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Discovery of antibiotics and their targets in multidrug-resistant bacteria

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Chapter 7

Summary and outlook

Summary

Antimicrobial resistance (AMR) is one of the greatest global health concerns of the 21st century.[1] In Chapter 1 this problem was introduced by taking a look at the cause of the problem, the problem itself, and efforts to solve the problem.

The discovery of antibiotics, followed by their widespread clinical and industrial use, improved worldwide health conditions in the 20th century. The use (and abuse) of antibiotics, however, has led to the equally widespread occurrence of AMR which today presents an ever-increasing problem, with many commonly used antimicrobial drugs having lost their efficacy

in recent decades. Concurrently, the supply of new therapies to circumvent AMR mechanisms has been slowly declining. The AMR problem can therefore be defined as a supply and demand problem, where the demand for new antibiotics is not being met by the dwindling supply. To meet the demand for new antibiotics, increasing efforts are necessary from medicinal chemistry programs. Antibacterial drug discovery is particularly challenging compared to regular drug discovery due to the fast resistance evolution in bacteria, and due to the strict requirements for drugs to enter the bacteria.[2,3]

The main goal of the work described in this thesis is to find and develop unique chemotypes to meet the demand for new antibiotics with a novel mode-of-action (MoA). Phenotypic screening provides the opportunity to discover compounds with an unprecedented MoA, and historically the mining of libraries of natural product extracts presented a fruitful strategy to search for new target-drug combinations. Today, however, the risk of re-discovery along with the challenges associated with the structure elucidation and chemical synthesis of complex natural products has led to a slowdown in antibiotic discovery from such sources.[4] As an alternative, phenotypic screening using libraries of small synthetic compounds ensures both synthetic accessibility and structural novelty.

Resistant forms of *Escherichia coli* and the ESKAPE pathogens are the bacteria that have been designed by the WHO as high and critical priority pathogens for which new antibacterial agents are urgently required.[5] The ESKAPE pathogens comprise a family of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. All pathogens that are widespread in hospital settings and have extensively reported resistance mechanisms.[6–8]

Chapter 2 describes a phenotypic screen of an in-house library consisting of 352 compounds for antibacterial activity. *E. coli* and methicillin-resistant *Staphylococcus aureus* (MRSA) were chosen as Gram-negative and Gram-positive models, respectively. This screen led to identification of hit **1** for MRSA, and hit **2** for *E. coli*: both hits had favorable physicochemical properties and had the highest antibacterial potency out of all tested compounds (Figure 7.1). These hits were subsequently resynthesized and biologically tested for hit validation.

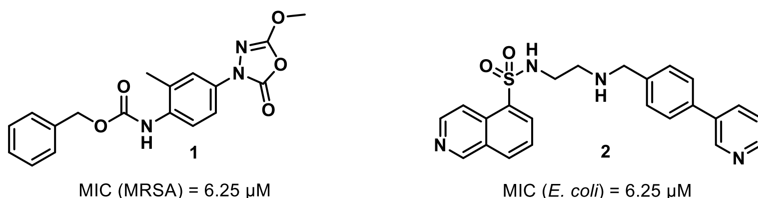

Figure 7.1 | An antibacterial screen of 352 compounds against MRSA and *E. coli* resulted in two clear hits: hit **1** (MRSA), and hit **2** (*E. coli*).

Chapter 3 describes a structure-activity relationship (SAR) study aimed to improve the antibacterial potency of hit **1** (Figure 7.2). Initially, the essential parts of hit **1** were determined by following a disjunctive approach, which simplified the scaffold. An additional 46 derivatives were synthesized leading to lead compound **3**, which had a submicromolar

minimum inhibitory concentration (MIC = 0.8 μM) against MRSA USA300. Further testing of **3** indicated that it had a favorable toxicity profile (>10x more toxic to human cells than to MRSA). Also, it showed high levels of potency against a variety of clinical isolates of MRSA, including vancomycin-resistant *S. aureus*, and it was able to time-dependently kill MRSA.

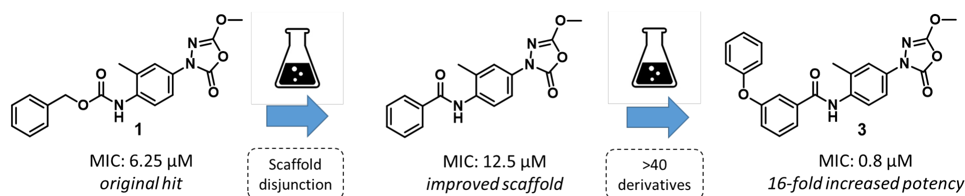

Figure 7.2 | Flow-chart from hit compound **1** to a simplified scaffold to the optimized lead compound **3**. MIC is measured against MRSA USA300.

In Chapter 4 the MoA of compound **3** was studied. An alkyne-functionalized chemical probe based on the structure of **3** was used, which can covalently bind target proteins and can subsequently be analyzed. Using MS-based competitive chemical proteomics, a set of target proteins of **3** were identified, most of them being cysteine and serine proteases (Table 7.1). Of these proteins FabH is the only protein essential for bacterial culture *in vitro*. Using a set of transposon mutants, these targets are then validated on SDS-PAGE.

Table 7.1 | Probe targets significantly outcompeted by compound **3** (edited from Table 4.1).

Protein	Description	Sequence length (aa)	Gene	Essential
FphE	Uncharacterized hydrolase	276	SAUSA300_2518	No
FabH	3-oxoacyl-[acyl-carrier-protein] synthase 3	313	*fabH*	Yes
FphH	Carboxylesterase	246	*est*	No
HZ1	Putative lysophospholipase	271	SAUSA300_0070	No
FphC	Hydrolase. alpha/beta hydrolase fold family	304	SAUSA300_1194	No
HH9	Putative lipase/esterase	347	SAUSA300_0641	No
FphB	Uncharacterized protein	322	SAUSA300_2473	No
FI2	Uncharacterized protein	275	SAUSA300_0321	No
IB7	Acetyl-CoA c-acetyltransferase	379	*vraB*	No
AdhE	Aldehyde-alcohol dehydrogenase	869	*adhE*	No

Resistance of MRSA to **3** was evolved over the course of several weeks, and these strains were then screened for abnormalities in general protein expression, as well as protein activity using the probe. The resistant strains showed significant increases in expression of FphE, AdhE, and FabH, while showing a highly significant reduction in AdhE activity.

The target inhibition profiles of active inhibitors were compared to that of inactive inhibitors via competitive chemical proteomics. Principal component analysis suggested AdhE and FphC as targets that are preferentially inhibited by active inhibitors. Interestingly, essential protein FabH was inhibited strongly by the inactive inhibitors, which suggested that FabH

inhibition alone is not responsible for the antibacterial effect. Also, inactive inhibitors were potentiated by testing them on AdhE and FphC transposon mutants. These data suggest a polypharmacological MoA in which FabH, FphC and AdhE inhibition play a role.

In Chapter 5, the discovery of a novel class of isoquinoline sulfonamides with potent antibiotic activity against clinically relevant Gram-negative bacteria was reported. A medicinal chemistry program was used to map the structure-activity relationships (SAR) of hit **2**. The initial SAR study comprised over 50 compounds that changed all parts except the linker length. This revealed steep activity cliffs, as most derivatives completely lost potency. Conformational restriction of the linker was then attempted, which led to a series of pyrrolidine-based diastereoisomers, of which the *cis*-2*R*,5*S* conformation was more active than the parent compound. This then led to the rational design of LEI-800, a compound with enhanced antimicrobial activity against *E. coli* and *K. pneumoniae*, and its closely related inactive isomer LEI-801.

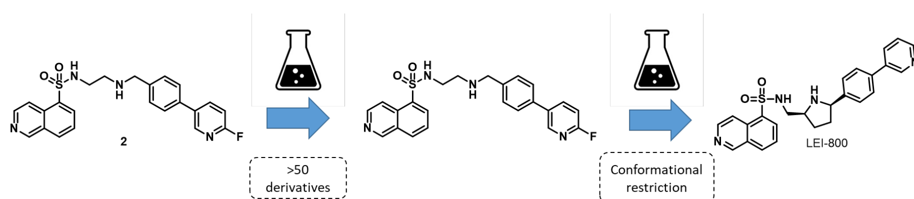

Figure 7.3 | Flow-chart from hit **2** to conformationally restricted lead LEI-800 via an extensive SAR study.

Chapter 6 describes target and MoA elucidation studies of LEI-800 in *E. coli*. Bacterial cytological profiling (BCP) in combination with whole-genome sequencing of resistant strains and biochemical studies led to the identification of DNA gyrase as the target responsible for antimicrobial activity. DNA gyrase is a well-validated essential bacterial topoisomerase that is targeted by ciprofloxacin (CIP), a fluoroquinolone for which widespread resistance has been observed in the clinic.[9,10] Importantly, LEI-800 did not show any cross-resistance with CIP or vice versa, and LEI-800 also showed activity against multidrug-resistant clinical isolates. LEI-800 was found to inhibit the supercoiling process by DNA gyrase 26x better than CIP (LEI-800 IC_{50} = 35 ± 13 nM; CIP IC_{50} = 925 ± 370 nM). Interestingly, LEI-800 had a lower antibacterial potency than CIP despite the more potent supercoiling inhibition. This was shown to be partially due to low cell permeability of LEI-800: both genetic and pharmacological membrane disruption increased the potency tenfold. Structural biology studies using cryo-electron microscopy revealed an unprecedented allosteric binding mode of LEI-800 at a site distinct from CIP and other gyrase inhibitors (Figure 7.4).

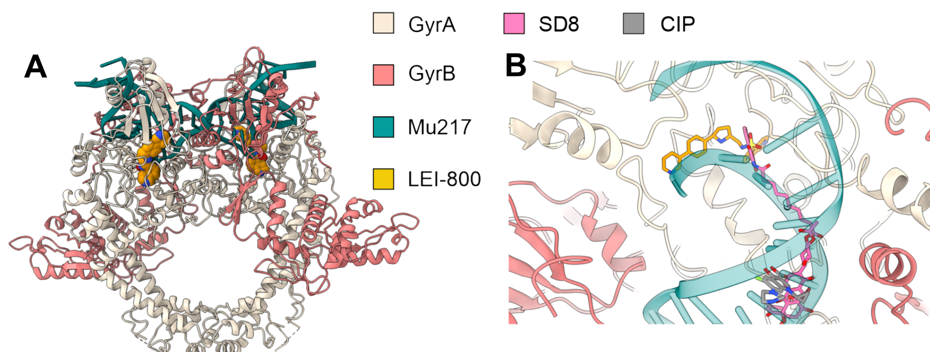

Figure 7.4 | LEI-800 occupies a unique binding pocket in DNA gyrase. A) The Gyr-Mu217-LEI-800 complex, earlier described in Chapter 6. Two molecules of LEI-800 observed in a single gyrase heteroteramer are shown as golden van der Waals spheres. **B)** A comparison of LEI-800, CIP, simocyclinone D8 (SD8) binding sites on GyrA DNA-binding surface as shown earlier in Chapter 6.

Outlook

New methods to address the threat of AMR are highly necessary, as the rate of resistance development is currently outpacing the rate at which novel therapies are brought to the clinic. There is a dearth of new antibiotics, especially those that work via mechanisms different than existing antibacterials.[11] Because of the low success rate in developing a target-based lead into a drug with antibacterial potency, generally a phenotypic screening approach is chosen in antibacterial research. The odds of finding a new chemotype with orthogonal antibacterial activity to existing antibiotics is largely dependent on the chemical diversity of the library and its size.

In the research described in this thesis, a relatively small library was chosen when comparing it to those used in high-throughput screening (HTS) campaigns. However, chemical orthogonality was assured due to the tested scaffolds being, for the most part, untested for antibacterial activity. Looking at the success of this small-scale effort, it seems very likely that there is a myriad of novel antibacterial compounds in libraries remaining to be discovered.

Phenotypic screening has been the primary source of new antibiotics since they were first discovered, but there is reason to believe that this might change in the future. More insight has been gained into the structural elements that facilitate bacterial membrane permeability, as well as the requirements to evade eviction by efflux mechanisms.[12–14] With enough knowledge, this should enable compounds to be more easily optimized for antibacterial potency. Recent examples of rational design show that this is a feasible method.[15,16] On the other hand, advances and improvements in computational models of bacterial targets with potential ligands will more accurately predict potent binders. This might then either lead to sufficiently strong inhibitors of known target sites, or the discovery of new druggable sites.

The future of oxadiazolone-based antibiotics. The antibacterial potency of oxadiazolone **3** is on par with the drugs in clinical use,[17] and this is also the case against multidrug resistant clinical isolates. Also, stable resistance was hard to induce, as a month of consistent culturing was necessary to generate highly resistant strains, of which the resistance stability remains to be tested.

The next step will be to investigate if these oxadiazolone compounds are active against biofilms, which consist of phenotypically diverse MRSA cells suspended in a distinct extracellular matrix.[18] Further efforts will be aimed at optimizing the performance of the oxadiazolones in human cells. It was found that addition of serum or BSA removed the activity of compound **3**, likely due to protein binding effects.[19] Compound **3** had a favorable toxicity profile in human liver and kidney cells, *in vivo* toxicity studies will have to be performed. Especially as the oxadiazolones have been shown to covalently bind human lipases.[20,21]

An interesting observation was that the series of oxadiazolones synthesized and tested was consistently more potent against the MRSA USA300 strain than the methicillin-susceptible *S. aureus* (MSSA) 29213 strain. Some of the compounds were completely inactive against the MSSA strain, while still showing high antibacterial activity against MRSA. As the genomes are highly comparable, it would make sense to investigate the differences in active proteome by the competitive ABPP method outlined in Chapter 4. If the MSSA-inactive compounds inhibit a protein significantly more in MRSA than in MSSA, then this protein can be highlighted a target contributing to the antibacterial activity.

Considering the multiple targets of compound **3**, it will likely be difficult to optimize for all the targets at the same time. Therefore, a single target-based approach to improve the potency of the compounds might not be effective. If a single target had to be chosen for further optimization, FabH is a clear candidate, as it is the only protein that is truly essential for MRSA growth *in vitro*,[22,23] and it has been reported as an antibiotic target.[24,25] There are doubts, however, about the clinical relevancy of FabH inhibition.[26] The fatty acids that FabH produces can, in some cases, be supplemented via the exogenous environment.[25]

The other proteins of which activity was significantly inhibited by compound **3** included AdhE, a large protein essential for anaerobic metabolism.[27–29] AdhE is a widely characterized bacterial protein, and has distant homologs in human cells, which are the aldehyde and alcohol dehydrogenases.[30] As AdhE contains two active sites that contain both of these functions via a catalytic cysteine, the site that our molecules target is still unknown. Furthermore, it will be interesting to study the effect of oxadiazolone antibacterials on anaerobically growing MRSA. The oxadiazolone probe that was developed can be potentially used to study activity levels of AdhE in MRSA under different conditions.

The oxadiazolones target a large part of the fluorophosphonate-binding hydrolases (Fph's), of which most do not have a clear function yet. They are not essential for growth, however FphB was found to be essential in *S. aureus* infections.[31] Further study of FphC and FphE is particularly interesting, as inhibition of FphC was correlated with antibacterial activity via competitive ABPP experiments, and FphC and FphE transposon mutants were able to

potentiate inactive oxadiazolones. Also, significant FphE upregulation was observed in oxadiazolone-resistant mutant strains combined with a reduced activity level. Now these Fph's are linked to antibacterial activity, there is more incentive to study these proteins.

The future of isoquinoline sulfonamide-based antibiotics. New antibiotics that are active against Gram-negative bacteria are scarce, as seen from the fact that no new class of Gram-negative antibacterials has been approved for clinical use in the 21st century.[32] This makes the discovery of the isoquinoline sulfonamides as a novel class of Gram-negative antibiotics particularly interesting. It was found that these compounds suffer potency loss because of the Gram-negative outer membrane (OM), as adding OM permeating agents, or choosing an OM deficient *E. coli* mutant, improves the potency of these compound series tenfold. In future studies, structures will be optimized based on OM permeability.

LEI-800, as well as several of its analogues, have been shown to bind to the DNA gyrase complex. DNA gyrase is one of the most thoroughly investigated antibiotic targets. It is the main target of the fluoroquinolone drugs, arguably the most successful antibiotic class beside the cephalosporins (Table S1.1), and 2 out of 4 current clinical candidates with a unique MoA are targetting the DNA gyrase complex.[33,34] Furthermore, there are many natural antibacterial products reported with gyrase activity, of which the aminocoumarins are the most noted.[35,36] DNA gyrase, thus, is a high-profile antibiotic target, for which multiple reasons can be outlined. 1) DNA gyrase is an essential protein for normal bacterial physiology. Removing its activity results in inability to properly supercoil DNA and to regulate DNA topology and DNA replication. 2) DNA gyrase lacks a clear human homologue. DNA gyrase introduces negative supercoils in DNA, a process that is not mimicked by any mammalian enzyme. 3) DNA gyrase is a large multimeric complex with many druggable sites. DNA gyrase consists of two GyrA subunits and two GyrB subunits that work together to fold DNA in the correct way, to introduce an ATP-binding site, and to form a DNA cleavage site. This is facilitated by many strict structural requirements, of which disruption can lead to inactivity.

LEI-800 is unique in its binding pose in comparison to other DNA gyrase inhibitors. Unlike other gyrase inhibitors, LEI-800 does not seem to have interactions with the DNA, but only with the gyrase structure itself. As is the case with all other antibiotics that target DNA gyrase, resistance formation was observed. To reduce the frequency of resistance, efforts can be made in optimizing the compound to also inhibit topoisomerase IV (Topo IV), the second type II topoisomerase in bacteria.[37] This target has high homology to DNA gyrase based on amino acid sequence, but has distinct functions. Fluoroquinolones and the clinical candidates zoliflodacin and gepotidacin all inhibit both DNA gyrase and Topo IV.

Preliminary *in vivo* studies make clear that LEI-800 will have to be optimized for use in infection models. LEI-800 was shown to have high serum binding, which resulted in removal of antibacterial activity. In the mouse studies performed, the pharmacokinetic properties of the compound were clearly an issue. The main problem observed in mice models is that LEI-800 is rapidly cleared from the blood ($t_{1/2} = 0.244 \pm 0.089$ h, n = 3). Furthermore, when the efficacy of LEI-800 to reduce the bacterial burden of *E. coli* in a mouse thigh infection model

was studied, there was no significant decrease observed compared to the vehicle control. Notably, intravenous (IV) administration of LEI-800 was tolerated up to 50 mg/kg, indicating that toxicity is not a direct issue. In conclusion, the pharmacokinetic parameters LEI-800 should be improved if the isoquinoline sulfonamides were to be further developed for potential clinical use.

Closing thoughts. The prospect of a post-antibiotic era, where bacterial infections are unable to be treated, is worrying. If AMR continues to grow at a similar rate as the last few decades, an antibiotic crisis is an unavoidable scenario. There are, however, exciting movements in the field of antibacterial research. The two projects outlined in this thesis are a significant addition to the ongoing medicinal chemistry efforts. The urgency of the antibiotic problem has been clearly communicated in the last decade, exemplified by reports from the Pew Charitable Trusts and the WHO,[38,39] and, as a result, research efforts have shifted towards antibacterial research.

The 21st century has seen a considerable development in fields of research such as structural biology, artificial intelligence, computational chemistry and automated chemical synthesis, of which the utility to antibiotic drug discovery is clear. However, large steps will still need to be taken to reach a stage where AMR is a problem of the past. Continued effort from scientists is required in order to combat AMR and contain its growth rate. The incentive for sufficient research effort is highly dependent on the regulatory and financial obstacles that are specific to antibiotic drug discovery and development.[40]

Besides classical antibiotics, there have been other developments in the field that will help to stunt the growth of AMR. Recent years have seen progress in other types of antibacterial strategies, including: virulence attenuation,[41] combination therapies,[42] bacteriophages,[43] vaccines,[44] and monoclonal antibodies.[45] It will have to be seen how well these different strategies will work at a practical level, as there are few clinically approved examples. These examples bode promising alternatives to the small molecule antibiotics. However, this thesis has shown that there is great potential for small molecule libraries to be mined for antibacterial potency. The Gram-negative antibiotic LEI-800 is particularly exciting, as there have been no new clinically approved classes of Gram-negative antibiotics in the past decades. Further development of LEI-800 will proceed with mechanistic studies of its DNA gyrase interactions, and this will lay the groundwork for further structure optimization studies.

To conclude, this thesis has elaborated on the challenges in antibiotic drug discovery, and has outlined examples of this process via two main projects. These projects were initiated via a phenotypic screen of a unique in-house compound library, which resulted in two clear hits. Via SAR studies, and target identification methods, these hits were developed into lead molecules, of which targets responsible for antibacterial activity have been revealed. Further efforts will have to determine the clinical potential of these compounds, and studies are currently ongoing. Regardless of future clinical successes, this research has expanded the knowledge in the field of antibiotic research, and has provided new tools to study highly pathogenic bacteria.

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