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Title: 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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Running title: Linezolid antagonism of cell wall active agents

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ABSTRACT

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

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

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

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

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

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

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

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

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

Salt Lake City, UT). Maximum concentration (Cmax) to MIC ratios, the percent time above the MIC (%T > MIC), and AUC_{0-24} to MIC ratios were calculated for each antibiotic and were compared to literature values.(22-25)

Changes in bacterial growth (log_{10} CFU/mL) at 4, 8, 24 and 48h and time to 99.9% kill were compared by analysis of variance with Tukey's post-hoc test. A p value of < 0.05 was considered significant.(7, 11) All statistical analyses were performed using SPSS Statistical Software (Release 20 SPSS, Inc., Chicago, IL).

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

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

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

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

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

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

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

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

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

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

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

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All named authors meet the ICMJE criteria for authorship for this manuscript, take responsibility for the integrity of the work as a whole, and have given final approval to the version to be published. The views expressed are those of the authors and do not necessarily represent the position or policy of the United States Department of Veterans Affairs.

Conflict of Interest

Megan Luther declares research funding from Pfizer and Cubist. Kerry LaPlante declares Cubist, Davol, Marvao Medical, and Pfizer research funding, advisor, and/or consultancy.

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	MIC (mcg/mL)	MBC (mcg/mL)	*f*Cmax/ MIC	%T>MIC	*f*AUC/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

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

MIC= minimum inhibitory concentration

MBC= minimum bactericidal concentration

*f*Cmax= maximum free concentration

AUC= area under the curve

%T>MIC= percentage of time above MIC

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)

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

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

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

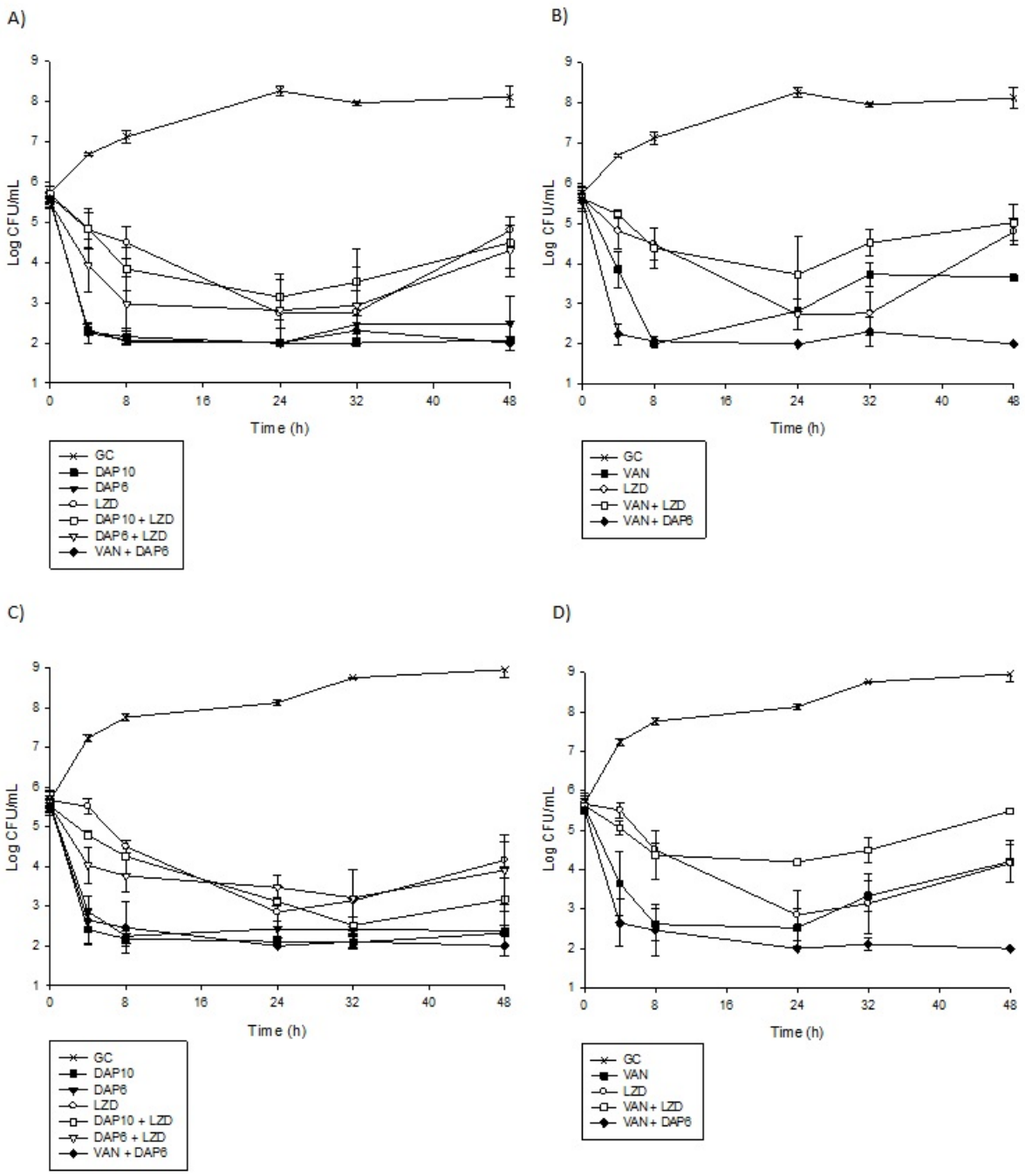
Enhancement: >2 $\log_{10}$ CFU/mL increase in kill over the most active component.

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

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

Figure 1. Activity of daptomycin and linezolid (A and C), or vancomycin and linezolid (B and D)combinations on planktonic MRSA L31 and L328 over 48h.

GC= growth control, DAP6= daptomycin 6mg/kg, DAP10= daptomycin 10mg/kg, VAN= vancomycin, LZD= linezolid


A)
B)
C)
D)
Log CFU/mL
Time (h)
GC
DAP10
DAP6
LZD
DAP10 + LZD
DAP6 + LZD
VAN + DAP6
VAN
VAN+ LZD
VAN+ DAP6

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