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Transcriptomic analysis of the *Clostridioides difficile*-targeting antimicrobial ridinilazole implicates disruption of energy-generation pathways as its mechanism of action

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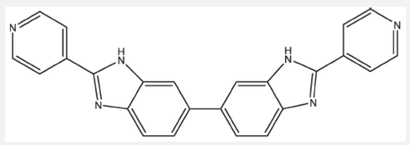
INTRODUCTION

- *Clostridioides difficile* is among the top 5 pathogens identified as urgent public health threats by the US Centers for Disease Control and Prevention[1].
- Ridinilazole (RDZ) is an investigational bisbenzimidazole oral antimicrobial being developed by Summit Therapeutics which targets *C. difficile* whilst sparing the gut microbiome and its ability to produce protective secondary bile acids[2,3].
- Whilst currently in clinical development, the precise mechanism by which RDZ exerts its bactericidal activity has not been fully elucidated.
- RDZ is a symmetrical small-molecule drug with a head-to-head bisbenzimidazole core (**Figure 1**) and has intrinsic fluorescence properties that are enhanced by interaction with DNA[4].
- We have previously shown that RDZ binding to polymeric dsDNA is specific for AT-rich molecules and tight binding was observed to dsDNA oligonucleotides with an AT-core. In these assays dissociation constants as low as 21 nM (10 ng/mL) were recorded[5] which is much lower than the minimal inhibitory concentration for RDZ against *C. difficile* (0.125 µg/mL in BHI broth)[6].

Figure 1. Structure of RDZ



- We present DNase I footprinting studies to further characterize the DNA-binding specificity of RDZ and transcriptomics to elucidate the cellular response of *C. difficile* vegetative cells to RDZ exposure.

METHODS

DNase I footprinting

- Synthetic dsDNA molecules were cloned into pUC18 or pUC19 and used to prepare fragments that were labelled at the 3'-end with ^{32}P[dATP].
- AT5a and AT5b contained all 16 arrangements of $(A/T)_5$ with CGTG/CACG spacers cloned in opposite orientations.
- HexA and HexARev together contained 32 symmetrical hexanucleotide sequences cloned in opposite orientations
- DNase I footprinting was performed[7] by mixing radiolabelled DNA with a titration of RDZ, incubating at 37°C for 1-30 min, digesting for 1 min with DNase I and the products separated on 8% denaturing polyacrylamide gels containing 8 M urea. Gels were fixed in 10% acetic acid, transferred to Whatmann 3MM paper, exposed to a storage phosphor screen overnight and scanned with a Typhoon phosphorimager.

RNAseq Transcriptomics

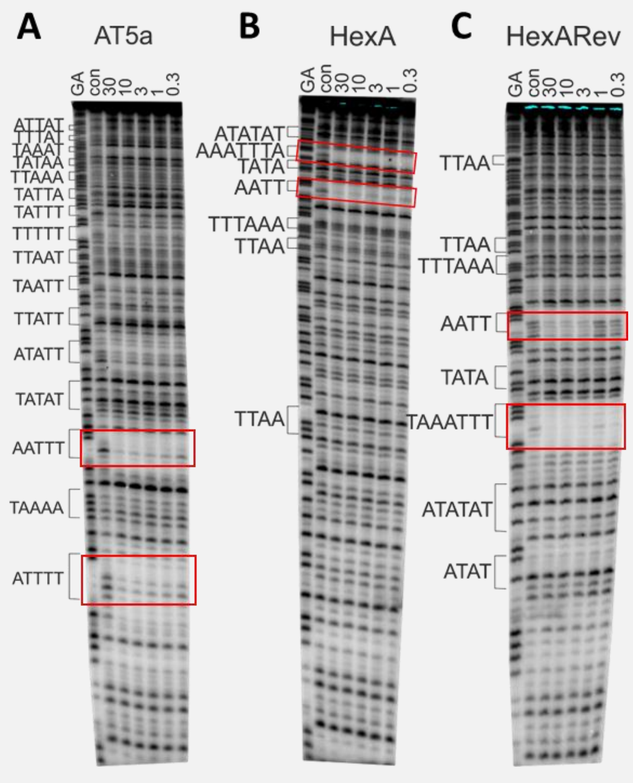
- *C. difficile* 630 was grown overnight (16 h) in BHIS + 0.1% L-cysteine + 0.1% sodium tauroholate under anaerobic conditions and subcultured 1 in 100 into BHIS + 0.1% L-cysteine. At A600nm~0.4 (5-6 h growth) the culture was diluted 1 in 10 to initiate quadruplicate 40 mL assay cultures for each condition (RDZ at 4x MIC or DMSO control) and timepoint (15 min, 1 h, 2 h, 3 h)
- At each timepoint bacteria were harvested by centrifugation, resuspended in 1 mL RNAprotect (QIAGEN), pelleted, supernatant aspirated and stored at -80°C prior to RNA extraction using the RNeasy® Mini Kit (QIAGEN).
- Ribosomal RNA was removed with the Ribo-Zero™ rRNA Removal Kit (Illumina), cDNA made using TruSeq® Stranded mRNA Library Prep (Illumina), normalised and pooled prior to sequencing on a NextSeq 550 sequencer (Illumina) with single-ended 76 cycles.
- Data quality was examined using FastQC (v0.11.9) and FastQ Screen (v0.14.1). Quality and adapter trimming was performed using Trim Galore (v 0.6.6; Cutadapt v3.4; Python v3.9.2). Reads were aligned to the *C. difficile* 630 genome (assembly name: ASM920v2) using Bowtie 2 (v 2.4.2). Alignment quality was examined using Picard tools (v2.25.0) and samtools (v1.12-2-gd515e1b). Aligned reads were assigned to genes using Subread featureCounts (v2.0.2).
- The RNA-seq count table was imported into R (v4.0.4), normalized by trimmed mean of M values (TMM) and transformed to log2 count-per-million (CPM) using edgeR (v3.32.1). Principal Component Analysis (PCA) was performed using the top 500 variable genes or all genes in the genome and two outlier samples (RDZ_2h_4 and dmso_2h_4) were removed. Differential expression analysis was performed using DESeq2 (v1.30.1). Enrichment of KEGG pathways in differentially expressed genes were analysed using limma (v3.46.0) and KEGG pathway maps were generated using pathview (v1.30.1). Expression of 163 genes that are involved in oxidative phosphorylation, bacterial chemotaxis, cationic antimicrobial peptide resistance and Stickland metabolism pathways, and the top 100 differentially expressed genes (based on fold-change at 15 min; padj < 0.01) were visualized in a heatmap generated using pheatmap (v1.0.12).

RESULTS

DNase I footprinting

- Using the AT5a template the clearest footprint was obtained for AATTT **(Figure 2),** persisting to [RDZ] below 1 µM. ATTTT was also a good binding site with ATATT, TAATT and TTTTT showing weaker attenuated cleavage at the highest concentrations.
- For the HexA and HexARev substrates, clear footprints were observed at AAATTTA (HexA) and TAAATTT (HexB). AATT was also a good binding site.
- These data confirm a clear preference for RDZ binding to AT-rich sequences with good binding sites lacking a TpA step in the middle of the sequence

Figure 2. DNase I footprinting

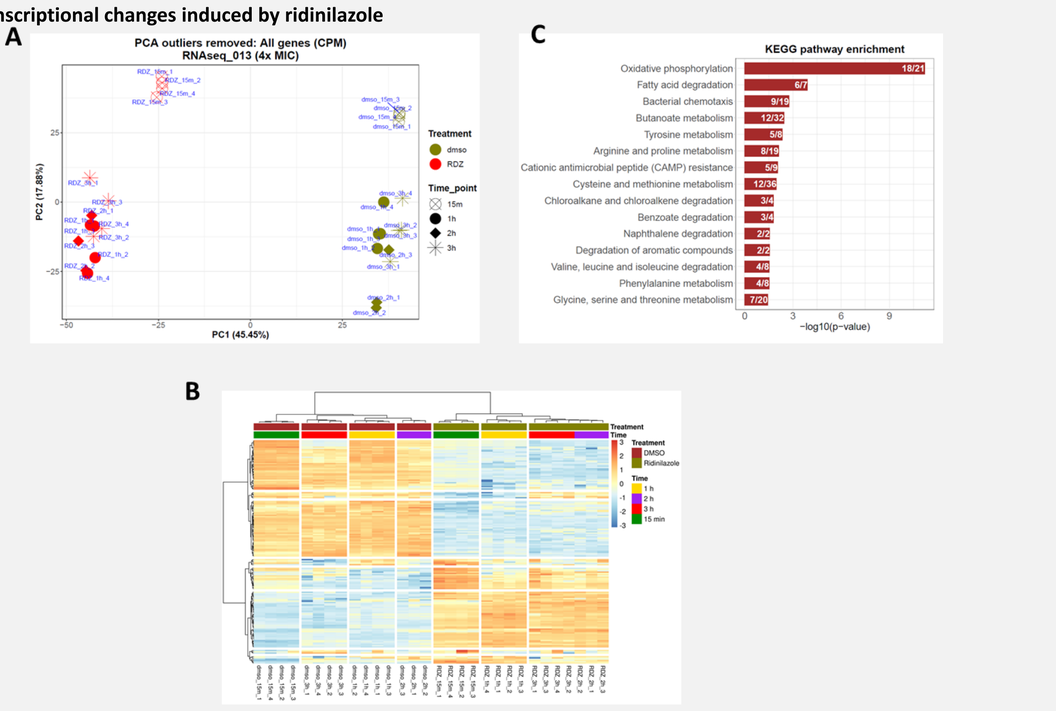


DNase I footprinting patterns for ridinilazole on three DNA AT-based templates. (**A**) AT5a; (**B**) HexA; (**C**) HexARev. The ligand concentration (µM) is shown at the top of each gel lane. Tracks labelled 'GA' are Maxam-Gilbert markers specific for purines, while 'con' indicates (control) cleavage without added ligand.
The position of each of the AT sites is indicated in brackets. Sequences located at the top of HexA are towards the bottom of HexARev and *vice versa*. Areas of proposed ridinilazole interaction are boxed in red.

Transcriptomics – ridinilazole induces pleiotropic transcriptional dysregulation including alteration of energy metabolism pathways

- RNAseq elucidated the transcriptional consequences of exposure of *C. difficile* to RDZ at 4x MIC.
- PCA and heatmap analysis showed that transcriptional changes were established by 15 min post-exposure and persist for at least 3 h (**Figure 3A & B**).
- Further analysis focussed on the 15 min timepoint where upregulation of 325 and downregulation of 407 genes was observed indicating a rapid pleiotropic response.
- The entire glycine reductase (GR) operon (*grdX trxB3 trxA2 grdEABCD*) was in the top 60 most upregulated genes.
- Genes in the proline reductase (PR) operon (*prdABDE prdE2 prdF*) were among the most downregulated.
- GR and PR are key components of the Stickland pathway which couples oxidation and reduction of amino acid pairs to the formation of ATP and is the major mechanism by which *C. difficile* generates energy.
- We also observed expression changes in other genes in the Stickland pathway (**Table 1**)**.**
- KEGG pathway analysis implicated 15 pathways in the response to RDZ exposure (**Figure 3C**), six of these being involved in amino acid metabolism, and all the genes encoding the ATP synthase were downregulated (**Table 1**). The ATP synthase generates ATP using proton motive force (PMF). PR is coupled to PMF generation by an uncharacterised mechanism[8].
- These alterations in key ATP-generating pathways may affect the ability of *C. difficile* to produce energy, ultimately resulting in bacterial cell death.
- The cationic antimicrobial peptide (CAMP) resistance pathway *dltDABC* operon, encoding D-alanine transferase, was downregulated. This adds D-alanine to the cell wall reducing sensitivity to host CAMPs[9]. If this downregulation also occurs *in vivo*, RDZ exposure may increase the susceptibility *C. difficile* to host defense mechanisms.

Figure 3. Transcriptional changes induced by ridinilazole



(A) PCA scores from all genes in *C. difficile* strain 630 treated with 4x MIC ridinilazole or DMSO at 15 min, 1 h, 2 h and 3 h post-exposure. PC 1 & 2 together account for 63.33% of variance in the dataset (**B)** Heatmap of 163 genes (top 100 differentially expressed genes and the genes involved in oxidative phosphorylation, bacterial chemotaxis, cationic antimicrobial peptide (CAMP) resistance and Stickland metabolism pathways). The rows represent genes and the columns represent samples from all four time points; Red: increased expression in RDZ *vs* DMSO; Blue: reduced expression (**C)** KEGG pathway enrichment at 15 min time point. The bars represent the negative logarithm (base 10) of the enrichment *P*-values. The numbers inside the bars represent the ratio of the number of differentially expressed genes in the query set and the total number of genes in the pathway.

Table 1. Energy-metabolism genes differentially expressed on 15 min ridinilazole exposure

Gene Name	UniProt Protein Name	log_2FC	padj
	Genes in the Stickland metabolic pathway		
grdB	Glycine reductase complex component B gamma subunit	9.18	2.23E-298
grdA	Glycine reductase complex selenoprotein A	8.94	0
grdC	Glycine reductase complex component C subunit beta	8.32	0
grdD	Glycine reductase complex component C subunit alpha	7.98	0
grdX	Putative glycine reductase complex component	6.63	0
adhE1	Aldehyde-alcohol dehydrogenase	3.65	6.91E-135
adhE	Aldehyde-alcohol dehydrogenase	3.16	2.10E-49
scrK	Fructokinase	0.55	3.76E-04
eutE	Ethanolamine acetaldehyde oxydoreductase	0.39	2.35E-01
ldhA	D-lactate dehydrogenase	-0.24	2.31E-01
CD630_01160	Putative ferredoxin/flavodoxin oxidoreductase,alpha subunit	-2.10	8.56E-25
ptb	Phosphate butyryltransferase	-3.06	9.18E-203
prdB	Proline reductase	-4.62	4.40E-121
prdF	Proline racemase	-4.64	2.36E-113
prdA	D-proline reductase proprotein prdA	-4.64	1.68E-181
prdE	Proline reductase PrdE	-4.91	2.56E-199
prdD	Proline reductase PrdD	-4.99	1.98E-121
prdR	Transcriptional regulator, sigma-54-dependent	-0.39	3.74E-03
	Genes in the oxidative phosphorylation pathway		
CD630_34050	Putative iron-only hydrogenase,electron-transferring subunit HymA-like	1.43	1.93E-39
CD630_34060	Putative iron-only hydrogenase,electron-transferring subunit HymB-like	1.35	1.14E-13
CD630_34070	Putative iron-only hydrogenase, catalytic subunit HymC-like	1.21	1.55E-15
CD630_29610	Uncharacterized protein	-2.07	5.23E-17
atpD	ATP synthase subunit beta	-2.53	1.18E-49
atpI	V-type ATP synthase subunit I	-2.58	3.86E-67
atpB	V-type ATP synthase beta chain	-2.65	6.77E-26
atpA	V-type ATP synthase alpha chain	-2.77	1.95E-47
atpD	V-type ATP synthase subunit D	-2.85	6.21E-44
atpK	V-type ATP synthase subunit K	-2.92	4.60E-26
atpC	V-type ATP synthase subunit C	-3.10	1.61E-38
atpF	V-type ATP synthase subunit F	-3.14	3.15E-34
atpE	V-type ATP synthase subunit E	-3.27	5.60E-20
atpC1	ATP hydrolase epsilon chain	-3.43	7.44E-75
ppaC	Pyrophosphate phospho-hydrolase	-3.82	6.84E-134
atpG	ATP synthase gamma chain	-3.86	1.26E-222
atpA	ATP synthase subunit alpha	-3.94	8.74E-232
atpE	ATP synthase subunit c	-4.04	1.53E-71
atpH	ATP synthase subunit delta	-4.34	2.34E-240
atpF	ATP synthase subunit b	-4.44	5.65E-124
atpA2 atpB	ATP synthase subunit a	-4.51	9.84E-223

CONCLUSIONS

- The small-molecule *C. difficile*-targeting antimicrobial ridinilazole which binds AT-rich DNA[5] has particular affinity for AATT, AATTT, AAATTTA and TAAATTT sites.
- Exposure of *C. difficile* to ridinilazole *in vitro* results in rapid, long-lasting and pleiotropic alterations in gene expression.
- Energy metabolism, specifically key enzymes in the Stickland fermentation pathway and the ATP synthase, is dysregulated on ridinilazole exposure.

REFERENCES

[1]CDC (2019) doi:10.15620/cdc:82532.; [2]Thorpe, C. M. *et al*. (2018) *PLoS One* doi:10.1371/journal.pone.0199810; [3]Qian, X. *et al.* (2020) *Am J Physiol Gastrointest Liver Physiol* doi:10.1152/ajpgi.00046.2020; [4]Chaudhuri, P. *et al.* (2007) *J Org Chem* doi:10.1021/jo0619433; [5]Mason, C. *et al.* (2021) *Open Forum Infect Dis* doi:10.1093/ofid/ofab466.1246; [6]Corbett, D. *et al.* (2015) *J Antimicrob Chemother* doi:0.1093/jac/dkv006; [7]Hampshire, A. J. *et al.* (2007) *Methods* doi:10.1016/j.ymeth.2007.01.002; [8]Jackson, S. *et al.* (2006) *J Bact* doi:10.1128/JB.01370-06; [9]McBride *et al.* (2011) *Microbiology* doi:10.1099/mic.0.045997-0

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Ridinilazole is an investigational compound that is not approved by any regulatory body.