7,10-Epoxyoctadeca-7,9-dienoic Acid: A Small Molecule Adjuvant That Potentiates β -Lactam Antibiotics Against Multidrug-Resistant *Staphylococcus aureus*

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Abstract The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections with multi-drug resistance needs effective and alternative control strategies. In this study we investigated the adjuvant effect of a novel furan fatty acid, 7,10-epoxyoctadeca-7,9-dienoic acid (7,10-EODA) against multidrug-resistant *S. aureus* (MDRSA) strain 01ST001 by disc diffusion, checker board and time kill assays. Further the membrane targeting action of 7,10-EODA was investigated by spectroscopic and confocal microscopic studies. 7,10-EODA exerted synergistic activity along with β -lactam antibiotics against all clinical MRSA strains, with a mean fractional inhibitory concentration index below 0.5. In time-kill kinetic study, combination of 7,10-EODA with oxacillin, ampicillin, and penicillin resulted in 3.8–4.2 log₁₀ reduction in the viable counts of MDRSA 01ST001. Further, 7,10-EODA dose dependently altered the membrane integrity ($p < 0.001$) and increased the binding of fluorescent analog of penicillin, Bocillin-FL to the MDRSA cells. The membrane

action of 7,10-EODA further facilitated the uptake of several other antibiotics in MDRSA. The results of the present study suggested that 7,10-EODA could be a novel antibiotic adjuvant, especially useful in repurposing β -lactam antibiotics against multidrug-resistant MRSA.

Keywords Furan fatty acids · 7,10-Epoxyoctadeca-7,9-dienoic acid · Synergistic antibacterial agent · β -Lactam antibiotics · Multidrug-resistant *Staphylococcus aureus*

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen associated with many clinical conditions [1, 2]. Development of multidrug-resistance in MRSA [3] severely impacts public health. According to World Health Organizations, MDRSA annually cause 19,000 deaths in US alone [4]. The increasing healthcare burden of MDRSA has led researcher to consider new control strategies. One attractive approach is to repurpose old antibiotics with unorthodox combinations of natural products against MDRSA. Antibiotic adjuvants have been reported to reduce the antibiotic resistance of pathogens [5]. Several plant extracts, food-products and essential oils have been used to repurpose various classes of antibiotics against MDRSA [6]. Nonetheless, huge clinical demand exists for novel non-antibiotic adjuvants with multiple health benefits.

Fatty acids are important structural and functional molecules in both prokaryotes and eukaryotes. The importance of various free fatty acids as antimicrobials in food, agriculture, and biomedical fields has been reviewed [7]. Natural furan fatty acids possess cardioprotective, neuroprotective, radical scavenging, anti-inflammatory and

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antifungal properties [8–12]. However, antimicrobial properties of furan fatty acids are not investigated and hence remained unknown. Recently, a novel furan fatty acid, 7,10-epoxyoctadeca-7,9-dienoic acid (7,10-EODA), was produced using a dihydroxy fatty acid [13]. Earlier, we reported the antimicrobial potential of 7,10-EODA with the ability of sub-lethal concentration to reduce the virulence factor production in MRSA [14]. Based on this report, we postulated that sub lethal concentration of 7,10-EODA could also exert adjuvant activity with commercial antibiotics against MDRSA. In the present study, we investigated the antibiotic adjuvant potential of 7,10-EODA with several commercial antibiotics. Further we describe the β -lactam antibiotic potentiating ability of 7,10-EODA against MDRSA and specific membrane targeting role of 7,10-EODA in this process.

Materials and Methods

Chemicals and Reagents

All Antibiotics and solvent (dimethyl sulfoxide, DMSO) used in the present study were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethidium bromide (EtBr), Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), 4',6-diamidino-2-phenylindole (DAPI), and Bocillin-FL were purchased from life technologies (Carlsbad, CA, USA). The 7,10-EODA (> 95% pure) was produced according to a previously described method [13].

Strains and Growth Conditions

Clinical isolates of MRSA strains 01ST001, 165, 018, and 093, 02ST 085, 136, 03ST 120, were obtained from National Culture Collection for Pathogen (Osong, South Korea), a member of world data center for microorganisms (WDCM 852). Methicillin-susceptible *S. aureus* (ATCC 29213) was obtained from American Type Culture Collection (ATCC, Baltimore, USA). *S. aureus* 1199 and 1199B (clinical isolates) were kind gift from Dr. G. W. Kaatz (Wayne State University, MI, USA). All strains were grown in either trypticase soy broth (TSB) or nutrient broth (NB) purchased from Becton–Dickinson (Cockeysville, MD, USA). Strains were stored at -80°C in NB containing 30% glycerol.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the test agents was determined according to CLSI guidelines [15]. Briefly, each test sample was prepared in a 2-fold serial dilution in Mueller–Hinton Broth

(MHB) in 96-well microtiter plates. Mid-log phase bacteria were diluted 10-fold with MHB (5×10^5 c.f.u/ml), and 0.1-ml aliquots were added to the wells and incubated at 37°C for 24 h. The optical density of cell growth was determined at 610 nm (OD_{610}). The minimum concentration required to prevent > 90% cell growth was considered as MIC.

Antibiotic Adjuvant Screening by Disc Diffusion Assay

For antibiotic adjuvant screening, TSA plates were seeded with 5×10^5 CFU/ml of MDRSA01ST001 and ATCC 29213 cultures and placed with two sets of sterile filter paper discs. One set was impregnated with antibiotics (1/4MIC), and the other set was impregnated with the mixture of antibiotics and 7,10-EODA (1/4MIC, 31.2 μg). Discs containing dimethyl sulfoxide (DMSO) or 7,10-EODA (1/4MIC) alone served as controls. The plates were incubated at 37°C for 24 h. Potential synergistic effect was inferred when the size of the zone of inhibition (ZOI) was ≥ 5 mm ($\geq 70\%$) around the antibiotic and 7,10-EODA containing discs.

Fractional Inhibitory Concentration Index Determination

The interaction between selected antibiotics and 7,10-EODA was evaluated by the checker board method [16]. Two-fold serial dilutions of 7,10-EODA and test antibiotics ($2 \times \text{MIC}$) from a stock were prepared in the columns and the rows of a 96-well microtiter plate to obtain combinations of 7,10-EODA and antibiotics at various concentrations. Then, test strains (5×10^5 CFU/ml) was added to the well of microtiter plate and incubated without shaking at 37°C for 24 h. At the end of the incubation, cell growth (OD_{610}) was determined using spectrophotometer (Tecan M200 Infinite Pro, Mannedorf, Switzerland). The FIC index (FICI) was calculated as follows: $\text{FICI} = \text{FIC A} + \text{FIC B}$, where FIC A is MIC of drug a in combination/MIC of drug a alone and FIC B is MIC of drug B in combination/MIC of drug B alone. FICI was as follows: ≤ 0.5 , synergy; > 0.5 to ≤ 1.0 , additive; > 1.0 to ≤ 4.0 , indifference, and > 4.0 , antagonism.

In-Vitro Time Kill Kinetics

Glass tubes containing β -lactam antibiotics (1/4MIC) or 7,10-EODA (1/8 and 1/4MIC) or a combination thereof in 10 ml of MHB were inoculated with of MDRSA01ST001 (5×10^5 CFU/ml). DMSO (10 μl) treated samples served as negative controls. The treatments were incubated at 37°C in a shaking incubator (150 rpm) for 24 h. At 0, 3, 6,

and 24 h, culture suspension (0.1 ml) was aseptically removed and serially diluted and plated on MHA plates and viable counts were determined.

EtBr Uptake Assay

The membrane integrity was determined according the method of [17] with slight modifications. Briefly, cell suspension of mid-log phase cells of MDRSA 001 (0.5 OD_{610}) was prepared in 1 ml of PBS (pH 7.4). Aliquots (0.1 ml) were transferred to the wells of microtiter plate containing 0.1 ml PBS (pH 7.4) containing 7,10-EODA (15.6–31.2 $\mu\text{g/ml}$). CCCP (A proton motive force disrupting agent) served as positive control. Finally EtBr (5 μM) was added to each well and the fluorescence intensity of cells was monitored at 360 nm (exi) and 616 nm (emi) wavelengths using fluorescence spectrophotometer (Tecan M200 Infinite Pro, Mannedorf, Switzerland) till 30 min. The relative final fluorescence was determined using the formula; $\text{RFF} = (\text{F}_{\text{compound}} - \text{F}_{\text{control}}) / \text{F}_{\text{control}}$, where $\text{F}_{\text{compound}}$ corresponds to the fluorescence of the EtBr accumulation curve at 30 min in the presence of a 7,10-EODA. $\text{F}_{\text{control}}$ is the fluorescence of the EtBr accumulation curve at 30 min in the absence of 7,10-EODA.

Visualization of Bacterial Cell Membrane Permeation

MDRSA 01ST001 cells ($2 \times 10^5 \text{ CFU/ml}$) was treated with 7,10-EODA (0–31.2 $\mu\text{g/ml}$) in MHB for 1 h. Cells were harvested by centrifugation at $5000 \times g$ for 5 min, washed twice with PBS (pH 7.4), and then incubated with DAPI/PI (5 μM) for 5 min. After harvesting and three washes with PBS, the cell pellet was resuspended in PBS, and a 10- μl aliquot of the sample was mounted on a 1% agarose pad and imaged using a Nikon Eclipse 80i microscope (Nikon Co., Tokyo, Japan) with an excitation wavelength of 494 nm.

Bocillin Binding Study

The effect of 7,10-EODA on the Bocillin-FL binding was studied according to the method of [18] with slight modifications. Briefly, the MDRSA 01ST001 in TSB medium and antibiotics were added with Bocillin-FL (2 $\mu\text{g/ml}$) or a combination of Bocillin-FL (2 $\mu\text{g/ml}$) and 7,10-EODA (15.6 or 31.2 $\mu\text{g/ml}$). After 30 min incubation, cultures were centrifuged at $9000 \times g$ for 4 min, washed four times with PBS and ground with 0.2 μm glass beads for 15 min and resuspended in a 0.1 ml of saline. The Bocillin-FL fluorescence was determined by microplate reader (Tecan M200 Infinite Pro, Mannedorf, Switzerland) operated at 485 nm (exc) and 530 nm (emi) wavelengths, respectively.

Statistical Analysis

Where applicable the data were analyzed for statistical significance by a two tailed student's *t* test for unpaired data using Graph Pad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

Results

7,10-EODA Exhibits Selective β -Lactam Potentiation Activity

MRSA 01ST001 shows resistance to multiple antibiotics (Fig. S1) and hence it is referred as MDRSA 01ST001 in this study. In viability assays, 7,10-EODA (125 $\mu\text{g/ml}$) was bactericidal (3.6 $\log_{10}$ reduction). However, sub MIC of 7,10-EODA (15.6, 31.2 $\mu\text{g/ml}$) showed no growth inhibition against MDRSA 01ST001 (Fig. 1). The results of antibiotic adjuvant screening suggested that 7,10-EODA (31.2 $\mu\text{g/ml}$) increased the size of ZOI of β -lactam antibiotics (oxacillin, ampicillin, and penicillin) by 92–135% when compared to antibiotic controls (Table 1). A representative synergistic interaction between 7,10-EODA and penicillin against MDRSA 01ST001 was represented in Fig. 2. Interestingly, 7,10-EODA could not sensitize β -lactams against methicillin-sensitive *S. aureus* (MSSA, ATCC 29213). All other antibiotics had nil to moderate activity (falling below 70% cutoff).

Fig. 1 Determination of susceptibility of MDRSA01ST001 to 7,10-EODA treatment. MDRSA 01ST001 cells ($5 \times 10^5 \text{ CFU/ml}$) were cultured in presence of 7,10-EODA (0–125 $\mu\text{g/ml}$) in MHB (10 ml) at 37 °C for 24 h. A 1 ml aliquot from each treatment was serially diluted in saline and 0.1 ml was placed on MHA for viable counts. Mean log CFU/ml $\pm$ SD value was given (n = 4). Student's *t* test: *** $p < 0.001$ compared with controls. The line spanning two adjacent bars are not significantly different (ns) from controls

Table 1 Antibiotic adjuvant effect of 7,10-EODA against methicillin-resistant and methicillin-sensitive *S. aureus*

Antibiotic ^a	MIC (µg/ml)		ZOI (mm) without/with 7,10-EODA ^b	
	01ST001	29213	MRSA01ST001	ATCC29213
STP	62.5	3	6.0 ± 0.0/12.9 ± 0.2	6.0 ± 0.0/11.4 ± 0.7
CIP	6.0	2.5	6.0 ± 0.0/13.2 ± 0.1	9.6 ± 0.2/13.2 ± 0.2
CPL	10	10	13.2 ± 0.4/13.9 ± 0.4	7.4 ± 0.2/7.21 ± 0.2
CPZ	62.5	125.0	7.2 ± 0.1/12.0 ± 0.5	6.0 ± 0.0/8.05 ± 0.5
RIF	5	5	18.3 ± 0.2/20.2 ± 0.1	21.0 ± 0.8/23.7 ± 1.2
IRG	0.1	0.1	12.4 ± 0.3/13.8 ± 1.1	11.1 ± 0.5/10.0 ± 0.3
TMP	7.8	7.8	6.0 ± 0.0/6.00 ± 0.0	8.9 ± 0.1/12.3 ± 0.2
OXA	62.5	0.04	6.0 ± 0.1/14.1 ± 0.2	8.3 ± 0.1/9.60 ± 0.4
AMP	1000	1.97	7.3 ± 0.2/14.2 ± 0.6	16.9 ± 0.3/17.0 ± 0.5
CEP	1000	1.97	10.1 ± 1.3/13.1 ± 0.6	15.3 ± 0.1/15.3 ± 0.2
PEN	1000	0.5	7.1 ± 0.1/13.6 ± 0.5	12.9 ± 0.4/13.2 ± 0.4

^aSTP streptomycin, CIP ciprofloxacin, CPL chloramphenicol, CPZ chlorpromazine, RIF rifampicin, IRG irgasan, TMP trimethoprim, OXA oxacillin, AMP ampicillin, CEP cephalexin, PEN penicillin

^bThe antibiotics and 7,10-EODA used for synergy was 1/4 MIC and 1/4 MIC (31.2 µg), respectively

Fig. 2 Representative adjuvant screening agar plate exhibiting a potential synergy between penicillin and 7,10-EODA in MDRSA01ST001. Disc *a* penicillin (5 µg), *b* penicillin (125 µg), *c* 7,10-EODA (31.2 µg), *d* Penicillin (5 µg) + 7,10-EODA (31.2 µg), *e* penicillin (125 µg) + 7,10-EODA (31.2 µg). All compounds were dissolved in DMSO (20 µl)

Fractional Inhibitory Concentration Index

In primary screening, 7,10-EODA was identified as a potentiator of β -lactam activity against MDRSA 01ST001. The interaction between the combinations of 7,10-EODA and β -lactam antibiotics was confirmed in clinically relevant MRSA strains using checker board assay. As summarized in the Table 2, the FIC indices of oxacillin, ampicillin, and penicillin were from 0.15–0.62, 0.18–0.62, and 0.28–0.74, in combination with 15.6 and 31.2 µg/ml of 7, 10-EODA against MRSA isolates. Specifically in 01ST001, the MIC of oxacillin (62.5 µg/ml) and ampicillin (1000 µg/ml) was reduced by 4 and 16 folds in the presence of 15.6 and

31.2 µg/ml of 7,10-EODA, respectively. Similarly, the MIC of penicillin (500 µg/ml) was reduced by 2 and 16 folds in the presence of 15.6 and 31.2 µg/ml of 7,10-EODA, respectively. 7,10-EODA exhibited indifference (FICI, 2.0) against *E. coli* and *S. typhimurium*. From primary screening, it was evident that 7,10-EODA was adjuvant with some non β -lactam antibiotics (> 70% increase in ZOI). From the checkerboard assays (Table 3), 7,10-EODA exhibited synergy (FICI range, 0.24–0.37) with streptomycin in 83% of the strains. In contrast 7,10-EODA with chlorpromazine (FICI range, 0.64–1.25) and ciprofloxacin (FICI range, 0.74–1.25) exhibited weak synergy or additive effect in most of the *S. aureus* strains tested.

Time Kill Kinetics

7,10-EODA/ β -lactam combinations showing synergy interaction in checkerboard assay were confirmed by time kill kinetics. The time kill kinetics of 7, 10-EODA-oxacillin, 7,10-EODA-ampicillin, and 7,10-EODA-penicillin combination against MDRSA 01ST001 are illustrated in Fig. 3a–c. In all cases, the untreated controls quickly reached the stationary phase (9.2–9.5 log c.f.u/ml) with in 6 h and remained stable thereafter during 24 h. 7,10-EODA at 15.6 µg/ml did not affect the growth of MDRSA 01ST001. 7,10-EODA (31.2 µg/ml), oxacillin (3.9 µg/ml, 1/16MIC), and ampicillin (62.5 µg) all had temporary growth inhibition by 3 h but the growth seems to be comparable to controls during 24 h incubation. Combination of 7,10-EODA (15.6 µg/ml) with oxacillin (3.9 µg/ml, 1/16MIC), ampicillin (62.5 µg/ml, 1/16MIC), and penicillin (31.2 µg/ml) exhibited additive effect (Range, 1.2–2.6 log reduction). Interestingly, combinations of 7,10-

Table 2 In vitro inhibitory activity of 7,10-EODA in combination with β -lactam antibiotics against methicillin-resistant *Staphylococcus aureus*

Strain ^a	Oxacillin					Ampicillin					Penicillin				
	MIC (μ g/ml)			FIC index		MIC (μ g/ml)			FIC index		MIC (μ g/ml)			FIC index	
	A ^b	B	C	b	c	A	B	C	b	c	A	B	C	b	c
01ST001	62.5	15.6	3.90	0.37	0.31	1000	125	62.5	0.24	0.25	500	250	31.2	0.74	0.31
01ST093	2000	1000	125	0.62	0.31	31.2	15.6	3.90	0.62	0.37	15.6	7.81	1.95	0.62	0.37
01ST165	31.2	7.81	3.90	0.37	0.31	2000	500	7.81	0.18	0.25	1000	500	31.2	0.62	0.28
02ST085	250	125	7.81	0.62	0.15	1000	500	62.5	0.62	0.31	125	31.2	7.81	0.37	0.31
02ST136	62.5	15.6	3.90	0.37	0.31	1000	125	15.6	0.24	0.26	1000	250	62.5	0.37	0.31
03ST120	250	125	31.2	0.62	0.37	250	31.2	7.81	0.25	0.28	62.5	15.6	3.91	0.37	0.31
ATCC 8739	200	200	200	1.25	1.50	0.65	0.65	0.31	1.25	0.72	125	125	62.5	1.25	1.00
KCTC 2515	250	250	250	1.25	1.50	0.65	0.65	0.65	1.25	1.25	250	250	250	1.25	1.50

^aThe MIC of 7,10-EODA against MRSA strains was 125 μ g/ml and against Gram negative strains (8739 and 2515) was > 500 μ g/ml

^bA, without 7,10-EODA. B to, C and b to c, 7,10-EODA at 15.6 and 31.2 μ g/ml for MRSA strains, and 125 and 250 μ g/ml for Gram negative strains, respectively

Table 3 In vitro synergy of 7,10-EODA in combination with non β -lactam antibiotics against clinical strains of *S. aureus*

Strain ^a	MIC of antibiotics (μ g/ml)														
	Streptomycin			FICI		Chlorpromazine			FICI		Ciprofloxacin			FICI	
	A ^b	B	C	b	c	A	B	C	b	c	A	B	C	b	c
01ST001	62.5	15.6	7.81	0.37	0.24	125	31.2	15.6	0.37	0.37	15.6	15.6	3.90	1.24	0.49
01ST018	2000	1000	250	0.67	0.37	62.5	62.5	31.2	1.12	0.49	7.80	7.80	3.90	1.12	0.74
01ST093	31.2	7.81	1.97	0.37	0.31	250	125	31.2	0.62	0.39	1.25	1.25	0.31	1.12	0.49
29213	6.25	1.56	0.78	0.37	0.37	31.2	7.81	15.6	0.37	0.64	0.24	0.24	0.12	1.12	0.74
SA1199	6.25	0.39	0.39	0.24	0.37	62.5	62.5	31.2	1.12	0.74	1.95	1.95	1.95	1.24	1.24
SA1199B	7.81	7.81	3.90	1.12	0.74	125	62.5	125	0.64	1.24	7.81	7.81	3.90	1.24	0.74

Boldface represents potential synergy (FIC index <1.0)

^a001, 018 and 093 are clinical MRSA. SA 1199 and SA 1199 B, and ATCC 29213 are methicillin-sensitive *S. aureus* reference strain

^bA, without 7,10-EODA. B to, C and b to c, 7,10-EODA at 15.6 and 31.2 μ g/ml, respectively. The MIC of 7,10-EODA is 125 μ g/ml

EODA/oxacillin (31.2 μ g/ml/3.9 μ g/ml), 7,10-EODA/ampicillin (31.2 μ g/ml/62.5 μ g/ml), and 7,10-EODA/penicillin (31.2 μ g/ml/31.2 μ g/ml) yielded 3.88, 3.89, and 4.27 log reductions suggesting a bactericidal interaction.

7,10-EODA Causes Membrane Integrity Loss in MDRSA 01ST001

From fluorimetric assay (Fig. 4), it was evident that cells treated with DMSO exhibited a small increase in RFF value (1.26 ± 0.11) over 30 min. A significant increase in the RFF value of EtBr (4.96 and 4.58 folds, $p < 0.001$) was evident in presence of 15.6 and 31.2 μ g/ml of 7,10-EODA, respectively. Positive control (CCCP, 5 μ M) exhibited a 2 fold increase in RFF of EtBr ($p < 0.05$). Further, microscopic visualization of MDRSA 01ST001 cells (Fig. 5) revealed that cells treated with increasing concentration of 7,10-EODA found to contained increased concentration of propidium iodide (PI).

Bocillin-FL Binding Study

Using fluorescent penicillin analog, bocillin-FL, the interaction between penicillin binding proteins and β -lactam antibiotic was studied. It was found that cells grown in the presence of 7,10-EODA (31.2 μ g/ml) had moderate, yet statistically significant binding of (31.7%, $p < 0.05$) of Bocillin-FL (Fig. 6). However, 7,10-EODA (15.6–31.2 μ g/ml) did not enhance the binding of Bocillin-FL in the case of ATCC 29213 (Fig. S2).

7,10-EODA May be a Potential Inhibitor of Efflux Pump Activity in MDRSA 01ST001

We studied the reversal of MIC of EtBr by 7,10-EODA in MDRSA01ST001, SA1199B (Nor A over expressing), and SA1199 (wild type) strains. From the checkerboard assay (Table 4), it is evident that the MIC of EtBr (25 μ g/ml) was reduced by 8, 4, and 4 folds in presence of 31.2 μ g/ml of

Fig. 3 Time-kill kinetics for MDRSA 01ST001 in presence of individual and combinations of β -lactam antibiotics. Cultures were incubated with **a** 3.9 $\mu\text{g/ml}$ of oxacillin; **b** 62.5 $\mu\text{g/ml}$ of ampicillin; **c** 31.2 $\mu\text{g/ml}$ of penicillin in combination with 7,10-EODA at

15.6 $\mu\text{g/ml}$ (E1) and 31.2 $\mu\text{g/ml}$ (E2). Controls with DMSO served as blank. Samples treated with only 7,10-EODA and antibiotics served as controls. Samples were obtained at various time points and plated for viable counts. The results are the mean $\pm$ SD ($n = 4$)

Fig. 4 Effects of 7,10-EODA on the fluorescent intensity of EtBr incubated with clinical isolate of MDRSA 01ST001 at 15.6 and 31.2 $\mu\text{g/ml}$. CCCP served as positive control. The results are the mean $\pm$ SD of two separate experiments with triplicates ($n = 6$). Student's t test: ** $p < 0.01$; *** $p < 0.001$ compared with controls

7,10-EODA against MDRSA 01ST001, SA 1199B, and SA1199, respectively and this effect was comparable with CCCP, an ionophore, which at 6.25 $\mu\text{g/ml}$, reduced the MIC of EtBr by 8 fold against test strains. Verapamil reduced the MIC of EtBr by 4–16 folds against the tested strains.

Discussion

MRSA has become resistant to vancomycin, teicoplanin, daptomycin, and linezolid [3]. Further, pharmaceutical companies with diminished profits, reduced its research on novel antibiotic discovery. Hence a clinical demand exists for safe non-antibiotic agents to against MRSA. The antimicrobial and virulence inhibitory role of 7,10-EODA was reported [14]. In light of these facts, the present study evaluated the adjuvant action of 7,10-EODA with conventional antibiotics against MDRSA 01ST001. 7,10-EODA at 125 $\mu\text{g/ml}$ is bactericidal against many *S. aureus* strains including MRSA. However, *In vitro* synergy was

Fig. 5 Confocal microscopic visualization of 7,10-EODA induced influx of propidium iodide (PI) in MDRSA 01ST001. DAPI was used as a counter stain. Each experiment was repeated two times with 3 replicates and representative image were presented. (Scale bar: 10 μ m)

Fig. 6 Biocillin-FL binding in *Staphylococcus aureus* in presence of 7,10-EODA. **a** Uptake of Bocillin-FL by MDRSA01ST001 and ATCC 29213. Each experiment was repeated 3 times with four replicates ($n = 12$). Student's t test: ** $p < 0.01$ in comparison with controls. **b** Confocal microscopy visualization of bocillin-FL uptake in MDRSA01ST001. Each experiment was performed two times with three replicates ($n = 6$) and representative images were presented

observed with 7,10-EODA and β -lactam antibiotics against clinical isolates of MRSA strains with MDR phenotype. Time-kill kinetics revealed that the combinations of 7,10-EODA and β -lactams are bactericidal against MDRSA 01ST001. However, to the best of our knowledge, we have described for the first time the β -lactam adjuvant potential of 7,10-EODA against MDRSA.

In general penicillin binding proteins (PBP2a) and β -lactamases confer β -lactam resistance in MDRSA. 7,10-EODA due to lipophilicity can accumulate and quickly alter the membrane integrity of MDRSA01ST001 in a non-lethal manner. In contrast, membrane integrity loss by several toxic lipids was lethal against *S. aureus* [19]. Considering the fact that membrane integrity is important

for cell wall synthesis and transport [20], we propose that the mechanism of β -lactam synergy by 7,10-EODA is a dual-step process with a fast-acting membrane damage step followed by a slow interference with PBP2a activity in MDRSA/MRSA. Therefore, 7,10-EODA and β -lactams combination would sequentially impact membrane integrity as well as cell wall biosynthesis. In support of our hypothesis, 7,10-EODA enhanced Bocillin-FL binding to PBPs in MDRSA01ST001 and did not synergize β -lactams in MSSA. Earlier a fatty acid derivative (HAMLET) potentiated β -lactam activity against a wide range of pathogens by virtue of membrane disruption [21].

Among the non- β -lactams tested for synergy, 7,10-EODA/streptomycin combination was the more potent in

Table 4 Effect of 7,10-EODA on antibiotic delivery in *S. aureus* with efflux pump activity

Drug (μg/ml)	MIC of EtBr (μg/ml)/(fold reduction)		
	01ST001	SA1199B	SA1199
Controls	25	25	6.25
7,10-EODA			
15.6	6.25/(4)	12.5/(2)	3.12/(2)
31.2	3.12/(8)	6.25/(4)	1.56/(4)
CCCP			
3.95	6.25/(4)	12.5/(2)	1.56/(4)
7.81	3.12/(8)	3.12/(8)	0.78/(8)
Verapamil			
62.5	3.12/(8)	6.25/(4)	3.12/(2)
125	1.56/(16)	1.56/(16)	1.56/(4)

Boldface represents increased accumulation of ethidium bromide in presence of efflux inhibitors

terms of FIC index against MRSA and MSSA. Besides being membrane active, lipids potentiate aminoglycoside activity by facilitating its uptake and inhibition of protein synthesis in *S. aureus*. These two mechanisms might have simultaneously acted to result in strong synergy observed with 7,10-EODA/streptomycin combination. These results are consistent with aminoglycoside potentiating activity of glyceryl monolaurate, tridecan-1-ol against *S. aureus* strains [22, 23]. In our study, chlorpromazine/7,10-EODA combination resulted in synergy against MRSA strains. We speculate that such combination might work sequentially on the β -lactam resistance factors. In support of our observation, chlorpromazine was shown to reduce *mec A* and *bla* genes expression in MRSA [24]. MRSA resistance to fluoroquinolones may be due to the activation of multidrug transporters such as *nor A* proteins [25]. 7,10-EODA/ciprofloxacin combination elicited a weak synergy against MRSA/MSSA and this effect was attributed to the efflux inhibitory potential of 7,10-EODA in MDRSA. MDRSA01ST001 is an efflux phenotype (data not shown) and we further shown that 7,10-EODA inhibited EtBr efflux in MDRSA 01ST001 and SA1199B (Nor A over expressing) strains. These results are consistent with previous studies pertaining to the efflux inhibitory activity of linoleic and oleic acids from purslane (32–64 μg/ml) potentiating erythromycin activity against MRSA [26].

The results of the present study suggested that 7,10-EODA has potential to synergize β -lactam antibiotics against clinical MRSA with MDR phenotype. The benefit of using 7,10-EODA as adjuvant is twofold, as it adds to the antimicrobial library and extends the use of β -lactam antibiotics. The concentration of 7,10-EODA used in this study can be clinically achievable. Further, the benefit of using anti-oxidant molecule, 7,10-EODA as adjuvant is

twofold, as it acts as a preservative in topical preparations as well as repurpose the use of β -lactam in patients with chronic secondary infections or sepsis. 7,10-EODA is a new chemical entity, therefore thorough investigation on the in vivo efficacy and molecular mechanism of antibiotic synergy is required.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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