Tolerance of *Pseudomonas aeruginosa* to *Melaleuca alternifolia* (tea tree) oil is associated with the outer membrane and energy-dependent cellular processes

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**Objectives**: The essential oil of *Melaleuca alternifolia* (tea tree oil) and its components have antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, fungi and viruses. The mechanism(s) by which *Pseudomonas aeruginosa* NCTC 10662 maintains a decreased susceptibility to tea tree oil and components was investigated.

**Results**: Ethylene diamine tetraacetic acid enhanced the antimicrobial activity of tea tree oil and terpinen-4-ol against stationary phase *P. aeruginosa* while polymyxin B nonapeptide enhanced the activity of tea tree oil and γ-terpinene. Pre-treatment with the protonophore carbonyl cyanide m-chlorophenyl-hydrazone increased the susceptibility of exponential phase cells to sub-inhibitory concentrations of tea tree oil, terpinen-4-ol and γ-terpinene, indicating that intrinsic tolerance to tea tree oil and components is substantially energy dependent.

**Conclusions**: Increased tolerance to tea tree oil in *P. aeruginosa* is directly related to the barrier and energy functions of the outer membrane, and may involve efflux systems.

Keywords: terpenes, efflux, PMBN, EDTA, CCCP

**Introduction**

Tea tree oil, the essential oil obtained from the Australian native plant *Melaleuca alternifolia*, is composed of more than 100 terpene hydrocarbons and their associated alcohols.1 It has broad-spectrum antimicrobial activity *in vitro*, making it ideal for incorporation into antiseptic creams and lotions for topical administration in cutaneous infections. A wide range of products is already available over the counter in many countries. Clinical trials using topical tea tree oil products have shown that it may be efficacious for a range of conditions including acne,2 oral candidiasis,3,4 herpes labialis,5 dandruff6 and tinea.7 The mechanisms of antimicrobial action elucidated so far reflect the terpenic hydrocarbon composition and indicate that cytoplasmic membrane integrity is compromised by treatment with tea tree oil or some of its major components.3,5 Alterations in eukaryotic cell membranes have also been observed with tea tree oil and terpinen-4-ol treatment.10,11

Documented MICs of tea tree oil range from 0.06 to 0.5% (v/v) for *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus* spp., and 2–>8% (v/v) for *Pseudomonas aeruginosa*.12–15 The reduced susceptibility of *P. aeruginosa* to an antimicrobial is not unprecedented as this organism is frequently less susceptible to a wide range of structurally and functionally diverse antimicrobials. Several mechanisms may facilitate this reduced susceptibility, including reduced outer membrane permeability and active efflux systems.16,17 The protonophore carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) depolarizes the cytoplasmic membrane, and has been used to successfully demonstrate a role for efflux in both antibiotic and organic solvent resistance in *P. aeruginosa*.18,19 Mann et al.20 suggested that permeabilization of the outer membrane of *P. aeruginosa* with
polymyxin B nonapeptide (PMBN) rendered it susceptible to γ-terpinene, a component that usually exhibits little antibacterial activity. While the exact mechanism of action of PMBN has not been elucidated, it is thought to bind to lipopolysaccharide (LPS) without causing its release. The outer membrane permeabilizer EDTA can disrupt the integrity of the outer membrane by chelating divalent cations, resulting in the release of LPS. The aim of this work was to further characterize the mechanisms responsible for the reduced susceptibility of *P. aeruginosa* to tea tree oil. These mechanisms may have a bearing on whether spontaneous resistance is likely to occur.

**Materials and methods**

**Antimicrobial agents and chemicals**

Tea tree oil was produced by steam distillation and provided by Australian Plantations Pty Ltd, Wyrrabalong, New South Wales, Australia. The levels of components quantified according to the international standard by gas chromatography–mass spectrometry were as published previously and included 38% terpinen-4-ol, 19.4% γ-terpinene and 3.0% cineole. Terpinen-4-ol (Sigma Chemical Co., St Louis, MO, USA) and γ-terpinene (Aldrich Chemical Co. Inc, Milwauke, WI, USA) were at least 97% pure. PMBN and CCCP were purchased from Sigma.

**Bacteria**

*P. aeruginosa* NCTC 6749 and *P. aeruginosa* NCTC 10662 were obtained from the culture collection of Microbiology, University of Western Australia, Crawley, Western Australia, Australia.

**Time–kill studies with EDTA and PMBN**

In preliminary experiments, the MICs of tea tree oil, terpinen-4-ol and γ-terpinene for *P. aeruginosa* NCTC 10662 were 4%, 2% and >8% (v/v), respectively. Time–kill studies were used to determine the bactericidal activity of tea tree oil, terpinen-4-ol and γ-terpinene against stationary phase *P. aeruginosa* NCTC 10662. Tests were carried out in the presence and absence of 5 mM EDTA. Two colonies of *P. aeruginosa* NCTC 6749 or NCTC 10662 from overnight cultures on blood agar [5% horse blood in a Columbia agar base (Oxoid)] were inoculated into 400 mL of Mueller–Hinton broth (MHB). Broths were incubated at 35°C for 18 h with shaking after which stationary phase bacteria were harvested by centrifugation at 10,000 g for 10 min at 4°C. The pellet was washed twice with phosphate-buffered saline (PBS; pH 7.4, NaCl 8 g/L) and suspended finally in PBS with 0.002% Tween 80 (v/v) (PBS-T). Suspensions were adjusted so that the optical density at 600 nm (OD600) of a 1 in 100 dilution was 0.1 ± 0.01. This corresponded to ~1 × 10^8 cfu/mL in the neat suspension. Viable counts were performed by serially diluting samples in PBS-T and spread-plating 0.1 mL samples on pre-dried nutrient agar in duplicate. After incubation at 37°C for 24 h, the number of colonies was counted.

For time–kill studies, volumes of 8.1 mL of bacterial suspension were placed in 50 mL conical flasks. A 0.1 mL sample was removed from each flask and the concentration of organisms determined by viable count. During all time–kill experiments, flasks were incubated at 35°C with shaking. For tests with and without EDTA, 1 mL of 50 mM EDTA in PBS-T or 1 mL of PBS-T, respectively, was added to the flask 10 min prior to the addition of tea tree oil, terpinen-4-ol or γ-terpinene. Treatment was initiated by adding 1 mL of stock concentrations of tea tree oil or components prepared earlier in PBS-T at 10-fold the final desired concentration. Tea tree oil, terpinen-4-ol and γ-terpinene were tested at final concentrations of 0.125–4%, 0.06–4% and 1–2% (v/v), respectively. PBS-T was added to control flasks. The flasks were mixed for 20 s, a sample removed at 30 s and the viable count determined as above. Further samples were taken from test flasks at 30, 60, 120 and 240 min and, where rapid killing occurred, samples were also taken at 2.5, 5, 10 and 15 min. Control flasks were sampled at 120 and 240 min. There are no established inactivating agents for tea tree oil or components, so dilution was used to arrest treatment and reduce carryover as described previously. The minimum dilution used was 1 in 10 and the minimum detection threshold was 3 × 10^3 cfu/mL. These experiments were repeated with PMBN at a concentration of 10 μg/mL replacing EDTA.

Using the method described above, neither EDTA nor PMBN pre-treatment elicited bactericidal activity from γ-terpinene. The results for PMBN contrasted with previous work by Mann et al. Consequently, these experiments were repeated using their method with slight changes. Briefly, *P. aeruginosa* NCTC 6749 or *P. aeruginosa* NCTC 10662 was grown in Iso-Sensitest broth (Oxoid) for 18 h (yielding ~1 × 10^8 cfu/mL) and then diluted in Iso-Sensitest broth to yield a final concentration of 1 × 10^6 cfu/mL. PMBN at a final concentration of 10 μg/mL was added 10 min prior to the addition of γ-terpinene at a final concentration of 0.1% (v/v). Iso-Sensitest broth was added to control flasks with and without PMBN. Flasks were incubated at 35°C with shaking during the experiment. Samples were taken immediately prior to treatment addition and at 0.5, 30, 60, 120 and 240 min. Samples were serially diluted and plated in duplicate to determine viable counts as described above. These experiments were repeated incorporating EDTA at 5 mM.

**Time–kill studies with CCCP**

To determine whether energy-dependent processes were involved in tolerance to tea tree oil, the effect of CCCP on susceptibility to tea tree oil, terpinen-4-ol and γ-terpinene was examined. In time–kill experiments conducted as described above with stationary phase organisms, the addition of CCCP did not alter the bactericidal activity of tea tree oil. Since growth phase may affect susceptibility, time–kill studies using exponential phase *P. aeruginosa* NCTC 10662 were carried out. The method used was similar to that described for experiments with EDTA and PMBN, except that exponential phase organisms were prepared. A 5 L flask was inoculated with a 6% inoculum from an overnight culture, in a final volume of 800 mL of MHB. Cells were incubated at 35°C and shaken at 150 rpm on an orbital shaker for 2 h, pre-determined as early exponential phase. After harvesting and washing once in PBS, cells were resuspended in PBS-T and adjusted so that the final OD600 of a 1 in 100 dilution was 0.1 ± 0.01. This corresponded to ~1 × 10^8 cfu/mL in the neat suspension. These suspensions were pre-treated with 250 μM CCCP for 10 min before tea tree oil, γ-terpinene or terpinen-4-ol was added. Norfloxacin was included as a positive efflux inhibition control at 3 × MIC (3 mg/L).

**Transmission electron microscopy**

Stationary phase cultures of *P. aeruginosa* NCTC 10662 were prepared by inoculating 10 mL volumes of MHB and incubating overnight. Organisms were harvested by centrifugation for 15 min at 4000 g and the pellets resuspended in PBS-T. Suspensions of *P. aeruginosa* were treated with 4% (v/v) tea tree oil or 2% (v/v) terpinen-4-ol in PBS-T for 10 or 60 min. Untreated controls in PBS-T were left on the bench for 60 min. All treatments were performed at room temperature.
After treatment, cell suspensions were centrifuged for 15 min at 4000 g, and pellets were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Fixed microbial pellets were processed in graded alcohols, propylene oxide and araldite, and cured for 48 h at 60°C. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a Philips 410 transmission electron microscope at an accelerating voltage of 80 kV.

Results

Time–kill studies with EDTA and PMBN

The MIC of tea tree oil (4%, data not shown), in the absence of EDTA, reduced the number of *P. aeruginosa* NCTC 10662 by $3.5 \log_{10}$ in 30 min and almost $4 \log_{10}$ by 60 min (Figure 1a). Concentrations of 1% and 2% reduced the number of organisms by $2.5 \log_{10}$ over 60 min (data not shown) and the effect of 0.5% was similar. At 0.25%, there was negligible bactericidal activity. The bactericidal activity of all concentrations of tea tree oil was greatly augmented by EDTA (Figure 1a). In the presence of 0.25% tea tree oil and EDTA, viability fell below the level of detection after 30 min. For 0.5% tea tree oil and EDTA, this occurred after 20 min and, for the remaining tea tree oil concentrations, in less than 10 min. Similarly, pre-treatment with $10 \mu g/mL$ PMBN amplified the bactericidal activity of tea tree oil, but was only effective in combination with 2% or 4% tea tree oil (Figure 1b) and caused no additional cell death at lower concentrations (data not shown).

In the absence of EDTA, terpinen-4-ol (MIC 2%, data not shown) reduced the viability of organisms very rapidly over the concentrations 0.25–4% (Figure 1c). A concentration of 0.25% reduced viability by $/C246 \log_{10}$ in 15 min although there appeared to be a $1 \log_{10}$ recovery by the end of the 4 h test period. The two lowest concentrations tested, 0.06% and 0.125%, had negligible effects on organism viability and the addition of EDTA did not alter the activity of 0.06% terpinen-4-ol (data for 0.06% terpinen-4-ol not shown). However, in the presence of EDTA, 0.125% terpinen-4-ol dramatically reduced organism viability. PMBN pre-treatment did not enhance the effect of terpinen-4-ol at 0.06% or 0.125% (data not shown).

$\gamma$-Terpinene at 1% or 2% did not show any significant bactericidal activity against stationary phase cells of *P. aeruginosa* NCTC 10662 in PBS-T in the presence or absence of EDTA or PMBN (data not shown). In contrast, using the method of Mann *et al.*, a $0.1%$ γ-terpinene in the presence of PMBN showed significant bactericidal activity against stationary phase cells of both *P. aeruginosa* NCTC 6749 and NCTC 10662 in Iso-Sensitest broth (Figure 2). After 30 min treatment with $0.1%$ γ-terpinene, PMBN-pre-treated cells of both strains showed a $2 \log_{10}$ decrease in viability. Beyond this time-point viability fell below the limit of detection. PMBN pre-treatment alone resulted in a $1.5 \log_{10}$ decrease for NCTC 10662 and a $0.5 \log_{10}$ decrease in viability for NCTC 6749 over the 4 h treatment period. In Iso-Sensitest broth alone or Iso-Sensitest broth with $0.1%$ γ-terpinene, the number of viable organisms increased by $\sim 1 \log_{10}$ over 4 h for both strains. When EDTA was substituted for PMBN, no sensitization to 0.1% γ-terpinene was seen. The presence of EDTA tempered the growth seen in Iso-Sensitest broth alone, and the addition of 0.1% γ-terpinene to EDTA-pre-treated cells failed to induce any significant bactericidal effect (Figure 2).
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Exponential phase \( P. \) aeruginosa NCTC 10662 cells were more susceptible to the antimicrobial action of tea tree oil than stationary phase cells. Cells treated with 250 \( \mu \)M CCCP alone did not show a decline in viability over the 4 h treatment period (data not shown). Bactericidal activity against cells pre-treated with CCCP and subsequently exposed to 2 or 3 \( \times \) MIC of norfloxacin (an antibiotic known to be effluxed from \( P. \) aeruginosa) was greater than that in the norfloxacin-only control after 2 h (data not shown). This indicated that depolarizing the cell membranes with CCCP sensitized the organisms to norfloxacin, possibly by inhibiting the function of efflux pumps. The addition of 0.25\% tea tree oil to CCCP-pre-treated cells resulted in a 3 log\(_{10}\) kill after 4 h, compared with a 0.7 log\(_{10}\) kill with 0.25\% tea tree oil alone (Figure 3a). Similarly, treatment with 0.125\% tea tree oil resulted in a 3 log\(_{10}\) kill over 4 h in CCCP-pre-treated cells, compared with no cell death in cells treated with 0.125\% tea tree oil only, a statistically significant difference (two-tailed Student’s t-test, \( P = 0.003\)). Additional killing was not observed for treatment with tea tree oil concentrations of >0.5\% for CCCP-pre-treated cells. Similar experiments using 2\% \( \gamma \)-terpinen-4-ol showed a 2 log\(_{10}\) kill after 2 h for CCCP-pre-treated cells but no change in the viability of the cells treated with \( \gamma \)-terpinen-4-ol only (\( P = 0.0009\)) (Figure 3b). The same trend was observed with 0.1\% \( \gamma \)-terpinene. CCCP pre-treatment was also able to augment the antimicrobial effect of terpinen-4-ol (Figure 3c). A statistically significant (\( P = 0.0005\)) 1 log\(_{10}\) kill over 4 h was observed in cells treated with CCCP and 0.0625\% terpinen-4-ol, compared with no loss of viability in cells treated with terpinen-4-ol only. Likewise, treatment with 0.125\% terpinen-4-ol caused an additional 0.7 log\(_{10}\) kill in CCCP-pre-treated cells compared with control cells (\( P = 0.04\)).

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\( P. \) aeruginosa cells treated for 60 min with terpinen-4-ol contained many electron-sparse inclusions (Figure 4a) while those treated for 10 min (not shown) and control cells had none (Figure 4b). Perturbations of the outer membrane, but no inclusions, were observed in tea-tree-oil-treated cells (Figure 4c).

Discussion

\textit{Pseudomonas} spp. have decreased susceptibility to tea tree oil compared with other bacterial species, and the mechanisms involved may have a bearing on the spontaneous development of resistance. Many of the components of tea tree oil have antimicrobial activity and it is possible that tea tree oil has multiple mechanisms of action. If so, spontaneous resistance may be less likely to occur. In this study, the antimicrobial activity of tea tree oil and/or components against \( P. \) aeruginosa was investigated in the presence of two outer membrane permeabilizers, EDTA and PMBN. The pre-treatment of stationary phase cells in PBS with EDTA rendered cells more vulnerable to the bactericidal properties of tea tree oil and terpinen-4-ol but not \( \gamma \)-terpinene. Similarly, PMBN pre-treatment rendered cells more vulnerable to the bactericidal effect of tea tree oil but not to terpinen-4-ol or \( \gamma \)-terpinene. The enhanced activity in the presence of EDTA or PMBN suggests that one or more target sites for tea tree oil and/or components against \( P. \) aeruginosa was investigated in the presence of two outer membrane permeabilizers, EDTA and PMBN. The pre-treatment of stationary phase cells in PBS with EDTA rendered cells more vulnerable to the bactericidal properties of tea tree oil and terpinen-4-ol but not \( \gamma \)-terpinene. Similarly, PMBN pre-treatment rendered cells more vulnerable to the bactericidal effect of tea tree oil but not to terpinen-4-ol or \( \gamma \)-terpinene. The enhanced activity in the presence of EDTA or PMBN suggests that one or more target sites for tea tree oil and/or terpinen-4-ol lie within the cell. The initial failure of either EDTA or PMBN to enhance the activity of \( \gamma \)-terpinene was in contrast to the findings of Mann et al. Interestingly, the bactericidal activity of \( \gamma \)-terpinene and PMBN was seen in Iso-Sensitest broth but not in buffer. The contrast in activity is noteworthy since there was little difference between the two methods apart from the medium used, suggesting that the permeabilization effect of PMBN or the bactericidal activity of \( \gamma \)-terpinene may require growth and an active metabolic state. Also noteworthy was the failure of EDTA to elicit bactericidal activity from \( \gamma \)-terpinene using either method.
Pre-treatment of *P. aeruginosa* with the electron-transport inhibitor CCCP resulted in increased susceptibility to otherwise sub-inhibitory concentrations of tea tree oil (0.125% and 0.25%), an effect that has also been shown in eukaryotic cells. Clearly, an energy-dependent process is involved in tolerance to tea tree oil. However, pre-treatment with CCCP followed by

**Figure 4.** Electron micrographs of *P. aeruginosa NCTC 10662* cells treated with 2% terpinen-4-ol for 60 min (a), 4% tea tree oil for 10 min (c) or after no treatment (60 min) (b). Magnifications: (a) 14400×; (b) 11700×; and (c) 10800×.

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**Figure 3.** Time–kill curves of exponential phase *P. aeruginosa* NCTC 10662 treated with (a) tea tree oil: filled triangles, 0.125%; crosses, 0.25%; filled squares, 0.5%; and (b) γ-terpinene: filled triangles, 0.1%; filled diamonds, 2%; or (c) terpinen-4-ol: filled triangles, 0.0625%; crosses, 0.125% with (open symbol, dotted line) or without (filled symbol, solid line) 250 μM CCCP pre-treatment. CCCP pre-treatment alone had no bactericidal activity (data not shown). The organisms were suspended in MHB-T. Each symbol indicates the mean±S.D. for at least three replicates. The lower detection threshold was 10³ cfu/mL.
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treatment with 0.5% or 1% tea tree oil had no additional bactericidal effect, suggesting that once a certain threshold is reached, the mechanisms that facilitate tolerance at low levels are overwhelmed.

The component γ-terpinene is frequently regarded as antimicrobially inactive.\(^{20,21}\) This work shows that PMBN or CCCP pre-treatment unmasks the antimicrobial activity of γ-terpinene, presumably by compromising various membrane barrier functions. Multi-drug efflux systems of *P. aeruginosa*, in particular the MexAB–PorM system, have been associated with intrinsic tolerance to organic solvents.\(^{18}\) Our results with CCCP suggest that energy-dependent processes, possibly efflux systems, are involved in the resistance of *P. aeruginosa* cells to γ-terpinene. Experiments with reserpine, a known inhibitor of efflux in Gram-positive\(^{26,27}\) and some Gram-negative bacteria, were unsuccessful since reserpine did not inhibit the efflux of norfloxacin by *P. aeruginosa* NCTC 10662 in positive controls (data not shown).

Further support for the role of efflux systems comes from the multiple, prominent electron-sparse inclusions seen in terpinen-4-ol-treated cells by electron microscopy. It is possible that they are globules of terpinen-4-ol that have accumulated in the cell due to efflux malfunction. However, their identity was not characterized further and this inference remains speculative. Extracellular electron-dense blebs and loss of cytoplasmic material have been observed in tea-tree-oil-treated *E. coli*\(^{26}\)\(^{28}\) and *S. aureus*,\(^{8}\) respectively. No blebbing or loss of electron-dense material was observed in either tea-tree-oil- or terpinen-4-ol-treated *P. aeruginosa* cells but perturbations of the outer membrane were observed in tea-tree-oil-treated cells.

*P. aeruginosa* is an important opportunistic pathogen well known for its resistance to conventional antimicrobials. Given the impact of this resistance, alternative antibacterial agents have long been sought, including those for topical treatment. This work has reiterated the role of the outer membrane in protecting *P. aeruginosa* against tea tree oil and components, and has shown that energy-dependent processes are involved in this increased tolerance. The role that efflux may play in this increased tolerance is currently being investigated.

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