6,7-Dihydroxyflavone Dramatically Intensifies the Susceptibility of Methicillin-Resistant or -Sensitive *Staphylococcus aureus* to β-Lactams

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We have demonstrated that 6,7-dihydroxyflavone by itself has only a weak antibacterial effect on methicillin-resistant *Staphylococcus aureus* (MRSA) but that at concentrations less than MIC it synergistically elevates the susceptibility of clinically isolated MRSA and methicillin-sensitive *S. aureus* strains to β-lactam antibiotics from 8- to 32,800-fold.

Infections by methicillin-resistant *Staphylococcus aureus* (MRSA), which occur in both hospitals and the community, present a major therapeutic problem (9). Vancomycin and its derivatives are currently used as antibacterial agents against infectious diseases due to MRSA. However, vancomycin-resistant *S. aureus* has emerged (2, 7, 18, 19). Thus, the development of a new anti-MRSA agents is urgently needed.

Recently, we found that apigenin, luteolin, and other flavonoids, all having similar chemical structures, are selectively toxic to MRSA and methicillin-sensitive *S. aureus* (MSSA) (13) and that these flavonoids elevated the susceptibility to β-lactam antibiotics in MRSA (5). We named these compounds intensifiers of β-lactam susceptibility in MRSA. Flavonoids are secondary plant metabolites present in fruit, vegetables, and beverages such as tea and wine, and they possess anti-inflammatory, antiallergic, antiviral, and anticarcinogenic properties (10).

Here we demonstrated that 6,7-dihydroxyflavone dramatically intensifies from 8- to 32,800-fold the susceptibility to β-lactam antibiotics in all clinically isolated strains of MRSA and MSSA examined.

S. aureus strains 1 to 22, 1003, 1010, 1020, and 1032 (clinical isolates) and COL, RN4220, and N315 were used. Some properties of the clinical strains of *S. aureus* used throughout the present study were determined previously (15).

The MICs of 6,7-dihydroxyflavone (Funakoshi Co. Ltd., Tokyo, Japan) and of antibiotics alone and in combination with 6,7-dihydroxyflavone against the MRSA and MSSA strains were determined as described previously (13). The fractional inhibitory concentration (FIC) was calculated as the MIC of oxacillin and 6,7-dihydroxyflavone in combination divided by the MIC of oxacillin or 6,7-dihydroxyflavone alone (1).

The viable-cell number was determined as follows. MRSA strain 5 (10^6 CFU/ml) was incubated at 37°C in cation-adjusted Mueller-Hinton (MH) broth containing either 8 μg of methicillin/ml, 25 μg of 6,7-dihydroxyflavone/ml, or both. Viability was confirmed by culturing the cells on MH agar plates for 24 h.

Detection of PBP2a was performed by Western blot analysis.

An antibody against PBP2a was prepared with a peptide of PBP2a (CGSKKFEKGMKKLGVGEDIPSDYPFC) (14), which was synthesized by Oriental Yeast Co. (Osaka, Japan) by a solid-phase method. The purity and molecular weight of the peptide were confirmed by reverse-phase high-pressure liquid chromatography and mass analysis (Voyager). An antibody against the peptide conjugated to bovine serum albumin was raised in rabbits by Oriental Yeast Co. MRSA 5 was incubated for 5 h at 37°C in a brain heart infusion broth containing 6,7-dihydroxyflavone (12.5 or 25 μg/ml), and the cell membrane was prepared as described by Oliver et al. (11). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8), proteins were transferred electrophoretically to a nitrocellulose membrane (Bio-Rad), essentially as described by Towbin et al. (17). PBP2a was identified by the luminescence method (ECL Plus; Amersham Biosciences) with the antibody against the peptide of PBP2a (6).

We found that 6,7-dihydroxyflavone (Fig. 1) has a weak antibacterial effect on MRSA but that, at a concentration less than its MIC, it elevates the susceptibility to oxacillin (Table 1). The FIC indices of the combination of oxacillin and 6,7-dihydroxyflavone against 20 isolates of MRSA were found to be between 0.251 and 0.504. This result indicates that the effect of the combination of oxacillin and 6,7-dihydroxyflavone is synergistic.

We also found that 6,7-dihydroxyflavone dramatically intensifies from 8- to 32,800-fold the susceptibility of MRSA strains to β-lactams such as methicillin, cephapirin, panipenem, and cefotaxime in all strains of MRSA and MSSA examined (Table 2). However, 6,7-dihydroxyflavone had no effect on the susceptibility to the other antibiotics indicated in Table 2 except that it also increased the susceptibility to streptomycin, but only weakly. Figure 2 shows the synergistic effects of β-lactams and 6,7-dihydroxyflavone in the growth of MRSA 5. In addition, 6,7-dihydroxyflavone dose dependently inhibited the growth of MRSA in the absence of β-lactams (Fig. 2).

We also examined the effects of 6,7-dihydroxyflavone and methicillin on MRSA viability. The growth rate of MRSA was slightly inhibited in the presence of 25 μg of 6,7-dihydroxyflavone/ml. Methicillin alone (8 μg/ml) had little effect on growth. In contrast, a combination of 25 μg of 6,7-dihydroxyflavone/ml and 8 μg of methicillin/ml, the concentration of which was

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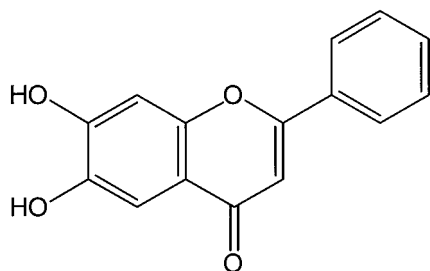
FIG. 1. Chemical structure of 6,7-dihydroxyflavone.

much lower than the MIC (1,024 μg/ml), greatly decreased the viable-cell number (Fig. 3). Therefore, these data clearly demonstrate that the combination of methicillin and 6,7-dihydroxy-flavone results in synergistic bactericidal activity against MRSA.

MRSA is resistant to all β-lactams because of the acquisition of a *mecA* gene cassette, which produces an additional transpeptidase called PBP2a. We therefore examined the effects of 6,7-dihydroxyflavone on the amount of PBP2a by using

TABLE 1. MICs and FIC indices of oxacillin in the presence of 6,7-dihydroxyflavone against MRSA and MSSA

	MIC (μg/ml)				
		Oxacillin			
Strain no.	6,7-Dihydroxy-flavone alone	Alone	With 6,7-dihydroxy-flavone at:		FIC index[a]
			12.5 μg/ml	25 μg/ml	
MRSA					
1	50	512	4	2	0.258
2	25	512	0.25	ND[b]	0.500
3	50	256	0.5	0.25	0.252
4	25	512	2	0.13	0.504
5	50	512	0.5	<0.06	0.251
6	50	128	0.5	0.25	0.254
7	25	512	0.25	ND	0.500
8	50	512	1	0.25	0.252
9	25	512	0.25	ND	0.500
10	50	128	0.25	<0.06	0.252
12	50	512	1	1	0.252
13	50	512	2	1	0.254
16	25	512	2	ND	0.504
17	25	128	0.5	ND	0.504
18	25	512	1	ND	0.502
19	25	512	1	ND	0.502
20	50	64	0.13	<0.06	0.252
21	50	128	0.13	0.13	0.251
22	50	128	0.25	0.13	0.252
COL	50	512	1	0.25	0.252
N315	50	64	<0.06	<0.06	0.251
MSSA					
1003	50	0.5	0.13	<0.06	0.510
1010	50	0.5	0.13	0.13	0.510
1020	50	0.25	<0.06	<0.06	0.490
1032	50	0.25	<0.06	<0.06	0.490
ATCC 6538	50	<0.06	<0.06	<0.06	1.250
RN4220	25	0.13	<0.06	ND	0.962

[a] FIC index ≤ 0.5, synergy effect; 0.5 < FIC index ≤ 1, additive effect.
[b] ND, not determined.
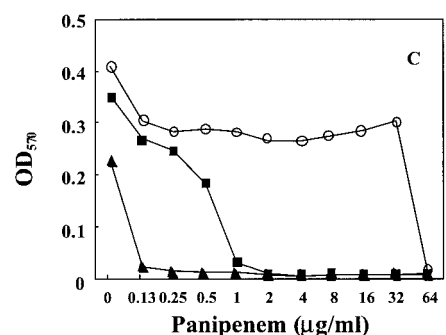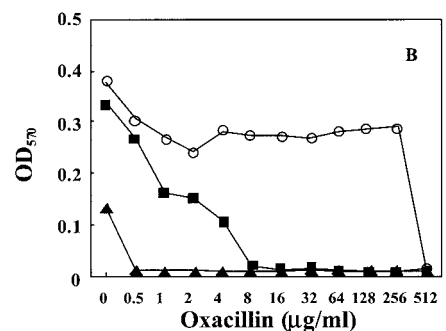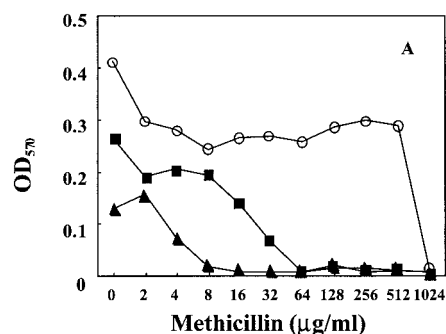
FIG. 2. Synergistic inhibition of growth by 6,7-dihydroxyflavone and methicillin (A), oxacillin (B), or panipenem (C) in MRSA 5. Bacteria were cultured at 37°C in cation-adjusted MH broth, to which was added increasing amounts of 6,7-dihydroxyflavone and the indicated concentrations of methicillin, oxacillin, and panipenem. The bacterial growth was detected as the change in optical density at 570 nm (OD_{570}) at 24 h. Levels of 6,7-dihydroxyflavone: 0 (control; ○), 12.5 (■), and 25 μg/ml (▲).

the antibody against the peptide of PBP2a. Figure 4 clearly demonstrates that 6,7-dihydroxyflavone had no effect on the amount of PBP2a in strain 5.

Guz et al. (4) have demonstrated that flavonolignan and flavone derivatives are potent inhibitors of the NorA multidrug resistance pump (12) in *S. aureus*. However, Table 2 clearly demonstrates that 6,7-dihydroxyflavone had no effect on the susceptibility to ofloxacin of MRSA strains. Furthermore, we have confirmed that 6,7-dihydroxyflavone has no effect on the uptake of oxacillin in MRSA 5 (Y. Miwa, H. Shibata, and T. Higuti, unpublished work).

Alternatively, we propose a model for the mechanism of high resistance of MRSA to β-lactams and the massive reduc-

TABLE 2. MICs of various antibacterial agents for MRSA and MSSA in the absence or presence of 6,7-dihydroxyflavone

Strain no.	MET −	MET +	HAP −	HAP +	PAPM −	PAPM +	CTX −	CTX +	VAN −	VAN +	STR −	STR +	OFX −	OFX +	TET −	TET +	ERY −	ERY +	KAN −	KAN +
MRSA																				
1	>1,024	4	128	0.063	128	0.031	64	0.25	1	0.5	32	8	16	16	0.5	0.5	>1,024	>1,024	>1,024	>1,024
2	>1,024	0.25	128	0.0078	512	0.0078	512	1	0.5	0.5	64	16	128	0.5	0.5	0.5	>1,024	>1,024	>1,024	>1,024
3	1,024	0.25	64	0.5	64	0.063	64	0.25	1	1	16	128	32	0.5	0.5	0.5	>1,024	>1,024	>1,024	>1,024
4	>1,024	4	64	4	64	0.13	64	16	2	2	32	8	64	128	128	128	>1,024	>1,024	>1,024	>1,024
5	>1,024	0.25	128	0.0039	64	0.0039	128	8	1	2	32	16	16	32	0.5	0.5	>1,024	>1,024	>1,024	>1,024
6	>1,024	8	64	0.25	64	0.031	64	0.25	1	0.5	64	32	64	32	0.5	0.5	>1,024	>1,024	>1,024	>1,024
7	>1,024	4	8	8	128	0.063	128	8	2	1	64	32	32	32	0.5	0.5	>1,024	>1,024	>1,024	>1,024
8	>1,024	4	128	8	64	0.13	64	16	2	1	64	16	32	32	0.5	0.5	>1,024	>1,024	>1,024	>1,024
9	>1,024	2	64	0.002	64	0.002	512	0.25	2	0.5	32	8	512	1	1	128	0.25	0.25	>1,024	>1,024
10	512	2	8	0.016	8	0.0078	512	0.25	1	1	32	16	8	256	0.5	0.5	>1,024	>1,024	>1,024	>1,024
12	>1,024	4	64	0.25	64	0.13	1,024	32	1	2	64	16	1	1	0.5	0.5	>1,024	>1,024	>1,024	>1,024
13	>1,024	4	8	2	128	0.13	1,024	32	2	2	64	16	1	32	0.5	0.5	>1,024	>1,024	>1,024	>1,024
16	>1,024	8	64	8	64	0.13	512	64	1	1	64	16	32	32	0.5	0.5	>1,024	>1,024	>1,024	>1,024
17	>1,024	16	32	1	32	0.031	512	4	2	2	32	16	1	1	128	128	>1,024	>1,024	>1,024	>1,024
18	>1,024	8	32	0.5	32	0.031	512	4	2	2	32	16	128	1	128	64	>1,024	>1,024	>1,024	>1,024
19	>1,024	32	32	0.25	32	0.13	1,024	8	1	2	64	8	1	32	128	128	>1,024	>1,024	>1,024	>1,024
20	64	2	16	0.031	2	0.0039	32	2	1	2	64	16	32	32	256	256	0.25	0.25	>1,024	>1,024
21	128	0.25	32	0.25	4	0.016	128	0.25	1	1	32	8	8	8	0.5	0.5	0.25	0.25	16	16
22	128	2	16	0.25	4	0.016	256	0.25	1	1	32	8	32	8	0.5	0.5	0.13	0.13	16	16
COL	>1,024	4	64	0.5	64	0.031	1,024	0.25	2	2	>1,024	>1,024	2	2	128	128	>1,024	>1,024	16	16
N315	8	2	8	0.002	8	0.002	256	0.25	0.5	0.25	32	4	32	1	0.25	0.25	0.25	0.25	512	512
MSSA																				
1003	2	0.25	0.063	0.063	0.016	0.016	2	0.25	2	0.5	32	8	1	1	0.25	0.25	0.13	0.13	8	8
1010	1	0.25	0.13	0.13	0.063	0.0078	2	0.25	1	1	32	16	1	2	0.25	0.25	0.25	0.25	>1,024	1,024
1020	1	0.25	0.13	0.002	0.031	0.0039	0.25	0.25	0.5	0.5	32	16	1	1	0.5	0.5	0.13	0.13	8	8
1032	1	0.25	0.25	0.0039	0.063	0.0039	2	0.25	2	2	32	32	32	32	0.5	0.5	0.25	0.25	16	16
ATCC 6538	1	0.25	0.13	0.0039	0.031	0.002	0.25	0.25	1	1	32	16	1	1	0.5	0.5	0.25	0.25	16	16
RN4220	2	0.25	0.13	0.002	0.031	0.002	0.25	0.0078	0.25	0.25	32	4	1	1	0.5	0.5	0.25	0.25	16	8

[a] MET, methicillin; HAP, cephapirin; PAPM, panipenem; CTX, cefotaxime; VAN, vancomycin; STR, streptomycin; OFX, ofloxacin; TET, tetracycline; ERY, erythromycin; KAN, kanamycin. −, alone; +, with 12.5 µg of 6,7-dihydroxyflavone/ml.
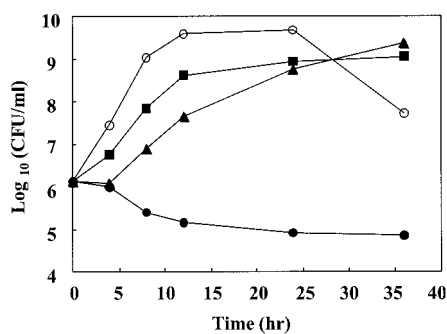
FIG. 3. Time-kill curves for 6,7-dihydroxyflavone and methicillin used against MRSA 5. Overnight cultures were diluted to approximately 10^6 CFU/ml with cation-adjusted MH broth containing either 8 µg of methicillin (■)/ml, 25 µg of 6,7-dihydroxyflavone (▲)/ml, or both (●). The control experiment (○) was performed by using culture medium without methicillin and 6,7-dihydroxyflavone. Bacteria were incubated at 37°C for 0, 4, 8, 12, 24, and 36 h, and viability was then confirmed by culturing the cells on MH agar plates for 24 h.

tion in the β-lactam MICs caused by transposons (16) or flavone and its derivatives. In this model, (i) PBP2a has a low affinity not only for the β-lactam ring of β-lactams but also for D-Ala-D-Ala in N-acetylmuramyl-pentapeptide because the β-lactam ring mimics the D-Ala-D-Ala moiety of the normal substrate. Thus (ii) PBP2a can cross-link between N-acetylmuramyl-pentapeptide and pentaglycine only when MRSA has been mutated such that there is an elevated concentration of N-acetylmuramyl-pentapeptide on the nascent cross wall (septum) as the main center of linear wall growth of the staphylococcal cell (3). Furthermore, (iii) the inhibition of peptidoglycan biosynthesis could decrease the concentration of N-acetylmuramyl-pentapeptide and/or pentaglycine on the nascent cross wall, such that PBP2a with low affinity for the D-Ala-D-Ala would not be able to participate in the cross-linking of peptidoglycans and such that the other four penicillin binding proteins with high affinity for the substrates would take part in the cross-linking. This could be a reason why flavone and its derivatives caused a massive reduction in the methicillin MIC even though they retained an intact PBP2a.

However, further in vitro and in vivo studies are required to confirm the model and also the relevancy of the combination therapy with 6,7-dihydroxyflavone and β-lactam antibiotics to multidrug-resistant MRSA infection.

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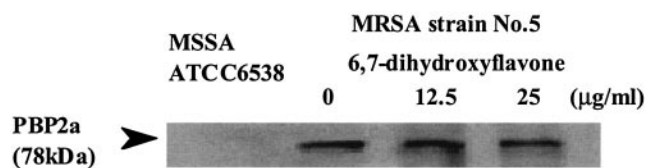

FIG. 4. Effects of 6,7-dihydroxyflavone on the amount of PBP2a in the membrane fraction of MRSA 5. MRSA 5 cells were collected at 5 h of incubation after adding a final concentration of 0, 12.5, or 25 µg/ml of 6,7-dihydroxyflavone, and the membrane fraction (10 µg of protein) was then loaded by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blotting analysis. PBP2′ was detected by use of ECL Plus.