Impact of Green Tea Catechins on Multi-Drug-Resistant Bacterial Pathogens

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Abstract

Catechin gallates have weak direct antibacterial activity but are able to substantially and reversibly modify the properties of multi-drug-resistant *Staphylococcus aureus*, a problematical opportunistic pathogen responsible for an enormous number of hospital-acquired and community infections worldwide. In particular, MRSA strains that have acquired resistance genes to β-lactam antibiotics, the penicillins and cephalosporins, compromise the therapeutic utility of these frontline drugs. Resistance to β-lactam agents can be completely abrogated by exposure of MRSA strains to catechin gallates, particularly (-)-epicatechin gallate (ECg); the compounds also prevent formation of biofilms and substantially reduce the virulence of MRSA. We have shown that the complex, ECg-induced staphylococcal phenotype is due to intercalation of ECg into the cytoplasmic membrane of the bacterial cells; incorporation of molecules deep within the hydrophobic core of the lipid palisade forces a reconfiguration of the membrane to provide a sub-optimal environment for proteins embedded in the staphylococcal membrane. In particular, the orderly function of the septal division machinery in MRSA, incorporating the penicillin binding protein PBP2a responsible for β-lactam resistance, is compromised leading to loss of resistance to all β-lactam antibiotics. Catechin gallates are attractive candidates as adjuncts to conventional antibacterial chemotherapy but improvements in stability, absorption and pharmacokinetic profile through chemical engineering would substantially increase their potential as agents for therapeutic intervention.

Introduction

The rising incidence of antibiotic resistance in both Gram-positive and Gram-negative bacteria poses a serious threat to global health, compounded by the paucity of new antibiotics in the drug development pipeline. The ESKAPE bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) have been highlighted as the primary threats to human health associated with antibiotic resistance and acquisition of multi-drug resistance appears to be intrinsic to their success (Boucher *et al.*, 2009). Multi-drug resistant bacteria have emerged due to the intense selective pressure on bacterial populations within hospitals and communities brought about by the use and overuse of frontline antibiotics. The emergence of resistance to conventional antibiotics, that kill or inhibit the growth of pathogens and commensals alike, is inevitable and provides a dramatic demonstration of Darwin's insights into evolution through natural selection. The spread of antibiotic resistance is further exacerbated by the capacity of many pathogens to acquire antibiotic resistance genes through horizontal gene transfer.

As a novel approach to the treatment of difficult-to-treat bacterial infections, we have examined the therapeutic potential of agents that do not directly kill the target bacterial population but modify them to produce a "less fit" phenotype with reduced capacity to survive at the site of infection (Taylor *et al.*, 2009). There are conceptual reasons to suppose that this approach will result in less selective pressure on the bacteria and delay the emergence of resistant genotypes (Tan *et al.*, 2000). We have focused on *S. aureus*, a leading cause of hospital-acquired and community-acquired infections worldwide; this highly successful opportunistic pathogen is a common component of the resident bacterial population (microbiota) of the upper respiratory tract and skin of healthy individuals but also causes a variety of infections ranging from minor skin conditions to life-threatening diseases such as endocarditis, septicaemia and toxic shock syndrome (Thwaites *et al.*, 2011). Multi-drug-resistant forms are typified by methicillin-resistant *S. aureus* (MRSA) that are represented by a limited number of successful clones that dominate worldwide in hospitals and

communities and are invariably resistant to all β-lactam antibiotics due to acquisition of *mecA* or *mecC* genes encoding the low-affinity penicillin binding protein PBP2a (Fuda *et al*., 2005; Paterson *et al*., 2014). MRSA strains are particularly adept at acquiring genes conferring resistance to a wide variety of antibiotics, considerably eroding their utility for the fight against serious infections caused by this pathogen.

Modulation of β-lactam resistance and other properties of MRSA by catechin gallates

Extracts of green tea (Hamilton-Miller, 1997) and their major individual components (Stapleton *et al*., 2004) have a relatively weak capacity to inhibit and kill a wide range of pathogenic bacteria at or slightly below typical concentrations found in brewed tea. However, green tea extracts have the capacity to reverse methicillin resistance in MRSA at concentrations much lower than those needed to produce inhibition of bacterial growth (Yam *et al*., 1998). Subsequent examination of the major bioactive components revealed that abrogation of β-lactam resistance was attributable to the catechin gallates, with (-)-epicatechin gallate (ECg) showing greater potency than either (-)-epigallocatechin gallate (EGCg) or (-)-catechin gallate (Cg); the effect was wide-ranging, with all forty MRSA isolates examined sensitised to a comprehensive selection of β-lactam chemical structures (Stapleton *et al*., 2004). Japanese groups also showed that ECg (Shiota *et al*., 1999) and EGCg (Zhao *et al*., 2001) significantly reduce the minimum inhibitory concentration (MIC) of methicillin, oxacillin and other β-lactam antibiotics in MRSA. ECg reduced the MIC of β-lactams for MRSA strains from full resistance (256-512 mg/l) to below the antibiotic breakpoint (~1 mg/l) where drugs are assumed to possess clinical efficacy (Stapleton *et al*., 2004; Yam *et al*., 1998), raising the possibility that such molecules could be used in combination with β-lactam agents to treat MRSA infections. We also found that ECg reduced the secretion by the common epidemic strain EMRSA-16 of a range of proteins such as the toxins and tissue-degrading enzymes the pathogen needs to spread through the body and cause harm (Shah *et al*., 2008), and prevented the formation of biofilms (Stapleton *et al*., 2007), an interfacial mode of growth essential for colonisation of natural and artificial surfaces. ECg also abrogates halotolerance in *S. aureus* by interference with cation ($Na^+[Li^+]/H^+$) ion-specific

antiporter systems in the cytoplasmic membrane (CM) (Stapleton *et al*., 2006a). These properties raise the intriguing possibility than ECg could function alone to "disarm" highly pathogenic staphylococci. Growth of MRSA in the presence of moderate concentrations of ECg has a profound impact on the appearance of the bacterial cells (Fig. 1): the compound increases the thickness of the staphylococcal cell wall and stimulates the formation of pseudomulticellular aggregates in which dividing cocci do not separate cleanly into daughter cells (Stapleton *et al*., 2007).

Interactions of ECg and MRSA cells

ECg and other galloyl catechins have a broad ranging impact on staphylococci due to their capacity to intercalate in non-lethal fashion into the bacterial CM (Bernal *et al*., 2010; Palacios *et al*., 2014). Their relative affinity for model lipid bilayers (Caturla *et al*., 2003; Kajiya *et al*., 2001; Kajiya *et al*., 2002) reflects both their partition coefficients in *n*-octanol-saline (Hashimoto *et al*., 1999) and their capacity to modulate β-lactam resistance (Stapleton *et al*., 2004). Thus, ECg binds more avidly than EGCg, Cg and the non-galloyl counterparts (-)-epicatechin (EC) and (-)-epigallocatechin (EGC); EC and EGC exert no β-lactam-modifying capacity but can enhance the membrane binding and bioactivity of ECg (Palacios *et al*., 2014; Stapleton *et al*., 2006b). Interactions between lipid bilayers and ECg are facilitated by an exposed hydrophobic domain in the region of the ester bond and C-ring, optimized by virtue of epi (*cis)* stereochemistry. We studied the binding of ECg and other catechins to artificial membranes modelled on the lipid composition of the staphylococcal bilayer and showed that ECg adopts a position deep within the lipid palisade, eliciting major alterations in the thermotropic and anisotropic behaviour of the bilayer. These data reflect the binding of ECg to the staphylococcal bilayer: the *S. aureus* CM is unusual (Haest *et al*., 1972; Mukhopadhyay *et al*., 2007) comprising three major phospholipids, negatively charged phosphatidylglycerol (PG) and cardiolipin in addition to positively charged lysyl-PG distributed with a high degree of asymmetry across the outer and inner leaflets of the lipid palisade (Mukhopadhyay *et al*., 2007; Rosado *et al*., 2015). Intercalation of ECg into the CM alters the expression of genes encoding membrane-embedded proteins and up-regulates genes belonging to the cell wall stress stimulon (Bernal *et al*., 2010), indicating that the

bacteria respond to ECg binding by taking steps to preserve and repair a compromised cell wall and membrane.

How does ECg binding to the CM elicit substantial changes to the structure and physiology of MRSA? ECg molecules rapidly enter the CM, inducing an immediate reduction in fluidity, but the bacteria respond by altering the composition of the membrane, incorporating a greater complement of branched chain fatty acids to re-establish a fluid phospholipid palisade whilst retaining membrane asymmetry (Bernal *et al.*, 2010; Rosado *et al.*, 2015). However, these changes result in a more fluid structure that is likely to provide a sub-optimal environment for integral membrane proteins. In the case of the $Na^+[Li^+]/H^+$ antiport, this would almost certainly result in a loss of ability to accumulate osmoprotective molecules and a loss of tolerance to salt. Similarly, efficient protein export requires an optimally-configured CM (Sibbald *et al.*, 2006). ECg reduces the net positive charge of the staphylococcal surface, in part due to its capacity to inhibit the attachment of D-alanine residues to peptidoglycan-associated wall teichoic acids (Bernal *et al.*, 2009); these anionic glycopolymers play crucial roles in cell shape determination, regulation of cell division, staphylococcal pathogenesis and antibiotic resistance (Brown *et al.*, 2013). Positive surface charge is also likely to be further reduced as a consequence of ECg-mediated reduction in the biosynthetic activity of membrane-anchored MprF, an enzyme that attaches positively-charged lysine to PG; ECg membrane intercalation elicits a large decrease in lysyl-PG in the lipid palisade (Bernal *et al.*, 2010). An increased negative surface charge leads to electrostatic repulsion from negatively charged surfaces and probably accounts for the biofilm-inhibiting properties of ECg.

Perturbation of orderly cell division, cell wall turnover and cell separation following growth in ECg-supplemented medium (Stapleton *et al.*, 2007) are in all likelihood due to changes in the secretion of staphylococcal autolysins (peptidoglycan hydrolases); these enzymes sculpt the shape, size and thickness of cell wall peptidoglycan and facilitate separation of daughter cells during and after cell division, often following secretion into the external milieu (Wheeler *et al.*, 2015). ECg-grown cells retained predominantly inactive autolysins within the thickened cell wall, with greatly

reduced amounts released into the external environment, partly as a consequence of decreases in the net positive charge of the cell surface (Stapleton *et al*., 2007). As ECg modulates the levels of PBP1 and PBP3 in the staphylococcal CM, the molecule induces a 5-10% reduction in peptidoglycan cross-linking without compromising cell integrity (Stapleton *et al*., 2007) resulting in a less dense appearance of the wall and greater packed cell volume of ECg-treated bacteria (Bernal *et al*., 2009).

Abrogation of resistance of MRSA to β-lactam antibiotics

Although the changes described above shed light on some aspects of the complex ECg-induced MRSA phenotype, they are unlikely to account for the large increases in β-lactam susceptibility induced by exposure to ECg. Low to moderate concentrations of ECg and other catechin gallates elicit complete restoration of β-lactam susceptibility, that we believe can only be a consequence of direct interference with the underlying antibiotic resistance machinery of the cell. The β-lactam resistance determinant PBP2a is localised during MRSA cell division at the septum within the macromolecular division machinery, the divisome (Fig. 2), a complex composed primarily of membrane-bound proteins but also including cytoplasmic components. Central to orderly staphylococcal division are the PBPs that transglycosylate and cross-link nascent peptidoglycan chains through transpeptidation reactions. Thus, PBPs catalyse the insertion and crosslinking of newly synthesized peptidoglycan precursors into the cell wall and are the targets for β-lactam antibiotics (Ehlert, 1999). These antibiotics function as analogues of D-alanyl-D-alanine peptidoglycan side chain termini, the normal substrates for the PBPs, to form a long-lived covalent acyl-enzyme complex that inactivates the enzyme, leading to loss of cell wall cross-linking, cell lysis and death.

Cell division is initiated by polymerisation into a filamentous Z-ring structure of the cytosolic tubulin homologue protein FtsZ, a self-activating guanosine triphosphatase (Adams and Errington, 2009). FtsZ recruits further divisome proteins to the septum, including the essential component PBP2, a bifunctional protein with spatially well-separated transglycosylase and transpeptidase domains that is essential for the expression of the β-lactam-resistant MRSA phenotype (Lovering *et*

al., 2007). In β-lactam susceptible *S. aureus* (MSSA), β-lactam drugs such as oxacillin prevent the recruitment of PBP2 to the septal site of cell division and the protein is dispersed over the entire surface of the viable cell (Pinho and Errington, 2005). The origins of SCC*mec*, the mobile genetic island staphylococcal cassette chromosome *mec* containing the *mecA* gene, are unclear but it may have been acquired by a MSSA strain from the animal pathogen *Staphylococcus sciuri* (Fuda *et al*., 2005). The *mecA* gene product PBP2a is a high molecular weight membrane-bound transpeptidase that is not susceptible to acylation by β-lactam antibiotics and functions cooperatively with PBP2 in the presence of these drugs to compensate for the loss of PBP2 transpeptidase activity due to active site acylation. Thus, MRSA continues to cross-link peptidoglycan chains in the presence of β-lactams albeit with reduced efficiency. We found that intercalation of ECg into the MRSA CM delocalised PBP2 from the cell wall biosynthetic machinery and led to its dispersal over the entire membrane (Bernal *et al*., 2010). It was not possible, for technical reasons, to determine if PBP2a was similarly dispersed but use of novel detergent-free extraction procedures provided strong evidence of the predicted physical association between PBP2 and PBP2a and of an altered spatial relationship between the two membrane-embedded proteins following exposure to ECg (Paulin *et al*., 2014). It appears highly probable, therefore, that ECg abrogates β-lactam resistance in MRSA by physical and functional disruption of the PBP2/PBP2a complex, decoupling transglycosylation and transpeptidation from the septal site of cell wall peptidoglycan biosynthesis, preventing peptidoglycan cross-linking and leading to loss of structural rigidity and cell lysis driven by the high internal turgor pressure of the staphylococcal cell. In support of this contention, we found no evidence that ECg interacts directly with PBP2a to reduce its capacity to compensate for loss of PBP2 function.

A role for catechin gallates in the treatment of MRSA infections?

The galloyl catechins possess substantial anti-oxidant properties but their biological activity is determined predominantly by their capacity to intercalate into lipid bilayers. Their safety profile is attractive and is underpinned by thousands of years of tea consumption. Catechins, like many

flavonoids, are subject to chemical transformation, both *in vitro* and *in vivo*. ECg and other catechin

gallates are unstable in alkaline solution, degrading in minutes (Zhu *et al*., 1997), but are more stable

in an acidic environment (Pomponio *et al*., 2003). EGCg degrades in phosphate buffer over the pH

range 6.5-7.8 to yield several products, including the dimeric compounds theasinensin A and

theasinensin D (Hatano *et al*., 2003), major oxidation products found in black tea. ECg was not

investigated by these authors but it is likely to show similar modification. These compounds

contribute to the distinctive dark colouration of black tea following a complex series of oxidative

structural modifications (Tanaka *et al*., 2010). Recovery by centrifugation of MRSA cells exposed to

ECg indicated that both intact cells (Fig. 3) and the CM fraction displayed a dark pigmentation as

little as 5 min after addition of the catechin gallate, suggesting that ECg incorporated into the CM

lipid palisade undergoes spontaneous oxidation or is subject to modification by CM enzymes. This

interesting observation should be explored further as it implies that the ECg-induced MRSA

phenotype results from membrane interactions involving metabolites or oxidation products of ECg

and not from the unmodified compound. In this context, it is interesting to note that Hatano and co-

workers demonstrated that EGCg-derived theasensin A is a potent modifier of resistance to oxacillin

(Hatano *et al*., 2003).

Catechin gallates are rapidly metabolised to inactive products by intestinal bacteria after

oral administration (Kohri *et al*., 2001) due to the presence of an esterase susceptible bond linking

the B-ring to the A-C fused ring system (for structures of naurally occurring and synthetic catechins,

see Palacios *et al*., 2014)). After *in vivo* removal of the galloyl group from EGCg, EGC undergoes

reduction, cleavage, dehydroxylation, further decomposition and lactonisation to form

hydroxyphenyl-γ-valerolactone products that are excreted in urine and faeces (Kohri *et al*., 2001).

We have replaced the hydrolytically susceptible ester bond in ECg with an amide linkage that

stabilises the molecule by preventing the initial cleavage step (Anderson *et al*., 2005a). Such

modifications, together with rearrangements of B-ring hydroxyl group substitutions (Anderson *et al*.,

2005b; 2011; 2014) that increase resistance-modifying potential, should improve the attractiveness

of these compounds as therapeutic adjuncts. In addition to substantial biotransformation, rates of absorption and bioavailability are low, at least in the case of orally administered EGCg. Systemic availability of EGCg in human volunteers increase with oral dose and low to moderate levels were detected in plasma (Chow *et al*., 2001); other catechins were not investigated in this study but administration of Polyphenon E, a commercial decaffeinated green tea catechin mixture, led to lower concentrations of EGC and EC in the blood compared to EGCg. Oral bioavailability of catechin gallates in human subjects is enhanced by administration after overnight fasting, which is likely to increase the biological response to the agent (Chow *et al*., 2005). Absorption, plasma bioavailability and biotransformation have also been investigated in rodents, with a complex and variable pharmacokinetic profile comparable to human data (Yang *et al*., 2008).

In spite of suboptimal structure-activity relationships and poor pharmacokinetic profile, EGCg has been examined for its antioxidant, anti-inflammatory and anti-atherogenic properties in randomised controlled human clinical trials with variable, conflicting and generally disappointing outcomes (National Cancer Institute, 2016; Mereles and Hunstein, 2011; Farrar *et al*., 2015). In the anti-infective domain, human trials support the anticaryogenic efficacy of green tea consumption (reviewed in Taylor *et al*., 2005), but no clinical trials have been undertaken to investigate the efficacy of green tea components in more serious microbial infections. Similarly, there have been very few attempts to examine green tea components in animal models of infection. Lee and colleagues examined the capacity of green tea extracts to resolve chronic induced experimental bacterial prostatitis in the adult rat with inconclusive results with regard to bacterial numbers at the infection site, chronic local inflammation, acinar change and intestinal fibrosis (Lee *et al*., 2005). In light of rapid biotranformation of catechin gallates after oral or systemic dosing, we were reluctant to undertake efficacy experiments in vertebrates with the native compounds but we recently examined the capacity of ECg to resolve MRSA infections in the zebrafish embryo (Stevens *et al*., 2015), a model of infection that would enable us to circumvent many of the pharmacokinetic issues described above. Embryos injected into the yolk sac 30 h after fertilisation with strain EMRSA-16

rapidly succumbed to lethal infection whereas the majority of embryos survived a similar challenge dose (1-5 × 10^3 CFU) injected into the circulation valley. No significant increases in survival were noted when infected embryos were maintained in 12.5-100 µg/ml ECg with or without oxacillin. However, when EMRSA-16 was grown in the presence of 12.5 µg/ml ECg and the bacteria used to infect embryos by either the circulation valley or yolk sac, there were significant increases in embryo survival in both the presence and absence of oxacillin. Thus, exposure to ECg prior to infection reduced the lethality of EMRSA-16, rendered cells more susceptible to elimination by immune processes and compromised their capacity to establish an inflammatory response, but the lack of capacity to alter the course of established infection with unmodified bacteria indicates that in this infection model ECg is unable to function either as a therapeutic that modifies staphylococcal virulence or as an adjunct to conventional β-lactam chemotherapy. Clearly, further investigations are needed to resolve the antibacterial potential of ECg and other catechin gallates.

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Legends

Figure 1: Scanning electron micrographs of mid-logarithmic growth phase EMRSA-16 (A) and EMRSA-16 grown in the presence of 12.5 mg/l ECg (B).

Figure 2: Proteins of the divisome recruited to the division septum of MRSA during cell division. Cytoplasmic synthesis of lipid II, the peptidoglycan precursor polymerised by PBPs, is also shown in the not-to-scale representation. A full description of the enzymatic steps involved can be found in the review by Barreteau *et al*. (2008). Figure kindly provided by Dr Sarah Paulin.

Figure 3: Appearance of pellets of EMRSA-16 cells after 5 min (A) and 4 h (B) growth in the presence and absence (control) of 12.5 mg/l ECg.