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In Vitro Activity of Ramoplanin against *Clostridium difficile*, Including Strains with Reduced Susceptibility to Vancomycin or with Resistance to Metronidazole

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We evaluated the in vitro activity of ramoplanin, an antimicrobial compound that inhibits cell wall synthesis by acting at the level of lipid intermediate formation, against *Clostridium difficile*. We included strains with reduced susceptibilities to vancomycin (vancomycin-intermediate [Van^i] strains) or with resistance to metronidazole (Mtz^r), in order to assess the potential utility of ramoplanin for the treatment of *C. difficile*-associated diarrhea. We tested the activity of ramoplanin against a total of 105 nonduplicate clinical isolates of toxigenic *C. difficile*, including 8 Van^i isolates and 6 Mtz^r isolates, obtained from our laboratory. Ramoplanin was active against all strains tested at concentrations ranging from 0.03 to 0.5 μg/ml (MICs at which 50 and 90% of isolates were inhibited, 0.25 μg/ml; geometric mean MIC, 0.22 μg/ml). All isolates, independently of their levels of susceptibility to vancomycin or metronidazole, were considered susceptible to ramoplanin (MICs, ≤0.5 μg/ml).

Rates of *Clostridium difficile*-associated diarrhea (CDAD) are increasing in hospitals worldwide as a consequence of the widespread use of broad-spectrum antibiotics, and the organism may also be an important cause of community-acquired diarrhea (1, 9, 14, 17). The drugs of choice for the treatment of CDAD are metronidazole (MTZ) and oral vancomycin (VAN).

Our group recently reported on the isolation of MTZ-resistant (Mtz^r) and VAN-intermediate (Van^i) C. *difficile* strains (19). The roles of these nonsusceptible strains in clinical failures and relapses remain unknown, but therapeutic alternatives must be sought.

Ramoplanin, a lipoglycodepsipeptide antibiotic obtained from the fermentation of an *Actinoplanes* strain (ATCC 33076), is being developed as an oral, nonabsorbable agent for the gastrointestinal decontamination of patients infected or colonized with VAN-resistant enterococci (12, 15, 24). No cross-resistance between ramoplanin and VAN has been so far described, due to differences in their structures and mechanisms of action (6, 21).

Our objective was to evaluate the in vitro activity of ramoplanin against *C. difficile*, with a special interest in those strains that have reduced susceptibilities to VAN (Van^i strains) or resistance to MTZ (Mtz^r strains), in order to assess its potential utility for the treatment of CDAD.

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MATERIALS AND METHODS

The activity of ramoplanin was tested against a total of 105 nonduplicate clinical isolates of toxigenic *C. difficile* obtained in our laboratory over a 9-year period (1994 to 2002). Eight of the strains had reduced susceptibility to VAN, and six strains were MTZ resistant. The MICs of VAN for the *C. difficile* Van^i isolates were 4 μg/ml (six strains) and 8 μg/ml (two strains); and the MTZ MICs for the Mtz^r isolates were 16 μg/ml (three strains), 32 μg/ml (two strains), and 64 μg/ml (one strain).

C. difficile isolates were presumptively identified by their colony morphology, yellow color, ground-glass texture, and characteristic horse dung smell and by Gram staining (16). Additional biochemical tests (Rapid ID 32A system; bio Mérieux, Marcy l'Etoile, France) were also used. All the strains with reduced susceptibilities to VAN and resistance to MTZ were further identified by molecular methods. A 270-bp fragment of the 16S rRNA gene was amplified with specific primers (10). The 16S rRNA gene sequences obtained were compared with those available in the GenBank database by use of the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The presence of *C. difficile* toxin B was determined by demonstrating a specific cytopathic effect on MRC-5 cells, as described previously (16, 20, 22), either directly from fecal samples or, if the fecal samples tested negative, from pure cultures of the microorganism (3). An enzyme immunoassay system (CdTOX A OIA; BioStar, Louisville, Ky.) was used to detect toxin A in the fecal samples. The test was repeated with pure cultures when a negative result was observed with a clinical specimen tested directly. Large clostridial toxins (LCTs) genes were detected by PCR assays (13, 23). All isolates included at this study were toxigenic as a result of the presence of both LCTs (TcdA and TcdB), as determined by phenotypic and genetic methods.

Ramoplanin (provided by Vicuron Pharmaceuticals) was prepared and stored according to the instructions of the supplier. Antimicrobial susceptibility testing was performed by the agar dilution method on brucella agar (Oxoid, Basingstoke, United Kingdom), according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (18). *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741 were always included as reference control strains for quality control for antimicrobial susceptibility testing. A collection strain of *C. difficile* (ATCC 9689) was also included to assess the reproducibility of the assay results.

Colonies were suspended in brucella broth (Becton Dickinson, Sparks, Md.) to a density equal to a 0.5 McFarland standard. The suspensions were applied to the antibiotic plates with a Steers replicator that delivered a final inoculum of

TABLE 1. Cumulative percentages of *C. difficile* isolates inhibited by each concentration of ramoplanin.

Strain group	No. of isolates	% Isolates inhibited by ramoplanin concn (μg/ml) of:				
		0.03	0.06	0.12	0.25	0.5
Total	105	0.95	2.85	16.15	99	100
Mtz^s and Van^{sa}	91	1.09	2.18	13.16	98.87	100
Mtzr	6	0	20	80	100	
Van^i	8	0	0	12.5	100	

[a] Strains susceptible to metronidazole and vancomycin.

approximately 10^5 CFU/spot. The plates were incubated in an anaerobic chamber incubator at 37°C for 48 h.

The MIC was defined as the lowest concentration of the agent that inhibited growth. The appearance of a barely visible haze was disregarded (18). Reference strains (*B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, and *C. difficile* ATCC 9689) were included as controls to monitor the results of the antimicrobial susceptibility tests and to assess the reproducibility of the assays. The breakpoints for MTZ were ≤8 μg/ml for susceptible, 16 μg/ml for intermediate, and ≥32 μg/ml for resistant. We considered the breakpoints for VAN to be ≤2 μg/ml for susceptible, 4 to 16 μg/ml for intermediate, and ≥32 μg/ml for resistant, as NCCLS has not defined breakpoint standards for VAN. A susceptibility breakpoint of ≤2 μg/ml was considered for ramoplanin, as preliminarily proposed (5).

RESULTS

The nucleotide sequences of a 270-bp fragment of the 16S rRNA genes of all the strains with reduced susceptibilities to VAN and resistance to MTZ showed identities of more than 99% with the *C. difficile* genome sequences in GenBank.

Ramoplanin was active against all strains tested at a concentration ≤0.5 μg/ml. Overall, the MICs ranged from 0.03 to 0.5 μg/ml, the MIC at which 50% of isolates were inhibited (MIC_{50}) and the MIC_{90} were both 0.25 μg/ml, and the MIC geometric mean was 0.22 μg/ml. The MICs for the isolates susceptible to VAN and MTZ (91 strains) ranged from 0.03 to 0.5 μg/ml, the MIC_{50} and MIC_{90} were both 0.25 μg/ml, and the geometric mean MIC was 0.23 μg/ml. The MICs for the Van^i isolates ranged from 0.12 to 0.25 μg/ml, the MIC_{50} and MIC_{90} were both 0.25 μg/ml, and the geometric mean MIC was 0.23 μg/ml. The MICs for the *C. difficile* Mtz^r isolates ranged from 0.06 to 0.25 μg/ml, the MIC_{50} and MIC_{90} were 0.12 and 0.25 μg/ml, respectively, and the geometric mean MIC was 0.14 μg/ml. The cumulative percentages of *C. difficile* isolates inhibited by each concentration of ramoplanin are shown in Table 1.

All isolates were considered susceptible to ramoplanin independently of their susceptibility to VAN or MTZ.

DISCUSSION

C. difficile susceptibility tests are not very often performed in microbiology laboratories because to date the first-line drugs, MTZ and VAN, have been considered universally active against the microorganism (4, 5, 9).

There are, however, a few reports of reductions in susceptibilities to MTZ and VAN (4, 7, 25). Our group has already registered a 6% rate of resistance to MTZ, and 3% of our *C. difficile* isolates have shown reduced susceptibility to VAN (19).

Published information (2, 5, 8) showing the activity of ramoplanin against all isolates of *C. difficile* is available for a limited number of strains, but all of those isolates were susceptible to MTZ and VAN. In this study, ramoplanin showed excellent in vitro activity against a large and heterogeneous collection of *C. difficile* isolates. It was also reported previously (19) that our nonsusceptible strains did not have a clonal origin. The activity of ramoplanin did not change when the level of susceptibility to either VAN or MTZ was reduced.

In vitro activity does not necessarily mean in vivo activity, and prospective clinical trials for the evaluation of ramoplanin for the treatment of CDAD are warranted. A recent report, presented in abstract form by Jabes et al. (11), showed the superior efficacy of ramoplanin treatment over that of standard VAN treatment for *C. difficile*-induced colitis in hamsters.

Our data indicate the need for further clinical studies of ramoplanin as a potential alternative treatment for CDAD.

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