Pharmacodynamic effect of clinical vancomycin exposures on cell wall thickness in heterogeneous vancomycin-intermediate Staphylococcus aureus

Warren E. Rose*, Ryan M. Knier and Paul R. Hutson

Pharmacy Practice Division, School of Pharmacy, University of Wisconsin, Madison, WI 53705, USA

*Corresponding author. Tel: +1-608-890-1917; Fax: +1-608-265-5421; E-mail: werose@pharmacy.wisc.edu

Received 11 April 2010; returned 23 June 2010; revised 9 July 2010; accepted 12 July 2010

Objectives: Heterogeneous vancomycin-intermediate Staphylococcus aureus (hVISA) have a higher predisposition to select for VISA with thickened cell walls upon vancomycin exposure, but the pharmacodynamic relationship of this occurrence with clinical doses is unknown. This study investigates the impact of clinical vancomycin dose simulations on cell wall thickness (CWT) and the emergence of resistance in hVISA in an in vitro pharmacodynamic model.

Methods: In an in vitro pharmacokinetic/pharmacodynamic model, we simulated 125–2000 mg of vancomycin every 12 h (fAUC/MIC 24–225) over a 72 h period against three clinical hVISA and two standard control S. aureus strains. Pharmacodynamic activity, susceptibility and resistance populations were assessed, and CWT was determined at the end of the exposure.

Results: Bactericidal activity occurred in hVISA only with vancomycin fAUC/MIC ≥164 exposures, but regrowth occurred after 24 h, regardless of initial activity. Following vancomycin exposure, CWT correlated with MIC increases (r=0.66; P<0.0001). A significant increase in CWT occurred in hVISA with any vancomycin simulation, including the high-dose fAUC/MIC 225 regimen (24.4% increase in hVISA versus 3.3% with control; P<0.001). Any vancomycin exposure in two of the three hVISA strains resulted in isolates with MICs ≥3 mg/L and as high as 8 mg/L, which corresponded with a more resistant VISA population profile.

Conclusions: High-dose vancomycin exposures in hVISA cannot prevent cell wall thickening, but prudent therapeutic strategies including treatment doses ≥1500 mg every 12 h (AUC/MIC ≥364) in conjunction with avoidance of long-term vancomycin exposure may avert further reduced susceptibility.

Keywords: glycopeptides, hVISA, pharmacokinetics, resistance

Introduction

The emergence of heterogeneous vancomycin-intermediate Staphylococcus aureus (hVISA) has become an increasing concern in the clinical setting. These strains are reported as having a vancomycin MIC ≤2 mg/L, but they contain subpopulations that are able to survive at concentrations above this susceptibility breakpoint. Multiple studies now suggest that the incidence of infections due to isolates with this susceptibility pattern is increasing.1–3 Patients with hVISA bacteraemia often receive insufficient vancomycin exposures, resulting in persistent, deep-seated infections that increase the risk of secondary complications and require prolonged durations of therapy.1,4 Further complicating treatment, hVISA strains have an increased probability for the emergence of resistance to adjunctive therapy, such as rifampicin.5

Exposure of hVISA isolates to subinhibitory concentrations of vancomycin (≤1 mg/L) in the laboratory has readily selected for vancomycin-intermediate S. aureus (VISA) with increased MICs in the range of 4–8 mg/L.7 Various genetic pathways are involved in the development of VISA, but these strains ultimately display thickened cell walls that inhibit the penetration of vancomycin to its active site in the cytoplasmic membrane as well as reduced peptidoglycan cross-linking, autolytic activity and acetate metabolism and longer doubling times.7–12 Clinical pharmacodynamic simulations with vancomycin in vitro and in vivo have previously demonstrated that hVISA isolates are difficult to kill even with supertherapeutic exposures, but there remains little understanding of the clinical relationship of vancomycin dosing and the primary cause of reduced susceptibility, cell wall thickening.13 To our knowledge, this study is the first to use an in vitro pharmacodynamic model approach to determine...
the clinical impact of simulated vancomycin exposures on both cell wall thickening and the emergence of resistance in hVISA.

Materials and methods

The isolates used in this study included Mu3, the first clinically identified hVISA from Japan, hVISA 21773 and hVISA 19936 (both provided by Alasdair P. MacGowan, Bristol, UK), and two vancomycin-susceptible strains (ATCC 25923 and ATCC 29213). All cultures were stored at −80°C and plated onto fresh medium prior to each experiment.

A previously described in vitro, one compartment pharmacokinetic/pharmacodynamic (PK/PD) model was used for simulated vancomycin exposures set to simulate the normal 6–10 h half-life of vancomycin. All experiments were performed in duplicate.

The pharmacodynamic effect of vancomycin was simulated using free drug concentrations in the model to account for the previously described 55% protein binding of this antibiotic. The following vancomycin regimens were administered and simulated over the full 72 h duration: 2000 mg every 12 h; 1500 mg every 12 h; 1000 mg every 12 h; 500 mg every 12 h; 250 mg every 12 h; and 125 mg every 12 h. Pharmacodynamic activity and results with the regimens and organisms were compared by fAUC/MIC exposure.

The initial vancomycin MIC was determined for all isolates by Etest methodology (AB bioMérieux, Solna, Sweden) by swabbing a 0.5 McFarland turbidity standard of the isolate from freezer storage (−80°C) onto Mueller–Hinton agar plates. All isolates evaluated in the PK/PD model were screened for changes in susceptibility throughout vancomycin exposure. At 0, 24, 48 and 72 h, bacterial cultures were collected from the model and spread onto brain heart infusion (BHI) agar containing vancomycin at 3- and 6-fold each organism’s MIC to screen for resistant mutants. The susceptibility changes were confirmed by Etest of (i) subsequent bacterial growth on vancomycin-screening plates or, if there was no growth on screening plates, then (ii) from samples taken directly from the model.

All isolates prior to vancomycin exposure were evaluated by population analysis profile (PAP). Following exposure in the PK/PD model, the isolates recovered as described above were evaluated by PAP determination and compared. A high organism inoculum of ~10^8–10^9 cfu/ml was standardized with a 2.0 McFarland turbidity standard and the dilutions were spot plated onto freshly prepared BHI agar containing 0–16 mg/L vancomycin. Plates were evaluated and colonies enumerated after 48 h of incubation at 37°C. The resulting cfu/mL inoculum at each vancomycin concentration tested was plotted for each strain. The AUC of the organism population was determined by the trapezoidal method with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) with a limit of detection of 10^2 cfu/mL. The test strains were evaluated in parallel to the original Mu3 isolate and a ratio was calculated by dividing the AUC of the test strain by the AUC of Mu3. The effect of vancomycin exposure was evaluated by the change in the population susceptibility as determined by the PAP:AUC ratio. The resulting ratio was also classified according to the following previously defined criteria: non-hVISA, <0.9; hVISA, ≥0.9 to <1.3; and VISA, ≥1.3.15,16

Cell wall thickness (CWT) was determined at the end of all 72 h simulations by transmission electron microscopy (TEM). Samples were collected for imaging from (i) subsequent bacterial growth on vancomycin-screening plates or, if there was no growth on screening plates, then (ii) from samples taken directly from the model. An organism pellet was immersion fixed overnight at 4°C in a solution of 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M sodium phosphate buffer at a pH of 7.4. The samples were post-fixed in 1% osmium tetroxide in this buffer for 2 h at 22°C. The samples were then dehydrated in a graded ethanol series, further dehydrated in propylene oxide and embedded in Spurr’s epoxy resin. Sectioning for TEM was performed using a Reichert–Jung Ultracut-E Ultramicrotome, and contrasted with Reynolds lead citrate and 8% uranyl acetate in 50% ethanol. Ultrathin sections were observed with a Philips CM120 electron microscope and images were captured with a MegaView III side-mounted digital camera. The resulting images were analysed and measured using ImageJ 1.39t software. The CWT was measured on a minimum of 25 cells per model using four separate quadrants of each cell.

Vancomycin pharmacokinetics in the PK/PD model were analysed using previously described HPLC methodology.17 The standard curve was linear over the vancomycin concentration range of 0.5–50 mg/L (r²=0.996). The fCmax, fCmin, half-life and fAUC values for each regimen were calculated from the derived values using the WinNonlin PK/PD modelling software program (Pharsight, Cary, NC, USA).

Antibacterial activity was compared among the strains for changes in cfu/mL at 24, 48 and 72 h, as well as time to 99.9% kill (T99.9) by two-way analysis of variance with Tukey’s post hoc test. ANOVA was also used to compare CWT changes among the strains and vancomycin regimens. Pearson correlation determined the linear relationship of susceptibility to CWT and PAP:AUC. A P value of ≤0.05 was considered significant. All statistical analyses were conducted using SPSS Statistical Software (Release 17.0, SPSS, Inc., Chicago, IL, USA).

Results

Pre-exposure MICs of vancomycin by Etest were 2 mg/L and were consistent with previous reports with these strains. The pharmacokinetic results and associated parameters of vancomycin achieved in the model were within the targeted range and represent appropriate free drug exposures for the simulated doses: 2000 mg every 12 h (fCmax 36.1 mg/L, fCmin 10.2 mg/L, t½ 8.9 h, fAUC/MIC 225); 1500 mg every 12 h (fCmax 25.1, fCmin 6.7, t½ 6.4, fAUC/MIC 164); 1000 mg every 12 h (fCmax 17.6, fCmin 4.9, t½ 6.8, fAUC/MIC 108); 500 mg every 12 h (fCmax 9.2, fCmin 3.3, t½ 8.3, fAUC/MIC 60); 250 mg every 12 h (fCmax 4.5, fCmin 2.2, t½ 11.6, fAUC/MIC 38); and 125 mg every 12 h (fCmax 2.5, fCmin 1.1, t½ 10.8, fAUC/MIC 24). Intermode variation for each duplicate regimen was within 10%.

The activity of vancomycin was similar for the three hVISA isolates displayed in Figure 1, but overall it was most active against hVISA 19936. The fAUC/MIC 225 regimen in all hVISA achieved bactericidal activity (99.9% kill), and was most rapid against hVISA 19936 at 11.0 h compared with at 18.5 and 31.5 h against Mu3 and 21773, respectively. A similar result emerged with fAUC/MIC 164 (data not shown). Bactericidal activity was not achieved for any hVISA with exposures ≤fAUC/MIC 108. Regardless of initial response, bacterial regrowth was a common occurrence in hVISA after 24 h of vancomycin exposure. The mean regrowth of all regimens in isolates 21773 and Mu3 was greater than that of 19936 (3.18 versus 2.26 log 10 cfu/mL, p = 0.001). Initial vancomycin activity was similar in the non-hVISA control strains, but resulted in reduced regrowth beyond 24 h of exposure in comparative regimens.

The simulated vancomycin regimens resulted in unique changes in CWT with each organism, but overall trends emerged. Figure 2 displays the CWT correlation with MIC in hVISA at the end of each vancomycin regimen. Overall, the CWT correlated with the MIC following vancomycin exposure (r=0.66; P<0.0001). For all hVISA isolates, an increase in CWT occurred with any vancomycin exposure, including the high-dose
AUC/MIC 225) regimen, which resulted in a mean 24.4% increase (range 18%–33%) in CWT with hVISA versus a 3.3% increase with control strains (P<0.001). In Mu3, the largest increase in CWT (78% above baseline) occurred with the AUC/MIC 164 regimen, while the lowest exposure (AUC/MIC 24) resulted in a 33% increase in CWT. A similar effect transpired with hVISA 19936, albeit its increase in CWT was not as prominent (data not shown). The largest increases in CWT in hVISA 21773, 60% and 77% above baseline, occurred with the AUC/MIC 60 and 38 simulations, respectively, while the lowest exposure (AUC/MIC 24) resulted in only a 29% increase. The CWT in the non-hVISA control strains did not significantly increase above baseline measurements in any of the vancomycin dosing regimens.

Vancomycin exposure in two of the three hVISA strains resulted in the detection of isolates with MICs ≥3 mg/L (Figure 2). In both Mu3 and 21773, MIC increases occurred with the highest simulated regimen (AUC/MIC 225) and continued to increase as the vancomycin exposures were reduced. Exposure of Mu3 to vancomycin in the range of AUC/MIC 60–225 resulted in an MIC of 3 mg/L. However, at lower vancomycin exposures the MIC for Mu3 increased to 8 mg/L, which corresponded to a considerable organism inoculum (>10^8 cfu/mL) at 72 h. For isolate 21773, vancomycin exposures in the range of AUC/MIC 108–225 resulted in a 3-fold MIC increase up to 6 mg/L. Lower vancomycin exposures produced mutant strains with MIC increases to 8 mg/L. No susceptibility changes were detected with hVISA 19936 or the non-hVISA control strains.

The hVISA phenotype was confirmed prior to vancomycin exposure in strains 21773 and 19936 with a PAP ratio to Mu3 of 1.11 and 0.96, respectively. In those regimens resulting in MIC increases, a notably more resistant population profile emerged (Figure 3). The AUC ratios and MICs correlated very well and confirmed the change in susceptibility (r=0.9; P=0.02). All vancomycin simulations in hVISA 21773 resulted in isolates with a PAP:AUC within a range of 1.70–2.04, which is consistent with the VISA phenotype. While there were minor increases in the AUC ratios of Mu3 following AUC/MIC 60–225 exposures, they were still within the range for hVISA classification. Lower exposures resulted in a population shift to VISA.

Figure 1. Pharmacodynamic activity of vancomycin in the PK/PD model over 72 h against three hVISA strains [(a) Mu3, (b) 21773 and (c) 19936] and one non-hVISA control [(d) ATCC 25923].
No significant change in PAP:AUC appeared with either hVISA 19936 or the control strains.

Discussion

In this present study, we describe the effect of vancomycin pharmacodynamic exposures on CWT changes and the emergence of reduced susceptibility in hVISA. The results of our pharmacodynamic analysis are consistent with previous reports of poor vancomycin activity with standard dosing. At vancomycin exposures equivalent to the often recommended regimen of 1 g every 12 h, bactericidal activity was not achieved in any of the hVISA strains in this study. At doses exceeding this range, vancomycin was initially slowly bactericidal, but was followed by considerable regrowth despite continued exposure. Our results confirm that even high-dose therapy with vancomycin is minimally effective against hVISA in vitro.

The thickened cell wall leading to reduced vancomycin activity represents a novel mechanism of antibiotic resistance in staphylococci. This mechanism has been reported in clinical VISA isolates recovered from patients who failed vancomycin therapy. However, initial MICs and CWT in hVISA can be highly variable and are often similar or only slightly greater than those of typical methicillin-resistant S. aureus (MRSA) strains. It is interesting to note in our study the variability in cell wall thickening changes among the hVISA strains. Vancomycin exposures ≥ fAUC/MIC 108 resulted in larger CWT increases in Mu3, a strain with a dysfunctional accessory gene regulator (agr), than in the functional agr strain 21773. A dysfunctional agr locus has correlated with reduced vancomycin activity and our observations indicate that it may be an important variable in cell wall thickening.

Our results demonstrate a significant correlation between CWT and MIC throughout the studied dosing ranges of vancomycin. Importantly, even high exposures of vancomycin ( fAUC/MIC
225; equivalent to 2 g every 12 h) produced significant increases in CWT in hVISA, regardless of the emergence of substantial MIC increases. Due to the effect of increased CWT on decreasing vancomycin activity, it is reasonable to expect that continued exposure beyond 72 h with high-dose vancomycin in our model would have resulted in strains with reduced vancomycin susceptibility. These findings have important therapeutic implications in the treatment of hVISA, since most serious infections require prolonged durations of vancomycin, even with high doses.

The acquisition of antibiotic resistance often comes at a considerable cost to bacterial fitness. In the case of VISA, increased CWT and reduced vancomycin susceptibility significantly correlate with prolonged replication times. Upon serial passage on vancomycin-free medium, VISA strains will revert back to hVISA or standard MRSA. Subinhibitory exposures of vancomycin can readily select for isolates with MICs in the VISA range of 4–8 mg/L, which have a high correlation with increased CWT. In the clinical setting, these isolates are mostly identified in unique cases of patients undergoing prolonged therapy with vancomycin and have rarely been transmitted to other patients or healthcare workers. Although our study found a significant correlation between CWT and MIC, we were intrigued to find this correlation was lower than previously reported and that the lowest vancomycin exposure did not result in the greatest increase in thickness. Rather, CWT was greatest either prior to or at the first expression of vancomycin resistance during the clinical dosing ranges of 250–1 500 mg every 12 h (AUC/MIC 38–164). We attribute this finding to the significant fitness cost that is required to maintain this resistance. We evaluated this in subsequent replication rate experiments with Mu3 and found that the mean doubling time was 58 min in mutants from our model with MICs of 8 mg/L compared with 34 min in post-vancomycin exposure isolates with MICs of 2–4 mg/L. Therefore, once reduced vancomycin susceptibility is acquired through this mechanism, VISA may regulate CWT to maintain intermediate vancomycin resistance without further compromising critical cellular function previously identified in strains with a higher level of resistance (MIC > 8 mg/L). Our study was limited in exploring this new finding in that we only evaluated CWT at two timepoints prior to and then after 72 h of vancomycin exposure. Further research is necessary to determine the relationship of these changes between these timepoints and with prolonged therapy.

In conclusion, this study provides the first clinical insight into the effect of simulated vancomycin pharmacodynamic exposures on cell wall thickening in hVISA. We demonstrated that hVISA has a higher propensity for cell wall thickening and resistance compared with standard MRSA, even with subtherapeutic dosing. The emergence of VISA from hVISA in our model with standard vancomycin dosing is concerning, since these strains may also be less susceptible to other treatment options for invasive S. aureus infections, further limiting effective therapies. The optimal vancomycin treatment regimen for hVISA has not been determined and its effectiveness in treating hVISA infections remains controversial. However, important considerations in minimizing cellular changes leading to the emergence of VISA revealed by this study are high vancomycin doses of >1500 mg every 12 h (AUC/MIC >164; total AUC/MIC >364), in conjunction with minimizing the duration of vancomycin exposure. Prudent monitoring of vancomycin-related adverse events is required when recommending these doses due to recent evidence of increased risk of nephrotoxicity when exceeding trough concentrations of 20 mg/L or cumulative doses of 4000 mg daily.

Acknowledgements
This study was presented in part at the Forty-ninth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 2009 (Abstract A1–1278). We would like to thank Dr William Craig (University of Wisconsin School of Medicine) for his study guidance and editorial review, Ben August (School of Medicine, Electron Microscope Facility) for his technical assistance, and Alastair MacGowan for supplying two of the hVISA strains.

Funding
This work was supported with internal funds through the Wisconsin Pharmacy Practice Research Initiative.

Transparency declarations
W. E. R. has received grant support and speaker honoraria from Cubist and has received grant support and served as a consultant for Astellas. All other authors have nothing to declare.

References


