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FULL PAPER

Gram-positive and Gram-negative Antibiotic Activity of Asymmetric and Monomeric Robenidine Analogues

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Supporting information for this article is given via a link at the end of the document.

Abstract: Desymmetrisation of robenidine **1**, *N'*,2-bis((*E*)-4-chlorobenzylidene)hydrazine-1-carboximidhydrazide, and the introduction of imine alkyl substituents gave good antibiotic activity. Of note was the increased potency of **17** and **20** against VRE with **20** the most active, MIC of 0.5 μg mL^{-1}. Analogues **2**, **14**, **17**, **19** and **20** were equipotent or more potent than the lead **1**. Introduction of an indole moiety, **30,** resulted in the most MRSA active robenidine analogue, MIC of 1.0 μg mL^{-1}. Imine C=NH isosteres (C=O / C=S) were inactive. Monomeric analogues, **33**–**35** were 16 – 64 μg mL^{-1} active against MRSA and VRE. Analogue **36**, lacking the terminal hydrazide NH moiety showed modest Gram-negative activity at 64 μg mL^{-1}. 4-*t*-Butyl **45** was Gram -positive and -negative active at of 16 – 64 μg mL^{-1}. Typically additional aromatic moiety modification was poorly tolerated, except with concomitant introduction of an imine C-alkyl moiety. The activity of these analogues against MRSA and VRE ranged from 8 μg mL^{-1} with **64** and **68**, to inactive (MIC > 128 μg mL^{-1}) with the naphthyl **69** and **70** and the indole **73**. Gram-negative activity was most promising with **62** and **68** at 16 μg mL^{-1} against *E. coli*. Against *Ps. aeruginosa*, the highest activity observed was with MIC values of 32 μg mL^{-1} with **62** and **64**. Combined, these findings support the further development of the (*E*)-2-benzylidenehydrazine-1-carboximidamide scaffold as a promising Gram-positive and Gram-negative antibiotic development.

Introduction

Pathogenic bacteria that are resistant to multiple classes of antimicrobials account for massive morbidity and mortality in humans worldwide, and poses both a significant public health concern and ever increasing health-care costs in many countries. A 2016 joint report from the European Center for Disease Prevention and Control and the European Medicines, based on 2007 data estimated that there were >25,000 bacterial infection associated deaths in Europe [1]. The US Centre for Disease Control and Prevention in 2013 estimated the US deaths from bacterial infections at >23,000 [2]. In Australia, there are an estimated 165,000 hospital acquired bacterial infections [3]. By 2050 The Review on Antimicrobial Resistance estimates bacterial infection will result in 10 million deaths globally [4].

Effective treatment of bacterial infections is becoming increasingly difficult with the emergence of resistance to multiple classes of antimicrobial drug. In particular, sepsis due to the multidrug-resistant (MDR) ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *E. coli*/*Enterobacter* species) contribute significantly to morbidity and mortality associated with antimicrobial resistance (700,000 deaths annually) worldwide [5-10]. Therefore, the discovery and development of new broad-spectrum antimicrobials with activity against pan-resistant resistant ESKAPE pathogens causing sepsis is one of the highest global priorities [11-13]. In 2010, the Infectious Diseases Society of America (IDSA) made an ambitious call for 10 new antimicrobial agents to be registered by 2020 [14]. However, since that call, only a single agent with a new mode of action (the narrow-spectrum drug fidaxomicin for *Clostridium difficile* infections) has been registered. The remainder represent modifications to existing drug classes targeting either Gram-positive organisms (telavancin, dalbavancin, tedizolid, oritavancin) or Gram-negative organisms (ceftolozane-tazobactam, ceftazidime-avibactam) [15-18]. Reiterating the call for novel agents with new modes of action, the World Health Organization (WHO) recently published a priority list of bacteria for which new antibiotics are urgently needed, in which MDR ESKAPE pathogens are prominent [19]. As of March 2017, there were 41 new antibiotics in clinical development within the US, but it is expected that less than 1 in 5 of these agents will enter the clinic. Baxdela (delafloxacin) and Mereopenen+Vaborbactam have NDAs submitted are potential treatments for the ESKAPE pathogens [20].

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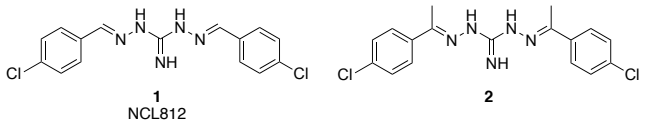
We recently looked to repurpose the anti-coccidial agent robenidine in this space (**1**; NCL812). Our preliminary reports show promising levels of bactericidal activity [21, 22]. Of these, **2** exhibits bactericidal activity against both *S. pneumoniae* (minimum inhibitory concentration (MIC) range 2-8 μg mL^{-1}) and *S. aureus* (MIC range 1-2 μg ml^{-1}) via disruption of the cell membrane potential. In the presence of polymyxin B nonapeptide, **2** also showed Gram-negative activity against *E. coli* and *P. aeruginosa* at 10 and 5 μM concentrations, respectively (Figure 1). Importantly, our preliminary analogues were less cytotoxic to a variety of mammalian cell lines than the parent compound, and were typically non-toxic to erythrocytes at the highest concentration tested (128 μg mL^{-1}). This suggests specificity for the prokaryotic cell membrane, which can be engineered by further modification of this scaffold, whilst identifying analogues with improved solubility, pharmacokinetics and potency against ESKAPE pathogens.



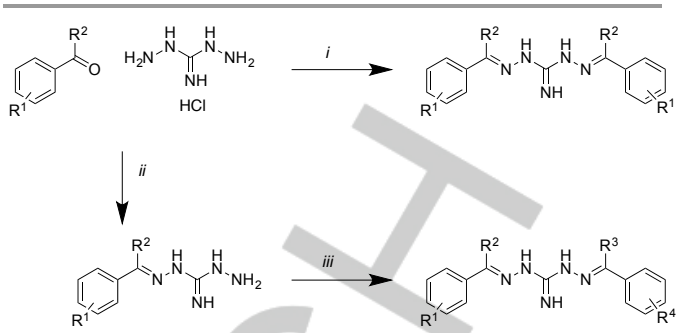
1 NCL812 **2**

Figure 1. Chemical structures of lead Robenidine analogues **1** (NCL812) and **2**.

In our initial report on the modifications of robenidine (**1**), our investigation was restricted to the SAR of symmetrical dimeric analogues and simple monomers. These preliminary SAR studies demonstrated a key role for the ring C2'-/C4'- halogens; that imine C-alkylation gives enhanced hydrolytic stability ($t^1/_2$ > 240 min in hepatic microsomal preparations); imine reduced analogues are inactive [21]; and N to O bioisosteric modifications abrogated activity. Herein we report on efforts to expand the known pharmacophore of robenidine analogues as potential antibiotic agents.

Results and Discussion

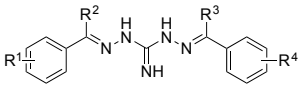
Access to robenidine analogues is rapidly accomplished via a condensation of an aldehyde or phenone with 1,3-diaminoguanidine hydrochloride. The use of two equivalents of the carbonyl entity affords symmetrically substituted analogues, while careful control of the reaction stoichiometry (one equivalent of C=O entity) allows isolation of the mono-adduct, which can be further reacted with a second equivalent of the same or a different aldehyde or phenone (Scheme 1).



Scheme 1. Reagents and Conditions: i) 2 equiv. 'C=O' (see Table 1 for detail), 1,3-diaminoguanidine hydrochloride, EtOH reflux; ii) 1 equiv. 'C=O' (see Table 1 for detail), 1,3-diaminoguanidine hydrochloride, EtOH reflux; and iii) as per (ii) with different 'C=O' as detailed in Table 1.

Our initial efforts examined the modification of one of the aromatic rings of the lead **1**, followed by the introduction of imine C-alkyl substituents. However, we note that the yields of this 'two-step' process were typically far lower than the equivalent one pot procedure. In the presence of the second equivalent of the C=O entity we typically observed a product distribution that related to an imine equilibrium in the resulting reaction mixture. Flash chromatography to isolate the mono adduct was possible, but on introduction of the mono-imine adduct to the reaction mixture, the initial equilibrium mixture was isolated. The outcome of subsequent antibiotic screening against methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococci* (VRE), *E. coli* and *Ps. aeruginosa* is presented in Table 1. Lead **1**, displayed good levels of activity against both MRSA and VRE with MIC values of 1-4 μg mL^{-1}. Reposition of one of the 4-Cl moieties to the asymmetrically substituted 2-Cl **3** resulted in a 2-4 fold reduction in activity (8 μg mL^{-1}), with the analogous 3-Cl **4** inactive (MIC >128 μg ml^{-1}). Isosteric replacement of a 4-Cl with a 4-CH_3 **5** or a 4-CF_3 **6** moiety saw a complete loss of activity. The 2-F **7** and 4-F **8** analogues were active against MRSA and VRE at 2-4 μg mL^{-1}. However the loss of activity with the introduction of a 2- or 3- or 4- CN (**9**-**11**) suggests that this is not purely a function of electronegativity at this position. The introduction of a second halogen to one aromatic ring saw a modest reduction in potency with **12** at 2-16 μg mL^{-1}, while the combination of a 2-F, 4-CF_3 and a 2-F, 4-Cl moieties with **13** and **14** saw excellent MIC values of 2-4 μg mL^{-1}. The symmetrically substituted 2-F, 4-Cl **15** saw a modest reduction in activity relative to **14**. Replacement of the 2-F of **15** with the H-bonding capable 2-NH_2, **16**, resulted in a 4-fold loss of activity suggesting that a hydrogen bonding (acceptor or donor) group in this position was not favoured.

Table 1. Inhibition of two isolates of *MRSA*, *VRE*, *E. coli*, and *Ps. aeruginosa* growth by 1,3-aminoguanidine Schiff Base analogues possessing mono- and di-substituted aromatic rings (**1**− **25**).



MIC mode (μg mL-1)

	R^1	R^2	R^3	R^4	MRSA	VRE	E. coli	Ps. aeruginosa
1	4-Cl	H	H	4-Cl	2	2	-[b]	-
2	4-Cl	Me	Me	4-Cl	2	2	-	-
3	4-Cl	H	H	2-Cl	8	4	-	-
4	4-Cl	H	H	3-Cl	-	-	-	-
5	4-Cl	H	H	4-Me	-	-	-	-
6	4-Cl	H	H	4-CF_3	-	-	-	-
7	4-Cl	H	H	2-F	8	4	-	-
8	4-Cl	H	H	4-F	4	4	-	-
9	4-Cl	H	H	2-CN	-	-	-	-
10	4-Cl	H	H	3-CN	-	-	-	-
11	4-Cl	H	H	4-CN	-	-	-	-
12	4-Cl	H	H	2,4-di-Cl	16	2	-	-
13	4-Cl	H	H	2-F, 4-CF_3	4	4	-	-
14	4-Cl	H	H	2-F, 4-Cl	2	2	-	-
15	2-F, 4-Cl	Me	Me	2-F, 4-Cl	8	4	-	-
16	2-NH_2, 4-Cl	H	H	2-NH_2, 4-Cl	32	32	-	-
17	4-Cl	H	Me	4-Cl	4	2	-	-
18	4-Cl	Me	H	4-CF_3	-	-	-	-
19	4-Cl	H	Me	4-CF_3	4	2	-	-
20	4-Cl	Me	Me	4-CF_3	4	0.5	-	-
21	4-CF_3	Me	H	4-CF_3	-	-	-	-
22	2-NH_2, 4-Cl	Me	Me	2-NH_2, 4-Cl	-	-	-	-
23	2-OH, 4-Cl	Me	Me	2-OH, 4-Cl	16	32	-	-
24	2-OH, 4-Cl	Et	Et	2-OH, 4-Cl	4	4	-	-
25	2-OH, 4-Cl	c-Pent	c-Pent	2-OH, 4-Cl	64	128	-	-

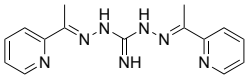
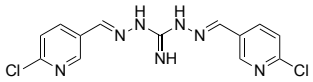
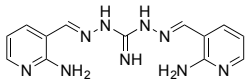
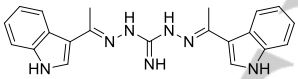
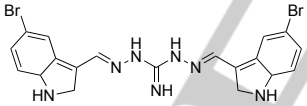
[a] compounds isolated as the HCl salts; [b] inactive at 128 μg mL^{-1}

The introduction of a CH_3 moiety at each of the imine carbons resulted, with **2**, in a moderate potency enhancement against MRSA and VRE, and as previously reported an *in vitro* stability enhancement [16]. Selective removal of one the imine carbon CH_3 moieties resulted in a modest decrease in MRSA but not VRE activity with **17**, but combined with the introduction of a 4-CF_3 moiety, abolished activity with **18**. With the 4-CF_3 analogues **19** and **20**, the loss of a CH_3 moiety was well tolerated with retention of the activity noted at 2-4 μg mL^{-1}, but with **20**, which retained both CH_3 moieties, activity was retained against MRSA with a concurrent 2-fold increase in potency against VRE (0.5 – 1 μg mL^{-1}). Bis-4-CF_3 **21** is inactive. This suggests that the position, and potentially the steric bulk, of the C-alkyl moiety, plays a key role in the observed antibiotic activity in combination with the nature of the aromatic substituent.

Given the retention of activity noted with **1** and **2** on introduction of the imine C-CH_3 moieties we looked to apply the same modification to the 2-NH_2, 4-Cl substituted **16**, however this removed all antibiotic activity (MIC > 128 μg mL^{-1}; **22**). The corresponding 2-OH **23** is more potent than either **16** or **22** with MIC values 4-32 μg mL^{-1}. Increasing the imine C-alkyl moiety to ethyl **24** gave a further 4-fold potency enhancement. However, the c-pentyl analogue **25** is only active against one of MRSA and one VRE strains examined. The 4-Cl, CH_2OH analogue **22** displays MIC values of 8 μg mL^{-1} against MRSA and VRE. None of these analogues show efficacy against the two exemplar Gram-negative bacterial strains examined, i.e. *E. coli* and *Ps. auruginosa*.

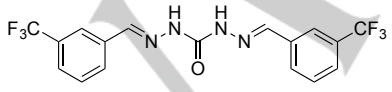
Phenyl ring modifications were investigated via the synthesis of the 2-pyridyl **26**, 3-pyridyl **27** and **28**, as well as the indole **29** and **30**. Subsequent screening of these analogues revealed that the pyridyl analogues resulted in a significant loss of activity with only 4-Cl-3-pyridyl **27** displaying modest activity against VRE. The 2-indole **29** displayed good activity against Gram-positive MRSA and VRE, 4 and 8 μg mL^{-1}respectively. The corresponding 5-bromoindole **30** was 4-fold more MRSA active while retaining the same 8 μg mL^{-1} activity against VRE.

Table 2. Inhibition of two isolates of MRSA, VRE, *E. coli* and *Ps. aeruginosa* growth by 1,3-aminoguanidine Schiff Base analogues possessing heterocyclic aromatic rings (**26** − **30**).

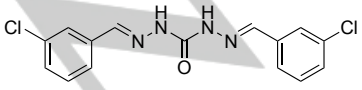
Compound	Structure	MIC mode (μg mL^{-1})			
		MRSA	VRE	*E. coli*	*Ps. aeruginosa*
26[a]		128	-[b]	-	-
27		128	128	-	-
28		-	-	-	-
29		4	8	-	-
30		1	4	-	-

[a] compounds isolated as the HCl salts; [b] in active at 128 μg mL^{-1}

Isosteric modification of the central imine C=NH to a C=O removed activity for all analogues excepting the 3-CF_3-substituted **31** (Figure 2), which retained modest potency against one of the VRE lines examined (32 μg mL^{-1}). In a similar manner a C=NH to C=S modification only afforded a single analogue, the 3-Cl substituted **32** (Figure 2), which retained activity against one of the VRE strains examined with a MIC of 32 μg mL^{-1} (Supplementary Data).



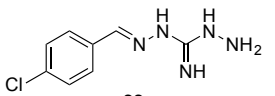
31



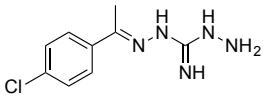
32

Figure 2. Chemical structure of **31** and **32**.

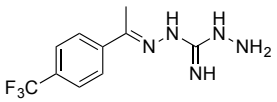
As noted above, the presence of asymmetrically di-substituted analogues retained, and in some cases enhanced activity against the Gram-positive strains examined. As a result of these findings we examined the activity of three simple monomeric compounds, the 4-Cl **33**, **34** and 4CF_3 **35** guanidine analogues, and the effect of removal of the terminal amino moiety of the guanidine hydrazine in the synthesis of 4-Cl **36** (Figure 3).



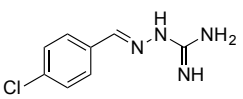
33



34



35



36

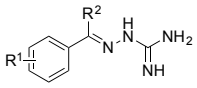
Figure 3. Chemical structures of monomeric analogues **33**-**36**.

Both **33** and **34** showed reduced Gram-positive antibiotic activity, with **33** active at 32 and 64 μg/mL against MRSA and VRE respectively, **34** showed >64 μg mL^{-1} activity against VRE only, the 4-Cl to 4-CF_3 modification with **35** saw and increase in VRE activity at 16 μg mL^{-1}, but similarly no activity against MRSA. Interestingly, **36** lacking one of the guanidine NH moieties showed

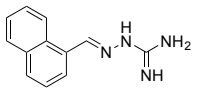
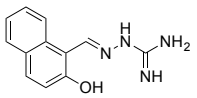
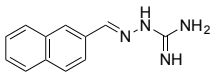
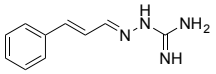
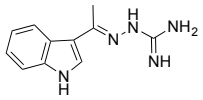
no Gram-positive activity (MRSA and VRE MIC values >128 μg mL-[1]), but did show promising and unexpected levels of Gram-negative activity with a 64 μg mL^{-1} MIC value for both *E. coli* and *Pseudomonas*.

Emboldened by the identification of Gram-negative activity of **36**, we explored the development of an additional focused compound library that saw further modification to the aromatic scaffold, but lacking one of the guanidine NH moieties. These synthesises were accomplished using a slight modification of the procedure described in Scheme 1. A total of 39 monomeric analogues **37**-**73**, and in many cases the corresponding C=S and C=O isosteres were generated, but only the parent analogues returned any noteworthy antibiotic activity (Table 3 and Supplementary Data). In most cases the removal of the second aromatic moiety removed all vestiges of antibiotic activity for analogues lacking an imine C-alkyl moiety, *viz* halogens **37**-**41**, small alkyl **42**-**44**, CN **46**-**48**, OCH_3 **49** and $N(CH_3)_2$ **50** moieties were inactive. However 4-*t*-Bu **45** afforded activity across Gram-positive (16 and 64 μg mL^{-1}) and Gram-negative bacteria (64 and 64 μg mL^{-1}). Of the trifluoromethyl substituted analogues **51** – **53** only the 4-CF_3 **53** was active. The 2-F,4-Cl **54** was very weakly active, as was the corresponding 2-F,4-CF_3 **55**, but this analogue showed low levels of Gram-negative activity as well.

Table 3. Inhibition of two isolates of MRSA, VRE, *E. coli*, and *Ps. aeruginosa* growth by monomeric 1,3-Aminoguanidine Schiff Base analogues (**37**−**73**).



			MIC mode (μg ml^{-1})			
	R^1	**R^2**	**MRSA**	**VRE**	***E. coli***	***Ps. aeruginosa***
37[a]	2-Cl	H	-[b]	-	-	-
38	3-Cl	H	-	-	-	-
39	2-F	H	-	-	-	-
40	3-F	H	-	-	-	-
41	4-F	H	-	-	-	-
42	2-CH_3	H	-	-	-	-
43	3-CH_3	H	-	-	-	-
44	4-CH_3	H	-	-	-	-
45	4-*t*-Bu	H	16	64	64	64
46	2-CN	H	-	-	-	-
47	3-CN	H	-	-	-	-
48	4-CN	H	-	-	-	-
49	2-OCH_3	H	-	-	-	-
50	4-$N(CH_3)_2$	H	-	-	-	-
51	2-CF_3	H	-	-	-	-
52	3-CF_3	H	-	-	-	-
53	4-CF_3	H	64	64	-	-
54	2-F,4-Cl	H	128	128	-	-
55	2-F, 4-CF_3	H	64	64	128	128
56	4-Cl	Me	>64	>64	>64	>64
57	4-*t*-Bu	Me	16	32	32	64
58	2-F,4-Cl,	Me	32	32	>128	128
59	4-Cl	Et	64	64	128	-[a]
60	4-Cl	Pr	32	32	64	128
61	4-Cl	*n*-Bu	32	32	64	>64
62	4-Cl	*n*-pent	8	8	16	32
63	4-Cl	*n*-hept	2	2	8	-

64	4-Cl	CH-cyclohexane	2	8	16	32
65	4-Cl	$(CH_2)_2$-cyclopentane	4	4	16	-
66	4-Cl	CH_2NHNH_2	32	64	>64	>64
67	H	COOH	>64	>64	>64	>64
68	2-OH,4-Cl	CH-cyclohexane	8	8	16	-
69			64	128	128	128
70			64	64	128	-
71			32	64	128	128
72			32	64	128	
73			-	-	-	-

[a] compounds isolated as the HCl salts; [b] inactive at 128 μg mL^{-1}

The parent imine C-alkyl analogue in this series **56** was inactive at 64 μg mL^{-1}, with the corresponding 4-*t*-Bu **57** 16-64 μg mL^{-1} active across all bacteria screened (Table 3). Introduction of the imine C-methyl to **53** gave **58**, which showed a 4-fold improved Gram-positive activity and some Gram-negative activity. Conducting a systematic evaluation of the effect of the C-alkyl moiety we examined the activity of a series of 4-Cl analogues through an ethyl **59**, propyl **60**, butyl **61**, pentyl **62**, CH-cyclohexane **64** and $(CH_2)_2$-cyclopentane **65**. Over this series, the antibiotic activity increased from methyl **64** to pentyl **70** with the latter analogue returning MIC values of 8 μg mL^{-1} against MRSA and VRE, and 16 and 32 μg μL^{-1} against *E. coli* and *Ps. aeruginosa* respectively. Further increasing in the size of the alkyl moiety was detrimental to activity.

Our findings within this compound series, that a reduction in molecular weight combined with an increase in polarity affords promising levels of Gram-negative activity, is in keeping with recent reports [23]. This is in part a consequence of reduced efflux liability [24], combined with the poor passive permeability through the outer membrane of gram-negative bacteria of hydrophobic molecules [25-27].

Having established the antibacterial activity of this class of compounds, we examined the activity of **64** and **63** against Gram-positive and Gram-negative isolates obtained from clinical cases of infection including, methicillin resistant *Staphylococcus aureus* (MRSA, n=20), *Escherichia coli* (n=20), *Enterobacter aerogenes* (n=16), *Enterobacter cloacae* (n=19), *Klebsiella pneumoniae* (n=20) and *Klebsiella oxytoca* (n=16). Both analogues (**64** and **63**) were bactericidal against all of the strains tested with MBC/MIC$_{90}$ ratios ≤ 2 (Table 4). The results demonstrated potent activity against major target pathogens in human isolates. Time−kill curve evaluation of **64** and **63** at 1x 2x and 4x their MIC against one susceptible *S. aureus* isolate (ATCC 25923), one clinical MRSA isolate (USA300) and one clinical *E. coli* isolate and one clinical *K. pneumoniae* ATCC 4352 confirmed their bactericidal activity, resulting in a >5-log reduction in bacterial cell number over an 0.5 h period across both analogue doses evaluated (Figure 4). After 24 h, no bacteria regrowth was observed with both compounds at all concentrations tested. Introduction of polar C-substituents was detrimental to activity, with CH_2NHNH_2 **66** and COOH **67** showing reduced activity. However introduction of a hydrogen bonding moiety to the aromatic ring with the 2-OH,4-Cl **68** was well tolerated with MIC values of 8-16 μg mL^{-1} observed. Increasing the size of the aromatic moiety to naphthyl with **69** – **71**, extension of the conjugation between the aromatic moiety and the amino warhead with **72**, or via an indole **73** all resulted in a loss of activity suggesting that the phenyl moiety maybe the optimal aromatic moiety in this compound series.

Table 4. MIC range, MIC$_{50}$, MIC$_{90}$, MBC values (μM) and MBC/MIC for **64** and **63** against *methicillin* resistant *staphylococcus aureus* (MRSA, n=20), *Escherichia coli* (n=20), *Enterobacter aerogenes* (n=16), *Enterobacter cloacae* (n=19), *Klebsiella pneumoniae* (n=20) and *Klebsiella oxytoca* (n=16) isolated from human clinical cases.

	MIC[a] values (μM) for **64**			MBC[d] range (μM)	MBC/ MIC	MIC values (μM) for **63**			MBC range (μM)	MBC/ MIC
	MIC range	MIC$_{50}$[b]	MIC$_{90}$[c]			MIC range	MIC$_{50}$	MIC$_{90}$		
MRSA (n=20)	6.8	6.8	6.8	6.8	1 (85%)	3.4-6.8	3.4	3.4	3.4	1 (90%)
E. coli (n=20)	27.3-218.6	109.3	109.3	27.3-437.2	1 (95%)	6.8-54.3	27.1	27.1	6.8-108.5	1 (85%)

E. aerogenes (n=16)	27.3-218.6	109.3	109.3	27.3-437.2	1 (88%)	6.8-54.3	27.1	27.1	6.8-108.5	1 (94%)
E. cloacae (n=19)	27.3-218.6	109.3	218.6	27.3-437.2	1 (89%)	6.8-108.5	27.1	108.5	6.8-217	1 (89%)
K. pneumoniae (n=20)	54.6-218.6	109.3	218.6	54.6-437.2	1 (95%)	6.8-108.5	27.1	54.3	6.8-217	1 (80%)
K. oxytoca (n=16)	54.6-218.6	109.3	109.3	54.6-437.2	1 (87%)	13.6-108.5	27.1	54.3	6.8-217	1 (94%)

[a] MIC = drug concentration required to inhibit bacterial growth, [b] MIC_{50} = drug concentration required to inhibit 50% of the bacteria isolates tested. [c] MIC_{90} = drug concentration required to inhibit 90% of the bacteria isolates tested, [d] MBC = drug concentration required to kill 99.9% bacteria.

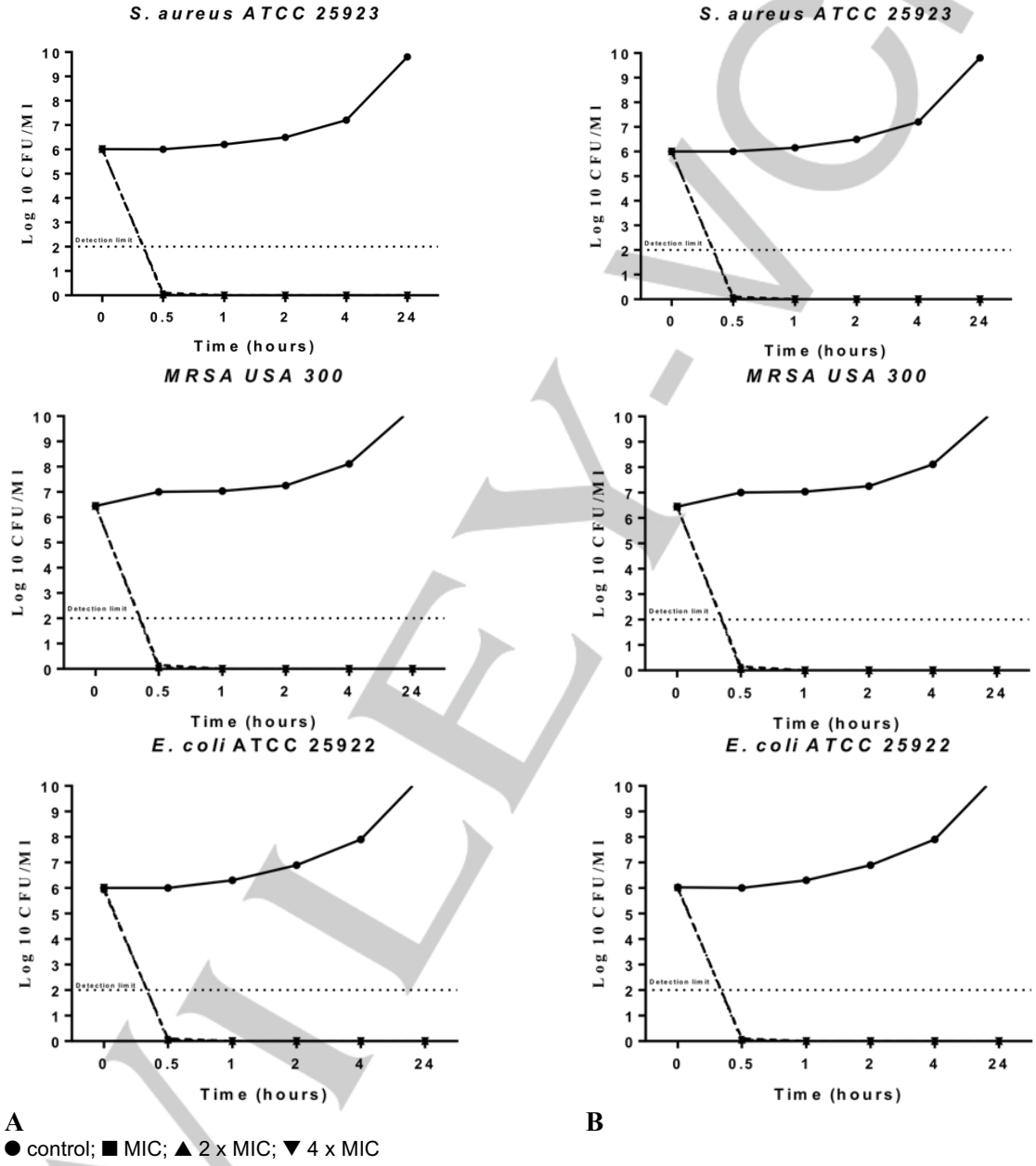


Figure 4. Time−kill assay of (**A**) **64** and (B) **63** against methicillin-sensitive *S. aureus*, methicillin-resistant *S. aureus* USA300, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 4352. Cultures were inoculated with 10^6 CFU/mL bacteria and exposed to increasing concentrations of compounds for 24 h. Samples were taken at 0.5, 1, 2, 4 and 24 h to determine viable cell numbers. After 30 min, all MIC data shows zero CFU, with all data points overlapping.

Conclusions

The robenidine core has proved to be amenable to multiple structural modifications that allowed retention, and enhancement, of antibiotic activity. In particular the introduction of asymmetry and the inclusion of imine C-alkyl substituents were well tolerated.

Of particular note was the increased potency of **17** and **20** against VRE with **20** the most active with an MIC of 0.5 μg mL^{-1}. In this initial library analogues **2**, **14**, **17**, **19** and **20** were equipotent or more potent than the lead **1**. The introduction of an indole moiety with **30** resulted in the most MRSA robenidine based analogue to date with an MIC of 0.5 μg mL^{-1}. In all instances imine C=NH isosteres were poorly tolerates; the C=O and C=S isosteres were inactive.

Simple monomeric analogues, e.g. **33** – **35** returned low levels of Gram-positive activity at ca. 16 – 64 μg mL^{-1}. Surprisingly removal of an NH moiety from the terminal hydrazide of **33** in the synthesis of **36** gave rise to modest Gram-negative activity at 64 μg mL^{-1}. Building on this finding, screening of a focused library revealed the 4-*t*-butyl **45** with broad spectrum Gram-positive and Gram-negative activity with MICs of 16 – 64 μg mL^{-1}. Other modifications to the aromatic substituent were poorly tolerated, but modifications in conjunction with the introduction of an imine C-alkyl moiety gave rise to more promising levels of both Gram-positive and Gram-negative antibiotic activity. Gram-positive MIC values ranged from 8 μg mL^{-1} with **64** and **68**, to inactive (MIC > 128 μg mL^{-1}) with the naphthyl **69** and **70** and the indole **73** based analogues. Gram-negative activity was most promising with **62** and **68** at 16 μg mL^{-1} against *E. coli*. Against *Ps. aeruginosa*, the highest activity observed was with MIC values of 32 μg mL^{-1} with **62** and **64**. As a result, we believe that the (*E*)-2-benzylidenehydrazine-1-carboximidamide scaffold is a promising development candidate targeting both Gram-positive and Gram-negative bacteria.

Acknowledgements

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Experimental

Microbiology

Bacterial isolates

A total of 20 MRSA clinical isolates, details listed in the Supplementary Data, were kindly provided by Professor Geoffrey Coombs (Department of Microbiology, Pathwest Laboratory Medicine WA). A total of *Escherichia coli* (n=20), *Enterobacter aerogenes* (n=16), *Enterobacter cloacae* (n=19), *Klebsiella oxytoca* (n=16) and *Klebsiella pneumoniae* (n=20) clinical isolates were kindly provided by Mrs Jan Bell (SA Pathology, Institute of Medical and Veterinary Science, South Australia). *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, MSSA strains ATCC 25922 and 25923 were obtained from the American Type Culture Collection. Isolates used in initial screening assay were sourced as follows: SCCmec type IV MRSA and VRE, multidrug resistant clinical isolates for initial screen of NCL812 analogues were kindly provided by Professor Mary Barton (University of South Australia).

Susceptibility testing

The MIC of all analogues was determined using a slightly modified microdilution method described in CLSI guidelines,[26] as follows: All analogues were dissolved in 100% DMSO to a concentration of 12.8 μg.mL^{-1}. Luria Bertani (LB) broth was used instead of CAMHB as it has been previously shown that **1** can chelate calcium ions.[27] In addition the antimicrobial dilutions of all analogues were completed in 100% DMSO, with 1 μL added to each well, as the compounds are hydrophobic. The assay was performed in a total volume of 100 μL with test concentration increasing 2-fold from 0.25 μg.mL^{-1} to 128 μg.mL in 96 well plates. MIC tests involving ampicillin were performed according to CLSI guidelines in CAMHB. Plates were incubated for 20 - 24 hours at 37°C before determination of the MIC. Similarly, the MIC_{50} and MIC_{90} for **63** and **64** were determined as described above.

Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) was determined according to the CLSI guidelines.[26] Briefly, the MIC assay was extended and after determination of the MIC 10 μL aliquots were taken from all wells above the MIC and spotted onto sheep blood agar. The plates were incubated for 24 and 48 hours before determination of the MBC as the lowest concentration where 99.9% of the final inoculum is killed.

Time kill assays

Time kill assays were performed in 20mL samples at 1×, 2× and 4× the MIC_{90} of each compound in glass flasks according to CLSI guidelines with LB broth replacing the CAMHB.[28] The compounds were serially diluted in 100% DMSO at 100x the final desired concentration and 200 μL of appropriate concentrations added to each flask. Cultures were incubated at 37 °C with samples taken at 0, 0.5, 1, 2, 4 and 24 hours.

Chemistry - General Methods

All reagents were purchased from Sigma-Aldrich, AK Scientific, Matrix Scientific or Lancaster Synthesis and were used without purification. All solvents were re-distilled from glass prior to use.

^{1}H, ^{13}C and ^{19}F (decoupled) NMR spectra were recorded on a Bruker Advance™ AMX 400 at 400.13, 100.62 and 376 MHz, respectively and Advance™ AMX 600 at 600.21 and 150.92 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm) measured relative to the internal standards. Coupling constants (*J*) are expressed in hertz (Hz). Mass spectra were recorded on a Shimadzu LCMS 2010 EV and Agilent 6100 series single quadrupole LCMS using a mobile phase of 1 : 1 acetonitrile :H_2O with 0.1% formic acid. The University of Wollongong, Australia, Mass Spectrometry User resource & Research Facility (MSURRF) analysed samples for High Resolution Mass Spectrometry HRMS Analytical HPLC traces were obtained using a Shimadzu system possessing a SIL-20A auto-sampler, dual LC-20AP pumps, CBM-20A bus module, CTO-20A column heater, and a SPD-20A UV/vis detector. This system was fitted with an Alltima™ C_{18} 5 μm 150 mm × 4.6 mm column with solvent A: 0.06% trifluoroacetic acid (TFA) in water and solvent B: 0.06% TFA in CH_3CN–H_2O (90 : 10). In each case HPLC traces were acquired at a flow rate of 2.0 mL min^{-1}, gradient 10–100 (%B), over 15.0 min, with detection at 220 nm and 254 nm. All samples returned satisfactory analyses. Compound purity was confirmed by a combination of LC-MS (HPLC), micro and/or high resolution mass spectrometry and NMR analysis. All analogues are ≥ 95% purity.

Melting points were recorded on a Büchi Melting Point M-565 instrument. IR spectra were recorded on a PerkinElmer Spectrum Two™ FTIR Spectrometer with the UATR accessories. Thin layer chromatography (TLC) was performed on Merck 60 F254 pre-coated aluminium plates with a thickness of 0.2 mm. Column chromatography was performed under 'flash' conditions on Merck silica gel 60 (230–400 mesh).

Compounds **3**, **4**, **8**, **9-11**, **15-17, 21-23**, **69**, **71**, **S1**, **S3**, and **S5-S7**, **S36**, **S37** were synthesized by an external contractor, EpiChem Pty Ltd (Melbourne, Australia).

General Method A

A suspension of aldehyde or phenone (2.2 equiv) and 1,3-diaminoguanidine hydrochloride (1.0 equiv) in EtOH (5 mL) was subjected to microwave irradiation (150 W) at 100 °C for 10 min. Most of the solvent was then removed *in vacuo*, Et_2O (5 mL) was added, and the flask was chilled to effect crystallization. The resulting precipitate was collected and washed with Et_2O (5 mL) to afford the carbonimidic dihydrazide.

General Method B

A suspension of 1,3-diaminoguanidine hydrochloride (3.0 equiv) in water/EtOH (50/50 v/v, 20 mL) was heated at 70°C. A solution of aldehyde or phenone (1.0 equiv) in Et_2O (2 mL) was added to the flask and heating was continued for 4 hr. The reaction mixture was filtered hot and the filtrate concentrated to near dryness. The residue was dissolved in Et_2O and the organic was washed with water and dried. The resulting solid was recrystallized from EtOH/water (50/50 v/v) to afford the mono-adduct.

A solution of the mono-adduct (1.0 equiv) and second aldehyde or phenone (1.2 equiv) in anhydrous DMSO (3 mL) was stirred at ambient temperature for 16 hr. The reaction mixture was taken up in CH_2Cl_2 and the organic washed with $NaHCO_{3(aq)}$. The organic was concentrated and the crude material was dissolved in Et_2O (~5 mL). Hydrochloric acid (2M in ether, 3 mL) was added and the resulting precipitate was collected, washed with Et_2O and dried to afford the unsymmetrical carbonimidic dihydrazide.

General Method C

A suspension of the appropriate aldehyde or acetophenone (2.2 equiv) and N,N-diaminoguanidine hydrochloride (1.0 equiv) in EtOH was heated at reflux for 16 h. The mixture was concentrated and Et_2O added to effect crystallization. The resulting precipitate was collected and washed with Et_2O to afford the carbonimidic dihydrazide.

General Method D

A suspension of the appropriate aldehyde or acetophenone (1.0 equiv) and aminoguanidine hydrochloride (1.0 equiv) in EtOH was heated at reflux for 16 h. The mixture was concentrated and Et_2O added to effect crystallization. The resulting precipitate was collected and washed with Et_2O to afford the carbonimidic hydrazide.

General Method E

Chlorobenzene (2.0 equiv) and aluminium chloride (1.0 equiv) were stirred together before the slow addition of the appropriate acid chloride (1.0 equiv), the reaction was stirred at room temperature overnight. The reaction mixture was added to ice and diluted with 10% HCl, and then extracted with 2 x $CHCl_3$. The combined organic phases were washed with brine, saturated sodium bicarbonate and then dried. The solvent was then removed to afford the desired substituted phenylketone.

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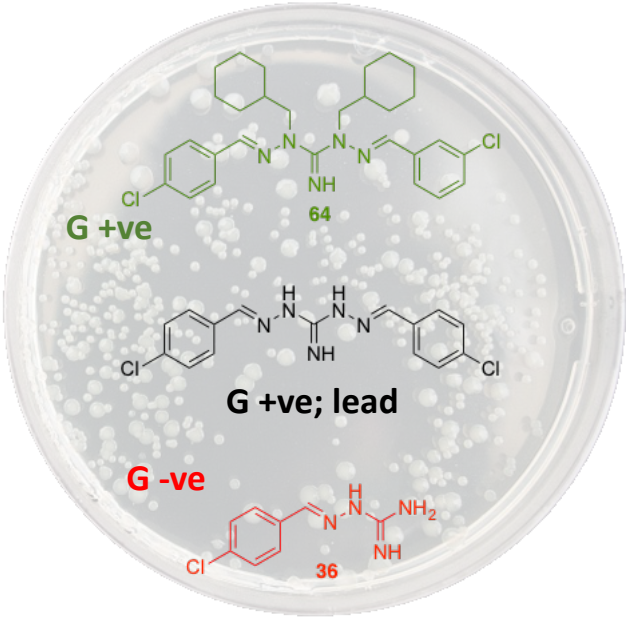

Repurposing Robenidine leads to novel Gram-positive and Gram-negative agents with activity against MRSA, VRE, *E. coli* and *Ps. Aeruginosa*. The most promising analogues return MIC values from 0.5-16 μg mL-1 against Gram-positive MRSA and VRE; and against the Gram-negative *E. coli* and *Ps. Aeruginosa* MIC values of 16 and 32 μg mL^{-1} were noted.