Triple Combinations of Lower and Longer Alkyl Gallates and Oxacillin Improve Antibiotic Synergy against Methicillin-Resistant *Staphylococcus aureus*[▽]

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Using liposome systems, we found that gallates with short alkyl chains were located in the external medium and those with longer alkyl chains were located in the surface region of lipid bilayer. Combinations of these gallates remarkably reduced oxacillin MICs against methicillin-resistant *Staphylococcus aureus* to below the antibiotic breakpoint (≤ 2 $\mu\text{g/ml}$).

We have shown that some alkyl gallates at sub-MIC concentrations, in combination with β -lactams, modulated the activities of antibiotics by reducing the concentration of antibiotics needed to inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) (6, 9). The effect, as well as the intrinsic antimicrobial activity, of alkyl gallates was associated with the length of the alkyl chain (9). An alkyl gallate is characterized molecularly by the amphiphilic property. We considered that such a property is presumably important for the establishment of a molecular interaction with the phospholipid acyl chains of the cell membrane of *S. aureus* (1).

In this study, we aimed to obtain some clue for demonstration of the location of alkyl gallates in the staphylococcal cell and to confirm whether or not combinations of alkyl gallates have a capability to improve the synergistic effect with oxacillin on MRSA strains.

Commercially unavailable alkyl gallates were synthesized as described previously (7, 9). 2-Anthroyloxystearic acid (2-AS), 12-AS, and phosphatidylcholine from egg yolk (egg PC) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were of the highest grade commercially available.

All strains of MRSA (19 strains) and methicillin-sensitive *S. aureus* (MSSA; seven strains) used throughout this work were recovered from our laboratory stock culture collection (8), including clinical isolates of MRSA (17 strains) and MSSA (6 strains), MRSA COL and Mu 3, and MSSA RN4220.

The MICs of the chemicals and antibiotics were determined as described previously (9). Fractional inhibitory concentration (FIC) indices for triple combinations were calculated as follows: $\text{FIC} = \text{FIC}_{\text{OXA}} + \text{FIC}_{\text{B}} + \text{FIC}_{\text{C}} = (\text{Ccomb}_{\text{OXA}}/\text{MIC}_{\text{OXA}}) + (\text{Ccomb}_{\text{B}}/\text{MIC}_{\text{B}}) + (\text{Ccomb}_{\text{C}}/\text{MIC}_{\text{C}})$, where

Ccomb_{B} and Ccomb_{C} are the concentrations of alkyl gallates tested; $\text{Ccomb}_{\text{OXA}}$ is the lowest concentration of oxacillin in the combination that inhibited growth; and MIC_{OXA} , MIC_{B} , and MIC_{C} are the MICs of the compounds when used alone. For combinations of two compounds, the term $\text{Ccomb}_{\text{C}}/\text{MIC}_{\text{C}}$ was omitted. An FIC index of ≤ 0.5 indicates synergy.

Liposome was prepared using egg PC in the presence of either 2-AS or 12-AS, as described previously (3–5) with modifications. The resulting small unilamellar vesicles were suspended in phosphate-buffered saline (PBS; pH 7.4). The final concentration of egg PC in the liposome solution was adjusted to 1 mg/ml. After incubation with an alkyl gallate for 10 min, the liposomes were purified by centrifugation and suspended with PBS. The fluorescence of 2-AS (excitation, 362 nm; emission, 446 nm) or 12-AS (excitation, 381 nm; emission, 446 nm) in the lipid bilayer was measured with a Hitachi fluorescence spectrophotometer F4500 (Hitachi, Ltd., Tokyo, Japan). The fluorescence intensity was calculated as a percentage of the fluorescence intensity of 2-AS or 12-AS in the absence of alkyl gallates.

The anthroyl moieties include in this study are linked to carbons 2 and 12 of the base fatty acid and report the location of alkyl gallates at a defined depth within the membrane bilayer. Hence, if an alkyl gallate is located on the surface of the lipid bilayers, it quenches the fluorescence of 2-AS. On the other hand, if it is located in the hydrophobic core of the lipid bilayers, it quenches that of 12-AS (3–5). In addition, the degree of fluorescence quenching is closely correlated with the amount of compound incorporated into the lipid bilayers (3, 4).

As shown in Fig. 1, gallic acid and gallates with the short alkyl chain, such as methyl, ethyl, propyl, and butyl gallates, had no quenching effect on the fluorescence of 2-AS or that of 12-AS, indicating that gallates with the short alkyl chain are located near the lipid-water interface. The results obtained using a model membrane system are quite suggestive of the behavior of alkyl gallates upon the staphylococcal cell membrane, even though there are some differences in membrane compositions between them: it would be probable that gallates

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FIG. 1. Effects of alkyl gallates on the fluorescence intensity of 2-AS or 12-AS. An alkyl gallate in 0.3 ml of PBS (2 mM) was added to 2.7 ml of liposomal solution containing 2-AS (open circles) or 12-AS (closed circles). After incubation of the mixture for 10 min at 20°C, the fluorescence intensity of 2-AS or 12-AS in the lipid bilayers was measured. The results are expressed as mean values of two independent experiments.

with short alkyl chains would act from the exterior part, which is near the lipid-water interface, of the staphylococcal cell membrane, while gallates with longer alkyl chains would act from the polar part of the phospholipid palisade in the staphylococcal cell membrane.

We predicted that such a difference in location of gallates with distinct lengths of alkyl chains would be reflected in their mechanisms of action, although the mechanisms are unknown. Consequently, the combination of the gallates with the distinct lengths of alkyl chains would cause a dramatic reduction in the MICs of β -lactams against MRSA strains. For this, we used propyl gallate (PG) and octyl gallate (OG) as representatives of a gallate with a short alkyl chain and one with a longer alkyl chain, respectively.

Table 1 summarizes MIC ranges, MIC₅₀s, and MIC₉₀s of oxacillin from testing the antimicrobial activities of mixtures of oxacillin, PG, and OG against 19 strains of MRSA and 7 strains of MSSA, as well as the resulting FIC indices. The oxacillin MICs against MRSA and MSSA strains examined in this study were 64 to 512 and 0.125 to 1 μ g/ml, respectively. The combination of PG, OG, and oxacillin showed a remarkable improvement of antimicrobial activity of the antibiotic against MRSA strains, and the FIC indices indicated strong synergy between oxacillin, PG, and OG against all MRSA strains. In particular, the combination of 25 μ g/ml PG and 6.25 μ g/ml OG with oxacillin is able to reduce the antibiotic MICs of MRSA strains from high levels of resistance (64 to 512 μ g/ml) to below the antibiotic breakpoint (≤ 2 μ g/ml).

The mechanism of alkyl gallate-mediated β -lactam resistance modulation is at present unclear; however, this study highlights the cooperative nature of the interactions between gallates with a longer alkyl chain and gallates with a short alkyl chain with regard to their membrane-associated biological ef-

TABLE 1. MICs of oxacillin in combination with PG and OG

PG concn (μ g/ml) ^a	OG concn (μ g/ml) ^b	MRSA				MSSA			
		Oxacillin MIC (μ g/ml) ^c			FIC index ^d	Oxacillin MIC (μ g/ml) ^c			FIC index ^d
		Range	50%	90%		Range	50%	90%	
0	0	64–512	256	512		0.125–1	0.5	1	
	1.56	32–512	256	512	0.83 $\pm$ 0.36 (0/19)	0.063–1	0.5	1	1.03 $\pm$ 0.50 (0/7)
	3.13	32–512	256	512	0.83 $\pm$ 0.26 (0/19)	0.125–2	0.5	2	1.35 $\pm$ 0.49 (0/7)
	6.25	32–512	256	256	0.97 $\pm$ 0.37 (0/19)	0.125–2	0.5	2	1.41 $\pm$ 0.49 (0/7)
	12.5	32–256	128	256	0.75 $\pm$ 0.25 (6/19)	0.063–0.5	0.25	0.5	0.89 $\pm$ 0.24 (0/7)
6.25	0	64–512	256	512	0.99 $\pm$ 0.16 (0/19)	0.063–1	0.5	1	1.14 $\pm$ 0.45 (0/7)
	1.56	32–512	256	512	0.85 $\pm$ 0.27 (1/19)	0.125–1	0.5	1	1.25 $\pm$ 0.37 (0/7)
	3.13	32–256	128	256	0.66 $\pm$ 0.21 (2/19)	0.125–1	0.25	1	0.99 $\pm$ 0.25 (0/7)
	6.25	32–128	128	128	0.46 $\pm$ 0.09 (16/19)	0.125–0.5	0.25	0.5	0.84 $\pm$ 0.26 (0/7)
	12.5	0.5–64	64	64	0.47 $\pm$ 0.12 (10/19)	0.125–0.5	0.25	0.5	0.89 $\pm$ 0.19 (0/7)
12.5	0	32–512	128	256	0.64 $\pm$ 0.28 (4/19)	0.125–1	0.5	1	1.04 $\pm$ 0.01 (0/7)
	1.56	32–128	64	128	0.46 $\pm$ 0.13 (12/19)	0.125–2	0.5	2	1.35 $\pm$ 0.49 (0/7)
	3.13	32–128	64	128	0.39 $\pm$ 0.13 (16/19)	0.125–1	0.5	1	1.24 $\pm$ 0.38 (0/7)
	6.25	1–32	32	32	0.31 $\pm$ 0.07 (19/19)	0.125–0.5	0.25	0.5	0.80 $\pm$ 0.24 (0/7)
	12.5	0.5–32	32	32	0.43 $\pm$ 0.07 (18/19)	0.063–0.5	0.125	0.5	0.68 $\pm$ 0.14 (0/7)
25	0	2–256	64	256	0.38 $\pm$ 0.15 (17/19)	0.125–0.5	0.5	0.5	1.07 $\pm$ 0.20 (0/7)
	1.56	2–128	128	128	0.49 $\pm$ 0.27 (10/19)	0.125–0.5	0.5	0.5	1.10 $\pm$ 0.20 (0/7)
	3.13	0.5–32	2	16	0.27 $\pm$ 0.08 (18/19)	0.125–0.5	0.25	0.5	0.92 $\pm$ 0.29 (0/7)
	6.25	≤ 0.031 –2	0.25	2	0.31 $\pm$ 0.06 (19/19)	0.063–0.5	0.125	0.5	0.70 $\pm$ 0.14 (1/7)
	12.5	≤ 0.031 –16	≤ 0.031	16	0.44 $\pm$ 0.07 (11/19)	≤ 0.031 –0.063	0.063	0.063	0.58 $\pm$ 0.17 (2/7)

^a For PG, the MIC range, MIC₅₀, and MIC₉₀ against both MRSA and MSSA are 62.5 to 125, 125, and 125 μ g/ml, respectively (9).

^b For OG, the MIC range, MIC₅₀, and MIC₉₀ are 15.6 to 31.3, 31.3, and 31.3 μ g/ml, respectively, against MRSA and 31.3, 31.3, and 31.3 μ g/ml, respectively, against MSSA (9).

^c 50% and 90%, MIC₅₀ and MIC₉₀, respectively.

^d Data are expressed as means $\pm$ standard deviations. Values in parentheses express the number of isolates for which drug combinations resulted in synergism/number of isolates tested.

fects. According to the report of the 28th Session of the Codex Committee on Food Additives and Contaminants (2), the temporary acceptable daily intake of PG in mg/kg of body weight is 0 to 1.4, whereas that of OG was not allocated. Therefore, further studies are needed to establish the pharmacokinetics and toxicity of octyl gallate.

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