The effect of a component of tea (Camellia sinensis) on methicillin resistance, PBP2' synthesis, and β-lactamase production in Staphylococcus aureus

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Extracts of tea (Camellia sinensis) can reverse methicillin resistance in methicillin-resistant Staphylococcus aureus (MRSA) and also, to some extent, penicillin resistance in β-lactamase-producing S. aureus. These phenomena are explained by prevention of PBP2' synthesis and inhibition of secretion of β-lactamase, respectively. Synergy between β-lactams and tea extracts were demonstrated by disc diffusion, chequerboard titration and growth curves. Partition chromatography of an extract of green tea on Sephadex LH-20 yielded several fractions, one of which contained a virtually pure compound that showed the above-mentioned activities, at concentrations above about 2 mg/L. The observed activities are novel and distinct from the previously reported direct antibacterial activity of tea extracts. Prevention of PBP2' synthesis offers an interesting possible new approach for the treatment of infections caused by MRSA.

Introduction

The antimicrobial activity of tea (an aqueous extract of Camellia sinensis) was recognized 90 years ago. This action is manifested both directly (as bacteriostatic and bactericidal effects) and indirectly (as inhibition of certain bacterial enzymes).

We report here, and explain, two further antibacterial properties of tea that occur at concentrations at least one order of magnitude lower than the bacteriostatic effect. The first of these, which we call ‘activity II’ (we call the previously reported direct inhibitory effect ‘activity I’), was noticed as synergy with methicillin and is specific for methicillin-resistant Staphylococcus epidermidis. The second, ‘activity III’, appears as synergy with benzylpenicillin against β-lactamase-producing Staphylococcus aureus. We also report the purification of a compound that we believe is responsible for activities II and III.

Materials and methods

Media

Unless otherwise stated, all media were from Unipath, Basingstoke, U.K.

Antibiotics

Benzylpenicillin (Crystapen), sodium methicillin and sodium flucloxacillin were from SmithKline Beecham (Betchworth, U.K) and vancomycin hydrochloride was from Sigma (Poole, U.K).

Bacterial strains

Methicillin-resistant S. aureus (MRSA) were from our collection of distinct strains from different countries and methicillin-sensitive S. aureus (MSSA) were separate recent clinical isolates, as were two strains of bl+$^+$ methicillin-resistant Staphylococcus epidermidis (MRSE). Indicator strains used in bioassays (see below) were: S. aureus Oxford (bla$^-$), S. aureus U S12 (an MRSA $^+$) and S. aureus 16084 (MSSA, bl+$^+$).

Strains of other species resistant to penicillin as a result of altered penicillin-binding proteins (PBPs), pneumococci, bl+$^-$ gonococci and Haemophilus influenzae, were recent clinical isolates.

Tea

Activities I, II and III were found in various Indian and China black teas, Oolong tea and green tea. We used...
green tea (Japanese Sencha) for our experiments, as it is chemically simpler.

Boiling water (100 mL) was added to 2 g of dry Sencha leaves that had been pulverized in a mortar, and the mixture was filtered after standing for 10 min. The resulting clear, light green liquid was either used at once or was freeze-dried and the powder stored at 4°C in the dark. This 2% nominal extract is referred to below as extract T.

Assays of activities I, II and III

Each of the activities was measured separately by bioassay. A test for activity I had to be diluted out before assaying II and III; the method for assaying activity I has been described. Activity II was assayed by growing bacteria overnight in broth, and removing excess fluid after 2 min. The agar contained benzylpenicillin (0.007–512 mg/L) in a similar way, except that no NaCl supplement was added to the medium and incubation was at 37°C for 24 h.

Isolation of active principle

Compound P, a compound showing high levels of activities II and III, but little activity I, was isolated from extract T using the chromatographic fractionation procedure previously described, which was continued until 2.8 L of eluate had been obtained. Five separate peaks showing a high absorbance at 280 nm were found. Respective fractions making up these peaks were combined, assayed for activities I, II and III and evaporated to dryness.

Synergy experiments

Forty strains of M RSA, 22 with high-level methicillin resistance (MIC ≥ 256 mg/L) and 18 with low-level resistance (MIC = 32–128 mg/L), and 20 strains of MSSA were tested using the checkerboard method for synergic action between extract T and methicillin. Methicillin concentrations were 1–1024 mg/L, and the tea extract was used diluted 40, 70, 100, 200 and 300 times (MIC for activity I was the 20-fold dilution). Both were incorporated into IsoSensitest agar supplemented with 2% NaCl then plates were inoculated with 10⁶ cfu and incubated at 30°C for 40 h.

Results

Crude extracts

Initial observations. Synergy was observed between extract T and methicillin against M RSA, and between extract T and benzylpenicillin against MSSA, by the paired hole technique, at concentrations much lower than...
those required to show activity I (direct inhibition). Extract T also showed synergy in this system with benzylpenicillin against MRSA. Thus, the combined effect of activities II and III converted an MRSA strain into one that was fully penicillin-sensitive. No synergy was observed between benzylpenicillin and extract T for a bla\(^{−}\) S. aureus strain, or penicillin-resistant pneumococci, gonococci or H. influenzae.

Chequerboard experiments; extract T and methicillin against MRSA. Significant synergy was found for all 40 MRSA strains tested at 40- to 200-fold dilutions of extract T. Maximum synergy for the individual strains ranged from ΣFIC values of 0.32–0.12 (mode 0.23, median 0.22) and was achieved at 100- and 200-fold dilutions of extract T for all but six strains. With the 300-fold dilution of extract T, all but two of the low-level resistant strains (89%), but only five of the high-level resistant strains (23%) showed synergy. Many strains were converted to methicillin sensitivity in the presence of various concentrations of extract T (Table I). In contrast, no synergy was seen when 20 MSSA were tested against various combinations of methicillin and extract T.

Chequerboard experiments, benzylpenicillin and extract T against bla\(^{−}\) S. aureus. Synergy was found between benzylpenicillin and at least one of the concentrations of extract T tested for all strains, for which benzylpenicillin MICs ranged between 4 and \(\geq 512\) mg/L. Maximum synergy ranged from ΣFIC of 0.54 to 0.08 (mode 0.42, median 0.32) and occurred most commonly (for 65% of the strains) with 70- and 100-fold dilutions of extract T. There were no obvious differences in the behaviour of the MRSA and MSSA strains. A few strains became ‘penicillin sensitive’ according to the conventional criterion (MIC ≤ 0.12 mg/L; Table I) although this required a decrease in MIC of at least seven dilution steps and, in one case (a low-level MRSA), 14 steps: the MIC of benzylpenicillin fell from 512 to 0.06 mg/L in the presence of 40-fold diluted extract T. This experiment shows the combined effect of activities II and III against the MRSA and of activity III alone against the MSSA.

Effects of extract T and methicillin or flucloxacillin on growth of MRSA. The growth of three strains of MRSA (Den1, Fin8 and AusF) in the presence of various combinations of dilutions of extract T and methicillin or flucloxacillin was monitored. While 8 mg/L methicillin alone and 100-fold diluted extract T alone had little effect on growth patterns, the combination almost completely suppressed growth for at least 24 h, and was considerably more active than methicillin 200 mg/L. Further, flucloxacillin 4 mg/L in combination with the 200-fold dilution of extract T was much more active than flucloxacillin 64 mg/L (Figure). In a separate experiment, viable counts carried out on strain Fin8 showed a slow bactericidal action for 100-fold diluted extract T plus methicillin 8 mg/L.

Action of extract T on PBP2\(^{−}\) production and \(\beta\)-lactamase production. In the strain that produced PBP2 constitutively (S. aureus 13136, bla\(^{−}\)), 25 mg/L of tea extract (equivalent to a dilution of \(\times 250\)-fold of extract T) inhibited synthesis of PBP2 by \(\geq 90\)% and induction of PBP2 was prevented in the inducible strain. The synthesis of PBP1env and, to some extent, PBP3 was inhibited; PBP2 was unaffected.

<table>
<thead>
<tr>
<th>Type of strain</th>
<th>n</th>
<th>40-fold</th>
<th>70-fold</th>
<th>100-fold</th>
<th>200-fold</th>
<th>300-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methicillin-resistant(^{a})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high-level</td>
<td>22</td>
<td>91</td>
<td>68</td>
<td>36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>low-level</td>
<td>18</td>
<td>94</td>
<td>89</td>
<td>89</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td>Penicillin-resistant(^{b})</td>
<td>49</td>
<td>16</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{a}\) Indication of activity II (anti-PBP2\(^{−}\)).

\(^{b}\) Indication of activity III (anti-\(\beta\)-lactamase).

**Table I.** Activities II and III manifesting as synergy between extract T and methicillin or benzylpenicillin against S. aureus
was most concentrated in fraction B, while the two latter were found in fraction D (Table II). Further, activity I was present in similar amounts in three of the fractions (suggesting involvement of more than one molecular species), a pattern that contrasted with that of activities II and III (which could not be separated). Compound P was obtained virtually pure by evaporation of fraction D; it showed activity II against ten strains of M R S A at concentrations between 1 and 3 mg/L, and against two strains of M R S E.

In a chequerboard experiment, compound P showed synergy with flucloxacillin against all 18 strains of M R S A tested (median value of minimum \( \Sigma F I C \) was 0.19). Synergy was also clearly demonstrated by growth curves for four M R S A strains: the combination of flucloxacillin 4 mg/L + compound P 20 mg/L was considerably more inhibitory than flucloxacillin 64 mg/L (Figure).

**Discussion**

The antimicrobial principles (activity I) of extract T differ from those reported\(^9\) for the essential oil of the 'tea tree' (*Melaleuca alterniflora*). The latter has no botanical or chemical relationship to *C. sinensis*. Essential oils are virtually insoluble in water, unlike the principles responsible for activity I of extract T which are highly soluble. *C. sinensis* also has essential oils (volatile flavour compounds), of which nerolidol has the greatest activity I potency,\(^10\) about 100-fold greater than that of 'tea tree' oil.

Previous work\(^2,4\) suggests that activity I in tea is a property of several low molecular weight (<450) catechins, based on the isoflavanol structure, together with dimers known as theaflavins. Compounds of the latter type are absent from green tea. This is consistent with our finding here that activity I was spread over several fractions, suggesting the involvement of different molecular species. It has been suggested\(^11\) that catechins kill bacteria by disrupting their membranes.

Contrary to popular belief, tea extracts do not contain tannin,\(^12\) a toxic, high molecular weight compound.

Activity II is highly selective, affecting only staphylococci that make PBP2. Other variations of 'normal' PBPs that can confer penicillin resistance on species such as pneumococci, gonococci and *H. influenzae* were not susceptible to activity II. Activities I and II were clearly separable. Activity II is more potent than activity I, as the former could still be detected in 300-fold diluted extract T (about 20 mg/L) in the synergy, PBP and growth curve experiments, while activity I disappeared when extract T was diluted more than 20-fold (c.300 mg/L). Compound P,

### Table II. Assay of activities I, II and III in chromatographic fractions obtained from crude extract of green tea

<table>
<thead>
<tr>
<th>Titre(^a) of activity in indicated fractions</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity I</td>
<td>0</td>
<td>38.5</td>
<td>7.1</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>Activity II</td>
<td>0</td>
<td>0</td>
<td>98</td>
<td>571</td>
<td>33</td>
</tr>
<tr>
<td>Activity III</td>
<td>0</td>
<td>0</td>
<td>154</td>
<td>555</td>
<td>33</td>
</tr>
</tbody>
</table>

\(^a\)Titre = 1/critical concentration' in g/L.

**Figure.** Effects of flucloxacillin, extract T and the purified component, compound P showing activity II, alone and in combination, on growth of M R S A Fin8. ●, control; □, 200-fold diluted extract T; ▲, flucloxacillin, 4 mg/L; ▼, flucloxacillin, 64 mg/L; ○, compound P, 20 mg/L; ■, flucloxacillin, 4 mg/L + 200-fold diluted extract T; Δ, flucloxacillin, 4 mg/L + compound P, 20 mg/L.
the active principle of extract T, showed activity down to about 2 mg/L.

Our experiments suggest that activities II and III may reside in the same molecule. Activity III clearly differs from suicide inhibitors such as clavulanate, as extract T did not inhibit the action of preformed staphylococcal β-lactamase. Extract T did not inhibit induction; rather, its target was the excretion of β-lactamase from the cell. The amount of synergy produced between extract T and benzylpenicillin shows that this has a profound effect on staphylococcal susceptibility.

It is not surprising that interference with the production of PBP2* (e.g. activity II) should also have some effect on β-lactamase activity (e.g. activity III), as mecA shares the same induction system as β-lactamase. It has been suggested that mecA arose by fusion of the β-lactamase regulatory region with a structural gene specifying a PBP with low affinity for β-lactams (see review23).

The ability of activity II to prevent MRSA making PBP2* a potentially important weapon against resistance. Thus, compound P, the component of extract T with activity II, should act as a molecular chaperone of the type envisaged by Levy (see reference 14). Activity II alone is not harmful to staphylococci, whether or not they are methicillin-resistant, so it would not exert any selection pressure. However, in the presence of a β-lactam antibiotic it renders MRSA sensitive.

Other compounds that mimic activity II in extract T have been described in the literature. Two non-ionic detergents, Triton X-100 (t-octylphenoxypolyethoxyethanol)15 and Polidocanol (dodecylpolyethyleneoxide ether),16 seem to reduce methicillin resistance in MRSA by involving autolytic enzymes rather than PBPs. The complex polyoxytungstates created when sodium tungstate and disodium hydrogen phosphate solutions are mixed and heated17 depress the formation of PBP2*.

A tripeptide composed of carbobenzoxy-diphenylalanine-proline-phenylalanine alcohol (LY 301621) acts, in mixed and heated states and disodium hydrogen phosphate solutions are mixed and heated17 depress the formation of PBP2*. The tripeptide composed of carbobenzoxy-diphenylalanine-proline-phenylalanine alcohol (LY 301621) acts, in four of its eight possible configurations, synergistically with methicillin,18 but the mechanism of synergy has not been investigated. Mirocide Pharmaceuticals19 have reported a series of synthetic compounds loosely based on a decalin nucleus (saturated naphthalene) that appeared to show activities analogous to activity II and III reported here for extract T. No chemicals of similar structure to this occur in tea.20 Little information is available on compounds in this series, and their toxicity is unknown. Tea, on the other hand, is a self-renewable resource, and the beverage has been drunk (c.5 x 108 L/day at present21) for hundreds of years by billions of people without any harmful effects—rather the reverse in fact.22

Aknowledgements

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References


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