Synergic, additive, indifferent and antagonistic effects in combinations of tea catechin and 20 antibiotics against MRSA.

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Summary
With the broth-dilution checkerboard technique, we examined the effects of epigallocatechin gallate (EGCg, a main constituent of tea catechins) in combinations with 20 antibiotics against methicillin-resistant Staphylococcus aureus (MRSA). Synergic, additive, indifferent and antagonistic effects were observed. The combinations of EGCg with all the tested β-lactams showed potent synergism against MRSA. EGCg even at 6.25 µg/ml apparently reversed the high level resistance of clinical isolates of MRSA to benzylpenicillin, ampicillin, methicillin, oxacillin, imipenem, cefalexin, cefmetazole and cefotaxime. Combinations between EGCg and the inhibitors of either protein or nuclear acid synthesis only showed additive or indifferent effects. These antibiotics include minocycline, tetracycline, chloramphenicol, gentamicin, streptomycin, kanamycin, erythromycin, ofloxacin and rifampicin. On the contrary, EGCg antagonized the anti-bacterial activities of glycopeptide antibiotics (vancomycin, teicoplanin and polymyxin B). The common property of those antibiotics is the peptide backbone structures, indicating a direct binding of EGCg with peptide structure of the antibiotics.

The above results suggest a possibility that tea catechins may affect the activities of antibiotics both positively and negatively.

Keywords
Epigallocatechin gallate (EGCg), Antibiotics, MRSA, Synergism, Antagonism

Introduction
Bacterial resistance to antibiotics is a very serious global problem. Especially MRSA has already made its way into the community not just limited to hospitalized patients and their care providers. Therefore, novel antimicrobials and/or new approaches to combat the problem are urgently needed. Natural products and traditional medicines are apparently the superior sources for new antimicrobial agents. With this in mind, our group has studied and demonstrated that tea (Camellia sinensis) and tea catechins have bactericidal activity against various Gram-positive and Gram-negative bacteria including methicillin-susceptible Staphylococcus aureus (MSSA) and MRSA. Among the tea catechins such as catechin (C), epicatechin (EC), epigallocatechin gallate (EGCg) and epigallocatechin gallate (EGCg), EGCg holds the strongest bactericidal activity. Furthermore, we found an interesting phenomenon that low concentration of EGCg reversed the resistance of clinical isolates of MRSA to β-lactams.¹⁻³

Vancomycin is also an inhibitor of bacterial cell wall synthesis and is the first choice in antibiotics for MRSA treatment. Nevertheless, there are increasing reports of vancomycin-resistant MRSA. We expected the same synergistic effect between EGCg and vancomycin against MRSA. Disappointingly, however, we observed an antagonistic tendency instead of the synergism. This result impelled us to investigate the combination effects of EGCg with 20 antibiotics. Tea as one of the most popular beverages is consumed every day by billions of people and capsules of tea catechins and EGCg are also becoming available for research and pre-clinical trial as cancer prevention, treatment of infectious diseases and others. Therefore, it is critical to pay attention to the interactions between tea components and medicines administrated to patients. Here, we present in vitro evidence that tea catechins affect the antibacterial activities of antibiotics in the ways of synergism, additivity, indifference and antagonism.

Materials and Methods
EGCg was extracted from green tea. Twenty antibiotics were purchased from commercial sources. Eight clinical isolates of MRSA were from Showa University Hospitals. All the strains were identified by both PCR analysis of meca gene expression and latex agglutination assay of PBP2' (Denka Seiken Co., Tokyo, Japan). E. coli ATCC 25922 was used to test EGCg-polymyxin B combination.
MICs were determined in Mueller-Hinton broth (MHB) using the broth microdilution method at a final inoculum of $> 5 \times 10^5$ CFU/ml and a stationary incubation at 35°C for 24 h. The growth inhibition was also performed in 3 ml MHB and the bacterial growth was detected using a spectrophotometer (OD at 600 nm). The combination effects were determined by checkerboard method and were evaluated by a fractional inhibitory concentration (FIC) index. FIC indices were interpreted as synergistic when values were $\leq 0.5$ and as antagonistic when values were $> 4$. If a combination with EGCg resulted in a reduction of the antibacterial activity of an antibiotic, but FIC index was still $< 4$, it was defined as an antagonistic tendency. The results between synergism and antagonistic tendency were defined as additive or indifferent.

**Results**

MIC of EGCg against MRSA strains was 100 μg/ml. When EGCg was combined with 20 antibiotics, EGCg-β-lactam combinations showed potent synergism. The β-lactams included natural penicillin (penicillin G), modified broad-spectrum penicillin (ampicillin), anti-penicillinase penicillin (methicillin and oxacillin), all generations of cephems (cephalexin, cefinetazole and cefotaxime) and carbapenem (imipenem). EGCg even at 6.25 μg/ml apparently reversed the high level resistance of clinical isolates of MRSA to the tested β-lactams. The FIC indices were $\leq 0.5$.

EGCg-vancomycin and EGCg-teicoplanin combinations showed an unexpected antagonistic tendency at certain concentrations and ratios. For example, EGCg at 12.5 μg/ml antagonized the activity of teicoplanin at 0.5 μg/ml, but EGCg at 6.25 and 25 μg/ml showed additive effect with teicoplanin at the same concentration. The antagonistic tendency was clearer when the bacteria were cultured at a fast-growing condition (shaking at 200 RPM) for 8 h or at early phase of stationary culture. Combination between EGCg and polymyxin B was also tested against *E. coli*. EGCg apparently antagonized the activity of polymyxin B against *E. coli*. MIC of polymyxin B increased from 0.25 μg/ml to 2 μg/ml in the presence of 12.5 μg/ml EGCg. The FIC indices were $> 4$.

Additive or indifferent effects were observed in combinations of EGCg with minocycline, tetracycline, chloramphenicol, gentamicin, streptomycin, kanamycin, erythromycin, ofloxacin and rifampicin.

**Discussion**

We characterized in vitro that there are four combination effects between EGCg and 20 antibiotics, that is, synergistic, additive, indifferent and antagonistic effects.

The most potent synergic interactions were observed between EGCg and β-lactams. We have recently demonstrated that the EGCg-induced damage of the bacterial cell wall and interference with its biosynthesis through direct binding with peptidoglycan are responsible for the potent synergism.(3)

The additive or indifferent interactions were observed between EGCg and the inhibitors of either protein or nuclear acid synthesis. The increased permeability of the cell wall/membrane to antibiotics due to the EGCg-induced damage of cell wall might be the main factor for the additive effects.

The synergistic effects between EGCg and β-lactams suggest a possible clinical use of EGCg to treat MRSA-infected patients especially with topical or digestive tract infections. When we attempted to enhance the antibacterial activities of vancomycin and teicoplanin against MRSA through combinations with EGCg, however, an antagonistic tendency between EGCg and the two antibiotics was observed. According to the mechanism of synergy between EGCg and β-lactams against MRSA, the two antibiotics should have shown synergism with EGCg. Vancomycin and teicoplanin are belonged to glycopeptide antibiotics and EGCg may directly bind to the peptide structure of the antibiotics and then interfere with their activities. The balances between the synergistic effect and the reduction of the antibacterial activities owing to their binding each other may result in the antagonistic tendency at certain ratios of the antibiotics and EGCg, but the additive or indifferent effects at the other ratios. To confirm this hypothesis, we detected the effect of EGCg-polymyxin B combination against *E. coli*. Polymyxin B is also a glycopeptide antibiotic and highly active in vitro against most Gram-negative bacilli. EGCg clearly antagonized the activity of polymyxin B (FIC index $> 4$), indicating the direct bindings between EGCg and glycopeptide antibiotics. Comparing to MIC of EGCg against *S. aureus* (100 μg/ml), the MIC of EGCg against *E. coli* was more than 800 μg/ml and there was no synergism between EGCg and ampicillin against *E. coli*. The lack of synergism between polymyxin B and EGCg against *E. coli* may explain the phenomenon that EGCg showed the strongest antagonism.
to the activity of polymyxin B.

Tea catechins including EGCg are structurally belonged to plant polyphenols and are generally known as tannins. EGCg at concentrations more than 100 μg/ml did precipitate proteins in broth, indicating a direct binding of EGCg with glycoproteins or polypeptides. These common properties of tannins also strongly support our explanation about the antagonistic tendency between EGCg and glycopeptide antibiotics.

It is hard to predict either synergistic or antagonistic effects in vivo just according to the presented in vitro evidence. Usually, EGCg concentration in tea beverage is 2-3 mg/ml. Compared to that, 6.25, 12.5 or 25 μg/ml EGCg is low concentration. However, it is difficult to estimate the concentration of bio-available EGCg in vivo after drinking tea or taking EGCg capsules. EGCg is absorbed through digestive tract and distributed to many organs of animal and human. In rat blood plasma, EGCg at 5.6 μg/ml was detected after orally administered with EGCg at 500 mg/kg body weight,(4) and total catechins at 15 to 112 μg/ml were detected at 2 h after orally administered with catechins at 5 g/kg body weight.(5) In human blood plasma, EGCg at 2 μg/ml was detected after 90 min of taking 525 mg EGCg capsules.(6) Thereby, tea and EGCg may affect the activities of antibiotics not only in vitro but also in vivo.

References