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**Bactericidal effects of Polyhexamethylene Biguanide against intracellular *Staphylococcus aureus* EMRSA-15 and USA 300**

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Running title: PHMB antimicrobial activity

## **ABSTRACT**

**Objectives:** The treatment of skin infections caused by *Staphylococcus aureus* (*S. aureus*) is limited by acquired antibiotic resistance and poor drug delivery into pathogen and host cells. Here we investigated the antibacterial activities of six topically used antimicrobials and a cationic polymer, polyhexamethylene biguanide (PHMB), against intracellular MSSA, strain RN4420 and MRSA, strains EMRSA-15 and USA 300.

**Methods:** The minimum inhibitory concentrations (MIC) of antimicrobials were determined against MSSA and MRSA strains, and the bactericidal activities of nadifloxacin and PHMB against intracellular MRSA were determined using infected keratinocytes. Fluorescein-tagged PHMB (PHMB-FITC) was used to study PHMB uptake, colocalisation with intracellular EMRSA-15 and retention in keratinocytes. The mechanism(s) of PHMB uptake into keratinocytes were studied using a dynamin inhibitor, dynasore.

**Results:** Gentamicin, nadifloxacin and PHMB showed the lowest MIC values against MRSA. Nadifloxacin at 10 mg/L killed 80% of intracellular EMRSA-15, but was not effective against USA 300. PHMB at 4 mg/L killed almost 100% of intracellular EMRSA-15 and USA 300. PHMB entered keratinocytes, colocalised with intracellular EMRSA-15 and was retained by the cells for over five hours. PHMB uptake and its intracellular antibacterial activities were inhibited by the dynamin inhibitor, dynasore.

**Conclusion:** PHMB kills intracellular MRSA via direct interaction with pathogens inside keratinocytes and host cell entry is dynamin dependent.

**Keywords:** topical antimicrobials, nadifloxacin, PHMB, intracellular *S. aureus*, keratinocytes

## INTRODUCTION

Skin infections caused by invasive pathogens start when outer skin structures are damaged due to injury, such as a burn,<sup>1</sup> or skin diseases, such as atopic dermatitis.<sup>2</sup> The majority of invasive skin infections are caused by *S. aureus*, including MRSA.<sup>3</sup> Invasive strains of *S. aureus* are difficult to manage therapeutically due to their thick cell barriers and acquired drug resistance. Additionally, *S. aureus* is able to gain entry and replicate within various host cell types, including keratinocytes, macrophages, endothelial cells, epithelial cells, fibroblasts, and osteoblasts, where antibiotic concentrations *in situ* are often sub-therapeutic.<sup>4</sup> Recent work by Lehar *et al*, demonstrated that the three major antibiotics that are currently administered for invasive MRSA infections (linezolid, vancomycin, and daptomycin) fail to kill intracellular *S. aureus* USA 300 in infected mice.<sup>5</sup> Consequently, intracellular invasion of mammalian cells by *S. aureus* can induce pyroptosis (cell death), followed by accumulation of bacteria, which can then breach out to infect nearby cells.<sup>6</sup> Therefore, drug resistant and intracellular *S. aureus* can result in incomplete eradication during therapy.

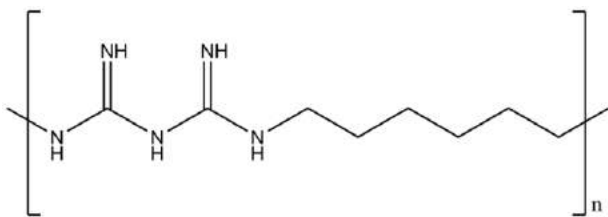
The control of *S. aureus*, particularly MRSA, is further complicated by the emergence of epidemic strains, which display increased virulence. These strains are reported to be highly invasive and able to cause bacteremia in patients.<sup>7</sup> In

the United Kingdom, epidemic methicillin-resistant *S. aureus* 15 (EMRSA-15) and EMRSA-16 strains constitute more than 60% and 35% of MRSA associated infections, respectively.<sup>7</sup> These two strains are considered to be the most successful strains at surviving, colonising and spreading in the hospital environment.<sup>8-10</sup> Similarly, in the United States (USA), strains USA 300 and USA 400 often cause community-acquired MRSA infections, with USA 300 being the predominant cause of skin-and soft tissue infections.<sup>11</sup> The USA 300 strain is widely disseminated across the USA and capable of causing clinical illness ranging from uncomplicated bacteremia to endocarditis, necrotising pneumonia, and osteomyelitis.<sup>12</sup>

The recommended treatments for skin infection caused by *S. aureus* includes administration of topical and systemic antimicrobials.<sup>13</sup> Topical administration is often needed to ensure drugs reach sites of infection. For example, in the case of burn injuries, damage to the local vascular system could limit oral antimicrobials distribution to infected sites. Therefore, topical antimicrobials such as mupirocin, fusidic acid, and bacitracin are frequently used.<sup>14</sup> Unfortunately, increasing resistance to these antimicrobials has reduced effectiveness.<sup>15</sup> Among the recently introduced topical antimicrobials, nadifloxacin, a fluoroquinolone, is effective against aerobic and anaerobic bacteria isolated from skin infections.<sup>16</sup> Importantly, nadifloxacin is effective against ciprofloxacin resistant strains.<sup>17</sup> Nadifloxacin is currently approved for acne treatment and skin infections.<sup>18</sup>

Synthetic cationic polymers are becoming widely used in clinics as antimicrobials due to their broad-spectrum bactericidal activities and high therapeutic index. For example polyhexamethylene biguanide (PHMB) (Figure 1) is a cationic polymer that provides potent topical antimicrobial effects against Gram-positive and Gram-negative bacteria,<sup>19,20</sup> fungi<sup>21,22</sup> and certain viruses.<sup>23</sup> PHMB is a relatively safe compound with no reported adverse reactions when used on the skin, eyes, epithelium of the nose and wounds.<sup>24,25</sup> It is available both as a disinfecting solution and impregnated into bio-cellulose dressings.<sup>26</sup> Although PHMB has been used in the clinic for more than 40 years, there are no reports of acquired resistance to this compound.<sup>24</sup> Due to its excellent antibacterial properties and safety profile, PHMB is often administered topically to prevent wound infection.<sup>27</sup>

Recently we observed that PHMB enters bacteria (*Staphylococcus aureus*, *Escherichia coli* and *Mycobacterium smegmatis*) and several types of mammalian cells grown in cell culture (including epithelial, macrophage and fibroblast cells).<sup>28,29</sup> On the basis of these findings, and the established antibacterial properties of PHMB, we hypothesised that PHMB may also possess previously unrecognised antibacterial activity against intracellular bacteria. Here, we investigated the potential antibacterial activities of six topical antimicrobials and PHMB against intracellular MRSA. We further characterised the route(s) of PHMB uptake into keratinocytes and the mechanism(s) of intracellular killing by PHMB.



**Figure 1 Structure of PHMB**

PHMB is a cationic polymer of repeating hexamethylene biguanide groups, with  $n$  average = 10-12 ( $n$  is the number of structural unit repeats) and molecular weight (mw) 3025 gram/mol.

## **MATERIALS AND METHODS**

### **Bacterial strains and growth conditions**

*S. aureus* strain RN4420 was obtained from Dr. Staffan Arvidson, Karolinska Institutet, Stockholm, Sweden,<sup>30</sup> strain EMRSA-15 was obtained from Dr. Sean Nair, University College London,<sup>31</sup> and strain USA 300 was obtained from Dr. Jonathan Otter, Imperial College Healthcare NHS Trust, UK.<sup>32</sup> Bacteria were grown in Mueller Hinton Broth (MHB, Sigma-Aldrich, UK) followed by incubation at 250 rpm (for liquid cultures), at 37°C for 18 hours.

### **Eukaryotic cell lines and growth conditions**

HaCaT cells were obtained from Dr. Amir Sharili, Queen Mary University of London and maintained in DMEM with 10% FBS (Sigma-Aldrich, UK), supplemented with 5% penicillin-streptomycin (Sigma-Aldrich, UK). Cells were maintained at 37°C in 5% carbon dioxide.

### **Determination of minimum inhibitory concentrations (MIC)**

All antimicrobials were purchased from Sigma-Aldrich, UK, except nadifloxacin, which was from Santa Cruz Biotechnology, UK. PHMB and fluorescein-tagged PHMB (PHMB-FITC) were from Tecrea Ltd, UK. All antimicrobials were prepared in stock solution at 10,000 mg/L. Fusidic acid, gentamicin, bacitracin and PHMB



were dissolved in sterile distilled water; mupirocin was dissolved in methanol; erythromycin was dissolved in ethanol and nadifloxacin was dissolved in 0.1M sodium hydroxide solution.

MICs were determined using the broth microdilution method.<sup>33</sup> Briefly, a range of concentrations of antimicrobials were prepared in a 96 well microplate, followed by inoculation of bacteria culture to yield  $\sim 5 \times 10^5$  cfu/mL in a 250  $\mu$ l final volume. The plate was then incubated at 37°C for 18 hours. The lowest concentration of antimicrobial that inhibited growth of bacteria was scored as the MIC.

### **Intracellular infection of keratinocytes by *S. aureus***

Intracellular infection of keratinocytes by *S. aureus* RN4420, EMRSA-15 and USA 300 were established using a gentamicin protection procedure.<sup>34</sup> Keratinocytes were seeded at  $1.2 \times 10^5$  cells/well in a 12 well plate and cultured overnight in DMEM with 10% FBS, without antibiotic. In parallel, all *S. aureus* strains were cultured overnight in MHB at 37°C in an incubator shaker. One mL of overnight bacterial culture was centrifuged at 8000 rpm for three minutes and the pellet was resuspended in phosphate buffered saline (PBS) (Sigma-Aldrich, UK). These steps were repeated three times to remove bacterial toxin residues. Bacteria were diluted to a final concentration of approximately  $10^7$  cfu/mL in DMEM with 10% FBS, without antibiotic. Aliquotes of bacteria (1 mL,  $10^7$  cfu/mL) were added to keratinocyte cultures after the original medium was removed.

Bacteria were coincubated with keratinocytes for three hours. 200 mg/L of gentamicin diluted in medium was added and incubated for three hours. Medium containing bacteria and gentamicin were removed and cells were rinsed with PBS. Rinse step was performed because this medium contained very high concentrations of gentamicin and therefore did not result in colony-forming units of bacteria when plated directly. Therefore, aliquots of PBS from the rinsing process were plated on nutrient agar to determine the remaining number of extracellular bacteria. Next, one mL of 0.5% Triton X-100 prepared in PBS was added to each well to lyse cells. Lysed cells were serially diluted in PBS and plated on nutrient agar (Sigma-Aldrich, UK) for enumeration of intracellular bacteria. Uninfected cells were also subjected to the lysis procedure to confirm sterility.

### **Visualisation of intracellular *S. aureus* within keratinocytes**

Keratinocytes were grown on glass cover slips in 12 well plates followed by EMRSA-15 infection as described above. Following gentamicin exposure to kill extracellular bacteria, cells were rinsed with PBS and fixed with 4% paraformaldehyde (Santa Cruz Biotechnology, UK). Cells were stained with 5 mg/L DAPI (Life technologies, UK) for nuclei staining and 5 mg/L Wheat Germ Agglutinin-conjugated Alexa Fluor 555 (WGA, Life technologies, UK) for membrane staining. Cover slips were mounted onto glass slides with FluorSave™ (Calbiochem, UK). Images were visualised using a Leica SP5 confocal microscope using Advanced Fluorescence Software (Leica

Microsystems, Milton Keynes, UK). Sequential scan Z-stacks (139 slices 1024 x 1024) were compiled at a line average of 96, using Volocity® software. Three dimensional (3D) Image Analysis Software was used for analysis and to produce 3D images.

### **Intracellular killing activity of EMRSA-15 and USA 300 by nadifloxacin and PHMB**

Keratinocytes were infected with EMRSA-15 and USA 300 followed by incubation with gentamicin to kill extracellular bacteria. Following gentamicin exposure, keratinocytes were rinsed with PBS, and antimicrobials (nadifloxacin and PHMB in medium) were added to the wells containing infected cells. Plates were incubated for another three hours to kill intracellular bacteria. Following this period, antibiotic solutions were removed, cells were rinsed and lysed. Lysed cells were serially diluted and plated on nutrient agar. For each experiment, gentamicin treated cells (without nadifloxacin or PHMB treatments) were used as controls.

### **Resazurin assay**

Keratinocytes ( $4 \times 10^4$  cells/well) were added to a 96 well plate and cultured with increasing concentrations of nadifloxacin or PHMB at 37°C for 24 hours. Non-treated cells and medium only were used as controls. Resazurin sodium salt (Sigma-Aldrich, UK) was prepared as a stock solution at 440 µM in PBS and

added to each well at 44  $\mu$ M final concentration and plates were incubated for an additional 48 hours. Optical density (OD) was then measured using a Tecan Infinite plate reader (Tecan group Ltd, Switzerland) at 550 nm and 630 nm. The OD value change (or % dye reduction) is proportional to the viable cell number and was used to calculate half maximal inhibitory concentrations ( $IC_{50}$ ) values based on the intercept theorem.

### **Visualisation and quantification of PHMB uptake into keratinocytes**

For both experiments, keratinocytes were grown on glass cover slips in a 12 well plate. PHMB-FITC was added to cultures at 4 mg/L for three hours. Cells were rinsed with PBS and stained as described above before confocal microscopy. To quantify PHMB uptake into keratinocytes, PHMB-FITC treated cells were incubated with 0.04% Trypan blue (Invitrogen, UK) in PBS for 15 minutes to quench membrane bounded PHMB-FITC, followed by flow cytometry (FACSBSD machine and BDFACSDiva<sup>TM</sup> software, BD Bioscience) using the FITC filter. For each sample, 10,000 gated cells were analysed.

### **Exocytosis of PHMB from keratinocytes**

Keratinocytes were seeded in 35 mm tissue culture treated dishes (Griener Bio-One, Austria). Keratinocytes were incubated with 4 mg/L of PHMB-FITC in medium for three hours. Following exposure, PHMB-FITC containing medium was removed and new medium containing 5 mg/L WGA was added. Fluorescent

quenching rates were determined and used to select an appropriate light intensity. Cellular fluorescence was monitored with images captured every 15 minutes for five hours using confocal microscopy. Mean fluorescent intensity (MFI) was calculated at the beginning and after five hours of experiment to assess retention of PHMB-FITC in the cells.

### **Statistical analysis**

All experiments were performed in triplicate. Statistical analysis was performed using one-way Analysis of Variance (ANOVA) followed by Tukey tests using the statistical packages Prism 6, Version 6.0 (GraphPad Prism 6.0, San Diego, CA). Data is presented as means  $\pm$  standard deviation. Differences were considered to be statistically significant where  $p \leq 0.05$ . For histogram and graphs, error bars represent standard deviations. \*( $p \leq 0.05$ ), \*\*\* ( $p \leq 0.001$ ), \*\*\*\* ( $p \leq 0.0001$ ), ns (not significant).

## RESULTS

### **Susceptibility of *S. aureus* strains to topically used antimicrobials and PHMB**

Six topically used antimicrobials were tested, and the MIC values for all six were higher for the clinical MRSA strains (EMRSA-15 & USA 300) than for the MSSA strain RN4420. In contrast, for PHMB, MIC values were similar for all strains. Overall, gentamicin, nadifloxacin and PHMB displayed the lowest MIC values against MRSA strains (Table 1).

Given that nadifloxacin and PHMB displayed low MIC values relative to the other antimicrobials tested, we chose to focus on these two compounds in further studies. There are no reports for nadifloxacin and PHMB activities against intracellular bacteria. Gentamicin is an antibiotic that is active against extracellular bacteria, but not against intracellular bacteria, due to its poor penetration into mammalian cells.<sup>35</sup> Therefore, we chose to further examine nadifloxacin and PHMB activities against intracellular MRSA, and gentamicin was not examined further.

**Table 1 MIC values for topical antimicrobials against *S. aureus* RN4420, EMRSA-15 and USA 300**

MIC (mg/L)			
Strains Antimicrobials	RN4420	EMRSA-15	USA 300
Mupirocin	0.125	32	2
Fusidic acid	0.25	32	0.5
Gentamicin	0.25	4	0.25
Nadifloxacin	0.125	2	2
Erythromycin	0.125	32	16
Bacitracin	16	>32	32
PHMB	1	1	1

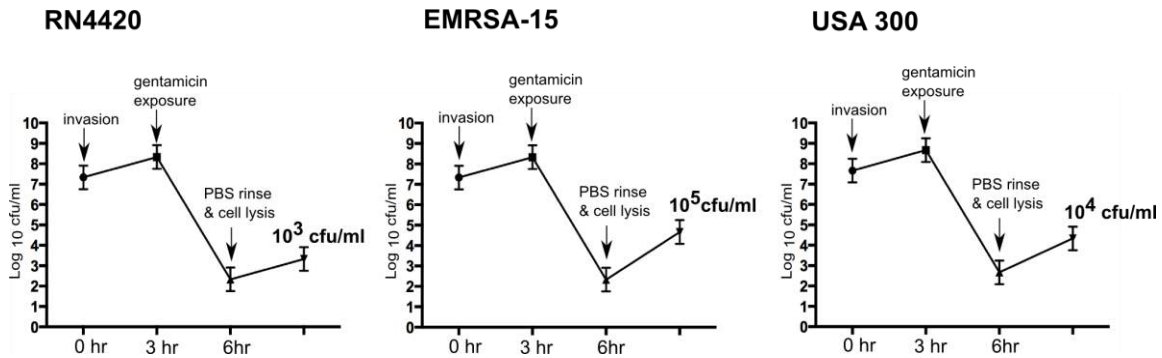
## **Intracellular invasion of keratinocytes by *S. aureus***

To determine the activities of nadifloxacin and PHMB against intracellular bacteria, we first established an *in vitro* intracellular infection assay using MRSA and HaCaT cells.<sup>34</sup> HaCaT is a well-established keratinocyte cell line that is often used experimentally as a host for intracellular *S. aureus*.<sup>6,36</sup> To establish conditions for *S. aureus* RN4420, EMRSA-15 and USA 300 invasion in keratinocytes, bacteria were incubated with keratinocytes, followed by co-incubation with 200 mg/L of gentamicin to kill extracellular bacteria. All strains were able to invade keratinocytes, as indicated by their ability to evade gentamicin treatment. Three hours of infection followed by three hours of gentamicin treatment resulted in reproducible infections (Figure 2a, b & c). Killing of mainly extracellular bacteria was evident for all three strains, where lysis of keratinocytes following gentamicin exposure released approximately  $10^3$  cfu/mL of *S. aureus* RN4420,  $10^5$  cfu/mL of EMRSA-15 and  $10^4$  cfu/mL of USA 300. These colony counts presumably reflected *S. aureus* that were inside keratinocytes and thus protected from gentamicin's extracellular antibacterial activities. As anticipated, the two clinical isolate strains, EMRSA-15 and USA 300, showed higher invasion levels than *S. aureus* RN4420.

*S. aureus* invasion of keratinocytes was visualised by confocal microscopy. Keratinocytes were infected with EMRSA-15 and stained with DAPI and WGA. Using 3D analysis, we observed localisation of EMRSA-15 (blue dots)

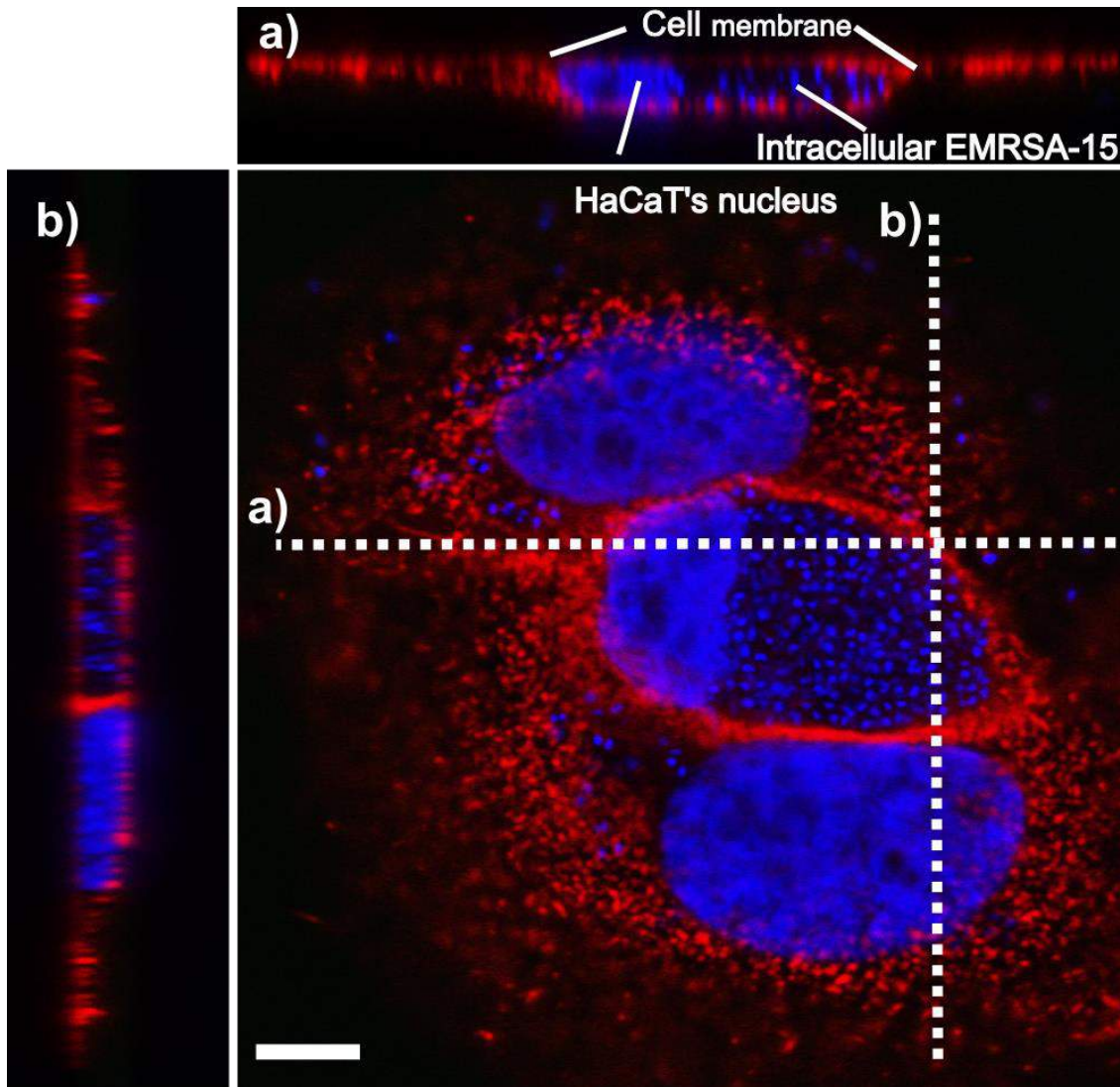


inside keratinocytes membranes (red) (Figure 3a & b), confirming invasion of EMRSA-15.



**Figure 2** *S. aureus* invasion in keratinocytes

Colony forming units (cfu) of *S. aureus* following gentamicin exposure. After gentamicin exposure, lysis of keratinocytes released approximately 10<sup>3</sup> cfu/mL of *S. aureus* RN4420, 10<sup>5</sup> cfu/mL of EMRSA-15 and 10<sup>4</sup> cfu/mL of USA 300.

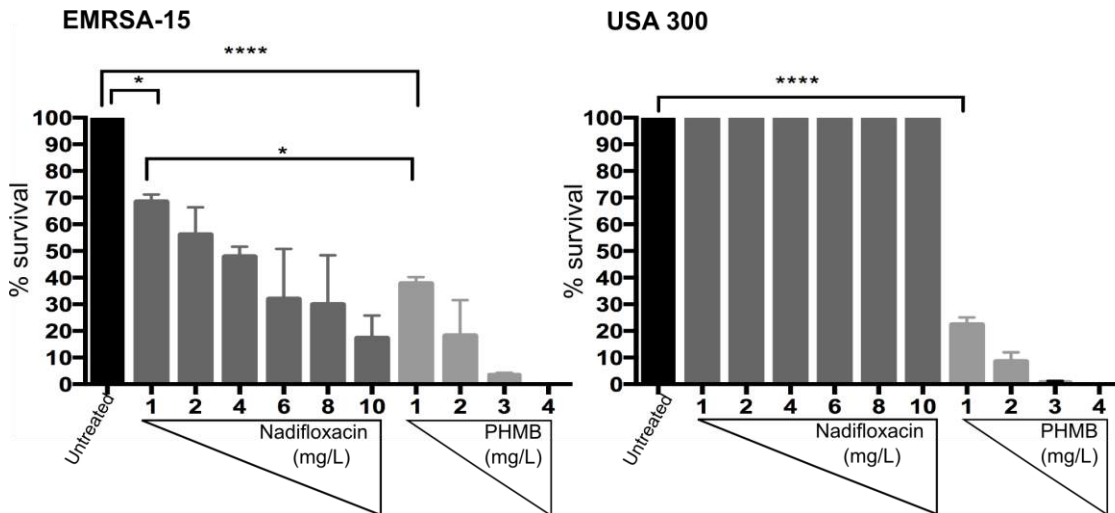


**Figure 3 Localization of *S. aureus* in keratinocytes**

Invasion of EMRSA-15 in keratinocytes was visualized using confocal microscopy. Prior to imaging, keratinocytes were stained with DAPI (blue) for keratinocytes and EMRSA-15 nuclei staining and WGA (red) for keratinocyte membrane staining. Confocal microscopy z-stack projection moved through 139 slices across keratinocytes. **a)** Horizontal cross-section of keratinocytes **b)** Ventricle cross-section of keratinocytes. The small blue dots are EMRSA-15 cells inside keratinocytes, indicating invasion. White scale bar is 7.5  $\mu\text{m}$ .

## **Susceptibility of intracellular EMRSA-15 and USA 300 to nadifloxacin and PHMB**

To investigate the potential of nadifloxacin and PHMB to kill intracellular MRSA, keratinocytes were infected with EMRSA-15 and USA 300, exposed to gentamicin to kill extracellular bacteria, followed by treatment with nadifloxacin or PHMB at 1, 2, 4, 6, 8, 10 mg/L for three hours and enumeration of cfu of surviving intracellular bacteria. Figure 4 shows the percentages of surviving intracellular MRSA after treatment with nadifloxacin and PHMB, relative to untreated infected cells. Nadifloxacin at 10 mg/L killed 80% of intracellular EMRSA-15, but failed to kill USA 300 after three hours of treatment. PHMB at 4 mg/L killed almost 100% of both strains as compared to 100% survival of the untreated infected cells. Therefore, while nadifloxacin displayed similar MIC values for both EMRSA-15 and USA 300; its activities were inconsistent against intracellular *S. aureus*, whereas, PHMB displayed consistent killing of both EMRSA-15 and USA 300.



**Figure 4 Bactericidal activities of nadifloxacin and PHMB against intracellular EMRSA-15 and USA 300**

Keratinocytes infected with EMRSA-15 and USA 300 were either untreated or treated with increasing concentrations of nadifloxacin or PHMB. Untreated cultures were used to establish cfu values corresponding to 100% survival.

## **Nadifloxacin and PHMB toxicity in keratinocytes**

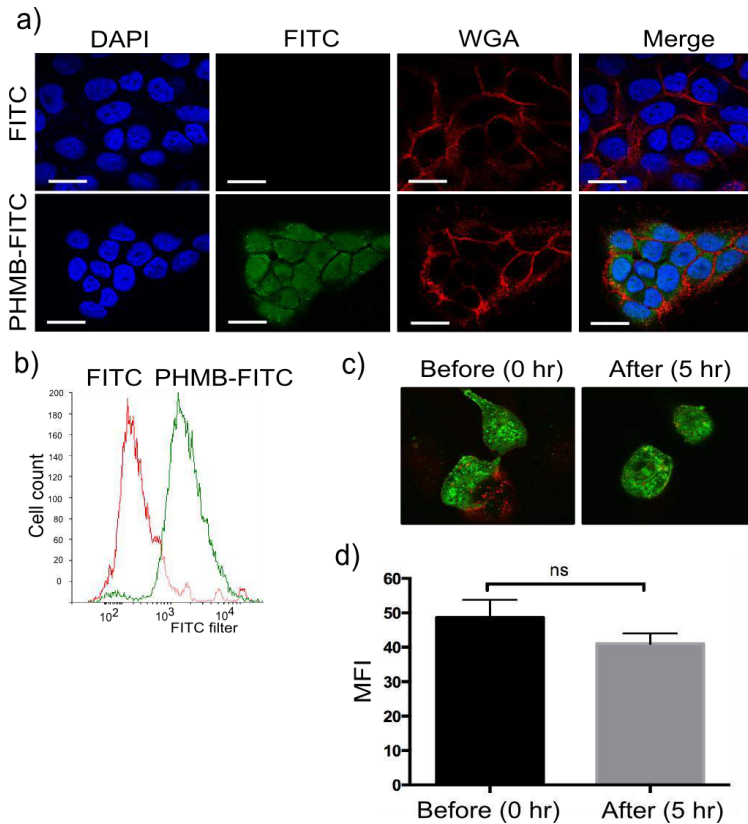
Resazurin assays were performed to determine the *in vitro* toxicity levels of nadifloxacin and PHMB against keratinocytes. The IC<sub>50</sub> values for nadifloxacin and PHMB were 1428 ± 247 mg/L and 19.7 ± 10 mg/L, respectively. Therefore, keratinocytes tolerated high concentrations of both compounds, which were far higher than the concentrations required to kill intracellular *S. aureus*; nadifloxacin (10 mg/L) and PHMB (4 mg/L). These results indicate that both compounds can be used to kill intracellular *S. aureus* with a high therapeutic index.

## **PHMB uptake into keratinocytes**

Killing of intracellular *S. aureus* by antimicrobials normally requires drug entry into host cells and direct contact with bacteria. To test whether or not PHMB can enter keratinocytes, we added PHMB-FITC to cultures and tracked its intracellular localisation using confocal microscopy. As can be seen in Figure 5a, a mix of predominantly diffuse and small-punctuated spots of PHMB-FITC were observed. The diffuse signals suggest localisation in the cytosol, and the punctuated spots suggest localisation in vesicles. To quantify PHMB entry into keratinocytes, we analysed treated cells using flow cytometry. Consistent with microscopy results, flow cytometry data indicate that PHMB-FITC entered 99% of cells (Figure 5b).

### **Exocytosis of PHMB-FITC from keratinocytes**

Antimicrobial-mediated killing of intracellular pathogens may benefit from host cell retention. To determine PHMB's host cell retention, we exposed keratinocytes to PHMB-FITC, rinsed the cells, added new medium and monitored cell-associated fluorescence over five hours. We observed only a minor reduction in fluorescence, with 90% of the fluorescence retained (Figure 5c & d). Therefore, the majority of PHMB was not excreted from cells, which may help to explain its potent killing of intracellular EMRSA-15 and USA 300.



**Figure 5 PHMB-FITC uptake and retention in keratinocytes**

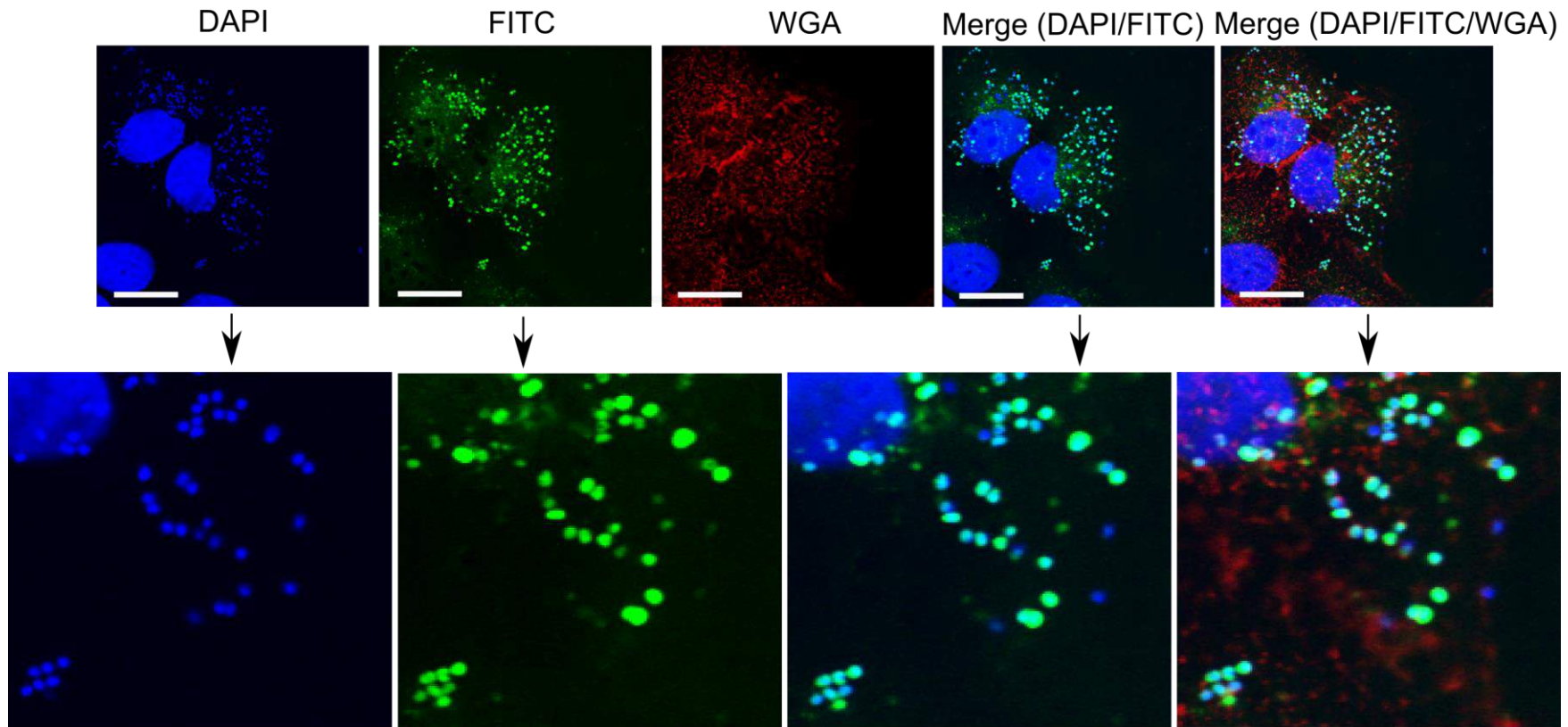
**a)** PHMB-FITC localization visualized by confocal microscopy. Free FITC was used as control. **b)** PHMB-FITC uptake was quantified by flow cytometry. The overlay histogram image illustrates the uptake of PHMB-FITC into keratinocytes. The red profile shows the population of keratinocytes treated with FITC and serves as a negative control. The green profile shows the population of keratinocytes treated with PHMB-FITC. Uptake of PHMB-FITC was observed in > 99% keratinocytes. **c)** Keratinocytes following treatment with PHMB-FITC and five hours after rinsing to remove extracellular PHMB-FITC. **d)** Mean fluorescence intensity (MFI) of PHMB-FITC before and after five hours of experiments.

## **Colocalisation of PHMB and EMRSA-15 in keratinocytes**

The results described above are most easily explained by a mechanism of direct killing of EMRSA-15 and USA 300, where PHMB enters keratinocytes and then kills bacteria. However, other indirect killing mechanisms are formally possible. To further characterise the PHMB-mediated killing mechanism(s), localisation of EMRSA-15 and PHMB-FITC inside keratinocytes was assessed using the gentamicin protection method together with microscopy. After gentamicin treatment, PHMB-FITC was added to cells, and after three hours of incubation, cells were rinsed and stained with DAPI and WGA. Cells were then fixed and imaged using confocal microscopy.

Colocalisation of blue-stained bacteria and green PHMB-FITC was apparent inside host cells, as shown in figure 6. Indeed, colocalisation of PHMB-FITC and EMRSA-15 in keratinocytes was apparent for the majority of intracellular bacteria in all cells examined. Therefore, PHMB-FITC appears to directly access intracellular EMRSA-15 inside keratinocytes, suggesting direct killing of intracellular bacteria.





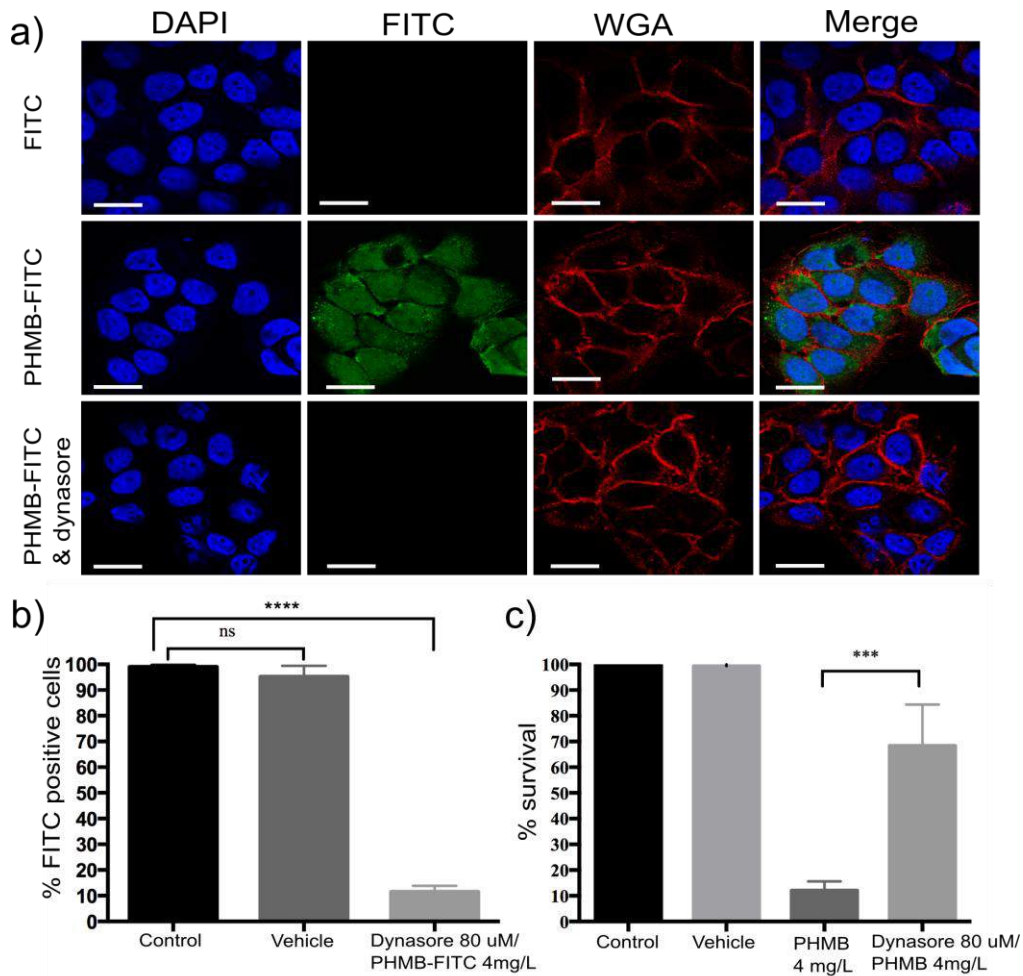
**Figure 6 Colocalization of PHMB-FITC with EMRSA-15 in keratinocytes**

Keratinocytes were infected with EMRSA-15 followed by treatment with PHMB-FITC (green). Prior to microscopy, keratinocytes were labeled with DAPI (blue) and WGA (red). Upper panels are images of infected cells visualized using filters for DAPI, FITC and WGA, and merged images. Lower panels are enlarged images that clearly show colocalization between PHMB-FITC (green) and EMRSA-15 (blue). White scale bar is 25  $\mu\text{m}$ .

## **Effect of dynamin inhibition on PHMB uptake and antibacterial activity in keratinocytes**

Cell entry pathways that transport PHMB and *S. aureus* to a common final destination in the cell could explain their colocalisation in keratinocytes. Recent work in our laboratory demonstrates that PHMB enters macrophage predominantly via a dynamin-dependent endocytic pathway.<sup>28</sup> This pathway requires dynamin (a GTPase) to excise newly formed vesicle from the membrane.<sup>37</sup> Without dynamin-mediated vesicle excision, vesicles do not form and enter cell.

If PHMB kills intracellular *S. aureus* via direct contact with bacteria in keratinocytes, as suggested by colocalisation results above, then inhibitors that block cell entry should reduce both PHMB uptake and intracellular killing. To test the effect of dynamin inhibition on PHMB uptake, we pre-treated keratinocytes with a dynamin inhibitor, dynasore before addition of PHMB-FITC. Using confocal microscopy, we observed that dynasore reduced PHMB-FITC uptake (Figure 7a). Quantification using flow cytometer confirmed that dynasore reduced PHMB-FITC uptake into keratinocytes by 85% (Figure 7b). Similarly, dynasore reduced PHMB-mediated killing of intracellular EMRSA-15 by approximately 50% (Figure 7c). These results suggest that PHMB entry and intracellular antibacterial activity in keratinocytes involves dynamin-dependent endocytic pathways.



**Figure 7 Effects of dynasore on PHMB uptake into keratinocytes and killing of intracellular EMRSA-15**

Uptake of PHMB and intracellular antimicrobial activities in dynasore pre-treated keratinocytes. Cells were pre-treated with dynasore (in 0.5% DMSO) for 30 minutes. Then cells were incubated with PHMB-FITC for uptake studies or PHMB for antimicrobial activity studies. “Control” samples were keratinocytes treated with PHMB-FITC alone. “Vehicle” samples were keratinocytes treated with PHMB-FITC in media containing 0.5% DMSO. Following treatment, keratinocytes were: **a)** visualized by confocal microscopy and **b)** analyzed by flow cytometer. **c)** Effects of dynasore on PHMB-mediated killing of intracellular bacteria.

## DISCUSSION

Intracellular bacteria are typically difficult to kill because they are shielded from host immune mechanisms and protected from antimicrobials. Here, we found that nadifloxacin and PHMB kill intracellular MRSA within keratinocytes, with PHMB being more potent. Nadifloxacin-mediated killing of intracellular *S. aureus* is perhaps expected, because it is a small molecule belonging to the fluoroquinolone group, a class of antibiotic that can enter and accumulate within mammalian cells.<sup>38</sup> However, nadifloxacin displayed inconsistent activity; being effective against EMRSA-15 but not against USA300. In contrast, the intracellular activity of PHMB was unexpected, because it is a relatively large molecule (average molecular weight around 3025 grams/mol)<sup>39</sup> and has only recently been reported to enter mammalian cells.<sup>28,29</sup> Nevertheless, our results clearly show that PHMB enters keratinocytes and is retained for several hours.

Intracellular pathogens such as *S. aureus* enter mammalian cells through phagocytosis (one of the endocytic pathways) via a zipper-uptake mechanism.<sup>40</sup> Inside host cells, the fate of *S. aureus* is to be killed by innate immune mechanisms within a phagolysosome, replicate inside an endosome, or escape into the cytosol.<sup>40</sup> Inside keratinocytes, the polymer PHMB-FITC localises mainly inside vesicles and in the cytosol. In this study, we observed colocalisation between PHMB-FITC and almost all intracellular EMRSA-15, suggesting direct interactions that could occur in both vesicles and the cytosol. These interactions may explain the potent intracellular bactericidal activities of PHMB. Furthermore,

inhibition of dynamin, a GTPase which pinches-off newly formed endocytic vesicles from the membrane,<sup>37</sup> impeded both PHMB-FITC uptake and its intracellular killing activity. Therefore, PHMB uptake into keratinocytes appears to be dynamin-dependent.

There are three main findings from this study. First, both nadifloxacin and PHMB are effective against intracellular *S. aureus* with PHMB being more potent against intracellular MRSA. Second, PHMB colocalises with intracellular MRSA in keratinocytes, indicating that killing occurs by direct interactions inside host cells. Third, PHMB's uptake and antibacterial activities inside keratinocytes appear to involve a dynamin-dependent cell uptake mechanism. Together, these findings suggest that nadifloxacin and particularly PHMB have previously unrecognised potential for treating skin infections caused by intracellular MRSA and possibly other intracellular bacteria.

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## **TRANSPARENCY DECLARATION**

Nothing to declare

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