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Antibacterial activity of epigallocatechin-3-gallate (EGCG) and its synergism with β-lactam antibiotics sensitizing carbapenem-associated multidrug resistant clinical isolates of *Acinetobacter baumannii*

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ABSTRACT

Background: Infections caused by *Acinetobacter baumannii* were responsive to conventional antibiotic therapy. However, recently, carbapenem-associated multidrug resistant isolates have been reported worldwide and present a major therapeutic challenge. Epigallocatechin-3-Gallate (EGCG) extracted from green tea exhibits antibacterial activity.

Purpose: We evaluated the antibacterial activity of EGCG and possible synergism with antibiotics in carbapenem-associated multidrug resistant *A. baumannii*. A potential mechanism for synergism was also explored.

Materials and methods: Seventy clinical isolates of *A. baumannii* collected from geographically different areas were analyzed by minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EGCG. Checkerboard and time-killing assays were performed to exam the synergism between EGCG and antibiotics. The effects of EGCG on a multidrug efflux pump inhibitor (1-[1-naphthylmethyl] piperazine; NMP) and β-lactamase production were also examined in *A. baumannii*.

Results: Sixty-three of 70 clinical isolates of *A. baumannii* carried carbapenemase-encoding genes with carbapenem-associated multidrug resistance. Levels of MIC and MBC of EGCG ranged from 64 to 512 μg/ml and from 128 to ≥ 1024 μg/ml, respectively among the clinical isolates. MIC_{90} and MBC_{86} levels were 256 μg/ml and 512 μg/ml of EGCG, respectively. Subinhibitory concentration of EGCG in combination with all antibiotics tested, including carbapenem, sensitized (MICs fall ≤ 1.0 μg/ml) all carbapenem-associated multidrug resistant isolates. Checkerboard and time-killing assays showed synergism between EGCG and meropenem (or carbenicillin) counted as fractional inhibitory concentration of < 0.5 and cell numbers' decrease per ml of > 2log10 within 12 h, respectively. EGCG significantly increased the effect of NMP but was unrelated to β-lactamase production in *A. baumannii*, suggesting EGCG may be associated with inhibition of efflux pumps.

Conclusion: Overall we suggest that EGCG-antibiotic combinations might provide an alternative approach to treat infections with *A. baumannii* regardless of antibiotic resistance.

Introduction

Acinetobacter baumannii is a Gram-negative glucose-non-fermenting nonmotile coccobacillus and a major human pathogen causing hospital-acquired infections, such as ventilator associated pneumonia, bacteremia, meningitis, urinary tract, and wound infections (De et al., 2016). The bacterium is also recognized as a cause of community-acquired pneumonia and wound infections in unusual situations such as victims of war- or earthquakes (Bachoumas et al., 2015). Until recently, these infections have been treated effectively with antibiotics such as β-lactams, aminoglyco-

Abbreviations: EGCG, Epigallocatechin-3-Gallate; h, hours; MIC, minimal inhibitory concentration; MBC, minimum bactericidal concentration; NMP, 1-[1-naphthylmethyl] piperazine; LB, Luria-Bertani; CLSI, Clinical and Laboratory Standards Institute; MH, Mueller-Hinton; CFU, colony forming units; FIC, fractional inhibitory concentration; ATM, aztreonam; CAR, carbenicillin; CAZ, ceftazidime; MEM, meropenem; CIP, ciprofloxacin; GEN, gentamicin; TET, tetracycline.

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sides, fluoroquinolones, tetracyclines, and rifampin. However, outbreaks of antibiotic resistance have been increasingly reported worldwide with carbapenem-associated multidrug resistance being the most problem in clinical isolates of *A. baumannii* (Durante-Mangoni and Zarrilli, 2011). Hospital-acquired infections with the carbapenem-associated multidrug resistant *A. baumannii* are associated with an increased risk of mortality from 8% to 40%.

Carbapenem antibiotics (e.g., imipenem and meropenem) are resistant to most β-lactamases such as cephalosporinases and penicillinases. Carbapenem antibiotics also exhibit broad spectrum for Gram-negative pathogens resistant to aminoglycosides, quinolones, and tetracyclines with good activity and low toxicity (Nowak and Paluchowska, 2016). Due to these reasons, they have been used to treat the multidrug resistant clinical isolates and are often administered as last resort or last-line agents in critically ill patients (Nowak and Paluchowska, 2016). However, carbapenem resistant *A. baumannii* has been increasingly reported worldwide in the last decade. In United States, carbapenem-associated multidrug resistant isolates have been increased from 20.6% (2002) to 49.2% (2008) (Mera et al., 2010) and are often resistant to all classes of antibiotics except to polymyxins (Durante-Mangoni and Zarrilli, 2011; Peleg et al., 2008). However, polymyxin-resistant clinical isolates have been also reported worldwide (Gales et al., 2011). Some carbapenem-associated multidrug resistant isolates are also not susceptible to almost all clinically available antibiotics (Lei et al., 2016). A variety of carbapenem resistance mechanisms have been identified in clinical isolates of *A. baumannii* including presence of carbapenem hydrolyzing β-lactamases, reduced-influx of carbapenem antibiotics by loss of outer membrane porins, efflux pumps, and alterations of penicillin-binding proteins (Peleg et al., 2008). Among the carbapenem resistant mechanisms, carbapenem hydrolyzing β-lactamases (class D or oxacillinases) are the most common in clinical isolates including three major types of acquired class D β-lactamases ($bla_{\text{OXA-23-type}}$, $bla_{\text{OXA-24-type}}$, and $bla_{\text{OXA-58-type}}$) and one intrinsic class D β-lactamases (IS*Aba1*-$bla_{\text{OXA-51-type}}$). The intrinsic class D β-lactamases are required for the IS*Aba1* to promote the gene (Nowak and Paluchowska, 2016; Peleg et al., 2008). The fact that carbapenem-associated multidrug or extensively drug resistant *A. baumannii* has spread globally, urgently demands development of novel prevention and treatment strategies.

Several non-antibiotic compounds have showed antibacterial activity. Epigallocatechin-3-gallate (EGCG) is a major polyphenolic compound extracted from the green tea leaves (Steinmann et al., 2013) and has shown antibacterial activity as well as a variety of potential human health benefits such as antioxidant, anti-inflammatory, anti-carcinogenic and cardioprotective activities (Reygaert, 2014). Antibacterial activity of pure EGCG has been tested against Gram-positive and Gram-negative bacteria including *Listeria monocytogenes, Staphylococcus aureus, Streptococcus mutans, Strepococcus pyogenes, A. baumannii, E. coli, Proteus mirabilis*, and *Pseudomonas aeruginosa* (Steinmann et al., 2013). The levels of antibacterial activity of EGCG have been reported to range from MICs 100 to $>$ 600 μg/ml. Mechanisms of antibacterial activity have been investigated in Gram-positive and Gram-negative bacteria. For example, EGCG damages cellular structures (e.g., peptidoglycan and cell membrane), inhibits cellular enzymes (dihydrofolate reductase, fatty acid synthetase, DNA gyrase, and ATP synthetase), and induces intracellular oxidative stress (Reygaert, 2014; Steinmann et al., 2013).

In *A. baumannii*, the antibacterial activity of EGCG has been reported by two groups to date (searched in PubMed). Osterburg et al. determined the MICs of 21 clinical isolates of *A. baumannii*, which ranged from MICs 78 to 625 μg/ml of EGCG and also showed synergism in combination with the topical agent of mafenide acetate (Sulfamylon) (Osterburg et al., 2009). Betts and Wareham reported MICs of EGCG ranging from 128 to 1024 μg/ml in 9 *A. baumannii* isolates and synergism in combination with curcumin, another plant polyphenol in some of the isolates (Betts and Wareham, 2014). In addition, the antibacterial activity of EGCG has not been studied in a large numbers of isolates, especially in carbapenem-associated multidrug resistant isolates from geographically diverse areas. The underlying mechanisms of antibacterial activity of EGCG and its synergism (if any) also remain to be clarified in *A. baumannii*.

In this study, we confirmed the antibacterial activity of EGCG in 70 clinical isolates of *A. baumannii* including 63 carbapenem-associated multidrug resistant isolates. Additionally, we found that all tested carbapenem-associated multidrug resistant isolates were synergistically sensitized by sub-inhibitory concentration of EGCG in combination with all antibiotics tested including β-lactam antibiotics. Our results suggest that EGCG may be associated with inhibition of efflux pumps to enhance antibiotic susceptibility in *A. baumannii*.

Materials and methods

Clinical isolates and culture conditions

A total of 70 clinical isolates of *A. baumannii* and the type strain ATCC 19606 were used in this study (Table 1). Among them, 32 isolates were reported previously (Nidha et al., 2015); 17 isolates were from Baylor college of Medicine (Houston, TX), 10 isolates were from The University of Washington, Medical Center (Seattle, WA), and 5 isolates were from SUNY Downstate Medical Center (Brooklyn, NY). Six clinical isolates carrying carbapenemase-encoding genes, AB0057 and H26 for $bla_{\text{OXA-23-type}}$, AA640 and AB1204 for $bla_{\text{OXA-24-type}}$, AB047 for $bla_{\text{OXA-58-type}}$, and MA309 for IS*Aba1*-$bla_{\text{OXA-51-type}}$ (Adams et al., 2008; Bratu et al., 2008; Lolans et al., 2006; Qi et al., 2008) were also included in this study. All clinical isolates of *A. baumannii* were routinely cultured in Luria-Bertani (LB) broth or LB agar plates.

Antibiotic susceptibility testing

Antibiotic susceptibility was determined by measuring Minimum Inhibitory Concentration (MIC) as guided by the Clinical and Laboratory Standards Institute (CLSI, 2006). Serial two-fold dilutions of antibiotics (0.125–256 μg/ml) were performed in divalent cation-adjusted Mueller-Hinton (MH) broth (Oxoid, pH 7.0). Fresh overnight cultures of each isolate were diluted in saline to an optical density at 600 nm of 0.1–0.12 (approximately 10^{8-9} cells/ml), and an inoculum of the adjusted cells suspension ($\sim 10^{6-7}$ cells) was inoculated. The inoculated cells were incubated overnight (16–18 h) at 37 °C. MICs were defined as the lowest concentration of each antibiotic that completely inhibited growth of the inoculum. MIC measurements were repeated and confirmed in three independent experiments. Antibiotic resistant breakpoints were used as described by Peleg et al. (2008).

Detection of carbapenemase-encoding genes and an insertion sequence element (ISAba1)

Carbapenemase-encoding genes from all clinical isolates were detected by a multiplex PCR method as described previously (Ellington et al., 2007). The 6 clinical isolates carrying carbapenemase-encoding genes mentioned above were used as the positive controls and *A. baumannii* ATCC 19606 was used as the negative control. The presence of an insertion sequence element (IS*Aba1*) and its location in the upstream of $bla_{\text{OXA-51-type}}$ was also examined by PCR methods as described (Turton et al., 2006).