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Observed antagonistic effect of linezolid on daptomycin or vancomycin activity against biofilm-forming methicillin-resistant *Staphylococcus aureus* in an *in vitro* pharmacodynamic model

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1 Title: Observed antagonistic effect of linezolid on daptomycin or vancomycin activity against
2 biofilm-forming methicillin-resistant *Staphylococcus aureus* in an in vitro pharmacodynamic
3 model

4

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6 Running title: Linezolid antagonism of cell wall active agents

7

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19

20

21 **ABSTRACT**

22 Pharmacodynamic activity in antibiotic combinations of daptomycin, vancomycin and linezolid
23 was investigated in a 48h in vitro pharmacodynamic model. Using free human-simulated
24 concentrations, activity against clinical biofilm-forming methicillin-resistant *Staphylococcus*
25 *aureus* isolates was evaluated. Linezolid antagonized vancomycin activity at 24 and 48h.
26 Linezolid antagonized daptomycin at 24 and 48h depending on dose and strain. Adding
27 daptomycin increased vancomycin activity at 48h ($p<0.03$). These results may be strain-
28 dependent and require further clinical investigation.

29

30 Keywords: methicillin-resistant *Staphylococcus aureus*, combination therapy, antagonism,
31 biofilm, linezolid, vancomycin, daptomycin, persistent bacteremia.

32 There is recent increased interest in the activity of protein synthesis inhibitors in combination
33 with cell wall active agents. Some combination regimens are being used clinically, but are
34 lacking data to support their combined use.(1) High-dose daptomycin and linezolid have been
35 recommended for use as combination therapy in the 2011 methicillin-resistant *Staphylococcus*
36 *aureus* (MRSA) treatment guidelines for persistent bacteremia or vancomycin failure.(2)
37 However, other in vitro studies have demonstrated antagonism with combinations of linezolid
38 and vancomycin.(3, 4) To date, there have been limited investigations with daptomycin and
39 linezolid in combination.(5, 6) The combined use of these agents prompted an investigation into
40 pharmacokinetic/pharmacodynamic activity and possible interactions when using combinations
41 of bactericidal and bacteriostatic antimicrobials, as previously described.(7, 8)

42
43 Two randomly selected clinical MRSA blood isolates (L31 and L328) from the LaPlante
44 Laboratory at the Providence Veterans Affairs Medical Center were selected for analysis. Both
45 are known biofilm-producing strains, previously isolated from patients with catheter-related
46 bloodstream infections.(9) Biofilm formation was previously determined as described.(9, 10)
47 Daptomycin (lot# CDC271; Cubist Pharmaceuticals, Inc., Lexington, MA), linezolid (lot#
48 11C10U10, 13F05U09; Pfizer, New York, NY), and vancomycin (lot# 12070DD, 382553A;
49 Hospira, Lake Forest, IL) were tested. Mueller-Hinton broth (MHB, Becton Dickinson, Sparks,
50 MD, USA) supplemented with calcium and adjusted to 25 mg/L calcium chloride (for daptomycin
51 studies 50mg/mL of calcium chloride; ionized Ca; 1.03-1.23 mmol/L) and 12.5 mg/L magnesium
52 was used for all minimum inhibitory concentrations (MICs), minimum bactericidal concentrations
53 (MBCs), and in vitro pharmacodynamic (IVPD) infection models.(11-13) Colony counts were
54 determined using Tryptic Soy Agar (TSA, Difco, Becton Dickinson).

55
56 A previously described IVPD model was used to evaluate several antibiotic regimens against
57 MRSA.(7) Briefly, a 0.5 McFarland standard of planktonic bacteria from overnight growth on

58 TSA was diluted in a one compartment model (250ml working volume) to a starting inoculum of
59 $\sim 10^6$ CFU/mL. Free concentrations of antimicrobials were evaluated. Daptomycin was
60 administered to simulate a 6mg/kg dose ($t_{1/2}$ 8h, C_{max} 98.6 μ g/mL, protein-binding 92%; fC_{max}
61 7.9 μ g/mL) or 10mg/kg dose ($t_{1/2}$ 8h, C_{max} 140 μ g/mL, protein binding 92%; fC_{max} 11.2 μ g/mL)
62 every 24 hours(q24h), (14); linezolid 600mg q12h, ($t_{1/2}$ 6hrs, C_{max} 21 μ g/mL, protein-binding
63 31%; fC_{max} 14.5 μ g/mL) (15); and vancomycin 1.25g q12h ($t_{1/2}$ 6hrs, C_{max} 45 μ g/mL, C_{min} 15-
64 20 μ g/mL, protein binding 55%; fC_{max} 20.3 μ g/mL). (16) Antibiotics were given as boluses into
65 the compartment and peristaltic pumps were used to achieve the desired half-lives and replace
66 media with fresh MHB. All model experiments were performed in duplicate to triplicate to ensure
67 reproducibility. In addition, simulations in the absence of antibiotics were performed to assure
68 adequate growth of organisms in the model. Samples were removed from each model at each
69 0, 4, 8, 24, 32 and 48 hour time point. Once removed, samples were immediately diluted, plated
70 on TSA, and incubated at 37°C for 24h before colony count enumeration. The limit of detection
71 for this method is 2.0 log₁₀ CFU/mL.(17) Antimicrobial carryover was minimized by serial dilution
72 (1:10-1:10,000) of plated samples in conjunction with vacuum filtration, if needed, as previously
73 described.(12)

74

75 MICs and MBCs of study antimicrobial agents were determined by Etest methodology and broth
76 microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines.(18, 19)
77 All samples were incubated at 37°C in ambient air for 24 hours. E-tests were used to assess
78 changes in MIC at 24 and 48h to detect resistance. Plates were examined for growth after 24h
79 of incubation at 37°C. Changes in MIC were confirmed with microbroth dilution MIC. Samples
80 were evaluated directly from the model to prevent changes in MIC from removing antibiotic
81 pressure and to optimize the detection of MIC changes.

82

83 Time-kill curves were plotted to determine reduction in \log_{10} CFU/mL over 48 hours. Bactericidal
84 activity (99.9% kill) was defined as a $\geq 3 \log_{10}$ CFU/mL reduction and bacteriostatic activity was
85 defined as a $< 3 \log_{10}$ CFU/mL change in colony count from the initial inoculum.(20) The time to
86 kill 99.9% of the bacteria present was determined by non-linear regression (using a minimum of
87 4 data points) if $r^2 \geq 0.95$ or by visual inspection. Enhancement of activity was defined as an
88 increase in kill of $\geq 2\text{-}\log_{10}$ CFU/mL by combination of antimicrobials versus the most active
89 single agent of that combination.(7) Improvement was defined as a 1 to $2\text{-}\log_{10}$ CFU/mL
90 increase in kill in comparison to the most active single agent, while combinations that resulted in
91 $\geq 1\text{-}\log_{10}$ bacterial growth in comparison to the most active single agent were considered to
92 represent antagonism.(7) The terms “improvement” and “enhancement” were used because our
93 simulations involve therapeutically obtained serum concentration and this does not permit the
94 mathematical modeling necessary to consider the standard terms “additivity” and “synergy” (7,
95 21). Indifference was defined as $<1\text{-}\log_{10}$ CFU/mL change in activity.

96

97 Samples for pharmacokinetic analyses were obtained through the injection port at 0, 0.5, 1, 2, 4,
98 6, 8, and 24h for verification of target antibiotic concentrations. All samples were stored at
99 -80°C until analysis. Daptomycin concentrations were determined by a previously described
100 and validated HPLC method (Center for Anti-Infective Research and Development, Hartford,
101 CT).(11) Vancomycin concentrations were determined by a homogeneous particle-enhanced
102 turbidmetric immunoassay (PETIA; Architect, Multigent®; Abbott Diagnostics Abbott Park, IL,
103 USA) at the Providence Veteran Affairs Medical Center.(11) The vancomycin assay has a
104 detection range of 0.5 to 80.0 $\mu\text{g/mL}$, and a between day sample precision and CV% of 1.6%
105 and $< 5.0\%$, respectively. Linezolid concentrations were evaluated using HPLC (Infectious
106 Disease Pharmacokinetics Laboratory; Charles Peloquin) as previously described.(7) The half-
107 life, AUC, C_{max} , and minimum concentration (C_{min}) of the antibiotics were determined by the
108 trapezoidal method utilizing PK Analyst software (Version 1.10, MicroMath Scientific Software,

109 Salt Lake City, UT). Maximum concentration (C_{max}) to MIC ratios, the percent time above the
110 MIC (%T > MIC), and AUC₀₋₂₄ to MIC ratios were calculated for each antibiotic and were
111 compared to literature values.(22-25)

112
113 Changes in bacterial growth (log₁₀ CFU/mL) at 4, 8, 24 and 48h and time to 99.9% kill were
114 compared by analysis of variance with Tukey's post-hoc test. A p value of < 0.05 was
115 considered significant.(7, 11) All statistical analyses were performed using SPSS Statistical
116 Software (Release 20 SPSS, Inc., Chicago, IL).

117
118 The MIC results are shown with MBCs and pharmacodynamic parameters obtained in Table 1.
119 Pharmacokinetic values obtained were within 8% of targeted values. The results of the IVPD
120 models are demonstrated in Figure 1 and Table 2.

121
122 Against both biofilm-forming isolates, all regimens, including monotherapy and combination,
123 demonstrated statistically significant kill (decrease in CFU/mL) by 8 hours as compared to
124 growth control (p<0.001). Linezolid demonstrated initial kill until 24h, with regrowth until 48h.
125 Vancomycin demonstrated bacteriostatic activity at 24h against L31, but bactericidal activity
126 against L328 at 24h. Vancomycin was bacteriostatic at 48h against both isolates. No increases
127 in MIC were found at 24 or 48h in any of the experiments.

128
129 For both isolates, daptomycin at 6mg/kg and 10mg/kg demonstrated bactericidal activity by 24h.
130 Daptomycin and vancomycin plus daptomycin were the only regimens to demonstrate sustained
131 bactericidal activity from 24 to 48h. Daptomycin alone was significantly more active than
132 linezolid at 48h (mean differences in log CFU/mL 1.78-2.73, p<0.04).

133

134 In combination studies, at 24h vancomycin plus daptomycin 6mg/kg and daptomycin 6mg/kg or
135 10mg/kg plus linezolid were not statistically significantly different from their most active
136 components. This is despite meeting the definition for antagonism against both isolates for
137 daptomycin 10mg/kg plus linezolid, and L328 for daptomycin 6mg/kg plus linezolid. Linezolid
138 plus vancomycin was the least active regimen. Linezolid plus vancomycin met the definition for
139 antagonism at 24h for both isolates, but was significantly different only for L328 (1.67, 95%CI
140 0.76-2.59, $p<0.01$).

141
142 Linezolid plus daptomycin 6mg/kg met the definition for antagonism at 24h for one isolate and
143 48 hours for both isolates, while the higher dose of daptomycin plus linezolid demonstrated
144 antagonism at 24h for both isolates and 48h for one. Against L31, the activity of daptomycin
145 6mg/kg or 10mg/kg alone was significantly greater than daptomycin (either dose) plus linezolid
146 at 48h (mean difference in log CFU/mL 1.82-2.43, $p<0.01$). The differences in activity between
147 linezolid containing regimens (linezolid alone, linezolid plus vancomycin, daptomycin plus
148 linezolid) were not statistically significant at 48h for both isolates, but linezolid alone was less
149 active than either dose of daptomycin alone (mean differences 1.78-2.73, $p<0.04$). Adding
150 daptomycin 6mg/kg improved the activity of vancomycin at 48h (mean difference in log CFU/mL
151 1.65-2.20, $p<0.03$), but was not significantly different than daptomycin alone.

152 Despite common concomitant clinical use of linezolid with bactericidal antibiotics,(1) we have
153 demonstrated in vitro antagonism at 24 and 48h using combinations of linezolid plus
154 vancomycin and linezolid plus daptomycin. The use of these combinations of antibiotics is
155 lacking both in vitro and clinical outcomes data to support their use. Combinations of two active
156 antibiotics are frequently excluded or not analyzed in clinical trials where single agents are the
157 main focus, due to small numbers of patients.(1, 26) Notably, a landmark study by Lepper *et al.*
158 demonstrated an increase in mortality in meningitis patients receiving tetracycline-penicillin
159 combination therapy over patients receiving the same penicillin dose alone.(27) The stasis
160 produced by protein synthesis inhibitors, including linezolid, likely inhibits the activity of cell wall
161 active antibiotics, which work best on actively-dividing bacteria. Antagonism has been
162 demonstrated in previous time-kill studies using static concentrations of combinations of
163 vancomycin and linezolid.(3-6) Linezolid has also demonstrated attenuation of activity of
164 aztreonam or ceftazidime against *Escherichia coli* isolates in an in vitro pharmacodynamic
165 model.(7) This highlights the importance of pharmacodynamic interactions with combination
166 therapy, even for antibiotics with a completely different spectrum of activity. Of interest, one
167 study has demonstrated activity of daptomycin and linezolid in combination against MRSA, but
168 in contrast to our study, this study tested formed biofilms on coupons.(28)

169
170 In our study, regrowth was noted between 24 and 48h for both strains though no increases in
171 MIC were noted using Etests. This could be due to biofilm formation of these planktonic strains
172 after 24h, increasing growth without susceptibility changes, since biofilms can withstand 10-
173 1000 times the concentrations of antibiotics compared to planktonic bacteria. According to
174 research by our group, approximately 50% of MRSA isolates from our institution form
175 biofilm.(29) Biofilm-forming isolates are known to cause persistent, difficult to treat infections
176 where combination therapy may be considered. The strains used in this study previously tested
177 positive for biofilm formation as noted above, using the same temperature and inoculum, with

178 similar media to this IVPD model. Over the 48h period tested, biofilm growth could seed
179 susceptible bacteria into the model during sampling, which would appear as regrowth.(9) A
180 previous study demonstrated a reduction in biofilm biomass, but no reduction in cell viability,
181 using combinations of linezolid and vancomycin against formed MRSA biofilms.(30)

182
183 Despite reaching the target of the estimated total AUC/MIC ratio for vancomycin of >400, and
184 with an estimated total vancomycin trough concentration of 15.5µg/mL, vancomycin did not
185 achieve bactericidal activity against L31 during the 48h period. This indicates that for an isolate
186 with a vancomycin MIC of 2mg/L, this regimen may not be adequate.

187
188 In regard to limitations, we evaluated two strains, and recognize that these observations may be
189 isolate-specific or dependent on the MICs of the isolates for each antibiotic.

190
191 In these daptomycin-, linezolid-, and vancomycin-susceptible strains of biofilm-forming MRSA,
192 regimens containing daptomycin were more active than those containing linezolid. Linezolid
193 antagonized the activity of vancomycin and daptomycin 6 mg/kg and 10mg/kg at 24 and 48h.
194 Adding linezolid to daptomycin 6mg/kg or 10mg/kg significantly decreased activity at 48h
195 against L31 versus daptomycin alone. The combination of vancomycin plus daptomycin 6mg/kg
196 or daptomycin 6mg/kg or 10mg/kg alone demonstrated sustained bactericidal activity through
197 the 48h period. Based on this data, combinations of linezolid with either daptomycin 6mg/kg,
198 10mg/kg or vancomycin should be investigated for the clinical implications of in vitro
199 antagonism.

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209 All named authors meet the ICMJE criteria for authorship for this manuscript, take responsibility for the
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213

214 **Conflict of Interest**

215 Megan Luther declares research funding from Pfizer and Cubist. Kerry LaPlante declares Cubist, Davol,
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- 312
- 313

314

	MIC (mcg/mL)	MBC (mcg/mL)	fCmax/ MIC	%T>MIC	fAUC/MIC	Estimated totalAUC/MIC
MRSA (L31)						
Daptomycin (6mg/kg)	0.5	1	17.13 ± 0.61	100%	170-181	2121-2262
Linezolid	1	>64	14.49 ± 0.66	100%	213	309
Vancomycin	2	2	10.77 ± 1.23	100%	181-185	402-411
MRSA (L328)						
Daptomycin (6mg/kg)	0.25	0.25	34.26 ± 1.22	100%	339-361	4243-4524
Linezolid	2	>64	7.24 ± 0.33	100%	107	155
Vancomycin	1	1	21.55 ± 2.45	100%	362-370	804-823

315

316

317 Table 1. MIC, MBC and pharmacodynamic parameters obtained from IVPD experiments using
318 free concentrations.

319 MIC= minimum inhibitory concentration

320 MBC= minimum bactericidal concentration

321 fCmax= maximum free concentration

322 AUC= area under the curve

323 %T>MIC= percentage of time above MIC

324

Regimen	MRSA Strain	Change in Log ₁₀ CFU/mL relative to 0h at:	
		24h	48h
Growth Control	L31	+2.52 ± 0.11	+2.37 ± 0.27
	L328	+2.46 ± 0.23	+3.29 ± 0.29
Daptomycin 6mg/kg	L31	-3.51 ± 0.08	-3.03 ± 0.68
	L328	-3.11 ± 0.32	-3.15 ± 0.28
Daptomycin 10mg/kg	L31	-3.54 ± 0.03	-3.48 ± 0.09
	L328	-3.45 ± 0.11	-3.24 ± 0.56
Linezolid	L31	-2.90 ± 0.47	-0.84 ± 0.43
	L328	-2.82 ± 0.69	-1.51 ± 0.54
Vancomycin	L31	-2.85 ± 0.15	-2.02 ± 0.15
	L328	-3.08 ± 0.52	-1.39 ± 0.57
Daptomycin 6mg/kg + Linezolid	L31	-2.62 ± 0.80 (inhibited 0.81 log CFU/mL, indifference)	-1.14 ± 0.68 (inhibited 1.82 log CFU/mL, antagonism)
	L328	-2.05 ± 0.35 (inhibited 1.04 log CFU/mL, antagonism*)	-1.62 ± 0.89 (inhibited 1.52 log CFU/mL, antagonism)
Daptomycin 10mg/kg + Linezolid	L31	-2.55 ± 0.58 (inhibited 1.14 log CFU/mL, antagonism)	-1.21 ± 0.66 (inhibited 2.43 log CFU/mL, antagonism*)
	L328	-2.40 ± 0.18 (inhibited 1.01 log CFU/mL, antagonism*)	-2.35 ± 0.83 (inhibited 0.85 log CFU/mL, indifference)
Linezolid + Vancomycin	L31	-1.88 ± 0.98 (inhibited 1.00 log CFU/mL, antagonism)	-0.60 ± 0.55 (inhibited 1.36 log CFU/mL, antagonism)
	L328	-1.43 ± 0.17 (inhibited 1.67 log CFU/mL, antagonism*)	-0.14 ± 0.17 (inhibited 1.27 log CFU/mL, antagonism*)
Vancomycin + Daptomycin 6mg/kg	L31	-3.57 ± 0.08 (no change, indifference)	-3.57 ± 0.08 (enhanced 0.48 log CFU/mL, indifference)
	L328	-3.51 ± 0.10 (enhanced 0.43 log CFU/mL, indifference)	-3.51 ± 0.10 (enhanced 0.39 log CFU/mL, indifference)

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333 Table 2. Activity of each antibiotic alone and in combination in an IVPD model at 24 and 48h.

334 *Significant antagonism from the most active component of the regimen ($p < 0.05$).

335 Improvement: 1-2 \log_{10} CFU/mL increase in kill over the most active component.

336 Enhancement: $>2 \log_{10}$ CFU/mL increase in kill over the most active component.

337 Antagonism: $\geq 1 \log_{10}$ CFU/mL increase in growth over the most active component.

338 Indifference: $< 1 \log_{10}$ CFU/mL change in activity from the most active component.

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342 Figure 1. Activity of daptomycin and linezolid (A and C), or vancomycin and linezolid (B and

343 D) combinations on planktonic MRSA L31 and L328 over 48h.

344 GC= growth control, DAP6= daptomycin 6mg/kg, DAP10= daptomycin 10mg/kg, VAN=

345 vancomycin, LZD= linezolid

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