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RESEARCH ARTICLE

Daptomycin exerts bactericidal effect through induction of excessive ROS production and blocking the function of stress response protein Usp2

Kathy Hiu Laam Po[1,2] | Hoi Yee Chow[3] | Qipeng Cheng[1,2] | Bill Kwan-wai Chan[1,2] | Xin Deng[4] | Shuping Wang[3] | Edward Wai Chi Chan[1,2] | Hang-Kin Kong[1] | Kin Fai Chan[1] | Xuechen Li[3] | Sheng Chen[2]

[1] State Key Lab of Chemical Biology and Drug Discovery, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong

[2] Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong

[3] State Key Lab of Synthetic Chemistry, Department of Chemistry, The University of Hong Kong, Kowloon, Hong Kong

[4] Department of Biomedical Science, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong

Correspondence
Xuechen Li, State Key Lab of Synthetic Chemistry, Department of Chemistry, The University of Hong Kong, Kowloon, Hong Kong.
Email: xuechenl@hku.hk
Sheng Chen, Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong.
Email: shechen@cityu.edu.hk

Kathy Hiu Laam Po and Hoi Yee Chow contributed equally.

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Abstract

Daptomycin, as a lipopeptide antibiotic, exhibits high potency in the treatment of infections caused by clinically relevant drug-resistant Gram-positive pathogens, such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*. However, its bactericidal mechanism of action remains controversial. In this study, we report that daptomycin kills bacteria through triggering overproduction of deleterious reactive oxygen species (ROS). This outcome is attributed to daptomycin binding to the universal stress response protein (Usp2) and subsequent blocking its function, triggering stress, and anti-ROS response. Based on these findings, we conclude that daptomycin causes bacterial cell death by simultaneously triggering ROS production through inflicting cell membrane damages and inhibiting antioxidant defense by blocking Usp2 function. This study depicts molecular mechanisms underlying the bactericidal effect of daptomycin, a combination of triggering ROS production and inhibiting anti-ROS response.

Key points:

- Novel bactericidal mechanism of daptomycin by ROS
- Identification of first bacterial protein target, Usp2, for daptomycin
- Deciphering the dual role of Usp2 on daptomycin mediated bacterial killing

KEYWORDS
bacteriacidal mechansims, daptomycin, ROS, stress response, Usp2

INTRODUCTION

Emergence of bacterial strains exhibiting novel antimicrobial resistance mechanisms results in marked increase in the risk of treatment failure worldwide as well as the cost of healthcare [1,2]. Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as one of the most problematic pathogens in both hospital and community-acquired infections [3]. It is also considered as one of the most important super-bugs, causing significant mobility and mortality. Daptomycin is a lipopeptide isolated from a *Streptomyces roseosporus* strain in a soil sample in Mount Ararat (Turkey) by scientists at Eli Lilly. Daptomycin has potent bactericidal activity against the otherwise antibiotic-resistant Gram-positive pathogens, including MRSA, vancomycin-resistant *Enterococci*, and vancomycin-resistant *S. aureus* [4]. Since daptomycin was approved for clinical use by the FDA in 2003, the elucidation of its antimicrobial mechanism has attracted extensive efforts. To date, different modes of action have been proposed, including induction of membrane pore formation, blockage of cell wall synthesis, and aberrant protein recruitment as a result of alteration in membrane curvature [5–7]. A well-accepted model is that daptomycin, in the presence of calcium ions, undergoes conformational changes upon association with bacterial membrane. Once daptomycin is inserted into the membrane, bacterial membrane leakage ensues [8]. However, one study confirmed that daptomycin did not lead to the formation of pores in the membrane of *Clostridium bacillus* and *Staphylococcus aureus*, but affected cell envelope synthesis by interfering with the fluidic membrane microdomains [5]. The most recent study showed that daptomycin targeted cell wall biosynthesis by forming a tripartite complex with undecaprenyl-coupled intermediates and membrane lipids [9]. All these models are supportive but inconclusive to explain how daptomycin exerts bactericidal effect. In this study, we used integrative chemical biology approach to depict a novel mechanism of action and bactericidal effect of daptomycin.

MATERIALS AND METHODS

Please see detail in the Supplementary Materials.

RESULTS AND DISCUSSION

Bactericidal effect of daptomycin was attributed to its ability to trigger overproduction of ROS in bacteria

To confirm the effect of daptomycin on membrane permeability, staining bacteria with SYTOX Green, a fluorescent dye that can only bind to chromosomal DNA and display fluorescence signals when cell membrane is disrupted, was performed. An increasing fluorescence signal could be detected over time upon incubation with daptomycin, suggesting that daptomycin could damage cell membrane and kill *S. aureus* (Figure S1). The data were in contrast to a recent finding that daptomycin did not result in the formation of pores in bacterial membrane [5].

We wondered whether bactericidal effect of daptomycin is resulted from the production of reactive oxygen species (ROS), which was recently reported as one of the mechanisms of action of antibiotics [10]. We then tested if the interaction of daptomycin with cell membrane could trigger redox-response and overproduction of ROS by a general oxidative stress indicator, CM-H2DCFDA. Our data showed that incubation of daptomycin with *S. aureus* for 3 h, indeed, triggered the production of a large amount of ROS, while the inactive daptomycin analogue, **D2**, which exhibited negligible antimicrobial activity, did not trigger any ROS response (Figure 1a1). Consistently, incubation of various concentrations of daptomycin with the dye did not lead to signal increase (Figure S2a), suggesting that daptomycin might kill bacteria through triggering the production of ROS. Furthermore, daptomycin was also shown to trigger overproduction of ROS in *Bacillus cereus*, *Streptococcus faecalis*, and *Enterococcus faecalis* in a dose-dependent manner (Figure 1a2–a4). To confirm that daptomycin specifically induces ROS production, other antibiotics were also tested for its ROS induction effect in the *S. aureus* ATCC 29213 strain. Our results showed that tetracycline did not induce ROS production at all, vancomycin induced a moderate level of ROS production, but such level was at least 1000-fold lower, even at 16 μg/ml, when compared to daptomycin. Teixobactin [11], a newly discovered cyclic peptide antibiotic, induced only very low level of ROS production (Figure 1b1–b3). Furthermore, colistin, a cyclic peptide antibiotic, was not found to induce ROS production in *E. coli* (Figure 1b4). These data suggest that induction of ROS production is likely specific to daptomycin.

To further confirm that triggering production of ROS is the major killing mechanism of daptomycin, thiourea, an antioxidant, was used to test its effect on suppressing the killing effect of daptomycin. In the presence of thiourea (100–200 mM), the ability of daptomycin to induce ROS production in *S. aureus* was reduced significantly ($p = 0.0056$ and $p = 0.0016$, respectively, and over 50 folds) when compared to the no-thiourea treatment (Figure 2a). Consistenly, incubation of various concentration of thiourea with dye only led to slightly decrease of dye signal (<1.5-fold), suggesting that thiourea, indeed, suppressed the ROS response induced by daptomycin (Figure S2b). Furthermore, incubation of thirourea with *S. aureus* ATCC 29213 strain did not lead to decrease (increase at some concentrations) of ROS production (Figures S2c and d), further confirming that the reduced production of ROS was supposed to be due to the neutralization of ROS by thiourea, which mimicked the anti-ROS response in *S. aureus*. However, as an antioxidant, how thiourea caused slightly increased production of ROS in *S. aureus* at high concentrations (100 and 200 mM) needs further investigation. It is speculated that high concentrations of thiourea may impose stresses on *S. aureus* leading to the production of ROS. The reduction in ROS production due to the presence of an increasing amount of thiourea (10, 100, and 200 mM) correlated well with a significant ($p = 0.0005$, $p < 0.0001$ and $p < 0.0001$, respectively) increase in the survival rate of *S. aureus* treated with 8 μg/ml daptomycin, confirming that triggering ROS production in *S. aureus* is the key killing mechanism of daptomycin (Figure 2b). Another dye, p-hydroxyphenyl

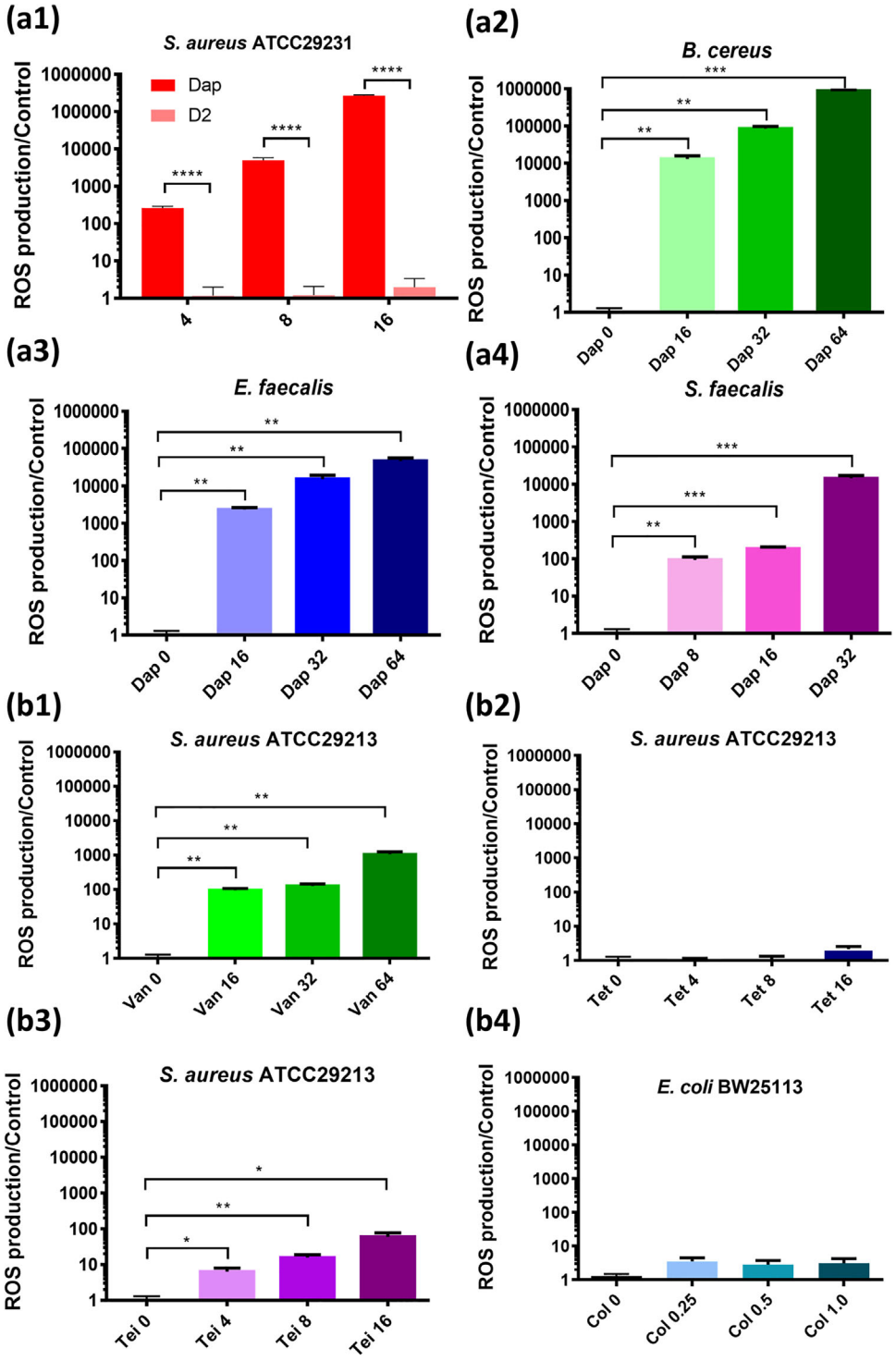

FIGURE 1 Daptomycin triggered overproduction of ROS in Gram-positive bacteria. (a1) ROS production in *S. aureus* induced by daptomycin and analogue D2; ROS production induced by different concentrations of daptomycin in *B. bacillus* (a2), *S. faecalis* (a3), and *E. faecalis* (a4). Effect of different antibiotics on triggering ROS production in *S. aureus* and *E. coli*. Induction of ROS production by different concentrations of vancomycin (b1), tetracycline (b2), and teixobactin (b3) in *S. aureus*; (b4) induction of ROS production by different concentrations of colistin in *E. coli*. Increased survival of *S. aureus* upon daptomycin treatment is due to neutralization of ROS or blockage of ROS production by preventing daptomycin to bind to the Usp2 protein. Intracellular ROS was determined using a general oxidative stress indicator, CM-H2DCFDA. X-axes represent the concentration of antibiotics, "Dap 16" (and similar labels) means 16 μg/ml. Unpaired two-sided Student's *t* test was performed for data analysis. Each data point was repeated for three times ($n = 3$). Data represent mean ± SEM. *–****, $p < 0.05$–$p < 0.00005$

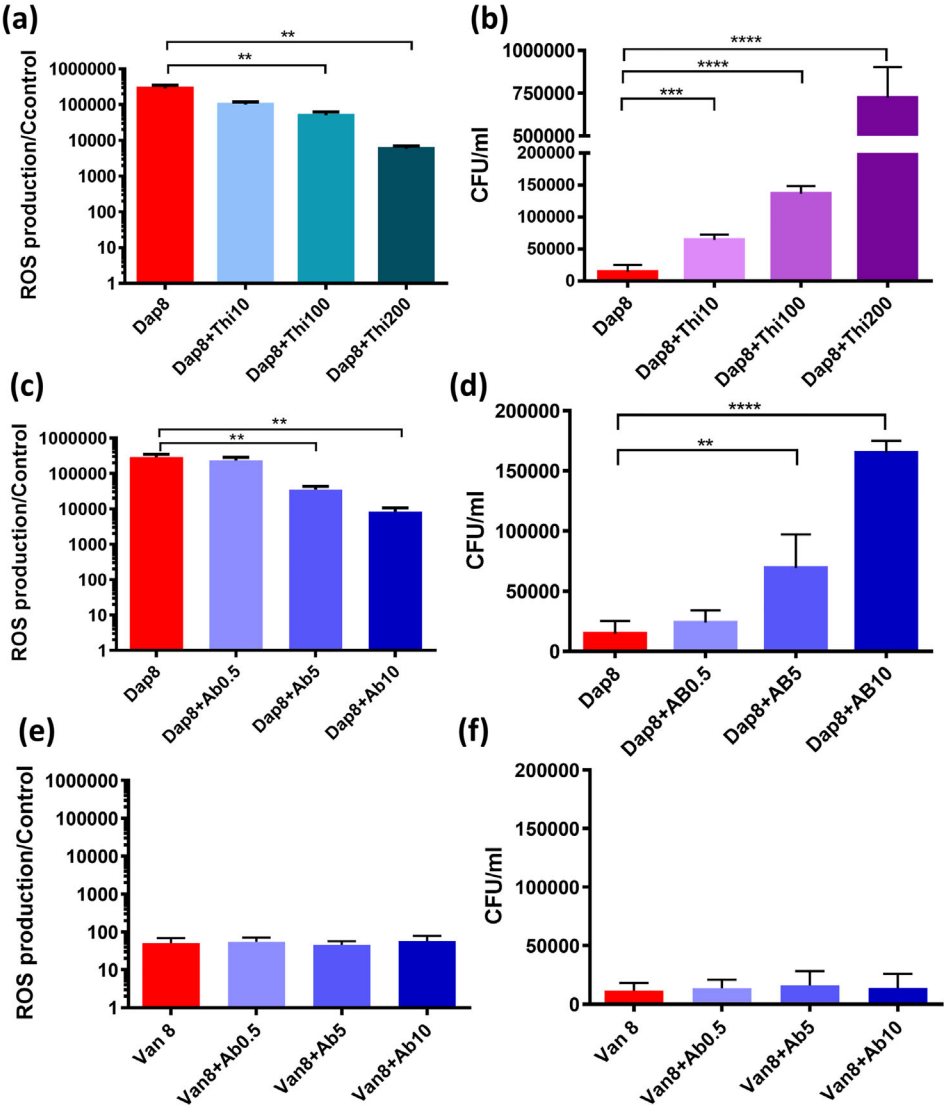

FIGURE 2 Daptomycin triggered overproduction of ROS in Gram-positive bacteria. (a) ROS production induced by daptomycin in *S. aureus* in the presence of 10, 100, and 200 mM of thiourea; (b) survival of *S. aureus* upon treatment with daptomycin in the presence of 10, 100, and 200 mM of thiourea; (c) ROS production induced by daptomycin in *S. aureus* in the presence of 0.5, 5, and 10 μg/ml of Usp2-specific polyclonal antibodies; (d) survival of *S. aureus* upon treatment with daptomycin in the presence of 0.5, 5, and 10 μg/ml of Usp2-specific polyclonal antibodies; (e), ROS production induced by vancomycin in *S. aureus* in the presence of 0.5, 5, and 10 μg/ml of Usp2-specific polyclonal antibodies; (f) survival of *S. aureus* upon treatment with vancomycin in the presence of 0.5, 5, and 10 μg/ml of Usp2-specific polyclonal antibodies. Intracellular ROS was determined using a general oxidative stress indicator, CM-H2DCFDA. X-axes represent the concentration of compounds, "Dap 8" (and similar labels) means 8 μg/ml. Unpaired two-sided Student's *t* test was performed for data analysis. Each data point was repeated for three times ($n = 3$). Data represent mean ± SEM. *–****, $p < 0.05$–$p < 0.00005$

fluorescein (HPF), was used to confirm the data with very consistent results as that of CM-H2DCFDA (Figure S3). It is noted that the signals obtained using HPF were about 10 times lower than CM-H2DCFDA, but both dyes are feasible for this study.

We have also tested the expression level of oxidative stress response genes, *katA* and *soda*, upon incubation with daptomycin. Our data showed that the expression level of these two genes was significantly increased in particular in the presence of 16 μg/ml of daptomycin. In the presence of thiourea, the ROS reduced gradually according to the increase in concentration of thiourea (Figure S4). The data suggested that daptomycin could induce the overproduction of oxidative stress response genes, while the overproduction of these genes seemed not enough to offset the ROS production triggered by high concentration of daptomycin.

Daptomycin binds to the universal stress protein (Usp2) in *S. aureus*

We have shown that the interaction of daptomycin with the cell membrane bilayer could lead to triggering ROS response. Next, we tried to investigate the specific induction of ROS response by daptomycin,

TABLE 1 MIC of daptomycin and its analogues tested against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus aureus* (SA) in μg/ml

	Bacterial strain				
	MRSA				SA
Compounds	SA11I4	SA11	SA86	SA88	29213
Daptomycin	2	0.25	0.25	0.25	0.25
D1	16	2	2	2	2
D2	≥32	≥32	≥32	≥32	≥32
D3	16	2	2	2	2
Probe 21	2	0.5	0.5	0.5	1
Probe 70	≥32	16	16	16	16

Abbreviations: CMR, coumarin; D1, Dap-Trp^{1}NBD; D2, Dap-Orn^{6}NBD; D3, Dap-Trp^{1}CMR; NBD, nitrobenzoxadiazole.
Note: Structures are shown in Table S1. MIC experiments have been repeated for at least three times.

while other cyclic peptide antibiotics, such as vancomycin, teixobactin, and colistin, not showing this effect. To probe whether there are protein targets involved in the bactericidal effect of daptomycin, we developed daptomycin-based photoaffinity probes based on the structure–activity relationship which we have established [12]. A daptomycin analogue named as probe **21** (Dap-Orn^{6}alkyne/diazirine) was chemically synthesized as bait to fish out the potential binding protein (Table S1). Probe **21** was shown to maintain the activity on *S. aureus*, suggesting that incorporation of the bifunctional group of alkyne and diazirine at Orn [6] did not affect the binding of this probe to the potential target (Table 1). Another probe, probe **70**, in which Orn [6] was substituted by a bifunctional amino acid (compound **S1**), exhibited much reduced antibacterial activity on *S. aureus* and was used as the control (Tables 1 and S1). When these probes were incubated with *S. aureus* cell lysate, followed by photo cross-linking and Click reaction with azide-rhodamine, a distinct fluorescence band unique to probe **21**, but not other controls, could be identified (Figure 3a). This band could be aligned to a 17 kDa protein band in the Coomassie blue-stained gel (Figure 3a). This protein band was cut out and subjected to the identification of protein ID by ESI ion-trap. Most of the peptide hits aligned to a protein named Usp2 (UspA) (Accession no. WP_000634176).

To confirm if Usp2 is the cell membrane target of daptomycin, in vitro biological assays were performed to test the binding affinity of daptomycin to a recombinant Usp2 protein, which was expressed in *E. coli* with the N-terminal His tag removed (Figure S2). First, binding of daptomycin to Usp2 in vitro was tested by incubating the recombinant Usp2 protein with a fluorescence-labeled daptomycin analogue. A coumarin-labeled daptomycin analogue, **D3** (Dap-Trp^{1}CMR), which exhibited similar activity as **D1**, was synthesized for this binding assay due to the high background of **D1** (Tables 1 and S1). No fluorescence signal was detectable upon incubating Usp2 alone. Compared to the assay containing analogue **D3** alone, much higher intensity of fluorescence could be detected when Usp2 and **D3** were incubated together, and the assay containing Usp2, **D3,** and calcium exhibited even higher fluorescence than that without calcium (Figure 3b). This observation suggested that **D3** could specifically bind to Usp2 with high affinity in the presence of calcium. The assay containing **D3** and daptomycin for competition purpose showed that **D3** bound with slightly lower affinity than a binding reaction with **D3** alone (Figure 3b). Next, florescent polarization (FP) assay was performed by incubating **D1** and recombinant Usp2. The results showed that the daptomycin analogue **D1** could bind to Usp2, with K_d being around 68.0 μM, while incubation of **D2** with Usp2 (or incubation of **D1** with BSA, data not shown) did not see any FP signal, suggesting that daptomycin, indeed, exhibited specific binding to the Usp2 protein (Figure 3c–e). Preincubation of daptomycin with Usp2 completely abolished FP, further confirming the specific interaction of **D1** with Usp2 (Figure 3f).

To further verify that daptomycin binds to Usp2 in the membrane of *S. aureus*, a FRET assay was performed. First, incubation of fluorescent daptomycin analogue **D1** with *S. aureus* indicated that daptomycin was localized on the cell membrane of *S. aureus*, while **D2** did not show any binding (Figures 4a and b). Next, the RFP-Usp2 fusion protein was expressed in *S. aureus* ATCC29213 and found to be present in abundance throughout the cell surface, with much higher intensity of signal detectable in the cell membrane (Figure 4c). This suggests that the Usp2 protein was localized in bacterial cell membrane and dispersed to some extent in the cytosol. To confirm that the RFP tag did not have effect on Usp2 localization, freshly prepared *S. aureus* ATCC29213 cells were fractionated to separate the membrane and cytosol fractions, followed by detection of the wild-type Usp2 protein by Western blot. The data showed that Usp2 could be detected at almost equal amount in the cytosol and cell membrane fraction, suggesting that RFP-Usp2 did not affect the localization of Usp2 (Figure 4d). FRET assay was then performed by incubating **D1** with *S. aureus* ATCC29213 expressing RFP-Usp2, with results showing that strong and modulating FRET signals (reflecting as orange and green color) were detectable in various *S. aureus* cells (Figures 4e and f). This finding suggests that daptomycin directly interacts with Usp2 on the membrane of *S. aureus*. It should be noted that no FRET signal was observed in the cytosol of *S. aureus*, suggesting that daptomycin was not internalized into the cytosol of *S. aureus*; such finding was consistent with the membrane location pattern of daptomycin observed.

Furthermore, we developed polyclonal antibodies, anti-Usp2 antibody, targeting to Usp2 using recombinant Usp2 protein produced in *E. coli* system (Figure S5). The anti-Usp2 antibody was shown to specifically recognize Usp2 protein from *S. aureus* ATCC29213 and recombinant Usp2 protein produced by *E. coli* (Figure S6). We then tested if it could block the binding of daptomycin to Usp2 on *S. aureus*. Our data showed that pretreatment of *S. aureus* strain with 5 μg/ml anti-Usp2 antibody could significantly reduce the binding of **D1** to *S. aureus* when compared to *S. aureus* that were treated with 0.5 and 0 μg/ml anti-Usp2 antibody, suggesting that anti-Usp2 antibody could specifically block the binding of daptomycin to Usp2. The data provide further evidence to support that daptomycin can specifically bind to Usp2 (Figure 5).

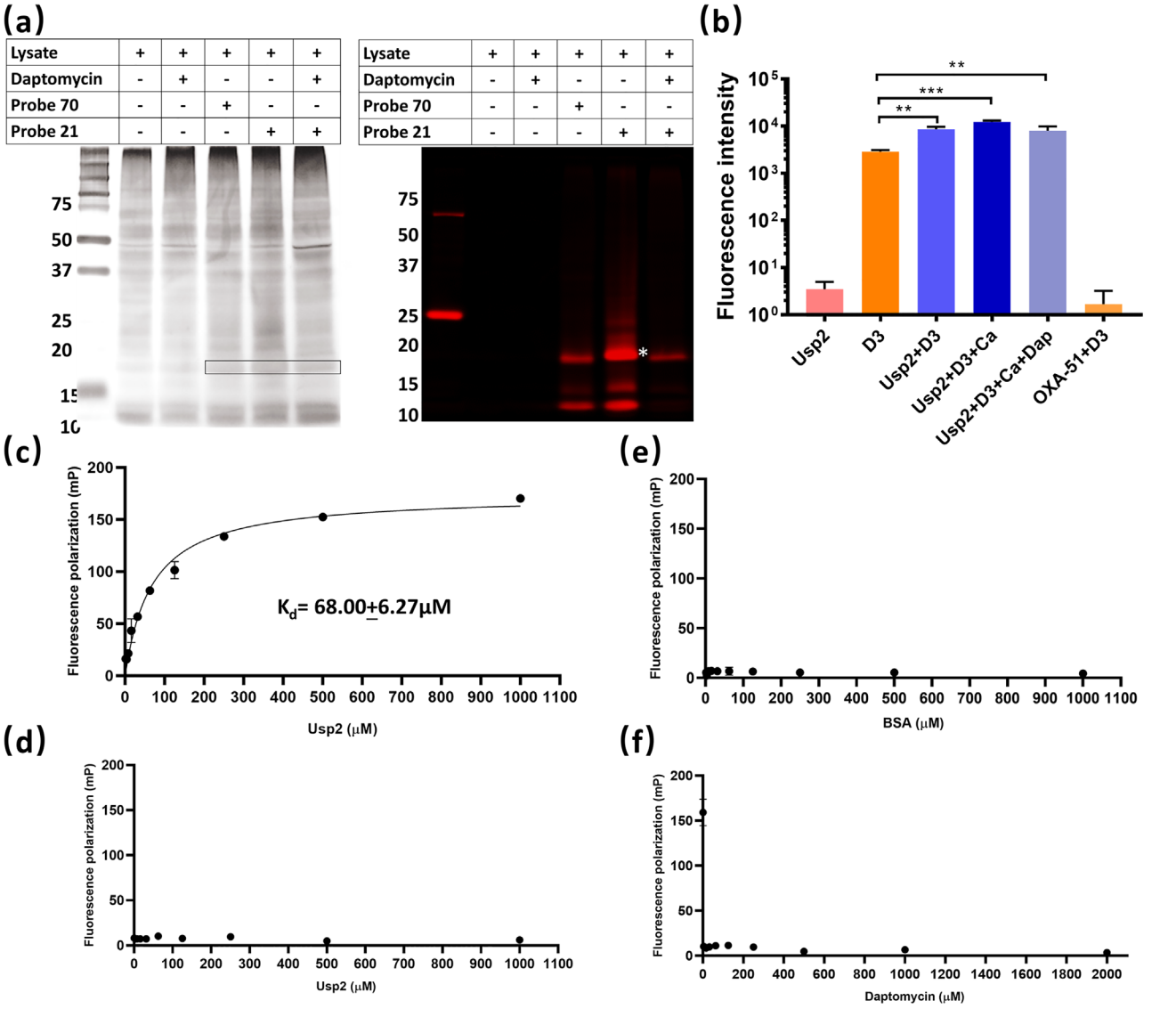

FIGURE 3 Usp2 as cell membrane target of daptomycin. Identification of daptomycin cell membrane target with rhodamine-labeled daptomycin, using a cross-linking approach. *S. aureus* lysates (3 mg/ml) were incubated with the indicated analogue (50 μM) with or without UV irradiation at 365 nm for 40 min. The labeled proteins were conjugated with rhodamine azide, resolved by SDS-PAGE, and visualized by fluorescence gel imaging. (a) Normal SDS-PAGE gel and SDS-PAGE visualized by fluorescence gel imaging. * represents the potential proteins that bind to daptomycin. Box area represents proteins band that was cut for protein ID. (b) Binding of daptomycin analogue, **D3**, with Usp2 in the presence of calcium. OXA-51, a β-lactamase, is used as negative control. (c) Measurement of binding between daptomycin analogue D1 and Usp2 through fluorescent polarization (FP) assay. (d) Binding of **D2** to Usp2 through FP assay. (e) Binding of daptomycin to BSA by FP assay. (f) Preincubation of Usp2 with daptomycin before FP assay. All experiments have been repeated for at least three times. Unpaired two-sided Student's *t* test was performed for data analysis. Each data point was repeated for three times ($n = 3$). Data represent mean ± SEM. *–****, $p < 0.05$–$p < 0.00005$

Binding of daptomycin to Usp2 blocks its role on anti-ROS response leading to excessive ROS production and bactericidal effect in *S. aureus*

To test if binding of daptomycin to Usp2 contributes to the ROS production, anti-Usp2 antibody was used to block the binding of daptomycin to Usp2, followed by assessment of effect of this blockage on ROS production. Different concentrations (0.5, 5, and 10 μg/ml) of anti-Usp2 antibody were incubated with *S. aureus* ATCC29213 before treatment with 8 μg/ml of daptomycin for 3 h. Significant ($p = 0.0074$ and $p = 0.0039$, respectively) reduction in ROS production was observed when the bacteria were preincubated with 5 and 10 μg/ml antibody (Figure 2c). Consistently, the survival rate of *S. aureus* was found to increase significantly ($p = 0.0064$ and $p < 0.0001$, respectively) upon preincubation with 5 and 10 μg/ml of antibody (Figure 2d). However, no reduction on ROS production and increase of bacterial survival for vancomycin were observed in the presence of up to 10 μg/ml of antibody (Figures 2e and f). These data suggested that

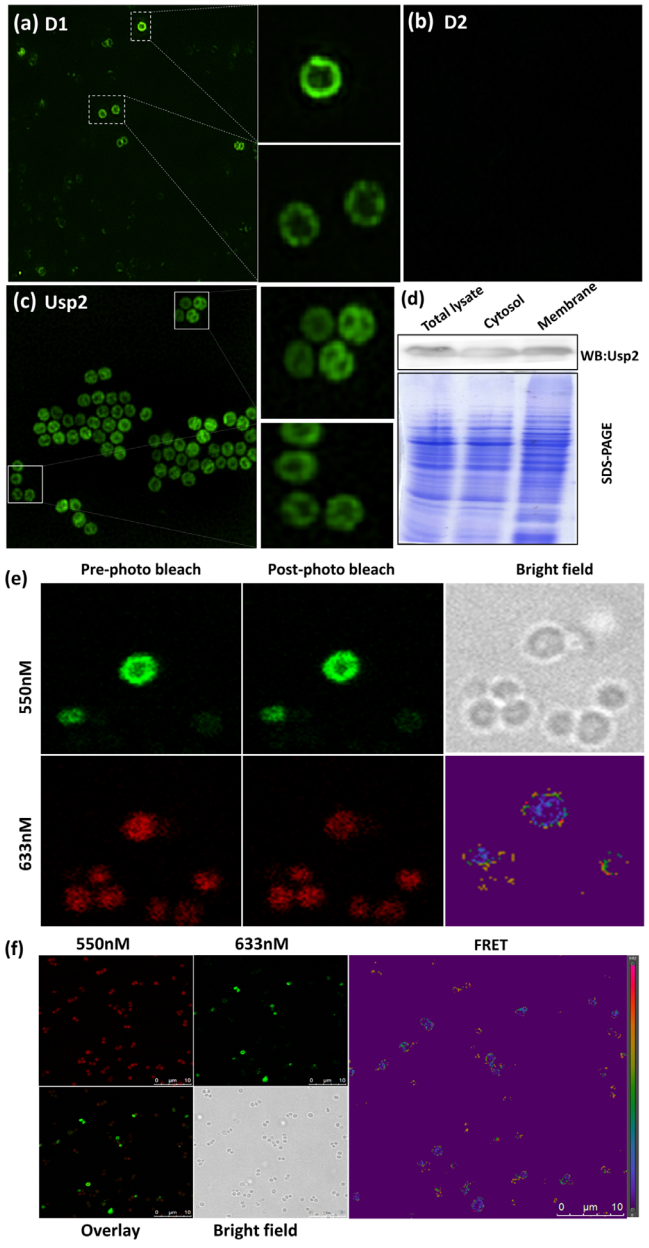

FIGURE 4 Membrane localization of daptomycin, Usp2, and Förster resonance energy transfer (FRET) between membrane Usp2 and daptomycin. Binding of fluorescence-labeled daptomycin, **D1**, (a) and its inactive analogue, **D2** (b) to *S. aureus* strain ATCC29213. Membrane localization (c) of RFP-Usp2 protein in *S. aureus* and fractionation (d) of wild-type Usp2 protein in *S. aureus* strain ATCC29213. FRET assay analyzed by confocal microscope with large (e) and small (f) magnitudes. RFP-Usp2 protein was expressed in *S. aureus* ATCC29213 and shown to be present in abundance throughout the cell surface, with much higher intensity of signal detectable in the cell membrane, suggesting that the Usp2 protein was localized in bacterial cell membrane and dispersed to some extent in the cytosol. Upon expression of the RFP-Usp2 protein in *S. aureus*, the fluorescent daptomycin analogue **D1** was incubated with bacterial cells expressing RFP-Usp2. FRET signals were analyzed and shown to be localized in the cell membrane. All experiments have been repeated for at least three times

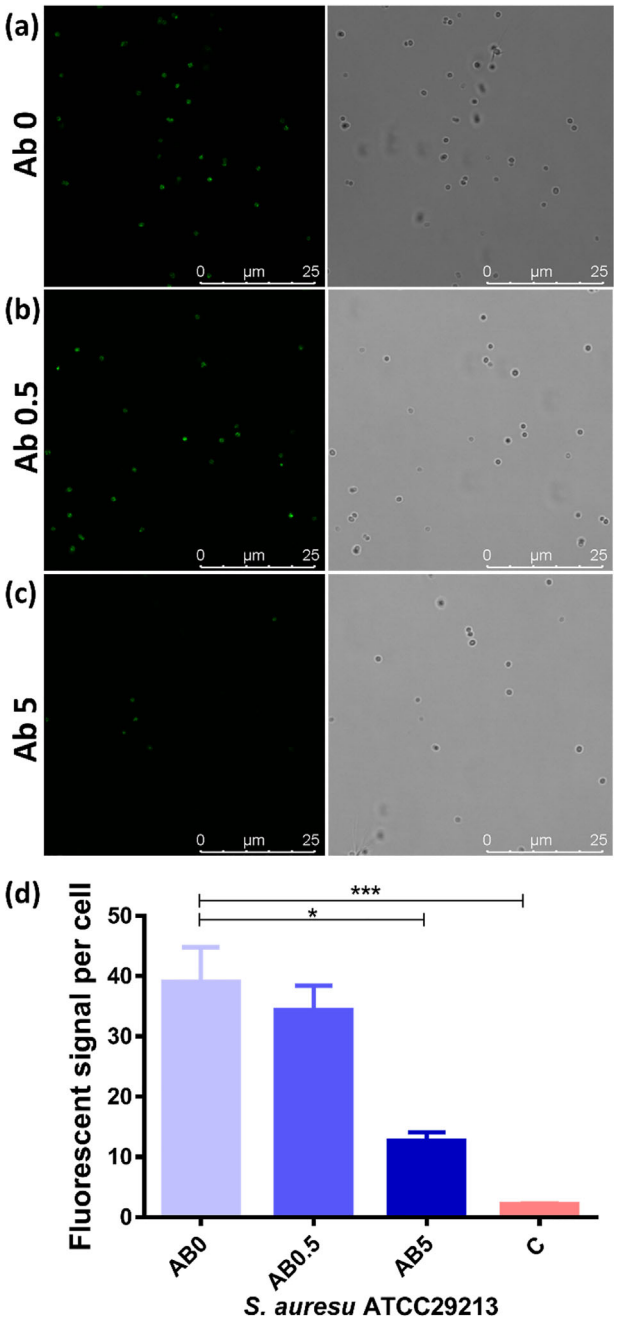

FIGURE 5 Anti-Usp2 antibody blocks binding of daptomycin to *S. aureus*. Binding of daptomycin analogue, **D1**, to *S. aureus* ATCC29213 in the presence of 0 μg/ml (a), 0.5 μg/ml (b), and 5 μg/ml (c) of anti-Usp2 antibody. Left panel, fluorescent *S. aureus* cells; right panel, bright field. (d) Quantification of fluorescence signals. Unpaired two-sided Student's *t* test was performed for data analysis. Each data point was repeated for three times ($n = 3$). Data represent mean ± SEM. *–****, $p < 0.05$–$p < 0.00005$

blockage of the binding of daptomycin to Usp2 significantly reduced ROS production and resulted in elevation in the survival rate of *S. aureus* upon daptomycin treatment. These data implied that blocking the interaction between Usp2 and daptomycin impaired ROS production and suppressed the bacterial killing effect of daptomycin, suggest-

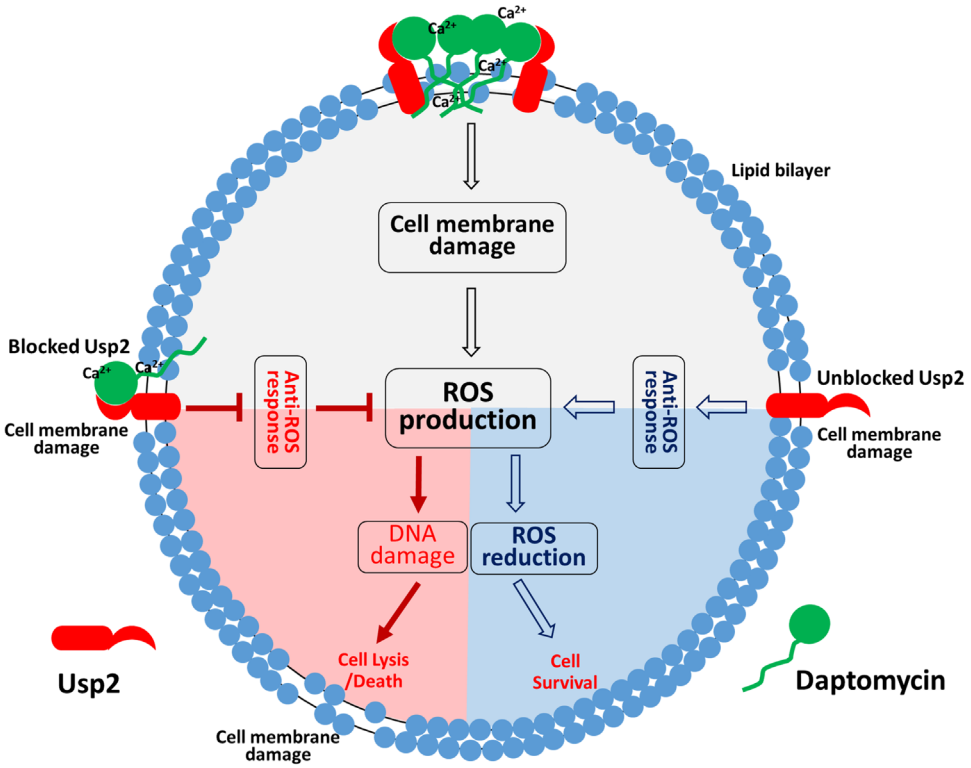

FIGURE 6 Mechanism of action of daptomycin. Daptomycin works by binding to Usp2 so that the hydrophobic lipid tail of the drug can be inserted to bacterial cell membrane. In the presence of calcium ions, aggregation of membrane-bound daptomycin occurs, resulting in membrane damage. These events in turn trigger overproduction of ROS. The binding to Usp2 protein, which is an essential protein that mediates onset of bacterial oxidative stress response, might block the function of this protein, rendering the organism unable to express anti-ROS response, thereby predisposing cell death due to excessive production of ROS

ing that binding to Usp2 is necessary for triggering ROS overproduction and *S. aureus* killing during daptomycin treatment. Usp2 is a universal stress protein (USP) that belongs to the USP family. There are six USPs, namely, Usp2, UspC, UspD, UspE, UspF, and UspG, in *E. coli*. The Usp2 protein has been found to be associated with different stress responses in bacteria, yet the exact functional role of these proteins has not been elucidated [12]. It was proposed that Usp2 played a protective role in mediating anti-ROS response as knockout of *usp2* in *E.coli* led to higher susceptibility to oxidative stress [13]. A similar phenomenon was also observed in *Acinetobacter baumannii* [14]. It was further reported that different Usp2 proteins in other bacterial species and plants were phosphorylated under stress conditions. For example, Usp2 of tomato was shown to be the phosphorylation target of protein kinase CIPK6, which is involved in ROS production in plant [15].

CONCLUSION

To conclude, our studies revealed the bactericidal mechanism of action of daptomycin. We showed that this drug works by triggering the ROS production through interacting with bacterial cell membrane. The presence of calcium ions also facilitates aggregation of membrane-bound daptomycin and promotes cell membrane damage. Our data further showed that the binding of daptomycin to Usp2 protein is necessary for triggering ROS overproduction and *S. aureus* killing during daptomycin treatment. Owing to the key role of Usp2 protein in mediating bacterial stress response, we speculated that the binding of daptomycin to Usp2 might suppress the function of this protein, rendering the organism unable to express anti-ROS response and hence extremely susceptible to the deleterious effect of excessive production of ROS (Figure 6).

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COMPETING INTEREST

The authors declare no competing financial interest.

AUTHOR CONTRIBUTIONS

Kathy Po: Conceptualization-Equal, Data curation-Equal, Investigation-Equal, Methodology-Equal. Hoi Yee Chow: Conceptualization-Equal, Data curation-Equal, Investigation-Equal, Methodology-Equal. Qipeng Cheng: Data curation-Supporting, Formal analysis-Supporting, Investigation-Supporting, Methodology-Supporting. Kwan Wai Chan: Data curation-Supporting. Xin Deng: Conceptualization-Supporting, Methodology-Supporting. Shuping Wang: Investigation-Supporting,

Methodology-Supporting. Edward Chan: Conceptualization-Supporting, Writing-review & editing-Supporting. Hang-Kin Kong: Resources-Supporting, Software-Supporting. Kin Fai Chan: Methodology-Supporting. Xuechen Li: Conceptualization-Equal, Formal analysis-Equal, Funding acquisition-Equal, Project administration-Equal, Writing-review & editing-Equal.

DATA AVALIBILITY STATMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

TRANSPARENT PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/ntls.10023.

ORCID

Xuechen Li https://orcid.org/0000-0001-5465-7727
Sheng Chen https://orcid.org/0000-0003-3526-7808

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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