Synergistic Effect of Kaempferol Glycosides Purified from *Laurus nobilis* and Fluoroquinolones on Methicillin-Resistant *Staphylococcus aureus*

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In a previous study, we reported that two kaempferol glycosides isolated from *Laurus nobilis* L., kaempferol-3-*O*-α-L-(2″,4″-di-*E*-*p*-coumaroyl)-rhamnoside (C2) and kaempferol-3-*O*-α-L-(2″-*E*-*p*-coumaroyl-4″-*Z*-*p*-coumaroyl)-rhamnoside (C3), showed strong antibacterial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci. Thereafter we found that these compounds greatly reduced the minimum inhibitory concentrations (MICs) of some fluoroquinolones in MRSA. In other words, C2 and C3 greatly potentiated anti-MRSA activity of fluoroquinolones. The effect of C2 and C3 with fluoroquinolones was found to be synergistic. The potentiation activity was observed with hydrophilic fluoroquinolones, such as norfloxacin and ciprofloxacin, but not with hydrophobic quinolones. We also found that norfloxacin reduced MICs of C2 and C3. The effect was synergistic. Possible mechanism of the synergistic effect was discussed.

Key words kaempferol glycoside; norfloxacin; synergistic effect; methicillin-resistant *Staphylococcus aureus*; topoisomerase IV

Infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious problem in health care units, especially for intensive care patients.[1)] Since the first report of MRSA from the U.K. in 1961, periodic outbreaks of MRSA have been observed in many hospitals in various countries. MRSA is at present the most commonly identified antibiotic-resistant pathogen. Moreover, MRSA infection rates have been swiftly increasing worldwide over the past decades as data from counting surveillance initiatives such as the National Nosocomial Infection Surveillance (NNIS) System and European Antimicrobial Resistance Surveillance System show.[2)] The proportion of MRSA in ICUs increased from 36% in 1992 to 64% in 2003 for hospitals in the NNIS system.[3)]

In general, nosocomial MRSA strains show multidrug resistance. Among the selected MRSA strains isolated in the U.K. between 1997 and 2000, EMRSA-17 and all of its variants showed resistance against β-lactams, fluoroquinolones, macrolides, aminoglycosides, tetracycline, rifampin and fusidic acid.[4)] In particular, fluoroquinolone resistance is a hallmark of nosocomial MRSA, although this was not always the case. Ciprofloxacin was initially perceived as an active agent against MRSA. A dramatic increase in the rate of ciprofloxacin resistance in many hospitals, however, has been observed within one year.[1)] For many years, vancomycin was the only effective treatment for serious MRSA infections. In 2002, however, vancomycin-resistant *Staphylococcus aureus* (VRSA) had been isolated from patients who were co-infected with MRSA and vancomycin-resistant enterococci (VRE).[5)]

Several strategies are possible for overcoming drug resistant bacteria. Development of new antibacterial drugs is very important. Development of inhibitors for bacterial drug resistance mechanisms can greatly increase activities of antimicrobial drugs and should be very useful.[6,7)] Such inhibitors would change drug resistant bacteria to susceptible ones, and can extend the life of currently used antimicrobial drugs. Inhibitors of bacterial virulence factors would be also useful.[8,9)] We have been trying to discover compounds showing such activities. We first reported several inhibitors of drug resistance systems in bacteria, for example epicatechin gallate,[10)] tellimagrandin I[11,12)] and corilagin.[13)] These compounds remarkably potentiated anti-MRSA activity of β-lactams. We found that these compounds inhibited penicillin binding protein 2a (PBP2a) in MRSA,[12)] which is a key enzyme primarily responsible for resistance of MRSA against β-lactams.[14)] We also reported baicalein which greatly potentiated anti-MRSA activity of tetracycline by inhibiting a tetracycline efflux pump.[15)] Recently, we found and reported that kaempferol glycosides, kaempferol-3-*O*-α-L-(2″,4″-di-*E*-*p*-coumaroyl)-rhamnoside (C2) and kaempferol-3-*O*-α-L-(2″-*E*-*p*-coumaroyl-4″-*Z*-*p*-coumaroyl)-rhamnoside (C3), showed strong antibacterial activity against MRSA.[16)] Here we report that C2 and C3 also showed synergistic activity with some fluoroquinolones against MRSA.

MATERIALS AND METHODS

Plant Material Leaves of *L. nobilis* L. (Laurel) were purchased from Toho TH2, Inc. (Kobe, Japan).

Extraction and Purification of C2 and C3 The C2 and C3 were purified as described previously.[16)] Briefly, constituents were extracted from leaves of *L. nobilis* (1.5 kg) with 70% acetone, and fractionated with hexane, ethyl acetate, successively. The ethyl acetate fraction was subjected to column chromatography over DIAION HP-20 (Mitsubishi Kasei Co.). Fractions showing anti-MRSA activity was collected and subjected to a Sephadex LH-20 (Amersham Biosciences) column, and eluted with 100% methanol. A fraction which showed the highest activity was subjected to

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preparative TLC using Kieselgel 60F254 plates (Merck). A zone showing the highest activity was collected (120 mg). Preparative HPLC was performed on an ODS-3 inertsil column (GL Science Inc.), eluted with methanol–H_2O (70 : 30) and effective compounds C2 (27.4 mg) and C3 (62.4 mg) were obtained.

Bacteria MRSA OM481, OM505, OM584 and OM623 were clinically isolated strains.[10] MRSA N315,[17] MRSA COL[18] are also clinically isolated strains.

Isolation of C2-Resistant Mutants Cells of MRSA N315, a parental strain, were cultured in Tryptic soy broth (TSB; Becton, Dickinson and Company) at 37 °C overnight, followed by plating (approximately 1×10^8 cells/plate) onto Mueller–Hinton (MH; Difco Laboratories) agar plates containing 4 μg/ml of C2 followed by incubation at 37 °C for 24 h. Mutants appeared on the plate were picked up, and single colony isolation was performed on the plate containing MH and 4 μg/ml of C2. We obtained many mutants, and they showed very similar MICs of C2 and C3. Therefore we used one of the representative mutants, NC23, as a C2-resistant mutant in this study. The frequency of isolation of the mutant was 4.9×10^{-8}. Judging from this frequency, it seems that the mutant possesses single mutation.

Drug Susceptibility Testing Minimum inhibitory concentrations (MICs) of antibacterial agents with MRSA, VRE, *S. marcescens* and *P. aeruginosa* were determined in cation-supplemented Mueller–Hinton broth (CSMHB), MH broth supplemented with $CaCl_2$ (50 μg/ml) and $MgCl_2$ (25 μg/ml), by a microdilution method.[19] The fractional inhibitory concentration (FIC) index was calculated as reported.[20] The effects of the drugs were interpreted to be indicative of synergy or indifferent when the index was $\leqq0.5$ or >0.5, respectively.

RESULTS AND DISCUSSION

Synergistic Activity of C2 and C3 During our studies on the anti-MRSA compounds C2 and C3, we noticed that these compounds also potentiated anti-MRSA activity of norfloxacin. The MICs of C2 and C3 in most of the *S. aureus* strains were between 1 and 2 μg/ml.[16] When we added 1/8 concentrations of MICs of C2 or C3 to the medium, we observed 8- to 16-fold reductions in the MICs of norfloxacin with MRSA OM481 and MRSA N315 (Table 1). The FIC indices for norfloxacin in combination with C2 or C3 were calculated to range from 0.19 to 0.25. These indices mean that the effects of C2 or C3 for norfloxacin against MRSA are synergistic. We tested the effects of C2 or C3 on various antibacterial drugs with various bacterial strains. In combinations with other antibacterial drugs and in other bacteria tested, C2 and C3 showed only a slight or no potentiation effect (data not shown). Thus, the potentiation activity of C2 or C3 is specific for norfloxacin, perhaps for fluoroquinolones, and *S. aureus*.

Effects of C2 or C3 with Fluoroquinolones on MRSA Based on the results described above, we tested the synergistic effects of C2 or C3 with several quinolones on several *S. aureus* strains (Table 1). In addition to norfloxacin, we tested the effects of ciprofloxacin, enoxacin and ofloxacin as hydrophilic quinolones, and of levofloxacin, nalidixic acid and sparfloxacin as hydrophobic quinolones. C2 and C3 lowered the MICs of norfloxacin and ciprofloxacin. Although the magnitude of potentiation on anti-MRSA activities for these fluoroquinolones depended on the strains, C2 and C3 potentiated the activity of norfloxacin by 4- to 16-fold against all MRSA strains tested (Table 1). These effects were all synergistic judging from the FIC indices. Regarding ciprofloxacin, C2 showed synergistic effects on OM481, OM584, N315, and C3 showed synergistic effects on 4 strains in Table 1 except for OM623 and COL. Anti-MRSA activity of enoxacin was also potentiated by C2 and C3 in a synergistic manner in the case of OM481. Such a synergistic effect, however, was not observed with other strains tested. Anti-MRSA activity of levofloxacin was slightly potentiated by C2 or C3 in the case of OM481 and N315. Regarding hydrophobic quinolones, anti-MRSA activities of nalidixic acid and sparfloxacin were not potentiated at all by C2 or C3 with any MRSA strain tested (Table 1).

Effect of C2 and C3 on C2-Resistant Mutant C2 and C3 show anti-MRSA activities themselves.[16] To understand how C2 and C3 potentiate the anti-MRSA activity of norfloxacin, it is important to investigate whether their anti-MRSA activity and norfloxacin-potentiating activity are due to the same action of C2 and C3 on a vital process of MRSA. To test this point, we isolated a C2-resistant mutant, NC23, from the parental MRSA strain N315. The MICs of C2 and C3 with the mutant NC23 were 4 and 8 μg/ml, respectively, which are 4- to 8-fold higher than those with the parental strain N315. We isolated the mutant as a C2-resistant mutant, and the mutant showed elevated MICs not only for C2 but also for C3. This result supports the view that the site of action of C2 and C3 in *S. aureus* is the same. This notion is very reasonable because C2 and C3 possess very similar structures.[16] We tried to isolate mutants showing MICs of C2 higher than 8 μg/ml from MRSA N315, but without success (data not shown). Thus, it is very difficult to isolate highly resistant mutants against C2, for unknown reasons. We tested whether there was a change in the synergistic effect of C2 or C3 with norfloxacin between the parent and the mutant. It should be noted that there was no change in MIC value of norfloxacin between parental N315 and mutant NC23 (Table 2). An eight-fold reduction in the MIC of norfloxacin was observed with both parental N315 and mutant NC23 in the presence of 1/8 MIC of C2 compared with its absence (Table 2). The FIC index was 0.25 in both cases, indicating that the effect is synergistic even in the mutant. When the same concentration of C2 (0.13 μg/ml) was added to the test medium of parental N315 and mutant NC23, reduction of the MIC of norfloxacin was smaller with NC23, reflecting the fact that the NC23 strain is a C2-resistant mutant. This suggests that the affinity of C2 to its target enzyme of the mutant NC23 became lower compared with that in the parent.

Effect of Norfloxacin on Anti-MRSA Activity of C2 and C3 We tested whether norfloxacin reduce MICs of C2 and C3 with some MRSA strains. We observed about 8-fold reduction in MICs of C2 and C3 with three MRSA strains tested (Table 3). The FIC indices were 0.25 to 0.38. Thus, the effect of norfloxacin for C2 or C3 against MRSA is synergistic.

Possible Mechanism of the Synergistic Action We previously reported that C2 and C3 showed anti-MRSA activity, and the site of action of C2 and C3 might be DNA gyrase and/or DNA topoisomerase IV.[16] The primary target of

Table 1. MICs of Various Quinolones for MRSA in the Absence or Presence of C2 or C3

Antibacterial agent/ Compound		MIC (μg/ml)					
		OM481	OM505	OM584	OM623	N315	COL
Norfloxacin	—	128	8	128	128	2	1
	+C2[a)]	8	2	16	16	0.25	0.25
	+C3[a)]	8	2	32	16	0.25	0.25
Ciprofloxacin	—	8	2	64	16	0.5	0.5
	+C2	1	1	16	8	0.13	0.25
	+C3	1	0.5	8	8	0.13	0.25
Enoxacin	—	64	8	128	64	1	1
	+C2	8	4	64	64	1	1
	+C3	16	4	64	64	0.5	1
Ofloxacin	—	32	16	128	128	16	8
	+C2	16	8	128	128	8	8
	+C3	16	16	128	128	8	8
Levofloxacin	—	2	0.5	8	8	0.25	0.25
	+C2	1	0.5	8	8	0.13	0.25
	+C3	1	0.5	8	8	0.13	0.25
Nalidixic acid	—	256	128	512	512	64	64
	+C2	256	128	512	512	64	64
	+C3	256	128	512	512	64	64
Sparfloxacin	—	0.25	0.13	8	8	0.13	0.13
	+C2	0.25	0.13	8	8	0.13	0.13
	+C3	0.25	0.13	8	8	0.13	0.13

a) The concentrations of C2 and C3 added to the medium are 1/8 of their MICs (see ref. 16).

Table 2. Effect of C2 and C3 on MICs of Norfloxacin for *S. aureus* N315 and C2-Resistant NC23

Antibacterial drug /Compound (concentration)		MIC (μg/ml)			
		N315		NC23	
Norfloxacin	—	2		2	
	+C2 (0.13 μg/ml)	0.25	(0.25)[a)]	1	(0.53)
	+C2 (0.5 μg/ml)	N.D.[b)]		0.25	(0.25)
	+C3 (0.13 μg/ml)	0.25	(0.25)	1	(0.51)
	+C3 (1 μg/ml)	N.D.		0.25	(0.25)

a) FIC index is shown in parenthesis. *b*) N.D., not determined.

Table 3. MICs of C2 and C3 for MRSA in the Absence or Presence of Norfloxacin

Compound/ Antibacterial agent		MIC (μg/ml)					
		S. aureus OM481		*S. aureus* OM584		*S. aureus* N315	
C2	—	1		2		1	
	+Norfloxacin[a)]	0.13	(0.25)[b)]	0.25	(0.25)	0.13	(0.25)
C3	—	1		2		1	
	+Norfloxacin	0.13	(0.25)	0.25	(0.25)	0.25	(0.38)

a) The concentrations of norfloxacin added to the medium are 1/8 of the MIC for each strain (see Table 3). *b*) FIC index is shown in parenthesis.

quinolones is DNA gyrase and DNA topoisomerase IV in bacteria. Takei and coworkers reported with *S. aureus* that the primary target of the type I quinolones was topoisomerase IV and that of the type II quinolones was DNA gyrase, whereas that of the type III quinolones were both topoisomerase IV and DNA gyrase.[22)] According to this grouping, quinolones that showed a synergistic effect with C2 or C3, norfloxacin and ciprofloxacin, belong to the type I quinolones that inhibit DNA topoisomerase IV as the primary target. On the other hand, the quinolone that did not show such an effect, sparfloxacin, belongs to the type II quinolones. Thus, it seems reasonable to assume that the primary site of action of C2 and C3 would be DNA topoisomerase IV. Recently we confirmed that the primary target of C2 and C3 is topoisomerase IV (manuscript in preparation). The topoisomerase IV consists of two subunits, ParC and ParE, and the DNA gyrase consists of two subunits, GyrA and GyrB. ParC and GyrA are catalytic subunits, and ParE and GyrB are energy supplying subunits showing ATPase activities. It has been reported that the flavonoid compounds quercetin and rutin, a quercetin analog possessing additional glucose-rhamnose moiety, inhibited topoisomerase IV,[23)] and quercetin[24)] and cathechins[25)] inhibited GyrB, a subunit of DNA gyrase, in *E. coli*. The ParE and GyrB of *S. aureus* N315 show 52% identity and 85% similarity in their amino acid sequences each other according to a homology search (GENETYX sequence analysis software, Software development Co., Tokyo). C2 and C3 are flavonoid glycosides. Therefore it seems that C2 and C3 might inhibit the ParE subunit of DNA topoisomerase IV. Thus, it is reasonable to suppose that norfloxacin binds to and inhibits the ParC subunit of the topoisomerase IV, and C2 binds and inhibits the ParE subunit of the topoisomerase IV, resulting in the synergistic effect of norfloxacin and C2. It has been reported that novobiocin which inhibits GyrB of DNA gyrase in *S. aureus*[26)] showed no additive effect with ciprofloxacin which primarily inhibits topoisomerase IV.[27)] We confirmed that novobiocin did not show any additive or synergistic effects with ciprofloxacin or norfloxacin on MRSA strains tested (data not shown). These results support the view that the sites of action of C2 and novobiocin are different.

In conclusion, both C2 and C3 are very interesting compounds towards development of drugs for therapy of MRSA infections, because 1) C2 and C3 show strong anti-MRSA activities,[16)] 2) C2 and C3 show synergistic anti-MRSA effects with norfloxacin and ciprofloxacin, and 3) it is very dif-

ficult to isolate mutants that are highly resistant to C2 and C3.

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