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# In Vitro Synergy of Daptomycin plus Rifampin against *Enterococcus faecium* Resistant to both Linezolid and Vancomycin

George Pankey,* Deborah Ashcraft, and Nalini Patel

*Ochsner Clinic Foundation, New Orleans, Louisiana*

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**In vitro synergy testing of daptomycin plus rifampin was performed against 24 unique isolates of *Enterococcus faecium* resistant to both linezolid and vancomycin. Synergy testing showed that 21/24 (88%) were synergistic and 3/24 (12%) were indifferent by the Etest method. Time-kill assays revealed synergy for 18/24 (75%) and indifference for 6/24 (25%).**

Therapeutic failure due to antimicrobial-resistant gram-positive pathogens, such as linezolid- and vancomycin-resistant *Enterococcus faecium*, is increasing. In an attempt to improve efficacy, an antimicrobial combination is frequently administered on an empirical basis. Many antimicrobial combinations have been studied for synergy in vitro and in vivo against enterococci (4, 9, 17, 19, 20), with no demonstrated correlation. We studied the in vitro combination of daptomycin (DAP) and rifampin (RIF) against linezolid- and vancomycin-resistant *E. faecium*.

Daptomycin has good in vitro activity against gram-positive bacteria, including vancomycin-resistant enterococci (1, 5, 8, 10–12, 21). Rifampin has shown synergy with other drugs in animal models for the treatment for gram-positive infections (2, 3, 7, 16, 17). In a synergy study with vancomycin-resistant *E. faecium* by Rand and Houck, it was suggested that, at subinhibitory daptomycin concentrations, daptomycin might bind to the bacterial cell, opening a channel that allows rifampin entry (19).

Standard laboratory powders of DAP (Cubist Pharmaceuticals, Inc., Lexington, MA) and RIF (Sigma-Aldrich, St. Louis, MO) were used in this study. Etest strips (AB Biodisk, Solna, Sweden) of daptomycin and rifampin were also used. The daptomycin Etest contained a concentration gradient of daptomycin with a standard amount of calcium throughout the strip.

Twenty-four unique clinical *Enterococcus faecium* isolates with distinct plasmid DNA (by pulsed-field gel electrophoresis) that were resistant to both linezolid (Etest MICs, 8 to >256 μg/ml) and vancomycin (Etest MICs, >256 μg/ml) were collected from throughout the United States. All strains were identified by the Vitek system (bioMerieux Inc., Hazelwood, MO). Isolates were stored frozen at −70°C in Columbia broth with 20% glycerol. *Enterococcus faecalis* ATCC 29212 was included as a quality control strain (6). Mueller-Hinton II broth (Becton Dickinson Microbiology Systems, Sparks, MD) was prepared in the laboratory and supplemented to the recommended 50 mg/liter calcium for the testing of daptomycin (15). Mueller-Hinton II agar plates (Becton Dickinson Microbiology Systems, Sparks, MD) were used for the Etest MIC determination and the Etest synergy method. Trypticase soy agar with 5% sheep blood (Becton Dickinson Microbiology Systems, Sparks, MD) was used for the colony counts in the time-kill assay.

Daptomycin and rifampin MICs were determined by broth microdilution (BMD) and Etest. The Clinical and Laboratory Standards Institute (CLSI [formerly NCCLS]) interpretive standards for rifampin and enterococci are as follows: ≤1 μg/ml, susceptible; 2 μg/ml, intermediate; ≥4 μg/ml, resistant; for daptomycin and enterococci, the CLSI standard is that ≤4 μg/ml implies susceptibility (6).

BMD MICs were determined following 2003 CLSI guidelines (15). Etest MICs for daptomycin and rifampin were determined in triplicate, and testing was performed according to the manufacturer's instructions. MICs between twofold dilutions were rounded up to the next twofold dilution for purposes of comparison with the BMD MIC.

Synergy testing was performed using an Etest method (18) and time-kill assay (TKA). The Etest method was performed in triplicate, the summation fractional inhibitory concentration (ΣFIC) was calculated for each set of MICs, and the mean ΣFIC was used for comparison to the TKA.

To evaluate the effect of the combinations, the FIC was calculated for each antibiotic in each combination. High-off-scale MICs (>256 μg/ml) were converted to the next twofold dilution (512 μg/ml). The following formulas were used to calculate the ΣFIC: (i) FIC of drug A = MIC of drug A in combination/MIC of drug A alone; (ii) FIC of drug B = MIC of drug B in combination/MIC of drug B alone; (iii) ΣFIC = FIC of drug A + FIC of drug B.

Synergy was defined by a ΣFIC of ≤0.5. Antagonism was defined by a ΣFIC of >4. Interactions represented by a ΣFIC of >0.5 but ≤4 were termed indifferent (1a).

The TKA was chosen to be compared with the Etest method for all isolates following guidelines set by the CLSI (14) and was performed as described in our previous study (18). Each isolate was tested against daptomycin and rifampin alone and in combination at a concentration equal to the MIC to correlate with the Etest. Bottles were incubated at 35°C in ambient air for 24 h. Samples (0.5 ml) were removed from each bottle at 0 h and 24 h. TKA results which were discordant to the Etest method results were repeated and confirmed the initial TKA

* Corresponding author. Mailing address: Ochsner Clinic Foundation, 1514 Jefferson Highway, New Orleans, LA 70121. Phone: (504) 842-4006. Fax: (504) 842-5433. E-mail: gpankey@ochsner.org.

TABLE 1. MICs by Etest and broth microdilution and synergy testing by Etest and time-kill assay

| *E. faecium* isolate ($n$ = 24) | MIC (μg/ml) of: | | | | Synergy testing | | |
|---|---|---|---|---|---|---|---|
| | DAP by: | | RIF by: | | Etest[b] | | TKA $\log_{10}$ change[a] |
| | Etest[b] | BMD | Etest[b] | BMD | ΣFICs | Mean ΣFIC | |
| 1 | 4 | 2 | 0.008 | 0.032 | 0.3, 0.5, 0.8 | 0.6 | −2.0 (SYN) |
| 2 | 4 | 1 | 16 | 8 | 0.1, 0.1, 0.1 | 0.1 | −2.0 (SYN) |
| 3 | 2 | 2 | 0.032 | 0.064 | 0.9, 0.9, 0.9 | 0.9 | −2.0 (SYN) |
| 4 | 2 | 2 | 0.016 | 0.064 | 0.2, 0.2, 0.3 | 0.2 | −4.0 (SYN) |
| 5 | 2 | 2 | 0.016 | ≤0.016 | 0.3, 0.2, 0.2 | 0.2 | −5.0 (SYN) |
| 6 | 1 | 2 | 16 | 16 | 0.2, 0.3, 0.2 | 0.2 | −2.4 (SYN) |
| 7 | 2 | 2 | 16 | 8 | 0.3, 0.3, 0.3 | 0.3 | −2.0 (SYN) |
| 8 | 2 | 2 | 0.016 | 0.032 | 0.3, 0.3, 0.2 | 0.3 | −2.1 (SYN) |
| 9 | 2 | 2 | >256 | >32 | 0.4, 0.4, 0.3 | 0.4 | −2.0 (SYN) |
| 10 | 2 | 1 | 8 | 1 | 0.2, 0.3, 0.4 | 0.3 | −2.0 (SYN) |
| 11 | 2 | 2 | 32 | 8 | 0.2, 0.2, 0.3 | 0.2 | −2.0 (SYN) |
| 12 | 2 | 4 | 32 | 16 | 0.2, 0.4, 1.0 | 0.5 | −2.6 (SYN) |
| 13 | 4 | 4 | 32 | 8 | 0.1, 0.4, 0.2 | 0.2 | −2.0 (SYN) |
| 14 | 2 | 2 | 0.016 | 0.032 | 0.4, 0.3, 0.4 | 0.4 | −2.5 (SYN) |
| 15 | 1 | 1 | 16 | 4 | 0.4, 0.4, 0.3 | 0.4 | −2.2 (SYN) |
| 16 | 2 | 0.5 | 16 | 4 | 0.3, 0.2, 0.2 | 0.2 | −2.0 (SYN) |
| 17 | 4 | 2 | 32 | 16 | 0.2, 0.2, 0.2 | 0.2 | −2.0 (SYN) |
| 18 | 4 | 1 | 0.032 | 0.032 | 1.0, 1.0, 1.0 | 1.0 | −2.0 (SYN) |
| 19 | 2 | 1 | 32 | 4 | 0.2, 0.2, 0.1 | 0.2 | −1.4 (IND) |
| 20 | 4 | 2 | 0.016 | ≤0.016 | 0.4, 0.3, 0.5 | 0.4 | −0.9 (IND) |
| 21 | 2 | 2 | 16 | 4 | 0.1, 0.2, 0.2 | 0.2 | −1.4 (IND) |
| 22 | 2 | 1 | 8 | 4 | 0.4, 0.4, 0.3 | 0.4 | −0.6 (IND) |
| 23 | 2 | 1 | 32 | 8 | 0.3, 0.3, 0.3 | 0.3 | −1.0 (IND) |
| 24 | 4 | 2 | 8 | 4 | 0.2, 0.2, 0.3 | 0.2 | −0.5 (IND) |

[a] Values represent the $\log_{10}$ change (CFU/ml) in the TKA after 24-h exposure to DAP and RIF when compared to the most active drug alone. Negative values indicate a decrease in colony count. SYN, synergy; IND, indifference.
[b] Performed in triplicate.

interpretation. Performing serial dilutions, plating with a spiral plater (which further dilutes and plates the sample), and using a concentration of drug equal to the MIC helped reduce the possibility of antibiotic carryover. Synergy was defined as a ≥2 $\log_{10}$ decrease in colony count at 24 h by the combination compared to the most active single agent, and the number of surviving organisms in the presence of the combination had to be ≥2 $\log_{10}$ CFU/ml below the starting inoculum (1a). Indifference was defined as <2 $\log_{10}$ increase in colony count at 24 h by the combination compared by the most active single agent. Antagonism was defined as a ≥2 $\log_{10}$ increase in colony count at 24 h by the combination compared with that by the most active single agent alone (13).

Some isolates showed resistance to rifampin: 15/24 (63%) by broth microdilution and 16/24 (67%) by Etest. All isolates were susceptible to daptomycin by both methods (Table 1).

The daptomycin Etest had 88% (21/24) ± 1 twofold dilution essential agreement with daptomycin BMD, suggesting adequate calcium content of the Etest strip when used with Becton Dickinson Mueller-Hinton II agar plates. The rifampin Etest had only 58% (14/24) essential agreement with rifampin BMD. The rifampin BMD MICs were lower for 17/24 (71%) isolates, higher for 5/24 (21%) isolates, and identical for 2/24 (8%) isolates when compared to rifampin Etest MICs.

Daptomycin and rifampin synergy (ΣFIC ≤ 0.5) was found in 20/24 (83%) strains when tested by Etest. The TKA revealed 75% (18/24) synergy and 25% (6/24) indifference. Concordance of the Etest synergy method and the TKA was demonstrated in 15/24 (63%) isolates. For three isolates, the Etest method ΣFICs were 0.6, 0.9, and 1.0 (indifference) but the TKA showed synergy. Six isolates showed synergy, with ΣFICs of 0.2, 0.4, 0.2, 0.4, 0.3, and 0.2 but were indifferent by TKA (−1.4, −0.9, −1.4, −0.6, −1.0, and −0.5 $\log_{10}$ change, respectively, in CFU/ml). The synergy occurred despite significant daptomycin activity against all isolates. No antagonism was detected by either method (Table 1).

Synergy testing methods are not standardized for reproducibility and interpretation, making comparison of results from different studies extremely difficult.

In the TKA for synergy, drug concentrations are fixed and do not decrease over time, as they would in vivo. In addition, there are no standard concentrations at which antibiotics are tested. The inoculum size and time frame of the TKA add more variability to the test. The time parameter of 24 h can limit or alter results of the experiment if regrowth occurs with one or both antibiotics. Regrowth can be caused by use of a subinhibitory concentration of antibiotics, emergence of resistant subpopulations, or bacteria that adhere to the surface of the bottle and are subsequently released in the media. Another factor affecting regrowth is inactivation of the antibiotics in vitro.

The Etest synergy method used a concentration equal to the MIC for each drug. Depending on the drug, concentration ranges vary on Etest strips. A MIC-to-MIC placement of the strips seems to give a more accurate diffusion of the two drugs and indication of the effects (if any) that each drug has on the other in combination against the organism (18). Several abstracts have been presented on the technique using gram-positive bacteria: G. Pankey and D. Ashcraft, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., abstr. C92, 2001; G. Pankey, D.

Ashcraft, and O. Prakash, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-1133, 2002; and G. Pankey, D. Ashcraft, and P. Pankey, Abstr. 41st Infect. Dis. Soc. Am., abstr. 229, 2003. The use of the Etest strips for synergy has yet to be standardized but has the potential to be a useful screening test for the determination of synergy.

Our Etest method was compared to TKA, but the two methods use totally different test systems, solid media versus liquid, respectively. However, both methods predict bactericidal activity in vitro. The Etest was able to detect slight hazes of growth and resistant subpopulations.

It is interesting that we could demonstrate in vitro synergy of daptomycin and rifampin against some *E. faecium* isolates. However, the mechanism of this in vitro synergy is unknown. The clinical benefit of in vitro synergy by daptomycin and rifampin against any strain of *E. faecium* remains speculation.

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