in vivo *22*: 667-672 (2008)

Development and Validation of a Multi-dose Neutropenic Rat Thigh Infection Model Using Real-time Monitoring of *Staphylococcus aureus* Growth *In Vivo*

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Abstract. *Background: Several animal models have been described using luminescent bacteria for non-invasive, real-time monitoring of infection in mice. In this study, a multi-dose rat thigh infection model with luminescent Staphylococcus aureus was developed for the evaluation of antibiotic efficacy. Materials and Methods: Bioluminescent imaging and bacterial loads of S. aureus infected rat thighs with or without vancomycin treatment at different time-points post-infection were compared. Results: Correlation between luminescence and bacterial load was observed based on the dose- and time-dependent activity of vancomycin in the model. Conclusion: While luminescence detection offered the advantage of monitoring an infection in live animals, limitations to this method included reduced sensitivity and a narrow dynamic range, as compared to a traditional tissue culturing method. Real-time luminescence monitoring of infection may be most appropriate for experiments where rapid in vivo assessment of compound efficacy is desired and absolute quantitation of colony forming units in infected tissue is not required.*

Animal infection models play a pivotal role in antimicrobial drug discovery and development by extending the *in vitro* cell-based potency of antibacterial agents to an *in vivo* setting, facilitating the prediction of antimicrobial activity in humans through the use of pharmacokinetic and pharmacodynamic (PK/PD) data. A wide variety of factors including, but not limited to dosing, stability, metabolism, excretion, tissue penetration, protein binding, and immune status cannot be completely simulated by *in vitro* systems, emphasizing the importance of animal infection studies during preclinical drug evaluation. In addition, the *in vivo* susceptibility of many bacteria to antibiotics can be markedly different from *in vitro* susceptibility due to the expression of a variety of virulence factors and animal models can better simulate the clinical relevance of these virulence processes. Many organisms exhibit decreased susceptibility to antibiotics during infection, or persist, as a result of subcellular compartmentalization (1), up-regulated efflux (2, 3), phenotypic variation (4) or biofilm formation within host tissues and foreign implants (5, 6). Thus, PK/PD information gained from animal infection studies has been shown to significantly reduce the risk in advancing antibacterial drug candidates into clinical trials (7, 8).

Recombinant luminescent bacterial pathogens have been utilized to monitor infections in live animals (9-11). As a result, the progression of infection in real-time can be monitored using sensitive imaging technology without the need to sacrifice animals. This technology has been used in a limited variety of infection models, the majority being mouse models (9-11) and only few real-time bacterial infection models have been described in rats (12). Because animal species differ in their intrinsic susceptibility to infection, disease pathology and metabolism, multiple animal infection systems are required to fully evaluate antibiotic efficacy *in vivo*. To further extend luminescent technology in the rat, a novel infection model using a luminescent strain of *Staphylococcus aureus* was developed to infect the soft tissue (thigh muscle) of neutropenic rats. Here, the experimental protocol is described, the validation of this model with multiple doses of vancomycin is presented, and the correlation and dynamic range of luminescence is compared with colony-forming units (CFUs).

Materials and Methods

Experimental animals. All animal experiment protocols were approved by the Institutional Animal Care and Usage Committee of Vertex Pharmaceuticals, Incorporated, Cambridge, MA, USA. Sprague-Dawley rats (100-130 g, Charles River Laboratories, Inc.

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Key Words: Rat thigh infection, *Staphylococcus aureus*, bioluminescence.

Wilmington, MA, USA) were rendered neutropenic by a single intraperitoneal injection of cyclophosphamide (150 mg/kg, Sigma-Aldrich, St Louis, MO, USA) three days before infection. A single dose of cyclophosphamide was comparable to the conventional use of two doses (13) in reducing total white blood cells (WBC) by >90% (data not shown) without any other adverse effects; the reduction in WBC counts was maintained for at least six days post-treatment, with no effect on red blood cell counts and body weight (data not shown).

Bacterial strains and inoculum preparation. Bacterial inocula were prepared by culturing luminescent *S. aureus* Xen29 (14) (Xenogen Corporation, Alameda, CA, USA) from frozen stocks of logarithmically grown cells, in fresh cation-adjusted Mueller Hinton Broth (MHB, Fisher Scientific, Pittsburgh, PA, USA) medium, for ~4 hours at 37˚C with aeration. The bacteria were washed twice in sterile phosphate-buffered saline, followed by resuspension in sterile saline to a final concentration of ~2.5×10^7 CFU/ml. An inoculum of ~5.0×10^6 CFU per thigh, delivered in 200 μl of sterile saline to each thigh, was experimentally determined to be optimal for initiating reproducible infections. Two controls for inoculum titer were performed for each experiment: (i) an aliquot of the inoculum was tested to confirm the CFU titer and (ii) a group of animals (n=3 to 4) were sacrificed at 1 hour post infection to confirm the initial titer of CFUs in the thigh.

Antibiotic treatment and quantitation of infection. Vancomycin (vancomycin hydrochloride; Hospira, Lake Forest, IL, USA) was administered subcutaneously (*s.c.*) at a dose of 100 mg/kg in a volume of 5 ml/kg. This dose had previously been shown to produce a maximal effect in a rat endocarditis model (15) and in our own results. The *in vitro* vancomycin minimal inhibitory concentration for *S. aureus* Xen29 was determined to be 1 μg/ml. The *s.c.* treatment was initiated 2 or 16 hours post-infection followed by twice-daily dosing (*bid*), as indicated in Figure 1A. At approximately 1, 20, 44 and 68 hours after infection, the animals were anesthetized with 2% isofluorane and bioluminescent images generated by the Xen29 infection in the dorsal side of the infected thighs of the live animals were recorded for five minutes using the IVIS imaging system (Xenogen Corporation). The total photon emission (relative light units, RLUs) from the defined regions of interest was quantified using LivingImage Software (Xenogen Corporation).

Parallel subgroups of animals at each time point were sacrificed for thigh CFU determinations. Total thigh muscle was removed and homogenized in 5 ml of sterile phosphate-buffered saline using a Powergen 35 homogenizer (Fisher Scientific). The homogenized tissue was serially diluted, plated on MHB agar plates and counted after 24 hours of incubation. The log reduction in CFU in thighs and the corresponding changes in photon emission were analyzed by analysis of variance (ANOVA) to detect the overall effect of vancomycin treatment, followed by Dunnett's pair-wise comparison between no-treatment controls and the treated group, and a least squares comparison method between treatment doses. The CFU value and photon emission value for each animal were calculated as a mean of the results from both thighs.

Results

As shown in Figures 1B and 1C, the cultured CFU values roughly correlated with the RLU values observed in all the groups. However, the CFU determination method appeared to be more sensitive than the RLU determination method, with a lower limit of detection of 10^2 CFU *versus* ~10^4 CFU, respectively (data not shown). In the untreated animals, a time-dependent increase in the RLU values correlated with an increase in the CFU values. When the animals were treated with 2 doses of vancomycin starting 2 hours post-infection, an ~2 log reduction in CFUs was observed 20 hours post-infection compared to the 1 hour time control. An additional 0.9 log reduction was observed at 44 hours (after 4 vancomycin doses), and the resulting 2.9 log reduction was maintained up to 68 hours (after 5 vancomycin doses). In contrast, in all the corresponding RLU determinations, a less than 1 log reduction was observed relative to the one hour control, indicating that the dynamic range for the detection of bacterial growth using luminescence was much smaller and less sensitive than the CFU method. However, regardless of the narrow dynamic range for luminescence detection, statistically significant bactericidal activity was observed, indicating that luminescence detection could *qualitatively* detect compound antibacterial activity. A treatment initiation time of 16 hours post-infection was determined to be too late in the infection to observe efficacy, since none of the animals showed a reduction in CFUs or RLUs, compared to the 1 hour control, following treatment up to 68 hours (Figure 1B and 1C).

To further evaluate the dose-dependent correlation of CFU values with RLU values in the infected animals, an experiment was conducted comparing different *s.c.* doses of vancomycin administered *bid*, with the treatment initiated 2 hours post-inoculation (Figure 2). The animals were imaged and the sub-groups were sacrificed for CFU determinations at 1, 20 (2 doses of vancomycin) and 68 hours (5 doses of vancomycin) post-infection, with the first dose initiated at 2 hours post-infection. A dose of 10 mg/kg *bid* was determined to be the static dose at 68 hours, as there was no reduction in the CFU levels in the thighs relative to the inoculum (Figure 2A). Doses of 30 and 100 mg/kg were bactericidal, producing 1.8 and 2.8 log reductions at 68 hours, respectively. An approximate correlation of CFU values with RLU values (Figure 2A and 2B) was observed, but CFU determinations were more sensitive. The captured images correlated well with these findings (Figure 3), showing dose-dependent decrease in thigh-specific luminescence.

Discussion

A bioluminescent multi-dose rat thigh *S. aureus* infection model was developed, extending previously reported luminescent thigh infection models in mice (9, 11) Adaptation of a mouse infection model to the rat is not always feasible since bacterial pathogens are often less virulent in rats as compared to mice. In addition, the greater tissue density in a rat can significantly reduce the

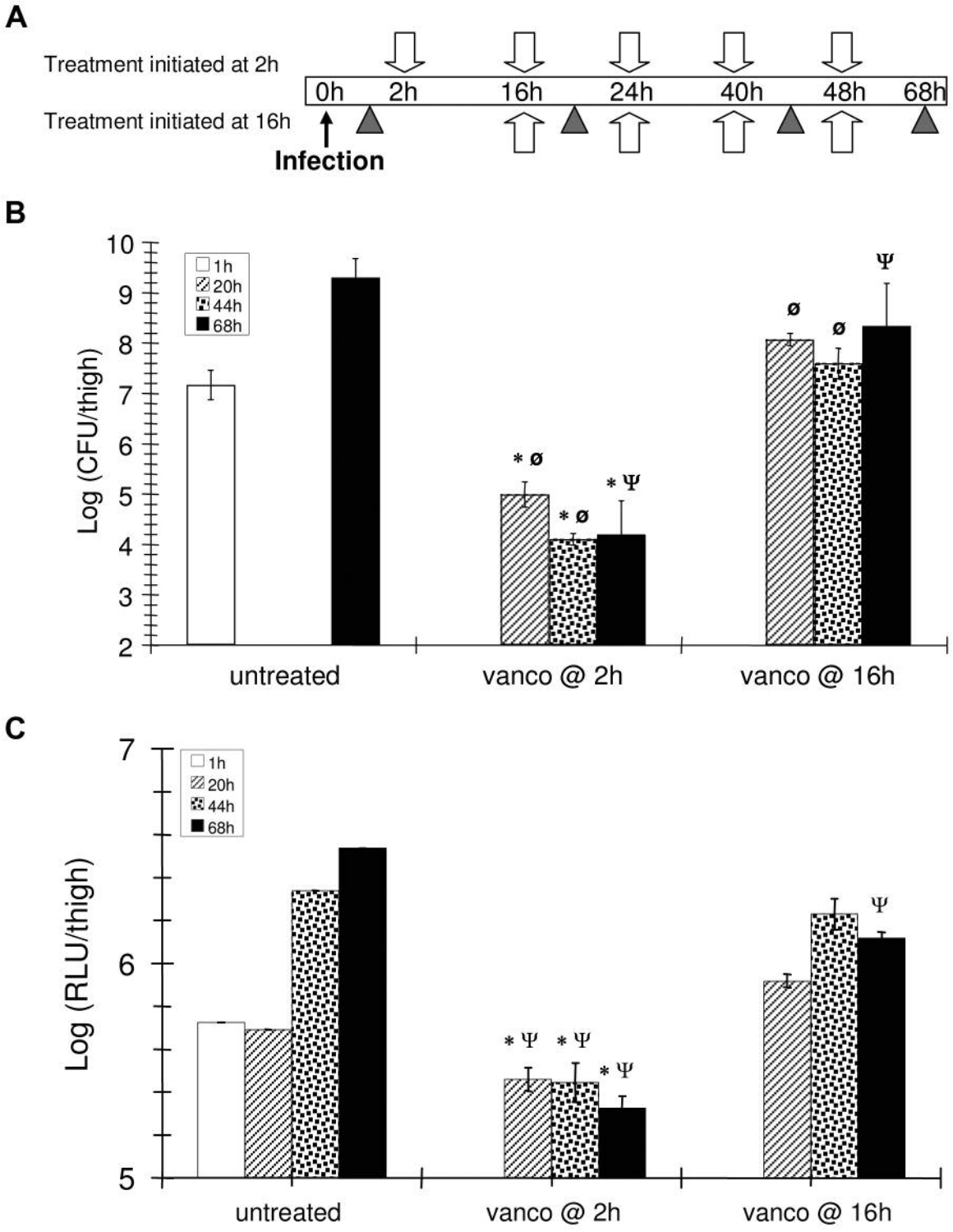

Figure 1. *Optimizing the time of treatment initiation with vancomycin. A) Schematic illustration of the dosing regimen: Rats (n=3-4 per group) were rendered neutropenic and infected in both thighs with S. aureus Xen29 (0 hours). Vancomycin treatment is indicated by open arrows. Thighs were imaged for luminescence and thigh tissue was harvested at 1, 20, 44 and 68 hours post infection (indicated by filled triangles). B) Bacterial load in the thighs of treated and untreated rats. The average log CFU/thigh ± SEM from each treatment group is shown. C) Quantitation of luminescence in infected thighs. The average log RLU/thigh ± SEM is shown. Treatment groups showing statistically significant ($p<0.05$) reductions in comparison to the 1-hour control group (*) and the same-hour control group (Ψ) are indicated. In some instances, the same-hour control data were not available to allow such a comparison (ø).*

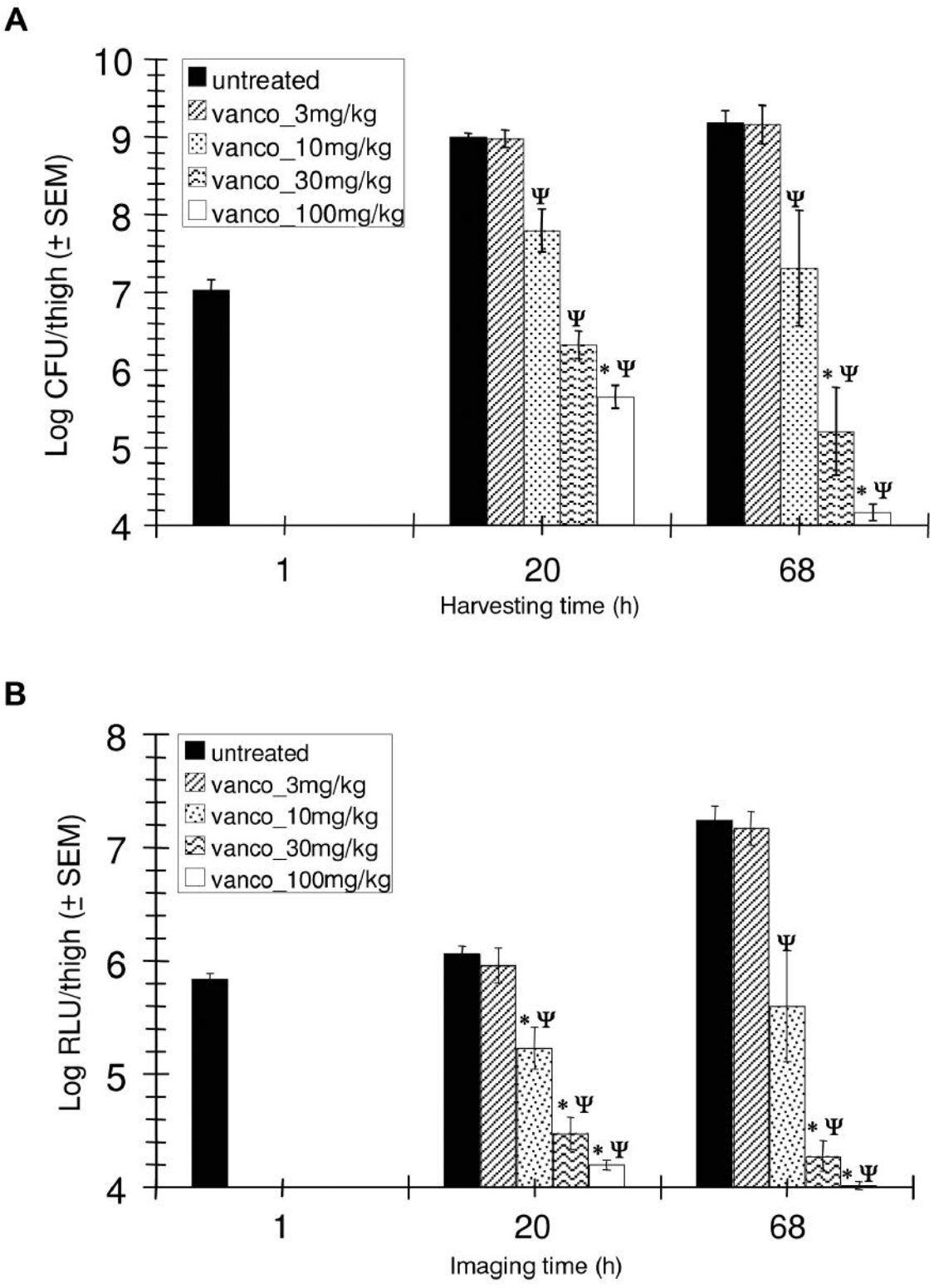

Figure 2. *Dose dependency of CFU and RLU reductions with vancomycin treatment. Rats (n=3-4 per group) were infected with S. aureus Xen29 in both thighs and vancomycin at 0 mg/kg (untreated), 3 mg/kg, 10 mg/kg, 30 mg/kg or 100 mg/kg was administered in 5 doses at 2, 16, 24, 40 and 48 hours post infection. Rat thigh tissue was harvested for CFU enumeration (A) and thighs were imaged (B) at 1, 20 and 68 hours post infection. Treatment groups that showed statistically significant ($p<0.05$) reductions in comparison to the 1-hour control group (*) and the same-hour control group (Ψ) are indicated.*

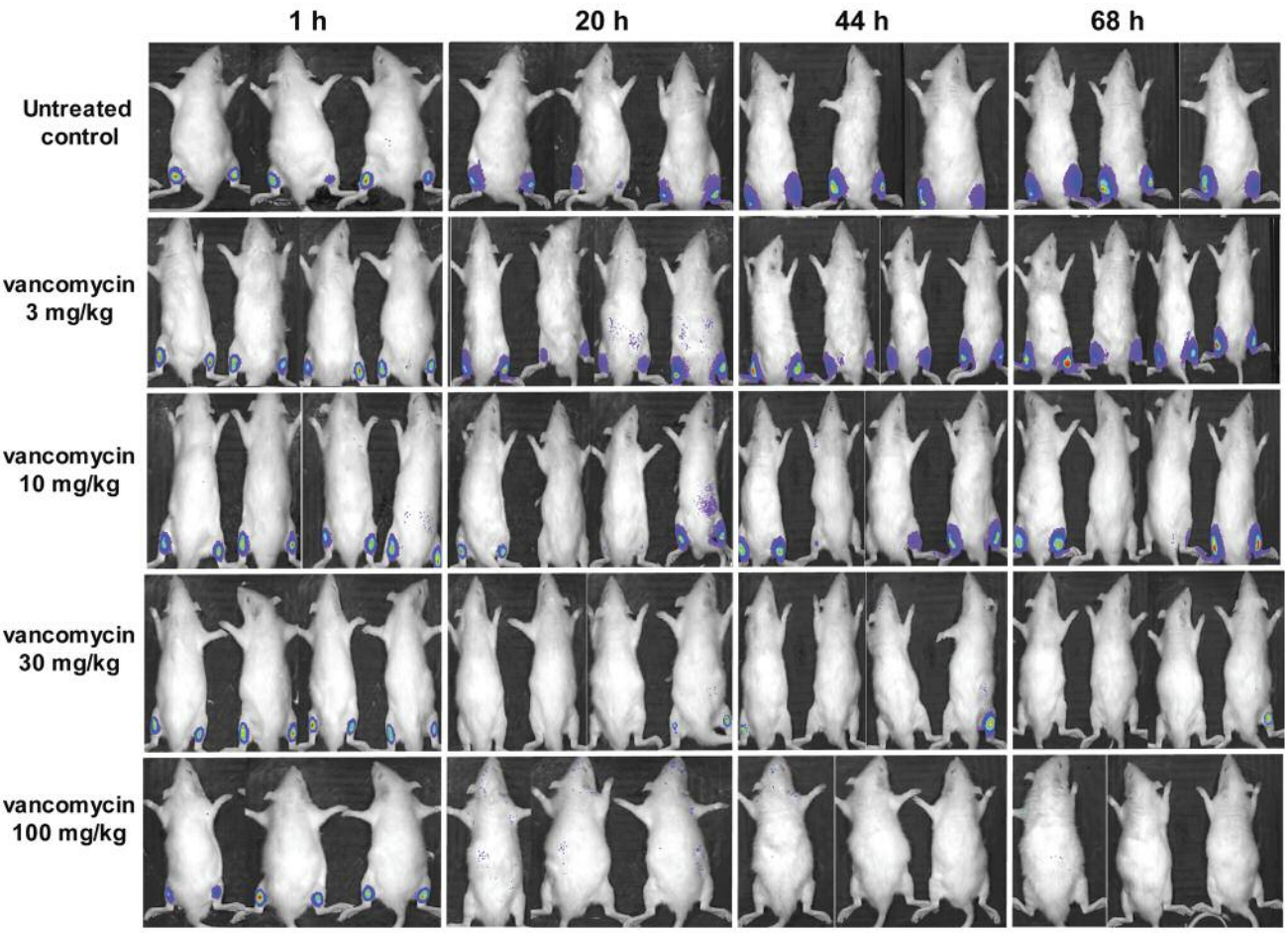

Figure 3. *Real-time monitoring of luminescence in S. aureus Xen29-infected rats treated with and without vancomycin. Bioluminescence generated by S. aureus Xen29 infection was detected in live animals using the IVIS imaging system, at different time-points.*

sensitivity of detection of a luminescence signal. For example, during our attempts to establish a rat lung infection with a luminescent *S. pneumoniae* strain, Xen10 (10), lower luminescence was consistently observed from the left lung in live animals, despite the confirmation of strong luminescence in both lobes of excised lungs (unpublished results. This finding was very different from what was reported for mice using the same strain of bacteria (10). A simple method for producing stable neutropenia in rats with a single dose of cyclophosphamide, enabling the evaluation of a variety of treatment regimens over the course of several days, was also introduced.

A major conclusion from the present findings is that quantitative CFU determination remains the most reliable method for demonstrating the extent of bactericidality of a given antibiotic, similar to a prior report by Kuklin *et al.* (11). It may be possible that the sensitivity of luminescence detection can be improved by developing a more luminescent strain of *S. aureus*. However, despite the lower sensitivity of luminescence detection *versus* CFU determination, a combination of both methods could be useful for screening novel antibacterial compounds for *in vivo* efficacy, real-time monitoring of PK/PD studies, *in vivo* resistance emergence studies and determining the post-antibiotic effects of antibacterial compounds. Having the ability to monitor the spread of infection in live animals during the course of an experiment provides valuable information not normally available using traditional pharmacology methods.

Acknowledgements

We appreciate the support of Mike Briggs, Paul Charifson, Eric Olson and Ann Kwong. We also thank Ann Kwong and Mark Namchuk for their helpful comments on the manuscript, and Julia Sun and Jingbo Cai for their assistance with media preparation. All the experiments were performed at Vertex Pharmaceuticals Incorporated.

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Received June 6, 2008
Revised August 1, 2008
Accepted August 26, 2008