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1 **Mode of Action and Bactericidal Properties of Surotomycin against**
2 **Growing and Non-growing *Clostridium difficile***

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23

24 **ABSTRACT**

25 Surotomycin (CB-183,315), a cyclic lipopeptide is in phase 3 clinical development for
26 the treatment of *Clostridium difficile* infection. We report further characterization of the
27 *in vitro* mode of action of surotomycin, including its activity against growing and non-
28 growing *C. difficile*. This was assessed through time kill kinetics, determining effects on
29 the membrane potential and permeability and macromolecular synthesis in *C. difficile*.
30 Against representative strains of *C. difficile*, surotomycin displayed concentration-
31 dependent killing of both logarithmic-phase and stationary-phase cultures at a
32 concentration that was ≤ 16 -fold more than the minimum inhibitory concentration (MIC).
33 Exposure resulted in the inhibition of macromolecular synthesis (DNA, RNA, protein and
34 cell wall). At bactericidal concentrations, surotomycin dissipated the membrane potential
35 of *C. difficile* without changes to the permeability of propidium iodide. These
36 observations are consistent with surotomycin acting as a membrane-active antibiotic,
37 exhibiting rapid bactericidal activities against growing and non-growing *C. difficile*.

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39

40 INTRODUCTION

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

51 For more than 30 years vancomycin and metronidazole have been the first-line
52 treatment choices for CDI (7). Metronidazole is prescribed for mild to moderate CDI,
53 while vancomycin is recommended for severe CDI (6, 8). However, rates of recurrence of
54 20-25% or higher in severe CDI are common, following treatment with metronidazole or
55 vancomycin (6, 9, 10). The mode of action of vancomycin is well established, involving
56 inhibition of the latter stages of peptidoglycan biosynthesis, which primarily kills rapidly
57 growing *C. difficile* (11). Metronidazole undergoes biochemical reduction to form
58 reactive species that target DNA and is potent in vitro, but only low concentrations reside
59 in the gastrointestinal tract (12-16). Fidaxomicin, which targets the bacterial RNA
60 polymerase inhibitor, has a narrower spectrum of activity than metronidazole and
61 vancomycin and is superior in the prevention of CDI recurrence (17, 18). However,

62 additional novel therapeutics are required to effectively treat CDI and reduce the rates of
63 recurrence following initial therapy.

64 Surotomycin is a minimally absorbed narrow-spectrum cyclic lipopeptide
65 antibiotic, which is in phase 3 clinical trials as a novel treatment for CDI. It is chemically
66 and structurally related to the antibiotic daptomycin that targets the bacterial membrane
67 thereby exhibiting bactericidal effects (19-21). Daptomycin has been shown to display
68 activities against stationary phase *Staphylococcus aureus* (21), which is a property that
69 would seem amenable to the action of surotomycin in mitigating the pathogenesis of *C.*
70 *difficile*. This organism produces spores and toxin (TcdA and TcdB), primarily in the late
71 logarithmic and stationary phases of growth (22). However, it is unreported whether the
72 bactericidal activity of surotomycin encompasses the non-growing stationary phase *C.*
73 *difficile*. Killing of stationary phase cells by membrane-active antibiotics has been shown
74 to lower toxin and spore numbers in vitro, which in principle could contribute to lowering
75 disease severity and rates of endogenous recurrence (11). The basis for surotomycin's
76 potent activity against *C. difficile* is thought to arise from dissipation of the bacterial
77 membrane as shown in *S. aureus* (19). However, direct studies determining if
78 surotomycin dissipates the membrane potential of *C. difficile* have not been reported.
79 Herein, we characterized the mode of action of surotomycin against *C. difficile*,
80 examining its bactericidal effects on logarithmic and stationary phase cells and associated
81 cellular effects linked to dissipation of the membrane potential in *C. difficile*. This study
82 was presented in part as a poster presentation at the 54th Interscience Conference on
83 Antimicrobial Agents and Chemotherapy (ICAAC).

84

85 **MATERIALS AND METHODS**

86 **Compounds, bacterial strains, and growth media.** Surotomycin and daptomycin were
87 provided by Merck and Co., Inc. All other antimicrobials were obtained from Sigma-
88 Aldrich (vancomycin, metronidazole, CCCP, ampicillin, fusidic acid, rifaximin and nisin)
89 or Enzo Life Sciences (gatifloxacin). The *C. difficile* strains BAA-1875 (ribotype 078)
90 and BAA-1803 (ribotype 027) were from the American Type Culture Collection (ATCC).
91 Strain R20291 (ribotype 027) was kindly provided by Dr. A. L. Sonenshein, Tuft
92 University, Boston USA. Strain IT0843 (ribotype 001) was kindly provided by Dr. Paola
93 Mastrantonio (Istituto Superiore di Sanità, Rome, Italy). Brain Heart Infusion (BHI; from
94 Oxoid) was used for all the experiments and was supplemented with calcium to a final
95 concentration of 50 mg/L for all experiments with surotomycin and daptomycin. To
96 supplement BHI to a final calcium concentration of 50 mg/L, calcium levels in
97 manufactured lots of BHI were determined by the Laboratory Specialists, Inc., OH. All
98 strains were routinely grown in pre-reduced BHI media under anaerobic conditions in a
99 Whitley A35 anaerobic workstation at 37°C.

100

101 **Determination of minimum inhibitory concentrations (MICs) and minimum**
102 **bactericidal concentrations (MBCs).** Minimum inhibitory and minimum bactericidal
103 concentrations of compounds against *C. difficile* were determined as described by Wu et
104 al. (11, 23). MICs were performed using $\sim 10^6$ cfu/mL inoculum of *C. difficile* in 24-well
105 microtitre plates containing 2-fold serial dilutions of compounds in a total volume of 1
106 mL. MICs were defined as the lowest concentrations of compounds inhibiting visible
107 growth after 24 h of incubation. MBCs were performed against both the logarithmic-

108 phase (MBC_{Log}) and stationary-phase (MBC_{Sta}) cells using logarithmic ($OD_{600nm} \approx 0.3$)
109 and 24 h old cultures, respectively. Briefly, in 24-well microtitre plates, cultures were
110 added to 2-fold diluted compounds in a total volume of 1 mL. After 24 h of incubation,
111 the number of viable cells was determined by plating aliquots onto pre-reduced BHI agar
112 containing activated charcoal (10 % w/v). MBCs were defined as the lowest
113 concentrations of compounds causing ≥ 3 log reduction in viable cells compared to the
114 starting inocula. The MICs and MBCs were determined from two independent starting
115 cultures.

116

117 **Time-kill kinetics**, Were evaluated against both the logarithmic and stationary phase
118 cultures as described by Wu et al. (11, 23). Logarithmic ($OD_{600nm} \approx 0.3$) and stationary-
119 phase cultures were exposed to 1, 4 and $16\times$ the MIC of compounds. Samples (100 μ L)
120 were taken at time 0, 1, 2, 4, 6 and 24 h after the addition of compounds and viable cell
121 counts were determined on BHI agar plates containing activated charcoal (10 % w/v).
122 Bacterial counts were enumerated after 24 h of incubation. This assay were determined
123 from two independent starting cultures.

124

125 **Effects on macromolecular biosynthesis**. Logarithmic cultures of R20291 and BAA-
126 1875 were grown to early logarithmic-phase ($OD_{600nm} \approx 0.3$) under anaerobic conditions
127 and aliquoted for subsequent analysis. To analyze the DNA, RNA, protein and cell wall
128 synthesis inhibition; 3H -Thymidine (2 μ Ci/mL), 3H -Uridine (2 μ Ci/mL), 3H -Threonine (2
129 μ Ci/mL) and 3H - N-acetyl-Glucosamine (2 μ Ci/mL) were used respectively.
130 Radiolabelled precursors were added 5 min before the addition of compounds at either

131 inhibitory (1×MIC) and bactericidal (16×MIC) concentrations. Gatifloxacin, rifaximin,
132 fusidic acid and ampicillin were used as controls for DNA, RNA, Protein and cell wall
133 synthesis inhibition, respectively. Against BAA-1875 strain, gatifloxacin, rifaximin,
134 fusidic acid, and ampicillin concentrations at 16×MIC, were 64 µg/mL, 0.96 µg/mL, 2
135 µg/mL, and 8 µg/mL, respectively. Against R20291, the concentrations at 16×MIC were
136 512 µg/mL, 2 µg/mL, 2 µg/mL, and 16 µg/mL, for the respective control drugs. Samples
137 (500 µL) were taken at specific time points (30, 60 and 120 min), spun down, and the cell
138 pellet collected and incubated on ice with 10% w/v ice-cold trichloroacetic acid (TCA)
139 for 30 min. Samples were then filtered through Whatman GF/C filters, washed twice with
140 5% w/v TCA and 95% ethanol. Filters were dried and scintillation counting performed.

141

142 **Determination of the membrane potential and permeability using FACS.** To assess
143 the effects of compounds on the membrane potential and permeability of *C. difficile*, we
144 adopted a fluorocytometric method relying on the use of DiBAC₄(3) to assess the
145 membrane potential and propidium iodide (PI) to assess membrane permeability. This
146 method was based on that reported by Nuding et al. (24) for anaerobic bacteria;
147 Diethyloxycarbocyanine iodide DiOC₂(3) was found to be inconsistent (*data not shown*).
148 Strains R20291 and BAA-1875 were used, and were exposed to different concentrations
149 of compounds as: 1, 4 and 16× the MIC of compounds. Briefly, cultures were grown
150 anaerobically to an OD_{600nm} ≈0.2 and 10 mL aliquots added to 20 mL serum vials.
151 Compounds were subsequently added and the vials crimped sealed with silicone bungs
152 and removed from the anaerobic chamber. After 10 min of adding compound, DiBAC₄(3)
153 was added via a 23G syringe needle to a final concentration of 5 µM. After an overall 30

154 min of exposing cells to compounds, at room temperature, fluorocytometric analysis was
155 performed using BD LSR II flow cytometer. DiBAC₄(3) was excited using the 488-nm
156 excitation laser and its fluorescence emission detected using FITC filters. As a positive
157 carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Sigma-Aldrich), which completely
158 dissipates the membrane potential was used; vancomycin was used as a negative control;
159 a minimum of three independent cultures was evaluated.

160 Membrane permeability assays were similarly performed using the protocol
161 described above, except that the membrane impermeant dye PI was added to a final
162 concentration of 5 μ M instead of DiBAC₄(3). After 30 min samples were analyzed in the
163 BD LSR II flow cytometer, with excitation at 488-nm and emission collected using the
164 PI-A filters. Nisin was used as a positive control for membrane damage and vancomycin
165 as a negative control. Daptomycin was also included as a control in these experiments.
166 Resazurin (0.001 gm/L) and sodium thioglycollate (0.5 gm/L) were added to the media to
167 act as an indicator of oxygenation and an oxygen scavenger, respectively. Histogram
168 plots of number of events against fluorescence of the population were comparatively
169 analyzed using FlowJo X 10.0.7.

170

171 **RESULTS**

172 **Surotomylin is bactericidal against both the logarithmic and stationary-phase *C.***
173 ***difficile*.** As shown in Table 1, surotomylin MICs against test strains ranged from 0.125
174 to 1 μ g/mL. The concentration of surotomylin required for bactericidal activities against
175 logarithmic and stationary phase cultures were similar and was 8-128 fold above the
176 MICs (Table 1), which corresponds to 2 to 16 μ g/mL. As expected the control

177 metronidazole was also bactericidal, killing both culture types at concentrations between
178 2 to 16 $\mu\text{g/mL}$, whereas vancomycin was bacteriostatic and was completely inactive
179 against stationary-phase *C. difficile*.

180

181 **Surotomylin kills *C. difficile* in a concentration-dependent manner.** Time kill kinetics
182 were subsequently performed to determine how rapidly surotomylin killed both
183 logarithmic and stationary phase cells. These assays revealed that surotomylin exhibited
184 a concentration dependent mode of killing against both the logarithmic- and stationary-
185 phase cultures of *C. difficile*. Against logarithmic BAA-1875, at 16 \times MIC (4 $\mu\text{g/mL}$),
186 surotomylin killed more than 99% of cells in 6 h, whereas 24 h was required to achieve a
187 similar reduction in culture viability, against stationary-phase cells (**Figure 1**). A similar
188 pattern of killing was observed against R20291, although, this strain seemed more
189 sensitive at 4 \times MIC of surotomylin, since a 99% reduction in viable numbers was
190 observed after 24 h against logarithmic-phase cultures (**Figure 2**). This suggests that
191 against R20291 in larger culture volume (10 mL) the MBC is 4 $\mu\text{g/mL}$, which differs by
192 4-fold from that obtained in 1 mL volumes for MBC determinations. Metronidazole was
193 also found to display concentration dependent killing (against both logarithmic and
194 stationary-phase cells), causing a $\geq 99\%$ reduction of viable cells at 16 \times MIC after 24 h;
195 whereas vancomycin demonstrated bacteriostatic effect and was completely inactive
196 against stationary-phase cultures. These observations broadly support the above findings
197 of the MBCs data.

198

199 **Surotomycin dissipates membrane potential without pore formation.** In order to
200 examine whether surotomycin dissipates the membrane potential of *C. difficile*, we
201 adopted the fluorescent probe DiBAC₄(3). The fluorescence of DiBAC₄(3) changes with
202 the membrane potential status of cells, with depolarized cells demonstrating enhanced
203 fluorescence, due to DiBAC₄(3) entering depolarized membranes and binding to lipid-
204 rich intracellular components, thereby exhibiting increased green fluorescence (24).
205 When *C. difficile* cultures of R20291 and BAA-1875 were treated with surotomycin or
206 daptomycin, increases in fluorescence were only observed 16× their MICs, compared to
207 the untreated cultures (**Figure 3**). These concentrations were bactericidal for both agents.
208 No changes in the fluorescence of cells were observed at lower concentrations of
209 surotomycin or daptomycin (i.e. 1 and 4×MIC). As expected, the negative control
210 vancomycin that inhibits peptidoglycan biosynthesis did not alter the membrane
211 potential, while CCCP that acts as a proton ionophore and disrupts the bacterial
212 membrane potential was shown to increase the fluorescence of R20291 and BAA-1875.
213 At the 30 min of treatment, dissipation of the membrane potential by surotomycin and
214 daptomycin at 16× their MICs did not result in membrane pore formation, as cells did not
215 show an increase in propidium iodide fluorescence compared to the untreated control and
216 vancomycin-treated cultures. In contrast, the pore-forming agent nisin caused membrane
217 pore formation, which was evident by an increase in the red fluorescence of cells (**Figure**
218 **3**).

219 As the above observations are based on 30 min incubation periods, we extended
220 out incubation times. Incubation of cultures with 1 and 4×MIC of surotomycin or
221 daptomycin for up to 2 h did not lead to an observable difference in the membrane

222 potential status of cells compared to untreated controls (*data not shown*). Similarly,
223 continued exposure to 16×MIC of these drugs did not produce further, measurable
224 increases in the dissipation of the membrane potential; extended incubation times at 1, 4,
225 and 16×MIC did not lead to increases in the permeability of cultures to propidium iodide
226 (*data not shown*).

227

228 **Surotomylin inhibits multiple macromolecular biosynthetic processes.** Exposure of
229 R20291 and BAA-1875 to inhibitory (MIC) and bactericidal (16×MIC) concentrations of
230 surotomylin resulted in the simultaneous inhibition of DNA, RNA, Protein and Cell Wall
231 (**Figure 4**). While it is expected that at the bactericidal concentration, all
232 macromolecular processes would be affected in dying cells, these processes were also
233 affected in cells exposed to inhibitory concentrations. This is consistent with the
234 membrane being the primary target for surotomylin action, thereby imposing multiple
235 cellular effects on processes that require membrane homeostasis (25).

236 **DISCUSSION**

237 Recent studies established that the membrane potential of *C. difficile* is critical to the
238 survival of logarithmic and stationary phase cells, making the clostridial membrane an
239 attractive target for agents to treat CDI (11). Dissipation of the membrane potential,
240 resulting in loss of viability in both growing and non-growing cell types has direct
241 relevance to *C. difficile* pathogenesis, as this organism produces its toxins and spores in
242 the late-logarithmic and stationary phases of growth (22). The cyclic lipopeptide drug
243 surotomylin represents the leading example of a membrane-active antibiotic for treating

244 CDI. In studies herein we validated that surotomycin dissipates the membrane potential
245 of *C. difficile* and this was not associated with the formation of pores at bactericidal
246 concentrations in two test strains. This observation is consistent with a prior report by
247 Mascio et al. (19) where surotomycin dissipated the membrane potential of *S. aureus*
248 without causing pore formation. Dissipation of the membrane potential of *C. difficile* was
249 evaluated using the fluoroprobe DiBAC₄(3) that other studies have adopted to measure
250 the membrane potential in anaerobes and as it appears more reliable than DiOC for
251 anaerobes (24). However, measurable disruptions of the membrane potential in *C.*
252 *difficile* were only observed at bactericidal concentrations. This might reflect that the
253 magnitude of the membrane potential in clostridia is low (26). Hence the marginal
254 lowering of the membrane potential of *C. difficile* upon exposure to inhibitory
255 concentrations of drug may be challenging to measure using DiBAC₄(3) and other
256 techniques may be required for lower concentrations.

257 The action of surotomycin against various strains of *C. difficile* resulted in
258 bactericidal activities against logarithmic and stationary phase cultures and imposed
259 multiple cellular effects as evident by widespread disruption of macromolecular
260 processes. In contrast, the cell wall synthesis inhibitor vancomycin was poorly active or
261 bacteriostatic against logarithmic cells and inactive against stationary phase cultures.
262 Metronidazole did reduce the viability of both cell types, but only low concentrations of
263 drug occur in the gastrointestinal tract as the drug is almost completely absorbed
264 following oral administration (27).

265 The concentration of surotomycin that was required to inactivate both logarithmic
266 and stationary phase cultures was typically 2-16 µg/mL. These levels are well within the

267 local concentrations of surotomycin ($>1,000 \mu\text{g/g}$) that is present in the colon of patients
268 following oral administration (data on file at Merck and Co., Inc. Kenilworth, NJ, USA).
269 It is therefore plausible that in the colon surotomycin is bactericidal against both
270 logarithmic and stationary phase cells. This property could reduce both toxin and spore
271 production in vegetative populations, as recently reported in the in vitro human gut model
272 (28). In Phase II clinical trials, lower rates of recurrence was associated with
273 surotomycin treatment compared to oral vancomycin; as 27.9% and 17.2% for
274 surotomycin at 125 mg and 250 mg twice daily, respectively; while 35.6% for
275 vancomycin given as 125 mg four times per day (29). From a microbiological
276 perspective, it is tempting to speculate that surotomycin's bactericidal activity and
277 narrower spectrum than vancomycin contributes to reducing recurrence. However, the in
278 vitro findings of this study do not provide a direct explanation for the superiority of
279 surotomycin compared to vancomycin in reducing recurrence.

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

286

287

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294

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386 **TABLES AND FIGURES**

387

388 **Table 1:** In vitro activities of surotomycin and other first-line drugs for the treatment of

389 CDI

	*Activity µg/mL								
	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

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391 *MBC_{LOG}, minimum bactericidal concentration against logarithmic cells; MBC_{STA}

392 minimum bactericidal concentration against stationary phase cells.

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404 **Figure 1:** Time-kill kinetics of antibiotics against logarithmic-phase (left) and stationary-
405 phase (right) BAA-1875 cultures. Various concentrations of SUR = surotomycin; MTZ =
406 metronidazole; and VAN = vancomycin are shown in the legend.

407

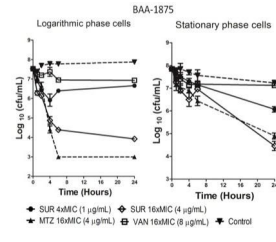
408 **Figure 2:** Time-kill kinetics of antibiotics against logarithmic-phase (left) and stationary-
409 phase (right) R20291 cultures. Various concentrations of SUR = surotomycin; MTZ =
410 metronidazole; and VAN = vancomycin are shown in the legend.

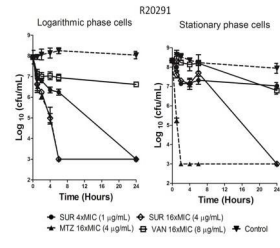
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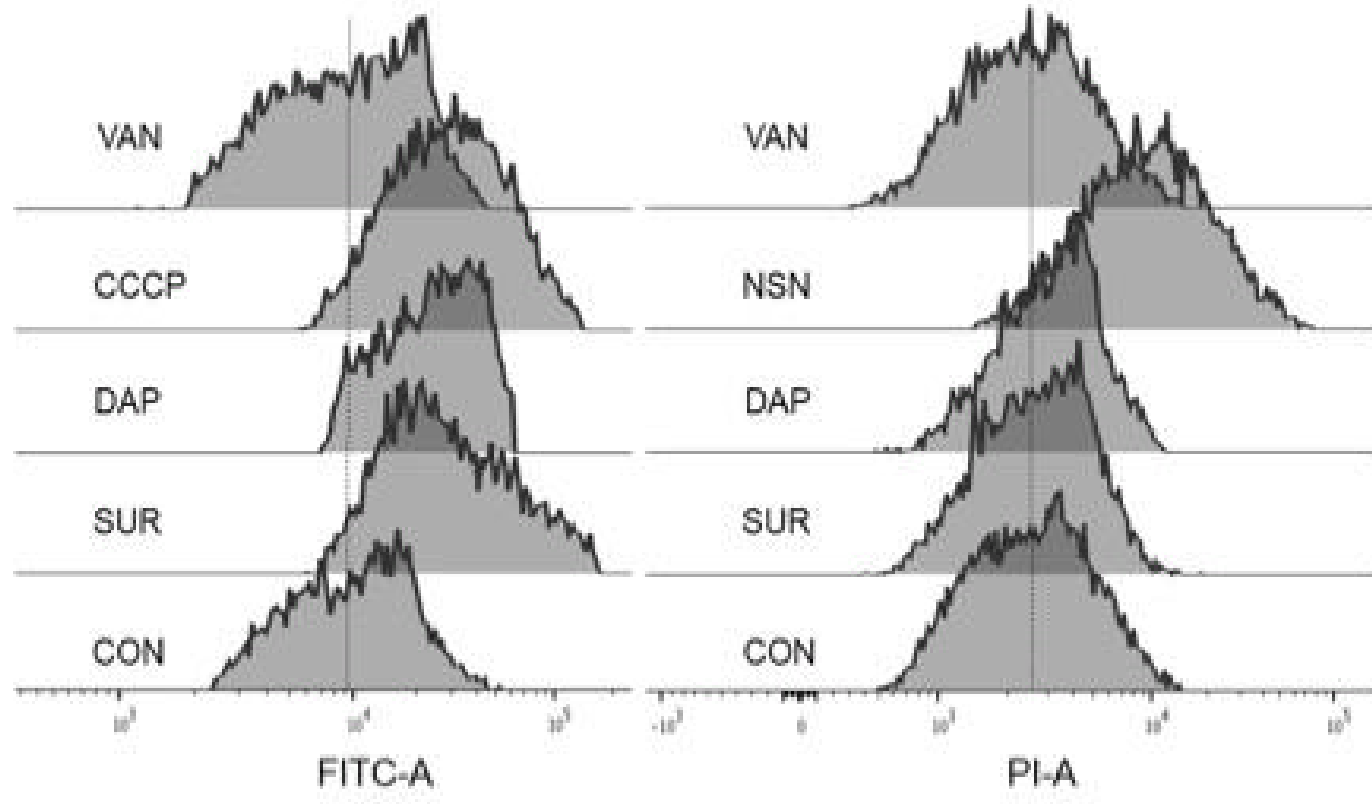
412 **Figure 3:** Dissipation of the membrane potential and effects on membrane permeability
413 of *C. difficile*, shown as histogram half overlays. Representative data from three
414 independent cultures of BAA-1875 is shown, following exposure to drugs at 16×MIC. **A.**
415 CCCP was used as a control for dissipation of membrane potential and in **B** nisin is used
416 as a pore forming control; vancomycin is a negative control. CON = Control, SUR =
417 Surotomycin (4 µg/mL), DAP = Daptomycin (16 µg/mL), CCCP = Carbonyl cyanide m-
418 chlorophenyl hydrazine (2 µg/mL), NSN = Nisin (8 µg/mL), VAN = Vancomycin (8
419 µg/mL). Filters for FITC = Fluorescein Isothiocyanate, PI = Propidium Iodide.

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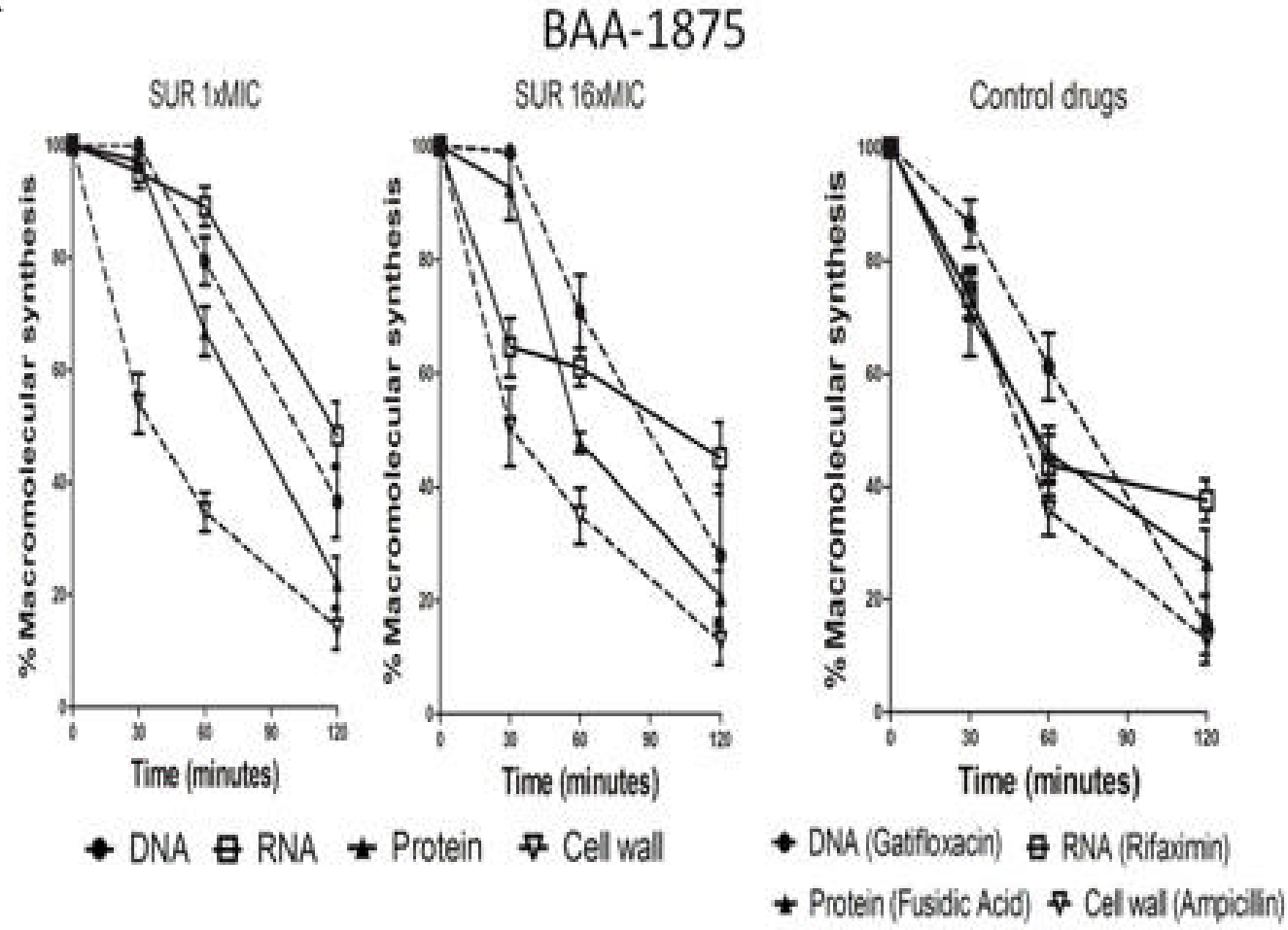
421 **Figure 4:** Effects of inhibitory and bactericidal concentrations of surotomycin (SUR) on
422 macromolecular biosynthesis in *C. difficile*. In **A**, effects against BAA-1875 is shown for
423 SUR (0.25 and 4 µg/mL); whereas in **B** effects against R20291 is shown for SUR (1 and
424 16 µg/mL). Control drugs are also shown in both **A** (Gatifloxacin = 64 µg/mL; Rifaximin
425 = 0.96 µg/mL; Fusidic Acid = 2 µg/mL; Ampicillin = 8 µg/mL) and **B** (Gatifloxacin =
426 512 µg/mL; Rifaximin = 2 µg/mL; Fusidic Acid = 2 µg/mL; Ampicillin = 16 µg/mL).







A



B

R20291

