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## 1,2,4-Triazolidine-3-thiones Have Specific Activity against *Acinetobacter baumannii* among Common Nosocomial Pathogens

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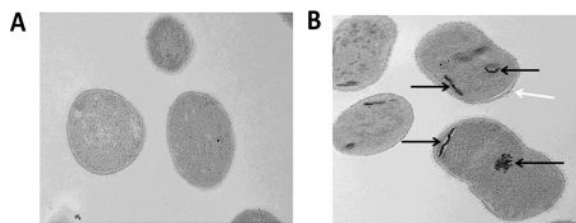
### Abstract

*Acinetobacter baumannii* are Gram-negative bacilli that pose a constant threat to susceptible patients because of increased resistance to multiple antibiotics and persistence in the hospital environment. After genome analysis, we discovered that *A. baumannii* harbors genes that share homology to an enzymatic pathway that elongates long-chain fatty acids (LCFA) in fungi. Previously, 1,2,4-triazolidine-3-thiones (T-3-Ts) were shown to inhibit hyphae production in fungi, and this same LCFA elongation pathway was implicated as the possible target. Therefore, we investigated if T-3-Ts also have activity against multidrug-resistant *A. baumannii*. Surprisingly, all of the clinical isolates of *A. baumannii* that were tested have susceptibility to ECC145 and ECC188 with MIC<sub>90</sub> values of 8.0 µg/mL. In contrast, reference strains and clinical isolates of other common nosocomial bacteria that lack the LCFA pathway also lacked susceptibility. Time-kill experiments revealed that both ECC145 and ECC188 have a bacteriostatic effect against *A. baumannii*. Mass spectrometry analysis suggested that exposure to T-3-Ts resulted in less LCFA production. Supplementation of media with either 0.02% w/v oleic or linoleic acid abrogated the bacteriostatic effect of the compounds, which again implicated LCFA elongation as the target. Our results suggest these molecules could be a promising start to further exploit what appears to be an important aspect of *A. baumannii* membrane function and integrity.

### Graphical abstract

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## Keywords

ESKAPE pathogens; fatty acids; antibiotic; antibacterial; fungi; Gram-negative membrane

To date, limited progress has been made in developing new antibacterial treatments for multidrug-resistant (MDR) and extremely drug-resistant (XDR) ESKAPE pathogens, despite the need for new treatments.<sup>1,2</sup> The acronym ESKAPE, which stands for *Enterococcus faecium* (E), *Staphylococcus aureus* (S), *Klebsiella pneumoniae* (K), *Acinetobacter baumannii* (A), *Pseudomonas aeruginosa* (P), *Enterobacter* species (E), is reflective of these bacterial species that have the ability to “escape” antibiotic treatment in clinical settings.<sup>3</sup> Of these species, perhaps the most troubling are the Gram-negative bacterial species *K. pneumoniae* and *A. baumannii* because of their rapid acquisition of resistance to antibiotic treatments<sup>4</sup> and because the mortality rates in susceptible patients can be high, sometimes >50%.<sup>5,6</sup> *A. baumannii* is particularly worrying as it has been shown that this species can also serve as a reservoir for antibiotic resistance genes for other bacterial species.<sup>7</sup>

The Dunn laboratory at the Uniformed Services University of the Health Sciences has spent many years evaluating the enzymes required for long-chain fatty acid (LCFA) synthesis and elongation in a variety of organisms. In 2009, it was discovered that Tsc10p from *Saccharomyces cerevisiae* had a mammalian homologue, FVT1, which could complement Tsc10p function in yeast, and that this protein is the 3-ketosphinganine reductase for mammalian cells.<sup>8</sup> In addition to this work, the homology of Tsc10p to prokaryotic genomes was also evaluated, with homologues discovered in multiple bacterial taxa and species; however, it was noted that, of these, *A. baumannii* was one of the only common nosocomial pathogens to have a homologue of this gene. The acquisition of fungal DNA by *A. baumannii* is a possible evolutionary event given that the *A. baumannii* genome is very plastic,<sup>9-11</sup> and these bacteria likely interact with<sup>12,13</sup> and potentially kill fungi<sup>14</sup> in the human body.

As genome sequencing has become more affordable and relatively easy to execute, multiple genomes of *A. baumannii* have now become available for analysis.<sup>10,11,15,16</sup> Therefore, we evaluated the *A. baumannii* genomes for genes that might be related to LCFA synthesis and elongation using BLAST and BLASTp.<sup>17</sup> Specifically, we discovered that many genes in the *Candida albicans* and *S. cerevisiae* LCFA biosynthetic pathway had homologues to genes in the AB5075 genome,<sup>18</sup> as well as an Ole1p homologue [(expected value =  $4.0 \times 10^{-51}$ , identities = 100/239 (42%), positives = 147/239 (62%)], which is annotated as a 9 stearoyl-CoA desaturase responsible for converting saturated fatty acyl-CoA substrates to monounsaturated fatty acids (GenBank: AKA30381.1).<sup>19,20</sup> Therefore, we performed a

larger BLASTp analysis against all *Acinetobacter* species and found that the Ole1p homologue was present in all sequenced *A. baumannii* strains as well as other *Acinetobacter* species linked to infection including *A. Iwoffii*, *A. junii*, *A. nosocomialis*, and *A. ursingii*.

The Ole1p homologue was specifically of interest because a previous study by Xu et al. had described a series of ChemBridge library compounds, 1,2,4-Triazolidine-3-thiones (T-3-Ts), that had whole-cell antifungal activity, and they had speculated that the target of these compounds was the gene *OLE1*, which makes the Ole1p protein.<sup>21</sup> Specifically, the *OLE1*<sup>±</sup> *C. albicans* strain had hypersensitivity to two of the T-3-Ts, ECC145 and ECC188 (Figure 1), which were shown to block hypha formation in *C. albicans*, a known mechanism of virulence.<sup>21</sup> Given that *A. baumannii* possessed an Ole1p homologue, we decided to explore if the same compounds examined by Xu et al., ECC145 and ECC188, had antibacterial activity against *A. baumannii* as well.

## RESULTS AND DISCUSSION

### Susceptibility

Both ECC145 and ECC188 were evaluated against type strains of ESKAPE pathogens and clinical isolates to include *S. aureus*, a Gram-positive pathogen, and *Escherichia coli*, which are also linked to hospital-acquired infections and have a relatively different set of genes with respect to metabolism and physiology when compared to *Acinetobacter*.<sup>22</sup> Only the *A. baumannii* strains showed susceptibility at concentrations below 128 µg/mL for either ECC145 or ECC188 (Table 1). Both compounds had activity against *A. baumannii* with MIC<sub>50</sub> and MIC<sub>90</sub> values of 8.0 and 16 µg/mL, respectively. In contrast, other ESKAPE pathogens had MIC values >128 µg/mL, and an MIC of 512 µg/mL was not uncommon (Table 1).

To further evaluate the activity of ECC145 and ECC188, we evaluated a diverse set of 44 different *A. baumannii* isolates, where the diversity of 33 of these isolates has been previously detailed.<sup>23</sup> For both compounds, the MIC<sub>90</sub> was 8.0 µg/mL for these isolates (Table 1). The range of MIC varied from 2.0 to 16 µg/mL for ECC145, and the range of MIC for ECC188 was between 1.0 and 8.0 µg/mL (Table 1). These results suggest that the target of ECC145 and ECC188 is shared among a multitude of different *A. baumannii* strains. Importantly, no strain, despite the diversity, displayed an inherent resistance to these compounds when using this assay.

### Time–Kill Assay

The observed inhibition was further characterized by the time–kill assay. AB5075, a reference strain,<sup>23</sup> was challenged with 1 or 4 times the determined MIC for either ECC145 or ECC188 (Figure 2). Cultures treated with ECC145 at 1 times the MIC showed no growth at 4 h with a mean CFU of  $1.27 \times 10^6$  CFU/mL, but grew to untreated levels of  $1.45 \times 10^9$  CFU/mL by 24 h, whereas cultures treated with 4 times the MIC had CFU/mL reduced to  $5.33 \times 10^5$  at 4 h post-treatment and were further reduced to  $2.19 \times 10^5$  by 24 h post-treatment (Figure 2A). Cultures treated with ECC188 at 1 times the MIC showed a similar initial bacteriostatic effect at 4 h, but reached a mean CFU/mL of  $4.22 \times 10^7$  after 24 h

(Figure 2B). Similarly, cultures treated with 4 times the MIC of ECC188 had CFU/mL reduced to  $5.87 \times 10^5$  by 4 h and were reduced nearly 1.0 log to  $1.17 \times 10^5$  CFU/mL at 24 h post-treatment. These results suggest the mechanism of action of T-3-Ts against *A. baumannii* is bacteriostatic.

### Transmission Electron Microscopy Analysis

To further understand the mechanism of action, we hypothesized that electron microscopy may be able to provide some insight. *A. baumannii* cells were treated with 8.0  $\mu\text{g/mL}$  ECC145 or 8.0  $\mu\text{g/mL}$  ECC188 for 1 h and were then compared to untreated controls utilizing thin-section transmission electron microscopy. Interestingly, the treated cells appeared to have aggregates or inclusion bodies (Figure 3B,C), whereas untreated bacteria did not harbor these darker areas of staining (Figure 3A). Also, the membrane in treated cells appeared to be disrupted, especially at the poles, where a portion of membrane extended from the rest of the bacterium (Figure 3B,C). Again, this phenotype was not observed in untreated cells (Figure 3A).

### Mass Spectrometry Analysis

On the basis of the EM data, we speculated that the negative impact on growth could be linked to overall deficiencies in the membrane, similar to what was seen in fungi when treated with ECC145 and ECC188.<sup>21</sup> To further evaluate the effects of T-3-Ts treatment on *A. baumannii*, we analyzed the overall fatty acid content of the membrane of the bacteria via mass spectrometry in the negative ion mode. AB5075 was exposed to ECC145 or ECC188 for 6 h at 1 times the MIC (8.0  $\mu\text{g/mL}$ ) or 4 times the MIC (32  $\mu\text{g/mL}$ ) and compared to untreated controls. The untreated group showed two major populations of phospholipids, a species at  $m/z$  716.5, phosphatidylethanolamine (PE), and a species at  $m/z$  747.5, phosphatidylglycerol (PG). Both of these species contain 16/18:1 fatty acids attached to the head groups. Treatment with ECC145 and ECC188 over time not only appeared to result in the incorporation of less 18:1 fatty acids to the membrane but also seemed to alter the overall amount of total PE in the samples (Figure 4). Interestingly, the samples treated at 4 times the MIC for 6 h show a large increase in the short-chain PG species indicated at  $m/z$  721.5. Additionally, there was a shift from  $m/z$  773.5 and 747.4 to the species at  $m/z$  775.5 and 749.5, respectively (data not shown). Both of these shifts indicate a change in fatty acid usage from saturated to unsaturated fatty acid and suggest treatment by T-3-Ts abrogates 16/18:1 fatty acid development as was also shown in *C. albicans*<sup>21</sup> and is a known function of Ole1p.<sup>19,20</sup>

### Fatty Acid Supplementation

Because the mass spectrometry and EM data implicated T-3-Ts in the disruption of fatty acid elongation with detrimental consequences to the bacteria, we hypothesized that perhaps *A. baumannii* could acquire LCFA from the environment to abrogate the effects of the T-3-Ts. To test this hypothesis in vitro, we supplemented CAMHB media used in the MIC assay with 0.02% linoleic acid. The addition of linoleic acid increased the observed MIC of ECC145 for *A. baumannii* 5075 from 8.0 to  $>256 \mu\text{g/mL}$ . This effect was confirmed in a time-kill assay (Figure 5). Whereas cultures treated with ECC145 at 4 times the MIC displayed the previously observed bacteriostatic activity, the cells treated with ECC145 with

0.02% linoleic acid in the CAMHB media showed growth 2 logs above initial inoculum to  $2.13 \times 10^8$  CFU/mL by 4 h post-treatment and reached a final CFU/mL of  $4.26 \times 10^8$  after 24 h, closer to that of untreated cultures ( $1.32 \times 10^9$  CFU/mL) (Figure 5). A similar result was seen when *A. baumannii* was treated with ECC188 (data not shown). Therefore, we tested the addition of other unsaturated fatty acids, specifically oleic acid and Tween 80 (polysorbate 80), which is a complex mixture of polyoxyethylene ethers of partial oleic acid esters of sorbitol anhydrides as well as other intermediates.<sup>24</sup> Both of these sources of unsaturated fatty acids also abrogated T-3-T activity in an MIC assay (Table 2), which suggests the target of T-3-Ts is a pathway that involves unsaturated fatty acid and elongation of fatty acids as predicted previously.<sup>21</sup>

### Targeting and Resistance

To determine a target for the T-3-Ts, a number of experiments were attempted. First, we analyzed our transposon mutant libraries of *A. baumannii*.<sup>18,23</sup> The gene encoding the Ole1p is annotated as a stearyl-CoA 9-desaturase, and the current identifier is ABUW\_0611 for the ABU5075 genome.<sup>18</sup> Given the experimental data from Xu et al. that seemed to implicate Ole1p as the target of T-3-Ts,<sup>21</sup> it should be an essential gene in *A. baumannii*, and any transposon mutant would not have survived when the mutants were first generated. To our surprise, we found multiple transposon mutants in the gene encoding the Ole1p homologue, and ABUW\_0611 does not appear to be an essential gene.<sup>18</sup> Nonetheless, we tested the ABUW\_0611 mutant in the case Ole1p homologue worked in concert with other enzymes to elongate fatty acids, which, in that case, an increase in MIC might have been detected in this mutant background. When the transposon mutants of ABUW\_0611 were exposed to ECC145 or ECC188, we did not see any abrogation of activity and the MIC remained 8.0  $\mu$ g/mL (data not shown). Furthermore, we evaluated the whole pool of transposon mutants in the presence of ECC145 at increasing concentrations of 8.0, 16, and 32  $\mu$ g/mL to identify mutants that were not susceptible to T-3-Ts. Although some colonies arose on these plates in the first 24 h, none of these bacteria were able to be further passaged, suggesting that if a specific transposon mutant was allowing survival in the presence of ECC145, it was not stable.

In another attempt to determine the target of T-3-Ts, we also checked for the development of spontaneous mutants at 0.5, 1, and 2 times the MIC of ECC145 on plates for 24 h. No colonies arose on any plates over this time period (data not shown). Lastly, we evaluated resistance by serial passaging *A. baumannii* in the presence of 2-fold increasing concentrations of ECC145 (2–32  $\mu$ g/mL). After 10 passages, growth was finally observed in the 32  $\mu$ g/mL tube (data not shown), suggesting a spontaneous mutant occurred that allowed for growth. However, again, we could not further passage from this point into fresh media, suggesting the mutant was not stable.

### Further Mechanistic Studies

Given that Ole1p does not appear to be essential in *A. baumannii* or the target of the compounds, we ran a number of additional experiments to determine whether these compounds could be acting as phenotypic Pan-Assay Interference (PAINS) compounds in our assays. First, given that fatty acid supplementation abrogated activity, we investigated

whether this phenomenon was due to simple disruption of ECC145 aggregation. In the presence of 0.01% Triton, we observed no change in MIC, and ECC145 inhibited AB5075 growth at 8  $\mu\text{g/mL}$ . We observed the same activity in the presence of other detergents, such that growth inhibition studies in media supplemented with either 0.1% PEG400 or 0.1% tyloxagol also returned MIC values of 8.0  $\mu\text{g/mL}$ . Thus, aggregation does not appear to play a role in the anti-*A. baumannii* activity of 3-T-3s.

Next, given the inability to generate stable resistant mutants, we probed whether the mechanism of action could simply be due to membrane disruption. We performed a BacLight assay on AB5075 using 0.5, 1, and 2 times the MIC of ECC145 and observed no significant membrane disruption at any of these concentrations. We also assessed the hemolytic potential of ECC145 and also observed no hemolysis (<3%) up to the highest concentration studied (512  $\mu\text{g/mL}$ ). Therefore, membrane disruption does not appear to underpin antimicrobial activity.

Finally, 3-T-3s may have some hydrolytic instability; however, the stability of the lead compound ECC145 has not been established in bacterial growth broth. We performed a stability study in CAMHB and observed steady degradation of ECC145 over a 24 h period (determined by LC analysis). On the basis of these data, we determined the half-life of ECC145 to be ca. 6–8 h under these conditions. Given that degradation is happening on the time scale of the experiment, one additional explanation is that the compounds are not the actual active species, but the degradation products are. To probe this, we allowed ECC145 to incubate in CAMHB for either 4 or 8 h prior to bacterial treatment. The 4 h pre-incubation still delivered a solution that was able to inhibit growth at 8  $\mu\text{g/mL}$  (original concentration); however, the 8 h pre-incubation was inactive, and we observed visible growth at the MIC (8  $\mu\text{g/mL}$  original concentration). Therefore, the degradation products due to hydrolysis of ECC145 do not appear to be the active constituents in this assay.

## DISCUSSION

In this study, we found that *A. baumannii* harbors some genes required for LCFA elongation similar to what is found in yeast and fungi, which could be an attractive target for drug design. Given this bacterial species is rapidly becoming resistant to all clinically used antibiotics, strides to further understand its physiology and what is required for its pathogenesis remain critical. The presence of this LCFA pathway appears to be somewhat unique to *A. baumannii* when compared to other common nosocomial pathogens, which also speaks to the unique metabolism and biochemistry pathways this bacterial species appears to have encoded in its genome. Whereas *P. aeruginosa* is often called a “cousin” of *Acinetobacter*, the genomes share only 58% homology.<sup>22</sup> When compared to the *E. coli* genome, the *A. baumannii* genome is missing enzymatic pathways, or there are significant changes in pathways, suggesting its somewhat unique metabolism.<sup>22</sup>

Separately, the versatility and plasticity of the *A. baumannii* genome was shown given the possible acquisition of these genes from the *C. albicans* or *S. cerevisiae*. As stated in the introduction, it is not completely unexpected given the potential interactions between fungi and *A. baumannii*. This would also not be the first time *A. baumannii* acquired DNA from



other organisms and hijacked the function if it suited the bacteria's pathogenic lifestyle requirements. For example, *A. baumannii* utilizes antibiotic resistance genes from various bacterial sources and incorporates this DNA into its chromosome.<sup>11</sup> It should also be noted that *A. baumannii* has a gene that is homologous to *Plasmodium* species (our unpublished data), and recently it was shown that bacteriophage DNA in its genome encodes small regulatory RNAs that the bacteria have usurped for regulatory functions.<sup>25</sup>

One drawback to our study is that we do not know if the Ole1p homologue or the other genes we found in the LCFA pathway are expressed and utilized by *A. baumannii*, and it is certainly possible that other bacterial species, related to *Acinetobacter*, were a precursor to other more developed genomes of eukaryotes such as fungi. Regardless, at least some of the LCFA genes in the pathway appear functional given the mass spectrometry data that suggest *A. baumannii* is generating 18:1 and 16:0 fatty acids for incorporation into the membrane (Figure 4). This result is also in agreement with a previous study that detected 18:1 and 16:0 fatty acids as the major lipid components of the *A. baumannii* membrane via gas chromatography analysis.<sup>26</sup> The fact that T-3-Ts can block the production and/or shorten fatty acids indicates that this enzymatic pathway can also be disrupted. However, at this point, the actual target or targets of the compounds are unclear, and all that can be concluded is that the membrane does not contain its typical fatty acid content after treatment with T-3-Ts. This conclusion is further supported by the electron microscopy data that indicates an apparent membrane disruption at the poles and the formation of inclusion bodies in the cytoplasm. Moreover, the supplementation of media with linoleum acid and oleic acid, which upon import by *A. baumannii*, abrogates the activity of T-3-Ts (Figure 5).

It is hoped that future efforts to find resistant mutants may identify the target of 3-T-3s in *A. baumannii*. Unexpectedly, the transposon mutant in the Ole1p homologue was still susceptible to T-3-T activity, and this result suggests that the Ole1p homologue is not the target of T-3-Ts. Possibly, T-3-Ts attack multiple enzymes in the pathway, or other desaturases present in the genome could compensate for a loss of function in this mutant background. Another possibility is that T-3-Ts may activate a reciprocal activity rather than block Ole1p function. Recently, a study identified an enzyme in *S. cerevisiae*, Sct1p, that competes with and antagonizes Ole1p activity.<sup>20</sup> Whereas a Sct1p homologue with an *E* value of <1.0 was not found in the *A. baumannii* genome (data not shown), it is still possible that there is another protein not detected by BLASTp analysis that could provide an antagonizing function.

Our work is certainly not the first time researchers have tried to exploit fatty acid synthesis as a target for antibacterials. In fact, many groups have developed inhibitors of FabI, the NADH-dependent enoyl reductase from the type II bacterial fatty acid biosynthesis pathway (FAS-II),<sup>27</sup> as well as targeting other enzymes in this pathway such as FabF and FabG.<sup>28</sup> However, depending on the bacterial species, the addition of exogenous fatty acids can abrogate the activity of LCFA synthesis inhibitors, and fatty acid import in the host can disrupt activity.<sup>28,29</sup> Interestingly, in some Gram-negative bacteria, a shunt pathway was also identified to bypass FAS-II.<sup>30</sup> When in vivo experiments are attempted in the future, we will need to be mindful of these possibilities in the host environment. Nevertheless, just like the



Fab genes, we believe the target of T-3-Ts, once identified, will be important when it comes to possible antibacterial development against *A. baumannii*.

Given that we have not identified the target of the 3-T-3s, we ran a number of experiments to determine whether the activity was simply due to a PAINS-type mechanism. Aggregation does not appear to drive activity, as ECC145 is equally active in the absence or presence of detergents (Triton, PEG400, or tyloxagol). Generalized membrane disruption also does not appear to drive antibacterial activity, as ECC145 does not significantly disrupt membrane permeation up to 2 times its MIC as determined by a BacLight assay, nor does it have hemolytic activity. The half-life of the compound was determined to be ca. 6–8 h in CAMHB, and activity does not appear to be driven by degradation products as pre-incubation of ECC145 with CAMHB for 8 h abrogated activity. Although this relative hydrolytic instability could be problematic in an in vivo setting, we note that there is ample precedent for hydrolytically unstable antibiotics (i.e., imipenem) that still show tremendous clinical utility.

In conclusion, we demonstrated that T-3-Ts have a pronounced bacteriostatic effect in vitro and that the most likely outcome of this treatment is blocking the production of LCFA required for complete membrane integrity and cell division. We showed that *A. baumannii* can bypass this antibacterial activity when in the presence of exogenous unsaturated fatty acids added to growth media, which further implicates 18:1 and 16:0 fatty acids being the affected membrane species. With this knowledge, we can try to identify the protein target(s) of T-3-Ts, design compounds with more activity using SAR and ADME analysis for in vivo testing, and begin to think of combinational therapies that synergize with blocking the synthesis and elongation of LCFA.

## MATERIALS AND METHODS

### Bacterial Strains and Antimicrobial Agents

The bacteria (a total of 83 strains) used in this study included 44 *Acinetobacter baumannii*, 10 *Klebsiella pneumoniae*, 10 *Escherichia coli*, 9 *Staphylococcus aureus*, and 10 *Pseudomonas aeruginosa* and can be found in Table 3. All strains were maintained and subcultured in lysogeny broth (LB) in liquid culture or on plates until utilized in various assays outlined below. T-3-Ts, ECC145 and ECC188, were procured from ChemBridge Co. (San Diego, CA, USA). Working stocks were prepared by dissolving the compounds in dimethyl sulfide (DMSO) to a final concentration of 25 mg/mL.

### Minimum Inhibitory Concentration (MIC)

MIC end points were determined according to the broth microdilution methodology recommended by the Clinical and Laboratory Standards Institute (CLSI). However, one slight modification is utilized; test strains were grown overnight at 37 °C in cation-adjusted Mueller–Hinton broth (CAMHB) and diluted with fresh CAMHB to yield an initial inoculum of approximately  $5.0 \times 10^5$  CFU/mL in 96-well plates as opposed to using single colonies. Each test strain was challenged with ECC145 or ECC188 across a concentration range from 512 to 1 µg/mL in 2-fold increments. Plates were incubated at 37 °C for 20–22 h

before the MIC end points were read. MIC<sub>90</sub> and MIC<sub>50</sub> are the lowest concentrations of the antibiotic at which 90 and 50% of the isolates were inhibited, respectively. Subsequent MIC analysis included the supplementation of media with either 0.02% w/v oleic, linoleic acid, or polysorbate 80 (Sigma-Aldrich). Additional MIC supplementation assays with detergents were performed with CAMHB as above with 0.01% Triton, 0.1% PEG400, or 0.1% tyloxagol.

### Time–Kill Assays

*A. baumannii* strain 5075 (AB5075) was grown overnight at 37 °C in CAMHB and diluted in 25 mL of fresh CAMHB to yield an initial inoculum of approximately 10<sup>6</sup> CFU/mL. ECC145 or ECC188 was added to a final concentration of 0, 1, or 4 times the MIC, and cultures were incubated with shaking at 37 °C. Samples were taken at 0, 4, and 24 h post addition of the tested compound. To test the ability of unsaturated fatty acids to abrogate the activity of ECC145, the growth medium was supplemented with 0.2% w/v linoleic acid (Sigma-Aldrich). CFU/mL was determined by dilution and plating utilizing a spiral plater (Advanced Instruments, Inc.) for each sample in triplicate.

### Electron Microscopy

AB5075 was grown overnight at 37 °C in CAMHB and diluted in 25 mL of fresh CAMHB to yield an initial inoculum of approximately 10<sup>6</sup> CFU/mL. ECC145 or ECC188 was added to a final concentration of 0 or 8 µg/mL, and cultures were incubated with shaking at 37 °C. After 1 h, samples were collected from each condition and (fixed using 4% formaldehyde and 1% glutaraldehyde). Samples were rinsed twice in 0.1 M phosphate buffer for 5 min. Samples were then subjected to En-Bloc staining (2% uranyl acetate aqueous) for 30–45 min. Samples were then dehydrated for 15 min in 50, 75, and 95% ethanol (aqueous), followed by two treatments with 100% ethanol, again for 15 min. Samples were then placed in 100% propylene oxide twice for 15 min, followed by 20% Epon and 80% propylene oxide for 60 min, 50% Epon and 50% propylene oxide overnight, and finally 100% Epon for 60 min. The samples were then embedded in preassembled Beem capsules and baked at 70 °C for 12–24 h. Thin sections were prepared on an Ultracut Microtome (Leica). Sections were imaged on a Jeol 1400 electron microscope.

### Total Lipid Extraction and Mass Spectrometry Analysis

Total membrane lipids were extracted from whole cells using a two-step Bligh–Dyer extraction protocol. Briefly, to a sample containing 50 mg of dry cells were added 200 µL of water, 250 µL of chloroform, and 500 µL of methanol to generate a monophasic solution. The solution was briefly vortexed and shaken for 3 h at room temperature. Subsequently, to each tube were added 250 µL each of water and chloroform to generate a biphasic solution, and the mixtures were vortexed and shaken for 30 min at room temperature. Phases were separated by centrifugation (3000 rpm for 10 min at room temperature). The lower chloroform phase was removed and evaporated under nitrogen.

Negative ion matrix-assisted laser ionization desorption–time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS) experiments were performed. Briefly, total lipids were solubilized in 100 µL of chloroform/methanol (2:1, v/v) and spotted (1 µL) directly

onto the MALDI sample plate, followed by 1  $\mu$ L of 100 mg/mL norharman MALDI matrix dissolved in chloroform/methanol/water (3:1.5:0.25, v/v/v). All experiments were performed using a Bruker Autoflex Speed MALDITOF/TOF mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). Each spectrum was an average of 500 shots and 50% laser power. ES Tuning Mix (Agilent, Palo Alto, CA, USA) was used as a calibrate standard, calibrated using Agilent Tuning Mix (Agilent Technologies, Foster City, CA, USA).

### Hemolysis Assay

Hemolysis assays were performed on mechanically defibrinated sheep blood (Hemostat Laboratories: DSB100). Defibrinated blood (1.5 mL) was placed into a microcentrifuge tube and centrifuged for 10 min at 10,000 rpm. The supernatant was then removed, and then the cells were resuspended in 1 mL of phosphate-buffered saline (PBS). The suspension was centrifuged, the supernatant was removed, and cells were resuspended two additional times. The final cell suspension was then diluted 10-fold. Test compound was added to aliquots of the 10-fold suspension dilution of blood. PBS was used as a negative control and a zero hemolysis marker. Triton  $\times$  (a 1% sample) was used as a positive control serving as the 100% lysis marker. Samples were then placed in an incubator at 37  $^{\circ}$ C while being shaken at 200 rpm for 1 h. After 1 h, the samples were centrifuged for 10 min at 10,000 rpm. The resulting supernatant was diluted by a factor of 40 in distilled water. The absorbance of the supernatant was then measured with a UV spectrometer at a 540 nm wavelength.

### Bacterial Membrane Permeabilization Assay

The BacLight assay (Invitrogen) was used to assess membrane permeability. AB5075 was grown overnight in CAMHB at 37  $^{\circ}$ C with shaking. The culture was diluted 1:40 in CAMHB and grown to an optical density at 600 nm ( $OD_{600}$ ) of  $\sim$ 1.0 ( $\sim$ 4 h of growth). The cultures were centrifuged at 10000g for 15 min, the cell pellet was washed once with sterile water, resuspended at 2 times the original volume, and aliquoted, and test compounds were added. Suspensions were incubated at 37  $^{\circ}$ C with shaking for 1 h and then centrifuged at 10000g for 10 min, washed once with sterile water, and resuspended in water. A 1:1 mixture of SYTO-9 and propidium iodide was added to the suspension (3  $\mu$ L/mL) and mixed well. One hundred microliters of the suspension was added to each well of a 96-well plate, and the plates were incubated in the dark for 15 min at room temperature. Green fluorescence (SYTO-9) was read at 530 nm, and red fluorescence (propidium iodide) was read at 645 nm (excitation wavelength, 485 nm). The ratio of green to red fluorescence was expressed as a percentage of the control.

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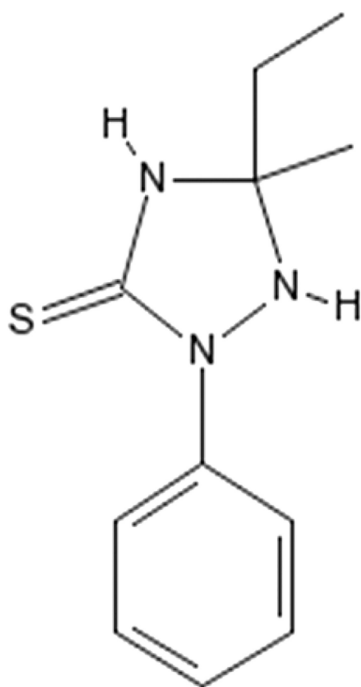
B.W.C., M.G.T., A.C.J., E.A.A.-A., and D.V.Z. were supported by W0136\_15\_WR, a grant from the Military Infectious Diseases Research Program. W.M.H., R.J.M., and C.M. were supported by NIH Grant GM055769.

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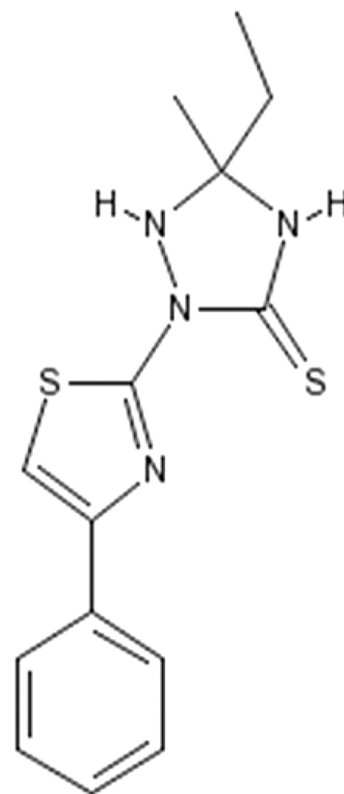
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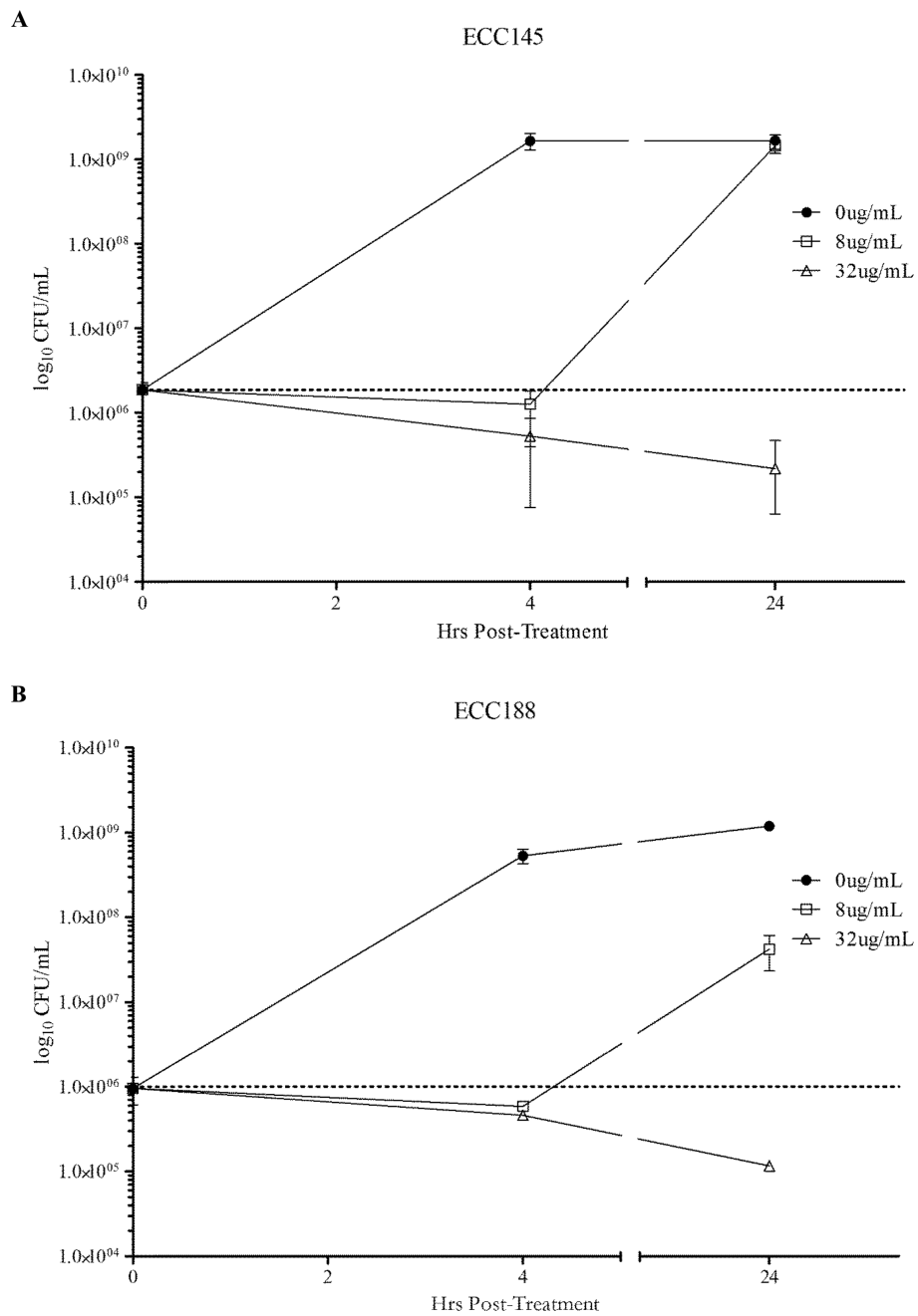
## ECC145



## ECC188

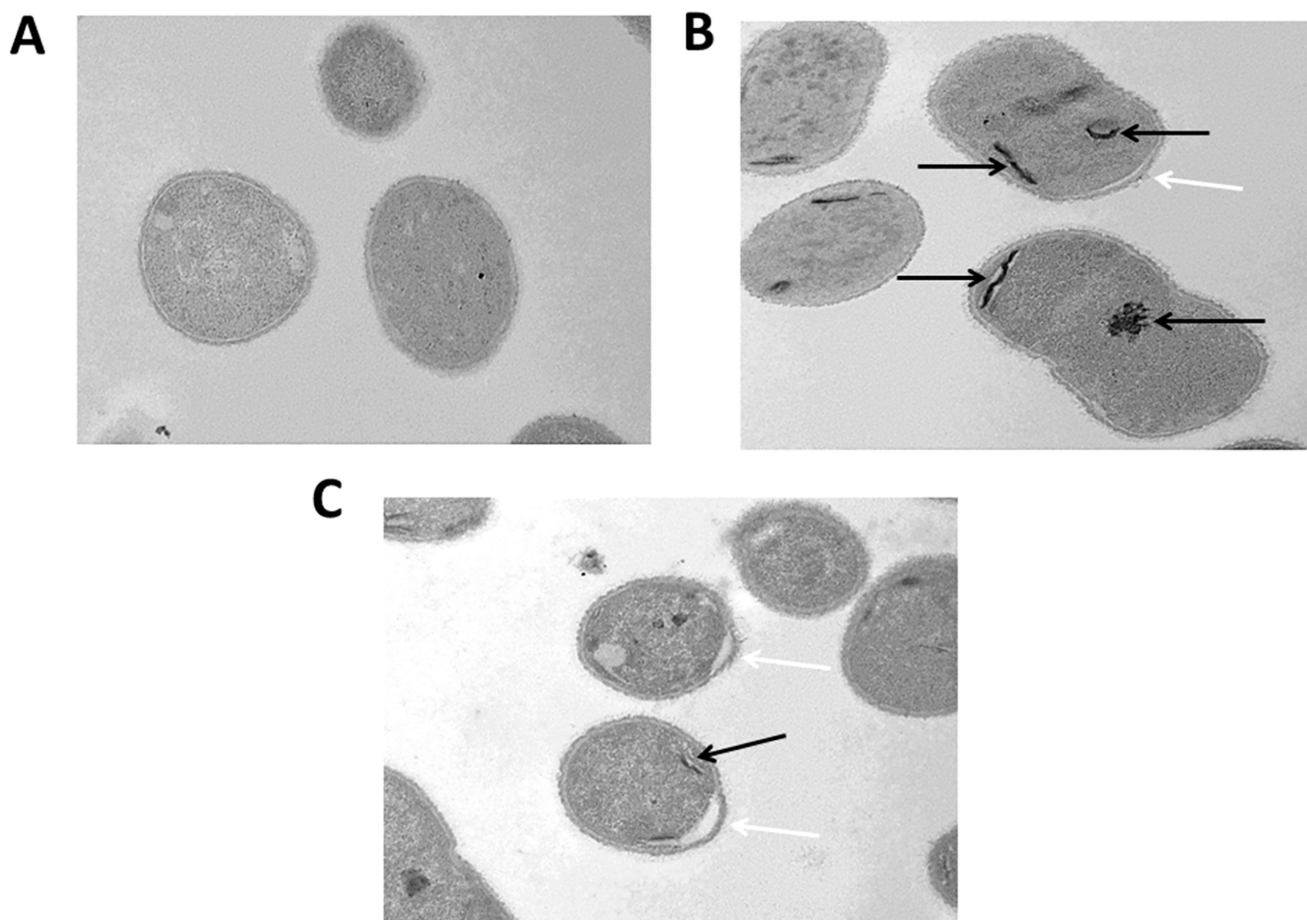


**Figure 1.**  
Chemical structures of the 1,2,4-triazolidine-3-thiones, ECC145 and ECC188, used in this study.

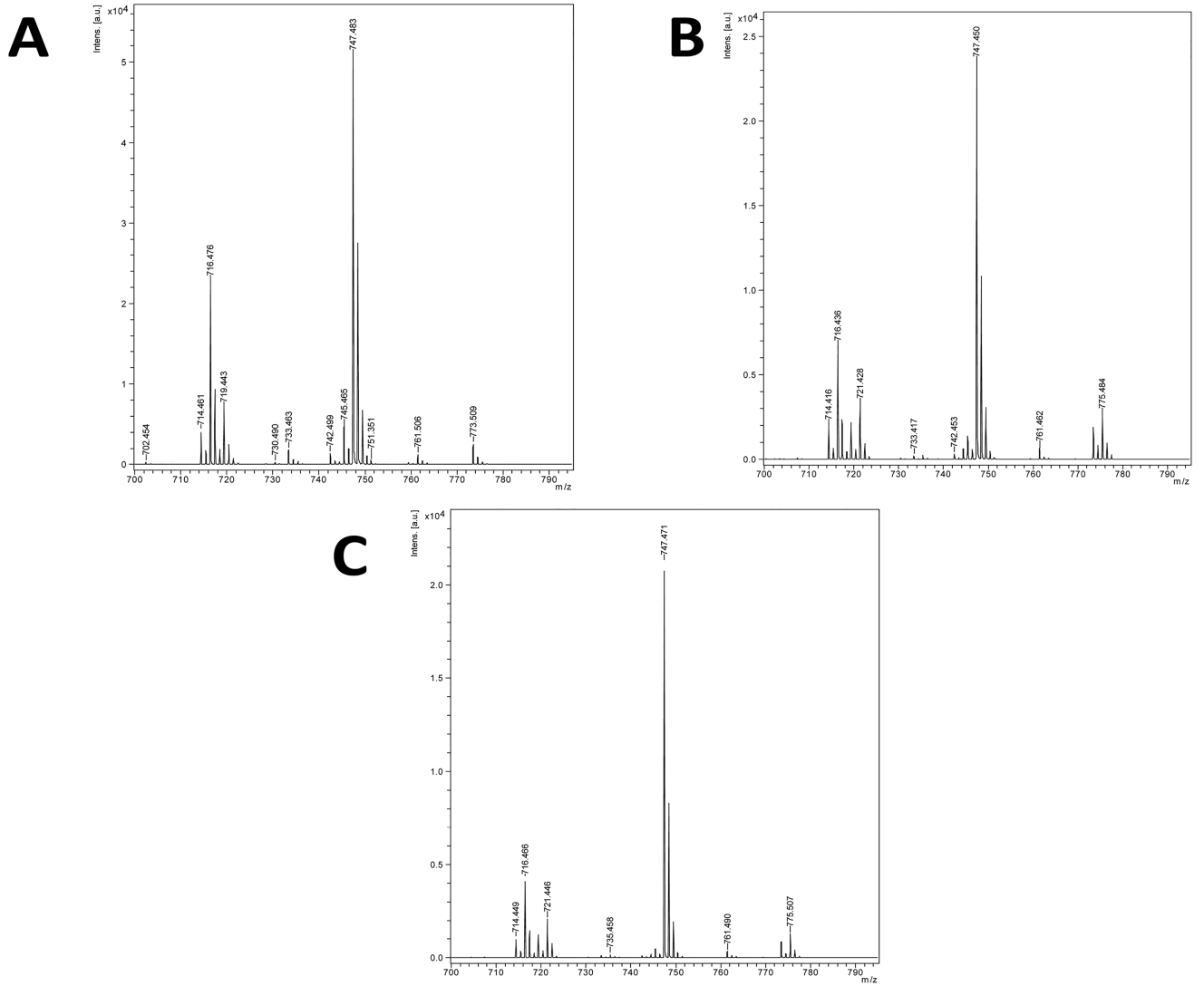


**Figure 2.** Time-kill assays displaying mean and standard error of the mean of CFU/mL at time of initial treatment and at 4 and 24 h post-treatment for ECC145 (A) and ECC188 (B) at 1 and 4 times the MIC determined via microbroth dilution.

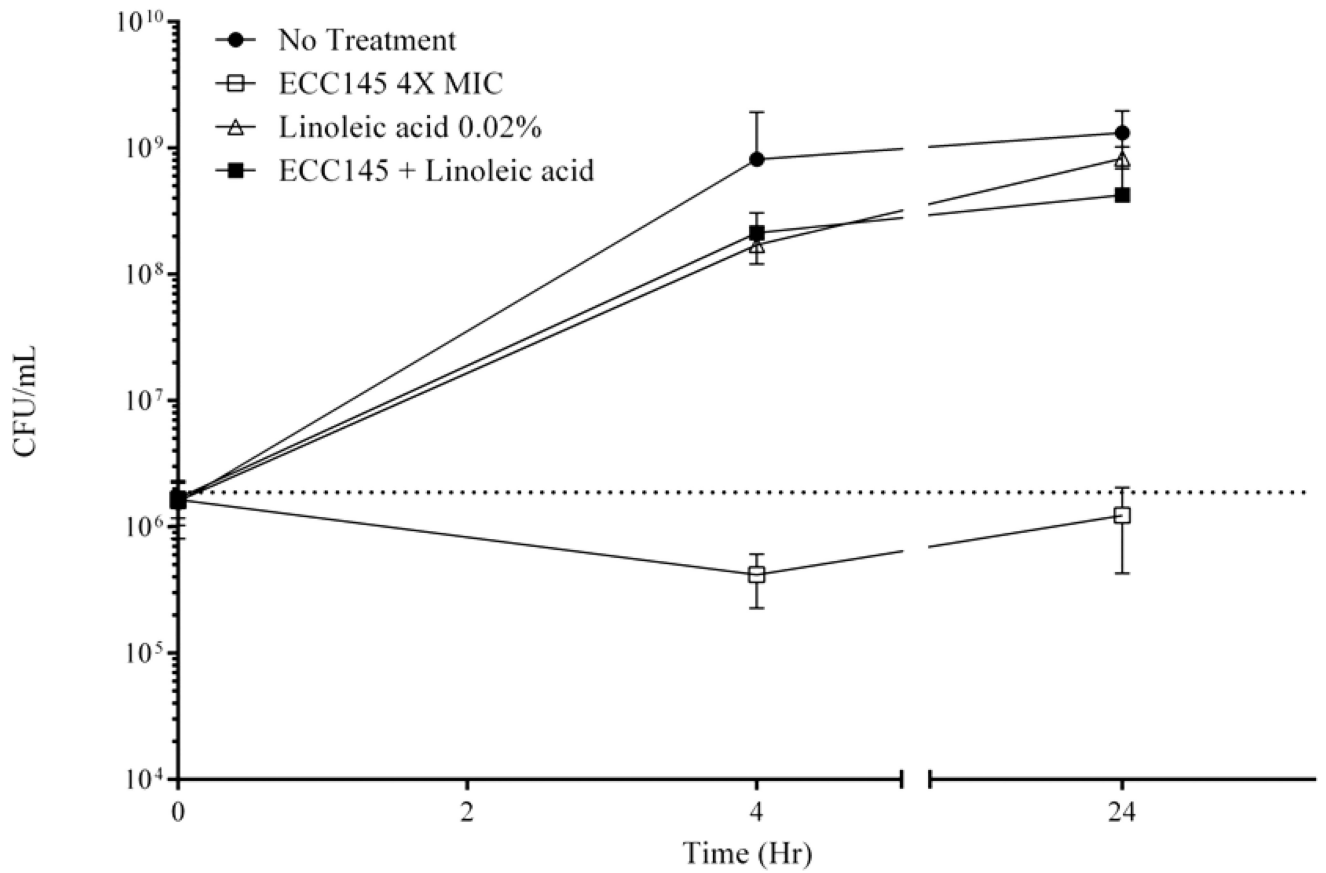




**Figure 3.** Thin-section transmission electron microscopy images (20,000 $\times$ ) of AB5075 untreated (A), exposed to ECC145 at 8.0  $\mu\text{g}/\text{mL}$  (B), or exposed to ECC188 at 8.0  $\mu\text{g}/\text{mL}$  (C) for 1 h. Aggregates are visible in the cytoplasm (black arrows), and membrane disruption (white arrows) is visible on the bacterial surface, distended from the cytoplasm, of the treated bacteria.



**Figure 4.** Mass spectrometry analysis of *A. baumannii* that was untreated (A) or treated with ECC145 for 6 h (B) or with ECC188 for 6 h (C).



**Figure 5.**

Time-kill assays displaying the mean and standard error of the mean of CFU/mL at time of initial treatment and at 4 and 24 h post-treatment for AB5075 treated with either ECC145 at 4 times the MIC, linoleic acid at 0.02% w/v, or a combination of ECC145 and linoleic acid.

**Table 1**  
MIC<sup>a</sup> of ECC145 and ECC188 against Common Nosocomial Bacterial Pathogens

organism (n)	MIC for ECC145 (µg/mL)			MIC for ECC188 (µg/mL)		
	50%	90%	range	50%	90%	range
<i>Escherichia coli</i> (10)	>512	>512	>512	>512	>512	256→512
<i>Staphylococcus aureus</i> (9)	256	256	128→512	256	512	128→512
<i>Klebsiella pneumoniae</i> (10)	>512	>512	>512	>512	>512	>512
<i>Acinetobacter baumannii</i> (44)	8	8	2–16	4	8	1–8
<i>Pseudomonas aeruginosa</i> (10)	512	512	128→512	512	512	256→512

<sup>a</sup>MIC in cation-adjusted Mueller–Hinton broth, 22 h.

**Table 2**MIC ( $\mu\text{g/mL}$ ) of ECC145 and ECC188 against AB5075  $\pm$  Unsaturated Fatty Acids

<b>fatty acid addition</b>	<b>ECC145</b>	<b>ECC188</b>
no addition	4	8
0.02% linoleic acid	256	256
0.02% oleic acid	512	512
0.02% polysorbate 80	512	512

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Table 3

## Sources of Bacterial Strains Used in This Study

bacterial species	strain <sup>d</sup>	source <sup>b</sup>
<i>Acinetobacter baumannii</i>	AB0057	Luis Actis
<i>Acinetobacter baumannii</i>	17978	Luis Actis
<i>Acinetobacter baumannii</i>	19606	Luis Actis
<i>Acinetobacter baumannii</i>	AB307-0294	Anthony Campagnari
<i>Acinetobacter baumannii</i>	A118	Luis Actis
<i>Acinetobacter baumannii</i>	ACICU	Luis Actis
<i>Acinetobacter baumannii</i>	AYE	ATCC
<i>Acinetobacter baumannii</i>	SDF	ATCC
<i>Acinetobacter baumannii</i>	LUH3876	Luis Actis
<i>Acinetobacter baumannii</i>	RUH134	Luis Actis
<i>Acinetobacter baumannii</i>	RUH876	Luis Actis
<i>Acinetobacter baumannii</i>	AB2828	MRSN
<i>Acinetobacter baumannii</i>	AB3340	MRSN
<i>Acinetobacter baumannii</i>	AB3560	MRSN
<i>Acinetobacter baumannii</i>	AB3638	MRSN
<i>Acinetobacter baumannii</i>	AB3785	MRSN
<i>Acinetobacter baumannii</i>	AB3806	MRSN
<i>Acinetobacter baumannii</i>	AB3917	MRSN
<i>Acinetobacter baumannii</i>	AB3927	MRSN
<i>Acinetobacter baumannii</i>	AB4025	MRSN
<i>Acinetobacter baumannii</i>	AB4026	MRSN
<i>Acinetobacter baumannii</i>	AB4027	MRSN
<i>Acinetobacter baumannii</i>	AB4052	MRSN
<i>Acinetobacter baumannii</i>	AB4269	MRSN
<i>Acinetobacter baumannii</i>	AB4448	MRSN
<i>Acinetobacter baumannii</i>	AB4456	MRSN
<i>Acinetobacter baumannii</i>	AB4490	MRSN
<i>Acinetobacter baumannii</i>	AB4498	MRSN

bacterial species	strain <sup>a</sup>	source <sup>b</sup>
<i>Acinetobacter baumannii</i>	AB4795	MRSN
<i>Acinetobacter baumannii</i>	AB4857	MRSN
<i>Acinetobacter baumannii</i>	AB4878	MRSN
<i>Acinetobacter baumannii</i>	AB4932	MRSN
<i>Acinetobacter baumannii</i>	AB4957	MRSN
<i>Acinetobacter baumannii</i>	AB4991	MRSN
<i>Acinetobacter baumannii</i>	AB5001	MRSN
<i>Acinetobacter baumannii</i>	AB5075	MRSN
<i>Acinetobacter baumannii</i>	AB5197	MRSN
<i>Acinetobacter baumannii</i>	AB5256	MRSN
<i>Acinetobacter baumannii</i>	AB5674	MRSN
<i>Acinetobacter baumannii</i>	AB5711	MRSN
<i>Acinetobacter baumannii</i>	AB3941 <sup>ColR</sup>	Glen Wortmann
<i>Acinetobacter baumannii</i>	AB3942 <sup>ColR</sup>	Glen Wortmann
<i>Acinetobacter baumannii</i>	AB4106 <sup>ColR</sup>	Glen Wortmann
<i>Klebsiella pneumoniae</i>	KP02615	MRSN
<i>Klebsiella pneumoniae</i>	KP02814	MRSN
<i>Klebsiella pneumoniae</i>	KP02843	MRSN
<i>Klebsiella pneumoniae</i>	KP04776	MRSN
<i>Klebsiella pneumoniae</i>	KP04843	MRSN
<i>Klebsiella pneumoniae</i>	KP05540	MRSN
<i>Klebsiella pneumoniae</i>	KP101436	Clinton Murray/BAMC
<i>Klebsiella pneumoniae</i>	KP101488	Clinton Murray/BAMC
<i>Klebsiella pneumoniae</i>	KP101712	Clinton Murray/BAMC
<i>Klebsiella pneumoniae</i>	KP105371	Clinton Murray/BAMC
<i>Pseudomonas aeruginosa</i>	PA4761	MRSN
<i>Pseudomonas aeruginosa</i>	PA4910	MRSN
<i>Pseudomonas aeruginosa</i>	PA4962	MRSN
<i>Pseudomonas aeruginosa</i>	PA5105	MRSN
<i>Pseudomonas aeruginosa</i>	PA5151	MRSN
<i>Pseudomonas aeruginosa</i>	PA105738	Clinton Murray/BAMC



bacterial species	strain <sup>a</sup>	source <sup>b</sup>
<i>Pseudomonas aeruginosa</i>	PA105777	Clinton Murray/BAMC
<i>Pseudomonas aeruginosa</i>	PA105819	Clinton Murray/BAMC
<i>Pseudomonas aeruginosa</i>	PA105857	Clinton Murray/BAMC
<i>Pseudomonas aeruginosa</i>	PA105880	Clinton Murray/BAMC
<i>Staphylococcus aureus</i>	MRSA5836	MRSN
<i>Staphylococcus aureus</i>	MRSA5971	MRSN
<i>Staphylococcus aureus</i>	MRSA5968	MRSN
<i>Staphylococcus aureus</i>	MRSA6048	MRSN
<i>Staphylococcus aureus</i>	MRSA6081	MRSN
<i>Staphylococcus aureus</i>	MRSA6139	MRSN
<i>Staphylococcus aureus</i>	MRSA103700	Clinton Murray/BAMC
<i>Staphylococcus aureus</i>	MRSA104161	Clinton Murray/BAMC
<i>Staphylococcus aureus</i>	MRSA107261	Clinton Murray/BAMC
<i>Escherichia coli</i>	EC5107	MRSN
<i>Escherichia coli</i>	EC5378	MRSN
<i>Escherichia coli</i>	EC5379	MRSN
<i>Escherichia coli</i>	EC5403	MRSN
<i>Escherichia coli</i>	EC5466	MRSN
<i>Escherichia coli</i>	EC5483	MRSN
<i>Escherichia coli</i>	EC5502	MRSN
<i>Escherichia coli</i>	EC105454	Clinton Murray/BAMC
<i>Escherichia coli</i>	EC105547	Clinton Murray/BAMC
<i>Escherichia coli</i>	EC109497	Clinton Murray/BAMC

<sup>a</sup>CoIR, colistin-resistant isolates.

<sup>b</sup>MRSN, Multidrug-resistant Organism Repository and Surveillance Network.