Poster# 1052

# Characterization of the DNA binding properties of ridinilazole, a phase III antibiotic for treatment of *Clostridioides difficile* Infection

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## INTRODUCTION

- *Clostridioides difficile* infection (CDI) brings a significant healthcare and economic burden (ranging from $5.4 to $6.3 billion per year [1]) and was responsible for nearly 13,000 deaths in the US in 2017.
- Ridinilazole, a selective investigational antibiotic currently in clinical development, demonstrated (in a Phase 2 study) a statistically significant increase in sustained clinical response compared to vancomycin (standard of care), 66.7% vs 42.4% respectively[2].
- Despite concerted efforts, the precise mechanism of action of ridinilazole has yet to be fully elucidated.
- Ridinilazole, belongs to the bis-benzimidazole class of compounds, and shares features with other members, such as Hoechst 33258, that have been described as DNA minor groove binders. These molecules have an overall crescent shape which aligns with the conformation of the minor groove in double stranded DNA. Similar to Hoechst dyes, ridinilazole also has intrinsic fluorescence.
- We now present data that reveals ridinilazole clearly co-localises with DNA in *C. difficile* and binds with high affinity to the minor groove of DNA.

## METHODS

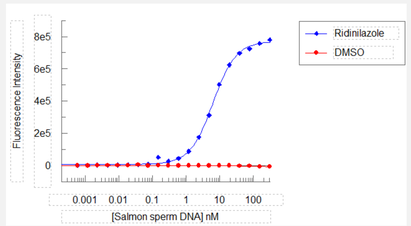
- DNA:ridinilazole binding was tracked with a fluorescent reader with excitation wavelength of 355±30 nm and an emission wavelength of 455±20 nm. Initial DNA binding of ridinilazole was demonstrated using a titration of salmon sperm DNA (figure 1).
- To test the binding specificity of ridinilazole, titrations of three double-stranded DNA polymers were used with a fixed concentration of ridinilazole (2 µM) (figure 2).
- Determining the binding affinity of ridinilazole to a predicted single binding site utilised short double stranded DNA oligonucleotides (previously used to characterise Hoechst 33258 binding[3,4]). Oligonucleotide was titrated with fixed ridinilazole concentrations (100-500 nM), and extrapolated dissociation constants ($K_d$) determined (figure 3).
- For 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 by molecular replacement through a collaboration with Domainex, UK (figure 4).
- Confocal imaging of *C. difficile* (CD630) was performed on fixed cells following exposure to ridinilozole (4xMIC, 2 hour).

## RESULTS

### Enhanced fluorescence of ridinilazole upon DNA binding

- Ridinilazole shows a DNA-dependent increase in fluorescence in the presence of salmon sperm DNA. Such 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).**

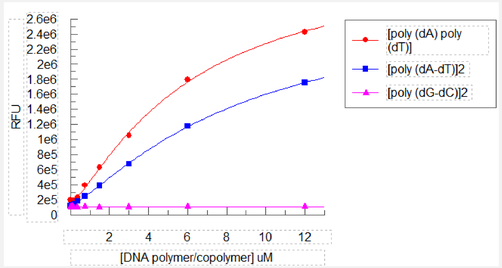



## RESULTS

### Ridinilazole demonstrates preference for AT-rich DNA sequences

- The two double-stranded DNA polymers containing adenine and thymine bases 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.

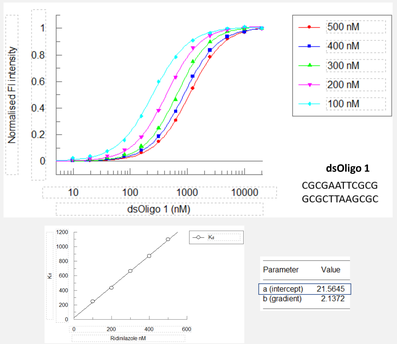
**Figure 2 : Titration of DNA polymers with 2 µM ridinilazole. No increase in fluorescence is observed for [poly (dG-dC)]2 DNA polymer. Enhanced fluorescence was observed with [poly (dA-dT)]2 and [poly (dA)-poly (dT)] polymers.**




### Tight binding of Ridinilazole to short double-stranded DNA oligonucleotides

- Binding curves for ridinilazole with a double-stranded oligonucleotide substrate are shown. The apparent $K_d$ for each ridinilazole-DNA titration was plotted on a linear fit to determine the extrapolated tight binding $K_d$ for the oligonucleotide. Potent binding of ridinilazole at concentrations significantly lower than MIC were observed, with a $K_d$ of ~20 nM for the oligonucleotide. The (CLSI) MIC of ridinilazole against *C. difficile* is 130-543 nM.

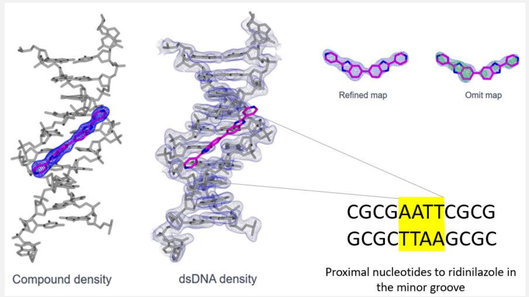
**Figure 3 : Titrations of double stranded DNA oligonucleotides with fixed concentrations of ridinilazole, with extrapolated dissociation constant plotted (below).**




### 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.
- These results demonstrate that ridinilazole directly binds to the minor groove of DNA in a sequence-specific manner.

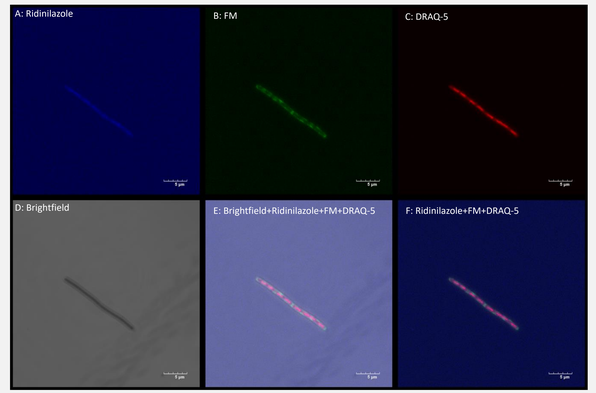
**Figure 4 : Solved crystal structure showing compound density and dsDNA density of dsOligo 1. ridinilazole binds in the minor groove with proximal nucleotides to ridinilazole highlighted.**




### Ridinilazole binds to and co-localises with DNA in *C. difficile*

- We have exploited the inherent fluorescence of ridinilazole to determine intracellular localization of the drug in *C. difficile* (Figure. 5A). Following exposure to ridinilazole, confocal microscopy revealed that the drug co-localised with a DNA-specific stain (DRAQ-5), indicating that it associates with intracellular DNA (Overlay (pink) shown in Figure. 5E and 5F). The cells also exhibit the elongated phenotype associated with ridinilazole exposure[5].
- These data support the hypothesis that ridinilazole elicits its antimicrobial action through direct binding of DNA in the target pathogen.

**Figure 5: Confocal microscopy images of *C. difficile* following exposure to ridinilazole. Cells were fixed post-exposure to ridinilazole (4xMIC, 2Hour). FM (FM4-64) membrane stain, DRAQ-5 far-red DNA stain.**




## CONCLUSIONS

- **Ridinilazole demonstrates potent binding, with AT sequence specificity, to the minor groove of DNA at sub-MIC concentrations.**
- **Ridinilazole binds to and co-localises with DNA in *C. difficile*.**
- **DNA binding is believed to be the primary mechanism through which ridinilazole exerts its bactericidal activity in *C. difficile*.**
- **Ongoing studies will look to determine the biological consequences of ridinilazole binding to genomic DNA in *C. difficile*.**

## REFERENCES

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Ridinazole is an investigational compound that is not approved by any regulatory body. Learn more at www.summittxinc.com