In vitro antibacterial activity of E-101 Solution, a novel myeloperoxidase-mediated antimicrobial, against Gram-positive and Gram-negative pathogens

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Objectives: E-101 Solution (E-101) is a novel myeloperoxidase-mediated antimicrobial. It is composed of porcine myeloperoxidase (pMPO), glucose oxidase, glucose as the substrate and specific amino acids in an aqueous vehicle. E-101 is being developed for topical application directly into surgical wounds to prevent surgical site infections (SSIs). The in vitro activity of E-101 was investigated.

Methods: MIC, MBC, time–kill and antimicrobial combination experiments were performed according to CLSI guidelines with modifications. Resistance selection studies were performed using a serial passage method.

Results: E-101 showed MIC90 values of 0.03, 0.5 and 0.5 mg pMPO/L for staphylococci (n=140), streptococci (n=95) and enterococci (n=55), respectively. MIC90 values ranged between 0.03–0.5 and ≤0.004–0.12 mg pMPO/L for Enterobacteriaceae (n=148) and Gram-negative non-Enterobacteriaceae (n=92) strains, respectively. There was no antimicrobial tolerance to E-101 for Staphylococcus aureus, Streptococcus agalactiae or Streptococcus pyogenes. Time–kill studies demonstrated a rapid (<30 min) bactericidal effect against S. aureus, Enterococcus faecalis, Escherichia coli and Pseudomonas aeruginosa in a concentration-dependent and time-dependent manner. There was no evidence of stable resistance to E-101 among staphylococci, enterococci, E. coli or P. aeruginosa strains and no evidence of E-101 interaction with antibiotics commonly used in clinical medicine.

Conclusions: E-101 shows potent and broad-spectrum in vitro activity against bacteria that are the causative pathogens of SSIs, thereby providing the impetus to test its clinical utility in the prevention of SSIs.

Keywords: topical antimicrobial, therapeutic enzyme system, singlet oxygen, surgical site infections

Introduction

E-101 Solution (E-101) is a defined formulated cell-free oxidant-generating enzyme system containing: (i) two therapeutic enzymes [porcine myeloperoxidase (pMPO) and glucose oxidase (GO)]; (ii) glucose, which is the substrate for GO; (iii) sodium chloride; and (iv) proprietary amino acids that enhance the activity of the system once it has been activated after mixing all of the components. The putative microbicidal mechanism of action of E-101 involves the binding of pMPO to the surface of target microorganisms where hypochlorous acid (HOCl) and singlet oxygen (¹O₂) exert direct oxidative damage.1–9 In the presence of hydrogen peroxide (H₂O₂), generated in situ by GO from glucose and oxygen, the microorganism-bound pMPO catalyses the oxidation of chloride ion to HOCl and facilitates the disproportionation of H₂O₂ to ¹O₂ at or near the surface of the target organism (Figure 1).

Myeloperoxidase (MPO) is a member of the family of highly conserved mammalian peroxidases and is found in high abundance in the azurophil granules in neutrophils. MPO is the principal haem protein of the neutrophil and is involved in the host defence mechanism against infection and is a key component of the oxygen-dependent antimicrobial system of the neutrophil phagosome.10 The ability to efficiently kill phagocytized microbes requires the activities of membrane-bound nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and MPO within the phagolysosome. The oxidase uses NADPH to reduce oxygen to generate the H₂O₂ that drives the MPO-dependent oxidation of chloride, yielding HOCl and ultimately ¹O₂. These end-products of MPO metabolism are potent host microbicidal
Materials and methods

Bacterial isolates

A total of 530 bacterial strains were selected to determine the spectrum of activity of E-101 by MIC and MBC testing. The bacterial strains included: 140 staphylococci [including 70 methicillin-resistant, 4 confirmed Panton–Valentine leucocidin (PVL)-positive isolates and 5 vancomycin-intermediate Staphylococcus aureus/vancomycin-resistant S. aureus (VISA/RVSA)]; 95 β-haemolytic streptococci; 55 enterococci (33 were vancomycin susceptible and the remaining 22 were vancomycin resistant); 148 Enterobacteriaceae strains (51 were ceftazidime resistant); and 92 non-Enterobacteriaceae strains (54 were cefazidime resistant). All clinical isolates were from the culture collection of Eurofins Medinet Anti-Infective Services (Chantilly, VA, USA). The strains were obtained from diverse geographical regions of North America and were originally isolated clinical specimens from symptomatic patients (Table 1). Escherichia coli ATCC 25922, S. aureus ATCC 25923, S. aureus ATCC 29213, Pseudomonas aeruginosa ATCC 27853 and Enterococcus faecalis ATCC 29212 were used as quality control strains for E-101 and to validate a new modified CLSI (formerly NCCLS) broth microdilution method described in this report.

Antimicrobial agents

E-101 is composed of two aqueous solutions designated as enzyme solution and substrate solution. They are packaged in two separate vials and mixed together to activate the system. The two stock solutions were prepared at AAI International (Charleston, SC, USA) and a range of concentrations of E-101 was prepared just prior to use. The enzyme solution contains pMPO, GO derived from Aspergillus niger and proprietary amino acids in an aqueous formulation vehicle consisting of 150 mM sodium chloride and 0.02% w/v polysorbate 80 in 20 mM sodium phosphate buffer pH 6.5. The stock concentrations of pMPO and GO were 1.2 mg/mL and 80 U/mL, respectively. The substrate solution contains 300 mM glucose in the same aqueous formulation as the enzyme solution. In this report, the concentration of E-101 is expressed only in terms of milligrams of pMPO per litre (mg pMPO/L) in the solution. The concentrations of GO and amino acids are directly proportional to pMPO (3:1 MPO/GO ratio). The concentrations of the other components are held constant.

Quality control agents included cefazolin and gentamicin from Sigma Chemical Co. (St Louis, MO, USA) and mupirocin from GlaxoSmithKline, Inc. (Philadelphia, PA, USA). All stock solutions were prepared immediately prior to testing. The pMPO test concentrations were: 0.004–8 mg pMPO/L E-101; 0.06–64 mg/L cefazolin; 0.5–32 mg/L gentamicin; and 0.03–32 mg/L mupirocin. Antibiotics used for drug interaction studies included cefazolin, ceftiraxone, cefazidime, ciprofloxacin, doxycycline, gentamicin, imipenem and vancomycin (GlaxoSmithKline, Inc.).

MIC determination

Broth microdilution and methods for determining bactericidal activity were performed using modifications to the CLSI-recommended procedures.20,21 Modifications were made to the standard broth microdilution method to accommodate the rapid in vitro activity of E-101. In the modified CLSI method, first the enzyme solution was diluted in Mueller–Hinton broth, resulting in doubling dilutions throughout the microdilution tray. Next, the bacterial suspension, prepared in the glucose substrate solution, was added to each well and mixed with the enzyme dilutions. This was done because of the rapidity of E-101 enzymatic reactions. Each antimicrobial agent and enzyme solution was diluted in double strength, cation-adjusted Mueller–Hinton broth (CAMHB) and dispensed in microdilution trays. All β-haemolytic streptococci were tested in double-strength CAMHB supplemented with 5%
In vitro activity of E-101 antibacterial solution

lysed horse blood. Isolates were prepared by suspending several colonies (four to six) from an overnight culture on Trypticase soy agar (TSA) with 5% sheep blood in sterile saline and the density was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard (≏10^8 cfu/mL). Standardized bacterial suspensions were further diluted in double-strength E-101 substrate solution previously described so that a suspension of 5×10^7 cfu/mL was mixed with serial drug dilutions. The microdilution trays were incubated in ambient air at 35°C for 18–24 h. The MIC of E-101 was determined by observing the lowest concentration of antimicrobial agent that completely inhibited the growth of the organism and was expressed as mg pMPO/L. The MIC₅₀ and MIC₉₀ represent, respectively, the concentrations at which 50% and 90% of the isolates were inhibited.

MRC ranged were established for E-101 by testing >20 replicates of one lot of E-101 against S. aureus ATCC 29213 and E. coli ATCC 25922. Quality control parameters were then determined for E-101 by testing three different lots of the enzyme solution (pMPO and GO) used for preparation of the final E-101 formulation. Testing was performed in duplicate against S. aureus ATCC 29213 and E. coli ATCC 25922. The effects of modifying the standard broth microdilution test were also assessed with antibiotics with known quality control limits to validate the CLSI broth microdilution method.21

MBC determination

MBCs were determined by first using the modified broth microdilution method described above for E-101 MIC. From the microdilution tray, the last drug-containing well with visible growth and each clear well thereafter were sampled. A 10 μL sample from each well was plated onto TSA with 5% sheep blood, incubated in ambient air at 35°C for 18–24 h and examined for growth and colony counts. The MBC was determined by observing the lowest antimicrobial concentration that demonstrated a ≥99.9% reduction in cfu relative to the number of cfu in the starting inoculum. The MBC₅₀ and MBC₉₀ represent, respectively, the concentrations at which 50% and 90% of the isolates were killed.

Time–kill assay

The bactericidal effect of E-101 was assessed by an adaptation of the CLSI microdilution time–kill assay.21 Test organisms included S. aureus ATCC 29213, E. faecalis ATCC 29212, E. coli ATCC 25922, and P. aeruginosa ATCC 27853. Several colonies (four to six), grown on TSA with 5% sheep blood overnight, were suspended in 3 mL of deionized water and suspensions were adjusted to a turbidity equivalent to that of a 0.5 McFarland standard (≏1×10^8 cfu/mL). This suspension was then diluted 1:10 in pre-warmed CAMHB and incubated for 1–4 h in a shaker incubator at 37°C. When the culture reached its logarithmic growth phase and the turbidity approximated that of a 0.5 McFarland standard, 100 μL was removed and added to 10 mL of E-101 substrate solution previously described to attain a final concentration of 5×10^8 cfu/mL. This suspension constituted the inoculum for the time–kill assay. The time–kill reaction wells contained 100 μL of enzyme solution prepared in CAMHB and inoculum (50 μL of enzyme solution and 50 μL of inoculum prepared in substrate solution). The final concentrations of pMPO in the enzyme solution were 16-fold dilutions of the highest concentration: 0, 0.06, 1, 16 and 256 mg pMPO/L. An identical reaction well containing broth and inoculum, but no enzyme solution, constituted the culture growth control. The final bacterial cell concentration was 5×10^8 cfu/mL. The microdilution trays were incubated at 35°C in ambient air and 10 μL of a 1% catalase solution was added to each time–kill well at 0, 5, 15, 30 and 60 min and 4 h to stop the antimicrobial action of E-101. Serial samples were obtained for quantification. A 100 μL sample was removed from each well at each timepoint and serial dilutions were prepared in sterile saline.

A 100 μL volume of each dilution was applied to duplicate TSA plates with 5% sheep blood and spread over the surface with a sterile inoculating loop. The plates at time zero functioned as the purity plates. Following overnight incubation at 35°C, colonies were manually counted and viable counts were calculated. The data are presented as log_{10} reduction in cfu/mL at designated timepoints compared with the original cfu/mL at the start of testing. Bactericidal activity was defined as a 99.9% or a 3 log_{10} cfu/mL reduction in the colony count from the initial inoculum.

Antimicrobial interactions

Three multidrug-resistant clinical strains were used to test for adverse interactions between E-101 and standard antibiotics by implementing a chequerboard titration method using 96-well microdilution trays.23 The test organisms included VISA Mu50 (Network on Antimicrobial Resistance in S. aureus isolate NR51),24 E. coli (Eurofins 1075701) and P. aeruginosa (Eurofins 1445536). E-101 and selected antibiotics were tested by the broth microdilution method with CAMHB and serially diluted (2-fold) alone and in combination. Each well in the chequerboard contained a unique combination of the two drug concentrations and two rows contained one drug alone. Drug concentration ranges for E-101 were 0.0005–0.5 mg pMPO/L against S. aureus, 0.008–8 mg pMPO/L against E. coli and 0.001–1.0 mg pMPO/L against P. aeruginosa. Concentration ranges of all antibiotics were ≤0.25–2 times their respective MIC for the tested isolate. The inoculum, prepared in substrate solution as described above, was added to each well of the microdilution trays and incubated in ambient air at 35°C. The MIC of each antimicrobial agent alone and in combination was defined as the lowest concentration(s) that demonstrated no visibly detectable growth after 18–24 h. The fractional inhibitory concentration index (FICI) was interpreted as follows: ≤0.5, synergy; >0.5–4.0, no interaction; and >4.0, antagonism.25

Resistance selection

To determine whether repeated exposure of organisms to subinhibitory concentrations of E-101 resulted in the emergence of resistance, a serial passage method was used in microdilution trays.26 Ten test strains included S. aureus (ATCC 29213 and Eurofins 1288199), E. faecalis (ATCC 29212 and ATCC 51299), E. coli (ATCC 25922, Eurofins 1075701, Eurofins 1337451 and Eurofins 1337019) and P. aeruginosa (ATCC 27853 and Eurofins 1077561). Baseline MICs of E-101 were determined as described above. Inocula for subsequent MIC tests were prepared from the well containing the highest concentration of E-101 that allowed growth. A fresh panel, containing E-101 serially diluted in CAMHB, was reinoculated with the new suspension prepared in substrate solution. Passages were continued for 21 consecutive days and E-101 MICs were determined following each serial passage. Subsequently, if the MICs showed an increase, stability studies were performed by three serial passages on drug-free agar (TSA) plates with new MICs being determined.

Results

MIC and MBC determination

Table 1 summarizes the MIC values of E-101 and comparators for 530 aerobic isolates. In general, E-101 demonstrated potent broad-spectrum activity against both aerobic Gram-positive and aerobic Gram-negative bacteria tested. Among the enterococci, MIC₅₀ and MIC₉₀ values were identical and were 0.5 mg pMPO/L for E. faecalis and 0.12 mg pMPO/L for Enterococcus faecium. No difference was noted in the activity of E-101 against vancomycin-susceptible compared with
vancomycin-resistant enterococci. E-101 was most active against the staphylococci. All MICs for S. aureus and Staphylococcus epidermidis strains, including methicillin-resistant S. aureus (MRSA) and methicillin-resistant Staphylococcus epidermidis (MRSE) were ≤0.06 mg pMPO/L; the MIC\(_{50}\) was ≤0.06 mg pMPO/L and the MIC\(_{90}\) was 0.03 mg pMPO/L. E-101 was highly active against PVL-positive and VISA/VRSA strains, demonstrating equivalent activity compared with the wild-types. The range of E-101 MIC values for PVL-positive isolates was ≤0.06 mg pMPO/L. E-101 displayed excellent activity against bacteria difficult to eradicate, such as S. aureus and S. epidermidis strains was several-fold higher than the E-101 MIC range. Two S. aureus and 10 S. epidermidis strains yielded a mupirocin MIC of >32 mg/L, whereas the maximum E-101 MIC for these strains was 0.06 mg pMPO/L. For the streptococci, E-101 and mupirocin demonstrated similar MIC\(_{50}\)s and ranges for Streptococcus agalactiae and Streptococcus pyogenes. There was, however, a clear trend of higher E-101 MIC for viridans Streptococcus groups C, F and G compared with the MIC for the other Gram-positive and Gram-negative bacteria tested (see Table 1). This may reflect the added 5% lysed horse blood, which is rich in catalase and competitive substrates for oxidation.

Table 1. *In vitro* activity of E-101 and comparator antimicrobials against 530 clinical isolates of aerobic Gram-positive and Gram-negative pathogens

<table>
<thead>
<tr>
<th>Organism (no. of strains)</th>
<th>E-101 MIC (mg pMPO/L)</th>
<th>Mupirocin or gentamicin MIC (mg/L)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC(_{50})</td>
<td>MIC(_{90})</td>
</tr>
<tr>
<td>Gram-positive organisms</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> (33)(^b)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td><em>E. faecium</em> (22)(^c)</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td><em>S. aureus</em> (109)(^d)</td>
<td>0.015</td>
<td>0.03</td>
</tr>
<tr>
<td><em>S. epidermidis</em> (31)(^e)</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td><em>S. agalactiae</em> (34)(^f)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Streptococcus group C (8)(^g)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Streptococcus group F (2)(^h)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Streptococcus group G (18)(^i)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. pyogenes</em> (33)(^j)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Gram-negative organisms</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Citrobacter freundii</em> (20)(^k)</td>
<td>0.6</td>
<td>0.12</td>
</tr>
<tr>
<td>Enterobacter cloacae (21)(^l)</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td><em>E. coli</em> (52)(^m)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Klebsiella pneumoniae (31)(^n)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Proteus mirabilis (24)</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td><em>A. baumannii</em> (29)(^o)</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (53)(^p)</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Aeromonas hydrophila (5)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pasteurella multocida (5)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable, total number of isolates <10.
\(^a\)MICs are of mupirocin for Gram-positive organisms and gentamicin for Gram-negative organisms.
\(^b\)E. faecalis vancomycin susceptible (n=27), E. faecalis vancomycin resistant (n=6).
\(^c\)E. faecium vancomycin susceptible (n=6), E. faecium vancomycin resistant (n=16).
\(^d\)S. aureus methicillin susceptible (n=55), MRSA (n=45), VISA/VRSA (n=5), S. aureus PVL positive (n=4).
\(^e\)S. epidermidis methicillin susceptible (n=6), S. epidermidis methicillin resistant (n=25).
\(^f\)CAMHB supplemented with 5% lysed horse blood was used for all streptococcal MIC studies.
\(^g\)C. freundii ceftazidime susceptible (n=11), C. freundii ceftazidime resistant (n=9).
\(^h\)E. cloacae ceftazidime susceptible (n=11), E. cloacae ceftazidime resistant (n=10).
\(^i\)E. coli ceftazidime susceptible (n=34), E. coli ceftazidime resistant (n=18).
\(^j\)K. pneumoniae ceftazidime susceptible (n=17), K. pneumoniae ceftazidime resistant (n=14).
\(^k\)A. baumannii ceftazidime susceptible (n=11), A. baumannii ceftazidime resistant (n=18).
\(^l\)P. aeruginosa ceftazidime susceptible (n=17), P. aeruginosa ceftazidime resistant (n=36).
MICs of cefazolin, gentamicin and mupirocin were compared between the standardized and modified CLSI reference broth microdilution methods. The in vitro test parameters of both modified and reference MIC methods were similar in respect of medium (CAMHB), pH (7.2), final inoculum (5 × 10^5 cfu/L) and incubation environment (35°C in ambient air). Cefazolin and mupirocin MICs for S. aureus ATCC 29213 and cefazolin and gentamicin MICs for E. coli ATCC 25922 were within previously published quality control ranges. The quality control range determined for E-101 for S. aureus ATCC 29212 and E. coli ATCC 25922 was 0.01–0.03 mg pMPO/L and 0.15–0.5 mg pMPO/L, respectively. Replicate testing performed with these organisms and results of testing three different lots of enzyme solution demonstrated all MIC values to be within the established quality control range.

The MIC and MBC results are shown in Table 2. The potent bactericidal activity of E-101 was confirmed by the range of MICs (0.015–2 mg pMPO/L) against Gram-positive and Gram-negative clinical isolates. E-101 was most active against all isolates of S. aureus with an MIC to MBC ratio of 1 (MIC_50 and MBC_50=0.015 mg pMPO/L; MIC_90 and MBC_90=0.03 mg pMPO/L).

**Bactericidal activity**

The killing kinetics of E-101 are shown in Figure 2. E-101 demonstrated bactericidal activity against S. aureus and P. aeruginosa at all concentrations tested. Against E. coli, E-101 was bactericidal at all concentrations tested except at 0.06 mg pMPO/L, which is 4-fold below its MIC. Against E. faecalis, E-101 was bactericidal at concentrations 32-fold above the MIC. The MICs for E. faecalis and P. aeruginosa were 0.5 and 0.06 mg pMPO/L, respectively. The rate of kill for all four bacterial strains was greater at higher concentrations of E-101 and the extent of kill increased with length of exposure time. Therefore, E-101 bactericidal activity under in vitro conditions was both concentration and time dependent. At E-101 concentrations of 256 and 16 mg pMPO/L, no detectable growth of all bacterial strains was observed within 30 min and 4 h, respectively. After 4 h of exposure to E-101, a >3 log_{10} reduction was achieved at 0.06 mg pMPO/L for S. aureus and P. aeruginosa, at 1.0 mg pMPO/L for E. coli and at 16 mg pMPO/L for E. faecalis.

**Antimicrobial interactions**

Antimicrobial interaction studies of E-101 with cefazolin, ceftazidime, ceftriaxone, ciprofloxacin, doxycycline, gentamicin, imipenem and vancomycin were performed and generated five to six checkerboard combinations for each of the three test bacterial strains (Table 3). The combinations were tested in duplicate and were highly reproducible (within one doubling dilution). All FICIs were interpreted as no interaction for each antimicrobial combination.

**Resistance selection**

The MIC results for S. aureus, E. faecalis and P. aeruginosa remained within one doubling dilution after daily passage in subinhibitory concentrations of E-101 for 21 days (Table 4). The MICs for three clinical strains of E. coli showed an increase of four or more doubling dilutions after only one passage from day 1 to day 2, and the MICs remained elevated for 21 days. The change in MICs over the study interval was 0.12 to 8 mg pMPO/L, 0.25 to 8 mg pMPO/L and 0.5 to 8 mg pMPO/L for each strain, respectively. After three passages of each strain on drug-free agar plates, the MICs were not stable upon retesting and decreased back to two or fewer doubling dilutions of the initial baseline MIC.

**Discussion**

E-101 represents an MPO-mediated investigational product for killing microorganisms that may contaminate the surgical incision site. A number of studies have demonstrated that haloperoxidase/H_{2}O_{2}/halide systems are microbicidal in vitro against a variety of bacteria, yeast, viruses and bacterial and fungal spores. The susceptibility data presented in this study demonstrated the wide spectrum of activity of E-101 against clinically important antibiotic-susceptible and multidrug-resistant bacteria, including those responsible for the majority of skin and skin structure infections, *E. coli* and *P. aeruginosa*.

**Table 2.** MICs and MBCs of E-101 for Gram-positive clinical isolates associated with skin and skin structure infections, *E. coli* and *P. aeruginosa*

<table>
<thead>
<tr>
<th>Organism (no. of strains)</th>
<th>MIC (mg pMPO/L)</th>
<th>MBC (mg pMPO/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC_50 MIC_90</td>
<td>range</td>
</tr>
<tr>
<td><em>E. faecalis</em> (7)*</td>
<td>NA NA</td>
<td>0.12–0.5</td>
</tr>
<tr>
<td><em>S. aureus</em> (16)*</td>
<td>0.015 0.03</td>
<td>0.015–0.03</td>
</tr>
<tr>
<td><em>S. agalactiae</em> (13)</td>
<td>0.5 0.5</td>
<td>0.12–0.5</td>
</tr>
<tr>
<td><em>S. pyogenes</em> (20)</td>
<td>0.5 0.5</td>
<td>0.12–0.5</td>
</tr>
<tr>
<td><em>E. coli</em> (5)*</td>
<td>NA NA</td>
<td>0.03–0.06</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (5)*</td>
<td>NA NA</td>
<td>0.03–0.06</td>
</tr>
</tbody>
</table>

NA, not applicable, total number of isolates <10.

*E. faecalis* vancomycin susceptible (*n*=5), *E. faecalis* vancomycin resistant (*n*=2).

*S. aureus* methicillin susceptible (*n*=8), MRSA (*n*=6), VISA/VRSA (*n*=2).

*E. coli* ceftazidime susceptible (*n*=1), *E. coli* ceftazidime resistant (*n*=4).

*P. aeruginosa* ceftazidime susceptible (*n*=2), *P. aeruginosa* ceftazidime resistant (*n*=3).
of SSIs. E-101 had greater activity against the staphylococci than did mupirocin and equivalent to greater activity against aerobic Gram-negative bacteria than did gentamicin, which is considered the ‘gold-standard’ topical antibiotic for these bacteria. Furthermore, E-101 demonstrated bactericidal activity against *S. aureus* and *S. pyogenes* (Table 2), two of the most common causative pathogens of SSIs. Historical tolerance has primarily been associated with β-lactam agents. The generally accepted definition of tolerance is a ratio of the MBC to the MIC of >32. This study showed that the MBC to MIC ratio for E-101 was 1–2, indicating no evidence of E-101 antimicrobial tolerance.

**Table 3.** Results of interaction studies between E-101 and other antimicrobial agents

<table>
<thead>
<tr>
<th>Organism</th>
<th>CFZ</th>
<th>CTX</th>
<th>CIP</th>
<th>DOX</th>
<th>GEN</th>
<th>VAN</th>
<th>CEF</th>
<th>IPM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>2 (NI)</td>
<td>2 (NI)</td>
<td>0.62 (NI)</td>
<td>0.56 (NI)</td>
<td>2 (NI)</td>
<td>0.75 (NI)</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1 (NI)</td>
<td>2 (NI)</td>
<td>0.75 (NI)</td>
<td>0.62 (NI)</td>
<td>0.62 (NI)</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>NT</td>
<td>NT</td>
<td>2 (NI)</td>
<td>0.53 (NI)</td>
<td>1 (NI)</td>
<td>NT</td>
<td>0.75 (NI)</td>
<td>1 (NI)</td>
</tr>
</tbody>
</table>

CFZ, cefazolin; CTX, ceftriaxone; CIP, ciprofloxacin; DOX, doxycycline; GEN, gentamicin; VAN, vancomycin; CEF, ceftazidime; IPM, imipenem; NT, not tested.

The FICIs were interpreted as follows: ≤0.5 = synergy; >0.5–4 = no interaction (NI); and >4.0 = antagonism.
than that of the H\textsubscript{2}O\textsubscript{2} that is formed alone in the absence of E-101 is several orders of magnitude more potent enzymatic catalytic activity. \textit{1}O\textsubscript{2} also reacts readily with unsaturated lipids, nucleic acids and sulphur-containing biochemical sites essential for metabolism and viability.\textsuperscript{7} E-101 also has the advantage of being equally effective against both susceptible and antibiotic-resistant bacteria. The low propensity of microbes treated with E-101 to develop resistance and the lack of drug–drug interaction (antagonism) further support the concept that E-101 may play an important future role in reducing SSIs.

The extent of bactericidal activity of E-101 appeared to increase with greater incubation time. This has important practical therapeutic implications for a topical antimicrobial in the prevention of SSIs since there is the need to rapidly kill potential pathogens on contact within the surgical field.

Unlike antibiotics that must first be incorporated into specific ‘intestinal’ bacterial targets before exerting their bacteriostatic or bactericidal effects, the \textit{1}O\textsubscript{2} generated from E-101 is an electrophilic oxygenating agent with a broad spectrum of reactivity. The ultimate effect of \textit{1}O\textsubscript{2} is to disrupt vital enzymatic processes or the structural integrity of microbes, thereby leading to their rapid death. \textit{1}O\textsubscript{2} can inhibit microbial enzymes by oxidizing specific amino acids essential to enzymatic catalytic activity. \textit{1}O\textsubscript{2} also reacts readily with amino acids associated with the catalytic sites of enzymes (e.g. histidine and tryptophan) and can react readily with unsaturated lipids, nucleic acids and sulphur-containing biological molecules.\textsuperscript{33} The pMPO:GO-mediated microbicidal action of E-101 is several orders of magnitude more potent than that of the H\textsubscript{2}O\textsubscript{2} that is formed alone in the absence of pMPO.\textsuperscript{7} The rapid rate of kill, as demonstrated by time–kill studies, further supports the proposition that \textit{1}O\textsubscript{2} accounts for the mechanism of action.

Resistance studies showed that E-101 does not select for stable resistance in \textit{S. aureus}, \textit{E. faecalis}, \textit{P. aeruginosa} or \textit{E. coli}. Owing to the mechanism of action of E-101 and the short exposure time required for microbial activity, the selective pressure on microorganisms exerted by E-101 is limited and reduced susceptibility should not convey resistance at concentrations proposed for clinical application. Moreover, in the drug interaction studies, E-101 did not interfere with the activity of conventional antibiotics. This is important since E-101 is intended to be used in conjunction with the standard-of-care measures in a surgical setting, including the use of prophylactic antibiotics.

In conclusion, E-101 showed excellent bactericidal activity. The distinct advantages of using E-101 in the prevention of wound infections are its demonstrated rapid action and its broad spectrum of activity. In fact, the rapid decrease in bacterial inocula following E-101 treatment should increase the effectiveness of any prophylactic antimicrobials used. E-101 attacks molecular sites essential for metabolism and viability.\textsuperscript{7} E-101 also has the advantage of being equally effective against both susceptible and antibiotic-resistant bacteria. The low propensity of microbes treated with E-101 to develop resistance and the lack of drug–drug interaction (antagonism) further support the concept that E-101 may play an important future role in reducing SSIs.

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### Transparency declarations
G. A. D. serves as a consultant for Exoxemis, Inc. P. G. is an employee of Eurofins, a Clinical Research Organization (CRO) focused on core general laboratory and microbiology services for clinical development. Eurofins was paid by Exoxemis, Inc. for access to its bacterial strains and research facilities and equipment for this study. P. O. serves as Executive Vice-President of Clinical Development and Regulatory Affairs of Exoxemis, Inc. J. T. S. serves as President and CEO of Exoxemis, Inc.

### References

### Table 4. Results of in vitro selection of E-101-resistant organisms and stability of resistance

<table>
<thead>
<tr>
<th>Organism/strain</th>
<th>E-101 MIC (mg pMPO/L)</th>
<th>baseline</th>
<th>day 21</th>
<th>3 day stability\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. aureus} 29213 and 1288199</td>
<td>0.015 and 0.015</td>
<td>0.03 and 0.03</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>\textit{E. faecalis} 29212 and 51299</td>
<td>0.25 and 0.25</td>
<td>0.5 and 0.25</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>\textit{E. coli} 25922</td>
<td>0.25 and 0.25</td>
<td>0.25</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>\textit{E. coli} 1337451</td>
<td>0.12</td>
<td>8</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>\textit{E. coli} 1337019</td>
<td>0.25</td>
<td>8</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>\textit{E. coli} 1075701 parent strain</td>
<td>0.5</td>
<td>8</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>\textit{E. coli} 1075701 putative mutant\textsuperscript{b}</td>
<td>0.5</td>
<td>8</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>\textit{P. aeruginosa} 27853 and 1077561</td>
<td>0.06 and 0.06</td>
<td>0.03 and 0.06</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

NT, not tested.
\textsuperscript{a}MIC after three passages on drug-free agar plates to determine the stability of increased MICs.
\textsuperscript{b}Represents repeat testing after an original 21 passages, resulting in an elevated MIC of 4 mg pMPO/L.


