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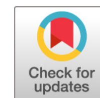

Influence of Inoculum Effect on the Efficacy of Daptomycin Monotherapy and in Combination with β-Lactams against Daptomycin-Susceptible *Enterococcus faecium* Harboring LiaSR Substitutions

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ABSTRACT *Enterococcus faecium* isolates that harbor LiaFSR substitutions but are phenotypically susceptible to daptomycin (DAP) by current breakpoints are problematic, since predisposition to resistance may lead to therapeutic failure. Using a simulated endocardial vegetation (SEV) pharmacokinetic/pharmacodynamic (PK/PD) model, we investigated DAP regimens (6, 8, and 10 mg/kg of body weight/day) as monotherapy and in combination with ampicillin (AMP), ceftaroline (CPT), or ertapenem (ERT) against *E. faecium* HOU503, a DAP-susceptible strain that harbors common LiaS and LiaR substitutions found in clinical isolates (T120S and W73C, respectively). Of interest, the efficacy of DAP monotherapy, at any dose regimen, was dependent on the size of the inoculum. At an inoculum of ~10^9 CFU/g, DAP doses of 6 to 8 mg/kg/day were not effective and led to significant regrowth with emergence of resistant derivatives. In contrast, at an inoculum of ~10^7 CFU/g, marked reductions in bacterial counts were observed with DAP at 6 mg/kg/day, with no resistance. The inoculum effect was confirmed in a rat model using humanized DAP exposures. Combinations of DAP with AMP, CPT, or ERT demonstrated enhanced eradication and reduced potential for resistance, allowing de-escalation of the DAP dose. Persistence of the LiaRS substitutions was identified in DAP-resistant isolates recovered from the SEV model and in DAP-resistant derivatives of an initially DAP-susceptible clinical isolate of *E. faecium* (HOU668) harboring LiaSR substitutions that was recovered from a patient with a recurrent bloodstream infection. Our results provide novel data for the use of DAP monotherapy and combinations for recalcitrant *E. faecium* infections and pave the way for testing these approaches in humans.

KEYWORDS combination therapy, daptomycin, PK/PD, VREfm

Enterococcus faecalis and *Enterococcus faecium* are important causes of hospital-acquired infections in the United States and are associated with a high degree of morbidity and mortality (1, 2).

Daptomycin (DAP) has *in vitro* bactericidal activity against vancomycin-resistant

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enterococci (VRE) (*E. faecalis* and *E. faecium*) and, due to this property, its favorable side effect profile, supportive clinical data, and ease of administration, it has become a key frontline drug against vancomycin-resistant *E. faecium* (VREfm) strains. Unfortunately, there are serious concerns about the breakpoints and emergence of resistance to DAP during therapy, as well as the identification of DAP-nonsusceptible isolates in patients who have never been exposed to the antibiotic (3–5). DAP is a lipopeptide antibiotic that exerts its mechanism of action by interactions with the bacterial cell membrane in a calcium-dependent manner, resulting in disruptions of cell membrane and cell division homeostasis (6–10). Enterococci are less susceptible to DAP than staphylococci and other Gram-positive organisms (current breakpoints are 4-fold higher than those of *Staphylococcus aureus*). It has been shown that much higher doses of DAP are required to achieve killing activity against enterococci similar to that observed with *S. aureus* (11–13). Furthermore, clinical observations suggest that underdosing of DAP (using doses approved for *S. aureus* and for acute bacterial skin and skin structure infections [ABSSSI] due to *E. faecalis*) is associated with worse clinical outcomes and may lead to development of DAP resistance (14).

DAP displays concentration-dependent bactericidal activity against most enterococci, including VREfm and linezolid-resistant strains. The area under the concentration-time curve over 24 h divided by the MIC (AUC_{24}/MIC) is the pharmacokinetic/pharmacodynamic (PK/PD) parameter that best predicts the *in vivo* efficacy of the antibiotic (15). We have previously demonstrated, in both *S. aureus* and enterococci, that emergence of DAP resistance can be delayed or prevented by increasing the DAP dose exposure (AUC_{24}/MIC) beyond the currently approved *S. aureus* bacteremia dose of 6 mg/kg of body weight/day (12, 16–19). Data from our laboratory suggest that DAP exposures and dosages of at least 10 mg/kg/day are needed to reduce the emergence of resistance under conditions of high bacterial burden (12).

Although the mechanism(s) of DAP resistance in enterococci is not fully understood, it is associated with major alterations in cell envelope phospholipid metabolism and structure that result in either repulsion of the drug from the cell surface (*E. faecium*) or "diversion" of the antibiotic from the septal principal target (*E. faecalis*) (20–25). In both *E. faecium* and *E. faecalis*, mutations in genes encoding a three-component regulatory system (LiaFSR) involved in cell envelope homeostasis are thought to be important mediators of DAP resistance, independent of the genetic background (23, 26). The most frequent substitutions observed in clinical isolates of VREfm strains are T120A and W73C in LiaS (histidine kinase) and LiaR (response regulator), respectively. These changes are often observed in VREfm strains with DAP MICs below the susceptibility breakpoint that, therefore, are considered susceptible by current standards (27). We have previously shown that such substitutions are associated with a marked decrease in the bactericidal activity of DAP *in vitro* (28) and DAP failure in patients treated with the antibiotic (29). Moreover, it is unclear if the use of higher doses of DAP (10 to 12 mg/kg) is effective against DAP-susceptible VREfm isolates that harbor these common LiaRS substitutions.

Recent data suggest that the combination of a β-lactam with DAP is highly synergistic against DAP-susceptible (DAP-S) and DAP-resistant (DAP-R) VREfm strains that contain LiaFSR substitutions (23–25, 30, 31), although this phenomenon is not observed in isolates with mutations in genes other than *liaFSR* (23). While these combinations appear to be promising for recalcitrant VREfm infections, there are few to no data to suggest which β-lactam works best with DAP and whether these combinations would reduce the use of high DAP exposures needed to prevent resistance emergence in VREfm strains, including those with LiaFSR substitutions. Achievement of improved pharmacodynamics with lower dose exposures of DAP would potentially increase therapeutic efficacy while reducing adverse effects and drug cost. Thus, there is an important need to determine the optimal dose exposure of DAP alone and in combination with β-lactams against VREfm strains, particularly those that are DAP "susceptible" by breakpoint definitions but have already developed mutations that may predispose them to DAP resistance *in vivo*. Here, using a well-characterized strain of

TABLE 1 MIC values

Drug[a]	MIC (mg/liter)
DAP	2
CPT	32
AMP	128
ERT	512
DAP + CPT	0.031
DAP + AMP	0.25
DAP + ERT	0.5

[a]DAP, daptomycin; CPT, ceftaroline; AMP, ampicillin; ERT, ertapenem.

DAP-susceptible *E. faecium* (HOU503 [DAP MIC, 2 mg/liter], harboring the T120S and W73C substitutions in LiaS and LiaR, respectively), we studied the efficacy of three different DAP dose regimes (6, 8, and 10 mg/kg) in monotherapy and in combination with ampicillin (AMP), ceftaroline (CPT), or ertapenem (ERT), using a simulated model of endocardial vegetations. We found that the response to DAP was dependent on the size of the inoculum, with higher inocula resulting in DAP failure and development of resistance. Furthermore, the inoculum effect could be demonstrated in an *in vivo* model of rat endocarditis. Finally, we show that the LiaRS substitutions predispose to DAP resistance and emergence of additional mutations when the pharmacodynamic target is not attained. The LiaRS changes also persisted in isolates recovered from a patient infected with a VREfm strain who failed DAP therapy.

RESULTS

Pharmacokinetics of DAP, AMP, CPT, and ERT. The DAP MIC for *E. faecium* HOU503 was 2 mg/liter. MIC results using broth microdilution are listed in Table 1. The DAP pharmacokinetics parameters achieved for a DAP regimen of 6 mg/kg in this model were a maximum concentration of drug in serum (C_{max}) of 89.86 ± 5.71 mg/liter (target C_{max}, 93.9 mg/liter) and a half-life ($t_{1/2}$) of 7.99 ± 0.53 h (target $t_{1/2}$, 8 h), an AUC from 0 to 24 h (AUC_{0-24}) of 985.19 ± 25.23 mg/liter, and an AUC_{24}/MIC of 492.59 ± 12.62. The simulated DAP 8-mg/kg model achieved a C_{max} of 123.43 ± 0.53 mg/liter and a $t_{1/2}$ of 8.22 ± 0.14 h, an AUC_{0-24} of 1,465.77 ± 44.29 mg/liter, and an AUC_{24}/MIC of 732.89 ± 22.15. Finally, the DAP 10-mg/kg simulated model achieved a C_{max} of 145.18 ± 0.71 mg/liter with a $t_{1/2}$ of 8.23 ± 0.2 h, an AUC_{0-24} of 1,725.92 ± 36.50 mg/liter, and an AUC_{24}/MIC of 862.96 ± 18.25. The AUC_{24}/MIC values are shown in Table 2. A high-performance liquid chromatography (HPLC) assay demonstrated an interday coefficient of variation between 0.6 and 7.3% for all DAP standards (assays were performed in duplicate).

The pharmacokinetics parameters for CPT in the model were a C_{max} of 21.39 ± 0.06 mg/liter (target C_{max}, 21 mg/liter) and a $t_{1/2}$ of 2.653 ± 0.11 h (target $t_{1/2}$, 2.66 h), an AUC_{0-24} of 80.53 ± 2.5 mg/liter, and an AUC_{24}/MIC of 2.52 ± 0.08. This CPT bioassay demonstrated an interday coefficient of variation between 2.76 and 5.14% for all standards. For ampicillin, the PK parameters in the model were a C_{max} of 74.14 ± 2.08 mg/liter (target C_{max}, 70 mg/liter), an AUC_{0-24} of 1,667.23 ± 26.21 mg/liter, and an AUC_{24}/MIC of 13.03 ± 0.2. The ampicillin bioassay interday coefficient of variation was between 0.67 and 1.68% for all standards. Finally, ERT achieved a C_{max} of 159.11 ± 0.52 mg/liter (target C_{max}, 155 mg/liter), an AUC_{0-24} of 1,146.85 ± 26.98 mg/liter, and an AUC_{24}/MIC of 2.24 ± 0.053. The ERT bioassay interday coefficient of variation was between 0.27 and 2.76% for all standards.

TABLE 2 AUC/MIC values for DAP, and DAP combinations with CPT, AMP, or ERT

	AUC/MIC (mean ± SD)			
Dose (mg/kg)	DAP	DAP + CPT	DAP + AMP	DAP + ERT
6	492.59 ± 12.62	31,780.32 ± 813.87	3,940.76 ± 100.92	1,970.38 ± 50.46
8	732.89 ± 22.15	47,282.90 ± 1,428.71	5,863.08 ± 177.16	2,931.54 ± 88.58
10	862.96 ± 18.25	55,674.84 ± 1,177.42	6,903.68 ± 146	3,451.84 ± 73

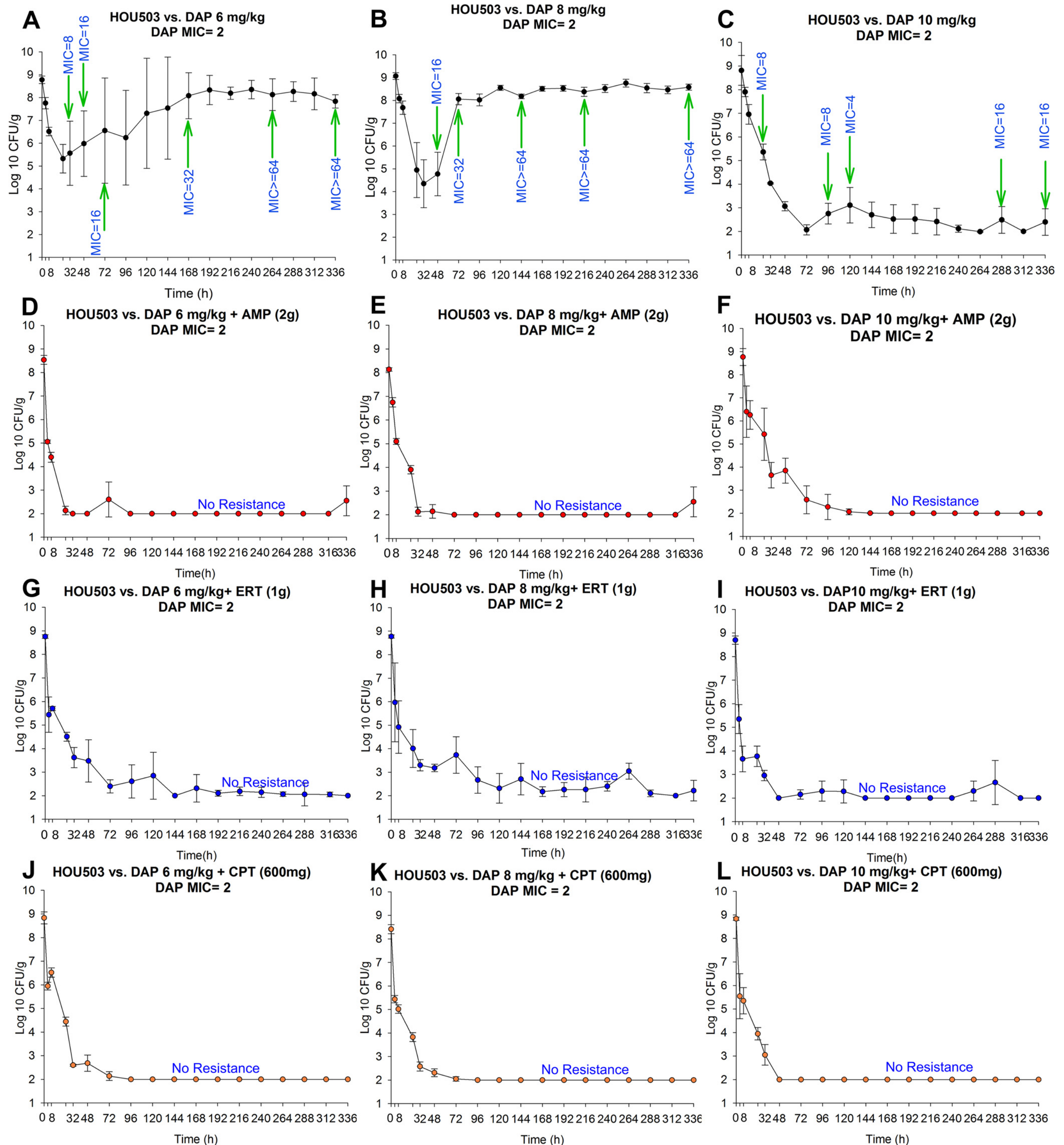

FIG 1 Comparison of daptomycin activity with and without AMP or ETP in various models. (A to C) DAP models. (D to F) DAP-plus-AMP models. (G to I) DAP-plus-ERT models. (J to L) DAP-plus-CPT models. The error bars indicate SD.

Pharmacodynamics. DAP 6- and 8-mg/kg/day regimens demonstrated initial bactericidal activity followed by significant regrowth close to the starting inoculum. DAP at 10 mg/kg/day killed at or slightly above the level of detection (2 $\log_{10}$ CFU/g) by 72 h with minor regrowth thereafter for the duration of the experiment. However, DAP resistance was detected in all three monotherapy regimens, with the MICs of DAP ranging from 8 to 256 mg/liter (an increase from baseline as high as 32-fold) (Fig. 1). In

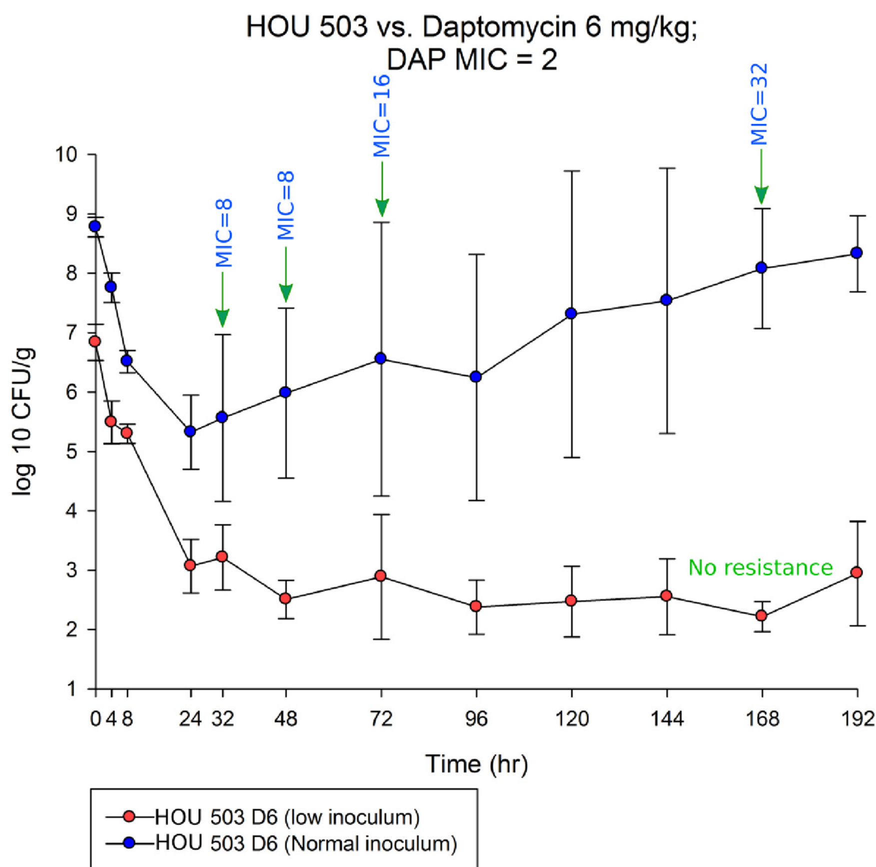

FIG 2 Comparison of the effects of high inoculum versus low inoculum. Antibiotic efficacy (eradication ability) increases at lower inocula. Reducing the initial inoculum from $10^{8.7}$ CFU/g to $10^{6.8}$ CFU/g caused $10^{3.5}$ more reduction in CFU per gram. Significant killing and no emergence of resistance were observed at lower inocula. The error bars indicate SD.

contrast, we were unable to detect emergence of resistance in the DAP combination regimens. The combination of DAP plus AMP, CPT, or ERT demonstrated enhanced killing activity compared to DAP alone, maintaining a reduction in $\log_{10}$ CFU per gram at or slightly above the level of detection during the 14-day experiments. Of note, emergence of DAP resistance was identified in one sample from one experiment with DAP at 8 mg/kg/day plus ertapenem at 264 h (MIC $>$ 64 mg/liter) but was not detected at any other sample point throughout the 14-day experiment. There was no resistance detected with DAP at 8 mg/kg/day plus AMP or CPT at any sample time point.

Effects of AMP, CPT, and ERT on the DAP MIC. To correlate the findings of the simulated endocardial vegetation (SEV) model, we tested MICs for DAP in the presence or absence of AMP, CPT, and ERT. HOU503 is resistant to AMP, ERT, and CPT. However, the DAP MIC in combination with β-lactams was reduced 8-fold for AMP, 64.5-fold for CPT, and 4-fold for ERT, as depicted in Table 1.

Efficacy of DAP against *E. faecium* 503 and development of resistance are inoculum dependent. Since DAP is a concentration-dependent antibiotic, we sought to evaluate the effect of the bacterial inoculum on the bactericidal activity of DAP using the SEV PK/PD model (Fig. 2). At an inoculum of approximately 10^9 CFU/g, DAP at a dose of 6 mg/kg/day killed up to 10^5 CFU/g at 24 h, followed by significant regrowth (up to 10^8 CFU/g at 192 h with minimal reduction of the total CFU per gram). Under these conditions, we were able to identify DAP-resistant derivatives with DAP MICs as high as 32 mg/liter at 192 h and $>$64 mg/liter at 336 h. In contrast, at an inoculum of $\sim 10^7$ CFU/g, DAP at 6 mg/kg/day reduced the inoculum to 10^3 CFU/g with no significant regrowth (10^3 at 192 h; total reduction of $\sim 10^4$ CFU/g). Most importantly, we

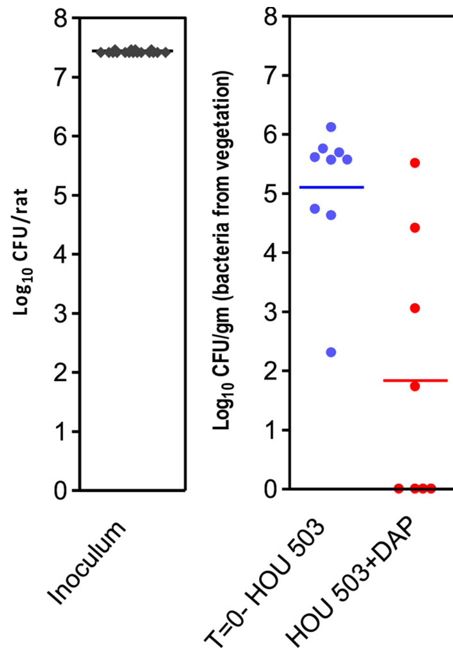

FIG 3 Rat endocarditis model. The inocula used (left) and bacterial counts are shown. DAP was used at a humanized dose of 6 mg/kg/day for 3 days. Blue, T_0 numbers; red, bacterial numbers after 3 days of DAP monotherapy.

were not able to find evidence of the emergence of resistance at 192 h in the model. These findings suggest that the killing activity of DAP is inoculum dependent, a finding with important clinical consequences.

In order to confirm the influence of the inoculum effect *in vivo*, we used a rat endocarditis model using a strategy that allowed us to administer DAP mimicking the pharmacokinetics in humans. For this purpose, we used an infusion pump attached to the animal and delivered DAP at a dose of 45.3 mg/kg/day, simulating the 6-mg/kg/day dose in humans, as described previously (32). A total of 17 animals were inoculated directly in the left ventricle with *E. faecium* HOU503 at >10 times the 90% infective dose (ID_{90}), i.e., 2×10^7 to 3×10^7 CFU/rat (Fig. 3). We postulated that this model would be ideal to assess the efficacy of DAP *in vivo* against HOU503 when bacterial counts in vegetations were similar to the “low-inoculum” state in our SEV model.

In the absence of antibiotics, at the time of therapy initiation (T_0), rats inoculated with the HOU503 strain (after 24 h) showed a count of 5.1 ± 1 $\log_{10}$ CFU/g (mean ± standard deviation [SD]) from aortic valve vegetations of infected rats (Fig. 3). Treatment with DAP alone for 3 days resulted in a significant reduction of 1.8 ± 2 $\log_{10}$ from aortic valve vegetations ($P = 0.0016$ versus T_0; 4 animals had sterile valves), indicating that DAP was active *in vivo* when used against *E. faecium* 503 at a bacterial density of 5.1 ± 1 $\log_{10}$ CFU/g (in the vegetation) (Fig. 3). Colonies recovered from the DAP-treated animals were screened for DAP resistance using an Etest method. We did not identify any derivative with an increased DAP MIC from this model, supporting the efficacy of the drug under conditions of a low inoculum and consistent with our SEV model findings.

The LiaRS substitutions persist in all DAP-resistant derivatives obtained *in vitro* and *in vivo* after DAP exposure. We initially performed whole-genome sequencing (WGS) in 16 DAP-resistant derivative isolates obtained from the SEV therapy models. They included 5, 5, and 6 isolates obtained from the 6-g/kg/day, 8-g/kg/day, and 10-mg/kg/day models, respectively. The DAP MICs ranged from 8 to >64 mg/liter, and times of isolation ranged from 48 to 336 h (see Table S1 in the supplemental material). Of note, the T120A and W73C substitutions in LiaR and LiaS, respectively, remained present in all DAP-resistant derivatives. Additional predicted substitutions found in the

resistant isolates are shown in Table S2 in the supplemental material. Changes in cardiolipin synthase, which have been previously identified in DAP-R isolates of both *E. faecalis* and *E. faecium* (33), were identified only in isolates recovered from the 8-mg/kg/day regimen. The majority of substitutions were present in hypothetical proteins not previously associated with DAP resistance and in mobile elements.

We had previously reported a case of DAP failure in a patient with bacteremia caused by a DAP-susceptible isolate (designated HOU668) with a DAP MIC of 3 mg/liter that harbored W73C and T120S substitutions in LiaR and LiaS, respectively (29). After the index isolate was recovered, four additional isolates genetically related to HOU668 (34) were obtained from the same patient, with DAP MICs ranging from 16 to 64 mg/liter (isolates HOU672, -673, -676, and -678) (see Table S3 in the supplemental material). We performed WGS of all the isolates recovered from this patient to determine possible genetic pathways associated with DAP resistance. Interestingly, similar to the *in vitro* situation, the substitutions in LiaS (T120A) and LiaR (W73C) persisted in all the isolates. No changes were found in any of 39 putative proteins previously associated with DAP-R in enterococci or staphylococci. Further, we found a total of 13 genes that harbored nonsynonymous single nucleotide polymorphisms (SNPs) compared to HOU668. However, none of the mutations found in DAP-R isolates from the patient were shared in the DAP-R isolates from the SEV despite both maintaining identical LiaRS substitutions. Nonetheless, proteins related to cell membrane phospholipid metabolism exhibited changes, such as cardiolipin synthase (*in vitro*-derived isolates), diacylglycerol kinase, phosphatidylglyceroltransferase, and undecaprenyl diphosphate synthase (the last was present in all *in vivo*-derived isolates). These findings support the notion that changes in LiaRS are the primary changes in the evolution of DAP resistance, with multiple pathways being followed after the initial changes.

DISCUSSION

As DAP has become a key antibiotic to treat multidrug-resistant enterococcal infections, questions remain about the optimal dosing scheme, a decision that has been complicated by uncertainties in the breakpoint (13, 35, 36). In particular, DAP-susceptible strains with MICs close to the susceptibility cutoff that harbor mutations in the *liaFSR* operon present a major therapeutic dilemma because bactericidal options other than DAP are scarce for deep-seated enterococcal infections and detection of the mutation is not routinely performed by clinical microbiology laboratories.

In the present study, using a prototypical strain of *E. faecium* that harbors the most common LiaRS substitutions found in clinical isolates (36) and no other changes previously associated with DAP resistance, we evaluated (in a simulated endocardial vegetation PK/PD model) different schemes of DAP as monotherapy in three dose regimens (6, 8, and 10 mg/kg/day). Additionally, we assessed the effect of DAP with combinations of three different β-lactams that have been previously shown to have synergistic activity with DAP (ampicillin, ceftaroline, and ertapenem). Our results support previous reports that DAP in combination with the tested β-lactams not only enhances killing, but also prevents emergence of resistance. Most importantly, we have now presented evidence of the novel concept that the efficacy of DAP as monotherapy against DAP-susceptible strains that are predisposed to develop resistance because of changes in LiaFSR seems to depend on the size of the infecting inoculum. Indeed, the SEV model showed that, at an inoculum of $\sim 10^7$ CFU/g, a dose of DAP as low as 6 mg/kg was sufficient to markedly reduce the infecting bacterial density to $\sim 10^3$ CFU/g with no significant regrowth, and we were unable to detect any emergence of resistance under these conditions. Moreover, we confirmed these findings using a "humanized" model of rat endocarditis mimicking the human DAP dose of 6 mg/kg/day. In this *in vivo* model, our results clearly show that DAP (at a dose of 6 mg/kg) can significantly reduce the bacterial load in vegetations, suggesting that enterococcal infections with lower inocula may respond to DAP monotherapy. However, such low inocula are unlikely to occur in deep-seated infections, such as human infective endocarditis, other endovascular infections, or high-grade bacteremia. Our results

indicate that, in the last case, development of resistance is frequent and therapeutic failure is highly likely.

The strategy to overcome the lack of DAP efficacy seems to be the use of combination therapy with selected β-lactams. We and others have previously reported MIC reductions in combinations of DAP plus β-lactams with both *E. faecalis* and *E. faecium* (23, 25). The compounds that seem to exhibit the best synergistic effects with DAP are ampicillin, ceftaroline, and ertapenem, as demonstrated in time-kill assays against enterococcal isolates (37). Although the molecular basis for the synergism and selectivity for certain β-lactam compounds is not known, it might be due to the differential affinity of β-lactams to enterocccal penicillin binding proteins (PBPs), similar to what has been shown previously in *S. aureus* (8, 11). Moreover, previous studies have shown that β-lactams such as ceftaroline may increase the binding of DAP, likely by enhancing the number of molecules that can oligomerize at the membrane level (38–40).

An important consideration is that DAP–β-lactam synergism does not appear to be a universal phenomenon seen in all multidrug-resistant clinical isolates of *E. faecium*. We have previously shown that the DAP-plus-ampicillin synergism occurs only in DAP-tolerant or -resistant strains that harbor substitutions in the LiaFSR system (in particular, the common T120S and W73C changes in LiaS and LiaR, respectively) (23). Recent data suggest that the W73C substitutions activate the LiaFSR system by increasing the oligomerization state of LiaR, enhancing the affinity for the promoter of target genes (33). Changes in LiaFSR seem to result in major alterations of the cell envelope, allowing additional changes (mostly in enzymes controlling cell membrane phospholipid homeostasis) to take place, resulting in DAP and antimicrobial peptide resistance. Here, using a PK/PD SEV model with a strain in which common substitutions in LiaFSR are present, we show that the best approach for prevention of the development of resistance is combination therapy with β-lactams, particularly in high-inoculum SEVs. Our data are supported by previous observations; Sakoulas et al. have shown that DAP-AMP combinations result in extensive killing *in vitro* of AMP/vancomycin-resistant *E. faecium* (24). They previously tested (in an *in vitro* model) whether simulated DAP doses of 4 to 10 mg/kg/day against the same strain resulted in bacteriostatic activity. In fact, addition of ampicillin (simulated 2 g every 4 h [q4h]) with DAP (simulated 4 to 10 mg/kg/day) provided better bacterial killing than high-dose (simulated 10 mg/kg/day) DAP monotherapy. They also reported that this combination delayed/prevented the emergence of DAP-resistant strains (24). Similarly, Hall Snyder et al. have reported that in an *in vitro* PK/PD model of SEV, DAP (equivalent to 6 to 12 mg/kg/day) plus ceftriaxone (equivalent to 2 g every 24 h) (30) was significantly more active *in vitro* than DAP alone against VRE strains; however, as mentioned above, the synergism was not consistently observed with other enterococcal strains, including a reported clinical case (41). Moreover, the previous studies did not take into account the genetic background of the tested strain.

Using whole-genome sequencing, we aimed to gain insights into the genetic pathways of DAP resistance in strains that harbor the typical LiaRS substitutions. Thus, we sequenced a total of 16 DAP-resistant derivatives of DAP-S HOU503, recovered from the SEV model under different dose schemes. Additionally, we performed WGS of derivatives of HOU668, a DAP-susceptible stain harboring the typical LiaRS substitutions recovered from the bloodstream of a neutropenic patient in whom DAP failed and selection for resistance occurred in multiple genetically related isolates (HOU672, HOU674, HOU676, and HOU678) (34). The most striking finding was that the LiaRS substitutions were maintained in all DAP-R derivatives, both *in vitro* and *in vivo*. Interestingly, different genes were mutated *in vitro* and *in vivo*, suggesting that distinct pathways of cell envelope adaptation are followed and seem to depend on the genetic background and on the conditions under which resistance emerges. Indeed, mutations in genes coding for enzymes involved in phospholipid metabolism were commonly seen *in vivo*, while they were less common in the DAP-R derivatives selected in the SEV model. Our findings support the notion that initial LiaFSR activation seems to be the

critical genetic event for the evolution of resistance both *in vivo* and *in vitro* and that after such occurrence, multiple pathways are plausible.

In summary, we present evidence that the success of DAP monotherapy against multidrug-resistant strains with LiaFSR substitutions is likely dependent on the infecting inoculum. These isolates are likely to develop resistance in deep-seated infections where bacterial density is higher and may lead to therapeutic failure. The combination of DAP (even at 6 mg/kg) with ampicillin, ceftaroline, or ertapenem seems to be very effective in infections with high bacterial density, preventing development of resistance. Our findings open the possibility of approaching severe enterococcal infections using a combination of DAP plus β-lactam antibiotics initially, as well as the potential to use lower DAP doses. This approach may also reduce the potential for daptomycin resistance emergence during therapy. In addition, the ability to lower the DAP dose may reduce the potential for unwarranted adverse effects. However, the addition of the second agent's impact on adverse reactions will also have to be taken into account in future clinical studies. It may also be possible to de-escalate to monotherapy with DAP once the patient responds to therapy (i.e., clears the bacteremia); however, this approach will need to be further investigated. In addition, these therapeutic approaches should be supported by genetic testing of specific LiaFSR substitutions to guide therapy where possible. Clinical studies are needed to test these novel approaches for the treatment of recalcitrant VRE infections.

MATERIALS AND METHODS

Bacterial strains. HOU503 is a clinical isolate of vancomycin-resistant and DAP-susceptible *E. faecium* (DAP MIC, 2 mg/liter) that harbors the T120S and W73C substitutions in LiaS and LiaR, respectively, and has been described previously (26, 42, 43). HOU668 is a DAP-susceptible *E. faecium* isolate (DAP MIC, 2 to 3 μg/ml) recovered from a patient with persistent bacteremia harboring the same LiaSR substitutions as HOU503 (29). Isolates HOU672, HOU673, HOU676, and HOU678 are DAP-resistant derivatives of HOU668 exhibiting a progressive increase in DAP MICs (up to >256 μg/ml) during the course of hospitalization and have been previously described (34).

Antimicrobial agents and media. DAP and ertapenem were obtained from Merck & Co., Inc. (Whitehouse Station, NJ); ampicillin powder was purchased from Sigma-Aldrich Co. (St. Paul, MN); and ceftaroline analytical powder was obtained from Allergan Pharmaceuticals (Parsippany, NJ). Mueller-Hinton broth II (MHB) (Difco, Detroit, MI) with 50 mg/liter calcium and 12.5 mg/liter magnesium was used for susceptibility testing. Due to the dependency of DAP on calcium for antimicrobial activity and calcium loss from the medium due to calcium binding to albumin, MHB supplementation with calcium to a concentration of 75 mg/liter was used in the *in vitro* SEV model experiments, as previously described (44). Colony counts were determined using brain-heart infusion agar (BHIA) supplemented with 50 mg/liter calcium.

Susceptibility testing. All MICs except for the animal models were determined in duplicate using the microbroth dilution method at ~10^6 CFU/ml, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (45). Due to raised MIC values of β-lactams for this organism, combination MIC values for daptomycin were determined by supplementing the broth with concentrations of β-lactam antimicrobials at their respective biological fC_{max} (free peak concentrations) of ampicillin (70 mg/liter), ceftaroline (17 mg/liter), and ertapenem (15.5 mg/liter) to validate the abilities of the corresponding β-lactams to reduce the DAP MIC. The daptomycin MIC fold reduction from baseline was calculated by dividing daptomycin MICs by the daptomycin MICs in the presence of the listed β-lactams.

***In vitro* PK/PD model.** A SEV PK/PD model was used for all antibiotic experiments (13, 46). Simulated vegetations were prepared as previously described (11), mixing cryoprecipitate as the source of fibrin, a platelet suspension (American Red Cross), and an organism suspension (final inoculum, 10^7 or 10^9 CFU/0.5 g). This methodology results in SEVs that contain approximately 3 to 3.5 g/dl albumin and 6.8 to 7.4 g/dl total protein (11). The resultant SEV mixture was then clotted using bovine thrombin in siliconized microcentrifuge tubes. The SEVs were removed from the tubes and suspended in the model via a sterile monofilament line. The model apparatus was prefilled with growth medium containing 3.5 g/dl human albumin. Fresh medium was continuously added to and removed from the model, along with the drug, via a peristaltic pump set to simulate the half-lives of the antibiotics. DAP was administered once daily via an injection port. The simulated DAP regimens with a targeted $t_{1/2}$ of 8 h were 6, 8, and 10 mg/kg/day with peaks of 93.9, 123.3, and 141.1 mg/liter, respectively. The DAP regimens were tested alone and in combination with ampicillin (2 g) continuous infusion (70 mg/liter) using freshly made ampicillin supplied daily, ertapenem (1 g) (C_{max} = 155 mg/liter; $t_{1/2}$ = 4 h) q24h, or ceftaroline at 600 mg q12h (C_{max} = 20.448 mg/liter; $t_{1/2}$ = 2.66 h) against HOU503 over 336 h. All the models were performed in duplicate to ensure reproducibility.

Pharmacokinetic analysis. Sampling for pharmacokinetic concentrations was performed by removing 1 ml of medium from each of the two SEV models at 0, 4, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, and 336 h in duplicate, as previously described (30). DAP PK evaluations were confirmed by a validated high-performance liquid chromatography assay that conformed to the guidelines set forth by

the College of American Pathologists (47). A bioassay method was applied for assessing concentrations of ceftaroline, ertapenem, and ampicillin against *Kocuria rhizophila* (formerly known as *Micrococcus luteus*) ATCC 9341. Disks containing standard concentrations, as well as unknown samples from different time points, were placed on an agar plate containing a 0.5-McFarland standard suspension of the test organism. Each sample was examined in duplicate. The antibiotic peak, AUC_{0-24}, and half-life were determined by the trapezoidal methods utilizing standard pharmacokinetic modeling software (PK Analyst version 1.1; MicroMath Scientific Software, Salt Lake City, UT).

Pharmacodynamic analysis. Two SEVs (clots) were removed from each of the two models (a total of 4 samples) at 0, 4, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264 and 336 h in duplicate. The SEVs were homogenized (SEV clots were transferred to 1-ml microcentrifuge tubes and vortexed for 1 h in trypsin), diluted in cold saline, and plated onto BHIA plates, and the colony counts were determined after 24 h of incubation at 35°C. For all samples, antimicrobial carryover was accounted for by serial dilution of the plated samples. If the anticipated dilution was at or near the MIC, the samples were processed by vacuum filtration and washed through a 0.45-μm filter (Pall Corp., Ann Arbor, MI) with normal saline to remove any DAP. The limit of detection for these samples was 1 $\log_{10}$ CFU/g. The results were plotted ($\log_{10}$ CFU per gram versus time [hours]) to determine antibacterial activity. A total reduction of $\geq$3 $\log_{10}$ CFU/ml was considered bactericidal, and $<$3-$\log_{10}$ CFU/g reduction from the initial inoculum was considered bacteriostatic. Enhancement of DAP by combination with β-lactams was defined as an increase in kill of $\geq$2 $\log_{10}$ CFU/g versus DAP alone at 24, 48, 72, and 96 h with no significant ($\geq$2 $\log_{10}$ CFU/g) regrowth for the remainder of the experiment. Improvement was defined as a 1 to 2 $\log_{10}$ CFU/g increase in DAP activity compared to daptomycin alone, as defined above (11, 48, 49).

Evaluation of emergence of DAP resistance. DAP resistance at each sampling point of the 14-day model was evaluated by plating model samples over 48 h on BHIA drug plates containing 3$\times$ the DAP MIC. Any colonies detected on the drug plates were tested for changes in the MIC using microbroth dilution MIC testing according to CLSI guidelines (36).

Humanized rat model of endocarditis. Aortic valve endocarditis was produced in anesthetized male Sprague-Dawley rats by placing a catheter into the right carotid artery and across the aortic valve into the left ventricle, which was heat sealed, ligated, and left for the duration of the experiments (50). After 24 h of catheter placement, various bacterial inocula were used to determine the infective dose (ID_{90} to ID_{100}). Jugular vein catheterization and placement was performed by inserting a catheter via the jugular vein in the superior vena cava, which was tunneled subcutaneously and brought to the skin of the interscapular region of the rat, where it was connected to a programmable infusion pump. The animals were inoculated with $>$10 times the ID_{90} via the carotid artery after 15 min of catheter placement. Therapy was initiated 24 h postinoculation. The baseline (T_0) numbers of CFU of bacteria in vegetations at the time of therapy initiation were determined by sacrificing 2 or 3 animals in each experiment and plating serial dilutions of homogenized aortic valves containing vegetations. DAP was delivered by a programmable infusion pump intravenously via the jugular vein at a dose of 45.3 mg/kg/day, for a total of 3 doses, as described previously (32). The animals were sacrificed $\sim$16 h after the last antibiotic dose, and numbers of CFU per gram of vegetation were determined as described above; the numbers of recovered CFU were compared with each other and with T_0 controls. The unpaired *t* test was used for statistics, using Prism 4 for Windows (GraphPad). The minimum detection limit was 10^1 CFU/g of tissue. The ID_{90}s were determined by the Reed and Muench method (51, 52). The rat endocarditis model and surgical procedures were performed in accordance with the institutional policies and the guidelines stipulated by the animal welfare committees, University of Texas Health Science Center at Houston (AWC, UTHSC), and Wayne State University, Detroit, MI . This study was reviewed and approved by the University Institutional Review Boards (AWC approval HSC-AWC-17-0008, WSU-AWC-17_11_0398).

Whole-genome sequencing and SNP analyses. DAP-resistant derivatives of HOU503 and HOU668 (34, 42) were subjected to whole-genome sequencing using an Illumina platform as described previously (5). Genomic-DNA libraries were prepared using a NexteraXT DNA sample preparation kit. Sequencing was carried out on a MiSeq desktop sequencer with 150-bp paired-end reads. Reads from each sequenced isolate were mapped against HOU503 and HOU668 parental genomes with BWA (53) for SNP analyses. Variant calling was performed with GATK (54), SAMtools (55), and a low-frequency-variant detector from CLC Genomics Workbench 8.5. Variants detected by three callers were selected and annotated with SnpEff (56) and then compared among the different groups of isolates.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00315-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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