New Evidence and Insights on Dalbavancin and Wound Healing in a Mouse Model of Skin Infection

Oriana Simonetti,a Guendalina Lucarini,b Gianluca Morroni,c Fiorenza Orlando,d Raffaella Lazzarini,b Antonio Zizzi,e Lucia Brescini,b Mauro Provinciali,d Andrea Giacometti,c Annamaria Offidani,a Oscar Cirioni

aClinic of Dermatology, Department of Clinical and Molecular Sciences, Polytechnic University of Marche, Ancona, Italy
bHistology, Department of Clinical and Molecular Sciences, Polytechnic University of Marche, Ancona, Italy
cClinic of Infectious Diseases, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy
dExperimental Animal Models for Aging Unit, Scientific Technological Area, IRRCS INRCA, Ancona, Italy
ePathologic Anatomy and Histopathology, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy

Oriana Simonetti and Guendalina Lucarini contributed equally to this work. Author order was determined in order of seniority.

ABSTRACT Dalbavancin is an effective antibiotic that is widely used to treat skin infection. Our aim was to determine the effect of dalbavancin administration on wound healing compared to that of vancomycin and to elucidate if epidermal growth factor receptor (EGFR), matrix metalloproteinase 1 (MMP-1), MMP-9, and vascular endothelial growth factor (VEGF) could be involved in its therapeutic mechanism. A mouse model of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) skin infection was established. Mice were treated daily with vancomycin (10 mg/kg) and weekly with dalbavancin at day 1 (20 mg/kg) and day 8 (10 mg/kg). After 14 days, wounds were excised, and bacterial counts were performed. Wound healing was assessed by histological and immunohistochemical staining, followed by protein extraction and immunoblotting. Our microbiological results confirmed that both dalbavancin and vancomycin are effective in reducing the bacterial load in wounds. The dalbavancin group showed a strong effect compared with infected untreated animals and the vancomycin-treated group. The wounds treated with dalbavancin showed robust epidermal coverage with reconstitution of the regular and keratinized epidermal lining and well-organized granulation tissue with numerous blood vessels, although slightly less than that in the uninfected group. While in the vancomycin-treated group the epithelium appeared, in general, still hypertrophic, the granulation tissue appeared even less organized. We observed elevated EGFR and VEGF expression in both treated groups, although it was higher in dalbavancin-treated mice. MMP-1 and MMP-9 were decreased in uninfected tissue and in both treated tissues compared with untreated infected wounds. This study showed faster healing with dalbavancin treatment that might be associated with higher EGFR and VEGF levels.

KEYWORDS EGFR, MMP-1, MMP-9, VEGF, dalbavancin, wound healing

A new classification of skin and soft tissue infections has recently been proposed, namely, acute bacterial skin and skin structure infections (ABSSSIs); superficial incisional site infections or SSIs (involving only skin or subcutaneous tissue of the incision) are recognized as a subgroup of ABSSSIs (1). In particular, SSIs constitute a significant burden for the health care system because of the associated morbidity, mortality, length of hospital stay, and higher probability of intensive care unit (ICU) admission with overall direct and indirect costs (2).

The most common pathogen responsible for SSIs is \textit{Staphylococcus aureus}, followed by coagulase-negative staphylococci and \textit{Enterococcus} species. In particular, methicillin-resistant...
resistant *Staphylococcus aureus* (MRSA) is considered a leading cause of human nosocomial infections (3).

Taking into account these considerations, respect of the principles of good antibiotic stewardship is mandatory for fighting antimicrobial resistance and, next to optimization of clinical outcomes, for the prevention of the spread of antibiotic-resistant strains (4). Dalbavancin is a novel antimicrobial agent belonging to the lipoglycopeptide family that is active against *S. aureus* (including MRSA), *Streptococcus pyogenes*, *Streptococcus agalactiae*, the *Streptococcus anginosus* group, *Enterococcus faecalis*, and *Enterococcus faecium*, and it is approved for the treatment of ABSSSIs caused by susceptible Gram-positive organisms (5). Moreover, it has a bactericidal effect and an extremely extended half-life that make it a valid alternative to conventional antibacterials such as vancomycin, the standard of care for treatment of Gram-positive skin and soft tissue infections (6).

Wound healing is a highly complex physiological process involving ordered events in which growth factors are critically important for coordinating cell-cell and cell-matrix interactions during normal injury repair. In particular, epidermal growth factor receptor (EGFR), a 170-kDa transmembrane protein belonging to the ErbB family of receptors, plays key roles in skin regeneration and wound healing by stimulating epidermal keratinocyte (KC) proliferation and migration (7). Among the many proteins essential for the restoration of tissue integrity, matrix metalloproteinases (MMPs) contribute significantly to reorganization of the extracellular matrix for numerous physiological functions, including morphogenesis, tissue remodeling, and wound healing (8, 9). Interestingly, high MMP-1 and MMP-9 activity correlates with poor healing outcomes (8).

Vascular endothelial growth factor (VEGF) has shown to be important in wound healing by promoting the early events in angiogenesis, particularly endothelial cell migration and proliferation (10). It is produced by endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, platelets, neutrophils, and macrophages (11), and, although in normal skin it is negligible, in injured skin it is markedly upregulated (10–12).

This study aimed to explore the therapeutic effects of dalbavancin on wound healing in an infected MRSA mouse model, using histological, immunohistochemical, and Western blot analysis, and to elucidate if EGFR, MMP-1, MMP-9, and VEGF could be involved in its therapeutic mechanisms.

**RESULTS**

**Bacterial counts.** Overall, data analysis showed that inhibition of bacterial growth was achieved in all treated groups. In detail, the mean number of bacteria in untreated group (7.51 ± 0.87 × 10^8 CFU/ml) was significantly higher than those recovered from all treatment groups (Fig. 1). Treatment with vancomycin reduced the mean number of bacteria to 8.04 ± 0.97 × 10^6 CFU/ml, while the dalbavancin group had a strong effect compared with those of the control infected untreated animal and vancomycin-treated groups (8.71 ± 0.92 × 10^5 CFU/ml).

**Histological evaluation.** To investigate the effects of the different treatments on wound healing, we analyzed the area of the wound, particularly reepithelialization, granulation tissue, collagen deposition, and inflammatory cells, according to wound healing scores (13) summarized in Table 1. The statistical analysis is reported in Table 2, and the morphological features are shown in Fig. 2.

The extent of newly formed epithelium and collagen deposition, the increase in the thickness of granulation tissue, and the decrease in the number of inflammatory cells were considered progress in the healing process.

Wound healing was still incomplete in the untreated groups, as observed by the partial regeneration of the epidermis, poor organization of dermis layers, immature granulation tissue (many cells and some fibers), and abundant inflammatory infiltrate. These mice showed the worst degree of healing (*P <* 0.05).

Based on a microscopic examination, the neoeptithelium developed significantly different patterns of regeneration among the treated groups. Interestingly, the reepi-
Epithelialization of the wounds treated with dalbavancin showed robust epidermal coverage, with a reconstitution of the regular and keratinized epidermal lining that was comparable to the reepithelialization of the uninfected mice, except in a small central area where the epithelium appeared to be hypertrophic. The wounds treated with vancomycin showed good reepithelialization, although the epithelium appeared, in general, still hypertrophic compared to those of the dalbavancin-treated and uninfected groups ($P < 0.05$).

The wounds treated with dalbavancin showed well-organized granulation tissue with numerous blood vessels, although it was slightly less organized than that in the uninfected group. In the wounds treated with vancomycin, the granulation tissue appeared even less organized.

The treated wounds presented a distinct pattern of collagen distribution and remodeling similar to that of uninfected skin; in addition, mild or moderate inflammation was observed in the underlying connective tissue.

**Immunohistochemical evaluation.** To investigate the influence of the dalbavancin and vancomycin treatment on the expression of VEGF, EGFR, MMP-9, and MMP-1 and in wound healing, an immunohistochemical evaluation was performed. The immunohistochemical data are summarized in Table 3.

VEGF expression was greater in the uninfected and treated infected groups compared to the untreated group ($P < 0.05$); the wounds treated with dalbavancin showed the highest VEGF values.

EGFR expression was significantly higher in the uninfected group and the infected group treated with dalbavancin compared to that in the other two groups ($P < 0.05$); the wounds treated with dalbavancin showed the highest EGFR expression.

MMP-9 and MMP-1 expression was high in the untreated infected wounds, while a significantly reduced expression was found in the other groups ($P < 0.05$); in particular, the treated wounds showed the lowest values.

**TABLE 1** A five-tiered grading system to evaluate wound healing

<table>
<thead>
<tr>
<th>Score</th>
<th>Reepithelialization</th>
<th>Granulation tissue</th>
<th>Dermis organization</th>
<th>Inflammatory infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Trace and modest keratinocyte migration</td>
<td>Trace</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1</td>
<td>Evident keratinocyte migration</td>
<td>Hypocellular and no vessels</td>
<td>Trace</td>
<td>Modest</td>
</tr>
<tr>
<td>2</td>
<td>Differentiation</td>
<td>Many cells and few vessels</td>
<td>Modest</td>
<td>Good</td>
</tr>
<tr>
<td>3</td>
<td>Hypertrophic and partial stratum corneum</td>
<td>Many fibroblasts, some fibers, and some vessels</td>
<td>Good</td>
<td>Evident</td>
</tr>
<tr>
<td>4</td>
<td>Complete and normal</td>
<td>More fibers and few cells</td>
<td>Evident</td>
<td>Absent</td>
</tr>
</tbody>
</table>
Analysis by Western blotting of the expression of EGFR, VEGF, MMP-1, and MMP-9. The expression of EGFR, VEGF, MMP-1, and MMP-9 was tested by Western blotting followed by densitometric analysis (Fig. 3). All proteins were detectable in uninfected, untreated infected, infected treated with dalbavancin, and infected treated with vancomycin tissue samples. The expression of EGFR and VEGF was significantly higher in infected treated with dalbavancin tissue than in all the other tissue samples ($P < 0.05$; Fig. 3). The VEGF protein expression level was significantly lower in the untreated infected group compared with those in the uninfected and treated infected groups ($P < 0.05$; Fig. 3). MMP-1 and MMP-9 protein expression levels were significantly higher in the untreated infected group compared to those in the other groups; in particular, the treatments with dalbavancin and vancomycin resulted in a significant decrease of protein expression levels ($P < 0.05$; Fig. 3).

**DISCUSSION**

Vancomycin is the first-choice antibiotic for the treatment of MRSA infection (14), but several papers have described infections sustained by vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA) strains. Different studies reported that the host immune system has difficulty in eradicating VISA/hVISA, leading to chronic, recurrent, and persistent infections (15).

Dalbavancin is an optimal alternative to conventional antibacterials for the treatment of ABSSSIs. Like other lipoglycopeptides, dalbavancin binds the C-terminal acyl-D-Ala-D-Ala subunit of peptidoglycan precursors. Furthermore, the positively charged C-terminal dimethylaminopropyl group may interact with the negative phospholipid head groups of the bacterial membrane (16). The fatty acyl group allows nonspecific protein binding that likely contributes to dalbavancin’s long plasma half-life and its ability to bind its target sites (16). This particular structure confers exceptional pharmacokinetic properties to dalbavancin and allows its administration in a two-dose regimen (5).

Dalbavancin demonstrated superior in vitro activity versus that of comparators, including that of vancomycin, with dramatically lower MICs and without evidence of resistance through direct selection or serial passage (5, 17). Our microbiological results confirmed that both dalbavancin and vancomycin are effective in reducing the bacterial load in wounds. These results are not surprising, considering that efficacy of these antibiotics was proven by widespread clinical use and numerous reports (18–20). Moreover, due to the long biological half-life, daily treatment did not result in better reduction of bacteria, as also previously demonstrated by other authors (21).

But, until now, there have been no published data comparing the effect of dalbavancin on wound healing with that of vancomycin.

Since there are obvious difficulties in sampling skin wounds in humans due to ethical concerns, in particular through histological assessment of reepithelialization through keratinocytes and dermal cell involvement, we studied wound healing by using mouse models. Some authors, over the past year, have stimulated discussion on the utility and validity of animal models (22); however, animal models are the only viable and fully intact biological systems that allow examination of clinically relevant

**TABLE 2 Analysis of wound healing in the different groups of mice**

<table>
<thead>
<tr>
<th>Model</th>
<th>Reepithelialization</th>
<th>Granulation tissue</th>
<th>Dermis organization</th>
<th>Inflammatory infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected (C0) (mean ± SD)*</td>
<td>3.85 ± 0.25</td>
<td>3.52 ± 0.22</td>
<td>3.85 ± 0.50</td>
<td>1.02 ± 0.02</td>
</tr>
<tr>
<td>Infected untreated (C1) (mean ± SD)*</td>
<td>0.50 ± 0.08</td>
<td>1.25 ± 0.08</td>
<td>1.80 ± 0.30</td>
<td>2.00 ± 0.25</td>
</tr>
<tr>
<td>Infected, treated with vancomycin (C2) (mean ± SD)*</td>
<td>2.85 ± 0.20</td>
<td>3.00 ± 0.25</td>
<td>3.20 ± 0.28</td>
<td>1.20 ± 0.03</td>
</tr>
<tr>
<td>Infected, treated with dalbavancin (C3) (mean ± SD)*</td>
<td>3.35 ± 0.10</td>
<td>3.20 ± 0.15</td>
<td>3.75 ± 0.18</td>
<td>1.2 ± 0.08</td>
</tr>
<tr>
<td>ANOVA test(s) performed</td>
<td>C0 vs C1, C2, C3; C1 vs C0, C2, C3; C3 vs C2</td>
<td>C0 vs C1, C2; C1 vs C2, C3</td>
<td>C0, C3 vs C0, C1; C2 vs C1</td>
<td>C1 vs C0, C2, C3</td>
</tr>
</tbody>
</table>

*Mean histological score.
hypotheses and studies to decipher underlying mechanisms of human biological phenomena (23–25). In humans, several tools for clinically assessing wounds have been described (26); however, these instruments can only measure macroscopic changes in wound healing and do not predict healing or measure histological and biomolecular wound characteristics (27).

After external skin injury, such as surgical intervention, the wound healing process involves a coordinated cascade of events that comprises inflammation, formation of granulation tissue, and dermis maturation (28, 29).

It has been shown that the bacteria are specifically able to alter stromal components and inflammatory cells and their responses. In fact, human stromal cells were demonstrated to be susceptible to soluble factors of S. aureus biofilms, decreasing cell

![Figure 2](image)

**Figure 2** Representative images of hematoxylin- and eosin-stained histological sections of wound healing tissues from uninfected mice, infected mice treated with dalbavancin or vancomycin, and infected untreated mice on day 14 after injury. Wound healing was still incomplete in the untreated groups, but both treated groups exhibited good reepithelialization; in particular, the wounds treated with dalbavancin showed robust epidermal coverage and well-organized granulation tissue (original magnification, ×100).
differentiation, viability, migration, and angiogenesis (30). In addition, S. aureus was shown to have a dramatic effect on keratinocyte proliferation rates, as well as to be an impediment to the migration of keratinocytes, evidenced by delayed wound closure (31).

As expected, our results confirmed incomplete wound healing in the untreated infected groups. In contrast, both treatments showed efficacy on the healing process. In particular, in the dalbavancin-treated group, we observed the reconstitution of a regular and keratinized epidermal lining, while in the vancomycin-treated group the epidermis was still hypertrophic, revealing that an epithelial healing problem is still occurring. In the treated groups, we also discovered elevated EGFR expression; in particular, it was higher in dalbavancin-treated mice. This increased expression appears to be a favorable factor for a good reepithelialization, since the epidermal keratinocytes are a rich source of EGFR ligands and EGFR signaling has a major effect on the proliferation and differentiation of keratinocytes. Therefore, EGFR plays a key role in skin development and homeostasis (32), and its expression in the epithelium is a good indicator of the healing potential, as previously observed (10, 33).

In our study, MMP-1 and MMP-9 were decreased in both treated tissues compared with the levels in untreated infected wounds. MMPs are upregulated during wound

**TABLE 3** Immunohistochemical expression of VEGF, MMP2, MMP9, MMP1, and EGFr in the different groups of mice

<table>
<thead>
<tr>
<th>Model</th>
<th>VEGF</th>
<th>EGFr</th>
<th>MMP-9</th>
<th>MMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected (C0) (mean ± SD)</td>
<td>30.0 ± 3.2</td>
<td>40.0 ± 2.5</td>
<td>20.3 ± 2.6</td>
<td>22.0 ± 1.2</td>
</tr>
<tr>
<td>Infected untreated (C1) (mean ± SD)</td>
<td>15.5 ± 2.6</td>
<td>31.0 ± 2.5</td>
<td>37.0 ± 1.6</td>
<td>45.0 ± 5.2</td>
</tr>
<tr>
<td>Infected, treated with vancomycin (C2) (mean ± SD)</td>
<td>31.6 ± 3.2</td>
<td>30.0 ± 2.0</td>
<td>16.2 ± 1.8</td>
<td>18.0 ± 3.6</td>
</tr>
<tr>
<td>Infected, treated with dalbavancin (C3) (mean ± SD)</td>
<td>40.0 ± 4.2</td>
<td>50.0 ± 2.2</td>
<td>15.0 ± 2.5</td>
<td>20.0 ± 3.0</td>
</tr>
<tr>
<td>ANOVA test(s) performed</td>
<td>C3 vs C0, C1, C2; C1 vs C0, C2</td>
<td>C3 vs C0, C1, C2; C0, C3 vs C1, C2; C2, C3 vs C0</td>
<td>C1 vs C0, C2, C3</td>
<td>C1 vs C0, C2, C3</td>
</tr>
</tbody>
</table>

*a*Expressed as percentage of positive cells.

FIG 3 Analysis of the expression of EGFR, MMP-1, MMP-9, and VEGF. Western blot and densitometric analyses of EGFR, MMP-1, MMP-9, and VEGF (a representative of the three performed experiments). Densitometric analyses of the immunoreactive bands are quantified as the ratio between bands relative to EGFR, VEGF, MMP-1, MMP-9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in corresponding samples, given in arbitrary units. Data are expressed as mean ± SD from analyses performed on uninfected, untreated infected, infected treated with vancomycin, and infected treated with dalbavancin tissue samples. *P < 0.05 for infected treated with dalbavancin versus uninfected, untreated infected, and infected treated with vancomycin groups. **P < 0.05 for untreated infected versus uninfected, infected treated with dalbavancin, and infected treated with vancomycin.
healing in the very early stages in epidermal cells, dermal cells, fibroblasts, and blood cells (8). In particular, it has been shown that MMP-1 expression peaks at day 1 after wounding in migrating basal keratinocytes at the wound edge, followed by a gradual decrease until reepithelialization is complete. Downregulation of MMP-1 seems to be important for normal tissue remodeling, as high levels of MMP-1 in chronic nonhealing wounds are described (34). Similarly, migrating keratinocytes at the leading edges secrete MMP-9, which modulates keratinocyte motility by degradation of proteins involved in cell-cell and cell-matrix adhesion, allowing reepithelialization to occur (34). Interestingly, MMP-9 knockout mice and MMP-9-deficient mice showed delayed wound closure with inhibition of cell proliferation (35, 36), whereas the persistence of a high level of MMP-9 has been associated with poor healing in several studies (37–39). MMPs play also a significant role in stimulation of angiogenesis in the proximity of wounds to accelerate recovery (34); in particular, MMP-9 modulates VEGF release (40, 41), contributing to wound repair (42). Previous studies have shown that keratinocytes express elevated VEGF as early as 1 day after injury and eventually in those cells that migrate to cover the defect. These levels remain elevated during the formation of granulation tissue and until epidermal coverage is complete (43). In our study, we observed that VEGF expression was highly expressed in the uninfected group and in both treated infected groups; in particular, the wounds treated with dalbavancin showed the highest values, underlining the positive effect of dalbavancin on angiogenesis.

In conclusion, it can be speculated that both treatments may have a positive effect not only on the bacterial load but also on tissue healing of infected surgical skin wounds by downregulation of MMP-1 and MMP-9 and an increase of EGFR and VEGF expression. In particular, this study showed faster healing after dalbavancin treatment that can be associated with a more efficient reduction of bacteria with respect to that after vancomycin treatment; however, we think that it also could be related to the role played by dalbavancin in levels of EGFR and VEGF, factors deeply involved in wound healing. The increase of these factors after dalbavancin treatment compared to levels in uninfected wounds, where bacteria are not involved, supports our opinion. In addition, in our previous study (37) using tigecycline and teicoplanin treatment, although we achieved a similar bacterial reduction, tigecycline showed better wound resolution related to an increased MMP-9 expression, confirming that the wound healing is driven not only by reduction of bacteria but also by specific factors. It is possible to hypothesize that the better effect of dalbavancin on wound healing could be related to its biochemical structure, which is designed to improve upon natural glycopeptides by the addition of lipophilic tails. On the other hand, our previous studies on lipopeptides showed better overall healing (33). Further investigations are needed to clarify the additional effects of dalbavancin on wound repair.

MATERIALS AND METHODS

Ethics. In vivo experiments were approved by the Institutional Animal Care Committee of the Ministry of Health and by the Animal Research Ethics Committee of IRRCS INRCA (Istituto di Ricovero e Cura a Carattere Scientifico–Istituto nazionale di Riposo e Cura per Anziani) under approval 767/2016 Pr 28/07/2016.

Bacterial strains and drugs. Our work is a preliminary study on the histological effects of dalbavancin in wound healing, and so we included only the MRSA strain ATCC 43300, a well-known strain that represents a model. Vancomycin and dalbavancin were diluted in accordance with the manufacturers’ recommendations, yielding 10 mg/ml stock solutions. Solutions were made fresh on the day of assay or stored at −80°C in the dark for short periods.

Mouse infection model. Six-month-old BALB/c mice weighting 28 to 30 g from the specific pathogen-free (SPF) animal facility of INRCA (Istituto nazionale di Riposo e Cura per Anziani, Ancona, Italy) were used for all the studies. All of the procedures involving animals were conducted in conformity with the institutional guidelines in compliance with national (legislative decree n. 26, 4 March 2014; authorization n.767/2016-PR, issued 28 July 2016, by the Italian Ministry of Health) and international law and policies (EEC Council Directive 2010/63/EU). Mice were individually caged in visual, auditory, and olfactory contact with other mice of the same experimental group (within ventilated cabinets cage systems) as well as in the presence of environmental enrichments consisting of nesting materials and wooden toys. Mice were kept in a 12-h light-dark cycle with food and drinking water ad libitum and were allowed to equilibrate in the phenotyping area allocated in the Scientific and Technological Pole of INRCA for approximately 1 month before starting the experiments. After surgical intervention, all mice
were housed individually under constant temperature (22 ± 2°C) and humidity. The animals were fed with standard pellet food (Harlan Laboratories, Udine, Italy) and given fresh daily tap water.

The study included a total of 48 animals divided into 4 groups (each composed of 12 mice), as follows: an uninfected group (C0, sham control); an infected but not treated group (C1); a vancomycin group (infected and treated daily with vancomycin; C2); and a dalbavancin group (infected and treated weekly with dalbavancin; C3).

MRSA ATCC 43300 cultures were grown in brain heart infusion and diluted in saline to a final concentration of 5 × 10^7 CFU/ml, prepared freshly at the time of intervention.

Mice were anesthetized by an intramuscular injection of ketamine (50 mg/kg of body weight) and xylazine (8 mg/kg of body weight), the hair on their back was shaved, and the skin was cleansed with 10% povidone-iodine solution (no animals dropped out due to infection or anesthetics). One full-thickness wound was established through the panniculus carnosus on the back subcutaneous tissue of each animal. A small piece of gauze was placed over each wound and then inoculated with 200 μl of previously diluted bacterial culture; in the control group, the gauze was soaked only with sterile saline solution. The pocket was closed by means of skin clips. This procedure resulted in a local abscess at 24 h. One wound was created per animal. The animals were returned to individual cages and thoroughly examined daily. After 24 h, the wound was opened and washed with saline, the gauze was removed, and treatment started (44). Group C0 did not receive any treatment. Groups C1 and C2 were treated with daily intraperitoneal administration of 200 μl of saline or intraperitoneal vancomycin (10 mg/kg), respectively. Group C3 was treated with intraperitoneal injection of 200 μl of dalbavancin at day 1 (20 mg/kg) and day 8 (10 mg/kg). Since there are no previous studies on skin infections in mice treated with dalbavancin, we decided to establish treatment with a dosage similar to that used in humans (two dalbavancin injection, 1,000 mg and 500 mg at days 1 and 8, respectively), considering that large and widely spaced doses had the most effectiveness (45).

After 14 days, animals were euthanized, and a 1 × 2-cm area of skin that included the wound was excised aseptically for histological and Western blot examination (see below) and for bacterial count. For the bacterial analysis, the samples were weighed and then homogenized in 1 ml phosphate-buffered saline (PBS) using a stomacher. Quantitation of viable bacteria was performed by culturing serial dilutions of the bacterial suspension on mannitol-salt agar plates at 37°C for 24 to 48 h. The limit of detection for this method was approximately 10 CFU/ml.

**Histological and immunohistochemical staining.** To understand the efficacy of the treatments in promoting wound healing, the reepithelialized tissue of each group was investigated histologically. Sections (5 μm thick) were stained with hematoxylin and eosin (H&E) according to a standard protocol. The sections were deparaffinized and rehydrated by sequential immersion in xylene, ethanol, and water. Antigen retrieval was carried out by heating the sections in 0.1 M citrate buffer solution (pH 6.0) at 98°C for 5 min via microwave. Then, they were incubated with monoclonal anti-mouse VEGF (clone C-1; Santa Cruz Biotechnologies, Santa Cruz, CA), MMP-1 (clone H-300; Santa Cruz Biotechnologies), MMP-9 (clone 2C3; Santa Cruz Biotechnologies) and EGFR (clone 1005; Santa Cruz Biotechnologies) at 4°C overnight and immunostained using the streptavidin-biotin peroxidase technique (Envision universal peroxidase kit; DakoCytomation, Milan, Italy). After incubation, the tissue sections were colored with 3,3-diaminobenzidine (DAB), stained with hematoxylin, and coverslipped with Eukitt mounting medium (Electron Microscopy Sciences, PA, USA).

Two investigators (G.L. and A.Z.), who were blind to the mouse outcome, performed all counts separately. We counted the number of marker-positive cells by using a light microscope (Nikon Eclipse E600; Nikon Instruments, Europe B.V., Kingston, Surrey, England) and scored according to a 5-tiered wound repair grading system based on epithelial presence, degree of stratification, degree of differentiation as well as maturational features of the granulation tissue, dermal tissue, and the presence of inflammatory cells (Table 1)(13).

In addition, identification of VEGF, MMP-1, MMP-9, and EGFR expression was carried out using immunohistochemistry of histological samples with a heat-mediated antigen retrieval process. Briefly, 5-μm paraffin sections were deparaffinized and rehydrated by sequential immersion in xylene, ethanol, and water. Antigen retrieval was carried out by heating the sections in 0.1 M citrate buffer solution (pH 6.0) at 98°C for 5 min via microwave. Then, they were incubated with monoclonal anti-mouse VEGF (clone C-1; Santa Cruz Biotechnologies, Santa Cruz, CA), MMP-1 (clone H-300; Santa Cruz Biotechnologies), MMP-9 (clone 2C3; Santa Cruz Biotechnologies) and EGFR (clone 1005; Santa Cruz Biotechnologies) at 4°C overnight and immunostained using the streptavidin-biotin peroxidase technique (Envision universal peroxidase kit; DakoCytomation, Milan, Italy). After incubation, the tissue sections were colored with 3,3-diaminobenzidine (DAB), stained with hematoxylin, and coverslipped with Eukitt mounting medium (Electron Microscopy Sciences, PA, USA).

Two investigators (G.L. and A.Z.), who were blind to the mouse outcome, performed all counts separately. We counted the number of marker-positive cells by using a light microscope (Nikon Eclipse E600; Nikon Instruments, Europe B.V., Amsterdam, Netherlands) at ×200 magnification in at least 10 fields/sample, and the number of positive cells were expressed as the mean value ± standard deviation (SD).

Images were captured with a Nikon DS-Vi1 digital camera (Nikon Instruments) connected to a computer. The area of each field (0.07 mm²) was standardized using NIS Elements BR v3.22 imaging software (Nikon Instruments).

**Protein extraction and immunoblotting.** Total proteins were extracted from wound tissue samples and immediately pulverized in liquid nitrogen. The pulverized skins were homogenized and lysed in ice-cold Radio immunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% sodium dodecyl sulfate, 1.0% Triton X-100, and 5 mM ethylenediaminetetraacetic acid (pH 8.0)) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for 45 min. The lysates were centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was collected and the protein concentration was determined. Protein concentration was determined using Bradford reagent (Sigma-Aldrich). Total protein extracts (30 μg) were reduced in 0.5 M dithiothreitol (DTT) for 10 min at 70°C, and samples were run on a 4 to 12% gradient precast NuPAGE Bis-Tris polyacrylamide gel for 1 h at 200 V. After electrophoresis, the proteins in the gel were transferred to a nitrocellulose membrane, and the blots were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 for 1 h at room temperature. Membranes were incubated overnight with primary antibodies anti-EGFR, anti-VEGF, anti-MMP-1, and anti-MMP-9 (Santa Cruz Biotechnology) and glyceralddehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) as the endogenous control, followed by incubation with
a secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The detection of the proteins using on the membrane was performed using the Clarity Western ECL substrate kit (Bio-Rad). The signals were captured using the Alliance Mini (UVITEC, Cambridge, UK) system. The UVITEC software carried out the quantization of the bands, and the intensity of each band of interest was normalized by comparing it to the housekeeping GAPDH protein, used as the loading control. Subsequently, the intensity of each tested band was compared with that of negative controls.

**Statistical analysis.** All results were expressed as mean values ± SD. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) tests, and P values were determined using Bonferroni’s test. Statistical analyses were performed using the SPSS 16 package (SPSS Inc., Chicago, IL, USA). Significance was set at P < 0.05.

**ACKNOWLEDGMENTS**

This work was supported by Progetto Scientifico di Ateneo 2016—Università Politecnica delle Marche “Study of new compounds and innovative strategies to control complicated bacterial skin infections.”

We have no conflicts to declare.

**REFERENCES**


24. Swaerengen JR. 2018. Choosing the right animal model for infectious...