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Restoration of antibacterial activity of β-lactams by epigallocatechin gallate against β-lactamase-producing species depending on location of β-lactamase

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Abstract

The combined effects of (−)-epigallocatechin gallate (EGCg) and β-lactams were investigated against various β-lactamase-producing clinical isolates, including 21 *Staphylococcus aureus*, 6 *Escherichia coli*, 3 *Klebsiella pneumoniae* and 8 *Serratia marcescens* strains. Penicillin in combination with EGCg at 12.5 μg mL^{-1} showed the most potent synergy against 100% penicillinase-producing *S. aureus*. However, cefotaxime or imipenem in combination with higher concentration of EGCg (100 μg mL^{-1}) only showed slight synergy against 2 of 17 Gram-negative rods. Similar to the effect on the penicillinase from *S. aureus*, however, EGCg also directly inhibited the extracted β-lactamases from the Gram-negative rods, thereby protecting β-lactams from inactivation. The different effects of the combinations on different β-lactamase-producing species were confirmed to be related to the cellular locations of β-lactamases. In contrast to a 32.7% extracellular fraction of total β-lactamase activity in a penicillinase-producing *S. aureus*, the fractions were 0.6%, 0.6% and 1.2% in a TEM-derived extended-spectrum β-lactamase-producing *E. coli*, an inhibitor-resistant β-lactamase-producing *K. pneumoniae* and an IMP-producing *S. marcescens*, respectively. In conclusion, the combination of penicillin with EGCg showed potent synergy against penicillinase-producing *S. aureus* in-vitro. The combinations of β-lactams and EGCg against β-lactamase-producing Gram-negative rods do indicate a limitation owing to the cellular location of β-lactamases.

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Introduction

The production of β-lactamases is a critical mechanism for the bacterial resistance to β-lactams (Medeiros 1984; McDougal & Thornsberry 1986). Penicillinase occurred in less than 5% of *Staphylococcus aureus* isolates at the time of penicillin's introduction into clinics in the 1940s, but has dramatically increased to 80–90% of isolates through plasmid transfer and strain selection (Lacey 1984; Rosdahl 1986). The recent emergence of bacterial strains producing inhibitor-resistant enzymes can be related to the frequent use of clavulanate (Thomson & Amyes 1992; Vedel et al 1992; Blazquez et al 1993; Lemozy et al 1995). Extended-spectrum β-lactamases (ESBLs), the mutant enzymes mostly derived from TEM or SHV (Jacoby & Medeiros 1991) and often found in *Escherichia coli* and *Klebsiella pneumoniae*, are threatening the value of expanded-spectrum cephalosporins and monobactams against enterobacteria (Heritage et al 1999). Carbapenems are stable to ESBLs, and imipenem has been used successfully in-vivo against many enzyme producers. Unfortunately, a new metallo-β-lactamase, IMP-1, poses a serious emerging threat to the use of carbapenems against *Serratia marcescens* and other Gram-negative rods (Watanabe et al 1991; Ito et al 1995). Presumably, the use and overuse of new antibiotics in clinical practice have selected and expanded such new variants of β-lactamases. Therefore, any effort to prevent β-lactams from being inactivated by β-lactamases is of particular significance.

We have previously reported the effects of combining various antibiotics and epigallocatechin gallate (EGCg, a main constituent of tea catechins) against penicillinase-producing and methicillin-resistant *S. aureus* (Takahashi et al 1995; Hu et al 2001, 2002a, b). Besides a direct effect on the bacterial cell wall (Zhao et al 2001), EGCg inhibits β-lactamase activity, thereby restoring the antibacterial activity of penicillin

against penicillinase-producing *S. aureus* (Zhao et al 2002). In an extended study, we observed the effects of combining β-lactams and EGCg against β-lactamase-producing Gram-negative rods such as *E. coli, K. pneumoniae* and *S. marcescens*.

Materials and Methods

EGCg and chemicals

EGCg extracted from green tea with a purity of 98% was kindly provided by Dr Yukihiko Hara (Tokyo Food Techno Co., Ltd, Tokyo, Japan). The following chemicals were purchased from commercial sources: ampicillin, cefoxitin, cefotaxime and penicillinase from *Bacillus cereus* (Sigma, St Louis, MO); penicillin, cefalexin, clavulanate (Wako Pure Chemical Industries Ltd, Osaka, Japan); aztreonam, imipenem (USP Rockville, MD); nitrocefin (Oxoid, Basingstoke, UK).

Bacterial strains and media

β-Lactamase-producing clinical isolates, including 21 *S. aureus*, 6 *E. coli*, 3 *K. pneumoniae* and 8 *S. marcescens*, were from specimens submitted for routine cultures in the Clinical Microbiology Laboratories, Showa University Hospital. A standard strain, *E. coli* ATCC 25922, was used as control. Mueller–Hinton broth (MHB; Becton Dickinson, MD) supplemented with Ca^{2+} (25 mg L^{-1}) and Mg^{2+} (12.5 mg L^{-1}) was used for all susceptibility tests.

Susceptibility test and confirmation of ESBLs

A broth microdilution method was performed on the basis of the proposed guidelines of the National Committee for Clinical Laboratory Standards (2000). Strains were inoculated into 100 μL of MHB containing various concentrations of antibiotics or EGCg (or both) with a bacterial inoculum of 5×10^5 colony-forming units per mL (CFU mL^{-1}). After culture at 35° for 24 h (*S. aureus*) or 18 h (Gram-negative rods), minimum inhibitory concentrations (MICs) were determined. The MIC of antibiotic or EGCg was defined as the lowest concentration at which no visible growth occurred. The effects of combinations were confirmed by the checkerboard method as described previously (Norden 1979). The two-fold serial dilutions of the β-lactams were tested in combinations with two-fold serial dilutions of EGCg. The results were evaluated by a fractional inhibitory concentration (FIC) index. FIC was calculated as MIC of β-lactam or EGCg in combination divided by MIC of the β-lactam or EGCg alone, and the FIC index was obtained by adding the FICs. FIC indices were interpreted as follows: ≤ 0.5, synergy; > 0.5 to 1, addition; and > 1, indifference.

Cefotaxime alone and in combination with clavulanate (4 μg mL^{-1}) was used for ESBLs confirmation based on the guidelines of the National Committee for Clinical Laboratory Standards (2000). A decrease in the MIC of $\geqslant 3$ two-fold dilutions in the presence of clavulanate is indicative of the presence of an ESBL.

β-Lactamase assay and *bla* gene detection

All the strains were identified by nitrocefin assay for β-lactamase expression (O'Callaghan et al 1972) and by polymerase chain reaction (PCR) analysis for *bla* gene presence. Specific primers for bla_Z (F 5′-ACT TCA ACA CCT GCT GCT TTC-3′ and R 5′-TGA CCA CTT TTA TCA GCA ACC-3′), bla_{TEM} (90 F 5′-TCG GGG AAA TGT GCG CG-3′ and 1062 R 5′-TGC TTA ATC AGT GAG GCA CC-3′), bla_{SHV} (103F 5′-CAC TCA AGG ATG TAT TGT G–3′ and 988 R 5′-TTA GCG TTG CCA GTG CTC G-3′) and bla_{IMP} (1241F 5′-CTA CCG CAG CAG AGT CTT TG-3′ and 1808R 5′-AAC CAG TTT TGC CTT ACC AT-3′) were designed as reported (Senda et al 1996; Pitout et al 1998; Martineau et al 2000). A 173-, 971-, 885- and 587-bp fragment should be amplified from bla_Z-, bla_{TEM}-, bla_{SHV}- and bla_{IMP}-bearing clinical isolates, respectively.

Preparation and location of β-lactamases

Penicillinase-producing *S. aureus* no. 226, TEM-derived ESBL-producing *E. coli* no. 5, inhibitor-resistant SHV- and TEM-β-lactamase-producing *K. pneumoniae* no. 1 and IMP-1-producing *S. marcescens* no. 8 were stationarily cultured in 5 mL of MHB without any inducer at 35 °C overnight. The cultures were diluted with 45 mL of fresh MHB and were incubated with shaking at 200 rev min^{-1} at 35 °C until 0.5–0.8 optical density units at 600 nm (OD_{600}) was reached. The bacteria were then centrifuged at 3000 rev min^{-1} for 20 min at 4 °C. After sterilization via filtration, the part of β-lactamase in the supernatants was considered as the extracellular enzyme. The pellets were washed three times with 0.1 M phosphate-buffered saline (pH 7.4). The harvested cells were then resuspended in 10 mL of MHB followed by sonication on ice. The enzyme in the solution was considered as intracellular (including the periplasmic space) enzyme. Insoluble particles were separated by centrifugation at 15 000 rev min^{-1} for 30 min at 4 °C.

Detection of β-lactamase activity

Crude extract of β-lactamase 100 μL and nitrocefin 10 μL (250 μg mL^{-1}) were mixed in a 96-well culture plate. After 10 min, the colour changes were recorded by detecting the optical density at 492 nm (OD_{492}) of each well with a spectrophotometer. The enzyme activity was determined graphically according to the standard curve of the purified β-lactamase from *B. cereus* under the same assay conditions.

For detection of the direct inhibition of enzyme activity by EGCg, β-lactamases and EGCg were pre-incubated in MHB for 30 min before the addition of nitrocefin. The concentration of EGCg required for 50% inhibition (IC50) of the enzyme activity was determined graphically.

Table 1 MICs ($\mu g\ mL^{-1}$) of β-lactams against β-lactamase-producing Gram-negative rods.

Strain	Ampicillin	Cefalexin	Cefoxitin	Cefotaxime (alone/plus clavulanate)	Aztreonam	Imipenem
E. coli						
1	>2048	>512	128	8/8	8	<0.25
2	2048	256	8	256/≤0.25	8	0.25
3	>2048	>512	128	4/2	8	<0.25
4	>2048	64	128	16/16	≤0.125	0.5
5	2048	256	8	512/≤0.25	8	<0.25
6	>2048	>512	256	64/64	64	0.5
K. pneumoniae						
1	>2048	512	512	16/16	0.25	1
2	>2048	>512	8	32/0.5	>128	0.5
3	>2048	512	8	2/≤0.25	>128	0.5
S. marcescens						
1	>2048	>512	>512	256/256	32	32
2	>2048	>512	>512	>256/>256	16	256
3	>2048	>512	256	>256/>256	32	2
4	>2048	>512	>512	256/256	16	64
5	>2048	>512	>512	>256/>256	8	256
6	>2048	>512	>512	256/256	4	64
7	>2048	>512	>512	>256/>256	64	256
8	>2048	>512	>512	>256/>256	64	256
E. coli ATCC 25922	4	8	2	0.125/0.125	<0.25	0.125

Protection of β-lactam from β-lactamase by EGCg

Susceptible *E. coli* ATCC 25922 (5×10^5 cells mL^{-1}), as the target cells, was inoculated in 100 μL MHB containing crude β-lactamase in the presence of β-lactam alone or plus EGCg. After culturing at 35 °C for 18 h, the MIC changes of β-lactams were observed as evidence of EGCg-induced protection of β-lactam activity from β-lactamase.

Presentation of data and statistical analysis

All experiments were performed three times to confirm the repeatability of MICs as well as the β-lactamase activity. The effects of EGCg on the antibacterial properties (MIC) of β-lactams were assessed using the Wilcoxon Signed Rank Test. In all cases $P < 0.05$ denoted significance.

Results

Susceptibility of β-lactams against β-lactamase-producing species

All the clinical isolates, including 21 *S. aureus*, 6 *E. coli*, 3 *K. pneumoniae* and 8 *S. marcescens*, were β-lactamase producers confirmed by nitrocefin test and *bla* gene analysis. MICs of β-lactams against β-lactamase-producing Gram-negative rods are summarized in Table 1. *E. coli* nos 2 and 5 and *K. pneumoniae* nos 2 and 3 were ESBL producers. *E. coli* nos 1, 3, 4 and 6 and *K. pneumoniae* no. 1 were inhibitor-resistant. All the *S. marcescens* strains were resistant to all the β-lactams tested.

Combination effects of β-lactams and EGCg against β-lactamase-producing strains

The chemical structure of EGCg is shown in Figure 1. The MIC of EGCg was 100 $\mu g\ mL^{-1}$ against *S. aureus* and

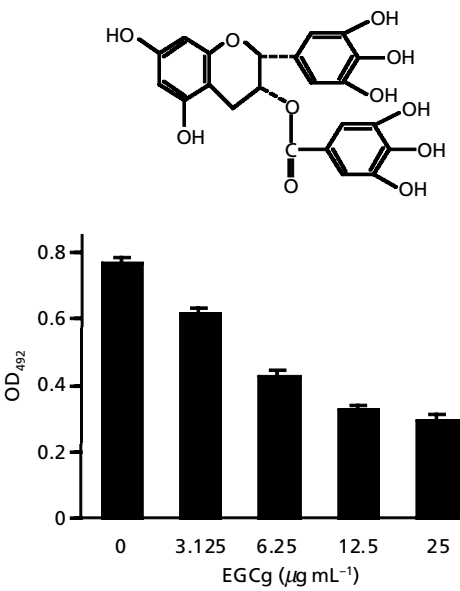

Figure 1 Chemical structure of epigallocatechin gallate (EGCg) and direct inhibition of IMP-1 activity by EGCg. The IMP-1 prepared from 1.8×10^6 cells (*S. marcescens* no. 8) was incubated with EGCg in 100 μL of MHB at 35°C for 30 min before the addition of nitrocefin as its substrate. OD_{492} was then detected using a spectrophotometer.

more than 400 $\mu g\,mL^{-1}$ against Gram-negative rods. The combination of penicillin with EGCg at 12.5 $\mu g\,mL^{-1}$ showed potent synergy against 100% (21/21) strains of the penicillinase-producing *S. aureus*. However, the combinations of cefotaxime or imipenem with the higher concentrations of EGCg (50 and 100 $\mu g\,mL^{-1}$) showed slight synergy against 2 and addition against 5 of 17 strains of *E. coli, K. pneumoniae* and *S. marcescens*. The remaining strains showed an indifferent effect (Table 2).

The cellular location of β-lactamases

Extracellular and intracellular β-lactamases were collected separately from the cultures of the strains and their activity was then detected. As shown in Table 3, in contrast to a 32.7% extracellular fraction of total β-lactamase activity in the penicillinase-producing *S. aureus* no. 226, the fractions were 0.6%, 0.6% and 1.2% in the TEM-derived ESBL-producing *E. coli* no. 5, the inhibitor-resist-

Table 2 MICs and FIC indices of β-lactams in combinations with epigallocatechin gallate (EGCg) against β-lactamase-producing strains.

Strain	MIC ($\mu g\,mL^{-1}$)			FIC index		Effect
	A	B	C	B	C	
S. aureus						
97	32	8	2	0.31	0.19	SN
226	128	64	16	0.56	0.26	SN
419	64	16	8	0.31	0.25	SN
1-152	128	32	16	0.31	0.25	SN
1-150	32	8	4	0.31	0.25	SN
1-813	128	32	16	0.31	0.25	SN
1-814	64	8	2	0.19	0.16	SN
1-820	64	8	2	0.19	0.16	SN
1-836	16	1	0.25	0.13	0.14	SN
1-909	16	1	0.5	0.13	0.16	SN
3-651	2	1	0.25	0.56	0.25	SN
5-28	128	8	8	0.13	0.19	SN
6-5	128	64	32	0.56	0.38	SN
6-42	8	1	1	0.19	0.25	SN
6-43	4	2	0.5	0.56	0.25	SN
6-78	64	8	2	0.19	0.16	SN
6-326	16	4	1	0.31	0.19	SN
6-339	8	2	0.25	0.31	0.16	SN
6-346	32	4	1	0.19	0.16	SN
6-350	16	4	1	0.31	0.19	SN
6-404	16	1	0.5	0.13	0.16	SN
E. coli						
1	8	4	4	< 1, > 0.5	< 1, > 0.5	AD
2	256	128	64	< 1, > 0.5	< 0.5	SN
3	4	4	4	> 1	> 1	ID
4	16	16	8	> 1	< 1, > 0.5	AD
6	512	256	128	< 1, > 0.5	< 0.5	SN
K. pneumoniae						
1	16	16	16	> 1	> 1	ID
2	32	32	16	> 1	< 1, > 0.5	AD
3	2	2	2	> 1	> 1	ID
S. marcescens						
1	32	32	32	> 1	> 1	ID
2	256	256	256	> 1	> 1	ID
3	2	2	2	> 1	> 1	ID
4	64	64	64	> 1	> 1	ID
5	256	256	256	> 1	> 1	ID
6	64	64	64	> 1	> 1	ID
7	256	128	128	< 1, > 0.5	< 1, > 0.5	AD
8	256	128	128	< 1, > 0.5	< 1, > 0.5	AD

A, β-lactam alone (penicillin for *S. aureus*, cefotaxime *E. coli* and *K. pneumoniae,* imipenem for *S. marcescens*); B, plus EGCg (6.25 $\mu g\,mL^{-1}$ for *S. aureus*, 50 $\mu g\,mL^{-1}$ for the others); C, plus EGCg (12.5 $\mu g\,mL^{-1}$ for *S. aureus*, 100 $\mu g\,mL^{-1}$ for the others). The MICs of EGCg alone were 100 $\mu g\,mL^{-1}$ for *S. aureus* and more than 400 $\mu g\,mL^{-1}$ for the others. SN, synergy; AD, addition; ID, indifference.

Table 3 Characteristics of β-lactamases.

Strain	*bla* gene	Type	Extracellular fraction (% of total)
S. aureus no. 226	bla_Z	PCase	32.7 ± 1.5*
E. coli no. 5	bla_{TEM}	TEM-derived ESBL	0.6 ± 0.1
K. pneumoniae no. 1	$bla_{SHV+TEM}$	Inhibitor-resistant β-lactamase	0.6 ± 0.3
S. marcescens no. 8	$bla_{IMP+TEM}$	IMP-1 and TEM	1.2 ± 0.5

* $P < 0.01$ compared with the data from *E. coli, K. pneumoniae* or *S. marcescens* (Student's t-test).

ant β-lactamase-producing *K. pneumoniae* no. 1 and the IMP-producing *S. marcescens* no. 8, respectively.

Inhibition of β-lactamase activity by EGCg

EGCg directly inhibited the activity of extracted β-lactamase in a dose-dependent manner (Figure 1). The concentration of EGCg required for 50% inhibition (IC50) of the enzyme activity prepared from 1.8×10^6 cells of *S. marcescens* no. 8 was 10.5 $\mu g\,mL^{-1}$.

Protection of β-lactams from β-lactamase by EGCg

The presence of extracted β-lactamases in culture media resulted in an increase of the β-lactam MIC against the susceptible strain of *E. coli* ATCC 25922. EGCg inhibited the enzyme activity and restored the β-lactam activity (Figure 2). For example, the MIC of imipenem rose from 0.125 to 8 $\mu g\,mL^{-1}$ in the presence of IMP-1 prepared from 3.6×10^6 cells of *S. marcescens* no. 8. EGCg blocked the β-lactamase activity and restored the MICs of imipenem from 8 $\mu g\,mL^{-1}$ to 4, 1 and 0.25 $\mu g\,mL^{-1}$ at concentrations of 6.25, 12.5 and 25 $\mu g\,mL^{-1}$, respectively (Figure2B).

Discussion

Inhibition of penicillinase by EGCg results in restoration of antibacterial activity of penicillin against penicillinase-producing *S. aureus* in-vitro. Disappointingly, no obvious restoration was observed by the combinations of β-lactams and EGCg against Gram-negative rods, even though EGCg directly inhibited the extracted β-lactamase from the rods and protected β-lactams from inactivation. The differences of the combination effects between the different β-lactamase-producing species were confirmed to be related to the cellular locations of the enzymes. The Gram-negative cell wall is capable of excluding certain substances and of trapping enzymes in periplasmic space. At the same time, the outer membrane is a very important permeability barrier to protect bacteria from various antibacterial materials (Nikaido & Vaara 1985). This physiological function of the outer membrane and the low affinity between EGCg and lipopolysaccharide (Zhao et al 2001) limited the penetration of EGCg into the periplasmic space, thereby reducing the effect of EGCg on β-lactamases there. Contrary to staphylococcal β-lactamase, which is extracellular, the β-lactamases of Gram-negative rods are regularly periplasmic, although some extracellular release may occur due to leakage rather than secretion. In this study, we compared the activity of extracellular and intracellular enzyme between *S. aureus* and various Gram-negative rods. The extracellular fractions of total β-lactamase activity clearly showed a correlation with the

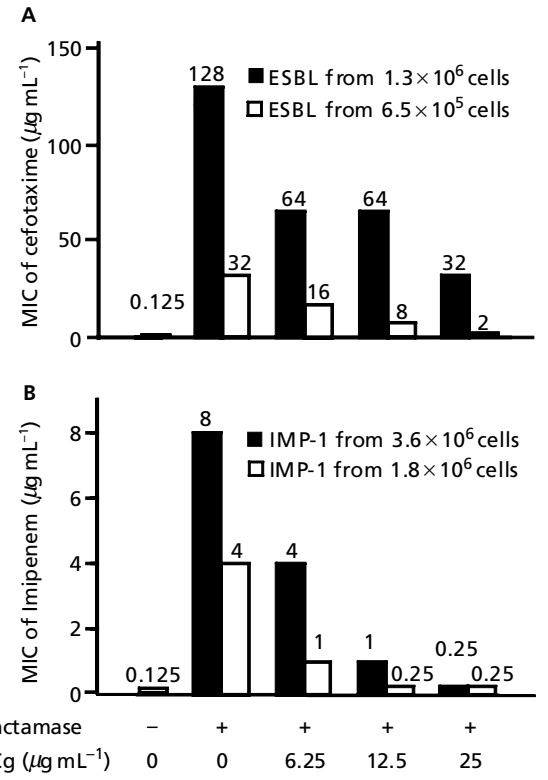

Figure 2 Protection of β-lactams by epigallocatechin gallate (EGCg). The susceptible *E. coli* ATCC 25922 was used as target cell (5×10^5 cells mL^{-1}). A. Cells were inoculated in MHB containing TEM-derived extended spectrum β-lactamases (ESBL) prepared from *E. coli* no. 5 in the presence of the two-fold serial dilutions of cefotaxime and EGCg. B. Cells were inoculated in the MHB containing IMP-1 prepared from *S. marcescens* no. 8 in the presence of two-fold serial dilutions of imipenem and EGCg. After incubation at 35 °C for 18 h, the MICs were determined.

restoration, by EGCg, of the antibacterial activity of β-lactams against β-lactamase-producing strains. Therefore, a synergic effect by the combinations of β-lactams and EGCg against β-lactamase-producing strains is largely dependent on the enzyme location.

Conclusion

Penicillin in combination with EGCg, at a possibly bioavailable concentration, showed potent synergy against penicillinase-producing *S. aureus* in-vitro. However, the combinations of β-lactams and EGCg against β-lactamase-producing Gram-negative rods do indicate a limitation owing to the cellular location of β-lactamase.

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