AAC Journals.ASM.org

Mode of Action and Bactericidal Properties of Surotomycin against Growing and Nongrowing *Clostridium difficile*

Mohammed Zahidul Alam,[a] Xiaoqian Wu,[a,b] Carmela Mascio,[c] Laurent Chesnel,[c] Julian G. Hurdle[a,b,d]

Department of Biology, University of Texas at Arlington, Arlington, Texas, USA[a]; Center for Infectious and Inflammatory Diseases, Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, Texas, USA[b]; Merck and Co., Inc., Kenilworth, New Jersey, USA[c]; Department of Microbial and Molecular Pathogenesis, College of Medicine, Texas A&M Health Science Center, Bryan, Texas, USA[d]

Surotomycin (CB-183,315), a cyclic lipopeptide, is in phase 3 clinical development for the treatment of *Clostridium difficile* infection. We report here the further characterization of the *in vitro* mode of action of surotomycin, including its activity against growing and nongrowing *C. difficile*. This was assessed through time-kill kinetics, allowing a determination of the effects on the membrane potential and permeability and macromolecular synthesis in *C. difficile*. Against representative strains of *C. difficile*, surotomycin displayed concentration-dependent killing of both logarithmic-phase and stationary-phase cultures at a concentration that was ≤16× the MIC. Exposure resulted in the inhibition of macromolecular synthesis (in DNA, RNA, proteins, and cell wall). At bactericidal concentrations, surotomycin dissipated the membrane potential of *C. difficile* without changes to the permeability of propidium iodide. These observations are consistent with surotomycin acting as a membrane-active antibiotic, exhibiting rapid bactericidal activities against growing and nongrowing *C. difficile*.

The Gram-positive spore-forming anaerobic bacterium *Clostridium difficile* is the leading cause of hospital-acquired diarrhea in North America and Europe (1, 2). Elderly hospitalized patients on broad-spectrum antibiotics are the main target population, but recent observations indicate there is an increase in the incidence of *C. difficile* infection (CDI) in the community without known risk factors (3, 4). In the United States in 2011, there were an estimated 500,000 cases of CDI resulting in 29,300 deaths (5), which reflects the devastating impact of CDI since the turn of the last century. Furthermore, the number of cases of severe CDI has escalated, coinciding with the emergence of epidemic ribotypes, such as BI/NAP1/027 (2, 6). BI/NAP1/027 is now responsible for a significant number of cases of hospital-acquired CDI in North America (5, 6).

For >30 years, vancomycin and metronidazole have been the first-line treatment choices for CDI (7). Metronidazole is prescribed for mild to moderate CDI, while vancomycin is recommended for severe CDI (6, 8). However, rates of recurrence of 20 to ≥25% in severe CDI are common following treatment with metronidazole or vancomycin (6, 9, 10). The mode of action of vancomycin is well established, involving inhibition of the later stages of peptidoglycan biosynthesis, which primarily kill rapidly growing *C. difficile* (11). Metronidazole undergoes biochemical reduction to form reactive species that target DNA and is potent *in vitro*, but only low concentrations reside in the gastrointestinal tract (12–16). Fidaxomicin, which targets the bacterial RNA polymerase inhibitor, has a narrower spectrum of activity than that of metronidazole and vancomycin and is superior in the prevention of CDI recurrence (17, 18). However, additional novel therapeutics are required to effectively treat CDI and reduce the rates of recurrence following initial therapy.

Surotomycin is a minimally absorbed narrow-spectrum cyclic lipopeptide antibiotic, which is in phase 3 clinical trials as a novel treatment for CDI. It is chemically and structurally related to the antibiotic daptomycin that targets the bacterial membrane, thereby exhibiting bactericidal effects (19–21). Daptomycin has been shown to display activity against stationary-phase *Staphylococcus aureus* (21), which is a property that would seem amenable to the action of surotomycin in mitigating the pathogenesis of *C. difficile*. This organism produces spores and toxins (TcdA and TcdB), primarily in the late logarithmic and stationary phases of growth (22). However, it has not been reported as to whether the bactericidal activity of surotomycin encompasses nongrowing stationary-phase *C. difficile*. Killing of stationary-phase cells by membrane-active antibiotics has been shown to lower toxin and spore numbers *in vitro*, which in principle might contribute to lowering disease severity and rates of endogenous recurrence (11). The basis for the potent activity of surotomycin against *C. difficile* is thought to arise from dissipation of the bacterial membrane, as shown in *S. aureus* (19). However, direct studies to determine if surotomycin dissipates the membrane potential of *C. difficile* have not been reported. Here, we characterized the mode of action of surotomycin against *C. difficile*, examining its bactericidal effects on logarithmic- and stationary-phase cells and the associated cellular effects linked to dissipation of the membrane potential in *C. difficile*.

(This study was presented in part as a poster presentation at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy [ICAAC], 5 to 9 September 2014, Washington, DC.)

Received 7 May 2015 **Returned for modification** 1 June 2015
Accepted 5 June 2015

Accepted manuscript posted online 8 June 2015

Citation Alam MZ, Wu X, Mascio C, Chesnel L, Hurdle JG. 2015. Mode of action and bactericidal properties of surotomycin against growing and nongrowing *Clostridium difficile*. Antimicrob Agents Chemother 59:5165–5170. doi:10.1128/AAC.01087-15.

Address correspondence to Julian G. Hurdle, jhurdle@ibt.tamhsc.edu.

doi:10.1128/AAC.01087-15

TABLE 1 *In vitro* activities of surotomycin and other first-line drugs for the treatment of CDI

Drug	Activity (μg/ml) of (ribotype)[a]:								
	BAA-1875 (078)			R20291 (027)			IT0843 (001)		
	MIC	MBC_{LOG}	MBC_{STA}	MIC	MBC_{LOG}	MBC_{STA}	MIC	MBC_{LOG}	MBC_{STA}
Surotomycin	0.25	4	2	1	8	16	0.125	8	16
Metronidazole	0.5	2	8	0.5	8	16	0.25	1	16
Vancomycin	0.5	>128	>128	2	>128	>128	2	>128	>128

[a] MBC_{LOG}, minimum bactericidal concentration against logarithmic-phase cells; MBC_{STA}, minimum bactericidal concentration against stationary-phase cells.

MATERIALS AND METHODS

Compounds, bacterial strains, and growth media. Surotomycin and daptomycin were provided by Merck and Co., Inc. All other antimicrobials (vancomycin, metronidazole, carbonyl cyanide *m*-chlorophenylhydrazone [CCCP], ampicillin, fusidic acid, rifaximin, and nisin) were obtained from Sigma-Aldrich or Enzo Life Sciences (gatifloxacin). *C. difficile* strains BAA-1875 (ribotype 078) and BAA-1803 (ribotype 027) were from the American Type Culture Collection (ATCC). *C. difficile* strain R20291 (ribotype 027) was kindly provided by A. L. Sonenshein, Tufts University, Boston, MA, USA. *C. difficile* strainIT0843 (ribotype 001) was kindly provided by Paola Mastrantonio (Istituto Superiore di Sanità, Rome, Italy). Brain heart infusion (BHI) agar (Oxoid) was used for all the experiments and supplemented with calcium to a final concentration of 50 mg/liter for all experiments with surotomycin and daptomycin. To supplement BHI to a final calcium concentration of 50 mg/liter, the calcium levels in manufactured lots of BHI were determined by Laboratory Specialists, Inc., OH. All strains were routinely grown in prereduced BHI medium under anaerobic conditions in a Whitley A35 anaerobic workstation at 37°C.

Determination of MICs and minimum bactericidal concentrations. Minimum inhibitory and minimum bactericidal concentrations of compounds against *C. difficile* were determined as described by Wu et al. (11, 23). The MICs were determined using ~10^6 CFU/ml of inoculum of *C. difficile* in 24-well microtiter plates containing 2-fold serial dilutions of compounds in a total volume of 1 ml. The MIC was defined as the lowest concentration of compound inhibiting visible growth after 24 h of incubation. Minimum bactericidal concentrations (MBCs) were determined against both logarithmic-phase (MBC_{LOG}) and stationary-phase (MBC_{STA}) cells using logarithmic (optical density at 600 nm [OD_{600}], ≈0.3) and 24-h-old cultures, respectively. Briefly, in 24-well microtiter plates, cultures were added to 2-fold-diluted compounds in a total volume of 1 ml. After 24 h of incubation, the number of viable cells was determined by plating aliquots onto prereduced BHI agar containing activated charcoal (10% [wt/vol]). The MBC was defined as the lowest concentration of compound causing a ≥3-log reduction in viable cells compared to the starting inocula. The MICs and MBCs were determined from two independent starting cultures.

Time-kill kinetics were evaluated against both the logarithmic- and stationary-phase cultures, as described by Wu et al. (11, 23). Logarithmic-phase (OD_{600}, ≈0.3) and stationary-phase cultures were exposed to 1, 4, and 16× the MIC of the compounds. Samples (100 μl) were taken at 0, 1, 2, 4, 6, and 24 h after the addition of compounds, and viable cell counts were determined on BHI agar plates containing activated charcoal (10% [wt/vol]). Bacterial counts were enumerated after 24 h of incubation. Two independent starting cultures were used for this assay.

Effects on macromolecular biosynthesis. Logarithmic cultures of R20291 and BAA-1875 were grown to early logarithmic phase (OD_{600}, ≈0.3) under anaerobic conditions and aliquoted for subsequent analysis. To analyze the DNA, RNA, protein, and cell wall synthesis inhibition, [^{3}H]thymidine (2 μCi/ml), [^{3}H]uridine (2 μCi/ml), [^{3}H]threonine (2 μCi/ml), and [^{3}H]*N*-acetyl-glucosamine (2 μCi/ml) were used, respectively. Radiolabeled precursors were added 5 min before the addition of compounds at either inhibitory (1× MIC) or bactericidal (16× MIC) concentrations. Gatifloxacin, rifaximin, fusidic acid, and ampicillin were used as controls for DNA, RNA, protein, and cell wall synthesis inhibition, respectively. Against the BAA-1875 strain, the gatifloxacin, rifaximin, fusidic acid, and ampicillin concentrations at 16× MIC were 64 μg/ml, 0.96 μg/ml, 2 μg/ml, and 8 μg/ml, respectively. Against R20291, the concentrations at 16× MIC were 512 μg/ml, 2 μg/ml, 2 μg/ml, and 16 μg/ml for the respective control drugs. Samples (500 μl) were taken at specific time points (30, 60, and 120 min), spun down, and the cell pellet collected and incubated on ice with 10% (wt/vol) ice-cold trichloroacetic acid (TCA) for 30 min. Samples were then filtered through Whatman GF/C filters and washed twice with 5% (wt/vol) TCA and 95% ethanol. The filters were dried and scintillation counting performed.

Determination of the membrane potential and permeability using fluorescence-activated cell sorting. To assess the effects of compounds on the membrane potential and permeability of *C. difficile*, we adopted a fluorocytometric method relying on the use of $DiBAC_4(3)$ [bis-(1,3-dibutylbarbituric acid)trimethine oxonol] to assess the membrane potential and propidium iodide (PI) to assess membrane permeability. This method was based on that reported by Nuding et al. (24) for anaerobic bacteria; 3,3′-diethyloxacarbocyanine iodide [$DiOC_2(3)$] was found to be inconsistent (data not shown). Strains R20291 and BAA-1875 were used and were exposed to different concentrations of compounds as 1, 4, and 16× the MIC of compounds. Briefly, cultures were grown anaerobically to an OD_{600} ≈0.2 and 10-ml aliquots added to 20-ml serum vials. Compounds were subsequently added and the vials crimped, sealed with silicone bungs, and removed from the anaerobic chamber. After 10 min of adding compound, $DiBAC_4(3)$ was added via a 23-G syringe needle to a final concentration of 5 μM. After an overall 30 min of exposing cells to the compounds at room temperature, fluorocytometric analysis was performed using the BD LSR II flow cytometer. $DiBAC_4(3)$ was excited using the 488-nm excitation laser and its fluorescence emission detected using fluorescein isothiocyanate (FITC) filters. As a positive control, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Sigma-Aldrich), which completely dissipates the membrane potential, was used; vancomycin was used as a negative control, and a minimum of three independent cultures were evaluated.

Membrane permeability assays were similarly performed using the protocol described above, except that the membrane-impermanent dye PI was added to a final concentration of 5 μM instead of $DiBAC_4(3)$. After 30 min, samples were analyzed in the BD LSR II flow cytometer, with excitation at 488 nm and emission collected using the PI-A filters. Nisin was used as a positive control for membrane damage and vancomycin as a negative control. Daptomycin was also included as a control in these experiments. Resazurin (0.001 gm/liter) and sodium thioglycolate (0.5 gm/liter) were added to the medium to act as an indicator of oxygenation and as an oxygen scavenger, respectively. Histogram plots of the number of events against fluorescence of the population were comparatively analyzed using FlowJo X 10.0.7.

RESULTS

Surotomycin is bactericidal against both the logarithmic- and stationary-phase *C. difficile*. As shown in Table 1, the surotomycin MICs against test strains ranged from 0.125 to 1 μg/ml. The concentration of surotomycin required for bactericidal activity

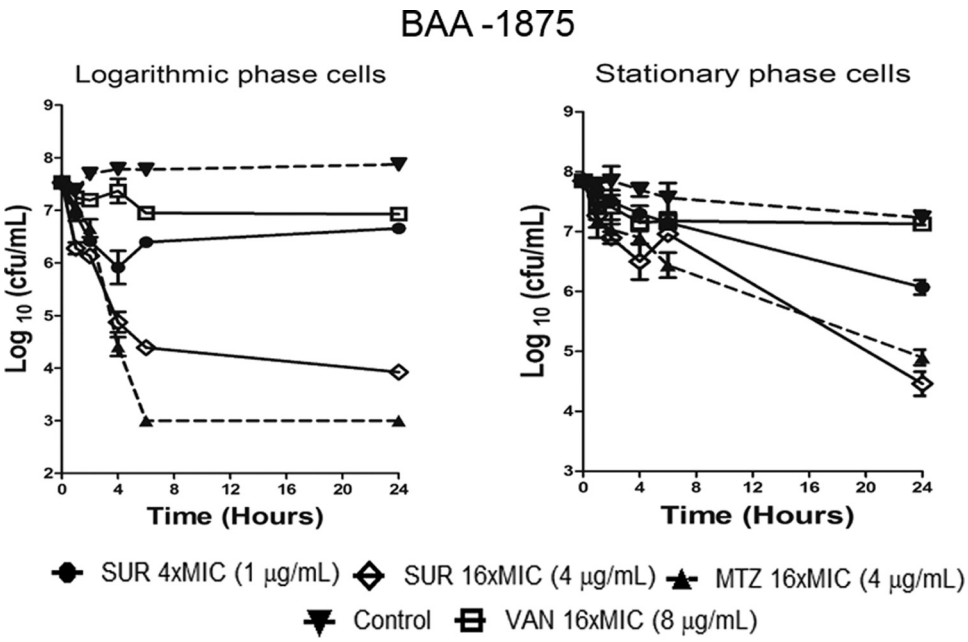

FIG 1 Time-kill kinetics of antibiotics against logarithmic-phase (left) and stationary-phase (right) BAA-1875 cultures. Various concentrations of surotomycin (SUR), metronidazole (MTZ), and vancomycin (VAN) are shown. Mean values are plotted, and error bars indicate standard deviations.

against logarithmic- and stationary-phase cultures was similar and was 8- to 128-fold above the MIC (Table 1), which corresponds to 2 to 16 μg/ml. As expected, the control metronidazole was also bactericidal, killing both culture types at concentrations between 2 and 16 μg/ml, whereas vancomycin was bacteriostatic and was completely inactive against stationary-phase *C. difficile*.

Surotomycin kills *C. difficile* in a concentration-dependent manner. Time-kill kinetics were subsequently performed to determine how rapidly surotomycin killed logarithmic- and stationary-phase cells. These assays revealed that surotomycin exhibited a concentration-dependent mode of killing against both the logarithmic- and stationary-phase cultures of *C. difficile*. Against logarithmic BAA-1875 at 16× the MIC (4 μg/ml), surotomycin killed >99% of the cells in 6 h, whereas 24 h was required to achieve a similar reduction in culture viability against stationary-phase cells (Fig. 1). A similar pattern of killing was observed against R20291, although this strain seemed to be more sensitive at 4× the MIC of surotomycin, since a 99% reduction in viable numbers was observed after 24 h against logarithmic-phase cultures (Fig. 2). This suggests that against R20291 in a larger culture volume (10 ml), the MBC is 4 μg/ml, which differs by 4-fold from that obtained in 1-ml volumes for MBC determinations. Metronidazole was also found to display concentration-dependent killing (against both logarithmic- and stationary-phase cells), causing a ≥99% reduction in viable cells at 16× the MIC after 24 h, whereas vancomycin demonstrated a bacteriostatic effect and was completely inactive against stationary-phase cultures. These observations broadly support the above findings of the MBC data.

Surotomycin dissipates membrane potential without pore formation. In order to examine whether surotomycin dissipates the membrane potential of *C. difficile*, we used the fluorescent probe $DiBAC_4(3)$. The fluorescence of $DiBAC_4(3)$ changes with the membrane potential status of cells, with depolarized cells demonstrating enhanced fluorescence, due to $DiBAC_4(3)$ entering depolarized membranes and binding to lipid-rich intracellular components, thereby exhibiting increased green fluorescence (24). When *C. difficile* cultures of R20291 and BAA-1875 were treated with surotomycin or daptomycin, increases in florescence were observed only at 16× their MICs compared to the untreated cultures (Fig. 3). These concentrations were bactericidal for both agents. No changes in the fluorescence of cells were observed at lower concentrations of surotomycin or daptomycin (i.e., 1 and 4× the MIC). As expected, the negative-control vancomycin, which inhibits peptidoglycan biosynthesis, did not alter the membrane potential, while CCCP, which acts as a proton ionophore and disrupts the bacterial membrane potential, was shown to increase the fluorescence of R20291 and BAA-1875. At 30 min of treatment, dissipation of the membrane potential by surotomycin and daptomycin at 16× their MICs did not result in membrane pore formation, as cells did not show an increase in propidium iodide fluorescence compared to that of the untreated control and vancomycin-treated cultures. In contrast, the pore-forming agent nisin caused membrane pore formation, which was evident by an increase in the red fluorescence of cells (Fig. 3).

As the above-described observations are based on 30-min incubation periods, we extended the incubation times. Incubation of cultures with 1 and 4× the MIC of surotomycin or daptomycin for up to 2 h did not lead to an observable difference in the membrane potential status of cells compared to that of the untreated controls (data not shown). Similarly, continued exposure to 16× the MIC of these drugs did not produce further measurable increases in the dissipation of the membrane potential; extended incubation times at 1, 4, and 16× the MIC did not lead to increases in the permeability of cultures to propidium iodide (data not shown).

Surotomycin inhibits multiple macromolecular biosynthetic processes. Exposure of R20291 and BAA-1875 to inhibitory (MIC) and bactericidal (16× the MIC) concentrations of suroto-

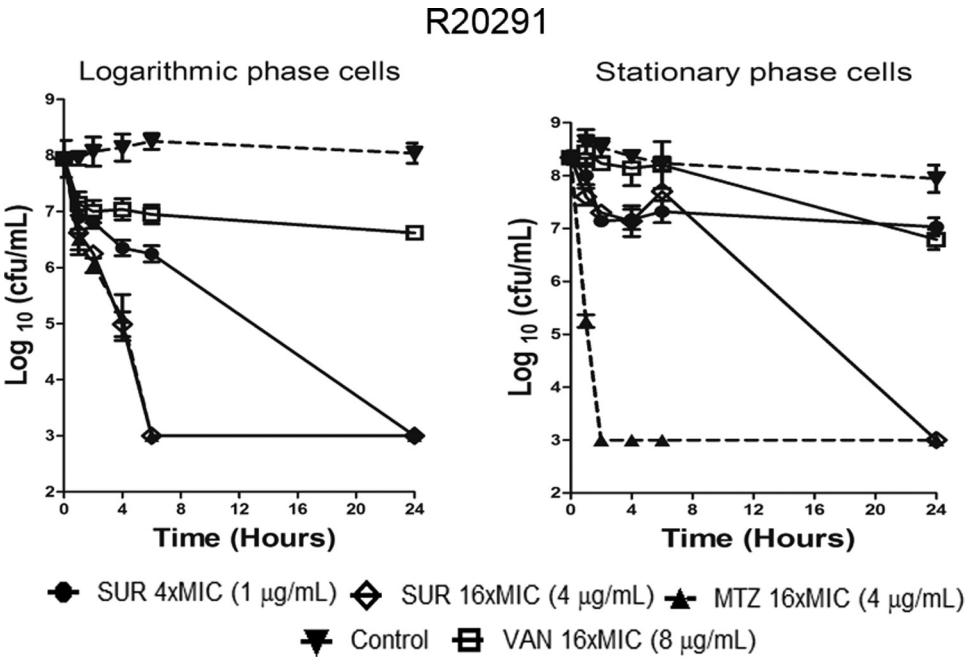

FIG 2 Time-kill kinetics of antibiotics against logarithmic-phase (left) and stationary-phase (right) R20291 cultures. Various concentrations of surotomycin (SUR), metronidazole (MTZ), and vancomycin (VAN) are shown. Mean values are plotted, and error bars indicate standard deviations.

mycin resulted in the simultaneous inhibition of DNA, RNA, protein, and cell walls (Fig. 4 and 5). While it is expected that all macromolecular processes would be affected in dying cells at the bactericidal concentration, these processes were also affected in cells exposed to inhibitory concentrations. This is consistent with the membrane being the primary target for surotomycin activity, thereby imposing multiple cellular effects on processes that require membrane homeostasis (25).

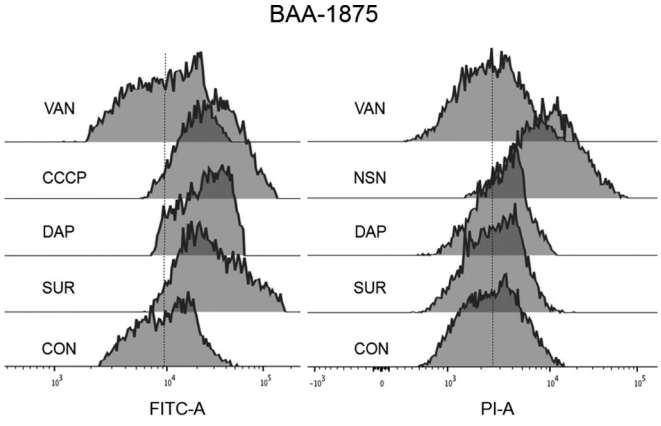

FIG 3 Dissipation of the membrane potential (left) and effects on membrane permeability (right) of *C. difficile*, shown as histogram half overlays. Representative data from three independent cultures of BAA-1875 are shown following exposure to drugs at 16× the MIC. Left, CCCP was used as a control for dissipation of membrane potential; right, nisin is used as a pore-forming control. Vancomycin was used as a negative control. CON, control; SUR, surotomycin (4 μg/ml); DAP, daptomycin (16 μg/ml); CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine (2 μg/ml); NSN, nisin (8 μg/ml); VAN, vancomycin (8 μg/ml). The filters were for fluorescein isothiocyanate (FITC) and propidium iodide (PI).

DISCUSSION

Recent studies established that the membrane potential of *C. difficile* is critical to the survival of logarithmic- and stationary-phase cells, making the clostridial membrane an attractive target for agents to treat CDI (11). Dissipation of the membrane potential, resulting in a loss of viability in both growing and nongrowing cell types, has direct relevance to *C. difficile* pathogenesis, as this organism produces its toxins and spores in the late-logarithmic and stationary phases of growth (22). The cyclic lipopeptide drug surotomycin represents the leading example of a membrane-active antibiotic for treating CDI. In this study, we validated that surotomycin dissipates the membrane potential of *C. difficile*, and this was not associated with the formation of pores at bactericidal concentrations in two test strains. This observation is consistent with a prior report by Mascio et al. (19) in which surotomycin dissipated the membrane potential of *S. aureus* without causing pore formation. Dissipation of the membrane potential of *C. difficile* was evaluated using the fluoroprobe $DiBAC_4(3)$, which other studies have adopted to measure the membrane potential in anaerobes, and it appears to be more reliable than DiOC for anaerobes (24). However, measurable disruptions of the membrane potential in *C. difficile* were observed only at bactericidal concentrations. This might reflect that the magnitude of the membrane potential in clostridia is low (26). Hence, the marginal lowering of the membrane potential of *C. difficile* upon exposure to inhibitory concentrations of drug may be challenging for measurements using $DiBAC_4(3)$, and other techniques may be required for lower concentrations.

The action of surotomycin against various strains of *C. difficile* resulted in bactericidal activities against logarithmic- and stationary-phase cultures and imposed multiple cellular effects, as evident by the widespread disruption of macromolecular processes. In contrast, the cell wall synthesis inhibitor vancomycin was

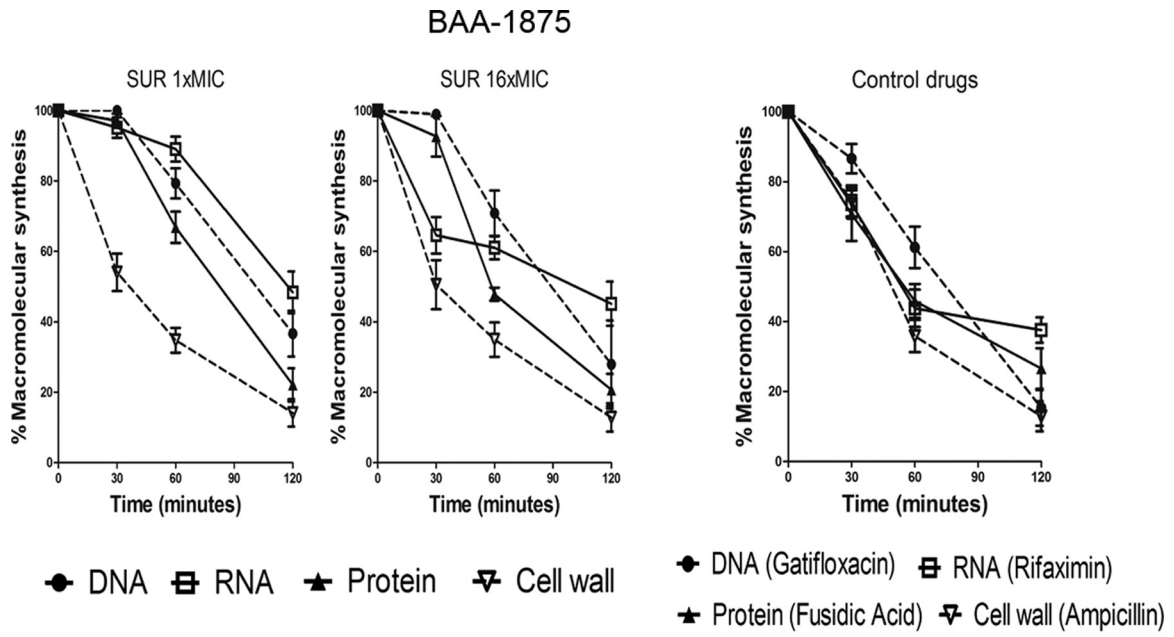

FIG 4 Effects of inhibitory and bactericidal concentrations of surotomycin (SUR) on macromolecular biosynthesis in *C. difficile*. The effects against BAA-1875 are shown for SUR (0.25 and 4 μg/ml). The control drugs and concentrations are as follows: gatifloxacin, 64 μg/ml; rifaximin, 0.96 μg/ml; fusidic acid, 2 μg/ml; and ampicillin, 8 μg/ml). Mean values are plotted, and error bars indicate standard deviations.

poorly active or bacteriostatic against logarithmic-phase cells and inactive against stationary-phase cultures. Metronidazole did reduce the viability of both cell types, but only low concentrations of drug occur in the gastrointestinal tract, as the drug is almost completely absorbed following oral administration (27).

The concentration of surotomycin that was required to inactivate both logarithmic- and stationary-phase cultures was typically 2 to 16 μg/ml. These levels are well within the local concentrations of surotomycin (>1,000 μg/g of fecal sample) that is present in the colon of patients following oral administration (data on file at Merck and Co., Inc., Kenilworth, NJ, USA). It is therefore plausible that in the colon, surotomycin is bactericidal against both logarithmic- and stationary-phase cells. This property might reduce both toxin and spore production in vegetative populations, as recently reported in the *in vitro* human gut model (28). In phase II clinical trials, lower rates of recurrence were associated with sur-

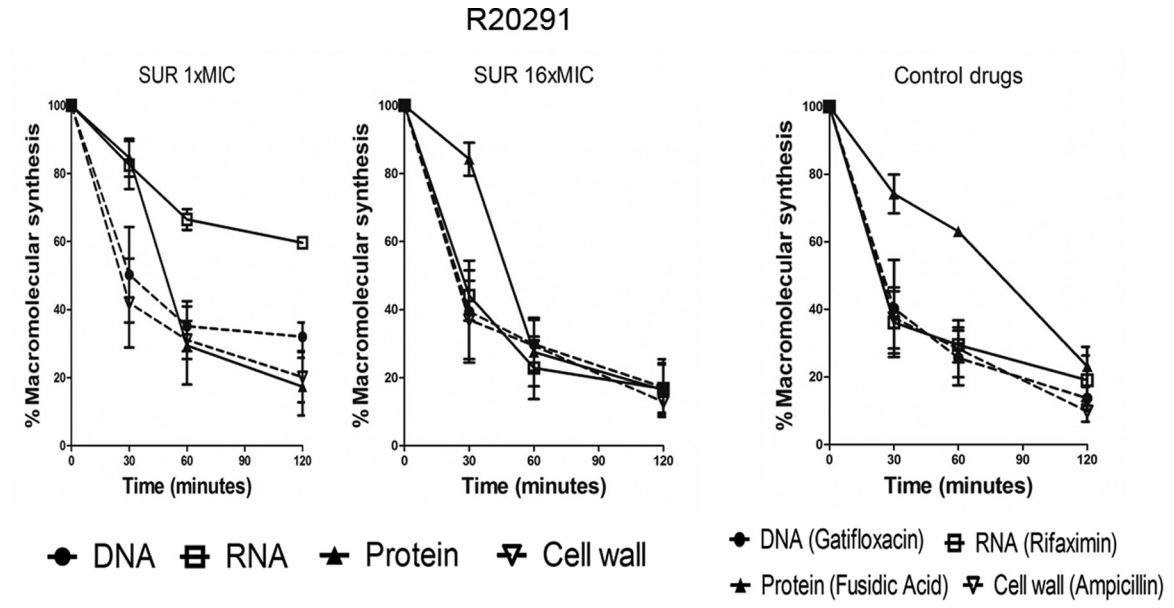

FIG 5 Effects of inhibitory and bactericidal concentrations of surotomycin (SUR) on macromolecular biosynthesis in *C. difficile*. The effects against R20291 are shown for SUR (1 and 16 μg/ml). The control drugs and concentrations are as follows: gatifloxacin, 512 μg/ml; rifaximin, 2 μg/ml; fusidic acid, 2 μg/ml; and ampicillin, 16 μg/ml. Mean values are plotted, and error bars indicate standard deviations.

otomycin treatment than those with oral vancomycin; 27.9% and 17.2% were seen for surotomycin at 125 mg and 250 mg twice daily, respectively, and 35.6% was seen for vancomycin given at 125 mg four times per day (29). From a microbiological perspective, it is tempting to speculate that the bactericidal activity of surotomycin and its narrower spectrum compared to that of vancomycin contribute to a reduction in recurrence. However, the *in vitro* findings of this study do not provide a direct explanation for the superiority of surotomycin compared to vancomycin in reducing recurrence.

The present study reported provides a solid framework from which to rationalize several recent findings on the *in vitro* activities against *C. difficile*. Indeed, reported observations that surotomycin is bactericidal against logarithmic-phase cultures with a long postantibiotic effect, reduces toxin and spore production, and has a low propensity to select for *de novo* resistance in *C. difficile* (19, 28) can be rationalized as being consistent with the membrane being the biological target.

ACKNOWLEDGMENTS

Julian G. Hurdle acknowledges the receipt of funding from Merck and Co., Inc., Kenilworth, NJ, USA, to support this study.

During the study, Carmela Mascio was an employee of Merck and Co., Inc. Laurent Chesnel is an employee of Merck and Co., Inc. This study was initiated by Cubist Pharmaceuticals.

REFERENCES

1. **Rupnik M, Wilcox MH, Gerding DN.** 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol **7:**526–536. http://dx.doi.org/10.1038/nrmicro2164.
2. **Freeman J, Bauer MP, Baines SD, Corver J, Fawley WN, Goorhuis B, Kuijper EJ, Wilcox MH.** 2010. The changing epidemiology of *Clostridium difficile* infections. Clin Microbiol Rev **23:**529–549. http://dx.doi.org/10.1128/CMR.00082-09.
3. **Gupta A, Khanna S.** 2014. Community-acquired *Clostridium difficile* infection: an increasing public health threat. Infect Drug Resist **7:**63–72.
4. **Khanna S, Pardi DS, Aronson SL, Kammer PP, Orenstein R, St. Sauver JL, Harmsen WS, Zinsmeister AR.** 2012. The epidemiology of community-acquired *Clostridium difficile* infection: a population-based study. Am J Gastroenterol **107:**89–95. http://dx.doi.org/10.1038/ajg.2011.398.
5. **Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, Holzbauer SM, Meek JI, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago BM, Fridkin SK, Gerding DN, McDonald LC.** 2015. Burden of *Clostridium difficile* infection in the United States. N Engl J Med **372:**825–834. http://dx.doi.org/10.1056/NEJMoa1408913.
6. **Kelly CP, LaMont JT.** 2008. *Clostridium difficile*—more difficult than ever. N Engl J Med **359:**1932–1940. http://dx.doi.org/10.1056/NEJMra0707500.
7. **Hedge DD, Strain JD, Heins JR, Farver DK.** 2008. New advances in the treatment of *Clostridium difficile* infection (CDI). Ther Clin Risk Manag **4:**949–964.
8. **Surawicz CM, Brandt LJ, Binion DG, Ananthakrishnan AN, Curry SR, Gilligan PH, McFarland LV, Mellow M, Zuckerbraun BS.** 2013. Guidelines for diagnosis, treatment, and prevention of *Clostridium difficile* infections. Am J Gastroenterol **108:**478–498, quiz 499. http://dx.doi.org/10.1038/ajg.2013.4.
9. **Duke PS, Fardy J.** 2014. Recurrent *Clostridium difficile* infection treated with home fecal transplantation: a case report. J Med Case Rep **8:**393. http://dx.doi.org/10.1186/1752-1947-8-393.
10. **Figueroa I, Johnson S, Sambol SP, Goldstein EJ, Citron DM, Gerding DN.** 2012. Relapse versus reinfection: recurrent *Clostridium difficile* infection following treatment with fidaxomicin or vancomycin. Clin Infect Dis **55**(Suppl 2)**:**S104–S109.
11. **Wu X, Cherian PT, Lee RE, Hurdle JG.** 2013. The membrane as a target for controlling hypervirulent *Clostridium difficile* infections. J Antimicrob Chemother **68:**806–815. http://dx.doi.org/10.1093/jac/dks493.
12. **Lau AH, Lam NP, Piscitelli SC, Wilkes L, Danziger LH.** 1992. Clinical pharmacokinetics of metronidazole and other nitroimidazole anti-infectives. Clin Pharmacokinet **23:**328–364. http://dx.doi.org/10.2165/00003088-199223050-00002.
13. **Musher DM, Aslam S, Logan N, Nallacheru S, Bhaila I, Borchert F, Hamill RJ.** 2005. Relatively poor outcome after treatment of *Clostridium difficile* colitis with metronidazole. Clin Infect Dis **40:**1586–1590. http://dx.doi.org/10.1086/430311.
14. **Freeman CD, Klutman NE, Lamp KC.** 1997. Metronidazole. A therapeutic review and update. Drugs **54:**679–708.
15. **Lamp KC, Freeman CD, Klutman NE, Lacy MK.** 1999. Pharmacokinetics and pharmacodynamics of the nitroimidazole antimicrobials. Clin Pharmacokinet **36:**353–373. http://dx.doi.org/10.2165/00003088-199936050-00004.
16. **Bolton RP, Culshaw MA.** 1986. Faecal metronidazole concentrations during oral and intravenous therapy for antibiotic associated colitis due to *Clostridium difficile*. Gut **27:**1169–1172. http://dx.doi.org/10.1136/gut.27.10.1169.
17. **Louie TJ, Cannon K, Byrne B, Emery J, Ward L, Eyben M, Krulicki W.** 2012. Fidaxomicin preserves the intestinal microbiome during and after treatment of *Clostridium difficile* infection (CDI) and reduces both toxin reexpression and recurrence of CDI. Clin Infect Dis **55:**S132–S142. http://dx.doi.org/10.1093/cid/cis338.
18. **Cornely OA, Crook DW, Esposito R, Poirier A, Somero MS, Weiss K, Sears P, Gorbach S, OPT-80-004 Clinical Study Group.** 2012. Fidaxomicin versus vancomycin for infection with *Clostridium difficile* in Europe, Canada, and the USA: a double-blind, non-inferiority, randomised controlled trial. Lancet Infect Dis **12:**281–289. http://dx.doi.org/10.1016/S1473-3099(11)70374-7.
19. **Mascio CT, Mortin LI, Howland KT, Van Praagh AD, Zhang S, Arya A, Chuong CL, Kang C, Li T, Silverman JA.** 2012. *In vitro* and *in vivo* characterization of CB-183,315, a novel lipopeptide antibiotic for treatment of *Clostridium difficile*. Antimicrob Agents Chemother **56:**5023–5030. http://dx.doi.org/10.1128/AAC.00057-12.
20. **Pogliano J, Pogliano N, Silverman JA.** 2012. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. J Bacteriol **194:**4494–4504. http://dx.doi.org/10.1128/JB.00011-12.
21. **Mascio CT, Alder JD, Silverman JA.** 2007. Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. Antimicrob Agents Chemother **51:**4255–4260. http://dx.doi.org/10.1128/AAC.00824-07.
22. **Merrigan M, Venugopal A, Mallozzi M, Roxas B, Viswanathan VK, Johnson S, Gerding DN, Vedantam G.** 2010. Human hypervirulent *Clostridium difficile* strains exhibit increased sporulation as well as robust toxin production. J Bacteriol **192:**4904–4911. http://dx.doi.org/10.1128/JB.00445-10.
23. **Wu X, Alam MZ, Feng L, Tsutsumi LS, Sun D, Hurdle JG.** 2014. Prospects for flavonoid and related phytochemicals as nature-inspired treatments for *Clostridium difficile* infection. J Appl Microbiol **116:**23–31. http://dx.doi.org/10.1111/jam.12344.
24. **Nuding S, Fellermann K, Wehkamp J, Mueller HA, Stange EF.** 2006. A flow cytometric assay to monitor antimicrobial activity of defensins and cationic tissue extracts. J Microbiol Methods **65:**335–345. http://dx.doi.org/10.1016/j.mimet.2005.08.004.
25. **Hurdle JG, O'Neill AJ, Chopra I, Lee RE.** 2011. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol **9:**62–75. http://dx.doi.org/10.1038/nrmicro2474.
26. **Okereke A, Montville TJ.** 1992. Nisin dissipates the proton motive force of the obligate anaerobe *Clostridium sporogenes* PA 3679. Appl Environ Microbiol **58:**2463–2467.
27. **Pepin J.** 2008. Vancomycin for the treatment of *Clostridium difficile* infection: for whom is this expensive bullet really magic? Clin Infect Dis **46:**1493–1498. http://dx.doi.org/10.1086/587656.
28. **Chilton CH, Crowther GS, Todhunter SL, Nicholson S, Freeman J, Chesnel L, Wilcox MH.** 2014. Efficacy of surotomycin in an *in vitro* gut model of *Clostridium difficile* infection. J Antimicrob Chemother **69:**2426–2433. http://dx.doi.org/10.1093/jac/dku141.
29. **Mascio CT, Chesnel L, Thorne G, Silverman JA.** 2014. Surotomycin demonstrates low *in vitro* frequency of resistance and rapid bactericidal activity in *Clostridium difficile*, *Enterococcus faecalis*, and *Enterococcus faecium*. Antimicrob Agents Chemother **58:**3976–3982. http://dx.doi.org/10.1128/AAC.00124-14.