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Comparison of the *in vitro* antibacterial activity of Ramizol, Fidaxomicin, Vancomycin and Metronidazole against 100 clinical isolates of *Clostridium difficile* by broth microdilution

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Abstract

Antibiotic drug development remains a major challenge with few candidates in clinical development. Ramizol, a first-in-class styrylbenzene antibiotic, is under development for the treatment of *Clostridium difficile* associated disease. Here, we investigate the *in vitro* antibacterial activity of Ramizol in comparison to fidaxomicin, vancomycin and metronidazole against 100 clinical isolates of *C. difficile* by the broth microdilution method. We show there is no apparent impact of ribotype, toxin-production, or resistance to fidaxomicin, vancomycin or metronidazole on the activity of Ramizol. Moreover, we show Ramizol has a narrower MIC range translating to potentially better control over the therapeutic dose. Together, these results support the further development of Ramizol for the treatment of *C. difficile* associated disease.

Introduction

Antibiotic resistance is a global health crisis exacerbated by a deficiently unhealthy antibiotic drug pipeline. By 2050, antibiotic drug resistance is predicted to overtake cancer as the leading cause of death from disease[1]. However, there are currently only 51 antibiotics (including combinations) in clinical development[2], compared to over 830 oncology drugs in clinical trials[3]. *Clostridium difficile* is a Gram-positive spore-forming anaerobic bacterium that colonises the gastrointestinal tract as a result of disturbance to the gut microbiota. *C. difficile*, which has been identified as an urgent threat by the Center for Disease Control and Prevention[4], claims over 14,000 lives annually in the US and is an increasing concern worldwide[5, 6]. Out of the 51 antibiotics in clinical development, only 7 have a *C. difficile* indication with over 50% of these belonging to an old antibiotic class[2]. With a less than 10% chance of any drug in clinical development getting to market[7], the current *Clostridium difficile*-associated disease (CDAD) pipeline would translate to 1 drug being commercialized at best, highlighting the need for further research in the area and the need for continuous development of new and effective treatment options. Ramizol, a first-in-class non-systemic styrylbenzene antibiotic, is in preclinical development for the treatment of *C. difficile* infections (CDI). The new class, identified using a combinatorial approach composed of *de novo* design and *in silico* docking, targets the mechanosensitive ion channel of large conductance (MscL)[8], a highly conservative transmembrane protein not present in the human genome. In a hamster *C. difficile* colitis infection model using the NAP1/027 strain, animals treated with Ramizol showed survival rates of 73% compared to 83% in the vancomycin treated group and no animals surviving in the untreated group; in addition, a significant reduction of spore counts in Ramizol-treated hamsters compared to the control was found[9]. Additionally, the drug has

shown no risk of resistance emergence in two strains of *C. difficile* when high inocula were tested at 4x and 8x MICs[9]. Here, we investigate the *in vitro* activity of Ramizol against a diverse collection of 100 clinical isolates of *C. difficile*, including those characterized for toxin production and ribotype, to determine if the antibacterial activity of the drug translates to other isolates and compare it's activity to fidaxomicin, metronidazole and vancomycin.

Materials and Methods

Chemicals. Ramizol was shipped to Micromyx and stored at room temperature, protected from light. The solvent for the preparation of the Ramizol stock solutions was 100% dimethylsulfoxide (DMSO; Sigma, St. Louis, MO). The Ramizol stock concentration was 5,120 µg/mL. Stock preparations of fidaxomicin (purchased from API) and metronidazole (purchased from Sigma) were made using a DMSO:water mixture (5% DMSO final) at concentrations of 320 µg/mL and 2,560 µg/mL, respectively. Vancomycin (purchased from Sigma) stocks (2,560 µg/mL) were prepared in water. Stock solutions were aliquoted and stored at 4 ºC until use.

Antimicrobial susceptibility testing. Test organisms for the assay were 100 *C. difficile* clinical isolates selected from the Micromyx collection (Table S1) as well as the American Type Culture Collection (ATCC) quality control organism *C. difficile* ATCC 700057. The clinical strains used were isolated in 2003, 2011, 2014, 2015 and 2016 with the majority isolated in 2011 and 2014. Of the 100 strains used, 31% of the isolates came from California, 61% from Indiana, 7% from New York and 1% from Michigan. Toxin positive strains formed 64% of the isolates (48% of which belong to a known ribotype), toxin negative strains formed 28% of the isolates and 8% were not identified.

The *in vitro* activity of the antibiotics was determined by broth microdilution in

accordance with guidelines from the Clinical and Laboratory Standards Institute[10, 11] with the exception that Reinforced Clostridial Medium was used as the primary test medium as previously reported[9]. After preparation of plates containing serially diluted Ramizol or comparator, plates were transferred into a Bactron II anaerobic chamber (Sheldon Manufacturing Inc., Cornelius, OR) and allowed to reduce for 1 – 2 hr before inoculating. After inoculation, plates were incubated in BD Gaspak EZ Anaerobe containers at 35 ºC for 46-48 hr. The MIC was read and recorded as the lowest concentration of drug that inhibited visible growth of the organism.

Results

The drug stocks of fidaxomicin, vancomycin, and metronidazole used in the assay were validated by MICs observed with *C. difficile* ATCC 700057 in supplemented Brucella agar, which were within the established CLSI QC range (results not shown).

The MIC results observed for Ramizol and the comparators are summarized in **Table 1**. For Ramizol, the MIC range was ≤0.12 – 8 μg/mL while for fidaxomicin, vancomycin and metronidazole, the MIC range was wider. Eight isolates were resistant to vancomycin and two to metronidazole, using the EUCAST ECOFF breakpoints (2 μg/ml for both). A breakpoint has not been defined for fidaxomicin. Two of the vancomycin-resistant isolates were noted to be much less susceptible to fidaxomicin (MIC > 8 μg/ml) but were susceptible to Ramizol with MICs of 2 μg/ml and 4 μg/ml. The two strains resistant to metronidazole were susceptible to Ramizol with an MIC of 2 μg/ml and the remaining six strains, which were resistant to vancomycin were susceptible to Ramizol at concentrations from 1 to 8 μg/ml, concentrations similar to the MICs of Ramizol against antibiotic sensitive *C. difficile* strains (Table S1) and MICs against toxin-positive and toxic-negative isolates (Table 1 and Table S1).

Table 1. Summary of the *in vitro* activity of Ramizol and comparators in reinforced Clostridial Medium by broth microdilution against clinical *C. difficile* isolates (N=100)

	Ramizol	Fidaxomicin	Vancomycin	Metronidazole
MIC range (µg/mL)	≤0.12 – 8	≤0.008 - >8	≤0.06 - 32	≤0.06 - >64
MIC range of toxin-positive isolates (n = 64)	≤0.12 – 8	≤0.008 - >8	0.12 - 32	≤0.06 - 1
MIC range of toxin-negative isolates (n = 36)	≤0.12 – 8	≤0.008 - >8	≤0.06 - 32	≤0.06 - >64
MIC_{50} (µg/mL)	2	0.015	0.5	0.12
MIC_{90} (µg/mL)	4	0.03	2	0.25

The resulting MIC distribution is shown in **Figure 1**, and a line listing of results by isolates is shown in **Table S1.** The MIC distributions of fidaxomicin and to a lesser extent metronidazole are positively skewed, while vancomycin is negatively skewed, and Ramizol displays more of a normal distribution. Ramizol activity was not notably impacted by the type of isolate, with similar MICs across the evaluated ribotypes and against toxin-positive isolates relative to toxin-negative isolates, **Table 1**. This was the same for the other drugs tested with the exception of metronidazole, which had higher MICs against toxin-negative isolates. Clinical isolates with reduced susceptibility to fidaxomicin, metronidazole or vancomycin, did not differ from the larger set in susceptibility to Ramizol. (**Table S1**).

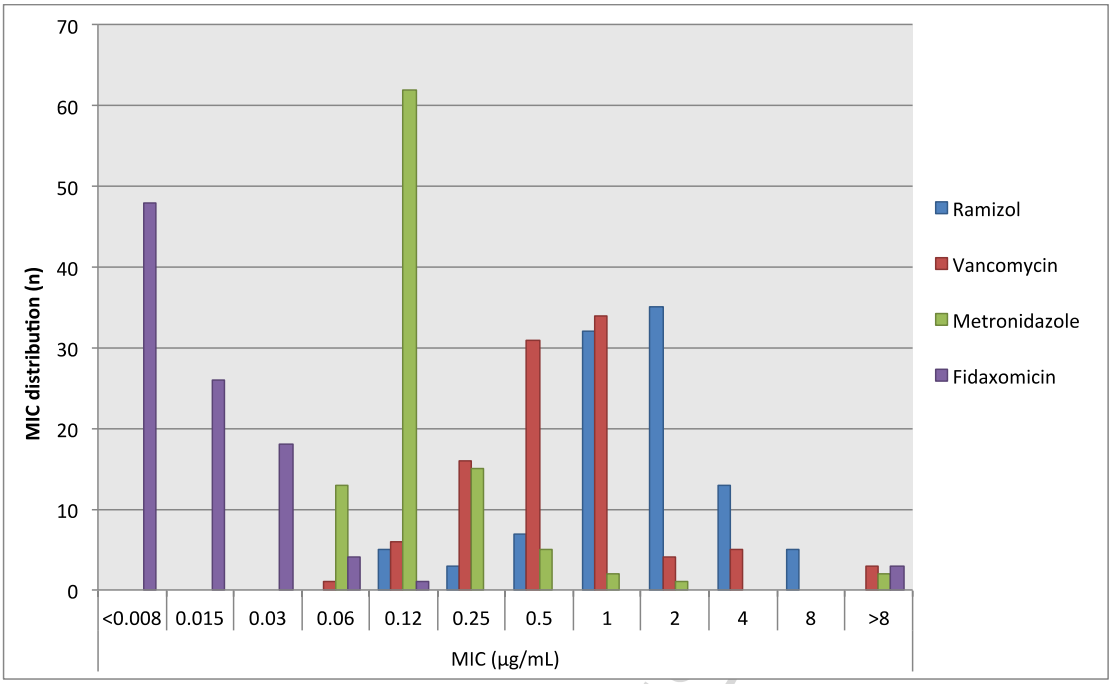

Figure 1. MIC distribution of Ramizol, vancomycin, metronidazole and fidaxomicin in Reinforced Clostridial Medium by broth microdilution against clinical *C. difficile* isolates (N=100)

Discussion

C. difficile infections remain the primary cause of diarrhoea from antibiotic administration, and are a serious problem in healthcare settings and the community. Other factors such as the use of proton pump inhibitors and the use of anticancer drugs also increase the risk of CDI[12]. Severity of the infection ranges from mild diarrhoea to severe life-threating pseudomembraneous colitis and toxic megacolon, an inflammation of the colon resulting from the overgrowth of *C. difficile*. *C. difficile* toxin-producing ribotypes carry virulence factors (toxins A and B) that stimulate epithelial tissue damage and inflammation in the host[13]. Some strains such as BI/NAP1/027 and ribotype 078 isolates produce an additional binary toxin which is prevalent in strains associated with severe disease[14]. The antibiotic of choice for the treatment of mild *C. difficile* infections remains metronidazole, mostly cost-driven, while vancomycin is the antibiotic of choice for severe infections[5, 12, 14]. In 14-27% of

cases however, they are not effective and do not prevent a relapsing infection. In addition, almost 100% of metronidazole is absorbed[14] from the intestine, with faecal concentrations of the drug at less than 10 μg/mL[12]. This concentration is below the MIC of some resistant isolates; the low faecal concentration is thought to contribute towards the development of resistance in *C. difficile*[12, 14]. Fidaxomicin, approved in 2011 for the treatment of CDI, is selective against *C. difficile* over other gut microbes, and has been more successful than vancomycin in reducing relapse caused by strains other than PCR ribotype 027[5, 15], an isolate with an elevated fidaxomicin MIC (16 μg/mL). However fidaxomicin still fails in approximately 1 out of 8 patients treated with the antibiotic[16] and in clinical trials, elevated MICs were observed[17]. Antibiotic resistance is still rare in *C. difficile*, but does occur. In a study investigating the *in vitro* susceptibility of fidaxomicin, metronidazole and vancomycin against 398 strains of *C. difficile* clinical isolates from 73 hospitals in 26 European countries, no resistance to any of the antibiotics tested was reported[6]. Susceptibility data compiled from a number of studies listed MIC ranges between 0.16 – 32 μg/mL, 0.016 – 16 μg/mL and <0.008 – 2 μg/mL for metronidazole, vancomycin and fidaxomicin, respectively[18]. While a link between failure of treatment in CDI patients and elevated MICs is unestablished, it is a contributing factor among others such as the pharmacokinetics of the drug, the health of the microbiota and the absence of recurrence of CDI after antibiotic excretion[12]. Failure of CDI therapy in these cases requires the development of novel antibiotics for *C. difficile* infections ensuring that new drugs are active against isolates with reduced susceptibility to antibiotics in current use. In our study, Ramizol was fully active against isolates with reduced susceptibility to fidaxomicin, metronidazole and vancomycin, suggesting that pre-existing resistance among *C. difficile* to these agents does not impact Ramizol

activity. This was also previously observed in other species where Ramizol maintained activity against resistant isolates[8]. The clinical implication of narrower MIC range for Ramizol is better control over the dose (and cost) required to achieve a successful clinical outcome. Considering the fast spreading nature of *C. difficile*, resistance emergence is a real concern, and with few treatment options currently available for CDI, there is a clear need for the development of new and effective treatments. Ramizol is a promising investigational drug for CDI that is advancing to first-in-man clinical trials. Future studies will include evaluation of susceptibility to more diverse clinical isolates and the effect of Ramizol treatment on intestinal microflora.

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Note: Ramizol is registered trademark in Australia.

References

1. Tackling drug-resistant infections globally: final report and recommendations. (The Review on Antimicrobial Resistance, 2016).
2. Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline, including tuberculosis. Geneva: World Health Organization; 2017.
3. 2015 Medicines in Development for Cancer. (Pharmaceutical Research and Manufacturers of America, 2015).
4. Antibiotic resistance threats in the United States 2013. 1-114 (Center for Disease Control and Prevention, Atlanta, GA, 2013).
5. P. Putsathit, M. Maneerattanaporn, P. Piewngam, D. R. Knight, P. Kiratisin and T. V. Riley, *Antimicrobial resistance and infection control*, 2017, **6**, 58.
6. S. B. Debast, M. P. Bauer, I. M. Sanders, M. H. Wilcox, E. J. Kuijper and E. S. Group, *The Journal of antimicrobial chemotherapy*, 2013, **68**, 1305-1311.
7. Clinical development success rates 2006-2015. (Biotechnology Innovation Organization, Biomedtracker and Amplion, 2016).
8. I. Iscla, R. Wray, P. Blount, J. Larkins-Ford, A. L. Conery, F. M. Ausubel, S. Ramu, A. Kavanagh, J. X. Huang, M. A. Blaskovich, M. A. Cooper, A. Obregon-Henao, I. Orme, E. S. Tjandra, U. H. Stroeher, M. H. Brown, C. Macardle, N. van Holst, C. Ling Tong, A. D. Slattery, C. T. Gibson, C. L. Raston and R. A. Boulos, *The Journal of antibiotics*, 2015, **68**, 453-462.
9. S. Rao, C. A. Prestidge, L. Miesel, D. Sweeney, D. L. Shinabarger and R. A. Boulos, *The Journal of antibiotics*, 2016, **69**, 879-884.
10. Clinical and Laboratory Standards Institute (CLSI). Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Eighth Edition. CLSI document M11-A8. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2012.
11. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Seventh Informational Supplement. CLSI document M100-S27. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2017.
12. S. D. Baines and M. H. Wilcox, *Antibiotics*, 2015, **4**, 267-298.
13. D. E. Voth and J. D. Ballard, *Clinical microbiology reviews*, 2005, **18**, 247-263.
14. A. M. Jarrad, T. Karoli, M. A. Blaskovich, D. Lyras and M. A. Cooper, *Journal of medicinal chemistry*, 2015, **58**, 5164-5185.
15. T. Pelaez, L. Alcala, R. Alonso, M. Rodriguez-Creixems, J. M. Garcia-Lechuz and E. Bouza, *Antimicrobial agents and chemotherapy*, 2002, **46**, 1647-1650.

16. D. W. Crook, A. S. Walker, Y. Kean, K. Weiss, O. A. Cornely, M. A. Miller, R. Esposito, T. J. Louie, N. E. Stoesser, B. C. Young, B. J. Angus, S. L. Gorbach, T. E. Peto and T. Study, *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 2012, **55 Suppl 2**, S93-103.
17. E. J. Goldstein, D. M. Citron, P. Sears, F. Babakhani, S. P. Sambol and D. N. Gerding, *Antimicrobial agents and chemotherapy*, 2011, **55**, 5194-5199.
18. D. Shah, M. D. Dang, R. Hasbun, H. L. Koo, Z. D. Jiang, H. L. DuPont and K. W. Garey, *Expert review of anti-infective therapy*, 2010, **8**, 555-564.

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Highlights

- Ramizol a first-in-class styrylbenzene antibiotic is effective against 100 strains of *C. difficile*
- *C. difficile* clinical isolates show resistance to vancomycin and metronidazole
- Vancomycin-resistant strains show elevated MICs (> 8 μg/mL) in the presence of fidaxomicin