Search and rescue: tackling antibiotic resistance with chemistry

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Chapter 6

Summary

With the rise of multi-drug resistant bacterial pathogens, the possibility of a post-antibiotic world is quickly becoming reality. It is therefore vital that research is focussed on overcoming the most challenging bacterial resistance mechanisms. To this end, the aim of the work described in this thesis was to develop novel strategies to combat resistant bacteria, with particular focus on the threat posed by gram-negative bacteria.

Chapter 1 reviews the overall problem of antibiotic resistance as well as the methods this thesis describes to address them. The natural phenomenon by which bacteria gain resistance to antibiotics has been known since antibiotics were first discovered, however it is only in recent decades that the number of fatalities associated with antimicrobial resistance have risen to significance. This is direct consequence of overuse and misuse of antibiotics, pushing us toward a post-antibiotic era. Several strategies can be used to address these problems, two of which are outlined in this chapter. The first involves recovering the activity of clinically important antibiotics, such as ß-lactams, by developing inhibitors against resistance enzymes which inactivate this class of antibiotic. In addition to this strategy, new antimicrobials can be developed against vital, unexploited bacterial pathways. Examples include the folding pathway for ß-barrel outer membrane proteins and the lipoprotein processing pathway. Both pathways contain essential enzymes, which if inhibited, would be fatal to the bacterial cell. These strategies form the basis of the work undertaken in this thesis to address antimicrobial resistance.

Chapter 2 describes mechanistic investigations that were undertaken to compare a panel of compounds reported as inhibitors of the bacterial resistance enzymes known as metallo-ß-lactamases (MBLs). Arguably one of the most destructive resistance mechanisms, MBLs catalyse the breakdown of ß-lactams, an important class of antibiotics used in most treatments of bacterial infections. MBLs contain active site zinc ions which activate a water molecule to hydrolyse the ß-lactam ring. Imipenemase-1 (IMP-1) and New Delhi metallo-ß-lactamase (NDM) are two clinically devastating members of the MBL family as they can inactivate the carbapenem class of ß-lactams, which are reserved as a last resort treatment.

The panel of compounds selected for evaluation covers a wide variety of structures and their inhibition profiles of MBLs were all reported individually. The work in this chapter aimed to provide a fair and robust comparison of their MBL inhibition activity through a series of mechanistic investigations. For each compound, the half-maximal inhibition constant (IC_{50}) was determined against IMP-1 and NDM-1 in the same biochemical assay. Furthermore, the

binding affinity of each MBL inhibitor for different divalent cations was measured. Finally, their ability to rescue meropenem's activity against carbapenem-resistant bacteria was also assessed. Comparing the results, one MBL inhibitor which stood out from the rest: the indole carboxylate (InC) derivate developed by Schofield and colleagues at Oxford University.[1] Despite showing no appreciable binding to any of the divalent cations tested, **1** was a potent inhibitor of both MBL enzymes tested and effectively re-sensitizes MBL-expressing bacteria to meropenem (**Figure 1**). The work concluded by suggesting that the Oxford compound and analogues thereof may be promising candidates for further drug development efforts aimed at overcoming MBL-expressing pathogens.

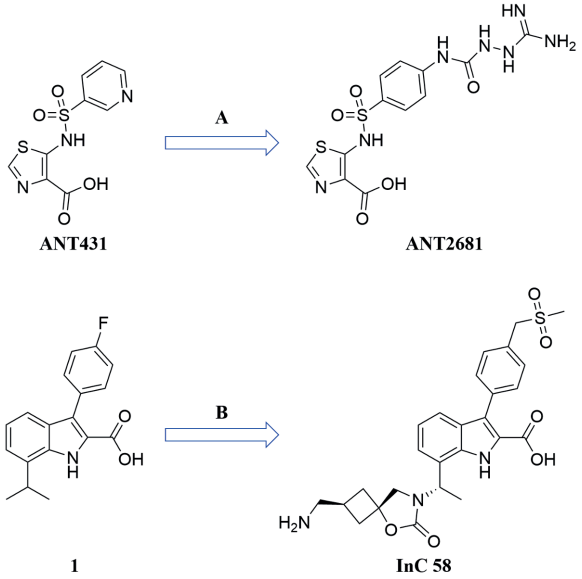

Figure 1. The structures of the two original MBL inhibitors developed as a result of high throughput screen hits which were included in the inhibitor panel of **chapter 2** are displayed on the left. The new lead compounds, displayed on the right, are a result of further structural development to increase the potency and pharmacokinetic properties of the compounds.

Since the publication of the work displayed in **chapter 2**, which concluded that strong zinc binding was not a necessity for MBL inhibition, there have been many interesting developments in the field of MBL inhibitors. The biopharmaceutical company Antabio have completed their lead optimization campaign to produce a preclinical candidate, ANT2681.[2] This compound has improved activity and physiochemical properties from their previous lead compound ANT431, which was included in the MBL inhibitor panel of **chapter 2**.[3] The Oxford

group have also continued to work on their InC derivatives. They have recently published their work on the development of InC inhibitors, and have identified compounds, such as InC 58, with greater potency than compound **1**.[4] They are actively progressing some of the InC derivatives towards clinical trials (**Figure 1**).

Chapter 3 describes the synthesis and evaluation of MRL-494, a ß-barrel assembly machinery (BAM) complex inhibitor originally reported by researchers at Merck. A synthetic route for MRL-494 was not disclosed in the original publication as it was an unintended by-product of a reaction which was isolated and stored for screening purposes.[5] We therefore developed a robust and modular synthetic route to MRL-494 which also allows for the preparation of analogues to probe the structure-activity relationship of the different functional groups present in MRL-494. The two guanidine groups in the structure were systematically replaced with a simple primary amine or amide functional group to remove the permanent positive charge. In doing so, three new analogues were produced, two of which contained one guanidine group and the third lacking both. Minimum inhibitory concentration (MIC) tests were carried out for MRL-494 and the three analogues on several clinically isolated gram-negative bacteria. This showed that only the parent compound, MRL-494, was lethal to bacteria. However, synergy assays with rifampicin indicated that only one guanidine group is necessary for potent synergistic effects. This can be explained by the findings from an outer membrane permeabilization experiment, which showed MRL-494 and single guanidine analogues can also disrupt the outer membrane. Lastly, all compounds were subjected to a stress response assay monitoring the regulation of capsular polysaccharide synthesis (Rcs). This response is particularly sensitive towards impaired functioning of the BAM complex, and evidence shows that MRL-494 induces this particular stress response.

The work reported in **chapter 3** (**Figure 2**) is likely to be of value in future studies aimed at identifying the binding mode of MRL-494 to the BAM complex. The synthetic route we developed to MRL-494 also opens the door for future research to be conducted using the compound. This work also highlights the necessity of both guanidine moieties for antibacterial activity as well as the ability of MRL-494 to activate the Rcs stress response, findings which should help guide further investigations into the mode of action of MRL-494.

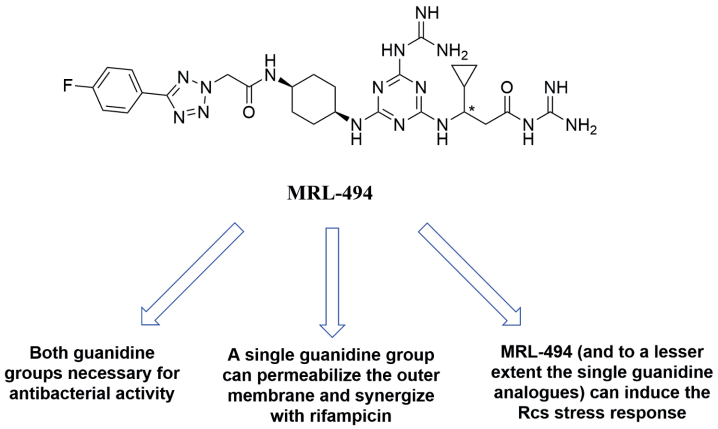

Figure 2. The main findings from **Chapter 3** associated with MRL-494.

Chapter 4 documents the implementation of an assay for monitoring the activity of the bacterial inner membrane protein, LspA, as well as the synthesis of FRET substrate for this enzyme. First described by Olatunji et al.,[6] the FRET assay used to characterize the activity of LspA relies on the principle of Förster resonance energy transfer (FRET) by employing a fluorophore and quencher at the N- and C- terminus of the peptide substrate. If the enzyme is active, the substrate will be cleaved, producing a measurable increase of fluorescence at the fluorophore's emission wavelength. Conversely, if the enzyme is inhibited, no fluorescence at this wavelength will be observed. The original substrate for the FRET assay contained an aminobenzyl (Abz) and 3-nitrotryosine (Tyr(3-NO_2)) FRET pair, which has excitation and emission wavelengths of 320 nm and 420 nm respectively. The work included in this chapter builds upon the original assay by producing a new substrate, utilising the EDANS/DABSYL FRET pair with excitation and emission wavelengths of 360 nm and 490 nm respectively (**Figure 3**). Validation studies revealed both the original and new FRET substrate to be recognized by LspA after which both substrates were then employed in determining the IC_{50} of a known pan-aspartyl protease inhibitor, pepstatin A. Also of note, access to these two distinct FRET substrates is expected to be of value in the screening of microbial extracts for novel LspA inhibitors, as it is possible that some extracts may contain compounds which interfere with the fluorescence readout of one or the other FRET substrates.

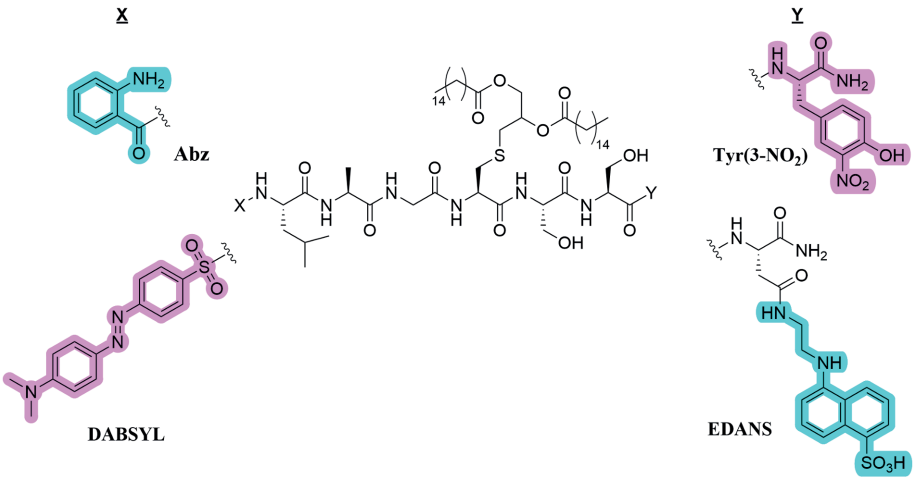

Figure 3. A depiction of the LspA substrates synthesised in **chapter 4**. The 6-mer amino acid core is flanked by a fluorophore and quencher pair. Fluorophore components are highlighted in blue, and the quenchers are highlighted in pink.

Chapter 5 describes the use of the FRET assay described in **chapter 4** to characterise the inhibition activity of a panel of novel peptidomimetic LspA inhibitors developed in our group. The compounds were designed to mimic the natural substrate of LspA, which plays a vital role in the lipoprotein processing pathway. The peptidomimetics contain the same amino acids on either side of the cleavage site found in the natural substrate, along with non-cleavable moieties at the cleavage site. These mimetics should block the active site of LspA for the natural substrate, causing an inhibition of the enzyme's function which in turn is lethal to gram-negative bacteria. Many non-cleavable motifs have been used in developing inhibitors of aspartyl protease enzymes, several of which were chosen for our initial investigations. The reduced amide, difluoroalcohol, hydroxymethylcarbonyl (HMC), and statin motifs were incorporated into the peptide sequence, replacing the glycine residue found at the site of cleavage. In addition, a simpler hydrophobic moiety (an octyl chain or benzyl group) was appended to the side chain of the Cys residue found in the LspA substrate in place of the more complex diacylglycerol group. An initial single concentration experiment was carried out to determine if any of the peptidomimetics could inhibit LspA and percentage inhibition was calculated in relation to the known LspA inhibitor, globomycin. Most compounds moderately inhibited LspA, but the stand-out result came from compound **2** which contained the HMC non-cleavable motif and the octyl chain hydrophobic moiety (**Figure 4**). Compound **2** showed

high inhibition of LspA at the single concentration tested and so a full half-maximal inhibitory concentration (IC_{50}) experiment was performed. The IC_{50} value of **2** against LspA was determined to be 181 μM, making it the best candidate for further modification to further improve the inhibition activity against LspA as well as improving pharmacokinetic properties.

It is expected that the work described in **chapters 4** and **5** will help lead to the development of an entirely new class of antibiotics, aspartyl protease inhibitors. Using the FRET assay described in **chapter 4**, microbial extract libraries can also be screened for natural products which can inhibit LspA activity. From **chapter 5**, peptidomimetic **2** is a candidate for further optimization and exploration for example by truncation or alanine/D-amino acid scans to improve the inhibition profile. Other modifications, such as swapping out the octyl chain for other also chains or hydrophobic moieties may also improve inhibition (**Figure 4**). This would lead to a first-in-class peptidomimetic inhibitor for the aspartyl protease bacterial enzyme, LspA.

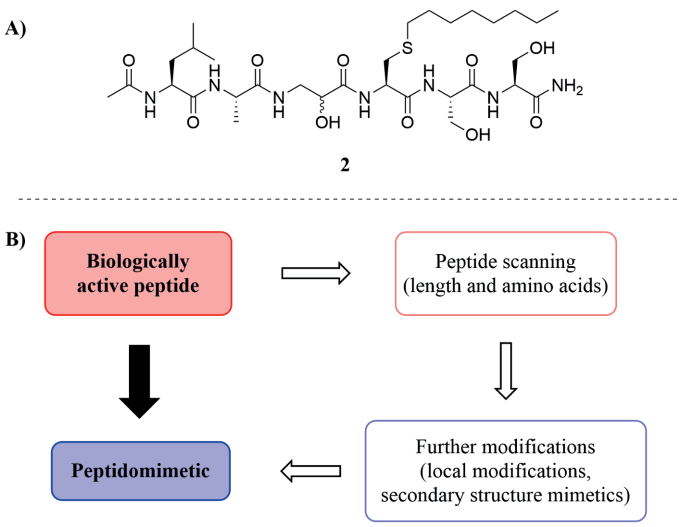

Figure 4. The workflow used to produce a peptidomimetic compound from a biologically active peptide.

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