free HK autophosphorylation inhibitors (Free) and HK autophosphorylation inhibitors loaded to silica-based mesoporous nanoparticles capped with ε-poly-L-lysine (Nano). HKs inhibitors part of the nanoformulations showed > 10 fold lower MICs for Gram negative strains compared to the free HKs autophosphorylation inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>MIC µg/ml</th>
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<th>E. coli CFT 073</th>
<th>E. coli DH5α</th>
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<td></td>
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<td>50</td>
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<td>&gt;500</td>
<td>&gt;100</td>
<td>100 &gt;500</td>
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<td>50</td>
<td>100 &gt;500</td>
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<td>&gt;100 &gt;500</td>
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<td>&gt;25</td>
<td>25 250</td>
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Table 3. Antibacterial activity of MCM-41, the control N.Rho and ePLL.

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<tr>
<td>MCM-41</td>
<td>&gt; 11</td>
<td>&gt; 11</td>
<td>&gt; 11</td>
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ePLLnano ePLL part of the nanoformulation N.Rho
ANTIBACTERIAL ACTIVITIES

The antibacterial activities of N.HKAIs, F.HKAIs, ePLL, MCM-41 and N.Rho were evaluated by determining the optical density at 600 nm (OD$_{600}$) of bacterial suspensions incubated overnight (16 to 24 h) in 96-well plates. The F.HKAIs did not inhibit bacterial growth at concentrations even as high as 500 µg/ml (MIC > 500 µg/ml) except for F.B14 and F.F1.8. F.B14 and F.F1.8 showed antibacterial effect for *E. coli* CFT 073 and *S. marcescens* 21639 with MICs of 500 and 250 µg/ml, respectively (Table 2). The N.HKAIs showed antibacterial effect for *E. coli* CFT 073 with MICs corresponding to 3 to 100 µg/ml HK inhibitors (Table 2) which in all cases is more than 10 times lower than the MICs of the F.HKAIs. To exclude the possibility that the observed antibacterial effect is due to the antibacterial action of the MCM-41 or of the ePLL in the nanoformulations, the MICs of MCM-41 and N.Rho were determined. MCM-41 and N.Rho did not inhibit bacterial growth, even at concentrations as high as 11 mg/ml (MIC > 11 mg/ml) and 10 mg/ml (MIC > 10 mg/ml), respectively. 10 mg/ml N.Rho corresponds to 1020 µg/ml ePLL (MIC$_{ePLL}$ nanoformulation > 1020 µg/ml, Table 3). The highest tested concentrations of MCM-41 and ePLL as part of N.Rho were higher than the MCM and ePLL content of the highest tested concentrations of the N.HKAIs. To exclude the possibility that the observed antibacterial effect of the N.HKAIs is due to a synergy effect of the HKAI and ePLL within the nanoformulations, synergy effects against *E. coli* CFT 073 were tested by the checkboard method and no synergy between ePLL and the free HK inhibitors was observed (FICs > 0.5). Therefore, the antibacterial effect of the N.HKAIs is solely dependent on the release of the NPs cargo (the HKAI) and not on the synergistic effect between the HKAIs and ePLL, or due to the presence of MCM-41 and/or ePLL in the nanoformulations.

ADVERSE EFFECTS ON HOST CELLS IN VITRO

To be used as effective drugs, antibacterials should not cause adverse effects such as cytotoxicity and immunotoxicity to host cells. Although silica is generally considered to be non-cytotoxic, the formulations of the silica-based NPs described here may affect their biocompatibility because of altered physicochemical properties. Therefore, to assess this we investigated the cytotoxicity and immunotoxicity of the NP formulations described in this chapter with and without incorporation of the HKAI to determine how it affected host toxicity of the HKAI.

Cytotoxicity

Viability of human colon carcinoma cells (Caco-2) incubated with MCM-41, N.Rho, N.HKAIs or F.HKAIs or ePLL was measured using the neutral-red uptake assay $^{39}$. The N.HKAIs showed lower cytotoxicity compared to the F.HKAIs (Table 4, Figure S3). The IC$_{50}$ (the concentration of test material that causes a 50% decrease in neutral-red uptake, relative to the solvent control) of the N.HKAIs corresponded to ≥ 100 µg/ml HKAI, whereas, the IC$_{50}$ of the F.HKAIs varied between 3 and 134 µg/ml. The selectivity index, i.e. mammalian cell cytotoxicity (IC$_{50}$/antibacterial effect
(MIC), was improved for the N.HKAIs compared to the F.HKAIs. This is an expected result because mesoporous NPs capped with cationic ePLL are designed to interact with the negatively charged OM of Gr- bacteria. Their proposed mechanism of action is attributed to ionic interaction of the positively charged ePLL capped NPs with the bacterial OM, followed by displacement and binding of ePLL to the Gr- cell membrane, thereby allowing the NKAs to be released. Furthermore, MCM-41 and ePLL were not cytotoxic at concentrations as high as 2742 µg/ml and 673 µg/ml, respectively, corresponding to the highest content of MCM-41 and ePLL present in the N.HKAIs. N.Rho was also not cytotoxic at 1000 µg/ml, corresponding to 102 µg/ml ePLL as part of the nanoformulation. This indicates that there is no synergistic effect between MCM-41 and the capping ePLL on cytotoxicity and suggests that ePLL capped mesoporous NPs are safe to be used for antibacterial drug delivery.

<table>
<thead>
<tr>
<th>IC₅₀ [µg/ml] Selectivity index</th>
<th>Caco-2 IC₅₀/MIC₅₀ E.coli*</th>
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<tr>
<td>Nano Free</td>
<td>Nano</td>
</tr>
<tr>
<td>B2</td>
<td>≥ 100 n.t.</td>
</tr>
<tr>
<td>B7</td>
<td>&gt; 100 105</td>
</tr>
<tr>
<td>B11</td>
<td>≥ 100 ≥ 500 ≥ 7.7</td>
</tr>
<tr>
<td>B13</td>
<td>≥ 100 29 ≥ 4</td>
</tr>
<tr>
<td>B14</td>
<td>≥ 50 3 ≥ 1</td>
</tr>
<tr>
<td>B15</td>
<td>&gt; 100 ≈ 500 ≥ 2</td>
</tr>
<tr>
<td>F1.8</td>
<td>&gt; 25 134 &gt; 4.2</td>
</tr>
<tr>
<td>F2.3</td>
<td>&gt; 25 30 &gt; 4.2 &lt; 0.06</td>
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</table>

*E. coli CFT 073

Table 4. Effect on cell viability of Caco-2 cells of free HKs autophosphorylation inhibitors (Free) and HKs autophosphorylation inhibitors loaded to silica-based mesoporous nanoparticles capped with ε-poly-L-lysine (Nano) evaluated by neutral-red uptake assay. IC₅₀ is the concentration at which 50% reduction in neutral-red uptake relative to the solvent control is observed. Detailed dose-effect curves are presented in Figure S3.
Activation of NO production by macrophages and macrophage viability

The high-output isoform of nitric oxide synthase (NOS2 or iNOS) lies at the interface between the innate and adaptive immune systems\textsuperscript{46}. Human NOS2 is most readily observed in monocytes or macrophages from patients with infectious or inflammatory diseases. Sustained production of NO endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminths, and tumor cells. Other macrophage products such as acid, glutathione, cysteine, hydrogen peroxide, or superoxide enhance the antibacterial and cytotoxic actions of NO. To investigate whether the NPs and the HKAI B13 interfered with NO production by immune cells we incubated cultured macrophages with MCM-41, N.Rho, N.B13 and F.B13 with and without LPS (1µg/ml) stimulation for 16 to 24 h and then measured NO production (Figure 4 and S4). Simultaneously, the effect of MCM-41, N.Rho, N.B13 and F.B13 on macrophage viability and TNFα protein levels were evaluated using the XTT assay and TNFα ELISA respectively (Figure 5, 6, and S4).

Figure 4. Dose effects of MCM-41 (A), N.Rho (B), N.B13 (C) and F.B13 (D) on NO production by cultured macrophages (Raw cells). NO production is not affected by MCM-41 (A), N.Rho (B), N.B13 (C), F.B13 (D) or the solvents (H2O and DMSO, E) with or without stimulation with 1 µg/ml LPS. The results are presented relative to the NO production induced by stimulation with 1 µg/ml LPS. The error bars present the SEM of at least three independent experiments in duplicate.
Figure 5. Dose effects of MCM-41 (A), N.Rho (B), N.B13 (C) and F.B13 (D) on cell viability of cultured macrophages (Raw cells). Cell viability is not affected by MCM-41 (A), N.Rho (B) or the solvents (H2O and DMSO, E) with or without stimulation with 1 µg/ml LPS. Reduction in cell viability by N.B13 (C) is observed only at the highest tested concentration (100 µg/ml B13) with stimulation with 1 µg/ml LPS. Reduction of cell viability by F.B13 (D) is observed at the highest tested concentration (100 µg/ml B13) with or without stimulation with 1 µg/ml LPS and at 20 µg/ml B13 with stimulation with 1 µg/ml LPS. The results are presented relative to the viability of the solvent controls (H2O or DMSO). The error bars present the SEM of at least two independent experiments in duplicate.

1 mg/ml MCM-41 or N.Rho did not cause significant changes in NO production by macrophages or in macrophage viability compared to the solvent control before or after stimulation with 1 µg/ml LPS. These results indicate that MCM-41 and N.Rho do not stimulate NO production by themselves and do not inhibit NO production induced by LPS. The production of NO before and after stimulation with 1 µg/ml LPS after incubation with N.B13 and F.B13 at concentrations corresponding to 100 µg/ml B13 was comparable to the solvent control. However, free HKAI F.B13 reduced viability of macrophages stimulated with LPS by more than 50% at a concentration of 100 µg/ml and LPS stimulated macrophage viability by more than 50% at a concentration of 20 µg/ml F.B13. This finding suggests that F.B13 might induce an increase in NO production by macrophages which is masked by its cytotoxic effect. The macrophage viability was decreased by N.B13 at concentrations corresponding to 100 µg/ml B13, implying that N.B13 also stimulates some NO production by macrophages. Nevertheless, these effects are observed at concentrations higher than the MIC of F.B13 for Gr+ bacteria and of N.B13 for Gr- bacteria (Chapter 2 and Table 2,
respectively). As the nanomaterials, MCM-41 and the control N.Rho did not cause significant changes in NO production or in macrophages viability, the N.B13 effect on NO production and macrophage viability can be attributed to release of the cargo, i.e. HKAI B13. This is in agreement with the cell viability study of free HKAI B13 and Caco-2 cells (Table 4 and Figure S3).

Production of tumor necrosis factor alpha by macrophages activated with NPs and F.HKAI

Tumor-necrosis factor alpha (TNFα) is involved in the process of inflammation and in cellular processes mediating protection to bacterial infections. Antibacterials ideally should not alter the level of TNFα production by activated and resting macrophages. Indeed, neither the NPs (MCM-41, N.Rho or N.B13) nor the HKAI F.B13 caused changes in TNFα secretion by Raw cells before or after stimulation with 1 μg/ml LPS (Figure 6).

ZEBRAFISH EMBRYOS CAN BE USED IN INFECTION STUDIES WITH MESOPOROUS SILICA-BASED NPS CAPPED WITH EPLL

To evaluate the toxicity of the NPs capped with ePLL and the suitability of zebrafish embryos for future infection studies, the toxicity of N.Rho to zebrafish embryos was evaluated. Given the low toxicity of the HKAI loaded NPs (N.HKAI) and their antibacterial activity we evaluated the potential to deliver antibacterials via nanoparticles to zebrafish embryos via dispersion in the water.

Zebrafish infection models are becoming a valuable tool in pre-clinical antibacterial drug discovery. Experimental candidate drugs can be injected via the yolk sack or the blood vessels using a microinjection device. However, microinjection is time-consuming, laborious and not always accurate. Ideally, antibacterials delivered via nanoparticles are dispersed in the water to test their uptake, distribution and adverse effects.

N.Rho was dispersed in the water where the zebrafish embryos were incubated. The dispersion of the N.Rho in the water did not result in signs of toxicity and lethality of the zebrafish embryos for up to 24h hours post incubation (Figure 7) at concentrations of 10, 20 and 50 μg/ml. Incubation with N.Rho led to concentration dependent increase of rhodamine fluorescence and increased over time in the intestine and in the yolk sac (Figure 7). These can be attributed to uptake of N.Rho via the gills or the skin, distribution in the blood and accumulation in the yolk sac and intestine. The gills have been suggested as the main site for antigen uptake in fish, however, the skin was shown to play a significant role in the uptake of antigens as well. If N.Rho is taken up via the gills, N.Rho might circulate through the vasculature to the yolk sac and the intestine. A similar distribution in the body was observed with liposomes delivered by immersion. Possible release of the rhodamine cargo due the encounter of the N.Rho with the yolk content or with Gr- negative bacteria normally present in the intestine may also be a factor influencing the results.
Figure 6. Dose effects of MCM-41 (A), N.Rho (B), N.B13 (C) and F.B13 (D) on TNFα levels of cultured macrophages (Raw cells). TNFα levels in Raw cells are not after incubation with MCM-41 (A), N.Rho (B), N.B13 (C), F.B13 (D) or by the solvents (H2O and DMSO, E) with or without stimulation with 1 µg/ml LPS. The results are presented relative to the TNFα levels after stimulation with 1 µg/ml LPS. The error bars present the SEM of at two independent experiments in duplicate.

The observations made after incubation of N.Rho with zebrafish embryos open up new targeted strategies for treating bacterial infections in aquaculture industry using mesoporous silica-based NPs capped with ePLL thanks to the simplicity, absence of toxicity and accuracy of the cargo delivery.
Figure 7. Incubation of zebrafish embryos with N.Rho. Rhodamine fluorescence in the yolk sack and the intestine is increased in concentration-dependent and time-dependent manner.

**SUMMARY**

By combining two novel strategies, loading of nanoparticles with antibacterials on the one hand, and targeting TCS signalling on the other hand, it was possible to achieve antibacterial effect against Gr- bacteria *in vitro*. The antibacterials-loaded NPs did not show adverse effects to mammalian cell viability, or immunotoxicity *in vitro*. The NPs did not cause adverse effects on zebrafish embryos. All together, these open promising possibilities for the treatment of Gr- infections and development of novel antibacterials and/ or delivery of antibacterials using nanoparticles.
REFERENCES

and antimicrobial resistance genes. Applied and environmental microbiology 2007, 73 (20), 6686-6690.


MATERIALS CHARACTERIZATION

MCM-41 as-synthesized, MCM-41 and the final N.Rho and N.HKAl (N.B2, N.B7, N.B11, N.B13, N.B14, N.B15, N.F1.8 and N.F2.3) synthesized materials were characterized through standard techniques. Figure S1 shows the X-ray diffraction (XRD) patterns of the nanoparticulated MCM-41 matrix as-synthesised, the MCM-41 calcined and the final N.Rho solid. (The rest of X-ray diffraction patterns of the antibacterial loaded solids are not shown due to the low amount of final solids obtained). The MCM-41 as-synthesised (curve a) displayed the four typical low-angle reflections of a hexagonal-ordered matrix indexed at (100), (110), (200) and (210) Bragg peaks. In curve b (MCM-41 calcined), a significant shift of the (100) peak in the XRD and a broadening of the (100) and (200) peaks are observed. These changes are due to the condensation of silanols in the calcination step, which caused an approximate cell contraction of 4 Å. Finally, curve c shows the N.Rho solid XRD pattern.

For this material, reflections (110) and (200) were mostly lost due to a reduction in contrast related to the functionalisation process and to the filling of mesopores with rhodamine B. Although these reductions, the intensity of the (100) peak in this pattern strongly indicates that the loading process with the dye and the additional functionalisation with N-[(3-Trimethoxysilyl)propyl]ethylenediamine triacetic acid trisodium salt and the covering with ε-poly-L-lysine did not modify the mesoporous MCM-41 scaffold nature.

Likewise, TEM analysis of the different prepared solids was performed. TEM images showed the typical channels of the MCM-41 scaffolding and can be visualised as alternate black and white stripes in which the typical hexagonal porosity of the MCM-41 calcined material can also be observed (see Figure 2). In the same way, TEM images of some of the final solids loaded with the antibacterial products are also shown in Figure 2. The remaining final solids also showed the same morphology under the TEM analysis. TEM images also show that the materials were obtained as spherical nanoparticles of ca 80 - 100 nm. Then, it can be concluded that the spherical shape and mesoporous nature of the inorganic matrix remains after the loading and functionalization processes.

Figure S2 (curve a) displays the N₂ adsorption-desorption isotherms of the MCM-41 calcined nanoparticles. This curve shows an adsorption step with a P/P₀ value between 0.2 and 0.35, due to a type IV isotherm, which is typical of mesoporous materials. This first step corresponds to nitrogen condensation in the mesopore inlets. With the BJH model on the adsorption curve of the isotherm, a narrow pore size distribution with an average pore diameter of 2.56 nm (see Figure S2 inlet) and a pore volume of 0.64 cm³ g⁻¹ were calculated. The absence of a hysteresis loop in this pressure range and the low BJH pore distribution is due to the cylindrical uniformity of mesopores. The total specific area was 916 m² g⁻¹, calculated with the BET model. The a₀ cell parameter 42.9 Å (d₁₀₀ = 37.17 Å), the pore diameter (2.56 nm) and the wall...
thickness value (17.31 Å) were calculated from the XRD, porosimetry and TEM measurements. Other important feature of the curve is the characteristic H1 hysteresis loop that appears in the isotherm at a high relative pressure (P/P₀ > 0.8) which can be closely associated with a wide pore size distribution. This hysteresis loop is due to the filling of the large pores among the nanoparticles (0.22 cm³ g⁻¹ calculated by the BJH model) because of textural porosity. In relation to the control synthesized N.Rho material, the N₂ adsorption-desorption isotherm of this solid was associated to the typical mesoporous systems with filled mesopores (see Figure S2, curve b). Thus, as it was expected, a lower N₂ adsorbed volume (BJH mesopore volume = 0.15 cm³ g⁻¹) and surface area (204.9 m² g⁻¹) were found, compared with the initial MCM-41 material. As it can be observed, this solid presents a curve with no gaps at low relative pressure values if compared to the mother MCM-41 matrix (curve a). Another important feature of N.Rho is that no maximum was found in the pore size distribution curve, which can be associated with the closed pores due to the functionalization process. Table S2 shows a summary of the BET-specific surface values, pore volumes and pore sizes calculated from the N₂ adsorption-desorption isotherms for MCM-41 calcined and N.Rho.

Thermogravimetric studies and elemental analyses of all final prepared solids were carried out, in order to quantify the organic contents of each material. In particular, the amount of the different loading products and functionalization with product f and ePLL were calculated. All the results are summarized in Table 1 in the body text.
Figure S1. Powder X-Ray diffractograms showing X-Ray patterns of MCM-41 scaffolding as synthesized (a), MCM-41 after the calcination process(b) and the final control solid N.Rho(c).

Figure S2. Nitrogen adsorption-desorption isotherms for (a) MCM-41 mesoporous material (b) N.Rho. Inlet: Pore size distributions of the MCM-41 mesoporous material.
Figure S3. Cell viability of Caco-2 cells assessed by neutral-red uptake after 24 h exposure to NPs or to free HKAI s. Error bars represent the SEM of at least two independent experiments in duplicate.
Figure S4. Dose effect of the NPs MCM-41 (A, B), N.Rho (A, B) or N.B13 (C, D) and F.B13 (C, D) on NO production and cell viability of macrophages before (A, C) or after stimulation with 1 µg/ml LPS (B, D). Results are expressed as the effect of the NPs or F.B13 relative to the effect of the solvent controls (water or DMSO, respectively). The error bars represent the SEM of at least two independent experiments in duplicate.
Table S1. Selected histidine kinase autophosphorylation inhibitors (HKAs) and their physicochemical properties

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*RB-rotatable bonds

Table S2. BET specific surface values, pore volumes and pore sizes calculated from the N\textsubscript{2} adsorption-desorption isotherms for selected materials.

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CHAPTER 5

COMPUTER-AIDED APPROACHES IN HIT OPTIMISATION

Nadya Velikova
ABBREVIATIONS

**QSAR** – quantitative structure-activity relationship  
**LEI** – ligand-efficiency index  
**SEI** – surface-efficiency index  
**BEI** – binding-efficiency index  
**TCS** – two-component systems  
**HK** – histidine kinase  
**RR** – response regulator
INTRODUCTION

Computational chemistry has been applied widely in the pharmaceutical industry for drug discovery, lead optimization, risk assessment, toxicity prediction and regulatory decisions\(^1\). Over the past four decades, quantitative structure–activity relationship (QSAR) modelling has completely changed the way the interaction of organic compounds with the various forms of life is studied\(^2\). It attempts to formulate the relationship between structure and activity as a mathematical model. Therefore, the binding affinity of a new molecule can be predicted using a QSAR model derived from the known inhibitors and their experimental bioassay values\(^3\). Using such an approach, a large library of possible drug candidates can be tested for their predicted selectivity and potency\(^4\), saving time and money in drug discovery research. Successful drug discovery requires not only identifying hits with desired biochemical properties (e.g. target affinity) but also hits with optimal physicochemical and ADMET (adsorption, distribution, metabolism, excretion and toxicity) properties\(^5\). Once a hit is identified its optimisation should be bidirectional. Ligand-efficiency indices (LEIs) connect the physicochemical properties of hits (e.g. MW and polar surface area [PSA]) with the biological target via the affinity parameter (IC\(_{50}\), enzyme activity or other related measures of affinity)\(^6,7\).

Herein we describe the application of computer-aided drug discovery approaches (including QSAR modelling, ligand-based similarity search and LEIs-based evaluation) for the identification of putative HK autophosphorylation inhibitors with antibacterial activity and low toxicity.
MATERIALS AND METHODS

QSAR PREDICTIVE MODEL BUILDING

The experimental *E. coli* PhoR autophosphorylation inhibition data (% inhibition at 2 mM) of 39 compounds (Chapter 3 and 4) were used as a dataset for building a quantitative structure–activity relationship (QSAR) model for inhibition of *E. coli* PhoR autophosphorylation (Table S1). The percentage inhibition range was from 6 to 85%. Predictive QSAR model was built automatically using the Auto-Modeller module of StarDrop (Optibrium, Cambridge).

LIGAND-BASED SIMILARITY SEARCHES

The National Cancer Institute Developmental Theraupeutics Programme database (DTP NCI/NIH) was searched for analogue structures of the query molecules B7, B11, B13, B15, F1.8, F2.4, F2 (Figure 1) using the Enhanced NCI database browser v 2.2. Molecules with more than 80% similarity to the query molecules (Tanimoto coefficient > 80%) and with predicted drug-like properties were saved for further scoring.

SCORING AND SELECTION OF COMPOUNDS FOR EXPERIMENTAL TESTING

Ligand-based similarity searches (LBSS) hits were evaluated using the StarDrop Oral non central nervous system (CNS) scoring profile (logS>1, importance 90%; positive HIA category, importance 85%; 0<logP<0.35, importance 60%; -0.2<BBB log<1, importance 55%; positive BBB category, importance 55%; P-gp category ‘no’, importance 50%; hERG IC50≤5, importance 50%; 2C9 pKi≤6, importance 30%; 2D6 affinity category ‘low to medium’, importance 30%; PPB90 category ‘low’, importance 20%).

Predicted *E. coli* PhoR autophosphorylation inhibition score was calculated using the QSAR Random Forest Regression model built with StarDrop. After visual inspection, 18 compounds with oral non-CNS score > 0.4 and predicted *E. coli* PhoR autophosphorylation inhibition higher than 0.45, were purchased for experimental testing.

The predicted *E. coli* PhoR autophosphorylation activity was used as input to calculate binding efficiency indices (nBEIs) and surface efficiency indices (NSEIs) using AtlasCBS and the following equations:

\[
\text{NSEI} = -\log_{10} \text{pREA/ NPOL}, \text{ where pREA stands for predicted residual enzyme activity in one concentration (2 mM) one time-point (30 sec) kinase assay experiments with PhoR, and NPOL is number of polar N and O atoms in the compound.}
\]
nBEI = - log10[pREA/NHEA], where NHEA is the number of non-hydrogen atoms in the compound.

Out of the 1012 LBSS hits, 36 were purchased for experimental testing following visual inspection.

CHEMICALS

All chemicals (Figure S1 and Table S2) were obtained from DTP NCI/NIH.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Strains used for antimicrobial susceptibility testing (Table 1) were propagated using standard microbiological procedures and Müller-Hinton (MH) agar or broth. For all microorganisms minimal inhibitory concentrations (MICs) were determined as previously described following a standard double-dilution method.\(^{10, 11}\)

PROTEIN EXPRESSION AND PURIFICATION

The catalytic portions (DHp and CA domain) of \(E.\ coli\) PhoR (Pho\(\text{R}^E\)) were expressed and purified as previously described.\(^{12}\) In brief the protein was expressed in \(E.\ coli\) RIL and purified by Ni-affinity and size-exclusion chromatography.

KINASE ASSAY

Kinase assay was performed as previously described.\(^{13}\) When comparing the inhibitory capacity of ligands and measuring IC\(_{50}\), the final DMSO concentration in the assay was 10% (v/v). Controls lacking ligands and containing an equal concentration of DMSO were carried out in parallel. Inhibition of HK\(_s\) autophosphorylation was determined by incubating 0.12 mg/ml (≈ 4 µM HK) and up to 20 mM of test compound in kinase buffer (50 mM Tris HCl, pH 8.5, 50 mM KCl, 5 mM MgCl\(_2\), 0.5 mM EDTA and 0.1 mM DTT). Autophosphorylation reactions were initiated by addition of 0.1 μCi/μl [γ\(^{32}\)P] ATP containing from 0.03 to 0.06 μM ATP (final concentrations). Autophosphorylation was stopped by addition of 2x SDS-PAGE sample buffer supplemented with 50 mM EDTA. Samples were applied without heating to 15% (w/v) Tris-glycine SDS-polyacrylamide gels. After electrophoresis, the bottom part of the gels were removed to lower the background signal from the unincorporated radiolabeled ATP. Gels were dried without staining on a Bio-Rad Gel Air drying system and the phosphorylated protein was quantified by phosphor-imaging using a Fluoro Image Analyzer FLA-5000 (Fujifilm, Japan) and evaluated with the MultiGauge software (Fujifilm, Japan). IC\(_{50}\) is the concentration at which 50% residual enzyme activity was observed compared to the negative control, DMSO. Prism GraphPad v.4 was used for curve fitting and statistical analysis.\(^{14}\)
BINDING MODE PREDICTION

The binding mode of selected ligands to the structure of the CA domain of *B. subtilis* WalK (PDB: 3SL2) was predicted by molecular docking using SwissDock\textsuperscript{15}. In Figure 2 the lowest energy binding pose is shown.
RESULTS AND DISCUSSION

SELECTION OF COMPOUNDS FOR EXPERIMENTAL TESTING

To facilitate the selection of putative histidine kinase inhibitors for experimental testing in the process of hit-to-lead optimization quantitative structure–activity relationship (QSAR) models were built using a trial version of StarDrop. The best among the generated models was the random forest regression QSAR model however, it was not of high merit with $r^2$ of 0.71 (Figure 1, Figure S2). In many QSAR studies the criterion $r^2$ greater than 0.9 is employed to decide whether a model is internally self-consistent. However, $r^2$ makes no assessment of the intrinsic precision or accuracy of the data itself. Nevertheless, we used this model to predict the E. coli PhoR autophosphorylation inhibitory capacity of 1012 hits from ligand-based similarity searches (LBSS) with previously identified histidine kinase autophosphorylation inhibitors as query molecules. The predicted autophosphorylation activities were used to evaluate the physicochemical properties of the selected LBSS hits using AtlasCBS. The hits fell into one of seven groups based on NSEI (surface efficiency index) with the group around B13 showing highest predicted surface efficiency (Figure S3). The following group in terms of NSEI grouped around the F1.8 but with nearly two-fold lower predicted NSEIs. In total 54 compounds were ordered for experimental testing based on the StarDrop and AtlasCBS evaluation and visual inspection of the hits for promising functional groups.

H30 AND H31 SHOW ANTIBACTERIAL EFFECT FOR BOTH GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

To evaluate the antibacterial effect of the selected hits, the MICs (minimal inhibitory concentrations) and MBCs (minimal bactericidal concentrations) for S. aureus DSM 20231, S. aureus CH3657, S. epidermidis DSM 20044 and E. coli CFT 073 were determined. H30 and H31 showed the strongest antibacterial effect. H30 inhibited S. aureus CH3657, S. epidermidis DSM 20044 and uropathogenic E. coli CFT 073 with MICs of 62.5, 500 µg/ml and 500 µg/ml, respectively (Table S4). H31 inhibits S. aureus CH3657, S. epidermidis DSM 20044 and E. coli CFT 073 with MICs of 62.5, 125 and 250 µg/ml, respectively. H12 and H27 also showed some antibacterial effect with MICs higher than 250 µg/ml. Therefore, only H30 and H31 were tested against a panel of Gram-positive and Gram-negative bacteria and the results are summarized in Table 1. H30 did not show antibacterial effect for any of the additional bacterial strains tested. H31 inhibited the growth of all tested bacterial strains, including the vancomycin resistant E. faecium strains with MICs of 125 µg/ml.
H30 inhibits *E. coli* PhoR autophosphorylation and is predicted to bind to the ATP-binding site of the CA domain of *B. subtilis* WalK

To confirm the autophosphorylation inhibitory capacity of H30 and H31 in vitro kinase assay with *E. coli* PhoR was performed. *E. coli* PhoR autophosphorylation activity was inhibited by H30 and H31 in a concentration dependent manner with IC$_{50}$ ≥ 1 and IC$_{50}$ > 2 mM, respectively. Both compounds showed good solubility in kinase buffer. As H30 showed antibacterial effect against *S. aureus* CH3657 and *S. epidermidis* DSM 20044 (Table 1) and not to the other tested Gram-positive strains, we hypothesized that it might interact with WalK. WalK belongs to the WalKR two-component system which is ubiquitous among Gram positive bacteria and in some species it is reported as essential for bacterial growth $^{16, 17}$. 

---

**Figure 1.** A) Computer-aided drug discovery approach B) Histidine kinase autophosphorylation inhibitors used as query molecules for ligand-based similarity search
Table 1. Antibacterial activities of H30 and H31. *E. faecium* Van and VanB strains are vancomycin resistant strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC [µg/ml] H30</th>
<th>MIC [µg/ml] H31</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH3657</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMS 20478</td>
<td>500</td>
<td>&lt; 4</td>
</tr>
<tr>
<td><em>Streptococcus epidermidis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMS 20444</td>
<td>500</td>
<td>125</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>&gt; 500</td>
<td>125</td>
</tr>
<tr>
<td>VanA</td>
<td>&gt; 500</td>
<td>125</td>
</tr>
<tr>
<td>VanB</td>
<td>&gt; 500</td>
<td>125</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFT 073</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
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<td>500</td>
</tr>
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</table>

H30 was docked to the structure of the catalytic and ATP-binding domain (CA) of *B. subtilis* WalK (PDB: 3SL2). The predicted binding mode (Figure 2) suggested that H30 interacts with the ATP-binding site of *B. subtilis* WalK and forms contacts with key residues involved in ATP-binding (Figure 2). In the same experimental settings H31 was not docked to *B. subtilis* WalK and this together with the *E. coli* PhoR IC$_{50}$ > 2 mM suggests that the antibacterial effect of H31 against the bacterial strains tested might be mediated via targets different than HKs.

NCI antitumor screen results suggest that H30 scaffold possess good safety profile. 400 mg/kg/injection did not cause death of mice on the toxicity evaluation day. Therefore, H30 might be a good starting point for the development of inhibitors of HK autophosphorylation using a structure-based approach.

100 mg/kg/injection of H31 caused 50% death of mice on the toxicity evaluation day in a NCI antitumor screen. This supports the hypothesis that H31 has (a) target(s) different than HKs.

In summary, we identified H30 as putative HK autophosphorylation inhibitors with weak antibacterial effect using a combined computer-aided drug discovery approach. H30 might serve as a good starting scaffold for the design of more potent HK autophosphorylation inhibitors.
**Figure 2.** A) predicted binding mode of H30 to the ATP-binding site of *B. subtilis* WalK (PDB:3SL2) B) Concentration-dependent effect of H30 on *E. coli* PhoR autophosphorylation C) Concentration-dependent effect of H31 PhoR autophosphorylation.
REFERENCES

9. Enhanced NCI Database Browser 2.2.
Figure S1. Compounds selected for experimental testing
Figure S2. QSAR model for inhibition of *E. coli* PhoR autophosphorylation

Figure S3. Ligand-efficiency indices facilitate the selection of hits for hit-to-lead optimisation
<table>
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<th>% Inhibition</th>
<th>st.error; n=4</th>
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<tr>
<td>B2</td>
<td>73%</td>
<td>7%</td>
</tr>
<tr>
<td>B3</td>
<td>29%</td>
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<td>36%</td>
<td>6%</td>
</tr>
<tr>
<td>B5</td>
<td>17%</td>
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</tr>
<tr>
<td>B6</td>
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<td>4%</td>
</tr>
<tr>
<td>B7</td>
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<tr>
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<tr>
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<td>F1.10</td>
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<tr>
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CHAPTER 6

PRELIMINARY BIOCHEMICAL STUDIES ON THE INTERACTION OF LACTOFERRICINB-DERIVED PEPTIDES WITH TWO-COMPONENT SYSTEMS

Nadya Velikova, Jerry Wells, Alberto Marina
**BACKGROUND**

LactoferricinB (LfcinB) is an antimicrobial peptide with broad-spectrum of antibacterial activity. It has been shown by *in vitro* and *in vivo* experiments that LfcinB inhibits the phosphorylation of the response regulators (RRs), BasR and CreB, and their cognate histidine kinases (HKs), BasS and CreC. Co-crystal structures of (peptide) inhibitors bound to their two-component system (TCS) targets would open up possibilities for the structure based drug design of more potent inhibitors. Here we investigated interaction of LfcinB-derived peptides with the *T. maritima* TCS HK853-RR468, for which X-ray structures of both components have been solved in different conformations.

**FINDINGS**

We show that LfcinB-derived peptides inhibit the phosphatase activity of *T. maritima* HK853-RR468 and seem to interact with *T. maritima* HK853 and *E. coli* PhoB. However, a possible mechanism of interaction is non-specific protein aggregation.

**CONCLUSIONS**

LfcinB has been proposed as a promising broad-spectrum inhibitor of bacterial TCS. LfcinB-derived peptides seem to be non-specific TCS inhibitors acting via protein aggregation. The latter would be in agreement with the broad-spectrum of antibacterial activity of LfcinB as protein aggregation would affect multiple targets.

**List of abbreviations:** TCS – two component systems; HK – histidine kinase; RR – response regulator; LfcinB – lactoferricin B

**Key words:** Lactoferricin B, antimicrobial peptides, two-component system inhibitors, antibacterials
INTRODUCTION

Antimicrobial peptides have been proposed as a promising alternative to fight resistant bacterial infections and to address the growing problem of multi-drug resistance. Eukaryotic cationic antimicrobial peptides (AMPs) are produced at sites of infection or inflammation in many different organisms. Typically they are peptides of 12 to 45 amino acids with a net positive charge and a high proportion of hydrophobic amino acids. There are four structural classes of AMPs, the most common being the β-sheet peptides stabilized by 2–4 disulphide bridges, and the unstructured peptides that fold into amphipathic α-helices upon contact with membranes. Interaction with the membrane may form pores, or act by thinning the membrane or by destabilizing the membrane bilayer. As net result interaction of AMPs and membranes increases permeability of the membrane, leads to loss of pH gradient and cell death. Some cationic peptides have been shown to interact with intracellular targets, but in most cases the exact mechanism of intracellular AMPs action remains unclear.

One of the AMPs proposed to possess intracellular targets is bovine lactoferricin (LfcinB). LfcinB is a typical cationic antibacterial peptide produced by gastric pepsin digestion of the N-terminal region of bovine lactoferrin and has been identified in vivo. The antibacterial core LfcinB includes six residues (positions 20 – 25 in the parental sequence) with the C-terminus amidated (peptide RRWQWR-NH₂). A physiologically diverse range of Gram-positive and Gram-negative bacteria have been found to be susceptible to inhibition and inactivation by LfcinB-derived peptides including LfcinB₂₀–₂₅ and LfcinB₁₇–₃₁. Concentrations of LfcinB required to cause complete inhibition of bacterial growth vary within the range of 0.3 to 150 µg/ml, depending on the strain and the culture medium used. The broad spectrum of antibacterial action of LfcinB suggests a common mechanism of action and/or a widespread intracellular target present in all susceptible strains.

It has been shown that LfcinB-derived peptides (Lfcin₁₇–₄₁, LfcinB₁₇–₃₁ and D-Lfcin₁₇–₃₁) enter the cytoplasm of E. coli and S. aureus. In a recent study the intracellular LfcinB protein targets were identified by hybridisation of biotin-labelled LfcinB to an Escherichia coli K12 proteome chip. In total 16 proteins were shown to interact with LfcinB, including two response regulators (RRs), BasR and CreB, belonging to the two-component systems (TCS) BasSR and CreCB. TCS are the predominant signalling systems in bacteria. TCS connect input stimuli and output responses with a core phosphotransfer between a membrane bound histidine kinase (HK) and a cognate response regulator (RR). Simply described, the signalling pathway is a series of steps including autophosphorylation of the HK, phosphotransfer to the cognate RR, and output modulation, usually via transcription regulation, mediated by the phosphorylated RR. TCS are highly conserved between bacterial species and TCS signalling inhibition by LfcinB seemed a logical common mechanism explaining the broad-spectrum antibacterial effect of LfcinB. It has been shown by in vitro and in vivo experiments that LfcinB inhibits the phosphorylation of the RRs (BasR and CreB) by their cognate histidine kinases (HKs), BasS and CreC but does not inhibit the DNA binding capacity of both RRs suggesting that LfcinB-derived
peptides most likely do not interact with the effector domain of RRs, but with the REC domain of RRs. Additionally, antibacterial assays have shown that LfcinB reduced the tolerance of *E. coli* to environmental challenges, such as excessive ferric ions and minimal medium conditions. TCS are known to be involved in the regulation of adaptive response to stress and logically TCS signalling inhibition could be correlated with reduced stress tolerance. Taken together, these results were the first indication that an AMP inhibits the growth of bacteria by influencing the catalytic activities of a TCS directly and seemed to provide a promising starting point for the development of broad-spectrum antimicrobial-peptide drugs targeted at TCS signalling. TCS have been proposed as attractive targets for novel antibacterial as they are well-conserved among bacteria and not present in higher eukaryotes.

The aim of this study was to investigate the interaction between LfcinB with TCS at atomic level by using X-ray crystallography. The structure of a LfcinB-RR complex would i) elucidate the molecular mechanism of RRs inhibition by LfcinB, ii) identify putative target sites in the RR which can further be exploited in structure-based drug design approaches, and iii) point to RR-peptide interactions and possible peptide modifications which would lead to stronger inhibitory effect. Altogether, these would facilitate the rational design of modified peptides with higher affinity of binding to TCS and may lead to the development of more potent peptide TCS inhibitors. For this purpose we have used the *T. maritima* HK853-RR468 which is a well-studied TCS and the X-ray structures of both components, HK853 and RR468, alone and in complex are known. *E. coli* PhoR-PhoB is a prototypical two-component system involved in the regulation of the *pho* regulon and structures of the RR PhoB are known. As a first step towards a structure of a RR in complex with LfcinB, we verified whether LfcinB-derived peptides inhibit the catalytic activities of *T. maritima* HK853-RR468 and *E. coli* PhoR-PhoB TCS.
RESULTS AND DISCUSSION

INHIBITION OF PHOSPHOTRANSFER AND PHOSPHATASE ACTIVITIES OF PURIFIED BACTERIAL TWO-COMPONENT SYSTEMS.

To check whether LfcinB-derived peptides inhibit the phosphorylation of RR468 and/or PhoB by their cognate HKs, HK853 and PhoR, respectively, phosphotransfer assays were performed in the presence of two LfcinB-derived peptides (LfcinB_{17-25} and LfcinB_{17-32}). The two peptides were chosen as both contain the antibacterial core sequence (positions 20 – 25 in the parental sequence)\(^{11,12}\). In the phosphotransfer assays the HKs were first auto-phosphorylated using \([\gamma^{32}\text{P}]\text{ATP}\)\(^{24}\). This leads to accumulation of \([\gamma^{32}\text{P}]\text{ATP}\)-autophosphorylated HK which in a SDS-PAGE gel appears as a well-defined band (Figure 1). The autophosphorylated HK was then diluted in ATP-free phosphorylation solution containing an equimolar amount (in terms of subunits) of RR and 0.5 mM of an analysed peptide. Upon mixture of the HK and the RR phosphotransfer occurs, and in a SDS-PAGE gel the intensity of the band corresponding to the HK becomes lower and a band corresponding to the phosphorylated RR appears. As several HKs have phosphatase activity (i.e. HK853)\(^{28,19,29,18}\), the band corresponding to the phosphorylated RR in the SDS-PAGE gel lower its intensity over time. The time course of the reaction (10 min) showed that in the absence of LfcinB-derived peptides the radioactive band corresponding to autophosphorylated HK853 almost completely disappeared by 1 min and coincided with appearance of a labelled band corresponding to phosphorylated RR468 indicating phosphotransfer from HK853 to RR468 (Figure 1A). However, the intensity of the bands corresponding to the phosphorylated RR468 at 1 min was lower than the intensity of the band corresponding to HK853, confirming dephosphorylation of the RR468 due to HK853 phosphatase activity. In the presence of LfcinB-derived peptides phosphotransfer from HK853 to RR468 did occur but to a lesser extent compared to the negative control evidenced by the slightly higher intensity of the bands corresponding to the autophosphorylated HK853. In the presence of Lfcin B_{17-25} or LfcinB_{17-32} a proportion of the autophosphorylated HK853 remained visible, indicating either inhibition of phosphotransfer to RR468, or inhibition of the phosphatase activity leading to putative shift in the phosphotransfer/ phosphatase reaction balance. Furthermore, in the presence of LfcinB_{17-25} and LfcinB_{17-32} the bands corresponding to the phosphorylated RR468 were with higher intensity compared to the negative control or Peptide 3 (a pentapeptide of different sequence) and with similar intensity over time, indicating that the phosphatase activity of HK853 was inhibited. In the case of PhoR-PhoB, the band corresponding to the autophosphorylated PhoR almost completely disappeared by 1 min and a band corresponding to the phosphorylated PhoB appeared. In the presence of LfcinB-derived peptides, the intensity of the bands corresponding to the autophosphorylated PhoR was similar to the assay in the absence of peptide indicating that phosphotransfer was not affected. In the absence of peptide, the intensity of the bands corresponding to the phosphorylated PhoB remained similar over time indicating no or very slow phosphatase activity. Intriguingly, in the presence
of both LfcinB<sub>17-25</sub> and LfcinB<sub>17-32</sub> the amount of phosphorylated PhoB was considerably lower. In the presence of control Peptide3 the intensity of the bands corresponding to autophosphorylated PhoR and phosphorylated PhoB was similar to the assay in the absence of peptides. As it appears that phosphotransfer activity of the PhoR-PhoB TCS is not affected by the LfcinB-derived peptides and the phosphatase activity in the absence of LfcinB-derived peptides is missing or very slow, the lower intensity of the bands corresponding to the phosphorylated PhoB could be explained by activating the PhoB dephosphorylation. Further experiments with in vitro phosphorylated PhoB are required to proof or discard this hypothesis.

**INHIBITION OF THE PHOSPHOTRANSFER AND PHOSPHATASE ACTIVITY OF HK853-RR468 IS LIKELY MEDIATED VIA INTERACTION WITH HK853**

Native polyacrylamide gel electrophoresis (native PAGE) was employed to check whether the observed inhibition of phosphotransfer and phosphatase activity of the HK853-RR468 TCS by LfcinB<sub>17-25</sub> and LfcinB<sub>17-32</sub> is due to interaction with the HK and/or with the RR. The RR was first phosphorylated in vitro by incubation with acetyl phosphate. The in vitro phosphorylated RR468 was combined with LfcinB<sub>17-25</sub> or LfcinB<sub>17-32</sub> or LfcinB<sub>17-25</sub> or LfcinB<sub>17-32</sub> together with the HK853. HK853 alone incubated with or without LfcinB<sub>17-25</sub> or LfcinB<sub>17-32</sub> or with Peptide 3 were run in parallel. In the absence of LfcinB-derived peptides in the native PAGE gel three well-defined bands corresponding to RR468 (lowest band), HK853 (intermediate band) and the complex HK853-RR468 (highest band; Figure 2) were evident. Addition of LfcinB<sub>17-25</sub> and LfcinB<sub>17-32</sub> resulted in loss of the band corresponding to HK853. Whereas, in the presence of LfcinB<sub>17-25</sub> and LfcinB<sub>17-32</sub> RR468 was only slightly influenced as a smear was observed. This suggests that the LfcinB-derived peptides interact with HK853 much stronger than RR468 (Figure 2) evidenced by the significant change in the mobility of HK853 in the native gel after incubation with LfcinB-derived peptides. The putative interaction of LfcinB-derived peptides with HK853 does not affect the formation of the complex with RR468 as evidenced by the band corresponding to the HK853-RR468 complex in the presence of the LfcinB-derived peptides. Furthermore, in the presence of LfcinB-derived peptides the complex HK853-RR468 is formed and phosphotransfer and dephosphorylation of the RR can still occur to some extend (Figure 1A). Nevertheless, the amount of complex formed in the presence of the LfcinB peptides was much less than that observed with peptide 3 and control samples (Figure 2). The events observed with HK853 and RR468 were specific to the LfcinB-derived peptides as no change in the mobility of HK853 or smear were evident after incubation with Peptide 3.
Figure 1. Phosphotransfer assays A) LfcinB17-25 and LfcinB17-32 inhibit the phosphorylation of RR468 by HK853 and the phosphatase activity of HK853 as evidenced by the higher intensity of the bands corresponding to autophosphorylated HK853 and the bands corresponding to the phosphorylated RR468, respectively, in the presence of the LfcinB-derived peptides compared to the negative control. B) LfcinB17-25 and LfcinB17-32 seem not to inhibit the phosphorylation of PhoB by PhoR as evidenced by the similar intensities of the bands corresponding to the autophosphorylated PhoR. The lower intensities of the bands corresponding to the phosphorylated PhoR in the presence of LfcinB-derived peptides point to the possibility that LfcinB-derived peptides stimulate dephosphorylation of PhoB.
Figure 2. LfcinB_{17-25} and LfcinB_{17-32} seem to interact with HK853 as evidenced by the disappearance of the band corresponding to HK853 in the presence of the LfcinB-derived peptides. LfcinB_{17-25} and LfcinB_{17-32} interact with RR468 to a lesser extent compared to HK853 as evidenced by smear of in the presence of the LfcinB-derived peptides. The strong interaction with HK853 and the weaker interaction with RR468 do not prevent the formation of the HK853-RR468 complex and the band corresponding to the complex appears even in the presence of the LfcinB-derived peptides. Nevertheless, the amount of the formed HK853-RR468 complex in the presence of LfcinB-derived peptides less than in the controls.

**LFCINB-DERIVED PEPTIDES INTERACT WITH PHO B IN A CONCENTRATION-DEPENDENT MANNER**

Native PAGE indicates that LfcinB-derived peptides interact with PhoB as a smear appeared after incubation of LfcinB-derived peptides with PhoB which had been or had not been *in vitro* phosphorylated (Figure S1 and S2). The interaction with the *in vitro* phosphorylated PhoB seemed to be concentration dependent as the smear was observed only at concentrations higher than 0.125 mM (Figure S2). The putative interaction was specific to the LfcinB-derived peptides as the mobility of *in vitro* phosphorylated PhoB in the native gel was not affected after incubation with Peptide3 (Figure S1).
SUMMARY

In summary, it seems the inhibition of phosphotransfer and phosphatase activities of the *T. maritima* TCS HK853-RR468 caused by LfcinB$_{17-25}$ and LfcinB$_{17-32}$ can be attributed to interaction with HK853 although some interaction with RR468 might also have contributed to this effect. The LfcinB-derived peptides seem to interact in a concentration dependent manner with *in vitro* phosphorylated *E. coli* response regulator PhoB. Changes in the mobility in native gels of PhoB after incubation with LfcinB-derived peptides indicate possible interaction with this protein. Further experiments are needed to check the hypothesis that incubation with LfcinB-derived peptides might activate the dephosphorylation of *E. coli* PhoB. The observed changes in the mobility of *T. maritima* HK853 and PhoB in native gels could be due to protein aggregation. However, further experiments such as size-exclusion chromatography and/or protein-aggregation evaluation by cross-linking and SDS-PAGE are required to confirm this hypothesis. Taken together, the results point to the possibility that the antibacterial effect caused by LfcinB-derived peptides might be attributed to interaction with multiple cellular targets after entering into the cell. This is in agreement with the fact that multiple *E. coli* proteins were shown to interact with LfcinB using proteomics. Furthermore, non-specific mechanism of action such as protein aggregation could not be excluded at this point. Attempts to solve the structure of *T. maritima* RR468 co-crystallized with LfcinB-derived peptides resulted in high-resolution twinned crystals of RR468 and the LfcinB-derived peptides were not encountered in the asymmetric unit (data not shown). It was not possible to obtain diffracting crystals of PhoB co-crystallized with LfcinB-derived peptides. No attempts have been made to co-crystallize LfcinB-derived peptides with any HK. However, before any future crystallization trials additional biochemical experiments are required to confirm that the mechanism of interaction of LfcinB-derived peptides with TCS is not mediated via non-specific protein aggregation.
MATERIALS AND METHODS

PEPTIDES

Lactoferricin B\textsubscript{17-25} and Lactoferricin B\textsubscript{17-32} were kindly provided by Paloma Manzanares, IATA, CSIC (Valencia, Spain).

PROTEIN EXPRESSION AND PURIFICATION

\textit{T. maritima} HK853 and RR468, \textit{E. coli} PhoR and PhoB were expressed and purified as previously described\textsuperscript{23, 22, 24, 25, 31, 31}. Shortly proteins were expressed in \textit{E. coli} and purified by anion-exchange (HK853 and RR468) or Ni-affinity chromatography (PhoR and PhoB), and size-exclusion chromatography.

PHOSPHOTRANSFER ASSAY

Phosphotransfer assays were performed as previously described\textsuperscript{22}. HK853 and PhoR were autophosphorylated in kinase buffer (50 mM Tris pH 8.0, 100 mM KCl, 10 mM MgCl\textsubscript{2}) with 0.1 μCi μl\textsuperscript{-1}[γ\textsuperscript{32}P] ATP (3000 Ci/mmol Perkin Elmer) for 30 min (exceeding equilibrium time) at room temperature. Then, each sample was aliquoted into three tubes and phosphotransfer reactions were initiated by the addition of equimolecular amounts of each RR (the final concentration of RRs and HKs in reaction was 2 μM) pre-incubated with 0.5 mM Lactoferricin B\textsubscript{17-25}, Lactoferricin B\textsubscript{17-32}, Peptide 3 or buffer. Aliquots were removed from each tube at different times (1, 2.5 and 10 min) and the reactions were stopped by adding 1/4 SDS-PAGE loading buffer (62.5 mM Tris pH 6.8, 30% glycerol, 0.01% bromophenol blue) supplemented with 50 mM EDTA. Afterwards, the samples were subjected to SDS-PAGE on a 15% gel to separate the protein from free nucleotide. After drying, the phosphorylated proteins were visualized by phosphorimaging using a Fluoro Image Analyzer FLA-5000 (Fuji).

NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS (NATIVE PAGE)

Purified PhoB and RR468 were autophosphorylated in kinase buffer (50 mM Tris pH 8.0, 100 mM KCl, 10 mM MgCl\textsubscript{2}) containing 12.5 mM acetyl phosphate for 1 h at room temperature. Phosphorylated PhoB and RR468 were mixed with 0.5 mM Lactoferricin B\textsubscript{17-25}, Lactoferricin B\textsubscript{17-32}, Peptide 3 or buffer. RR468 mixed with 0.5 mM Lactoferricin B\textsubscript{17-25}, Lactoferricin B\textsubscript{17-32}, Peptide 3 or buffer was incubated with or without equimolar amounts of HK853. After 30 min of incubation at room temperature Native polyacrylamide gel electrophoresis (Native-PAGE) loading buffer was added and samples loaded. Coomassie blue staining was used for protein visualization.
REFERENCES

LACTOFERRIN AND LACTOFERRICIN AGAINST ORAL STREPTOCOCCI. ROMANIAN BIOTECHNOLOGICAL LETTERS 2010, 15 (6), 5788-5792.


SUPPLEMENTARY MATERIAL CHAPTER 6

Figure S1. LfcinB\textsubscript{17-25} and LfcinB\textsubscript{17-32} interact with \textit{E. coli} PhoB as evidenced by the smear observed after incubation of PhoB with the LfcinB-derived peptides and not in the negative control or after incubation with Peptide 3.

Figure S2. Interaction of LfcinB-derived peptides with \textit{E. coli} PhoB. LfcinB-derived peptides seem to interact with \textit{in vitro} phosphorylated PhoB in a concentration dependent manner as only incubation with LfcinB-derived peptides at concentrations higher than 0.025mM cause change in the mobility of PhoR evidenced by a smear and/ or reduction of the band corresponding to PhoB.
CHAPTER 7

EXPRESSION, PURIFICATION, (CO-)CRYSTALLIZATION AND PRELIMINARY X-RAY DIFFRACTION ANALYSIS OF TWO-COMPONENT SYSTEMS AND TWO-COMPONENT SYSTEM-INHIBITOR CO-CRYSTALS

Nadya Velikova, Alberto Marina
7.1. **C**O-**C**RYS**T**ALLIZATION OF H**I**STIDINE-**K**INASE AUTOPHOSPHORYLATION INHIBITORS WITH *THERMOTOGA MARITIMA* CHEA

**INTRODUCTION**

Obtaining the structure of a putative target histidine kinase (HK) and a histidine-kinase autophosphorylation inhibitor (HKAI) would allow the rational structure-based design of more potent and specific inhibitors. The structure of *Thermotoga maritima* CheA CA domain has been previously crystallized in complex with the native ligand ATP/ADP (PDB: 1I58, 1I59) to atomic resolution (1.6 Å), and the conditions to obtain high-resolution well-diffracting crystals are well-known¹,². Thus the aim of this study was to obtain X-ray structures of *T. maritima* CheA in complex with the HKAI discovered in this thesis (Chapter 2, 3, and 5).

The structure of the CA domain of *T. maritima* CheA contains the characteristic ATP-binding Bergerat fold including the ATP-lid (Figure 1). The ATP-lid is a loop variable in sequence and in length crucial for nucleotide binding and catalytic activity³,⁴. The ATP-lid is proposed as promising feature for the design of specific HK autophosphorylation inhibitors with reduced adverse effects to host cells⁵,⁶.

*Figure 1. Catalytic and ATP-binding (CA) domain of *T. maritima* CheA (PDB: 1I58, chain A). Residues are coloured based on their conservation score calculated with ConSurf. ATP is shown in sticks. The ATP-lid is a variable loop that can be used to design more specific HK inhibitors.*
**MATERIALS AND METHODS**

**PROTEIN EXPRESSION AND PURIFICATION**

*T. maritima* CheA (CA) was expressed and purified as previously described\(^1,2\). In brief, protein was expressed in *E. coli* RIL and purified by Ni-affinity and size-exclusion chromatography.

**CO-CRYSTALLIZATION AND SOAKING WITH HISTIDINE-KINASE AUTOPHOSPHORYLATION INHIBITORS**

Crystals of CheA (25-50 mg/ml) were obtained by the hanging-drop method in solution containing 28-33 % PEG 8000, 0.6M Ammonium acetate, and 0.065 M sodium acetate pH 4.5. Co-crystals with histidine kinase autoprophosphorylation inhibitors (HKAs) were obtained by incubating CheA with 100 mg/ml of the HKAI for at least 16h at 4°C. In some cases (B13) co-crystals were additionally soaked in crystallization solution containing 100 mg/ml HKAI for at least 3 h.

**DATA COLLECTION AND PROCESSING**

Single crystals were harvested with a loop and vitrified in liquid nitrogen. Diffraction data of CheA-HKAI crystals (Table 1) were collected at the synchrotrons Diamond Light Source (Didcot, United Kingdom), European Synchrotron Radiation Facility (Grenoble, France), and ALBA (Barcelona, Spain), or at IBV-CSIC (Valencia, Spain) using SuperNova (Agilent). Diffraction images were indexed and integrated using Mosflm\(^7\), XDS\(^8\), or CrysAlisPro (Agilent) and the data were scaled using Scala\(^9\). Data collection and processing statistics of representative crystals are summarized in Table 2. Structures were solved by molecular replacement using as template the previous solved structure of the CheA CA domain (PDB: 1I58, Chain A) and the software MolRep\(^10\). All models were improved by alternating manual building using Coot\(^11,12\) with automatic refinement using Refmac\(^12\) or Phenix Refine\(^13,14,15\).
RESULTS AND DISCUSSION

Crystals of the CA domain of *T. maritima* CheA co-crystallized with the histidine-kinase autophosphorylation inhibitors (HKAs, Table 1) diffracted at very high resolution between 1.5 to 2.5 Å (Table 2). In the case of inhibitors A5, B13, B14, F1, F1.5, F2, F2.2, F2.4, and H30 (Table 1) the CheA-inhibitor structures, solved by X-ray crystallography, revealed electron density in the ATP-binding site (Figure 2). However, the structures of the HK inhibitors could not be fully resolved, probably due to partial occupancy and/or multiple conformations. In all cases the ATP-lid was not visible.

**Table 1.** Co-crystals of CheA with inhibitors

<table>
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<th>Inhibitor</th>
<th>Data set(s) collected at:</th>
<th>Electron density in the ATP-binding site</th>
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<tr>
<td>A1</td>
<td>DLS</td>
<td>No</td>
</tr>
<tr>
<td>A5</td>
<td>DLS, Agilent</td>
<td>Yes/No</td>
</tr>
<tr>
<td>A6</td>
<td>DLS</td>
<td>No</td>
</tr>
<tr>
<td>A11</td>
<td>DLS</td>
<td>No</td>
</tr>
<tr>
<td>B7</td>
<td>ALBA</td>
<td>No</td>
</tr>
<tr>
<td>B11</td>
<td>ALBA</td>
<td>No</td>
</tr>
<tr>
<td>B13</td>
<td>ALBA</td>
<td>Yes</td>
</tr>
<tr>
<td>B14</td>
<td>ALBA</td>
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</tr>
<tr>
<td>F1</td>
<td>DLS, Agilent, ALBA</td>
<td>Yes/No</td>
</tr>
<tr>
<td>F1.5</td>
<td>DLS</td>
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</tr>
<tr>
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</tr>
<tr>
<td>F1.7</td>
<td>DLS</td>
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</tr>
<tr>
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</tr>
<tr>
<td>F2</td>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>F2.4</td>
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<tr>
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</tr>
<tr>
<td>F2.7</td>
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</tr>
<tr>
<td>H30</td>
<td>ALBA</td>
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</tr>
<tr>
<td>H31</td>
<td>ALBA</td>
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</tr>
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Table 2. Data collection and processing statistics for representative CheA-HKAI crystals

<table>
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<tr>
<th></th>
<th>CheA-F2</th>
<th>CheA-B13</th>
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<td>Synchrotron</td>
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<tr>
<td>Wavelength (Å)</td>
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<td>1.07217</td>
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<tr>
<td>Space group</td>
<td>P 2₁</td>
<td>P 2₁</td>
</tr>
<tr>
<td>Cell (Å, °)</td>
<td>a=40.7730</td>
<td>a=41.01</td>
</tr>
<tr>
<td></td>
<td>b=60.2130</td>
<td>b=59.24</td>
</tr>
<tr>
<td></td>
<td>c=65.6980</td>
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<tr>
<td></td>
<td>α=90.0</td>
<td>α=90.0</td>
</tr>
<tr>
<td></td>
<td>β=98.2850</td>
<td>β=97.68</td>
</tr>
<tr>
<td></td>
<td>γ=90.0</td>
<td>γ=90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>28.60 – 2.16</td>
<td>66.20 – 1.85</td>
</tr>
<tr>
<td></td>
<td>(2.28-2.16)</td>
<td>(1.95 – 1.85)</td>
</tr>
<tr>
<td>R merge</td>
<td>0.029</td>
<td>0.06</td>
</tr>
<tr>
<td>I/δ(I)</td>
<td>27.0</td>
<td>11.4</td>
</tr>
<tr>
<td>No. of reflections (observed/unique)</td>
<td>62363/16957 (9120/2454)</td>
<td>86960/27188 (12139/3946)</td>
</tr>
<tr>
<td>Completeness</td>
<td>99.6 (99.6)</td>
<td>99.3 (99.2)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>3.7 (3.7)</td>
<td>3.2 (3.1)</td>
</tr>
</tbody>
</table>

The data for the outer shell is shown in parenthesis.
Figure 2. *T. maritima* CheA-HKAI structures solved by X-ray crystallography. A) Co-crystals of *T. maritima* CheA (CA domain) and the initial hits from the structure-based virtual screening (A5, Chapter 2) and the fragment-based screening by differential scanning fluorimetry (F1 and F2) were obtained by co-crystallization with 100 mM HKAI. B) Co-crystal of *T. maritima* CheA and the most promising HKAI described in the thesis B13 (Chapter 2) was obtained by co-crystallization followed by soaking with 100 mM B13. The 2Fo-Fc (blue) and Fo-Fc (green) electron density maps contoured to 1 and 2.5 σ, respectively, showed extra density in the active site that was not possible to explain by the protein model and were ascribed to the HKAI. However, the HKAI could not be fully resolved, probably due to partial occupancy and/or multiple conformations.
INTRODUCTION

Copper is an essential trace element for organisms living under aerobic condition \(^16\). It is required in fundamental cellular processes such as oxidative phosphorylation, photosynthesis, and free radical control. However copper is highly toxic when it is present in excess, thus, many organisms have developed homeostatic mechanism to tightly regulate its cellular concentration \(^17\). Cyanobacteria are an attractive model to investigate the systems involved in copper homeostasis since they are unique bacteria with internal copper requirement for two proteins: the blue-copper protein plastocyanin, and the caa\(_3\)-type cytochrome oxidase \(^18\). These two proteins are localized in the thylakoids, a special internal structure where photosynthesis and respiration take place in cyanobacteria. Cyanobacteria are model microorganisms for the study of photosynthesis, carbon and nitrogen assimilation, evolution of plant plastids, and adaptability to environmental stresses. *Synechocystis* sp. PCC 6803 is one of the most highly studied types of cyanobacteria as it can grow both autotrophically or heterotrophically in the absence of light. Copper resistance in cyanobacteria has been mainly investigated in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), which involves a two-component system, CopRS, a protein of unknown function, CopM, and an HME-RND exports system, CopBAC \(^19,17\). These proteins are encoded by two operons: *copMRS*, and *copBAC*. Both *copM* and *copBAC* are regulated by the CopRS two-component system, and are specifically induced by the presence of Cu\(^{2+}\) \(^19\). CopM is an unknown protein that contains a predicted periplasmic signal domain and a high number of histidine (8) and methionine (23) residues, which are usually implicated in direct metal binding in proteins. Furthermore, CopM has two domain of unknown function DUF305, which is present in other secretion proteins in bacteria and belongs to the ferritin superfamily \(^20\). Herein, we report the purification, crystallization and preliminary X-ray diffraction analysis of *Synechocystis* CopM.
MATERIALS AND METHODS

CLONING, EXPRESSION AND PURIFICATION

A 519-bp band (from ORF sll0788) coding for the CopM<sub>25-196</sub> periplasmic domain was PCR amplified from genomic DNA with oligonucleotides ACOPMSTF-COPMSTR (Table 1), digested with KpnI and SacI, and cloned into pET51 digested with the same enzymes. CopM<sub>25-196</sub> was expressed in E. coli BL21. An overnight preculture was used to inoculate Luria broth medium and was grown to an optical density at 600 nm of 0.6. The culture was cooled down on ice for 20 min, protein expression induced by addition of 0.2 mM isopropyl-b-D-thiogalactopyranoside, and incubation continued for 4 h at 25°C. Cells were harvested by centrifugation and frozen at -80°C. Frozen pellets were re-suspended in buffer S (100 mM Tris HCl pH 8, 150 mM NaCl, 1 mM BCSA, 1 mM EDTA, and 2 mM Tris (2-carboxyethyl)-phosphine) and broken by sonication. The suspension was centrifuged 30 min at 30,000 g at 4°C and the supernatant was loaded into a 5-ml streptavidin beads (IBA GmbH) column equilibrated in buffer S. Beads were washed with 50 mL of buffer S and CopM<sub>25-196</sub> was eluted with 1x Strep-Tag elution buffer (IBA GmbH). CopM<sub>25-196</sub> was further purified by gel filtration in a Hi-Load 75 (GE-Healthcare) column equilibrated with 20 mM Tris HCl pH 8, 150 mM NaCl. The purified protein was concentrated using a 3K Vivaspin concentrator and store at -20 °C until use.

CRYSTALLIZATION

Initial sitting-drop vapour-diffusion crystallization trials to identify promising crystallization conditions for CopM were performed using the commercial crystallization screens JCSG+ (Qiagen, Germany), JBC I and JBC II (Jena Bioscience, Germany). In an attempt to obtain crystals of both the apo and Cu<sup>2+</sup>-bound CopM initial crystallization conditions screenings were performed in the presence of 1 mM EDTA and 1 mM CuSO<sub>4</sub>, respectively. Crystallization drops of 0.6 µl (protein:precipitant ratio of 1:1) were set up in 96-well plates containing 75 µl reservoir solution. Initial hits were obtained after 4-6 days in multiple conditions (Table S1). Optimization of the crystallization conditions was attempted by varying the concentration of PEGs and salts under the conditions that initially generated diffracting crystals.
DATA COLLECTION AND PROCESSING

For data collection, the crystals were soaked in cryoprotectant (reservoir solution supplemented with 40% PEG 3350) for 5-10 sec. A single crystal was harvested with a loop and vitrified in liquid nitrogen. Diffraction data were collected the synchrotrons at Diamond Light Source (Didcot, United Kingdom) or European Synchrotron Radiation Facility (Grenoble, France). Diffraction images were indexed and integrated using Mosflm \(^7\) or XDS \(^8\) and the data were scaled using Scala \(^9\). Data collection and processing statistics are summarized in Table 2.
RESULTS AND DISCUSSION

Recombinant CopM was cloned from genomic DNA, overexpressed in E. coli and purified to homogeneity using streptavidin-affinity chromatography and size-exclusion chromatography (SEC). The SEC elution profile showed a large peak (data not shown) eluting at apparent molecular mass between 17 and 44 kDa. This suggested that recombinant CopM with a theoretical molecular mass of 22.65 kDa is a monomer or a dimer in solution. Analysis of the purified CopM by SDS-PAGE and Coomassie staining showed a purity of at least 95% (Figure 1). From a 2 L culture, 4.5 mg pure CopM was obtained. CopM was concentrated to 6.5 mg/ml and used in initial crystallization conditions screenings. Crystals with different shapes grew in various conditions (Table S1). In JBS I C7 (25% PEG 4000, 0.1 M Na MES pH 6.5, 0.2 M MgCl2) rectangular crystals of CopM diffracting at 4 Å were obtained. In JCSG H9 (25% PEG 3350, 0.1 M Bis-Tris pH 5.5, different salts) in the presence of EDTA CopM crystals diffracting at 2.9 Å and C2 space group were obtained (Figure 2). Based on these initial results optimization of the crystals was attempted by varying PEG, salt and/or buffer concentrations in the presence or absence of EDTA or Cu2+. Crystallization in the presence of EDTA was performed in order to eliminate any possible interacting ions and to obtain unbound CopM crystals (CopM apo). As CopM is related to copper resistance and it is hypothesized that it might interact with CopM we tried to obtain CopM bound to Cu2+ (CopM Cu2+). CopM was overexpressed in E. coli and during the expression and purification it might have bound to ions present in the medium and crystallization of the overexpressed CopM might have resulted in CopM bound to unknown ligands (CopM medium). The optimization of the crystallization conditions resulted in crystals with different shapes diffracting between 2.5 – 2.75 Å with P21 (CopM Cu2+) and P212121 space group (CopM apo). Complete datasets from 2.5 to 3 Å resolution were collected from single crystals (Table 2). Data-collection and processing statistics are summarized in Table 2. Preliminary diffraction data analysis using POINTLESS 21 suggested the presence of 5 (CopM EDTA, space group C2), 1 (CopM medium, space group P21) or 2 (CopM Cu2+, space group P212121) molecules per asymmetric unit. Attempts of structure solution of CopM by molecular replacement using the coordinates of a fragment from Deinococcus radiodurans DUF305 (PDB: 3BT5) and Streptomyces coelicolor DUF305 (PDB: 2QF9) as search models and/or with MRBUMP 22 automatic mode were not successful. D. radiodurans DUF305 and S. coelicolor DUF 305 share 26.5 and 21.9% sequence identity with CopM, respectively.
Figure 1. CopM purity assessed by SDS-PAGE

Figure 2. CopM crystals

Table 1. Oligonucleotides used in this work.

<table>
<thead>
<tr>
<th>Name</th>
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<td>ACOPMSTF</td>
<td>caggtaccgtatgcaatacttcc</td>
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<td>COPMSTR</td>
<td>atgagcttcactgaccataccagt</td>
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Table 2. Data collection and processing statistics for CopM crystals

<table>
<thead>
<tr>
<th></th>
<th>CopM apo</th>
<th>CopM Cu²⁺</th>
<th>CopM medium</th>
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<td>ESRF</td>
<td>ESRF</td>
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<tr>
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<td>0.87260</td>
<td>0.87260</td>
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<td>Space group</td>
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<td>P 2₁ 2₁ 2₁</td>
<td>P 2₁</td>
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<tr>
<td>Cell (Å)</td>
<td>a=199.29 b=54.09 c=139.38 β=125.970</td>
<td>a=64.43 b=85.35 c=99.12</td>
<td>a=29.84 b=86.49 c=52.18 β=96.21</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>52.6 - 2.92 (3 - 2.92)</td>
<td>49.56 - 2.90 (3.08 - 2.9)</td>
<td>51.87 - 2.70 (2.83 - 2.70)</td>
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<tr>
<td>R merge</td>
<td>0.062</td>
<td>0.062</td>
<td>0.124</td>
</tr>
<tr>
<td>I/δ(I)</td>
<td>11.3</td>
<td>11.1</td>
<td>8.4</td>
</tr>
<tr>
<td>No. of reflections (observed/unique)</td>
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<td>51235/12654 (8263/2002)</td>
<td>53148/7219 (5678/880)</td>
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<tr>
<td>Completeness</td>
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<td>99.9 (100)</td>
<td>99.0 (93.3)</td>
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<tr>
<td>Multiplicity</td>
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<td>4.0 (4.1)</td>
<td>7.4 (6.5)</td>
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</table>

The data for the outer shell is shown in parentheses.
DLS – Diamond Light Source
ESRF – European Synchrotron Radiation Facility
REFERENCES


### Table S1. Crystallization screenings results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Screening, position, morphology</th>
<th>Condition</th>
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<td>CopM glycerol</td>
<td>JBS I, A11</td>
<td>25% PEG 1000, 0.1 M Na Hepes pH 7.5</td>
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<td>JBS I, C11 very small</td>
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<td>20% glycerol ethoxylate, 3% polyethylene imine</td>
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<tr>
<td>CopM</td>
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<td>30% glycerol ethoxylate, 0.2 M Amm Acetate pH 6.5, 0.1 M MES-NaOH</td>
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<td>CopM EDTA</td>
<td>JBS I, G2</td>
<td>10% PEG 8000, 0.1 M Na Hepes pH 7.5, 0.2 mM Na Acetate</td>
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<td>CopM EDTA</td>
<td>JBS I, C8</td>
<td>25% PEG 4000, 0.1 M Tris HCl 8.5, 0.2 M CaCl2</td>
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<td>CopM EDTA</td>
<td>JCSG+, from H6 to H11</td>
<td>25% PEG 3350, 0.1 M Bis-Tris pH 5.5, different salts</td>
</tr>
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<td>CopM EDTA</td>
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<td>30% Jeaffamine ED 2001, 0.1 M Heps pH 7</td>
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<td>CopM EDTA</td>
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<td>1M Tri-Na citrate, 0.1 M Na cacodylate pH 6.5</td>
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<td>CopM EDTA</td>
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<td>0.2 N NaCl, 0.1 M Na cacodylate pH 6.5, 2M AmSO4</td>
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<td>CopM EDTA</td>
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<td>JBS I, F4</td>
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<td>CopM</td>
<td>JBS I, B12</td>
<td>12% PEG 4000, 0.1 M Na Hepes pH 7.5, 0.1 M Na Acetate</td>
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CHAPTER 8

WALK, THE PATH TOWARDS NEW ANTIBACTERIALS WITH LOW POTENTIAL FOR RESISTANCE DEVELOPMENT

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ABSTRACT

Resistance to antibiotics used in the treatment of bacterial infectious diseases is a global health problem. More than a decade ago, two-component systems such as WalKR were proposed as ideal targets for the development of new antibiotics. Biochemical screens for WalKR inhibitors using compound libraries have identified many hits, some of which were shown to have non-specific effects. The recently published structures of the S. mutans and B. subtilis WalK provide the opportunity to study inhibitors of WalK autophosphorylation at the atomic level and means to design compounds with improved specificity and affinity using a structure-based approach.
Recently scientists have been watching in awe as bacteria develop resistance to the latest antibiotics. Multidrug resistant tuberculosis (MDR-TB), penicillin-resistant *Streptococcus pneumoniae*, and hospital-acquired infections with vancomycin-resistant enterococci (VRE) or methicillin-resistant *Staphylococcus aureus* (MRSA) are regularly making headline news. The problem is not only one of resistance to front line drugs and the fear that one day, infections will be incurable, but also the increased cost of patient care and the prospect of future inability to perform trivial medical procedures without significant risk.

The scarcity of new antibiotics has been put down to dwindling returns on screening natural sources but also poor incentives for companies to invest in this sector as opposed to treatment for cancer and chronic diseases. However, the challenge of developing anti-infective drugs has been taken up by small enterprises and academia with the support of governments and other funding organizations.

The genomics revolution has accelerated finding new targets for anti-infective drugs, but designing drug-like inhibitors against specific targets remains highly challenging, costly, and not without significant risk of failure.

Against this somewhat gloomy perspective, there are some promising developments unfolding, one of which concerns bacterial two-component systems (TCSs) as drug targets. Bacteria respond and adapt to a large variety of environmental and intracellular signals via TCS signal transduction. The minimum components of a TCS are a sensor histidine kinase (HK) and an effector response regulator (RR) (Figure 1). Typically, the sensor histidine kinase (HK) is membrane-bound, and signal recognition alters the phosphorylation state of a cognate response regulator (RR). HK sensing of specific signals occurs via a variable domain that is commonly exposed to the extracellular milieu, whereas the remaining protein domains are generally conserved, cytoplasmic, and required for signal transduction (8) (Figure 1). RRs are usually transcription factors of which the DNA-binding capacity and consequently, gene transcription is determined by their phosphorylation state. TCSs may directly or indirectly control numerous genes, including those involved in the regulation of metabolism, cell physiology, virulence, persistence, and resistance to antibiotics or antimicrobial peptides. Targeting virulence is a promising antibacterial drug discovery strategy as it exerts less selective pressure on the pathogen and resistance development will be slower.

TCSs were proposed as attractive targets more than 20 years ago because they are absent in mammals and essential or conditionally essential for viability in several important bacterial pathogens. WalKR system (a.k.a., YycGF, VicKR, MicAB) is an obligate essential regulatory system in Firmicutes including MRSA, VRE, and some other notorious pathogens. Other TCSs might not be essential for growth in vitro, but in vivo they can be necessary for survival or persistence (e.g., the DosRS system in *Mycobacterium*). Besides, some bacteria possess as many as 200 TCSs depending on
Figure 1. TCS signaling. TCS signaling is triggered by the recognition of the signal (1) by the HK sensor variable domain, which regulates the autophosphorylation of a histidine residue in the conserved portion of HK (2). Signal is then transduced to the cognate RR by the transfer of the phosphoryl group from the histidine to a conserved aspartic residue in the RR (3). Typically, RRs are transcription factors, and their phosphorylation state regulates their DNA-binding capacity (4) and, consequently, gene transcription (5)(1-4).

their lifestyle and requirements for adaptation and metabolism in different environments. Presumably the combined effect of inhibiting all or multiple TCSs and thus the ability of a bacterium to adapt to changing physiological conditions would also greatly weaken their ability to cause infections. Given that the first TCS inhibitors were described more than a decade ago, one might think that finding TCS inhibitors is an intractable problem, and it might be better to focus on other essential pathways in bacteria. Nevertheless, there is no doubt that TCSs are good antibacterial drug targets because they have a high degree of conservation in the active sites of their catalytic domains. This fact implies that inhibitors of one TCS may in fact block multiple TCS regulatory networks, effectively incapacitating the ability of bacteria to adapt to environmental and physiological changes.

Perhaps the lack of drug candidates against WalKR or other TCSs is due to the limitations of the previously adopted approach.
The pioneering work by the Utsumís group identified imidazole and zerumbone derivatives as the first inhibitors of WalK. Afterward, the same group developed biochemical and genetic high-throughput (HTP) screening methods resulting in the discovery of a number of WalKR inhibitors. However, some of these inhibitors were not exclusively selective to WalKR suggesting that HTP screening approach may favor the identification of compounds that inhibit through mechanisms that are not specific for TCSs and may be toxic. A more promising avenue of research seems to be structure-based virtual screenings (SBVS) with tailored libraries or rational structure-based drug design. Targeting WalKR following structure-based approach is expected to help identify specific inhibitors of WalKR and general inhibitors of TCSs. Both these will expedite the urgently needed development of novel antibacterial drugs.

The more druggable component of TCS is the HK due to the presence of the catalytic ATP-binding domain (CA domain). The CA domain contains a well-defined and partially conserved pocket, which accommodates the ATP required for autokinase activation, i.e., initiation of signal transduction (Figure 2A,B). This pocket is hydrophobic in nature but presents two conserved polar residues, aspartic acid (Asp) and asparagine (Asn), which are responsible for ATP selectivity and Mg$^{2+}$ chelation, respectively. In addition, three conserved structural water molecules increase the pocket polarity and are involved in hydrogen bonds between the ATP and the conserved Asp and Asn.

The major bottleneck for structure-based discovery of WalK inhibitors has been the lack of high-resolution structures of WalK. Instead, structural homology models of the CA domain of *S. pneumoniae* and *S. epidermidis* WalK based on the structures of *Thermotoga maritima* HK853 (PDB:2C2A) and *E. coli* EnvZ (PDB:1BXD) were used for SBVS. These screenings yielded WalK autophosphorylation inhibitors belonging to different classes of chemical structures, such as imidazole analogues and derivatives of furan, thiophene, thiazolidine, benzamide, and pyrimidinone. This suggests that the ATP-binding pocket can accommodate a variety of ligands, a feature that can be exploited to design potent and selective HK inhibitors with a desired ADMET (absorption, distribution, metabolism, elimination, and toxicology) profile. Notably, unfavorable ADMET properties are one of the major reasons for high attrition rates of candidate molecules in drug discovery.

WalK autophosphorylation inhibitors identified by SBVS inhibited the growth of *S. pneumoniae* and *S. epidermidis* and showed bactericidal effects toward both planktonic and biofilm cells. Furthermore, some of them decreased the mortality of mice infected with *S. pneumoniae* in an in vivo sepsis model, supporting the idea that SBVS is a viable tool for the identification of WalK inhibitors with therapeutic effect. Nevertheless, the design of potent ATP-competitive inhibitors specific for WalK is challenging, even more so when structural homology models are used. This issue is crucial when attempting to generate a WalK inhibitor with higher specificity and affinity in the hit-to-lead optimization phase. Although the overall fold of the CA domain as well as the catalytic residues is generally conserved, there are large
Figure 2. Structure of WalK. (A) S. mutans WalK (PDB:4I5S) is a long-rod dimer anchoring a HAMP signal-transducer domain (green) and a PAS sensor domain (blue) directly connected to the catalytic DHp (yellow) and CA (pink) domains. (B) B. subtilis WalK CA domain (PDB:3SL2). The ATP-binding site (semitransparent surface, ATP shown as sticks) is generally conserved except for the variable ATP-lid (magenta). More potent and specific WalK inhibitors can be redesigned by optimizing the interactions of previously identified hits with the ATP-binding site, particularly with the conserved structural water molecules (blue spheres) and the ATP-lid. (C) Close up view of S. mutans WalK PAS domain shows the putative ligand-binding pockets with a large cavity and a unique tunnel; these structural characteristics can be exploited to identify WalK PAS domain ligands.

variations in size and sequence in the ATP-lid, a HK distinctive flexible loop that covers the ATP-binding pocket and is crucially involved in the autophosphorylation reactions (8)(Figure 2A,B).

Recent elucidation of WalK structures of the entire intracellular portion from Streptococcus mutans (PDB:4I5S) (Figure 2A,C) and the CA domain from Bacillus subtilis (PDB:3SL2) (Figure 2B) might provide the key to design improved WalK inhibitors. These structures have not only revealed specific characteristics of the WalK CA domain but also insights into the molecular mechanism of WalK autokinase activation. These WalK structures might be used to generate potent and selective WalK inhibitors by the rational redesign of the previously identified hits by optimizing interactions with the ATP-binding site and particularly with the ATP-lid. Moreover, the structure of S. mutans WalK has also revealed specific folds of the HAMP transducer, the PAS sensor, and the DHp catalytic domains of WalK (Figure 2), opening up new possibilities to explore these domains for structure-based drug design.
PAS (Per-Arnt-Sim) domains are small sensor modules that bind a chemically diverse range of small molecules and present a conserved three-dimensional architecture but a divergent primary sequence. The *B. subtilis* PAS domain is required for WalK activity and localization to the divisome during cell division. The structure of *S. mutans* WalK shows that the putative ligand binding site forms unique pockets for a variety of ligands to bind (Figure 2C) suggesting that WalK PAS domain might bind ligands differently from other HK PAS domains. Therefore, the WalK PAS domain appears to be a promising target for novel antimicrobials, and its structure might also facilitate the rational design of specific and potent WalK inhibitors. Furthermore, WalK PAS ligands remain unknown, and their identification might generate chemical reagents to facilitate mechanistic studies.

In summary, we argue that discovery of new WalKR, and in general TCS, inhibitors, which will be further developed into antibacterial drugs, is still a realistic scenario. However, the focus should be on structure-based approaches. Certainly, ongoing efforts to solve the structures of HK from other important pathogens will help to design inhibitors with optimized activity. The high homology between TCSs and their wide distribution among bacteria imply that such inhibitors can be developed into broad-spectrum antibiotics. The road ahead is still challenging and arduous, but given the importance of the problem of drug resistance, we are convinced it is a road we should take.
REFERENCES


CHAPTER 9

GENERAL DISCUSSION

Nadya Velikova
THE NEED OF NEW ANTIBACTERIALS AND THE IDEAL ANTIBACTERIAL TARGETS

The growing problem of bacterial multi-drug resistance (MDR) requires the urgent development of novel antibacterials with different mechanisms of action from known antibiotics\(^1\),\(^2\). At the outset of this thesis bacterial two-component systems (TCS) were selected as targets for the discovery of novel lead candidates for the development of antibacterial drugs. At this point it seems prudent to revaluate TCS suitability as drug targets based on the results presented in the thesis and developments published in the literature. Recent reviews\(^3\),\(^4\),\(^5\),\(^6\),\(^7\),\(^8\) from opinion leaders in the field suggest that from pharmaceutical point of view novel antibacterial targets should fulfil the following criteria:

1. Have no human homologues or structurally similar proteins.
2. Be highly conserved among various bacterial species to assure broad-spectrum of antibacterial action.
3. Be strictly essential for bacterial viability such that their inhibition would lead to bacterial death but as targeting essentiality might increase the rise of resistance, targeting virulence targets has been proposed as an alternative.
4. Be “assayable”, i.e. should have easy measurable activity, assays amenable to high-throughput screening (HTS), target structural data should be available or easy to disclose and genetic tools should be available to validate the target in a key species.
5. Be patentable.

The suitability of TCS as targets for novel antibacterials is discussed below in relation to the first four criteria listed above.

1. HOMOLOGUES OF TWO-COMPONENT SIGNALLING SYSTEMS ARE ABSENT IN HIGHER EUKARYOTES

In all living organisms cellular processes are regulated via signal transduction. Both prokaryotes and eukaryotes depend on the ability to rapidly sense and respond to changes in intracellular and extracellular signals to adapt and survive. Two-component systems (TCS) are the major mode of signal transduction in bacteria\(^9\). They are attractive antibacterial (drug) targets because multiple TCS are found in nearly all bacteria and homologues have not been identified in mammals, including humans\(^10\),\(^11\),\(^12\). TCS signalling involves autophosphorylation of a membrane-bound histidine kinase (HK), phosphotransfer of the phosphoryl group to a cognate response regulator (RR), and ultimately modulation of the expression of target genes (Figure 1)\(^13\). The
Figure 1. Two-component system domain organization and signalling. A) A two-component system consist of a membrane-bound HK composed of a sensor domain (periplasmic sensor domain, Sp, and/or cytosolic sensor domain, Sc), transmembrane helices (TM1 and TM2) and the DHp and CA domains, and of an RR composed of a REC and an effector domain. ATP is used to first phosphorylate a His in the DHp domain and the phosphoryl group is then transferred to an Asp residue in the RR REC domain. The composition of the Sc can vary and is not always present in all HK, whereas the DHp and the CA domain are present in all HKs (modified from 13). B) HK domain organisation exemplified by S. mutans WalK (PDB: 4I5S).

phosphorylation levels of the RR are tightly regulated by the phosphatase activity of the HK, the RR or a partner protein 13, 14. HK autophosphorylation is mediated via the catalytic and ATP-binding (CA) domain, which binds ATP and phosphorylates the HK at conserved histidine (His) residues in the dimerization and histidine phosphotransfer (DHp) domain. The CA and DHp domains are conserved and present in all HKs, whereas the remaining sensor domains (periplasmic, PAS; GAF, HAMP) are variable and not present in all HKs (Figure 1) Furthermore, the ATP-binding site of the CA domain is a well-defined and highly conserved binding pocket (Figure 2). The conserved features of the CA domain and its essential role in signal transduction highlight it as an attractive target for structure-based virtual screening and phenotypic screening of biochemical inhibitors13. Moreover, the high degree of sequence conservation in the CA catalytic site implies that inhibitors targeted against this site will possess broad-spectrum of antibacterial activity. Altogether, this makes the CA domain the most attractive HK target site for the discovery and development of broad-spectrum antibacterials.
The only caveat with targeting the CA domain is the presence of the ATP-binding Bergerat fold which is shared with GHKL family of proteins, including mammalian proteins as Hsp90 or MutL[4] (Figure 3). The Bergerat fold includes four conserved motifs common to the GHKL family of proteins. Three of the four motifs correspond to the conserved N, G1 and G2 boxes in the HK CA domain. These similarities might lead to off target inhibitory effects of putative HK autophosphorylation inhibitors (HKAI) and therefore toxicity to mammalian cells. Indeed, the novel HKAI described in this thesis (Chapter 2 and 3) were cytotoxic to Caco-2 cells. The observed IC50 values for cell viability were mostly lower than the observed minimal inhibitory concentrations (MICs) against the bacterial strains tested suggesting off-target activities. Nevertheless, these were small molecule hits and there is considerable potential to improve their specificity and target selectivity through structure-based drug design and medicinal chemistry approach.

The similarity between the CA domain of bacterial TCS and eukaryotic proteins containing the Bergerat fold is exemplified by the demonstration that the eukaryotic Hsp90 inhibitor radicicol is an inhibitor of PhoQ autophosphorylation and was co-crystallized with the CA domain of PhoQ. However, other GHKL family member protein inhibitors, such as novobiocin and geldanamycin, could not be co-crystallized successfully with PhoQ and had no inhibitory effect on PhoQ autophosphorylation. The differences between radicicol and the other Hsp90 inhibitors with respect to PhoQ inhibition were attributed to differences in the putative interaction with the ATP-lid and the ATP-lid conformation in the CA domain. The ATP-lid (Figure 1) is a variable loop that connects the G1 and G2 boxes or the corresponding motifs in other GHKL family members. The ATP-lid is crucially involved in autophosphorylation. Although HKs are structurally similar to eukaryotic proteins belonging to the GHKL family, the differences in the ATP-lid can be a valuable feature for designing inhibitors that are specific to HKs with limited effect on eukaryotic GHKL proteins and therefore with low toxicity to host cells.
Figure 2. The ATP-binding site of HK CA domain is a well-defined and highly conserved pocket and allows the discovery and design of HK autophosphorylation inhibitors with broad-spectrum antibacterial activity following structure-based approaches. A) Structure of *B. subtilis* WalK CA domain (PDB: 3SL2). The conserved N-, G1- and G2-boxes are shown in red, blue and green, respectively. The variable ATP-lid is shown in pink. B) *B. subtilis* WalK CA domain (PDB:3SL2) coloured by conservation. The conservation scores were calculated using ConSurf. The ATP-binding pocket was calculated using PyMol and is shown as a semi-transparent yellow surface. ATP is shown as ball-and-stick in A and B. C) Alignment of WalK CA domains from different organisms. D) Alignment of the CA domains of different *E. coli* HKs.
Discovery of Inhibitors of Bacterial Histidine Kinases

Figure 3. The ATP-lid is a flexible loop variable in length and in sequence playing a critical role in ligand-binding and catalytic activity of members of the GHKL-superfamily of proteins. Features of the ATP-lid can be exploited to design histidine-kinase autophosphorylation inhibitors with higher specificity to bacterial histidine kinases and reduced off-target effects to mammalian members of the GHKL-superfamily, i.e. with lower toxicity. Conservation scores were calculated using ConSurf. Ligands are shown in sticks.

2. TCS ARE HIGHLY CONSERVED IN THE BACTERIAL KINGDOM AND TCS INHIBITORS ARE EXPECTED TO SHOW BROAD-SPECTRUM OF ANTIBACTERIAL ACTIVITY AND LOWER POTENTIAL FOR RESISTANCE DEVELOPMENT

TCS and especially the HK CA domains are highly conserved among various bacterial species (Figure 1). The high conservation on the one hand assures broad-spectrum of antibacterial activity and on the other hand simultaneous inhibition of multiple targets. Simultaneous inhibition of multiple targets (drug polypharmacology) has been proposed as a strategy to slow down resistance development to drugs including to novel antibacterials. As bacteria possess multiple sometimes as many as 160 TCSs, inhibitors of the highly conserved CA domain are likely to shut down multiple signaling pathways compromising the ability of the bacteria to rapidly adapt to environmental changes including those encountered in the host during infection. For some bacteria TCS inhibition may not be bactericidal but it is likely to compromise efficient growth, especially under the physiological stresses encountered by pathogens in the host.
The notion that inhibitors of TCS could inhibit multiple TCS targets is supported by the activity studies performed on the novel histidine kinase autophosphorylation inhibitors (HKAs) described in this thesis (Chapter 2 and 3). Promising HKAs were discovered by structure-based virtual screening (SBVS) and fragment-based screening (FBS) for putative ligands of three or two different HKs, respectively. Inhibition of autophosphorylation was then shown in biochemical assays with HKs not used for screening, indicating the potential to identify inhibitors with a broad-spectrum of HK autophosphorylation inhibitory activity using such approaches. The broad spectrum of activity against HKs was also reflected in the capacity of these inhibitors to prevent growth of several species of Gram-positive bacteria (Chapter 2 and 3). Furthermore, the most promising inhibitors were effective against multi-drug resistant (MDR) clinical isolates, including MRSA strains resistant to a wide range of β-lactam and non-β-lactam antibiotics. These results suggest that the HKAs possess different mechanism of action than the known antibiotics and/or the mechanisms of resistance present in the MDR strains are not active against the HKAs. The activity of the novel HKAs against Gram-negative bacteria was initially found to be low (minimal inhibitory concentrations (MICs) ≥ 63 µg/ml for the active compounds, and > 500 µg/ml for the majority of the tested inhibitors) but when combined with ε-poly-L-lysine capped nanoparticles (Chapter 4) the activity increased more than 10-fold. The antibacterial activity of the nanoparticles was only observed when combined with the HKAs, suggesting that the cell envelope of Gram-negative bacteria is most likely limiting intracellular uptake of the HKAs. Despite the evidence stated above, it remains possible that the antibacterial activity is due to inhibition of targets other than TCS HKs. Until this is proven we cannot unequivocally conclude that inhibition of multiple HKs is the reason for their antibacterial activity of the identified HKAs.

3. SOME TCS ARE ESSENTIAL FOR BACTERIAL GROWTH AND/OR INVOLVED IN THE REGULATION OF VIRULENCE

Ideally novel antibacterials should target essential proteins or processes to assure bactericidal effect but most TCS are not essential for viability of bacteria in their normal habitat. This has been demonstrated by the systematic knock out of TCS in different bacteria. However, this approach does not rule out the possibility that simultaneous inhibition of multiple TCS would cause bacterial death. In only a few organisms, more than one TCS has been genetically deleted or inactivated and this is restricted to typically just two or three TCS. One TCS that is known to be essential for bacterial viability is WalKR, which is highly conserved among Gram-positives with a low G+C content in their genomic DNA (Figure 1). In this thesis (Chapter 3) inhibitors of *S. pneumoniae* WalK autophosphorylation with moderate antibacterial effect for Gram-positive strains, including *S. pneumoniae* and MRSA, and Gram-negative strains, including multidrug resistant *Acinetobacter baumannii* were discovered by FBS for putative ligands of *S. pneumoniae* WalK and then ligand-based similarity searches (LBS) with the two best hits. The SBVS hits, A5 and A6 (Chapter 2), also inhibited *S. pneumoniae* WalK autophosphorylation *in vitro* and showed antibacterial effect for Gram-positive bacteria. Nevertheless it is difficult
to demonstrate that the antibacterial effect is due to inhibition of WalKR even more so when multiple-target inhibition is expected. Possible approaches to demonstrate target specificity and to elucidate the mechanism of action of the novel antibacterial HKAI{s} include overexpression of the putative target and checking of inhibitor-induced changes in the growth rate or the morphology of the mutant strain \(^{27}\), transcriptomics signatures \(^{28}\), transcriptional profiling of conditional mutants \(^{29}\). Furthermore, some of the identified fragment-like HKAI{s} (Chapter 3) showed antibacterial effect against the Gram-negative \(A.\ baumannii\), \(S.\ maltophilia\) and/or \(E.\ coli\). WalKR is not present in Gram-negative bacteria. The high IC50 values of the fragment-like HKAI{s} for WalK autophosphorylation inhibitors (≥ 2 mM) and their antibacterial effect for Gram-negative strains where WalKR is not present indicate that the identified HKAI{s} inhibit more HK targets and/or possess mechanism of action different than HK autophosphorylation inhibition.

It has been suggested that inhibiting essential targets would lead to fast(er) development of resistance than inhibition of virulence mechanisms because there would only be selection for resistance in pathogens causing an infection in the host \(^{25},\ 30\). High throughput screening for ligands of \(E.\ coli\) QseC periplasmic domain identified Led209 as a promising hit for the development of antibacterials that act via inhibiting virulence. QseCB is a TCS involved in the response to host adrenergic signals and in quorum sensing mediated via AI-2 \(^{31},\ 32,\ 33,\ 34\). Led209 was shown to inhibit the binding of signals to QseC, preventing its autophosphorylation and consequently inhibiting QseC-mediated activation of virulence gene expression \(^{30}\). Altogether, this led to inhibition of virulence \textit{in vitro} and \textit{in vivo} of several susceptible pathogens such as UPEC and \(S.\ maltophilia\) \(^{30}\). The results obtained with Led209 highlight the potential of targeting virulence for the development of novel antibacterials. Other TCS such as PhoQP and PhoRB involved in responses to environmental stimuli have also been linked to regulation of virulence \(^{35},\ 36\) and therefore might also be promising targets for the discovery of antibacterials reducing virulence. Interestingly the HKAI{s} identified in this thesis (Chapter 2 and 3) inhibited autophosphorylation activity of \(E.\ coli\) PhoR and \(S.\ aureus\) PhoR. In the future it would be interesting to study their specific effects on the regulation of genes controlled by the PhoRB TCS under phosphate limiting conditions in order to demonstrate that these inhibitors act on the targets that are inhibited \textit{in vitro}. Furthermore, evaluation of the effect of these HKAI{s} in \textit{pho} knockout strains will give crucial information about the specificity of the compounds.

An example of a promising antimicrobial target that is crucial for virulence and persistent infections is the Mycobacterium DosRST composed of the RR DosR and the HKs DosS and DosT. DosRST plays an essential role in triggering and maintaining dormancy and in enabling resuscitation in tuberculosis \(^{37},\ 38,\ 39,\ 40\). The metabolic adaptations occurring in non-duplicating, drug resistant dormant \(M.\ tuberculosis\) are triggered by hypoxia and respiratory poisons (i.e. NO, CO), and are regulated by the DosR RR. DosR is activated by phosphorylation catalyzed by two HKs, DosS and DosT. Due the conservation of the CA domains of HKs a future possibility is to test the activity of the discovered HKAI{s} (Chapter 2 and 3) against DosS and DosT and their antimicrobial effect against \(M.\ tuberculosis\). In a genomics-based \textit{in silico} target identification pipeline for \(M.\ tuberculosis\) incorporating a network analysis of the protein-protein interactome, a flux balance analysis of the reactome, a structural
assessments of targetability as well as experimentally derived phenotype essentiality data, DosS was selected as a high-confidence target for dormant mycobacteria.

4. A VARIETY OF DRUG-DISCOVERY APPROACHES CAN BE APPLIED TO TCS INHIBITORS DISCOVERY

The first published TCS inhibitors were discovered by high-throughput screenings of natural products or chemical libraries for biochemical inhibition of the target. With the elucidation of TCS structures, structure-based virtual screenings were employed to identify TCS inhibitors hits and some of the hits have been further improved by rational design. Drug design strategies for identifying TCS inhibitors with broad spectrum of inhibition as well as specific TCS inhibitors is facilitated by the available structural and genomic information for TCS as well as the large body of research on their role in bacterial physiology and virulence. Virtual structure-based or in vitro fragment-based screenings with multiple targets (Chapter 2 and 3, respectively) and selecting for common hits facilitates the discovery of broad-spectrum inhibitors, whereas selecting hits specific to particular target will facilitate the discovery of narrow-spectrum inhibitors. In this case high-resolution structures of TCS targets in complex with their inhibitors are still needed to facilitate the rational design of improved inhibitors against specific TCS targets that are either essential or controlling key virulence factors. With the advance of high-throughput crystallography, it seems feasible to crystallize the kinome of a bacterial pathogen or promising HK targets from different pathogens and use the structural information to design more potent and/or specific inhibitors following structure-based design approach. Complementary approaches should include the structural determination of co-crystals of putative target HK CA domains that has been previously crystallized (e.g. WalK from B. subtilis, PDB:3SL2) with fragment libraries or known kinase inhibitors and co-crystals with mammalian members of the GHKL protein family (e.g. Hsp90) to facilitate structure-based design of inhibitors with improved affinity and specificity.

APPROACHES FOR TCS INHIBITORS DISCOVERY

STRUCTURE-BASED VIRTUAL SCREENINGS USING MULTIPLE HK STRUCTURES INCREASES THE CHANCES OF IDENTIFYING HK INHIBITORS WITH BROAD SPECTRUM OF INHIBITION

Molecular docking and receptor-based virtual screening have been an indispensable component within structure-based drug discovery, including TCS inhibitors discovery, for hits identification and hit-to-lead optimization. Although, ligand flexibility can be handled by variety of algorithms in current docking implementations, receptor flexibility remains a major outstanding challenge in the practice of molecular docking-based virtual screening. The
challenge is mainly because of the high dimensionality of the conformational space and the complexity of scoring energy function. It is generally accepted protein flexibility to be often coupled to ligand binding in numerous experimental and theoretical studies. Two kinds of ligand-binding mechanisms have been well discussed. The first is conformational selection, which assumes that the ligand binds to a pre-existing receptor conformation in an equilibrated ensemble; and the second is induced fit, which presumes that the binding of the ligand induces conformational changes in the receptor. In both cases, structural conformations of the receptors need to be taken into account in molecular docking studies and, ideally, (multiple) ligand-bound structures should be used instead of apo-structures.

Various modeling methods have been developed to account for receptor flexibility in molecular docking and structure-based virtual screening. Soft docking, which docks ligands to a rigid receptor with a soft scoring function tolerating some steric clashes, has been reported to be worse for identifying known ligands than the hard scoring function when multiple receptor conformations were used. Several docking programs limit protein flexibility to side chains by exploration of rotamer libraries and make the problem of protein flexibility less computationally demanding. However, this approach cannot deal with backbone movement or other major structural rearrangements. Another implementation is docking of ligands to multiple receptor conformations, which may either be obtained experimentally by X-ray crystallography and NMR spectroscopy or computationally by molecular dynamics, normal-mode analysis, and other techniques.

In Chapter 2 we applied an approach combining structure-based virtual screenings for putative ligands of three different HK X-ray structures followed by ligand-based similarity searches with the most promising hits as query molecules. The problem of receptor flexibility in the docking approach was addressed by using three different receptor structures. The use of multiple structures in the docking experiments increases the chance of finding broad-spectrum ligands because it implicitly considers the receptors plasticity, sequence and conformation variability in the docking approach. This approach mimics an ensemble based docking which has been shown to improve the overall performance of virtual screening experiments.

Screening by molecular docking for putative ligands of three different HKs successfully identified two hits, A5 and A6, which inhibited the autophosphorylation activity of multiple HKs and showed weak antibacterial effects. A5 and A6 inhibited the autophosphorylation of three HKs not employed in the virtual screenings (i.e. E. coli and S. aureus PhoR, and S. pneumoniae WalK). Therefore, A5 and A6 were used as query molecules in ligand-based similarity searches (LBSS). The LBSS successfully identified B13 as a promising lead candidate. B13 (R,S)-4-[1-Ethyl-2-(4-fluorophenyl)butyl]phenol inhibited the autophosphorylation of E. coli PhoR and S. aureus PhoR with IC50 values of 16 and 212 µM, respectively (Chapter 2). The fact that B13 inhibited the autophosphorylation of multiple HKs including targets not used in the structure-based virtual screening supports the concept that use of multiple structures in the virtual screening by molecular docking, facilitates the identification of broad-spectrum inhibitors. Furthermore, even the next generation hits identified by other methods, in this case ligand-based similarity search (LBSS), possessed broad-spectrum inhibitory capacity. The putative broad spectrum HK autophosphorylation
inhibitory capacity of B13 was reflected in its broad-spectrum antibacterial effect (Chapter 2). B13 showed antibacterial effect for a range of Gram-positive bacteria, including MRSA clinical isolates. The MICs for the different Gram-positive strains ranged between 1 and 16 µg/ml.

Antibacterials should not cause adverse effects to host cells. Furthermore, one of the main causes of high attrition rates in drug discovery is so called adverse "off-target" effects. Therefore, as early as possible in the developmental program the toxicity of the hits has to be evaluated. B13 inhibited cell viability of Caco-2 cells and macrophages with IC₅₀ values of 29 µg/ml and > 20 µg/ml, respectively (Chapter 2 and 4). The selectivity index (IC₅₀/MICs) > 1 indicates that B13 is a promising candidate for hit-to-lead optimisation in the preclinical development of antibacterials for Gram-positive bacteria. B13 showed no antibacterial effect for Gram-negative bacteria. We hypothesized that this was most probably due to inability of B13 to cross the outer membrane of Gram-negative bacteria and explored the possibility to use nanoparticles for enhanced delivery to Gram-negative bacteria (Chapter 4). Indeed, as part of a nano-formulation for delivery of antibacterials to Gram-negative bacteria B13 showed antibacterial effect for the uropathogenic *E. coli* CFT 073 with MIC of 25 µg/ml (Chapter 4). The latter is in agreement with B13 autophosphorylation inhibitory capacity for HKs from Gram-negative bacteria (Chapter 2). This is encouraging because it might be possible to enhance the permeability of next generation hits based on B13 using medicinal chemistry approaches.

The other two promising hits coming from the LBSS following SBVS (Chapter 2), B7 and B14, showed lower HK autophosphorylation inhibitory capacity, weaker antibacterial effect and stronger cytotoxicity to Caco-2 cells than B13. Nevertheless, B7 and B14 are worth considering as starting points for the design of improved HK inhibitors because they are predicted to interact with the ATP-lid. The ATP-lid is a highly flexible and variable in sequence component of the ATP-binding site of HKs and is critically involved in autophosphorylation. The variability of the ATP-lid has been proposed as a valuable feature for the design of inhibitors that are specific to bacterial HKs with limited off-target effects on eukaryotic proteins sharing the ATP-binding Bergerat fold (the so called GHKL proteins, including Hsp90 and MutL) or for the design of strain-specific HK inhibitors.

**Fragment-based screening (FBS)**

**Fluorimetry is a potent tool for the discovery of HK autophosphorylation inhibitors with desired spectrum of inhibition**

So far, TCS inhibitors have been discovered mainly by high-throughput screenings (HTS) or by structure-based virtual screenings. Fragment-based screening (FBS) is a more recent approach and the methodology behind it is complementary to HTS. Fragments are small molecules with low affinity (usually Kd > 0.1 mM) following the rule of three (MW < 300, ClogP < 3, number of hydrogen bond donors and hydrogen bond acceptors < 3, number of
rotatable bonds < 3) \textsuperscript{74, 75}. Fragments serve as starting point for the elaboration of more potent inhibitors by rational design \textsuperscript{76}. The main advantage of FBS over HTS is that fragment libraries are much smaller than conventional compound libraries \textsuperscript{77, 78, 79}. It has been estimated that there are only \textasciitilde{} \(10^7\) molecules of up to 11 heavy atoms which are also fragment-like (MW \textless{} 300 Da), compared to \textasciitilde{} \(10^{63}\) molecules of molecular weight less than 500 Da, i.e. drug-like. This means that a library of \(10^4\) fragments covers 0.01\% of the fragment-like chemical space compared to \(10^{-58}\)\% of the chemical drug-like space.

The low molecular weight of fragments (typically \textless{}300Da) and their corresponding reduced functionality implies that they will generally bind a target protein with weak affinity (Kd > 0.1 mM), if at all. Despite their weak affinities, fragments possess high intrinsic binding energy to overcome a large entropic barrier upon binding \textsuperscript{80} and often exhibit high ligand efficiency (\(\Delta G\) of binding per non-hydrogen atoms) \textsuperscript{81}. Therefore, fragments are suitable starting points for elaboration into larger, more potent inhibitors.

Considering the advantages of FBS over HTS mentioned above, we performed fragment-based screenings (FBS) by differential scanning fluorimetry (DSF) for ligands of multiple HKs (Chapter 3). By choosing the hits identified with different targets we were able to identify HKAIs that inhibited the autophosphorylation of HKs that were not used in the screens, showing that the use of multiple targets selects for inhibitors with broad-spectrum inhibitory capacity. The hits identified by FBS by DSF were used as query molecules in ligand-based similarity searches (LBSS) and the LBSS hits showed broad-spectrum antibacterial effect against multi-drug resistant Gram-positive and Gram-negative strains (Chapter 3). Probably due to their small size, the fragment-like HKAIs showed lower affinity to HKs (indicated by their IC\textsubscript{50}s for \textit{E. coli} PhoR autophosphorylation) and higher cytotoxicity to Caco-2 cells compared to the hits identified by SBVS and LBSS in Chapter 2. The antibacterial activity of the FBS hits was comparable to the less potent hits identified by SBVS and LBSS, B7 and B14 (Chapter 2). The comparable antibacterial effect despite of the lower affinity to HKs and the higher cytotoxicity might be related to off-target effects as these hits are fragment-like and most likely bind also to multiple prokaryotic and eukaryotic targets. The structural similarity with previously published TCS inhibitors (F1 and F1.6, \textsuperscript{33, 46, 47}) and the limited options for further improvement of the carbazoles from drug discovery perspective (F2.1, F2.3 and F2.8) makes these hits unattractive for further investigation. Nevertheless, F1.8 (4-N-(4-bromophenyl)pyrimidine-2,4-diamine) seems to present a promising starting point for structure-based drug design of HK inhibitors with broad-spectrum antibacterial effect. F1.8 possess weak HK autophosphorylation inhibitory capacity, however, it shows broad-spectrum antibacterial activity including against multi-drug Gram-negative strains. The latter suggests that F1.8 scaffold is attractive for the development of antibacterials with improved permeability for Gram-negative bacteria.
COMPUTER-AIDED APPROACHES CAN FACILITATE HIT IDENTIFICATION AND OPTIMISATION PROVIDED SCREENINGS ARE PERFORMED BY STANDARDISED METHODS

Nowadays early-stage antibacterial drug discovery is largely done by academic organisations or SMEs. It is unlikely that such organisations would have the resources for extensive screening. However, if screening and *in vitro* evaluation of screening hits are performed under standardized conditions in different laboratories and the information is made (publicly) available different computational methods can be exploited to speed up the discovery and optimisation of hits. These include predictive quantitative structure-activity relationship (QSAR) models and ligand-efficiency indices (LEIs) approaches (Chapter 5).

MOLECULAR MODELING CAN GUIDE RATIONAL OPTIMISATION OF HITS IN THE ABSENCE OF EXPERIMENTAL TARGET-INHIBITOR STRUCTURES

The hits identified by SBVS, FBS, LBSS, QSAR and LEIs approaches (Chapter 2, 3 and 6) were co-crystallized with the CA domain of *T. maritima* CheA HK as a model system (data not shown). In some cases, such as A5, B13, B14, F1, F2, F2.3, H30 the CheA-inhibitor structures, solved by X-ray crystallography, revealed electron density in the binding site, although the structures of the HK inhibitors could not be fully resolved, probably due to partial occupancy and/or multiple conformations. Unfortunately, these structures could not be used to guide rational design of more potent inhibitors following structure-based drug design approach. Therefore, the putative binding mode of the HK inhibitors was predicted by molecular docking to the structure of the CA domain of *T. maritima* HK853 or *B. subtilis* WalK. The predicted binding modes of the HKAs (Chapter 2 and 3) resembled the binding mode of the natural substrate ATP/ADP. The most potent inhibitors, e.g. B13, were predicted to form contacts with conserved residues in the N-, G1- and G2-boxes in the ATP-binding site. Some inhibitors, such as B7, were predicted to interact with the variable ATP-lid and showed significant differences in the autophosphorylation inhibitory capacity for different HKs. Other inhibitors predicted to interact with the ATP-lid, e.g. F1.8 (Chapter 3), showed broad spectrum HK inhibitory activity and antibacterial action. Although the ATP-lid is more variable compared to the remaining parts of the ATP-binding site of the CA domains (Figure 2), it contains conserved residues, such as Arg, Ile or Leu, and Tyr (Figure 2B). These residues are conserved among HKs from different species or different HKs from the same bacteria (Figure 2C and 2D). The binding modes predicted by molecular modeling can serve as starting point for the structure-based design of more potent and specific or broad-spectrum inhibitors with lower off-target effects by exploring the features of the ATP-lid.
ANTIMICROBIAL PEPTIDES AND TWO-COMPONENT SYSTEMS INHIBITION

Eukaryotic cationic antimicrobial peptides (AMPs) are produced at sites of infection or inflammation in many different organisms. Usually, AMPs increase the permeability of the bacterial membrane, leading to loss of pH gradient and cell death. Some AMPs have been shown to interact with intracellular targets but in most cases the exact mechanism of action of intracellular AMPs remains unclear.

INHIBITION OF \( T. \) \( \text{MARITIMA} \) HK853-RR468 BY LACTOFERRICIN B-DERIVED PEPTIDES IS NOT SUITABLE FOR STRUCTURAL STUDIES ON TCS INHIBITION BY AMPs

LactoferricinB (LfcinB) is an AMP with broad-spectrum antibacterial activity. It has been shown by in vitro and in vivo experiments and bioinformatics methods that LfcinB inhibits the phosphorylation of the RR (BasR and CreB) by their cognate histidine kinases (HKs). We showed that LfcinB-derived peptides inhibit the phosphotransfer and phosphatase activity of \( T. \) \( \text{MARITIMA} \) HK853-RR468. However, the LfcinB-derived peptides seemed to induce aggregation of \( T. \) \( \text{MARITIMA} \) HK853 and \( E. \) \( \text{COLI} \) PhoB. Therefore, \( T. \) \( \text{MARITIMA} \) HK853-RR468 and \( E. \) \( \text{COLI} \) PhoRB might not be suitable for structural studies of TCS inhibition by LfcinB-derived peptides. Furthermore, the described results taken together with the broad-spectrum of antibacterial activity of LfcinB point to the possibility that the antibacterial effect caused by LfcinB and LfcinB-derived peptides might be attributed to non-specific protein aggregation and might act on multiple cellular targets after entering into the cell. On this basis further work on LfcinB-derived peptide inhibitors was abandoned.

SYNERGY EFFECT BETWEEN ANTIMICROBIAL PEPTIDES AND TCS INHIBITORS

Antimicrobial peptides have been proposed as a way to overcome the lower permeability of Gram-negatives cell envelope. Therefore, we tested the synergistic effect of three frog AMPs with antibacterial effect against Gram-negative bacteria and B13, as the most potent HK inhibitor described in this thesis. The AMPs tested were [E4k]alyteserin-1c, [E6K]hymenochirin-1B and [K20K27]esculentin-2Cha. Unfortunately, no synergistic antibacterial effect against \( E. \) \( \text{COLI} \) CFT 073 was observed (data not shown). \( \varepsilon \)-Poly-L-lysine (ePLL) is a homo-polypeptide with demonstrated antibacterial effect. Similar to the frog AMPs, ePLL showed no synergistic effect with HK inhibitors on antibacterial activity against \( E. \) \( \text{COLI} \) CFT073 (Chapter 4). The MICs against \( E. \) \( \text{COLI} \) CFT073 of the tested frog AMPs and ePLL
were \( \geq 63 \) µg/ml. Combinations of more potent AMPs and/or more potent HKAI might be required to achieve synergistic effect and ultimately to observe antibacterial effect against Gram-negative pathogens.

**HK inhibitor spectrum can be broadened and the cytotoxicity reduced via the use of nanotechnology**

Although the best hits described in Chapter 2 and 3 have weak or no activity (MICs in the range of 125-500 µg/ml) against Gram-negative pathogens, they do inhibit autophosphorylation of HKs from Gram-negative bacteria suggesting permeability of the Gram-negative cell envelope is limiting their anti-bacterial effects. This assumption was shown to be correct using histidine kinase autophosphorylation inhibitors (HKAI) loaded mesoporous silica-based nanoparticles capped with \( \varepsilon \)-poly-L-lysine as a strategy for effective drug delivery (Chapter 4). Whereas the nanoparticles themselves had no antibacterial activity, the MICs of HKAI combined with nanoparticles was decreased more than 10-fold (Chapter 4). The free HKAI showed relatively low toxicity to human cells and even lower toxicity when combined with nanoparticles. Taken together these data indicate high potential for developing a new structural class of antibacterial lead compounds with activity against multiple targets in several MDR Gram-positive and Gram-negative pathogens.

**Comparison of the novel HK inhibitors with other published TCS inhibitors**

Structure-based virtual screenings for *S. epidermidis* WalK ligands yielded inhibitors which have been subsequently further optimized by rational design. The last generation of *S. epiderimidis* WalK inhibitors had MICs for *S. epidermidis* and *S. aureus* lower than 3.1 µM, corresponding to lower than 1.66 µg/ml. B13, the most potent hit described in this thesis (Chapter 2), possess MICs for *S. epidermidis* laboratory strains and clinical isolates in the range of 1 to 8 µg/ml. B13 MICs for reference strains of *S. aureus* and clinical isolates of MRSA are in the range of 8 to 16 µg/ml (Chapter 2). Therefore, in terms of antibacterial activity B13 is comparable to the last generation of published *S. epidermidis* WalK inhibitors. *S. epidermidis* WalK inhibitors inhibited *S. epidermidis* WalK autophosphorylation with \( IC_{50} \) values in the range of 24.2 to 71.2 µM. B13 has not been tested for its autophosphorylation inhibitory capacity for any WalK, however, it inhibits *E. coli* PhoR and *S. aureus* PhoR with \( IC_{50} \) of 16 and 212 µM, respectively (Chapter 2). Considering the differences in the experiments used to determine the \( IC_{50} \)s of the *S. epidermidis* WalK inhibitors and B13 and the fact that different HKs Km differ in the range 2 and 200 µM, it is difficult to conclude how B13 compares to the last generation of published *S. epidermidis* WalK inhibitors. The cytotoxicity of the *S.*
epidermidis WalK inhibitors was evaluated with Vero Cells using the MTT assay and the IC\textsubscript{50} were higher than 100 µg/ml \textsuperscript{47} corresponding to > 60 selectivity index (IC\textsubscript{50}/Viability/MIC). B13 cytotoxicity was evaluated with Caco-2 cells using the neutral-red uptake assay (Chapter 2) and with macrophages in cell culture (Raw cells) using the XTT assay (Chapter 4) and the IC\textsubscript{50} were 29 µg/ml and ≤ 20 µg/ml, respectively, corresponding to selectivity index ≥ 1.25 for the different S. epidermidis and S. aureus strains tested (Chapter 2). Even though the cell viability was evaluated using different cell lines and methods, it is clear that B13 selectivity is worse than the selectivity of the last generation published S. epidermidis WalK inhibitors and needs to be improved. However, B13 (MW 272.4) is a relatively smaller compound compared to the last generation S. epidermidis WalK inhibitors (MW between 498.02 and 534.02) implying that it possesses higher ligand efficiency and more possibilities for further improvements using an iterative structure-based drug design approach.

**FUTURE CHALLENGES AND PERSPECTIVES OF THE DISCOVERY OF TCS INHIBITORS**

Both SBVS and FBS seem to be promising tools for the discovery of TCS inhibitors. The application of multidisciplinary screening approach (including SBVS, FBD, and LBSS) allowed us to identify HK inhibitors with activity against multiple HKs and a broad-spectrum antibacterial effect. Furthermore, the identified inhibitors are predicted to interact with different parts of the ATP-binding sites. The latter together with the relatively small size of the identified HK inhibitors (MW < 300) provides the possibility to design improved inhibitors by combining chemical moieties from the different hits to generate inhibitors with stronger affinity and higher specificity to bacterial HKs.

Although a very promising starting point for development of novel antibacterials, the identified HKAI s have a number of shortcomings that need to be addressed in the hit-to-lead optimisation process:

- The affinity to model HKs such as T. maritima CheA is most likely not high enough and/or multiple conformations of the inhibitors are possible and therefore, the CheA-inhibitors structures cannot be resolved and used for structure-based drug design. This liability can be addressed by discovering more potent HK autophosphorylation inhibitors and/or by solving the structures of the discovered inhibitors with other putative target HKs, such as the recently published B. subtilis and S. mutans WalK (PDB: 3SL2 and 4I5S) or E. coli/T. maritima EnvZ (PDB: 4KP4) \textsuperscript{89, 104}.

- The identified HKs inhibitors are not sufficiently permeable to Gram-negative bacteria. This can be overcome by using nanoparticles (Chapter 4). The latter however, raises additional issues related to the following steps of the drug discovery process related to the safety of such complex therapeutic agents. Therefore, it is desirable to apply medicinal chemistry approaches to design inhibitors with improved permeability for Gram-negatives \textsuperscript{69}. Addition of functional groups to the identified
HKAs, given their small size, could increase the permeability in Gram-negatives. Nevertheless, increasing the specificity of the identified inhibitors to Gram-positive targets such as the obligatory essential WalKR can be a promising strategy to develop narrow-spectrum antibacterials against Gram-positive bacterial infections.

The identified HKAs showed some cytotoxicity to Caco-2 cells evaluated by neutral-red uptake assays (Chapter 2 and 3) and B13 showed cytotoxic effect to macrophages (Raw cells) evaluated by the XTT assay (Chapter 4). The most promising and most potent HK inhibitor B13 possesses a selectivity index for Gram positives (IC₅₀ viability/ MICs) ≥ 1.25. Increasing the potency and the selectivity of the HK inhibitors will most likely lead to decrease in their cytotoxicity. To facilitate the discovery of more selective HK inhibitors, the next generation of HK inhibitors should be tested also for their inhibitory activity on the structurally closely related mammalian members of the GHKL superfamily.

In summary, following structure-based and ligand-based virtual screenings and fragment-based in vitro screenings, we have successfully identified HK autophosphorylation inhibitors (HKAs) that can serve as a starting point for the design of HKAs with improved target affinity and selectivity, which would lead to stronger antibacterial effect and lower toxicity.
The growing problem of bacterial multi-drug resistance requires the urgent establishment of pipelines for discovery of novel antibacterials. Two-component systems (TCS) have been proposed for years as promising novel antibacterial targets. Inhibitors of TCS, especially autophosphorylation inhibitors targeted at the CA domains of multiple histidine kinases (HKs) are expected to show broad-spectrum of antibacterial activity and low toxicity to host cells.

Using structure-based virtual screenings (SBVS) for putative ligands of three different HKs followed by ligand–based similarity search with the most promising SBVS hits (Chapter 2) we identified HK autophosphorylation inhibitors of multiple HKs from both Gram-positive and Gram-negative bacteria. The inhibitors showed broad-spectrum antibacterial activity for Gram-positive bacteria, including clinical isolates of MRSA strains resistant to a wide range of β-lactam and non-β-lactam antibiotics. The most promising hit, B13, showed relatively low cytotoxicity and hemolytic activity and therefore presents a promising starting point for hit-to-lead optimisation using a structure-based drug design approach. The predicted binding modes of the other less potent hits, B7 and B14, point to possibilities to design more potent and selective inhibitors by combining chemical moieties belonging to the different hits.

In Chapter 3 fragment-based in vitro screenings (FBS) by differential scanning fluorimetry (DSF) were performed for ligands of two different HKs followed by ligand-based similarity searches with the two identified hits. Weak HK autophosphorylation inhibitors with broad spectrum of antibacterial activity were identified. Compounds F1.8 and F2.3 showed antibacterial effect for both Gram-positive and Gram-negative strains, including clinical isolates of MRSA and multi-drug resistant Acinetobacter baumannii and Stenotrophomonas maltophilia. The identified hits present a promising starting point for the design of HK autophosphorylation inhibitors with improved permeability.

In Chapter 5 a computer-aided approach was used in an attempt to identify more promising hits for HK autophosphorylation inhibitors using Nanoparticles delivery was employed to show that the most potent HK inhibitors, e.g. B13, possess antibacterial effect against Gram-negative bacteria (Chapter 4). Mesoporous silica-based nanoparticles loaded with HK autophosphorylation inhibitors showed 10-fold lower MICs compared to the free HK autophosphorylation inhibitors for UPEC E. coli CFT 073 and Serratia marcescens. Despite the lower MICs of HK autophosphorylation inhibitors loaded in nanoparticles for bacteria they were less cytotoxic than the free inhibitors at equivalent concentrations.

Lactoferricin (LfcinB) has been shown to inhibit TCS from E. coli. We demonstrated that LfcinB-derived peptides interact with T. maritima HK853-RR468 and E. coli PhoRB however, the mechanism of interaction was most likely non-specific and due to protein aggregation. (Chapter 6). Therefore efforts to co-crystallize these peptides with TCS for the rational structure-based drug design of improved inhibitors was not pursued further.

Co-crystallization of the HK autophosphorylation inhibitors with T. maritima CheA is described in Chapter 7.1. Furthermore, the purification, crystallization and
preliminary X-ray analysis of the periplasmic domain of CopM, a protein suggested to be involved in copper resistance, is presented in Chapter 7.2.

Chapter 8 is a discussion on the possibility to develop novel antibacterials with low potential for resistance development by targeting TCS signalling in general and the essential WalKR in particular.

Chapter 9 is a discussion of the main results of this thesis in the context of current literature on antibacterial drug discovery and published research on discovery of TCS inhibitors.
SAMENVATTING
Het toenemende probleem van bacteriële multidrug resistentie vereist het vlotte opzetten van onderzoeks-structuren om nieuwe antibiotica te ontdekken. Enkele jaren geleden werd voorgesteld dat twee-component systemen (TCSen) veelbelovende nieuwe antibacteriële doelwitten zouden kunnen vormen. Het is de verwachting dat remmers van TCSen, vooral autofosforylatie remmers gericht op de zogenoemde "CA" domeinen van histidine kinases (HKs), een breed spectrum van antibacteriële activiteit en lage toxiciteit voor gastheercellen zullen vertonen.

Met behulp van structuur-gebaseerde virtuele screenings (SBVS) voor vermeende liganden van drie verschillende HKs, gevolgd door zoeken naar liganden die het sterkst lijken op de meest veelbelovende SBVS treffers (Hoofdstuk 2) identificeerden we HK autofosforylatie remmers van HKs van zowel Gram-positieve als Gram-negatieve bacteriën. De remmers toonden breed-spectrum antibacteriële activiteit ten aanzien van Gram-positieve bacteriën, inclusief klinische isolaten van MRSA-stammen met resistentie tegen diverse β-lactam en niet-β-lactam antibiotica. De meest veelbelovende treffer, B13, liet een relatief lage cytotoxiciteit en hemolytische activiteit zien en lijkt daarom een veelbelovend uitgangspunt voor de zogenoemde "hit-to-lead" optimalisatie waarbij een structuur-gebaseerde drug ontwerp aanpak wordt gevolgd. De voorspelde bindingscapaciteit van twee minder krachtige treffers, B7 en B14, biedt mogelijkheden om krachtiger en selectievere remmers te ontwerpen, door het combineren van chemische groepen die behoren tot de verschillende treffers.

In hoofdstuk 3 is beschreven hoe fragment-gebaseerde in vitro screenings (FBS) door differentiële scanning fluorimetrie (DSF) werden uitgevoerd om liganden van twee verschillende HKs te zoeken, gevolgd door het zoeken naar chemische verbindingen die een ligand-gebaseerde gelijkenis vertonen, met als uitgangspunt de twee in hoofdstuk 2 geïdentificeerde treffers. Zwakke HK autofosforylatie remmers met een breed spectrum van antibacteriële activiteit werden gevonden. De verbindingen F1.8 en F2.3 toonden antibacteriële werking tegen zowel Gram-positieve als Gram-negatieve stammen, met inbegrip van klinische isolaten van MRSA en multiresistente Acinetobacter baumannii en Stenotrophomonas maltophilia. De geïdentificeerde treffers bieden een veelbelovend uitgangspunt voor het ontwerp van HK autofosforylatie remmers met verbeterde doorlaatbaarheid voor bacteriemembranen.

In hoofdstuk 5 werd een computer-gebaseerde benadering gebruikt in een poging om meer veelbelovende treffers van HK autofosforylatie remmers te vinden.

Nanodeeltjes werden gebruikt om te laten zien dat de meest potente HK-remmers, bijvoorbeeld B13, een antibacteriële werking hebben tegen Gram-negatieve bacteriën (Hoofdstuk 4). Mesoporeuze silica-gebaseerde nanodeeltjes gevolgd met HK autofosforylatie remmers toonden 10-voudig lagere MICs in vergelijking met de MIC van de pure HK autofosforylatie remmers; deze remmers hadden aantoonbare activiteit tegen UPEC E. coli CFT 073 en Serratia marcescens. Ondanks het feit dat de in nanodeeltjes verpakte HK autofosforylatie remmers een lagere MIC hadden wanneer toegepast om bacteriën te bestrijden, waren deze verpakte remmers minder cytotoxisch dan de pure remmers bij dezelfde concentraties.

Het is aangetoond dat Lactoferricine (LfcinB) TCSen uit E. coli kan remmen. We toonden aan dat van LfcinB afgeleide peptides activiteit bezitten tegen T. maritima HK853-RR468 en E. coli PhoRB, en dat het mechanisme van interactie
hoogstwaarschijnlijk niet specifiek was maar werd bepaald door eiwit aggregatie (Hoofdstuk 6). Pogingen om deze peptiden samen te kristalliseren met TCSen om betere remmers te vinden via een rationele structuur-gebaseerde drug ontwerp benadering werden daarom niet doorgezet.

Het tegelijk kristalliseren van HK autofosforylatie remmers met het T. maritima CheA TCS wordt beschreven in hoofdstuk 7.1. In hoofdstuk 7.2 worden eveneens de zuivering, kristallisatie en voorlopige röntgenanalyse van het periplasmatische domein van CopM, een eiwit dat gedacht wordt betrokken te zijn bij koper resistentie, beschreven.

Hoofdstuk 8 presenteert een discussie over de mogelijkheid om nieuwe antibacteriële middelen met een lage kans op ontwikkeling van resistantie te ontwikkelen, door middelen te ontwikkelen die het doorgeven van cellulaire signalen via TCSen in het algemeen, en in het bijzonder de essentiële WalKR, blokkeren.

In hoofdstuk 9 worden de belangrijkste resultaten van dit proefschrift en hun inbedding in de huidige literatuur over antibacteriële drug ontdekking in het algemeen, en de specifieke literatuur over de ontdekking van TCS-remmers, besproken.

Translated from English to Dutch by Peter van Baarlen
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3 hours to thank people I have met for 30 years ... I thank also everyone else who is not mentioned due to dehydration, my short memory or “blindness” to their impact, and last but not least laziness to read the text again ...
PERSONALIA

CURRICULUM VITAE

LIST OF PUBLICATIONS
Nadya Velikova (Надя Великова) was born on 17th of January 1984 in Burgas, Bulgaria. She finished her secondary school education in the High School for Mathematics and Natural Sciences “Akad. Nikola Obreshkov”, Burgas, Bulgaria, in 2002. In the same year she was among the winners of the National Olimpiad in Biology, the winner of the National Contest for High School Students Projects in Ecology and Environmental protection and participant in the finals of the National Competition in Chemistry. The first granted her admission to the Bachelor of Science programme in Molecular Biology at the Faculty of Biology, Sofia University “St. Kliment Ohridski”, Sofia, Bulgaria. In parallel to her BSc studies she worked in Eugene Semeonov’s group at the Department of Molecular Neurobiology, Institute of Molecular Biology, Bulgarian Academy of Sciences, on ivermectin resistance. After finishing her BSc studies in 2007, Nadya continued her education in the Master of Science programme in Medical Biotechnology at Wageningen University, The Netherlands. She did a MSs thesis at the Department of Toxicology under the supervision of Dr. ir. Jac Aarts, and an internship at the Toxicology and Bioassays Business Unit at RIKILT-Institute of Food Safety, under the supervision of Dr. ir. Toine Bovee. Both the thesis and the internship were related to design, development and implementation of reporter-gene assays for nuclear-hormone receptors ligands. In 2010 In 2010, Nadya continued her education and research experience in Dr. Alberto Marina’s group in the Institute of Biomedicine of Valencia, Spanish National Research Council, Valencia, Spain, working on the structure-based discovery of two-component system inhibitors as part of the EU FP7-funded Marie Curie ITN STARS – Scientific Training in Antimicrobial Research Strategies. The project included secondments at InhibOx, Oxford, United Kingdom, and at the Department of Biochemistry, United Arab Emirates University, Al Ain, United Arab Emirates. In 2014 Nadya continued her PhD studies as an external PhD candidate at the Host-Microbe Interactomics Group at Wageningen University under the supervision of Prof. dr. Jerry Wells.
PUBLICATIONS RELATED TO THE CURRENT THESIS

OTHER PUBLICATIONS
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