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Announcement

Opal's technology presented at the Australian Society for Microbiology Annual Meeting

Melbourne, 16 July 2015 Australian infectious disease therapy and vaccine development company BioDiem Ltd and its subsidiary company Opal Biosciences, is pleased to announce a presentation on Opal's technology at the annual Australian Society for Microbiology conference in Canberra on the 13th July.

Doctor of Philosophy (PhD) student Mr Michael Radzieta presented the poster "Investigating the Mechanism of Action of the Novel Antimicrobial BDM-I" for which he won a best poster prize. Mr Radzieta, under the supervision of A/Professor Slade Jensen, has been investigating the mechanism of action of BDM-I against priority disease causing "superbugs" Vancomycin-resistant Enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA).

This exciting work is continuing at the Ingham Institute for Applied Medical Research and University of Western Sydney.

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About BioDiem Ltd
BioDiem is an Australian biopharmaceutical company that is focused on developing and commercialising vaccines and infectious disease therapies. BioDiem's business model is to generate income from partnerships including with other vaccine and infectious disease treatment companies through existing and new licences to its LAIV vaccine and other technologies. Income comes from licence fees and royalties on sales.

BioDiem's antimicrobial technology, BDM-I, is being developed through its subsidiary, Opal Biosciences Ltd. For additional information, please visit www.biodiem.com.

About Opal Biosciences Ltd
Opal Biosciences is an Australian biotechnology company and an innovative player in infectious disease treatment. The unmet need for new anti-infectives is due to increasing resistance to existing antibiotics, more widespread and common difficult-to-treat infections, and the paucity of upcoming new treatments. This need has spurred the EU and US to introduce significant financial incentives to encourage development of new anti-infectives. Opal is currently seeking funding to support the next stage of development of our products:

- Opal-I, an injectable product, and
- Opal-T, which can be applied to the skin.

For more information, please visit www.opalbiosciences.com.

About Ingham Institute/UWS/AMREG
The ARMEG was founded by the Microbiology and Infectious Diseases Unit, UWS School of Medicine and its laboratory is located within the Ingham Institute for Applied Medical Research, which is a new purpose-built institute that serves as the centre of medical research in South West Sydney. The group's core research projects are centred on the evolution of antibiotic resistance in ESKAPE pathogens, particularly MRSA and VRE, but also has projects that examine the role of

biofilms in hospital-acquired infections and the clinical utility of whole genome sequencing in an infectious disease context. Key members of the group are Dr. Slade Jensen, Dr. Björn Espedido, Dr. Sebastiaan van Hal and Prof. Iain Gosbell.

Further information

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Investigating the Mechanism of Action of the Novel Antimicrobial BDM-I

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Introduction

The continuing emergence and spread of antibiotic-resistant pathogens has coincided with a significant decrease in the research and development of novel antimicrobial compounds[3]. Two species of primary concern are the opportunistic pathogens *Enterococcus faecium* and *Staphylococcus aureus*, representing the two most common causes of hospital-acquired infections worldwide[2,3,7]. Currently, complicated infections for both species are treated with the glycopeptide vancomycin. However, through the acquisition of the *van* operon, vancomycin-resistant strains are now commonly observed in *E. faecium*; while vancomycin-intermediate *S. aureus* (VISA) strains are beginning to emerge due to *de novo* mutations.

BDM-I (figure 1) is a novel anti-infective belonging to the class of benzyl nitroalkenes[5] that is currently in development at the Australian Biotechnology company Opal Biosciences LTD. Preliminary data suggests that BDM-I acts as a tyrosine phosphatase inhibitor and is effective *in vitro* against a broad range of microorganisms: protozoa, fungi, bacteria[1]; most importantly, multi-drug resistant bacteria such as vancomycin-resistant enterococci (VRE) and methicillin-resistant *S. aureus* (MRSA).

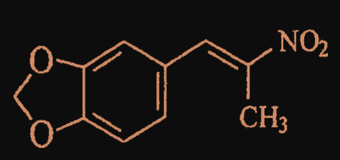

Figure 1: Molecular Structure of BDM-I[6]

The aim of this project was to determine the mechanism of action (MOA) of BDM-I using whole genome sequencing (WGS) and proteomics.

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Methods

***In vitro* mutant generation:** Mutants of VRE isolate Efm0008 were generated through the long-term exposure (approximately 70-80 days) of each of the isolates to increasing concentrations of BDM-I (in triplicate; series 1-3). As growth was observed, the concentration of BDM-I was gradually increased by increments of 1-2 µg/mL until an end point was reached. The increased resistance of Efm0008 to BDM-I was confirmed by determining the minimum inhibitory concentration (MIC) of selected mutant colonies as previously described (CLSI M07-A9, 2012).

WGS: DNA was extracted from three colonies selected from each series (9 in total), and were used to prepare 400 bp barcoded-libraries. The DNA samples were then loaded onto an Ion 318™ v2 chip and sequenced using the Ion Torrent PGM (Thermo Scientific), with generated sequencing reads being analysed using CLC genomics workbench ver. 7.0.3 (Qiagen).

Proteomics: The whole proteomes of VRE BDM-I treated isolates and derived mutants underwent analysis via 2D electrophoresis for protein separation, and subsequent mass spectrometry for protein identification.

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Results

BDM-I-Resistant VRE developed shortly after BDM-I exposure: Following the identification of the MIC of Efm0008 (7 µg/mL), induction experiments commenced by exposing Efm0008 to increasing concentrations of BDM-I. As seen in figure 2, sharp increases in BDM-I MIC were observed around day 10. Increased MICs (12-16 µg/mL) were maintained in Efm0008 until the end of the experiment (80 days), with final MICs being more than double that of the sensitive progenitor strain.

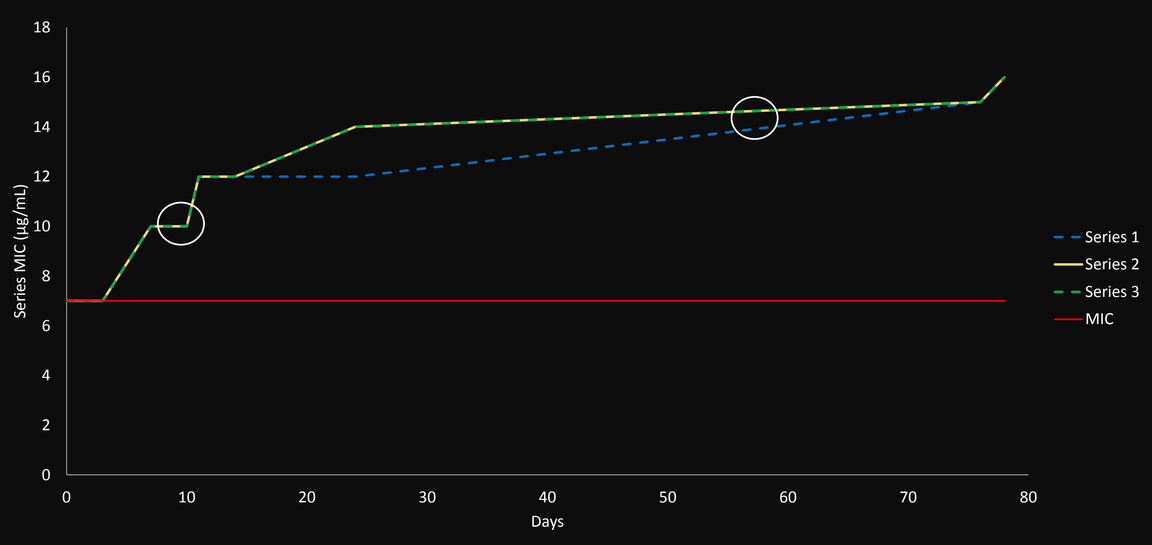

***Figure 2: Changes in BDM-I MIC within Efm0008 following 80 days of continuous treatment**. Regions circled indicate isolates that were utilised for whole genome sequencing.*

Mutations identified in ATP Synthase Genes of VRE series sequenced following 8 and 58 days of BDM-I exposure: Three parallel Efm0008 series were subjected to WGS at each time point indicated in figure 2. Following variant analysis, mutations were identified within the ATP synthase operon of all series sequenced (figure 3). Within the day 8 isolates, five of the nine colonies contained IS*1251*-like insertion sequences within the *atpE, atpG, atpF* and *atpB* genes. Single nucleotide polymorphisms (SNPs) resulting in amino acid changes were also identified within the *atpD* gene of two of the colonies, as well as a frameshift mutation within *atpD* of a single colony sequenced.

***Figure 3: ATP synthase operon:** Arrows indicate the locations of SNPs, circle-tipped arrow indicates the location of a frameshift, diamond-vertical lines indicate the location of IS*1256*-like insertion sequences and horizontal line indicates an IS*1256*-like mediated deletion. The colour of each mutation correlates with the series from which it was obtained (see figure 2).*

BDM-I Treatment Results in Altered Protein Expression within VRE: The soluble and membrane bound protein fractions of BDM-I treated Efm0008 (MIC_{50}), and the Efm0008$_{\Delta atpACDG}$ mutant were analysed using 2D electrophoresis. Within the membrane bound fraction, two protein subunits of ATP synthase were identified to be down-regulated as shown in table 1. Additionally, within the deletion mutant, a pyruvate dehydrogenase complex was identified as being up-regulated, possibly indicating that an alternate pathway of ATP production was being utilised.

Table 1: Proteins identified with altered expression

Gene	Protein	Expression[1]	
		MIC_{50}	$\Delta_{atpACDG}$
atpD	ATP synthase β-subunit	=	↓
atpH	ATP synthase δ-subunit	↓	↓
GMD4E_07358	Pyruvate dehydrogenase complex	-	↑
ptsH	Phosphocarrier protein	↓	=
asnB	Asparagine synthetase	↑	↑
ugpC-1	Glycerol-3-phosphate transporting protein	↑	-

[1]Protein expression level: ↓, decreased; ↑, increased; =, no change; -, absent

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Current and Future Work

Currently, further genomic studies are being completed on staphylococcal isolates with increased resistance to BDM-I. Similar induction experiments were completed on three clinical MRSA isolates with varying degrees of vancomycin resistance. A slight increase in BDM-I MIC was observed in a single VISA isolate and was further studied by WGS. Preliminary results identified SNPs within the gene *walK* in all series sequenced (figure 4); the significance of these mutations are currently being explored.

Additionally, ATP assays will be undertaken on mutant VRE and MRSA isolates as well as BDM-I treated isolates to examine possible changes in cellular ATP levels. Phosphoproteomics will also be utilised to examine changes in the phosphorylation state of the proteome to explore the proposed MOA of BDM-I as a tyrosine phosphatase inhibitor.

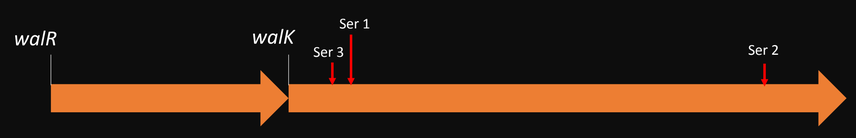

***Figure 4: SNPs identified within walK.** Red arrows indicate the positions of SNPs identified within three staphylococcal series sequenced. Two series (1 and 3) contained SNPs within the extracellular PAS domain, and one series contained a SNP within the HATPase_c domain (series 2).*

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Conclusions

Results obtained through WGS and proteomic analysis of BDM-I treated isolates, as well as *in vitro*-derived mutants indicate that ATP synthase is affected by BDM-I. Consistent within all sequenced VRE isolates were mutations within the ATP synthase operon, primarily SNPs within *atpD*. These results in combination with the apparent decrease in expression of *atpH* indicate that ATP synthase is important to the BDM-I MOA.

The role of ATP synthase is further reinforced by the difficulty in generating *S. aureus* isolates with increased resistance to BDM-I, as ATP synthase is essential in *S. aureus*[2]. Thus, this limits the ability to develop resistance by altering the structure of ATP synthase.

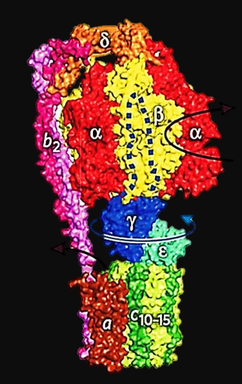

***Figure 5:** Model of bacterial ATP synthase. Image sourced from Weber, 2010*

Acknowledgments

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References

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