Caprazamycins: Promising lead structures acting on a novel antibacterial target MraY

Bhautikkumar Patel,^{a,b} Philip Ryan,^{a,b} Vivek Makwana,^{a,b} Matthew Zunk,^{a,b,c} Santosh Rudrawar,^{a,b,c†} Gary Grant^{a,b†}

^a*School of Pharmacy and Pharmacology, Griffith University, Gold Coast, QLD 4222, Australia*

^b*Quality Use of Medicines Network, Gold Coast, QLD 4222, Australia*

^c*Menzies Health Institute Queensland, Griffith University, Gold Coast, QLD 4222, Australia*

[†]Corresponding authors:

1) Dr Santosh Rudrawar

E-mail address: s.rudrawar@griffith.edu.au

2) Dr Gary Grant

E-mail address: g.grant@griffith.edu.au

Abstract: The present status of antibiotic resistant requires an urgent invention of novel agents that act on clinically unexplored antibacterial targets. The enzyme Mray (phospho-MurNac-pentapeptide translocase), essential for bacterial cell wall synthesis, fulfils this criterion as it has not been explored as a target in a clinical context. Specifically, the enzyme is involved in the lipid-linked cycle of peptidoglycan biosynthesis and is reportedly targeted by naturally-occurring nucleoside antibiotics. The antimicrobial ‘caprazamycin’ class of nucleoside antibiotics targets *Mycobacterium tuberculosis* and clinically relevant Gram-negative bacteria such as *Pseudomonas aeruginosa* besides various drug resistant strains and is therefore an eligible starting point for the development of novel agents. In this review, we aim to summarise the structure-activity relationships of the natural, semi-synthetic as well as synthetic analogues of nucleoside antibiotic caprazamycins. This review highlights caprazamycins as promising lead structures for development of potent and selective antimicrobial agents that target Mray, the bacterial enzyme involved in the first membrane-dependent step in bacterial peptidoglycan assembly.

Keywords: Antibacterial; antibiotics; Mray; nucleoside natural product; uridylpeptide; caprazamycins.

Accelerating antibiotic resistance has become a global public-health problem [1-2]. Multidrug resistance is rising to dangerously high levels in all parts of the world, threatening our ability to treat common infections, especially multidrug-resistant Gram-negative bacteria (MDR GNB). Consequently, there is an urgent need for new classes of antibiotics with clinically unexploited modes of action [3]. Bacterial cell-wall peptidoglycan layer biosynthesis is a well-recognised antibacterial target for clinically useful β -lactam (penicillin) and glycopeptide (vancomycin) classes of antibiotics [4], however these well-established antibiotics target the later, lipid-linked extracellular steps in peptidoglycan assembly. The recent advance in the availability of peptidoglycan biosynthetic intermediates generated significant interest towards the earlier, membrane-associated core steps of peptidoglycan formation for the development of antibacterial agents [5]. Bacterial cell wall assembly remains an attractive antibacterial target for drug design [6] and so the integral membrane protein involved in the first step of bacterial peptidoglycan assembly (**Figure 1**), MraY (phospho-*N*-acetylmuramoyl-pentapeptide-transferase [EC:2.7.8.13]), has consequently become a novel drug target for antibacterial agents [7].

The naturally-occurring caprazamycin class of nucleoside antibiotics target the MraY enzyme, unlike antibiotics from those of existing peptidoglycan inhibitors (including penicillins and vancomycin) [8, 9]. This new class of antibiotics targeting MraY are active against MDR GNB [9-10]. Interestingly, candidates from the mureidomycin class of nucleoside antibiotics are active against clinical isolates of β -lactam and quinolone-resistant strains of *P. aeruginosa* [11]. The caprazamycin-derived antibiotic CPZEN-45, exhibits potent antibacterial activity against several multidrug-resistant strains of *Mycobacterium*, including extensively multi-drug-resistant TB (XDR-TB) [12], qualifying it as a promising antitubercular drug candidate [13]. MraY-targeting caprazamycins are therefore speculated to become promising, novel, antibacterial leads effective against drug-resistant bacterial pathogens. Several review articles have enriched the knowledgebase recently, focussing on isolation, biosynthesis, chemical synthesis, modelling and biological activity of nucleoside antibiotics [7, 10, 14-24]. Herein, we highlight the structure and function of naturally occurring caprazamycin and discuss its synthetic derivatives' competencies as broad-spectrum antibacterial agents.

2. MraY –novel antibacterial target

The peptidoglycan cell wall layer is found in both Gram-positive and Gram-negative bacteria. Peptidoglycan biosynthesis is a well-established target for antibacterial action [25] and takes place across three biosynthetic stages; Stage I: peptidoglycan precursor synthesis in the cytosol, Stage II: transportation of cytoplasmic precursor across the membrane to the periplasm, and Stage III:

peptidoglycan cross-linking *via* transpeptidation [26]. The MraY enzyme is a member of the polyprenyl-phosphate *N*-acetyl hexosamine 1-phosphate transferase (PNPT) superfamily [27-28]. The enzymes' role in bacterial peptidoglycan assembly is to transfer the UDP-*N*-acetylmuramoyl (UDP-MurNAc) pentapeptide (-L-Ala₁-D-γ-Glu₂-Lys/DAP₃-D-Ala₄-D-Ala₅-COOH) (Park's nucleotide) to the membrane-soluble C₅₅ isoprenoid carrier lipid known as bactoprenol-phosphate (bactoprenol-P), resulting in the formation of lipid I (undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide) [27]. The MurNAc-pentapeptide is subsequently glycosylated with an *N*-acetylglucosamine (GlcNAc) residue forming lipid II, which is then flipped to the outer surface of the cytoplasmic membrane before being further elongated *via* cross-linking (**Figure 1**). The catalytic activity of MraY is an essential prerequisite for the transfer of peptidoglycan synthesis building blocks to the outer surface of the membrane. As such, MraY is often labelled a translocase. Gene knockout experiments in *Escherichia coli* and *Streptococcus pneumoniae* identified the involvement of MraY in the second stage of bacterial cell wall biosynthesis as an essential process for bacterial cell viability and growth [29-30]. Given the critical role of the peptidoglycan layer in the survival of bacteria, potent disruption of its formation *via* inhibition of essential enzymes like MraY offers a novel antibacterial strategy [31]. Furthermore, as MraY enzyme is not present in eukaryotes, selective inhibitors should be free from mechanism-based toxicity, qualifying it as a promising target for development of next generation antibiotics [7].

Figure 1. Peptidoglycan biosynthesis pathway, showing the site of action of the natural product nucleoside antibiotic-caprazamycins [34]

3. Natural product nucleoside antibiotics – *MraY* inhibitors

MraY is the target of many of nucleoside natural product antibiotics including liposidomycins, caprazamycins, capuramycins, muraymycins and uridylpeptides [10, 14, 19]. It is believed that these natural products compete with UDP-MurNAc-pentapeptide as *MraY* substrates [32]. Naturally occurring *MraY* inhibitors exhibit a wide range of biological actions, specifically antibacterial, antifungal, antiviral, insecticidal, immunostimulative, immunosuppressive and antitumor activities [15]. Fortunately, the availability of X-ray crystal structures of *MraY*-inhibitor complexes [apo (PDB: 5JNQ) (*MraY* from *Aquifex aeolicus*), tunicamycin- (PDB: 6BW5) (*MraY* from *Clostridium bolteae*), and muraymycin-bound (PDB: 5CKR) (*MraY* from *Aquifex aeolicus*)] have provided significant insight into the structural prerequisites for selective inhibition of *MraY* [27, 33-35]. As such, there now exists an exciting opportunity for structure-based design and these natural products provide a promising starting point for the discovery of next generation antibacterial agents with increased potency [10, 14, 19].

Antibacterial nucleoside antibiotics can be further divided into four subfamilies based on their chemical structures: *i*) ribosamino-uridines; *ii*) tunicamycins; *iii*) uridylpeptides and *iv*) capuramycins (**SI Figure 1**) [19]. Members of these families are often referred to as uridine-based nucleosides due to their shared structural scaffold. Caprazamycins, like other ribosamino-uridines (i.e. muraymycins and liposidomycins), share an identical aminoglycosylated 5'-*C*-glycyluridine core, making it a common structural motif within the class [19, 36].

Members of the caprazamycin (**CPZ**, **1**) class were first reported in 2003 by Igarashi *et al.* via the isolation of caprazamycin B (**CPZ-B**, **2**) from the cultured broth of the *Streptomyces* sp. MK730-62F2 [37]. These compounds have similar structures to liposidomycins though they differ in the 2"-sulfate group of the aminoribose and bear a permethylated L-rhamnoside β -glycosidically linked to the 3-methylglutaryl moiety (**Figure 3**) [21]. Various analogues of naturally occurring caprazamycins A-G (**2-8**) were isolated from a common source and were characterized in 2005 by Igarashi *et al* [38]. They were found to have very similar chemical functionalities, yet differed in the length of their lipophilic side chains. The caprazamycin structure can be divided into four structural subunits: uridine (*subunit A*), 5"-aminoribose (*subunit B*), diazepanone ring (*subunit C*) and variable fatty acyl side chain (*subunit D*). Caprazol was characterized through X-ray crystallographic study to reveal the presence of the key subunits **A**, **B** and **C** [38]. The caprazamycins 'western zone' consists of an L-rhamnoside attached to the fatty acyl side chain (*subunits D*) via a β -methylglutarylether [39] (see Figure 2).

Figure 2: Structural features of caprazamycin

4. Structure-activity relationships

In this section, we analyse key pharmacophores within the structures of bioactive caprazamycins and summarise critical structural elements required for translating their potent antibacterial activity across to novel, rationally designed analogues.

4.1. Natural product caprazamycins A-G

The natural analogues of caprazamycins A-G (**2-8**) have displayed an excellent anti-mycobacterial profile *in vitro* against both drug-susceptible and multidrug-resistance *Mycobacterium tuberculosis* strains without any observation of significant toxicity against mouse models (**Table 1**) [37, 40].

Table 1: Chemical structures of naturally occurring caprazamycins and their antimicrobial spectrum

Caprazamycin	R	Antimicrobial spectrum
3 CPZ-A		<i>M. smegmatis</i> (MIC = 0.78-1.56 µg/mL) ^[63]
		<i>M. phlei</i> (MIC = 1.56 µg/mL) ^[63]
		<i>M. vaccae</i> (MIC = 0.2 µg/mL) ^[63]
		<i>M. fortuitum</i> (MIC = 6.25 µg/mL) ^[63]
		<i>M. tuberculosis</i> (MIC = 1.56 µg/mL) ^[58, 63]
		<i>M. avium</i> (MIC < 0.025 µg/mg) ^[63]
		<i>M. intracellulare</i> (MIC = 0.78 µg/mg) ^[63]

147			<i>M. smegmatis</i> (MIC = 1.56-3.13 µg/mL) ^[63]
148			<i>M. tuberculosis</i> (MIC = 3.13-12.5 µg/mL, 1.56 µg/mg) ^[58, 63]
149			<i>M. avium</i> (MIC = 6.25-50 µg/mL, < 0.025 µg/mg) ^[58]
150			<i>M. intracellulare</i> (MIC = 1.56-25 µg/mL, = 0.78 µg/mg) ^[58]
151	2	CPZ-B	<i>M. bovis</i> (MIC = 3.13 µg/mL) ^[58]
152			<i>M. phlei</i> (MIC = 3.13 µg/mL) ^[63]
153			<i>M. vaccae</i> (MIC = 0.39 µg/mL) ^[63]
154			<i>M. fortuitum</i> (MIC = 50 µg/mL) ^[63]
155			<i>S. aureus</i> (MIC = 1.56-6.25 µg/mL) ^[63]
156			<i>M. luteus</i> (MIC = 3.13 µg/mL) ^[63]
157			<i>B. anthracis</i> (MIC = 0.78 µg/mL) ^[63]
158			<i>B. subtilis</i> (MIC = 6.25-12.5 µg/mL) ^[63]
159			<i>B. cereus</i> (MIC = 3.13 µg/mL) ^[63]
160			<i>C. bovis</i> (MIC = 3.13 µg/mL) ^[63]
161			<i>E. coli</i> (MIC = 100 µg/mL) ^[63]
162			<i>M. smegmatis</i> (MIC = 0.78-1.56 µg/mL) ^[63]
163			<i>M. phlei</i> (MIC = 1.56 µg/mL) ^[63]
164			<i>M. vaccae</i> (MIC = 0.39 µg/mL) ^[63]
165	4	CPZ-C	<i>M. fortuitum</i> (MIC = 12.5 µg/mL) ^[63]
166			<i>M. tuberculosis</i> (MIC = 0.78 µg/mg) ^[63]
167			<i>M. avium</i> (MIC < 0.025 µg/mg) ^[63]
168			<i>M. intracellulare</i> (MIC = 0.78 µg/mg) ^[63]
169			—
170	5	CPZ-D	<i>M. smegmatis</i> (MIC = 0.39-1.56 µg/mL) ^[63]
171			<i>M. phlei</i> (MIC = 1.56 µg/mL) ^[63]
172			<i>M. vaccae</i> (MIC = 0.39 µg/mL) ^[63]
173			<i>M. fortuitum</i> (MIC = 12.5 µg/mL) ^[63]
	6	CPZ-E	<i>M. tuberculosis</i> (MIC = 0.78 µg/mg) ^[63]
			<i>M. avium</i> (MIC < 0.025 µg/mg) ^[63]
			<i>M. intracellulare</i> (MIC = 0.78 µg/mg) ^[63]
			—
			<i>M. smegmatis</i> (MIC = 0.78-1.56 µg/mL) ^[63]
			<i>M. phlei</i> (MIC = 1.56 µg/mL) ^[63]
			<i>M. vaccae</i> (MIC = 0.78 µg/mL) ^[63]
	7	CPZ-F	<i>M. fortuitum</i> (MIC = 12.5 µg/mL) ^[63]
			<i>M. tuberculosis</i> (MIC = 1.56 µg/mg) ^[63]
			<i>M. avium</i> (MIC = 0.1 µg/mg) ^[63]
			<i>M. intracellulare</i> (MIC = 1.56 µg/mg) ^[63]
	8	CPZ-G	—

175 Despite their potent antibiotic activity, the naturally occurring caprazamycins are not considered
 176 eligible drug candidates due to: *i*) their sourcing limitations and *ii*) complex caprazamycin scaffold,
 177 which demands challenging multi-step synthesis with numerous poor yielding steps [41-42].
 178 Increasingly, medicinal chemistry is being employed to generate synthetic analogues of lesser
 179 structural complexity that retain comparable or even superior function [17]. Evaluation of each
 180 subsites' structural requirements facilitates the truncation of the caprazamycin scaffold by removal
 181 of the non-essential components to generate novel derivatives with increased potency or
 182 druglikeness. Simplified analogues produced *via* synthetic or semisynthetic protocols are described
 183 in the following sections according to the subsite explored.

184 4.2.1. Modifications on uridine and 5''-aminoribosyl moieties (subunit A and B)

Figure 3: Modifications on uridine and 5-aminoribosyl moieties (subunit A and B)

187 Initial studies focussed on probing *subunits A and B* (9) (Figure 3). The preliminary investigation
 188 was carried out by synthesis of *O*- β -D-ribofuranosyl nucleoside to evaluate the minimal structural
 189 requirement responsible for antibacterial activity against MraY (from *Escherichia coli*) (SI Table 1)
 190 [43]. Following biological evaluation, a moderately active anti-MraY scaffold (10, $IC_{50} = 50 \mu M$)
 191 was identified. It was hypothesized that activity could be further improved by introducing a chiral
 192 centre in between the two sugars, decreasing conformational flexibility. Accordingly,
 193 hydroxymethyl analogues of *O*- β -D-ribofuranosyl nucleoside having both the (*R*) and (*S*)
 194 stereochemistry were designed. Notably, the *S* isomer (11) was found 10 times more active than the
 195 parent structure (10) while the *R* isomer suffered reduced inhibitory activity. Subsequent
 196 investigation found the primary amino group, besides the conservation of uridine, were vital for
 197 retaining caprazamycin's activity [44]. Finally, while the 3'' hydroxyl proved essential, removal of
 198 the 3' hydroxyl improved antibacterial potency by five-fold relative to the parent scaffold [45].

Complete removal of subunit A (**12**, MIC₅₀ = >100 µg/mL) or subunit B (**13**, MIC₅₀ = >100 µg/mL) resulted in a complete loss of antibiotic potency against *M. tuberculosis*, highlighting the indispensable nature of both the 5''-aminoribose and uridine functions within the caprazamycin scaffold [46].

4.2.2. Modifications on diazepanone ring (subunit C)

The central diazepanone ring in caprazamycin (**14**, **Figure 4**) plays a critical anchoring role, orienting the other subunits and facilitating their highly specific interactions within MraY's active site [47]. As the diazepanone ring adds further complexity to synthesis however [48-55], its isosteric substitution with both cyclic and acyclic, or complete removal, are being investigated for use as diazepanone substitutes.

Figure 4: Structures of caprazamycin -subunit C and cyclic/acyclic bioisostere derivatives

Matsuda and co-workers investigated relatively simple diketopiperazine motif as a diazepanone substitute (**SI Table 2**) [47]. Cyclic and acyclic analogues bearing palmitoyl side chains (**15**, **17**, **19**, **Figure 4**) exhibited moderate antibacterial activity against Gram-positive bacterial strains (MIC = 12.5-50 µg/mL). The reduced activity of substrates **15** and **19** was reasoned to be due to their acyclic nature, perhaps not fulfilling the criteria for peripheral subunit orientation. The reduced activity of **17** was assigned to the absence of the amino and carboxylic acid functionality present in native caprazamycins, groups potentially forming critical interactions with MraY. Compounds

218 lacking a lipophilic acyl side chain (e.g. palmitoyl) (**16, 18, 20, 21, 22**) were found inactive (MIC >
 219 100 µg/mL), likely due to a reduced penetration through the bacterial cell wall. SAR analysis
 220 suggested that the cyclic ring, lipophilic side chain, amino and carboxylic acid groups primarily
 221 contributed to the antibacterial activity of the parent natural product.

222 Further investigation into decoding the role of the diazepanone ring led to the generation of acyclic
 223 caprazamycin analogues (**23-28, Figure 5**) lacking in any ring-substitution. These analogues bore
 224 the lipophilic side chain linked, either directly or *via* aryl substitution, to one of either the *C*-7''' or
 225 *N*-5''' positions *via* either an amide or urea linkage. A diverse array of acyclic analogues were
 226 generated including *C*-amide (**23, 24**), *N*-amide (**25, 26**) and urea derivatives (**27, 28**) (SI Table 3)
 227 [56]. The moderate activity of these acyclic analogues demonstrated that the diazepanone ring was
 228 not essential – though it was contributing to caprazamycin's antibacterial activity. This result further
 229 strengthened the diazepanone's proposed role in proper orientation of subsites A, B and D.

Figure 5: Structures of acyclic caprazamycin analogues

230 Matsuda and co-workers hypothesized that the moderate activity displayed by their acyclic scaffold
 231 could be due to their increased flexibility relative to caprazamycin. They therefore aimed to reduce
 232 the number of rotatable bonds, thus restricting the number of conformers. To achieve this, the
 233 sequential rotatable bonds through *N*-5'''–*C*-6'''–*C*-5'–*O*-5'–*C*-1'' were incorporated into a single
 234
 235
 236

237 oxazolidine ring which restricted all dihedral angles while retaining caprazamycins relative spatial
 238 orientation simultaneously (**Figure 6**) (**SI Table 4**) [56]. Additionally, relatively simple
 239 oxazolidine ring system enhanced synthetic accessibility.

Figure 6: Oxazolidine analogues of caprazamycin natural product

242 Oxazolidines bearing free carboxylic acids (**29**) failed to show antibacterial activity (MIC up to 64
 243 $\mu g/mL$). Analogues **30** and **31** exhibited significant antibacterial activity against drug-susceptible
 244 *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium* (MIC = 2-64 $\mu g/mL$) as
 245 well as drug-resistant bacterial strains such as MRSA and VRE (MIC = 2-64 $\mu g/mL$). Notably, the
 246 *tert*-butyl esters **31** were the most effective against both the drug-susceptible and drug-resistant
 247 *Enterococcus faecalis* (MIC = 2-4 $\mu g/mL$) with potency like that of desmethylpalmitoylcaprazol. It
 248 was suggested that the differences in antibacterial activity between acid **29** and ester analogues **30**,
 249 **31** might be due to their permeability through the bacterial cell membrane. Synthetic azide-bearing
 250 intermediates were also evaluated (not depicted) however they suffered from significantly reduced
 251 activity, confirming the critical role of the amino functionality. SAR analysis using the function-
 252 oriented synthesis [57] suggested the cyclic scaffold, the amino functional group and the lipophilic
 253 side chain present in the natural product are very critical. This systematic structural activity study
 254 highlighted that the diazepanone ring could be replaced with a simple heterocycle as long as the
 255 three essential pharmacophores were oriented appropriately.

256 Subsequently, Gravier-Pelletier and co-workers introduced structural diversity by introducing a
 257 triazole linker in between the 5' position of aminoribosyl-*O*-uridine and the lipophilic side chain
 258 (**Figure 7**) (**SI Table 5**) [58].

Figure 7: The most potent caprazamycins analogues from the series of triazoles

All of the synthesized compounds with benzophenone-substituted side chains exhibited moderate inhibitory activity against MraY enzyme (from *Bacillus subtilis*) ($IC_{50} = 50$ – $125 \mu M$). Their second-generation 5'-methylene triazole analogues (e.g. **34**, **Figure 7**), linked through either a C- or N-triazole, possessed increased flexibility due to presence of an additional methylene group, proposed to aid their positioning within MraY's active site (MraY from *Bacillus subtilis*) (**SI Table 6**) [59]. Of their initial library of 5'-triazoles, their most effective inhibitors contained either a benzophenone moiety (e.g. **32**, $IC_{50} = 50$ – $125 \mu M$) or an alkyl chain (not depicted) ($IC_{50} = 140$ – $150 \mu M$). A shorter alkyl chain bearing a polar functional group exhibited decreased inhibitory activity against MraY (from *Bacillus subtilis*) ($IC_{50} > 1000 \mu M$). It was interesting to note that the anti-MraY activity could be improved by increasing the length of the hydrophobic side chain. The inhibition was competitive as well as irreversible against the nucleotide substrate.

Both the 5'-methylene linked C- and N-triazole-containing analogues were found to be more effective inhibitors than directly linked 5'-triazoles, with 5'-methylene linked C-triazoles exhibiting the most potent activity. Notably, an increase in the length of the hydrophobic chain on either of the 5'-methylene linked C- or N-triazoles correlated with increased anti-MraY activity (MraY from *Bacillus subtilis*) ($IC_{50} = 15$ – $25 \mu M$ for 5'-methylene linked C-triazoles, 25 – $100 \mu M$ for 5'-methylene linked N-triazoles). As observed previously [58], the presence of a bulky benzophenone group was well tolerated with analogues exhibiting promising inhibitory activity ($IC_{50} = 20$ – $25 \mu M$ for 5'-methylene linked C-triazoles, 25 – $100 \mu M$ for 5'-methylene linked N-triazoles). During *in cellulo* studies against Gram-positive and Gram-negative pathogens, 9 compounds from a library of 33 were found to have antibacterial activity (MIC = 8–64 $\mu g/mL$) against three Gram-positive and Gram-negative *Pseudomonas aeruginosa*. Decyloxybenzophenone functionalised triazoles exhibited the most potent antibacterial activity (**33**, $IC_{50} = 100 \mu M$, MIC = 8 $\mu g/mL$; **34**, $IC_{50} = 25 \mu M$, MIC = 8 $\mu g/mL$) against *Staphylococcus aureus* MRSA from the series of all synthesized triazoles (**Figure 7**). It was interesting to note that although the triazoles were not as structurally

complex, their antibacterial activity was comparable with the natural nucleoside antibiotic Mray inhibitor muraymycin [60].

Matsuda and co-workers employed a scaffold hopping approach to identify the isoxazolidine ring system as an isosteric replacement for the diazepanone and aminoribose moieties (**SI Table 7**) [61-62]. A series of isoxazolidine analogues decorated with the critical aminoalkyl and fatty acyl motifs were evaluated against a range of bacterial pathogens. Synthetic analogue **35**, containing opposite stereochemistry to caprazamycins at the 5'-position, was found to be inactive against all the evaluated bacterial strains except for *Haemophilus influenzae* ATCC 10211 (MIC = 1 µg/mL). Analogue **36**, retaining the same stereochemistry as the natural product, displayed significant antibacterial activity against *Haemophilus influenzae* ATCC 10211 (MIC = 0.25-0.5 µg/mL) and moderate activity against vancomycin resistant *Enterococcus faecalis* SR7914 (MIC = 4-8 µg/mL) (**Figure 8**). This study demonstrates effectively the contribution made by the 5'-positions' stereochemical configuration to its antibiotic properties.

Figure 8: Structures of isoxazolidine-containing caprazamycin analogues

The fact that anti-MraY activity was reported to tolerate a simpler isoxazolidine scaffold encouraged the design of simpler analogues. In continuation of the efforts to explore simpler yet bioequivalent scaffolds, Ichikawa and co-workers synthesized uridine analogues containing a six membered piperidine scaffold (**Figure 9**) (**SI Table 8**) [63]. This piperidine scaffold facilitated investigation into the importance of the caprazamycins stereochemistry at the diazepanone's C-6''' and at the uridine's C-5'. Additionally, it was proposed that the more constrained six member ring, relative to caprazamycins seven-member diazepanone ring, might translate to an increased activity against MraY due to the fewer possible conformational isomers. Finally, the piperidine scaffold is significantly more synthetically accessible. Following synthesis of a library of piperidine-based analogues, inhibitory activity against purified MraY (from *Staphylococcus aureus*) was evaluated in a fluorescence-based MraY assay [64]. It was observed that the piperidine analogues **37** and **38**, with opposite stereochemistry at C-5' to caprazamycin, were found to be weak inhibitors (IC₅₀ value >2700 nM). The remaining analogues with the same stereochemistry at C-5' (**39** and **40**), exhibited good inhibitory activity. Substrate **39**, which retained the natural products' 6'''R chirality, was found to be 8.6-fold stronger (IC₅₀ = 24 nM) than its diastereomer **40** (IC₅₀ = 206 nM). The strong

316 anti-MraY activity of these analogues represented a successful transformation from diazepanone
 317 ring to the six-membered piperidine scaffold.

Figure 9: Structures of piperidiny analogues of caprazamycins

320 Piperidine-based acetate-containing analogues (not depicted) (**SI Table 8**) failed to show any
 321 evidence of MraY (from *Staphylococcus aureus*) activity, suggesting lipophilicity played a
 322 significant role in enzyme inhibition. Substrates boasting a palmitoyl group as lipophilic side chain
 323 (**37-40**) exhibited significant antibacterial activity against a range of Gram-positive bacterial strains
 324 such as *Staphylococcus aureus* SR3637 (MRSA) and *Enterococcus faecium* SR7917 (VRE) (MIC =
 325 2-32 μ g/mL). Similar trends of enzyme inhibition were observed regarding antibacterial activity,
 326 with 5'S isomeric substrates **39** and **40** (MIC = 2-16 μ g/mL) exhibiting increased activity over the
 327 5'R analogue, **37** and **38** (MIC = 8-32 μ g/mL). Once more, analogue **39**, retaining both 6'''R and 5'S
 328 natural product stereochemistry, exhibited the strongest antibacterial activity (MIC 2-16 μ g/mL).
 329 Subsequent examinations of **39**'s metabolic stability showed it was unaffected by human or mouse
 330 liver microsomes. These compounds were found cytotoxic against human hepatocellular liver
 331 carcinoma (HepG2) cells ($IC_{50} = 23$ to 35 μ M) however, limiting their utility.

332 The observed cytotoxicity was presumed to be due to the increased hydrophobicity incurred from
 333 truncating polar functional groups such as the carboxylic acid and the amide group. To resolve this,
 334 Ichikawa and co-workers designed model compounds bearing shorter lipophilic groups such as

lauroyl (**41**) and myristoyl groups (**42**) (**Figure 9**) (**SI Table 9**) [63]. They also speculated that toxicity was due to micelle formation and so, to reduce the likelihood of micelle formation, analogues with branched lipophilic side chains **43** were designed (**SI Table 9**). Analogues **41** and **42**, bearing shorter lipophilic side chains, displayed decreased activity relative to the palmitoyl-containing parent compound **39**. The branched chain analogues (e.g. **43**) retained their antibacterial activity against Gram-positive bacterial strains (MIC = 4 to 8 µg/mL) however, while also showing a complete loss of cytotoxicity against HepG2 cells up to 100 µM. Author suggested that though further optimization is essential for evaluating impact of branching on toxicity, the branched analogue **43** could be a lead structure because of its high therapeutic index. This study demonstrates the successful transformation from the diazepanone to a simplified piperidine ring while also highlighting the importance of the stereochemistry at the C-5' and C-6''' positions.

In an attempt to improve the practicality of their synthesis, Ichikawa and co-workers designed additional, simplified caprazamycin analogues aided by their implemented function-oriented synthesis (FOS) strategy [57]. They propose that the function of structurally complex natural products can be mimicked or even improved upon by appropriately linking the essential structural components through simpler structural entities. Synthetic accessibility can then be prioritised, facilitating the construction of larger analogue libraries promising an enriched perspective on the natural product's SARs. By applying FOS principles, the researchers designed and synthesized diverse analogues of lactam-fused isooxazolidine core structures (**SI Table 10**) [65]. Such a scaffold permitted modulation of the three-dimensional orientation of key structural units, either by alteration of stereochemistry or by modifying the size of the ring.

Two types of lactam-fused isoxazolidine derivatives were investigated: the first bearing a linear substituent linking the lipophilic side chain with an accessory arginine group (Type I), and the second, a branched variant with each substituent anchored to the central lactam ring (Type II) (e.g. **44**, **Figure 10**) [65].

Figure 10: The most active structure from a series of lactam-fused isooxazolidines

Generally, many lactam-fused isooxazolidine analogues exhibited moderate activity against a range of Gram-positive drug-resistant bacterial strains including *Staphylococcus aureus* SR3637 (MRSA)

and *Enterococcus faecium* SR7917 (VRE) (MIC = 8-64 $\mu\text{g/mL}$). Notably, the branched analogues (Type II) boasted increased activity over linearly substituted (Type I) compounds. Amongst all the synthesised lactam-fused isooxazolidines, compound **44** was found to be the most potent analogue (Figure 10).

4.2.3. Modifications on western zone fatty acyl chain (subunit D)

Figure 11: Modifications on western zone fatty acyl chain (subunit D)

Caprazamycins western zone is composed of three structural subunits - a fatty acyl moiety, a 3-methylglutaric acid tether and an *O*-methylated-L-rhamnoside (Figure 11) [39]. The naturally occurring caprazamycins are classified into seven analogues (CPZ A-G) according to their structural diversity within western zones. Though highly potent, their limited sourcing from nature necessitates investigation into the SARs of potent semisynthetic analogues to aid identification of eligible drug candidates. Furthermore, difficulties arise when investigating the natural

caprazamycins because of their challenging isolation from complex mixtures. Adding further complexity to the endeavour, conventional methods (e.g. high-performance liquid chromatography, HPLC) aren't reliable within this context because of the caprazamycins' poor water-solubility profile, due in part to their western zone. Due to its complex nature, modulation or replacement of the western zone is an appealing strategy.

One such strategy circumventing this entire issue was to treat the complex mixture of natural caprazamycins with an acid to afford caprazene (CPZEN) (**46**), made up of subunits A, B and C (**Figure 11**) [12]. Though its isolation from the complex mixture of natural products was drastically simplified, the absence of any fatty acyl moiety in caprazene rendered it biologically inactive. Following isolation, functionalisation at the diazepinone with some lipophilic chain was expected to restore antibacterial activity [12]. Substitution at the 5"-amino group of D-ribose was also considered, however the location of the carboxylic acid group was preferred due to its position adjacent to the native fatty acyl linkage site. Besides that, the availability of the free amino group afforded some control over water solubility *via* acid salt formation. Semisynthetic caprazamycin analogues bearing 1'''-alkylamide, 1'''-anilide and 1'''-ester substitutions were generated and evaluated subsequently (**SI Table 11**).

From the series of semi-synthetic caprazamycin analogues, the 4-butyranilide substituted derivative of caprazene (CPZEN-45, **47**) was identified to be the most active inhibitor (**Figure 11**). It displayed superior inhibition over the natural product CPZ-B (**2**) with excellent activity against diverse *Mycobacterium* strains (MIC = 0.20-50 µg/ml). However, the reduction of antibacterial activity of CPZEN derivatives against various Gram-positive and Gram-negative bacterial strains (MIC = 3.13-100 µg/mL) in comparison with caprazamycin B (**2**) reflected their obvious difference in selectivity toward a bacterial spectrum. Further investigation confirmed that unlike natural caprazamycin, the caprazene derivatives inhibit WecA enzyme, which is involved in biosynthesis of mycolyl arabinogalactan [66]. Since long-term chemotherapy is required for TB treatment, the CPZEN derivatives were studied for selectivity, specificity and a narrow-range antimicrobial spectrum to evaluate their suitability for extended therapy. CPZEN-45 (**47**) was found to be the most active CPZEN derivative, with an MIC range, MIC₅₀ and MIC₉₀ for drug-sensitive *Mycobacterium tuberculosis* clinical isolates of 0.78-12.5, 1.56 and 3.13 µg/ml, respectively. Moreover, the selectivity, negligible haemolysis and excellent water solubility of **47** qualified it as a promising lead in clinical trials for use as both a new anti-TB and a novel anti-MAC agent [13, 67]. As acid-catalysed hydrolysis of caprazamycin yielded caprazene analogues, base-catalysed hydrolysis of natural product afforded biologically inactive caprazol (**48**) (**Figure 11**).

To restore the antibacterial activity of caprazol (**48**) a simple palmitoyl residue was introduced as the lipophilic side chain, generating palmitoyl caprazol **49** (**Figure 11**) (**SI Table 12**) [9]. Compound **49** exhibited significant antibacterial activity against *Mycobacterium smegmatis* (MIC = 6.25 µg/mL) with a potency in the range of the caprazamycins. Absence of the methyl group at the *N*-5'''-position, as in the case of desmethyl palmitoyl caprazol **50**, led to a 4-fold reduction in antibacterial potency (MIC = 25 µg/mL). The observed reduction of antibacterial activity might be due to the difference in lipophilicity contributed by the methyl group (**SI Table 12**). The author suggested that the lipophilic nature of the molecule might be essential for its permeation through the exceptionally thick and tight mycobacteria cell wall. However, unlike *Mycobacterium smegmatis*, both **49** and **50** exhibited similar potency against drug resistant bacteria such as MRSA and VRE strains (MIC = 3.13-12.5 µg/mL). This study demonstrated the successful replacement of the complex substituents and stereocenters of the western zone with a simple palmitoyl substitution. Further precise optimization of the lipophilic side chain could promote permeation through bacterial cells.

Though the palmitoylcaprazol scaffold afforded strong potency, palmitoyl caprazol (**49**) was not considered a viable lead structure due to its complex synthesis and its β -acyloxy ester linkage, a function speculated to hinder metabolic stability *via* β -elimination. To overcome this instability, 'carbaprazamycin' analogues [e.g. **51** (**Figure 11**)], functionalised with hydrophobic chains linked directly to the diazepanone *via* C-C bonding, were designed (**SI Table 13**) [68]. Both epimers, differing at the 3''' position of diazepanone ring, were employed to probe the chiral centres influence on bioactivity. Evaluation against *MraY* (from *Staphylococcus aureus*) during a fluorescence-based *MraY* assay [64] suggested that the carbaprazamycins retained strong *MraY* inhibitory activity (IC_{50} = 2.6-6.9 nM). Subsequent antibacterial evaluation highlighted the moderate potency of carbaprazamycins against drug-resistant bacterial pathogens such as MRSA and VRE (MIC = 4-16 µg/mL). In both the cases of enzymatic inhibition (IC_{50} = 2.6-6.9 nM) and antibacterial activity (MIC = 4-16 µg/mL), the carbaprazamycins were found 4-6 times less effective than the palmitoylcaprazole (IC_{50} = 1.2 nM, MIC = 0.5-2 µg/mL) however this was speculated to be due to the replacement of the ester moiety, increasing the lipophilicity of entire molecule. Once more, methyl substitution at *N*-5''' of carbaprazamycin translated to an improved potency relative to the desmethyl analogue (4-8 µg/mL *vs* 8-16 µg/mL), a trend correlating with previous results from the palmitoylcaprazole study (**SI Table 13**) [9]. Interestingly, epimer **51** (*S* configuration, MIC = 4-8 µg/mL), possessing unnatural stereochemistry the 3''' position, displayed an increased activity relative to that of the epimer reflecting the native stereochemistry (*R*

configuration, MIC = 8-16 µg/mL). The author suggested further optimization of the lipophilic side chain to increase the antibacterial activity of carbacaprazamycins. Research pursuit to enrich our understanding of caprazamycins' anti-MraY activity has resulted in the generation of potent, broad-spectrum antibiotics. Their promising antibiotic properties have qualified caprazamycin analogues as platforms upon which potent drug-like scaffolds may be designed and optimised. SAR-based analysis of the critical pharmacophores has proved invaluable in this endeavour. Herein, we have compiled the trends that should be considered when aiming to improve bioactivity and drug likeness: *i*) conservation of both uridine and 5"-aminoribose structures is essential [43, 46]; *ii*) stereochemistry at both C-5' and C-6''' positions must be considered [62-63]; *iii*) a cyclic core orienting the key subunits in specific stereochemical arrangement is essential [47, 56]; *iv*) regarding the core cycle, simpler rings than the complex diazepanone are well tolerated [47, 56, 58-59, 62-63, 65]; *v*) the western zone, though structurally complex in the natural caprazamycins, can be replaced by simple lipophilic side chain [9, 12, 68].

5. Concluding remarks

The global status of antimicrobial resistance amongst currently employed agents outlines the need to explore novel targets for antibacterial action. Bacterial cell wall biosynthesis has been heavily exploited since the discovery of penicillin [6]. Despite having promising activity for bacterial cell wall inhibitors, in the present era of increasing resistance there is a need to explore novel targets which will translate to extended drug utility before resistance develops. Indeed, most of the enzymes involved in peptidoglycan biosynthesis have been targeted by clinical antibiotics, except the translocase I enzyme (MraY). MraY as an antibacterial target has gained research interest in the past few years due to the existence of diverse nucleoside antibiotics that act specifically [14, 19]. Naturally occurring nucleoside antibiotics have not yet proved useful within the clinical context however, mainly due to the lack of three-dimensional structural insight into the inhibition of MraY. Recently, structural determination of the MraY enzyme, along with its complex with natural nucleoside antibiotics, has opened possibilities for structure-based drug design against MraY [27, 34, 69].

Unlike existing cell wall inhibitors (i.e. penicillins), nucleoside antibiotics (capuramycin analogues) have shown bactericidal activity against nonreplicating (dormant) *Mycobacterium* TB [70]. Caprazamycins as a subclass of nucleoside antibiotics have exhibited a wide range of *in vitro* anti-mycobacterial activity against both drug-susceptible and multi drug-resistant *Mycobacterium* TB strains without conferring toxicity in mice [37]. One semisynthetic caprazamycin analogue (CPZEN-45) is under preclinical study for antitubercular activity [67]. Additionally, the caprazamycins class of nucleoside antibiotics have also shown their promising activity against

diverse Gram-positive strains including both drug-sensitive as well as drug resistant MRSA and VRE. Their antibiotic properties also include their potent activity against Gram-negative bacterial strains such as *Pseudomonas aeruginosa* and *Haemophilus influenzae*. With these excellent antibiotic properties against both Gram-positive as well as Gram-negative bacterial strains, caprazamycins are expected to be a promising lead structures for development of broad-spectrum antibacterial agent. This SAR (see **SI Figure 2**) study was carried out to reveal the essential pharmacophores present in the natural caprazamycin scaffold. We hope this work will contribute to the development of urgently required novel antibacterial agents.

Appendix A. An example appendix

A.1. Example of a sub-heading within an appendix

Supplementary Material

Detailed information on antimicrobial profile of synthetic caprazamycin derivatives is available on the Journal's website.

Conflicts of Interest

The authors declare no conflicts of interest.

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- Nucleoside antibiotics caprazamycins are the most promising lead structures acting on a novel antibacterial target *MraY*.
- A systematic review on the structure-activity relationships (SAR) of caprazamycins was carried out.
- The SAR study revealed the essential pharmacophores present in caprazamycins nucleoside antibiotics.
- Antibacterial spectrum of the natural, semi-synthetic and synthetic analogues of caprazamycins are described.

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Caprazamycins: Promising lead structures acting on a novel antibacterial target MraY

Bhautikkumar Patel, Philip Ryan, Vivek Makwana, Matthew Zunk, Santosh Rudrawar, Gary Grant

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