Synergists from *Portulaca oleracea* with macrolides against methicillin-resistant *Staphylococcus aureus* and related mechanism

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KEY MESSAGES

1. Two unsaturated fatty acids—linoleic acid and oleic acid—were identified from *Portulaca oleracea* that acted synergistically with erythromycin in vitro against methicillin-resistant *Staphylococcus aureus* RN4220-pUL5054, possibly by inhibiting the methionine sulfoxide reductase A multidrug efflux pump of the bacteria.
2. By comparing a panel of linoleic acid and oleic acid analogues, unsaturated fatty acids in salt form with cis configuration and an increase in number of double bonds were found to increase the antibacterial activity against RN4220-pUL5054 when used alone or in combination with erythromycin.
3. The salt forms of linoleic acid and oleic acid with erythromycin could significantly reduce the bacterial count in the lungs of mice infected with RN4220-pUL5054.

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Introduction

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) remain a major problem worldwide. The pharmaceutical arsenal available to control MRSA is limited. Vancomycin is the mainstay treatment for MRSA, but its overuse has generated fully resistant MRSA strains. Screening of natural products for antibacterial effects, especially efflux pump inhibitors, is attracting increasing attention. Using high-speed counter-current chromatography, 18 fractions were obtained by fractionation of *Portulaca oleracea* (PO). The non-polar fraction no.18 (F18) showed synergistic activity with four antibiotics (ampicillin, ciprofloxacin, erythromycin, and gentamicin) against intermediate level vancomycin-resistant MRSA strain ST239. F18 only exhibited synergism with erythromycin against MRSA RN4220/pUL5054 that the methionine sulfoxide reductase A (MsrA) is resistant to 14-membered macrolides via an adenosine triphosphate (ATP)–binding cassette pump, and could lower the minimal inhibitory concentrations (MIC) of erythromycin by three-fold at 32 μg/mL.[1] No synergism of F18 was found against *S aureus* NorA 1199B (resistant to quinolones and harbouring a multidrug resistance pump) or aminoglycoside-resistant MRSA strains APH2"–AAC6', APH3', and ANT4'. These results suggest that F18 may contain putative efflux pump inhibitors and restore the antibacterial activity of erythromycin by inhibiting the ATP-binding cassette efflux pump but not NorA from MRSA. This study aimed to identify the active ingredients from F18 of PO that synergise with macrolides against MRSA and to determine the mechanisms of resistance and to confirm their synergistic effects with antibiotics in a murine pneumonia model.

Methods

The study was conducted from April 2012 to March 2014. The ethanol extract of PO was fractionated using a high-speed counter-current chromatography TBE-1000. MRSA strains of ST239, 1199B, RN4220-pUL5054, APH2"-AAC6', APH3', and ANT4' were used. ATCC25923 served as a control. To identify the synergistic interaction of PO active ingredients with erythromycin against SA-RN4220/pUL5054, checkerboard arrays with multiple dilution combinations of two different antimicrobial agents in a concentration range from below to above the MIC were performed in a 96-well microtitre for 24 hours at 37°C. An efflux inhibitory assay was performed to confirm whether the synergistic mechanisms of the PO-isolated compounds were mediated through inhibition of *S aureus* efflux pumps. A murine lung infection model was used to validate the *in vivo* efficacy of the isolated compounds of PO that showed promising activity against MRSA. The animal study protocols were approved by the Animal Experimentation Ethics Committee of

The Chinese University of Hong Kong (reference: 11/055/MIS).

Results

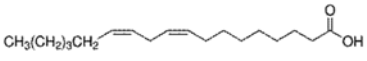
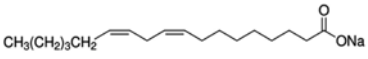
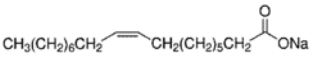
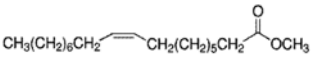
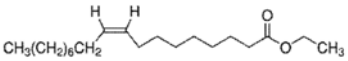
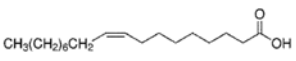
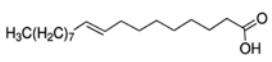
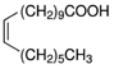
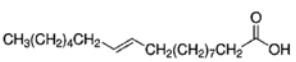
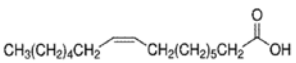
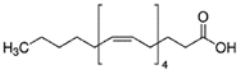
Synergism of linoleic acid and oleic acid with erythromycin against RN4220-pUL5054

Eighteen fractions were obtained by fractionation of PO. A synergistic effect was observed when F18 (32 μg/mL) was combined with erythromycin (32 μg/mL). Growth of the ATP-binding cassette efflux pump of overexpressed strain of RN4220-pUL5054 was inhibited with a fractional inhibitory concentration index (FICI) of 0.38. Using gas chromatography mass spectrometry, 13 compounds were identified. When combined with erythromycin, both linoleic acid and oleic acid inhibited the growth of the bacteria synergistically. Linoleic acid at 16 μg/mL and oleic acid at 32 μg/mL could reduce the MIC of erythromycin from 256 to 16 μg/mL (8-fold reduction) and from 256 to 32 μg/mL (4-fold reduction), respectively. The respective FICI were 0.12 and 0.18, and the respective estimated percentage yields in PO extracts were 0.093 and 0.043%.

Structural activity of linoleic acid and oleic acid analogues against RN4220-pUL5054

To explore the structural activity of the synergistic antibacterial activity of fatty acids, a panel of fatty acid analogues related to oleic acid and linoleic acid was selected for checkerboard assay using RN4220-pUL5054 (Table).[2] Sodium linoleate and sodium oleate were more potent than linoleic acid and oleic acid in inhibiting the growth of RN4220/pUL5054, and acted synergistically with erythromycin, probably due to better water solubility of these salt forms. These results suggest that improved solubility of linoleic acid and oleic acid in the form of ionic salt might enhance their antibacterial activity. When oleic acid was in an ester form, ie methyl or ethyl oleate, antibacterial activity was abolished (Table).[2] The influence of the stereochemistry was investigated. Elaidic acid, the stereoisomer of oleic acid in *trans* configuration, was not active against RN4220-pUL5054 (FICI>0.5). Similar results were also observed for vaccenic acid in *cis* and *trans* configurations, a fatty acid with 18 carbons, as in

TABLE. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI) of oleic acid analogues and related compounds with erythromycin against methicillin-resistant *Staphylococcus aureus* RN4220/pUL5054 (n=2). FICI ≤0.5 is defined as synergistic, >0.5 to ≤1.0 as additive, >1.0 to ≤2.0 as indifferent, and >2.0 as antagonistic.

Chemical structure of the fatty acid	Agent	MIC		FIC	FICI/ outcome
		Alone	Combined		
	1. Linoleic acid	256/256	16/16	0.06/0.06	0.12/0.12
	Erythromycin	256/256	16/16	0.06/0.06	Synergy
	2. Sodium linoleate	128/128	8/8	0.06/0.06	0.07/0.07
	Erythromycin	256/256	1/1	0.01/0.01	Synergy
	3. Sodium oleate	128/128	4/4	0.03/0.03	0.06/0.06
	Erythromycin	256/256	8/8	0.03/0.03	Synergy
	4. Methyl oleate	>256/>256	256/256	0.5/0.5	1.5/1.5
	Erythromycin	256/256	256/256	1.0/1.0	Indifferent
	5. Ethyl oleate	>256/>256	256/256	0.5/0.5	1.5/1.5
	Erythromycin	256/256	256/256	1.0/1.0	Indifferent
	6. Oleic acid,	>256/>256	32/32	0.06/0.06	0.12/0.18
	Erythromycin	256/256	16/32	0.06/0.13	Synergy
	7. Elaidic acid,	>256/>256	256/256	0.5/0.5	0.75/0.75
	Erythromycin	256/256	64/64	0.25/0.25	Additive
	8. cis-Vaccenic acid,	>256/>256	32/64	0.06/0.12	0.09/0.15
	Erythromycin	256/256	8/8	0.03/0.03	Synergy
	9. trans-Vaccenic acid	>256/>256	128/128	0.25/0.25	0.5/0.5
	Erythromycin	256/256	64/64	0.25/0.25	Synergy
	10. Palmitoleic acid,	256/256	16/16	0.06/0.06	0.09/0.09
	Erythromycin	256/256	8/8	0.03/0.03	Synergy
	11. Arachidonic acid	32/32	8/8	0.25/0.25	0.28/0.28
	Erythromycin	256/256	1/1	0.03/0.03	Synergy

oleic acid, but a different unsaturation position (6 and 11, respectively). The MIC of the *cis* form of vaccenic acid was two to three fold lower than that of the *trans* isomer. The MIC and FICI values of oleic acid and vaccenic acid against RN4220/pUL5054 were similar, suggesting that the position of a double bond was not a critical factor that would affect the antibacterial activity of unsaturated fatty acids. Nonetheless, the presence of a double bond appeared to be essential for antibacterial activity. Arachidonic acid, with four unsaturations, was the most potent fatty acid among all tested compounds against RN4220/pUL5054 (MIC alone, 32 μg/mL; FICI=0.25, Table).[2]

Resistance inhibition and cytotoxicity of linoleic acid and oleic acid

In the ethidium bromide efflux inhibitory assay, both sodium linoleate and sodium oleate (32 and 64 μg/mL, respectively) inhibited the fluorescence loss after 30 minutes of incubation and washing, compared with the drug-free control (Fig 1).[2] The inhibitory effects of sodium linoleate (64 μg/mL) were comparable to the positive control reserpine (64 μg/mL). When the fluorescence signals of the compounds over time were quantitatively expressed as the area under the curve (Fig 1), the values of both sodium linoleate and sodium oleate were significantly larger than the drug-free control. In contrast, palmitic acid (PA) had no significant modulating effect on the fluorescence signal of ethidium bromide–loaded cells. Neither linoleic acid nor oleic acid was toxic to human peripheral mononuclear cells, human Caco2, or human skin fibroblasts at concentrations of 32 to 128 μg/mL (data not shown).

Animal studies

In a murine pneumonia model, the amount of inoculated RN4220-pUL5054 for infection was $1x10^8$ colony-forming units (CFU) per mouse. Symptoms of severe illness such as lethargy, hunched posture, ruffled fur, and weight loss were observed after infection. The mean log CFU value for all treatment groups was reduced when compared with no treatment group (8.03±0.21, Fig 2). Vancomycin was the most efficacious among all tested agents (4.73±0.08), with a >3 log reduction in bacterial counts. Since suboptimal dosages of sodium linoleate, sodium oleate and erythromycin were used due to toxicity and solubility issues, their antibacterial activity was mild. When combined erythromycin with sodium linoleate or sodium oleate, the *in vivo* antibacterial activity against RN4220-pUL5054 was enhanced, and their mean log CFU values were 6.51±0.15 and 5.80±0.14, respectively, with an almost 2-log reduction in bacterial count when compared with the no treatment group. Tissue sections from lungs infected with RN4220-pUL5054 revealed recruitment of leukocytes, inflammation of the lung parenchyma, bronchial epithelial damage, and tissue necrosis, compared with a phosphate-buffered saline control. Figure 2 shows the histological changes in the lungs of mice treated with sodium linoleate and erythromycin, sodium oleate and erythromycin, and vancomycin alone. The tissue profile of these treatment groups revealed fewer lesions, infiltration of leukocytes and erythrocytes in the airspace, and preserved alveolar structure. For mice treated with sodium linoleate, sodium oleate and erythromycin alone, the improvement in tissue profile was less significant.

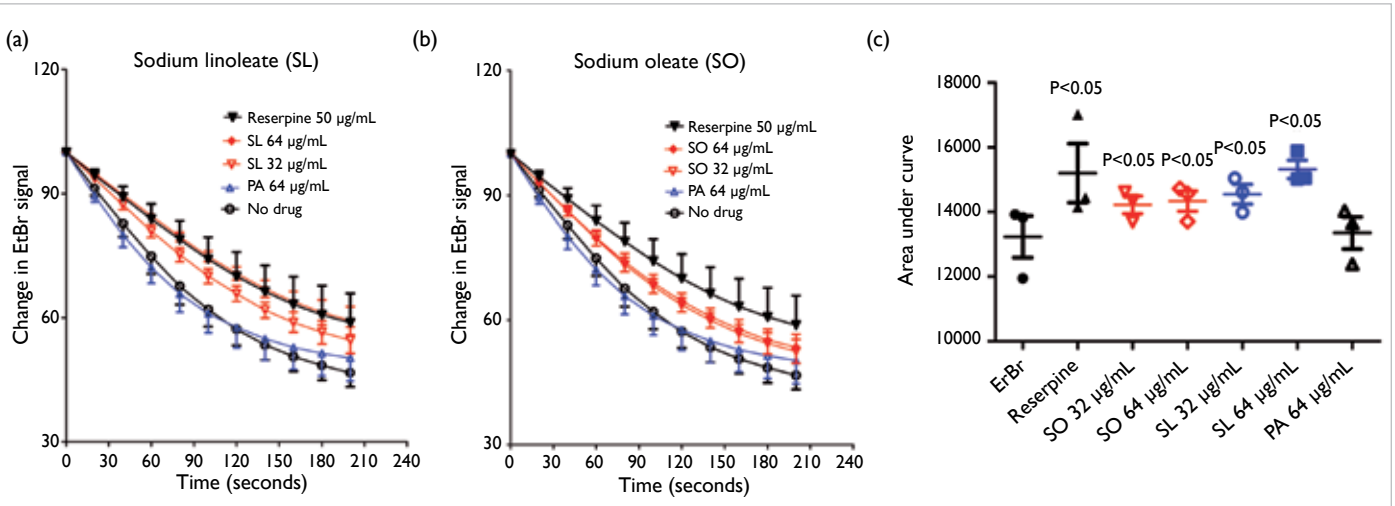


FIG 1. Effects of (a) linoleic acid and (b) oleic acid on ethidium bromide efflux (EtBr) from methicillin-resistant *Staphylococcus aureus* RN4220/pUL5054. Reserpine at 50 μg/mL was used as a positive control, and palmitic acid (PA) was used as negative a control. (c) Area under the curve of the fluorescence against time of sodium linoleate (SL), sodium oleate (SO), reserpine, and PA was compared with the drug-free control. Data are expressed as mean±standard error of mean (n=3).

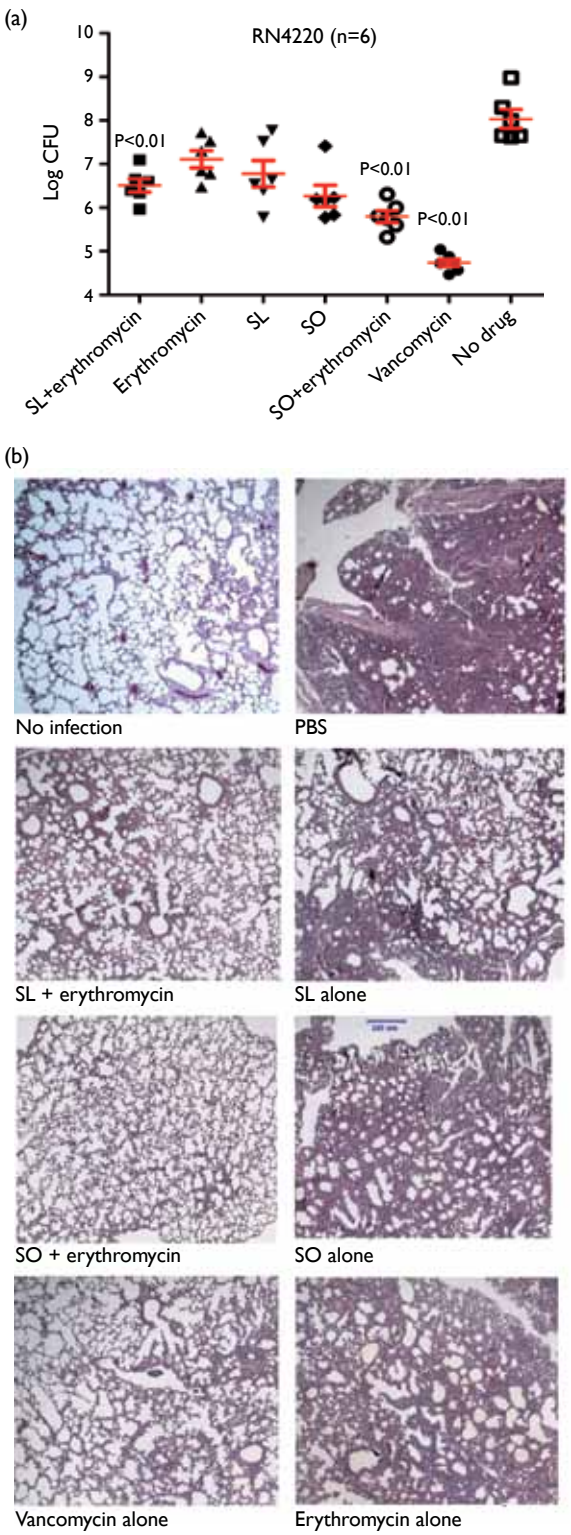


FIG 2. (a) Bacteria counts in log colony forming units (CFU) recovered from the left lung of mice infected with RN4220-pUL5054 (1×10^8 CFU) and treated with sodium linoleate (SL) and erythromycin, erythromycin alone, sodium linoleate (SL) alone, sodium oleate (SO) alone, sodium oleate (SO) and erythromycin, vancomycin alone, or phosphate-buffered saline (no drug) for 48 hours. (b) Histology of lung tissue in normal mice without infection, and mice infected with RN4220-pUL5054 and treated with phosphate-buffered saline (PBS), SL + erythromycin, SL alone, SO + erythromycin, SO alone, vancomycin alone, or erythromycin alone.

Discussion

We identified two unsaturated fatty acids, namely linoleic acid and oleic acid, from PO F18 with mild antibacterial activity against msrA overexpressed RN4220-pUL5054. The synergistic antibacterial activity was observed when combined with erythromycin. Linoleic acid, with two carbon double bonds, is more active than oleic acid that has one carbon double bond in its fatty acid chain.

With regard to the mechanism of resistance, both fatty acids may interfere with the msrA pump and restore the antibacterial effects of erythromycin against RN4220-pUL5054. MsrA is a 488-amino-acid protein with two ATP-binding motifs and functions independently when cloned in SA-RN4220. Linoleic acid and oleic acid may interfere with the activity of the MsrA pump and restore the activity of erythromycin. In a systematic study of the antibacterial effect of fatty acids against *S aureus* RN4220,[3] oleic acid was not active against the growth of RN4220 when used alone, whereas palmitoleic acid was the most potent growth inhibitor against RN4220 with rapid membrane depolarisation and disruption of all major branches of macromolecular synthesis on the bacterial cells. The study suggested that the absence of teichoic acids, a reduction in the level of D-alanine modification, and the absence of major surface protein IsdA could all increase the sensitivity of *S aureus* to fatty acids. There are also studies of multi-drug resistance ATP-binding cassette transporters that are associated with the multi-drug resistance mechanism of cancer cells. In our preliminary study using an ATP-binding cassette p-glycoprotein overexpressed human cancer cell line HepG2, incubation of oleic acid (32 μg/mL) enhanced the rhodamine 123 (a specific substrate to p-glycoprotein transporter) uptake of cells (data not shown). This finding suggests that use of oleic acid may be extended to the development of multi-drug resistance cancer adjuvant therapy.

For *in vivo* studies, sodium linoleate and sodium oleate at a suboptimal dosage could significantly inhibit the growth of RN4220-pUL5054 and ATCC25923, although the effect was inferior to that of vancomycin. This may mainly be due to the possible side effect (acute respiratory distress syndrome) of the unsaturated fatty acid that may worsen in bacteria-induced pneumonia. The dosage of both fatty acids could not be further increased to examine a potentially stronger antibacterial activity in animals. In this regard, the mice pneumonia model may not be an ideal model to evaluate the antibacterial effects of unsaturated fatty acids. We aim to confirm the antibacterial activity of linoleic and oleic acids using skin and wound infection models and examine their potential therapeutic effects in skin and soft tissue infections with MRSA in future studies.

Conclusions

Two active ingredients, namely linoleic and oleic acids, were identified from F18 of PO with synergistic antibacterial activity with erythromycin against MRSA RN4220/pUL5054 *in vitro* and *in vivo*. The effect is likely mediated through inhibition of the efflux pumps of bacteria cells.

Acknowledgement

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References

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3. Parsons JB, Yao J, Frank MW, Jackson P, Rock CO. Membrane disruption by antimicrobial fatty acids releases low-molecular-weight proteins from *Staphylococcus aureus*. J Bacteriol 2012;194:5294-304.