Poster 03404

Identification of the mechanism of action for ridinilazole, a phase III antibiotic for treatment of *Clostridioides difficile*

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Introduction

- *Clostridioides difficile* infection (CDI) was responsible for nearly 13,000 deaths in the US in 2017 and brings a significant economic burden (ranging from $5.4 to $6.3 billion per year [1]). **Ridinilazole, an investigational, selective antibiotic currently in phase III clinical trials for CDI, demonstrated a statistically significant increase in sustained clinical response compared to vancomycin (standard of care) in Phase II** [2]. The precise mechanism of action of ridinilazole has yet to be fully elucidated.
- Ridinilazole, a bis-benzimidazole, shares features with other members of this compound class that have previously been described as DNA minor groove binders, such as Hoechst 33258. Both molecules have an overall crescent shape which aligns with the conformation of the minor groove in double stranded DNA. Like Hoechst dyes, ridinilazole has intrinsic fluorescence properties.
- We now present data that shows ridinilazole binds with high affinity and sequence specificity to the minor groove of DNA. This interaction occurs at sub-MIC concentrations and is predicted to have consequences on cellular functions within *Clostridioides difficile*.

Methods

- DNA: ridinilazole binding studies were performed using a fluorescent plate reader or by visualisation under UV light following agarose gel electrophoresis. To measure fluorescence, we used an excitation wavelength of 355±30 nm and an emission wavelength of 455±20 nm. Initial experiments were performed with ultra pure salmon sperm DNA (figure 1). To visualise ridinilazole binding to *C. difficile* DNA, a 1.2 kb amplicon was incubated with ridinilazole prior to gel electrophoresis (figure 2). To test the binding specificity of ridinilazole, 3 polymers, that are double stranded DNA models used for confirmational studies of DNA structure and drug interactions, were titrated with a fixed concentration of ridinilazole of 2 µM (figure 3). Binding affinity of ridinilazole to a predicted single binding site utilised two short double stranded DNA oligonucleotides, previously used to characterise Hoechst 33258 binding [3,4]. Oligonucleotides were titrated with fixed ridinilazole concentrations (100-500 nM), and extrapolated dissociation constants (K_d) determined (figure 4). For the x-ray crystallography a short double stranded DNA oligonucleotide (dsOligo) was co-crystalised with ridinilazole. Crystals were sent for x-ray diffraction (DLS, Oxford) and the co-crystal structure solved through collaboration with Domainex, UK (figure 5).

Results

Enhanced fluorescence of ridinilazole upon DNA binding

The intrinsic fluorescence of ridinilazole shows a DNA concentration dependent increase in fluorescence in the presence of salmon sperm DNA. The enhanced fluorescence upon binding DNA is a described feature for certain bis-benzimidazoles, such as Hoechst dyes, which occurs due to suppressed rotational relaxation of the molecule.

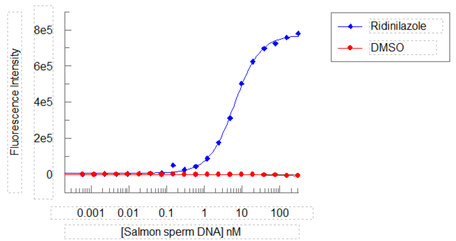


Figure 1 : Fluorescence intensity plot of salmon sperm DNA with a fixed concentration of ridinilazole (500 nM) or DMSO (control).

UV Visualisation of ridinilazole binding to a C. difficile amplicon

Through the enhanced fluorescence displayed by ridinilazole upon DNA binding we were able to directly visualise, under UV illumination, ridinilazole binding to the amplicon from *C. difficile* following agarose gel electrophoresis. DNA-ridinilazole fluorescence can be visualised well below the concentrations required to inhibit growth of *C. difficile*.

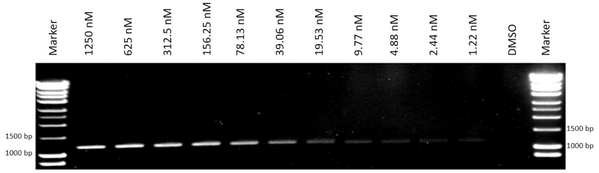


Figure 2a: UV visualisation of ridinilazole (1250-1.22 nM) bound to DNA (2 µg C. difficile amplicon), CLSI MIC of ridinilazole (130-543 nM) against C. difficile.

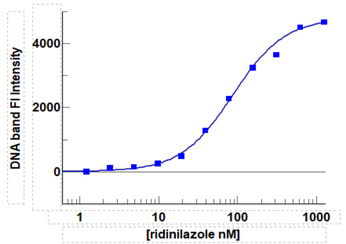


Figure 2b: Densitometry plot of the fluorescent bands seen in figure 2a. An apparent K_d of 87 nM was determined.

Ridinilazole demonstrates preference for AT-rich DNA sequences

The two polymers containing adenine and thymine show concentration dependent increase in fluorescence with ridinilazole, whereas the polymer containing only guanine and cytosine shows no concentration dependent increase in fluorescence. Ridinilazole shows a propensity to bind only AT-rich DNA.

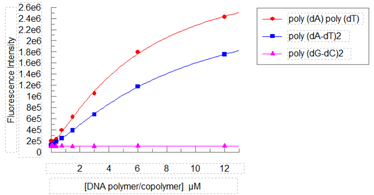
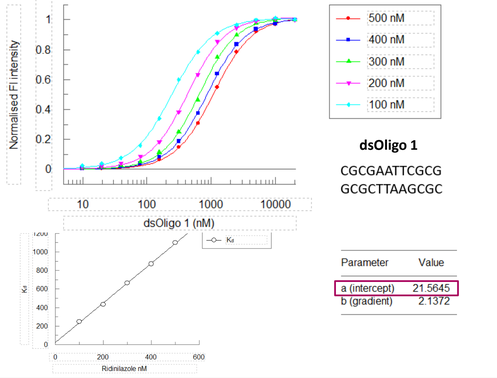


Figure 3 : Titration of DNA polymers with 2 µM ridinilazole. No increase in fluorescence is observed for poly (dG-dC)2 DNA polymer. Enhanced fluorescence was observed with poly (dA-dT)2 and poly (dA) poly (dT) polymers.

Results

Ridinilazole shows tight binding to short double-stranded DNA oligonucleotides

Figure 4 shows tight binding curves for ridinilazole and two different double-stranded oligonucleotides. The apparent K_d for each ridinilazole-DNA titration was plotted on a linear fit to determine the extrapolated tight binding K_d for each oligonucleotide. Potent binding at ridinilazole concentrations significantly lower than MIC were observed, with a K_d of ~20 nM for each oligonucleotide.



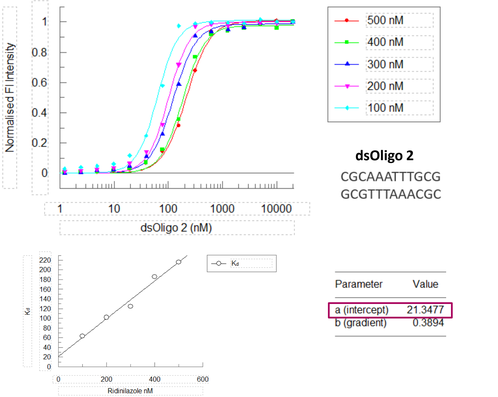


Figure 4 : Titrations of two double stranded DNA oligonucleotides with fixed concentrations of ridinilazole. Extrapolated dissociation constants plotted below for each.

Ridinilazole binds to the minor groove of double-stranded DNA

A crystal structure of ridinilazole bound to the double-stranded oligonucleotide (dsOligo 1) was solved by X-ray diffraction analysis to a resolution of 2.2 Å. Strong compound density was observed in the minor groove of the DNA double helix. The AATT nucleotides proximal to bound ridinilazole are highlighted

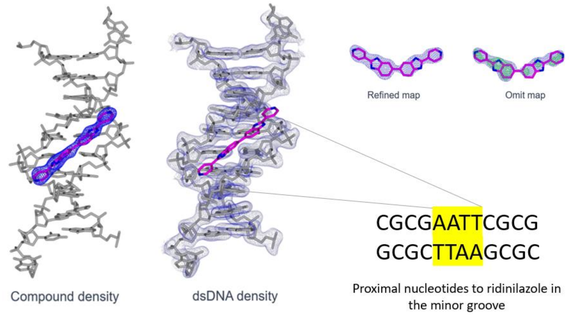


Figure 5 : Solved crystal structure showing compound density and dsDNA density of dsOligo 1. ridinilazole binds in the minor groove with proximal nucleotides to ridinilazole highlighted.

These results add strong evidence to support that the primary mechanism of action is through sequence specific DNA binding.

Conclusions

- Ridinilazole demonstrates potent binding, with AT sequence specificity, to the minor groove of DNA at sub-MIC concentrations.
- DNA binding is believed to be the primary mechanism through which ridinilazole exerts its bactericidal activity in *C. difficile* .
- We are currently carrying out further studies to determine the downstream effects of ridinilazole binding to genomic DNA in *C. difficile* and what cellular functions are subsequently affected.
- Ridinilazole is currently being evaluated in two Phase 3 studies for the treatment of CDI and the reduction of recurrence (www.ricodifytrial.com/eu).

References

1) Balsells *et al.* J Glob Health 2019. 2) Vickers *et al.* The lancet 2017 3) Teng *et al.* Nucleic acids research, 1988. 4) Vega *et al.* European journal of biochemistry, 1994.

Acknowledgments

- Structural studies conducted in partnership with Domainex

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Learn more on the Phase 3 at www.ricodifytrial.com/eu

Ridinazole is an investigational compound that is not approved by any regulatory body

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