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Short communication

Synergistic interactions of epigallocatechin gallate and oxytetracycline against various drug resistant *Staphylococcus aureus* strains *in vitro*

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ABSTRACT

Epigallocatechin gallate (EGCG), the major catechin contained in tea leaves, is known to possess the synergistic anti-staphylococcal activity in combination with various β-lactam antibiotics and tetracycline. In the present study, we explored the *in vitro* combinatory effect of EGCG in combination with oxytetracycline against eight standard strains and clinical isolates of *Staphylococcus aureus*, including erythromycin, methicillin and tetracycline resistant strains. The minimum inhibitory concentrations were determined by the broth microdilution assay and the data were evaluated according to the sum of fractional inhibitory concentrations ($\sum$FIC). Our results showed synergistic and additive interactions against all *S. aureus* strains tested ($\sum$FIC 0.288–0.631), two of which were multidrug resistant. According to our best knowledge, it is the first report on the EGCG synergy with oxytetracycline. Considering its significant synergistic antimicrobial effect and low toxicity, we suggest EGCG as a promising compound for the development of new anti-staphylococcal formulations.

Introduction

The bacterial resistance is a phenomenon inevitably connected with the use of antimicrobials (French 2010). It has become the major global problem in the treatment of infectious diseases, thus creating a continuous need for new therapeutic options (Jordheim et al. 2012). Among bacterial pathogens, *Staphylococcus aureus* is one of the most serious ones due to its potential for rapid acquisition of drug resistance (French 2010). One of the recently adopted strategies for fast development of new antimicrobials effective against resistant pathogens is the combination of approved drugs (Jordheim et al. 2012). Another option to overcome the bacterial resistance is the combination of currently used antibiotics with compounds derived from plants traditionally used by humans for food or medicinal purposes (Wagner and Ulrich-Merzenich 2009). Epigallocatechin gallate (EGCG) (Fig. 1), the major catechin contained in tea [*Camellia sinensis* (L.) Kuntze] leaves, is an example of such a kind of synergistically acting antimicrobial agent. Besides the recent report on its synergy with imipenem against *Klebsiella pneumoniae* (Cho et al. 2011) it has been reported to potentiate the anticandidal effect of amphotericin B (Hirasawa and Takada 2004) and the activity of β-lactams and tetracycline against methicillin and tetracycline resistant *S. aureus*, respectively (Abreu et al. 2012). This anti-staphylococcal activity is attributed to the EGCG effect on the bacterial cell wall (Zhao et al. 2001), inhibition of penicillinase activity (Zhao et al. 2002), and inhibition of tetracycline efflux (Roccaro et al. 2004).

Despite the fact that the ability of EGCG to effectively increase the anti-staphylococcal effect of tetracycline has previously been described, its interaction with other tetracycline antibiotics has poorly been studied. Therefore, we decided to evaluate the *in vitro* synergistic effect of EGCG with oxytetracycline, a combination selected as the most promising result of the initial screening of EGCG with representatives of eight major antibiotic classes (Novy et al. 2009), against various drug resistant strains of *S. aureus*.

Materials and methods

Chemicals

Bacteria were grown in cation adjusted Mueller-Hinton broth (MHB; Oxoid, Basingstoke, UK). Tris-buffered saline used for the MHB equilibration was purchased from Sigma–Aldrich (Prague, CZ) as well as the antimicrobials tested: EGCG, oxacillin and oxytetracycline. Dimethyl sulfoxid, hydrochloric acid (Lach-Ner, Neratovice, CZ) and deionised water were used as solvents.

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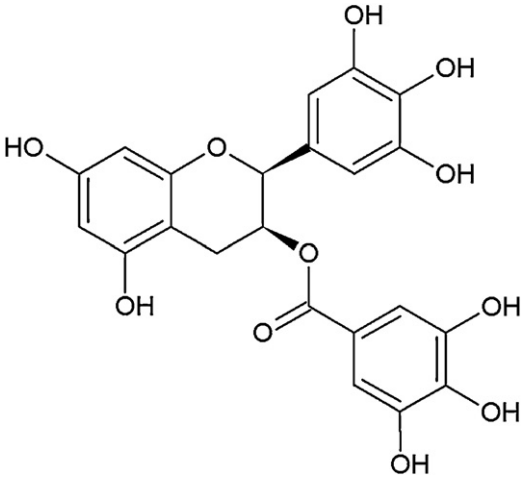

Fig. 1. Chemical formula of epigallocatechin gallate.

Bacterial strains

Eight staphylococcal strains were tested in this study. Two standard strains, methicillin sensitive (MSSA) ATCC 29213 and MRSA ATTC 43300, were purchased from Oxoid (Basingstoke, UK). Another two standard MRSA strains CCM 7112 and CCM 7115 and four drug resistant clinical isolates, including epidemic MRSA strain EMRSA-15, were obtained from the Motol University Hospital in Prague, Czech Republic.

Antimicrobial assay

The minimum inhibitory concentrations (MICs) were performed by the broth microdilution method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI 2009) using 96-well microtiter plates. Briefly, 2-fold serial dilutions of antimicrobials in MHB (100 ml) were inoculated with staphylococcal suspension to reach the final concentration of 5×10^5 cfu/ml and the results were evaluated after 24 h incubation at 35 °C. Turbidity was measured by a Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA) at 405 nm. The MIC was defined as the lowest concentration causing ≥80% growth inhibition of tested strain. All tests were carried out in triplicate in three independent experiments and *S. aureus* ATCC 29213 was used as a quality control strain for antibiotic susceptibility testing. Oxacillin and oxytetracycline were used as markers of methicillin and oxytetracycline resistance, respectively.

Synergy evaluation

The fractional inhibitory concentrations (FICs) were evaluated by the checkerboard assays. Two-fold serial dilutions of oxytetracycline prepared in horizontal rows of microtiter plate were subsequently cross-diluted vertically by two-fold serial dilutions of EGCG. The combinatory effects were then determined based on the ∑FICs according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2000) criteria for synergy as follows: ∑FIC ≤ 0.5 = synergy; ∑FIC > 0.5 – 1 = additivity; ∑FIC > 1 to <2 = indifference; ∑FIC ≥ 2 = antagonism.

Results and discussion

The individual MICs of oxytetracycline and EGCG against staphylococcal strains as well as the MICs of its combinations with corresponding ∑FICs are summarised in Table 1. EGCG (average individual MIC 104 mg/l) potentiated the activity of oxytetracycline against all *S. aureus* strains tested whereas synergistic and additive effect was obtained against 7 and 1 out of 8 staphylococcal strains tested, respectively. The synergistic and additive interactions against 6 and 2 strains, respectively, were obtained even at the lowest EGCG concentration tested of 4 mg/l. The ∑FICs for this EGCG concentration ranged from 0.288 to 0.527 whereas 2–4-fold reduction in oxytetracycline MICs was observed. The combination profiles are presented graphically in Fig. 2. The isobole curves clearly show the potentiating effect against all *S. aureus* strains tested whereas the synergistic interactions can be read according to the curve indicating the borderline synergy.

Table 1
Sensitivity of staphylococcal strains to reference antibiotics.

S. aureus strain	MIC (μg/ml)		
	TET	OXA	ERY
ATCC 29213	0.4 ^{S}	0.3 ^{S}	0.3 ^{S}
ATCC 43300 (MRSA)	0.3 ^{S}	16.0 ^{R}	>512.0 ^{R}
EMRSA-15	1.0 ^{S}	32.0 ^{R}	>512.0 ^{R}
CCM 7112 (MRSA)	16.0 ^{R}	256.0 ^{R}	>512.0 ^{R}
CCM 7115 (MRSA)	16.0 ^{R}	256.0 ^{R}	>512.0 ^{R}
TR 12001 (TRSA)	8.0 ^{R}	0.5 ^{S}	0.3 ^{S}
TR 12002 (TRSA)	12.0 ^{R}	0.5 ^{S}	0.3 ^{S}
ER 12001 (ERSA)	0.3 ^{S}	0.5 ^{S}	>512.0 ^{R}

^{S}, sensitive; ^{R}, resistant; MIC, minimum inhibitory concentration; TET, tetracycline; OXA, oxacillin; ERY, erythromycin; MRSA, methicillin resistant *S. aureus*; EMRSA, epidemic MRSA; TRSA, tetracycline resistant *S. aureus*; ERSA, erythromycin resistant *S. aureus*.

The most sensitive strain in this study was the epidemic MRSA (EMRSA-15) with 4–12-fold reduction in oxytetracycline MICs (∑FICs 0.313–0.583), followed by two standard strains (ATCC 29213, 43300), and one oxytetracycline resistant isolate (TR 12001). The synergy against these three strains was observed at all EGCG concentrations tested whereas up to 8-, 9-, and 5-fold reduction in oxytetracycline MIC was obtained, respectively. Synergy was obtained against all MRSA strains exerting also high-level resistance to erythromycin (see Table 2 for the sensitivity of tested strains to selected reference antibiotics).

Out of four tetracycline resistant (TRSA) strains, the oxytetracycline resistance was completely reversed in one multidrug-resistant (MDR) MRSA isolate CCM 7115 at all EGCG concentrations tested with 3–10-fold reduction in oxytetracycline MICs (∑FICs 0.363–0.6). The resistance in the remaining TRSA strains (TR 12001, 12002, and MDR MRSA CCM 7112) was decreased to intermediate level at EGCG concentration of 16, 32 and 4 mg/l with ∑FICs 0.338 and 0.631 and 0.363, respectively. The EGCG ability to reduce the oxytetracycline resistance in TRSA strains suggests that EGCG might interfere with some mechanisms of *S. aureus* resistance. As has been previously reported, the synergy between tetracycline and EGCG is probably dependent on the direct binding of EGCG to peptidoglycan (Zhao et al. 2001) and on the inhibition of Tet(K) and Tet(B) effluxes (Roccaro et al. 2004) that typically export also oxytetracycline and chlortetracycline (Poole 2005). Thus, it is possible that these mechanisms can also be responsible for the synergy between EGCG and oxytetracycline. Since the ATCC strains tested in our study (and perhaps also the remaining oxytetracycline sensitive strains) are not carrying Tet genes, we suppose that the EGCG interference with the bacterial cell wall (Cui et al. 2012) might be responsible for the majority of positive anti-staphylococcal interactions obtained in this study. We can further deduce that the inhibition of an efflux pump could contribute to the synergy against the TRSA isolates. The low sensitivity of the TRSA strain TR12002 is possibly caused by another mechanism of resistance, *e.g.* the production of ribosome protection proteins (Thaker et al. 2010), or the strain possess an efflux pump that EGCG does not suppress.