Review

Antibiotic Discovery in the Age of Structural Biology – A Comprehensive Overview with Special Reference to Development of Drugs for the Treatment of *Pseudomonas aeruginosa* Infection

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Abstract. *Due to the persistence and spread of antibiotic resistance, the discovery and exploitation of new antibiotic targets should be the subject of intensive research. Effective strategies are required to develop antibiotic alternatives. Antibiotics that act on new targets or via novel mechanisms have the greatest likelihood of overcoming resistance. In particular, there is a lack of specific antibiotics for Pseudomonas aeruginosa, one of the leading causes of healthcare-associated infections, exhibiting high resistance levels. Herein we describe how structure-based drug design can be used to achieve new antibiotics for the treatment of Pseudomonas aeruginosa infection, using an essential enzyme of the fatty acid synthesis pathway from P. aeruginosa as an example.*

Infectious diseases are of major concern in head and neck medicine. The sources of infection are manifold, *e.g.* injuries of the skin (1), bacteriaemia (2), or odontogenic (3, 4). Furthermore, surgical procedures in the head and neck region, such as tracheostomy, almost inevitably pave the way for infections of the airways (5). Bone reconstruction of the facial skeleton are at risk of becoming infected and subsequently destroyed (6). Furthermore, oral microbes appear to be a risk factor for general diseases such as cardiovascular disease (7). These risks of head and neck

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infections and infectious diseases are due to the unavoidable exposure of the tissues to environmental microbes entering the upper aerodigestive tract and contaminating the skin and mucosa. Whereas *Streptococcus* and *Staphylococcus species* are accepted as the major pathogens of the head and neck region, problematic germs such as *Pseudomonas* spp. are increasingly coming into the focus of antimicrobial therapy for head and neck infections (8, 9). Physicians and microbiologists have long recognized the ever increasing resistance of *Pseudomonas* spp. to antibiotics in the treatment of oral and maxillofacial infections (10). Improvement of antibiotic treatment in these frequently multi-resistant mixed infections is difficult to achieve (11). Furthermore, several drugs appear to facilitate certain bacteria such as *Pseudomonas* spp. to adhere to bone constituents (12). These bacteria are subsequently able to disintegrate the bone (13). In addition, the interaction of these problematic species with locally resident wires is poorly understood (14).

However, since their discovery in the early 20th century, antibiotics have contributed to a significant decrease of mortality from infectious diseases, which has led to a sharp rise in life expectancy (15). On the other hand, the overuse and abuse of antibiotics in developed countries, combined with improper antibiotic treatments on a global scale, has led to the rapid emergence and spread of antibiotic resistance amongst bacteria (16). Especially important human and animal pathogens have become resistant within a few years of the introduction of a new antibiotic (Figure 1A) (17, 18). This rapid development of antibiotic resistance poses severe healthcare and economic problems (17). At the same time, pharmaceutical companies have found it unprofitable to pursue the development of new antibiotics, either as chemical derivatives of existing compound classes or as novel antibiotic
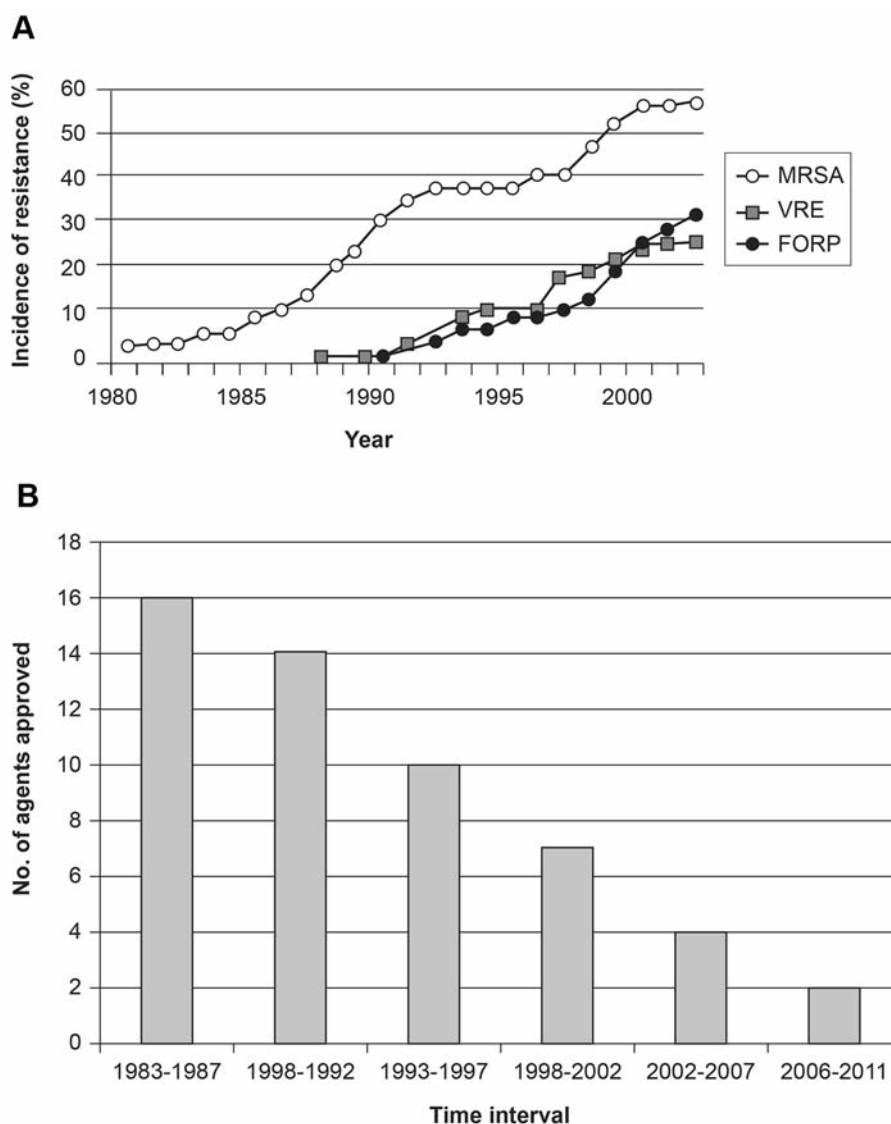
Figure 1. *A: Incidence of antibiotic resistance in three common major infections: methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant Enterococcus (VRE) and fluoroquinolone-resistant Pseudomonas aeruginosa (FQRP). B: The number of new antibacterial agents approved for clinical use in the USA (Food and Drug Administration-approved) in five-year intervals since 1983. Both graphs adapted from (11).*

classes (Figure 1B). Intensive care units are faced with the fast occurrence and spread of resistant strains. Healthcare-associated infections, also known as nosocomial infections, are infections acquired by patients during the course of their receiving treatment in a hospital or healthcare service unit. Bacteria with high resistance rates are *Staphylococcus aureus* (methicillin-resistant, MRSA), *Enterococcus faecium* (vancomycin-resistant, VRE), *Streptococcus pneumonia*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Escherichia coli* and *Pseudomonas aeruginosa* (19). This combination of factors has led to antibiotic resistance becoming a major threat to public health.

Strategies for antibiotic discovery have been refined over the years. The most common antibiotic classes used today were found in the middle of the last century as natural products. Since these antibiotic classes employ a relatively small set of chemical scaffolds, they have been modified over the past decades by synthetic tailoring to combat resistance (16). Today antibiotic discovery is generally based on two methods: discovery of compounds from natural sources, especially from fungi and the soil bacteria *Actinomyces*, and the development of synthetic molecules (20). The main antibiotic classes affect a limited range of bacterial physiology: cell wall biosynthesis, cell membranes,
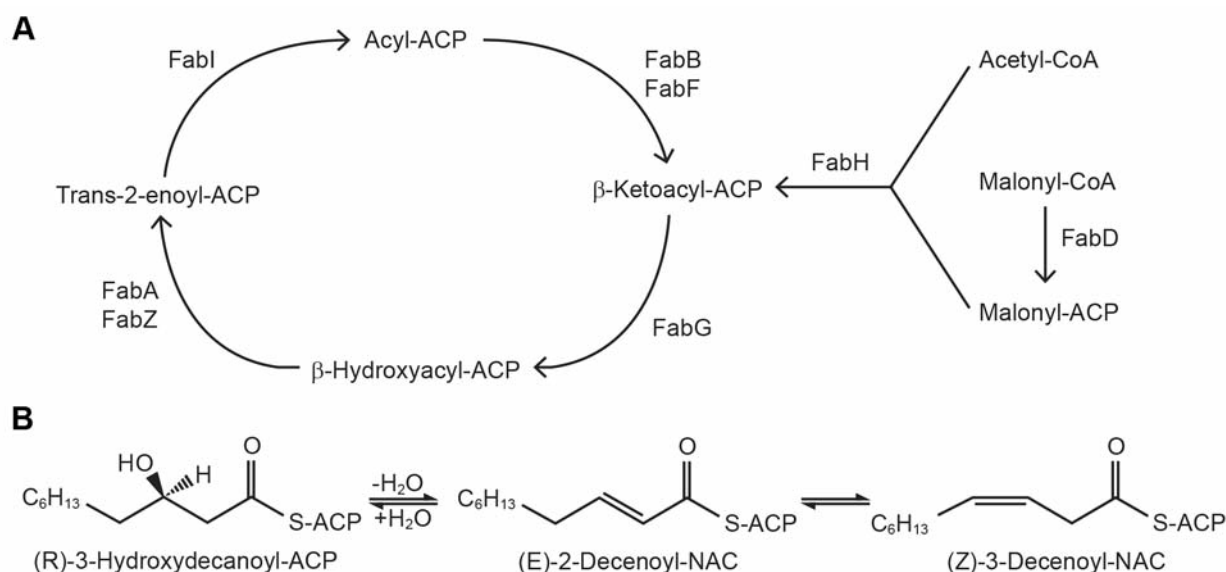
Figure 2. *A: Enzymes of fatty acid elongation in Escherichia coli (35). The production of fatty acids in bacteria is catalyzed by a series of proteins, encoded by separate genes, which carry out the individual enzymatic steps. B: Catalytic reactions of FabA (37): biological reactions of dehydration and isomerization.*

type II topoisomerases, ribosomes, transcription and folate biosynthesis (21, 22). Bacterial resistance mechanisms are caused by reduced accumulation of the drug, associated with decreased permeability of the bacterial membrane or increased efflux of the drug; modification (inactivation) of the drug or mutations in bacterial target proteins (23).

P. aeruginosa is a rod-shaped, Gram-negative, none-spore forming bacterium, which belongs to the class of γ-proteobacteria (24-26). In one survey, *P. aeruginosa* was the second most common cause of infections in intensive care units, causing 30% of pneumonia cases, 19% of urinary tract infections and 10% of bloodstream infections in Europe (27). Clinical isolates of *P. aeruginosa* have been shown to be resistant to a variety of commonly used antibiotics (β-lactams, fluoroquinolones and aminoglycosides) (28, 29). Resistance mechanisms include low permeability of the outer membrane, production of antibiotic-inactivating enzymes and the constitutive expression of several multidrug efflux pumps (30). As a consequence, there is a significant interest in generating new antibiotics for new targets in *P. aeruginosa*.

Especially attractive targets for antibiotic research are proteins essential for bacterial survival, which are identified and then characterized structurally and biochemically. In the case of enzymes as potential targets, the mechanism(s) of catalysis are analyzed in detail. This newfound knowledge is then combined with atomic resolution structural data (derived from experiments using X-ray crystallography or nuclear magnetic resonance, NMR) to find inhibitors of the enzyme (*e.g.* competitive or allosteric). Structure elucidation of the target molecule is the starting point of structure-based drug design, which holds promise of giving detailed insight into the mode of action of potential new drugs and efficient de-novo drug design.

To hold potential as a target protein, a protein should not only be essential to bacterial survival but also not be replaceable by another enzyme from the same or similar pathway. This would lead to an easy circumvention of the newly-developed drug. An indispensable metabolic pathway is that of fatty acid metabolism. Fatty acids are essential for all living organisms. They are stored as triacylglycerol through an ester linkage to glycerol. Consequently, they are an important source of energy in the cell. Fatty acids are important components of biological membranes, forming the building blocks for phospholipids and glycolipids. Moreover, they can modify many proteins by their covalent attachment. Fatty acid derivatives serve as intracellular messengers and hormones (31). Generally, two related but distinct biosynthetic systems synthesize fatty acids. Type I fatty acid synthase (FAS I) is located in the mammalian cytosol and in fungi. In humans, the synthesis of fatty acids primarily takes place in the cytoplasm of adipose and liver tissue, as well as in the central nervous system and lactating mammary gland (31). Type II fatty acid synthase (FAS II) is found in plants, bacteria and other microorganisms, and is especially involved in membrane formation. This synthesis is carried out by a series of discrete enzymes (Figure 2A) (32, 33). Although the reactions catalyzed by FAS I and II are the same, there are important structural differences between the enzymes (34).

In bacteria, one of these enzymes is the β-hydroxyacyl-Acyl-Carrier-Protein (ACP) dehydratase FabA (35), where FabA is one of nine subunits of *Escherichia coli* FAS (36). FabA carries-out the dehydration and the isomerization of *trans*-2- to *cis*-3-decenoyl-ACP and thereby controls the divergence of the metabolic pathways of saturated and unsaturated fatty acid synthesis (Figure 2B) (35, 37). Without FabA, bacteria would not be able to synthesize unsaturated fatty acids themselves and studies have shown that mutational inactivation of the *fab*A gene results in an unsaturated fatty acid auxotroph phenotype (38, 39).

FabA, therefore, fulfilled the above-mentioned requirements for a potential drug target and we decided to investigate further. The first step was the heterologous production of large quantities of highly pure enzyme. In the case of FabA, activity assays had long been established for *E. coli* FabA by other groups (40) and the assay of Schwab *et al.* (40) was used to study activity. The structure of wild-type FabA in *P. aeruginosa* (PaFabA) was determined by Moynié *et al.* in 2013 (41). A good way to inhibit an enzyme is to use a competitive inhibitor, which binds the active site preferential to the natural substrate. To rationalize the design such of an inhibitor, a detailed understanding of how the substrate binds to the active site of the enzyme is required. One way to better-understand the interactions involved is to crystallize the enzyme in the presence of its substrate and determine the atomic-resolution structure of the complex. Since most enzymes turn-over substrates quickly and crystal growth is slow, an inactive version of the enzyme or a non-processable substrate analog are required. In the case of a mutant enzyme, changes to the protein should be conservative and minimal so as to allow it to bind substrates as the wild-type enzyme does but be unable to turn it into product. The residues of the active site of FabA were predicted to be histidine 70 (H70) and aspartate 84 (D84) and conserved enzyme mutants were produced (H70N, H70Q, D84N, and H70N/D84N; N=asparagine, Q=glutamine) to probe the catalytic site of the protein.

To validate the prediction of the active site residue, biochemical essays were performed using in-house-synthesized substrate analogs 3-hydroxydecanoyl-*N*-acetylcysteamine and (E)-2-decenoyl-*N*-acetylcysteamine. In order to define the activity of the wild-type protein and mutants, activity assays were performed using UV spectrophotometry. While the mutant proteins were inactive, which corroborated our predictions, we wanted to investigate if the mutations had an effect on overall stability of the protein. If the mutant protein was inherently unstable it might still be competent to perform catalysis but simply be misfolded. In such a case, our mutations would have to be altered to obtain relevant biochemical and structural data. An easy way to test protein stability is by a fluorescence-based thermal shift assay. In such an assay, the protein of interest is mixed with a dye which only fluoresces when the protein is unfolded. The protein dye mix is then subjected to a temperature gradient and the melting temperature of the protein (the temperature at which it denatures) is recorded. Such an assay was subsequently used to determine the thermal stability of the mutants compared to wild-type FabA of *P. aeruginosa* of to investigate if the mutations had an impact on overall protein stability, which was only slightly affected (Figure 3C). After confirmation that the mutant enzymes were stable but unable to perform catalysis, they were used with the substrate analogs in crystallization experiments. The complex structure of *P. aeruginosa* FabAH70N and 3-hydroxy-decanoyl-NAC were solved and provided the desired and detailed insight into FabA catalysis in *P. aeruginosa* (Figures 3A and B) (41).

At this point, we could have chosen the path of purely rational inhibitor design – designing potential new antibiotic lead chemicals based on the observed interactions between enzyme and substrate. We however chose to screen a library of small chemical compounds for interactions with *P. aeruginosa* FabA by NMR to find initial lead structures (Figure 3D). Libraries of small molecules are commercially available and standard tools of the pharmaceutical industry. The small molecules give a starting point around which to build a more complex and specific molecule. Here, a variety of NMR techniques were used to identify 'hits'. Hits are small molecules interacting with the enzyme, which is a prerequisite for a potential target-specific drug in general, and antibiotic in this case. The identified compounds would then be used for further crystallization trials to understand how and where the small molecule bound the enzyme. The structures of *P. aeruginosa* FabA with four small molecules, which were hits in NMR screening, were determined (41). The three different types of FabA structures originating from this study can be used for comparison to direct further steps. The structure of native FabA without a binding partner serves as a baseline of the enzyme and can be compared to the structure of mutant enzyme bound with the substrate. Changes resulting from binding of the substrate, as well as the specific interactions between the substrate and enzyme (*e.g.* hydrogen bonds), are important guides for further compound development. The third type of structure, bound with a small molecule (which serves as a starting point for inhibitor design) can then be compared to the enzyme bound with substrates (Figure 4A). It allows for determination of whether the small molecule is a good mimic of the substrate, or if it forces changes in the enzyme that might be undesirable for compound design. The differences in the enzyme in these three states can then be used to tailor the lead compound to give tighter binding variants of the initial hit (Figure 4B). These may then be used as fatty acid biosynthesis inhibitors in further studies, such as anti-bacterial screens, to validate specific antibiotic activity and pharmacokinetics.
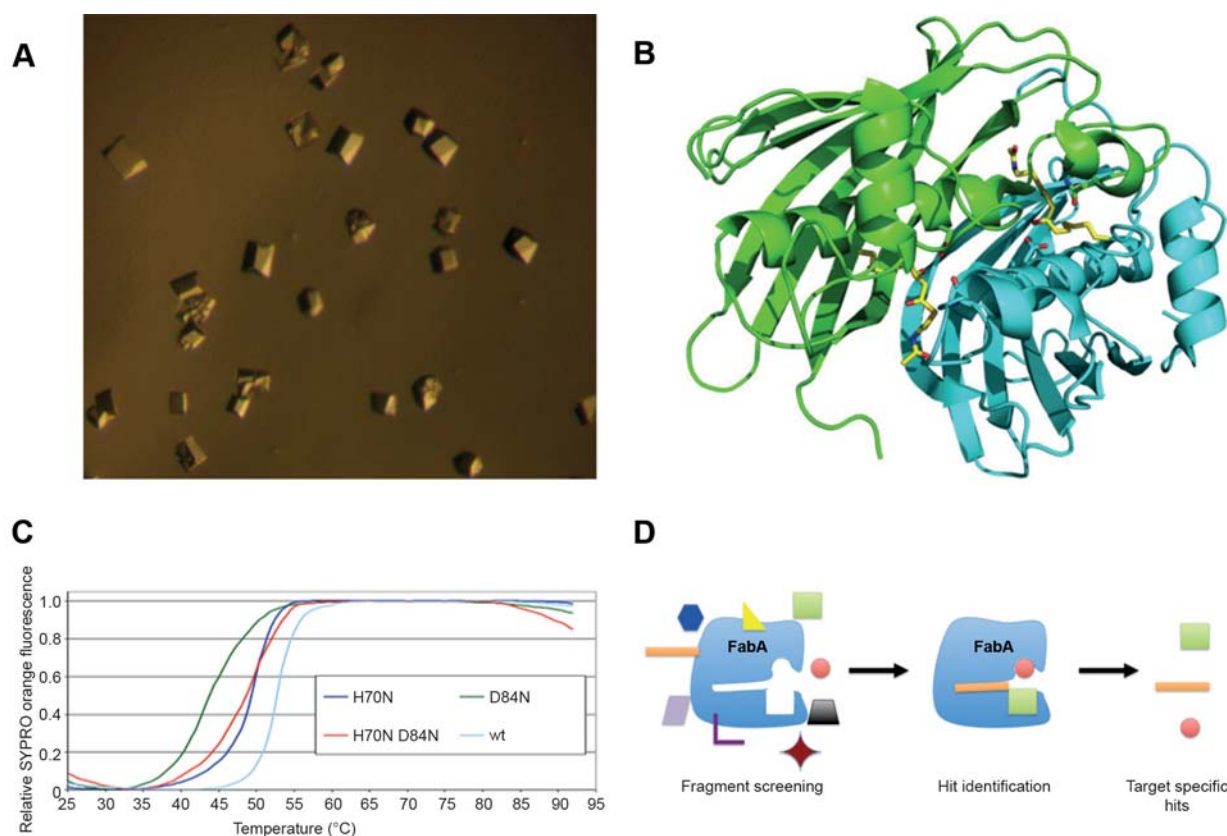
Figure 3. *A: Protein crystals of Pseudomonas aeruginosa FabA mutant H70N with 3-hydroxy-decanoyl-NAC. B: Cartoon representation of the overall structure of the P. aeruginosa FabAH70N dimer, with catalytic residues shown as sticks. Pseudosubstrate 3-hydroxy-decanoyl-NAC is shown as yellow sticks: C: Thermal shift assay results for mutant and wild-type P. aeruginosa FabA. The wild-type PaFabA (cyan) has the highest stability. Mutant D84N (green) is slightly less stable than mutants H70N/D84N (red) and H70N (dark blue). D: Fragment screening by nuclear magnetic resonance. A mixture of small molecules is added to the protein and specific binders (hits) are identified by a variety of NMR techniques. Specific binders are then used as starting points for antibiotic development.*
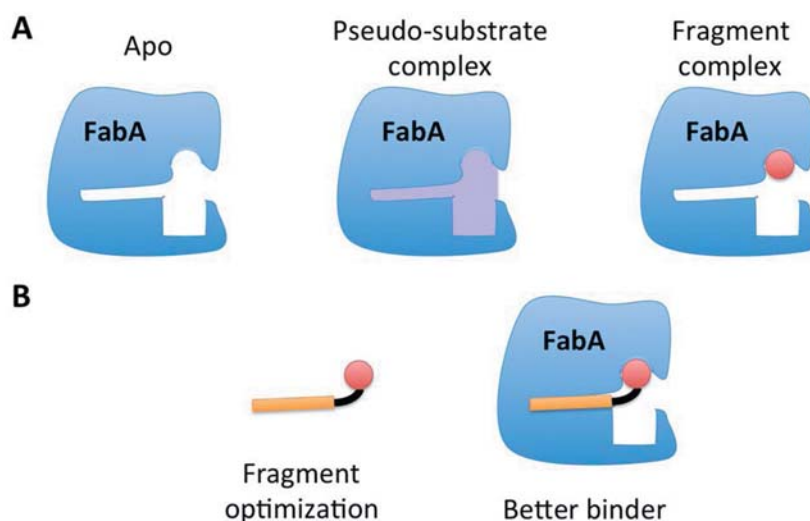

Figure 4. *A: Schematic representation of the three enzyme state structures were determined. Comparison of these structures guides fragment optimization. B: Example of a first compound optimization step. A covalent linkage (black line) between two smaller fragments creates a larger molecule, which may have better properties than either small fragment on its own (e.g. through tighter binding). Apo: Apoenzyme; FabA: Fragment antigen binding A.*

While this process may seem tedious, it is an important step towards the development of new antibiotics, with the option of making rational alterations. Despite the large amount of work required to reach this stage, the compound can prove worthless at any point along the pathway towards clinical application, *e.g.* if its pharmacokinetics are insurmountably poor. The authors are of the opinion that lead compound testing on laboratory strains of *P. aeruginosa* can be misleading, and that clinical isolates of these bacteria are vital to give a more realistic view of a compound's true antibiotic potential.

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