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Why Antibacterial Minor Groove Binders Are a Good Thing

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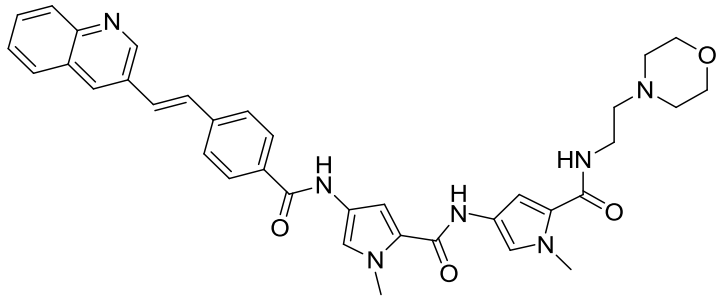
Why Antibacterial Minor Groove Binders Are a Good Thing

Graphical Abstract

MGB-BP-3, a resilient and effective antibiotic against Gram-positive bacteria.



Abstract: The challenge of antimicrobial resistance is well understood. Combining potency with resilience is unlikely to be met using the standard medicinal chemistry paradigm of single drug, single target, single effect. Our approach using specially designed minor groove binders for DNA (Strathclyde MGBs), whilst formally attacking a single molecular target, in practice disrupts many biological processes such that the emergence of resistance can be expected to be low. The first example of this approach to reach the clinic, MGB-BP-3, is highly effective against Gram positive bacteria and has been successfully completed a Phase 1 clinical trial against *Clostridium difficile* infections by our development partner, MGB Biopharma. Mechanism of action studies with *S. aureus* as the target organism provided evidence consistent with the expectation. RNAseq experiments show that there are substantial changes in gene expression such that the bacterium faces multiple metabolic challenges to its survival. In particular processes associated with cell wall integrity and energy production are affected. Attempts to generate resistant strains have failed. Taken together, these properties identify Strathclyde minor groove binders as significant new compounds in the fight against antibacterial resistance.
Keywords: antibacterial, minor groove binder, DNA, antimicrobial resistance, mechanism of action,

Introduction

MGB-BP-3 (Figure 1) is a novel antibiotic candidate derived by chemical-synthesis and SAR from Distamycin - a natural product antibiotic that acts by binding to the minor groove of DNA. It that has very strong antibacterial activity against all susceptible and multi-resistant Gram-positive pathogens tested, including methicillin-resistant and susceptible *Staphylococcus* species, pathogenic *Streptococcus* species, Vancomycin-Resistant and susceptible *Enterococcus* and *Clostridium difficile.*

The oral formulation of MGB-BP-3 has successfully completed a clinical Phase I study for the treatment of *C. difficile* infections and through our development partner, MGB Biopharma, has been granted qualified infectious disease product (QIPD) status by the U.S. FDA for the treatment of *Clostridium difficile*-associated Diarrhoea (CDAD).

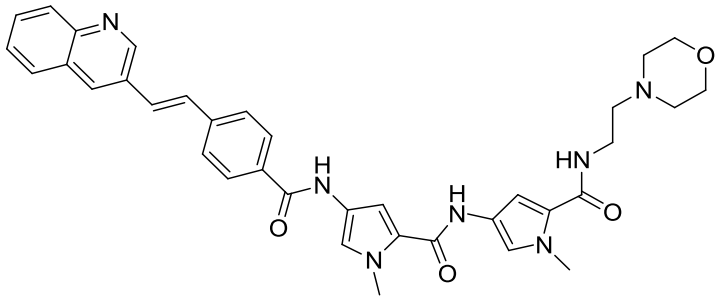


Figure 1. Structure of MGB-BP-3

Resilience to the generation of resistance is very important in a new antibiotic. The discovery concept for MGB-BP3 and related minor groove binders from Strathclyde (S-MGBs) was to design compounds that have low toxicity to mammalian cells but, by targeting bacterial DNA, cause multiple disruption to bacterial metabolism thereby minimising the probability of the development of resistance. This study investigates the mode of action of MGB-BP-3 and applies RNA-sequencing transcriptomics to determine the effect of drug on global gene expression of *Staphylococcus aureus* and demonstrates high resilience to the development of resistance.

The antibacterial drug, MGB-BP-3

The general antibacterial properties of MGB-BP-3 are introduced in the next three slides.

MGB-BP-3 is broadly active against Gram-positive bacteria (Table 1) but is only weakly active at non-clinically useful levels against Gram-negative bacteria. It is probable that the lack of activity against Gram-negative bacteria is associated with the function of efflux pumps. MGB-BP-3 is fluorescent allowing its uptake into cells to be observed (Figure 2). Uptake is clearly different in different cell types; *S. aureus* cells can be seen to light up but the mouse melanoma cell line, B16F0/*luc*, does not. This provides evidence for the intracellular activity of MGB-BP-3 and for its selective toxicity.

The data in Table 1 show that the MICs of MGB-BP-3 are 1 mg/L or less for many Gram-positive bacteria. These numbers are approximately equivalent to sub-micromolar for the test substance, the bis-trifluoroacetate salt (MW 859.7) and allow a comparison with concentration dependence of viability of mammalian cell lines with the MICs (Figure 3). This comparison indicates that MGB-BP-3 has an *in vitro* safety index of up to 500 fold depending upon the cell line used.. Evidently at high concentrations mammalian cells are affected but this is well above the expected clinical concentration to be used.

Questions can now be posed.

Is the multiple target multiple effect design plan real? Addressed by RNAseq analysis.

What effects does binding to DNA by MGB-BP-3 have? Also addressed by RNAseq analysis.

Are these effects consistent with a reasonable interpretation of the biology of BP3? Addressed by RNAseq analysis, PCR evaluation of changes in protein levels, and DNA footprinting of MGB-BP-3 itself.

Is MGB-BP-3 resilient to the development of resistance?

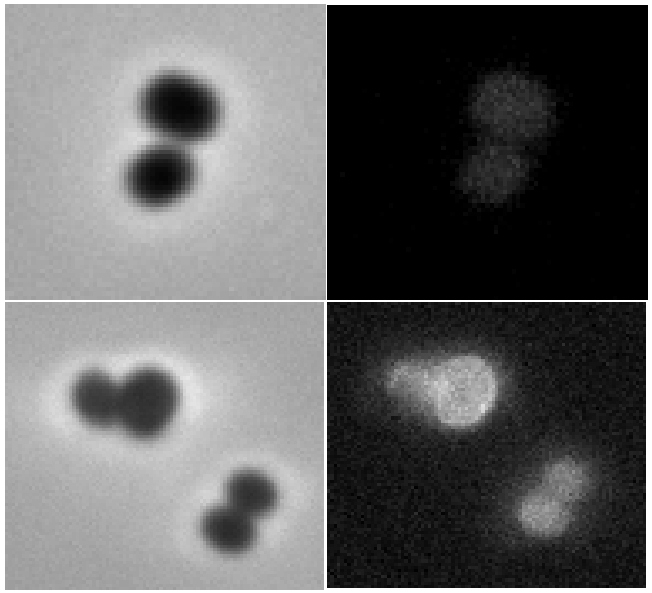
Examples of species selectivity of MGB-BP3 (Table 1)

Organism	MGB-BP-3				
	n=	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MBC_{50} (mg/L)	MBC_{90} (mg/L)
Group B Streptococci	15	0.25	1	0.25	1
Group C Streptococci	15	0.25	1	0.5	1
Group G Streptococci	15	0.5	0.5	0.5	0.5
Methicillin-resistant *Staphylococcus aureus*	15	1	2	1	2
Methicillin-resistant *Staphylococcus epidermidis*	15	0.25	0.5	0.5	2
Methicillin-susceptible *Staphylococcus aureus*	15	0.5	1	1	2
Methicillin-susceptible *Staphylococcus epidermidis*	15	0.25	0.5	0.25	2
Streptococcus constellatus	15	0.25	0.5	0.5	1
Streptococcus mitis	15	0.5	2	0.5	2
Streptococcus pyogenes	15	0.25	0.5	0.25	2
Vancomycin-resistant *Enterococcus faecalis*	15	2	2	>32	>32
Vancomycin-resistant *Enterococcus faecium*	15	1	2	>32	>32
Vancomycin-susceptible *Enterococcus faecalis*	15	1	2	>32	>32
Vancomycin-susceptible *Enterococcus faecium*	15	1	2	>32	>32

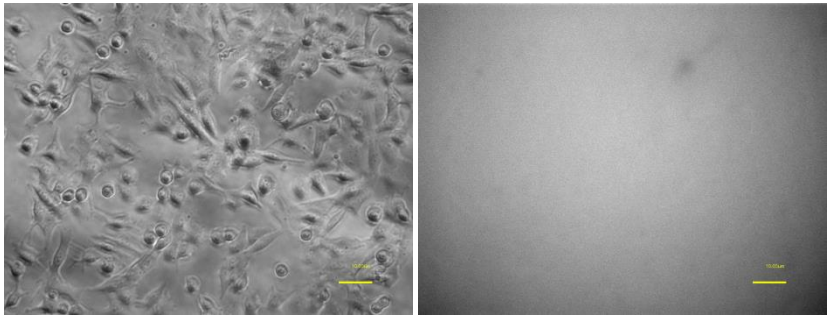
MGB Biopharma
Innovative Development Company

Selective uptake of MGB-BP-3 by bacterial and mammalian cells

S. aureus without MGB-BP3
Brightfield under UV



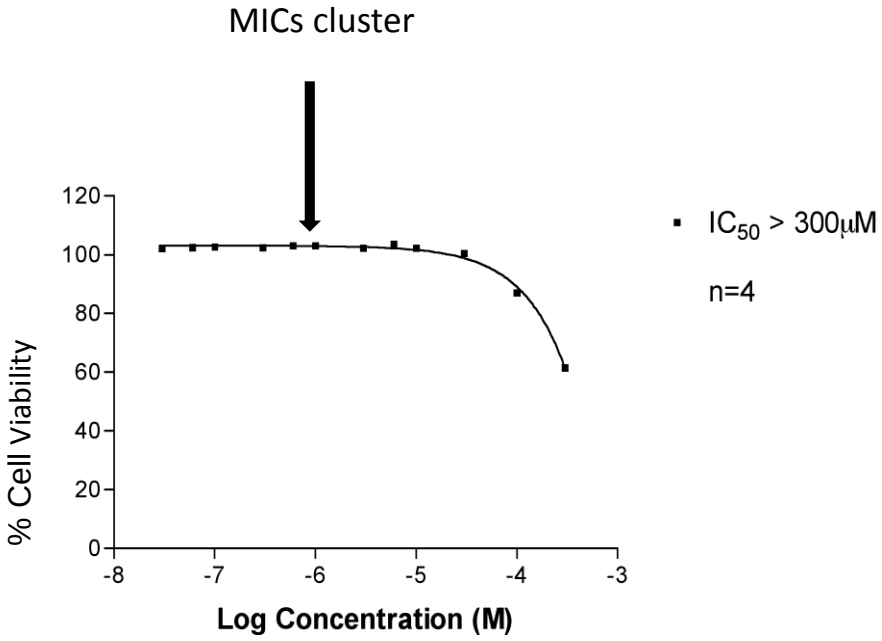
S. aureus with MGB-BP3
Brightfield under UV



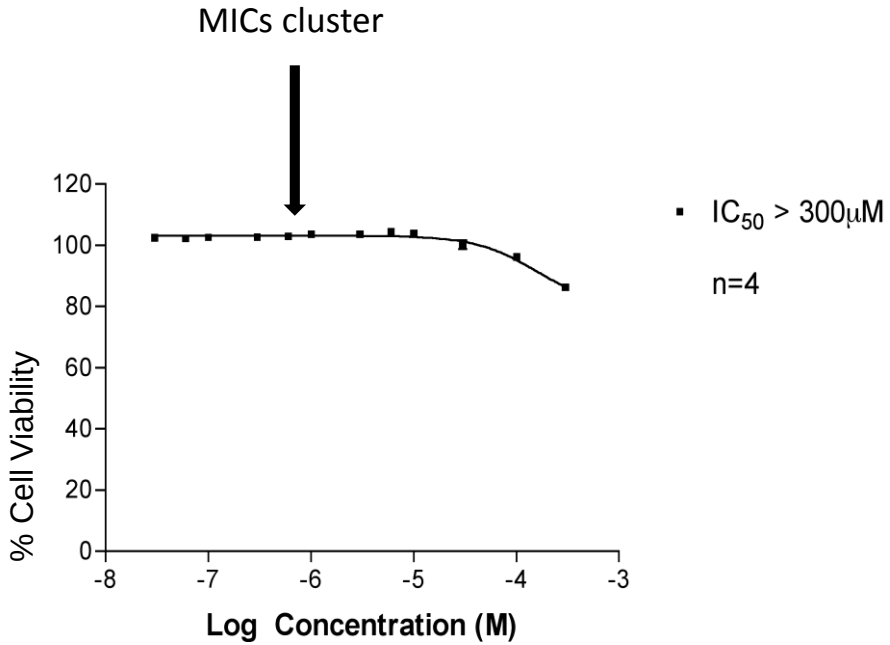
B16FOluc cells with MGB-BP3
Brightfield under UV

Figure 2

Low toxicity of MGB-BP-3 to mammalian cell lines



MGB-BP-3 on HS27, human fibroblast cells



MGB-BP-3 on L929, mouse fibroblast cells

Figure 3. Data obtained by Carol Clements, University of Strathclyde

MGB-BP-3 as a drug

MGB-BP-3 is a lipophilic molecule that is known to undergo self-association in aqueous solution. Self association of MGB-BP-3 and its relatives has been studied in detail by NMR spectroscopy (Parkinson *et.al. Med. Chem. Commun.,* **2013**, *4*, 1105-1108), which showed that a head-to-tail dimer was especially stable in aqueous solution; this dimer is believed to bind to DNA intact leading to exceptionally tight binding (Suckling *et.al. J. Med. Chem.* **2007**, *50*, 6116-6125).

Rheological measurements show time an temperature dependent molecular aggregation. Dynamic light scattering of dilute solutions shows presence of two aggregate particles (radii ~ 5 nm to 100 nm). Clear solutions, colloidal phases, and gel phases have all been observed. The type of solution observed depends upon pH, composition, concentration, and temperature. Consistent with self-aggregation, the pK_a of the morpholino tail group is unusually low (Figure 4). These properties lead to non-absorption from the GI tract making MGB-BP-3 ideal for treatment of *Clostridium difficile* infections for which it has been developed by our partner company, MGB Biopharma.

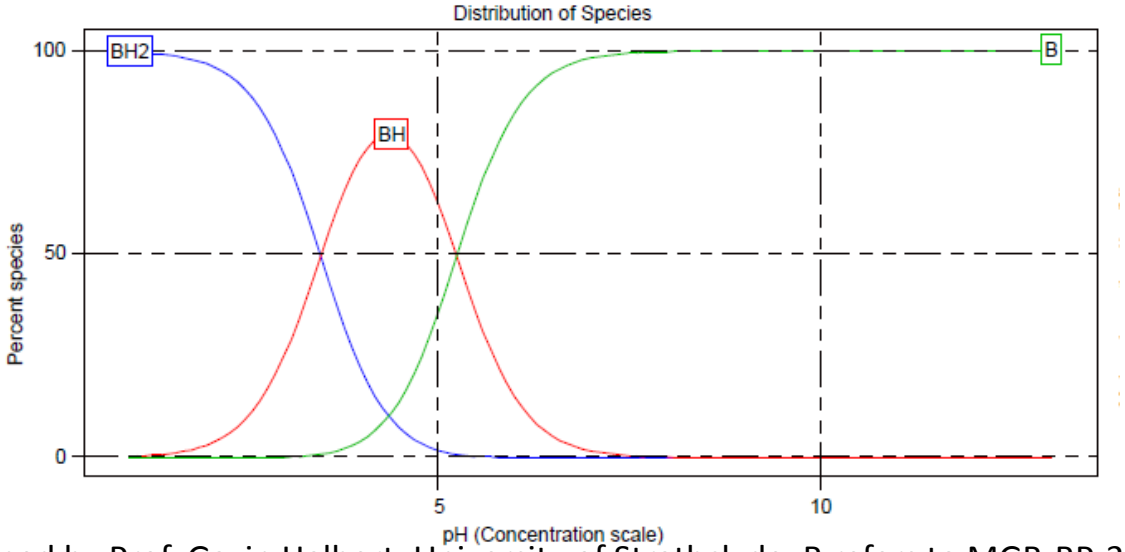


Figure 4. Data obtained by Prof. Gavin Halbert, University of Strathclyde. B refers to MGB-BP-3.

Results and discussion

The protocol for the RNA-seq experiments was as follows:
RNA-seq analysis was undertaken on *S. aureus* following challenge with 0.5 x MIC (0.1 µg/mL) MGB-BP-3. Triplicate samples of RNA were extracted at 10 min after challenge (Figure 5). Approximately 5 - 7.5 million sequencing reads were obtained per sample (average length between 100 - 170 bp) using an Ion Torrent PGM.

Culture *Staphylococcus aureus* with and without MGB
(3 biological replicates)

→ QC check

Isolate high quality total RNA:

RNA protect bacterial reagent
Mechanical cell lyses with zirconia beads (0.1 mm)
RNA isolation with phenol/chloroform extraction
Silica filter purification of total RNA
DNase I treatment
Removal of DNase I and divalent cations

→ QC check

total RNA concentration per sample standardized to 4.5 µg in 26 µl

Depletion of ribosomal RNA (ribozero kit)

→ QC check

RNA-seq. library preparation

→ QC check

Barcoded libraries pooled at equal conc. (ctrl vrs. treated)
ctrl and MGB treated RNA-seq. samples diluted to 20 pM

Sequencing with Ion Torrent PGM

Results from RNA-seq experiment

The data were analysed using CLC Genomics Workbench 7.5.1 software (Qiagen). The 'Empirical Analysis of DGE' (Differential Gene Expression) Tool was used, with Bonferroni-adjusted p-value, to trimmed reads.

The biological replicates were found to group together in a PCA plot (Figure 6).

RNA-Seq analysis identified 698 transcripts showing significant changes in expression profile (Figure 7). Key enzymes of glycolysis were enhanced whereas the pentose phosphate pathway was reduced; flux through the TCA cycle was likely reduced significantly as citrate synthase and isocitrate dehydrogenase were reduced. These changes are associated with energy depletion. In addition, biosynthesis of nucleotides and certain amino acids were altered but it is not yet clear if the changes are directly due to MGB action or an indirect effect (Figure 8).

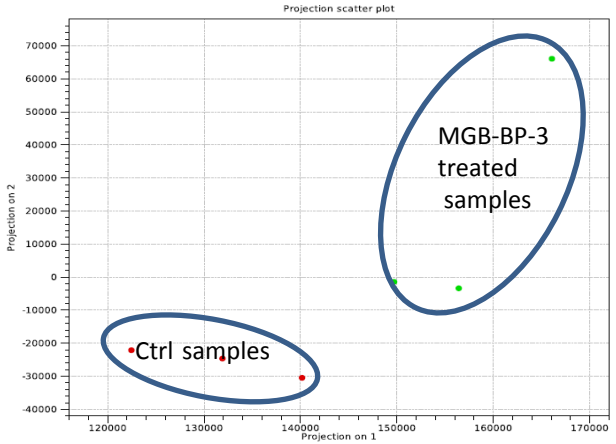


Figure 6.

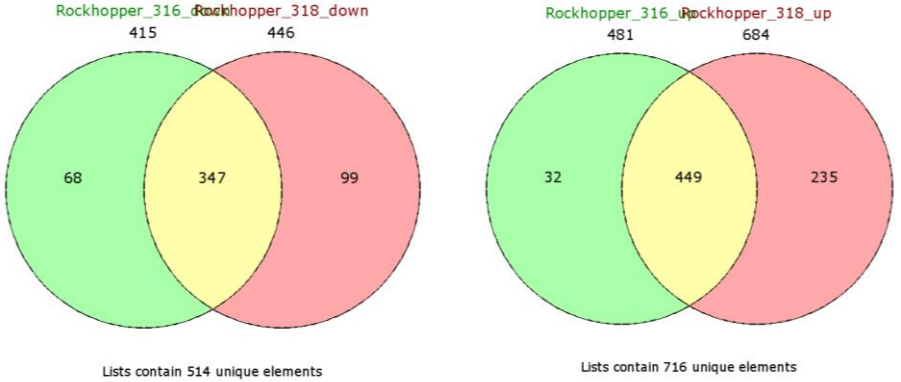


Figure 7.

Genes significantly downregulated

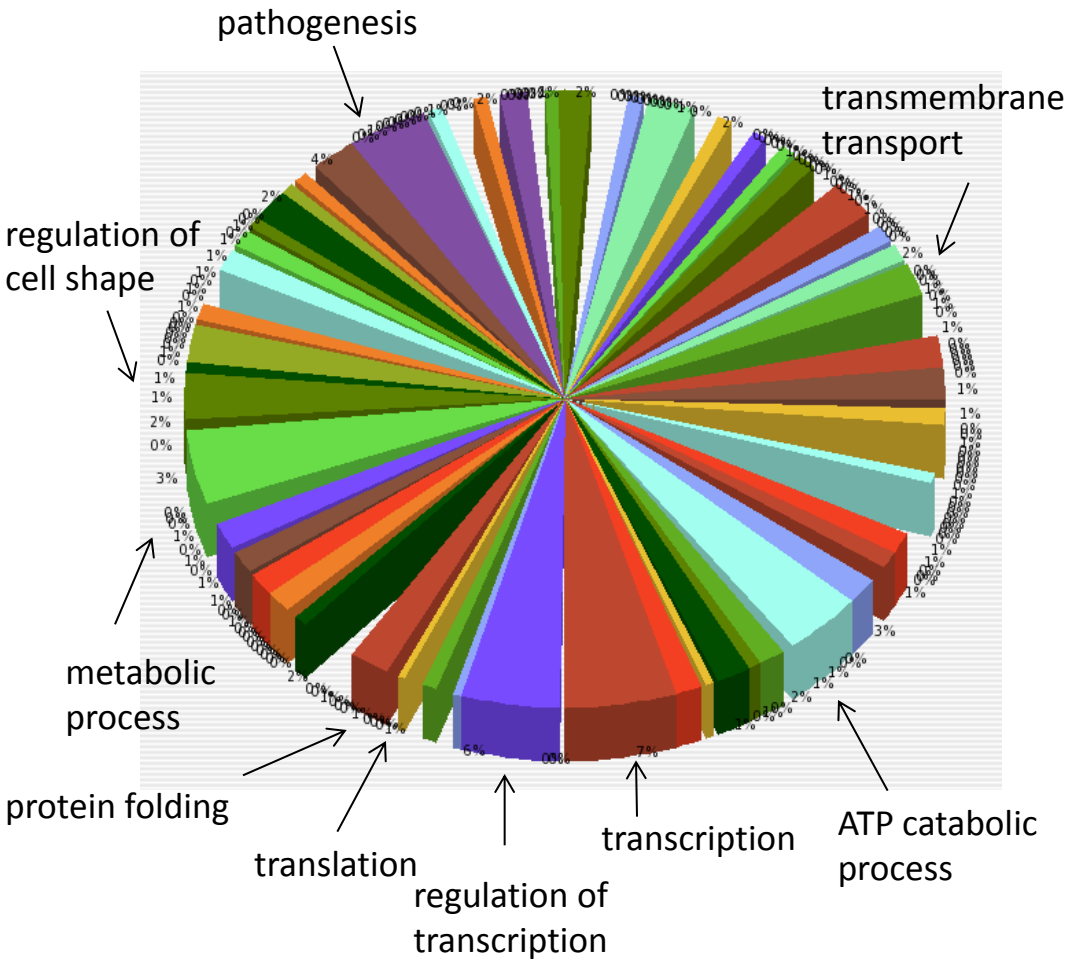
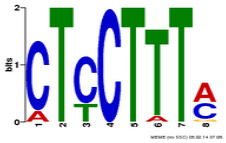


Figure 8.

Target binding sites suggested

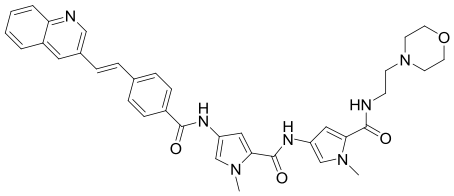
Scrutiny of the RNAseq results suggests a number of consensus binding sites for MGB-BP-3 on genomic DNA from *S. aureus* (Figure 9). These are AT rich, consistent with the known binding preferences of MGB-BP-3 as determined by footprinting (Prof Keith Fox, University of Southampton Figure 10).

- 3.1e+001
- 14 sites



- 4.6e+002
- 12 sites

Figure 9.



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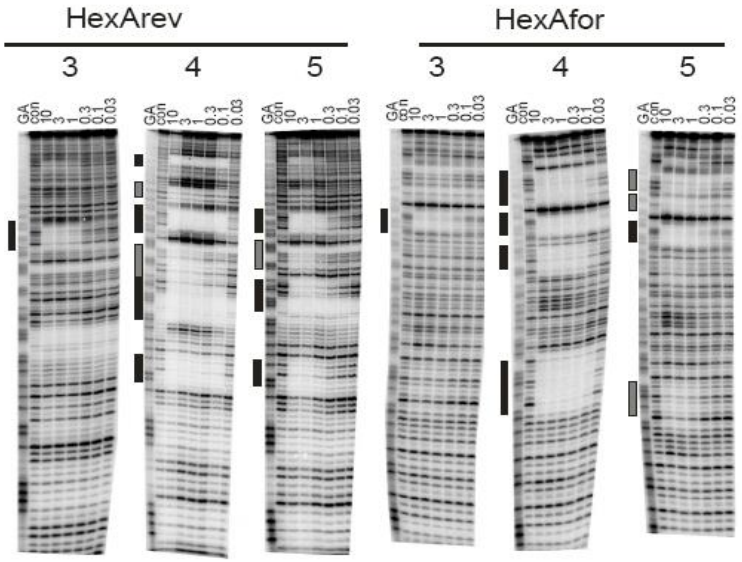

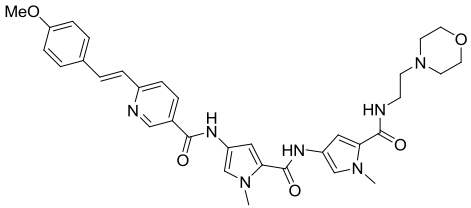

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Figure 10.

Possible significance of target binding sites

RNA-seq reveals differential gene expression in *S. aureus* with single nucleotide resolution from which some promoter sequences can be identified (Figure 11, red highlights). Interestingly, many of these contain target sequences for MGB-BP-3. Figure 12 shows the sequence of the polynucleotide, HexA, used as substrate for footprinting with 3 of the preferred binding sites for MGB-BP-3 that match the promoter sequences highlighted in yellow boxes. The combined evidence clearly demonstrates that MGB-BP-3 has multiple binding sites and can therefore be expected to have multiple effects.

Figure 11.

Figure 12.

Translation of gene effects into metabolism

Figures 13 and 14 show metabolic maps in which the effects of MGB-BP-3 are highlighted in red.

Figure 13, downregulated genes, shows substantial effects on nucleotide and cofactor metabolism (top right). Impaired input to the tricarboxylic acid cycle is also indicated together with downregulation of the electron transport chain; these effects would be expected to cause significant problems in energy production for the bacterium, which is consistent with the catastrophic death observed in kill curves caused by MGBs.

Figure 14 shows what could be considered to be the bacterial response to the challenge of MGB-BP-3. Clearly it is widespread. Glycolytic enzymes are seen to be upregulated ,perhaps to restore energy production. A shunt across the tricarboxylic acid cycle is highlighted. Lipid metabolism is also enhanced, perhaps suggesting that attempts to reinforce the cell wall are being made. Effects are also seen in nucleotide and cofactor metabolism.

Scientifically, it is important to confirm at least in some cases that the pattern emerging carries through to gene expression. This was done for 6 enzymes using qRT-PCR. The results (Figure 15) confirm the interpretation of the RNA-seq data.

Multiple effects on metabolism shown by downregulated genes

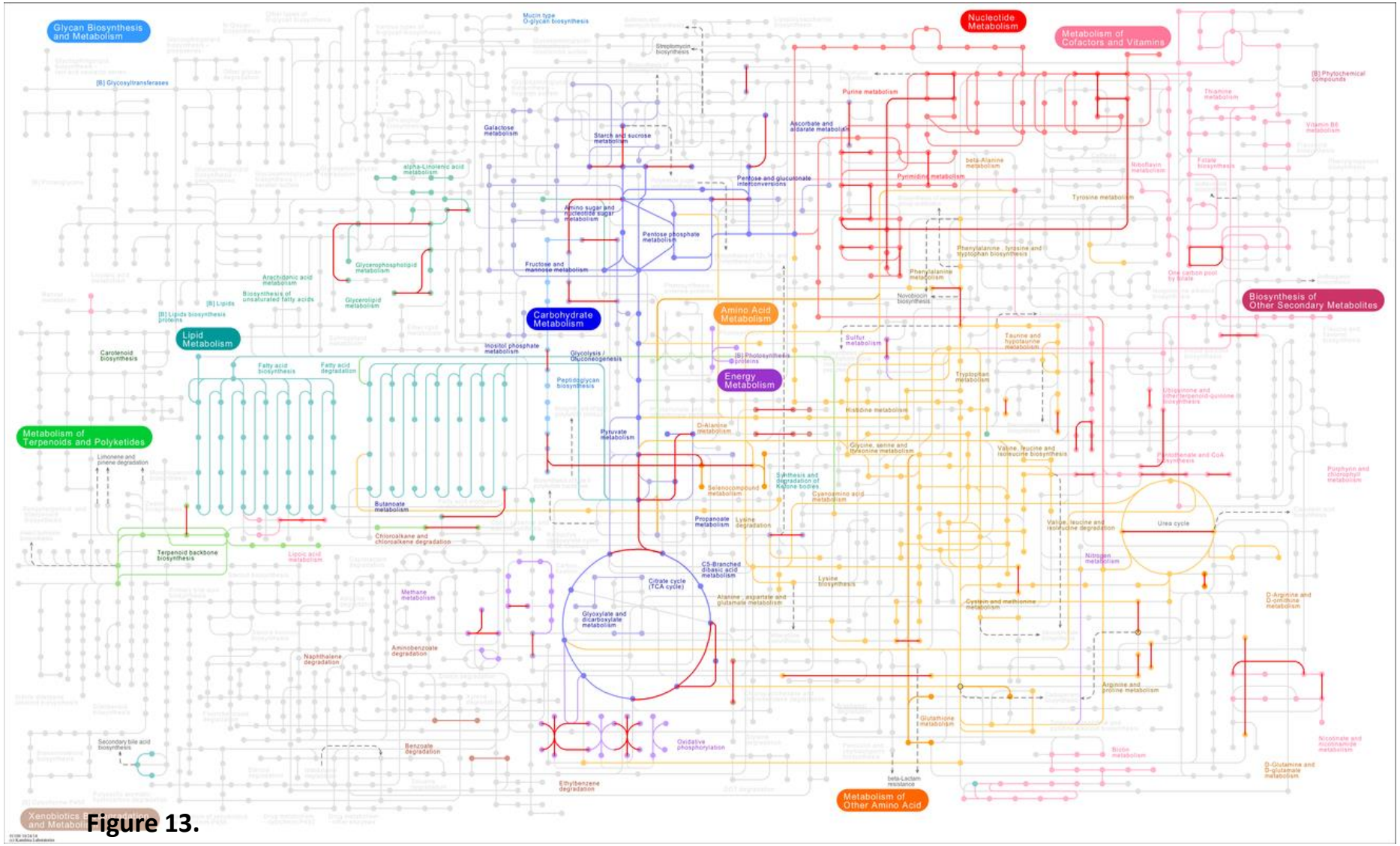

Figure 13.

Multiple effects on metabolism shown by upregulated genes

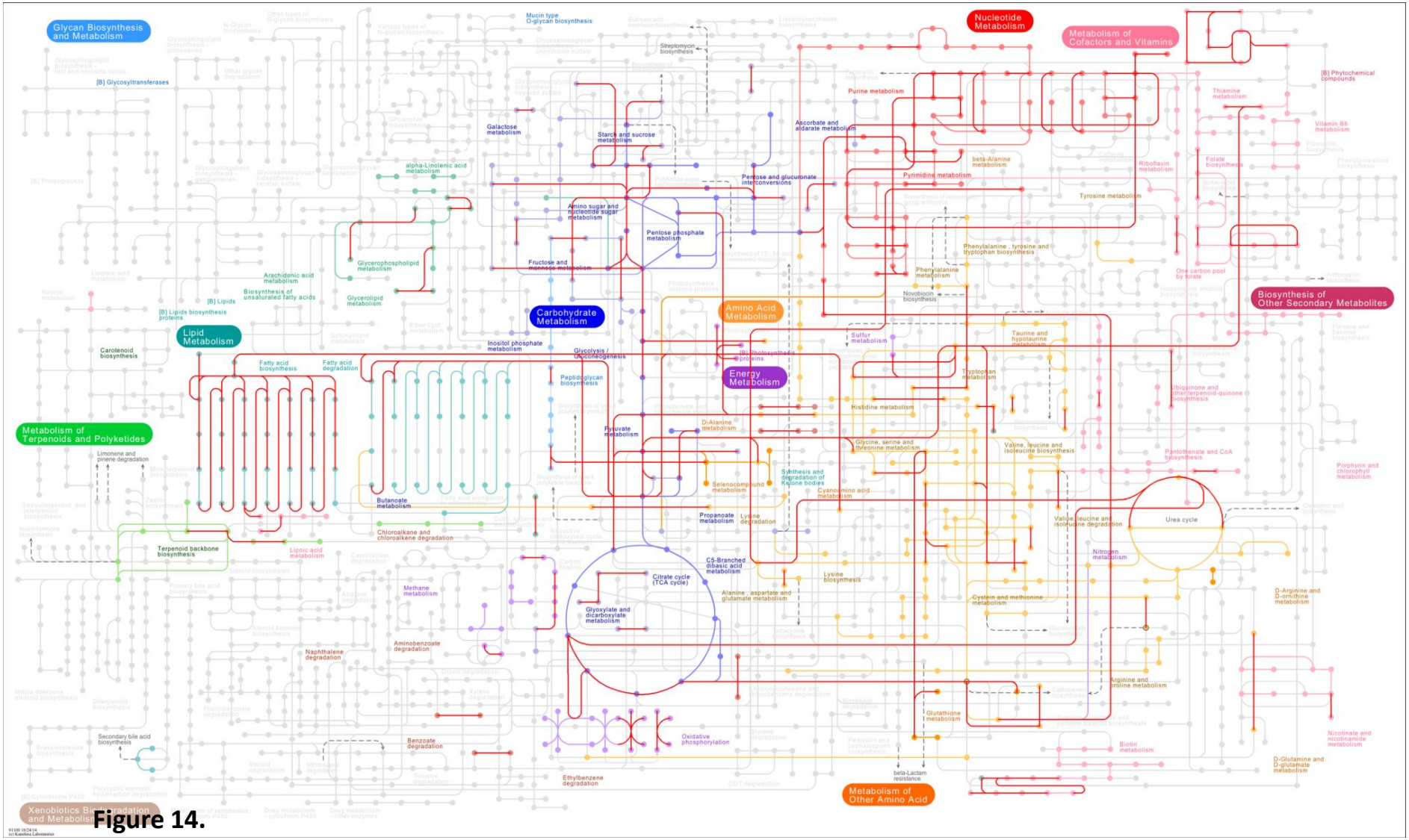

Figure 14.

PCR confirmation of RNA-seq output

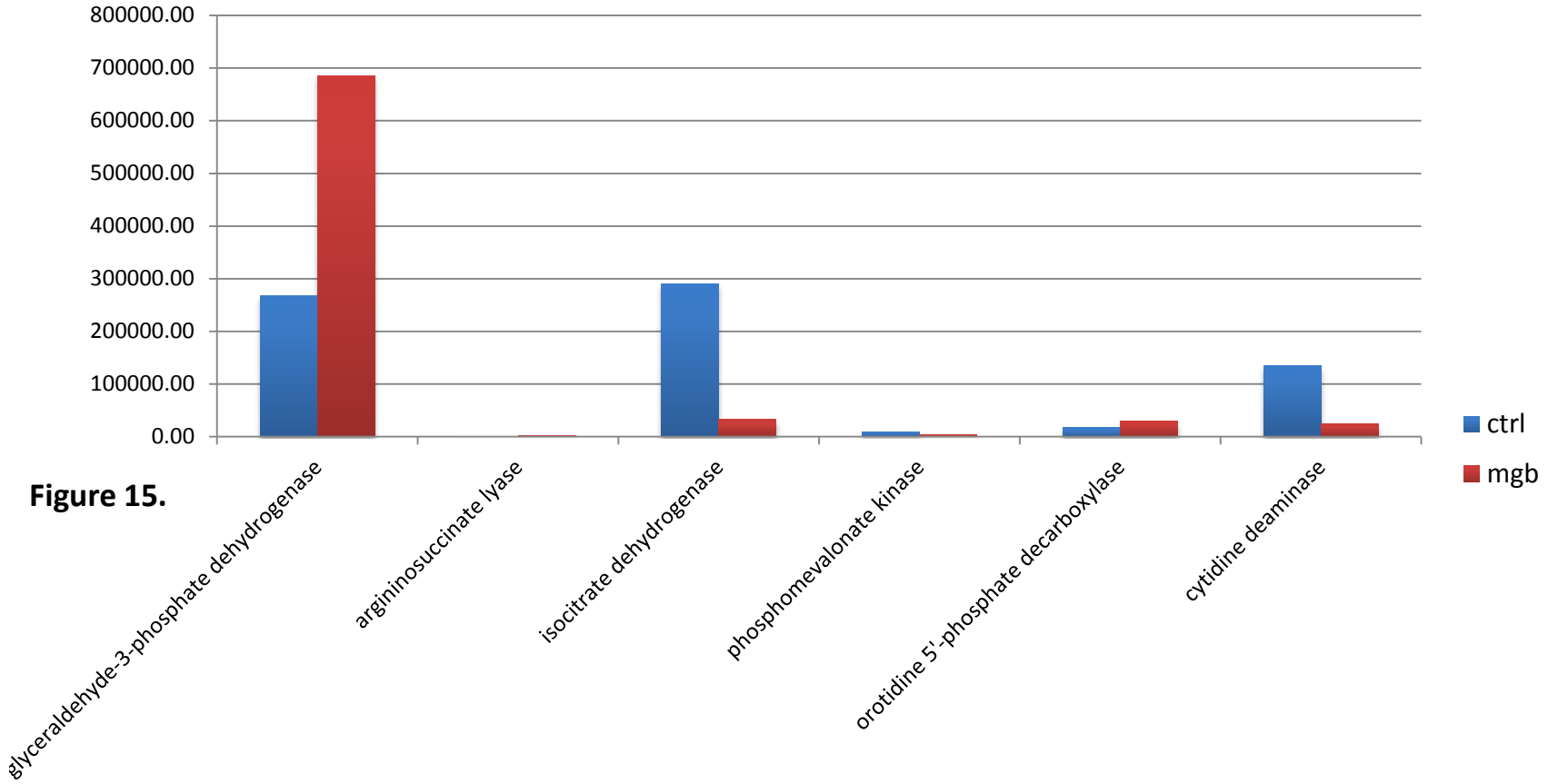

Figure 15.

To confirm the significance of RNA-seq data, qRT-PCR studies were undertaken with respect to selected genes, including both up and downregulated genes (Figure 15). It was found that the qRT-PCR data matches that from RNA-seq.

RNA-seq in the context of MGB-BP-3 as a resilient antibiotic

RNA-seq demonstrates that the design concept and expectations with MGB-BP-3 are supported by experimental results: there are multiple effects that contribute to the antibacterial action. In this context it is not possible to identify a single lethal event. Nevertheless, the PCR confirmation supported two genes of importance, namely those coding for phosphomevalonate kinase and isocitrate dehydrogenase; both have been identified as essential genes in *S. aureus* (Forsyth et al., *Molecular Microbiology* **2002**, *43*, 1387-1400; Christiansen et al., *PLOS One* **2014**, *9*, e89018.

In a further experiment, when *S. aureus* was grown at sub-MIC80 concentrations of MGB-BP-3 no resistance to BP3 was observed even after passaging for more than 200 generations. In contrast, resistance was observed to develop with rifampicin used under the same conditions. This is strong evidence for the resilience of MGB-BP-3 to drug induced resistance.

Conclusions

- **MGB-BP-3** is a new antibiotic with a wide range of biological effects on its target Gram-positive bacteria.
- Treatment of *S. aureus* with MGB-BP-3 causes a large number of changes in gene expression many associated with energy production.
- The binding sites of MGB-BP-3 to DNA in *S. aureus* as shown by RNA-seq experiments are consistent with those observed by footprinting using the HexAB sequences developed by Fox (Hampshire and Fox, *Biochimie* **2008**, *90*, 988-998) All binding sites are AT rich but single G or C bases are accommodated.
- There is a similarity between the sequences identified by RNA-seq and footprinting with the recognition sites for a number of transcription factors in *S. aureus*.
- Together these observations support the concept that MGB-BP-3 is a single molecule with multiple targets on DNA that lead to multiple biological changes in *S. aureus*, the combination of which is lethal.
- Attempts to induce resistance in *S. aureus* by multiple passages of MGB-BP-3 have failed to generate a resistant strain.

MGB-BP-3 can therefore be described by a profile that implies an intrinsic resilience and avoidance of future bacterial resistance, a major potential benefit in the clinic.

Acknowledgments

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The differing biological fates of DNA MGBs. 2013-2014, MRC Confidence in Concept.

The differing biological fates of DNA minor groove-binding (MGB) antibiotics in Gram-negative and Gram-Positive bacteria. 2014 – 2017, BBSRC BB/N007999/1, £369,782.

Accelerating clinical introduction of novel antibacterial drugs. 2016 – 2017, Chief Scientist Office (Scotland).