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Antibacterial activity and mode of action of *tert*-butylhydroquinone (TBHQ) and its oxidation product, *tert*-butylbenzoquinone (TBBQ)

N. Ooi[1], I. Chopra[1], A. Eady[2], J. Cove[1], R. Bojar[3] and A. J. O'Neill[1]*

[1]*Antimicrobial Research Centre and School of Molecular and Cellular Biology, University of Leeds, Leeds, UK;* [2]*Syntopix Group plc, University of Bradford, Bradford, UK;* [3]*Evocutis plc, Sandbeck Lane, Wetherby, UK*

*Corresponding author. Tel: +44-113-3435600; Fax: +44-113-3435638; E-mail: a.j.oneill@leeds.ac.uk

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Objectives: The antioxidant *tert*-butylhydroquinone (TBHQ) is a food additive reported to have antibacterial activity, and may therefore have application in the healthcare setting. This study sought to characterize the antibacterial activity and mode of action of TBHQ and its oxidation product, *tert*-butylbenzoquinone (TBBQ).

Methods: The stability of TBHQ/TBBQ was studied in buffer. Susceptibility testing was performed by broth microdilution, and killing and lytic activity were evaluated by viable counting and culture turbidity measurements. Mode of action studies included following the incorporation of radiolabelled precursors into macromolecules. The effect of TBHQ/TBBQ upon bacterial and mammalian membranes was assessed using the *Bac*Light™ assay and by monitoring the haemolysis of equine erythrocytes.

Results: TBHQ underwent oxidation in solution to form TBBQ. When oxidation was prevented, TBHQ lacked useful antibacterial activity, indicating that TBBQ is responsible for the antibacterial activity attributed to TBHQ. TBBQ demonstrated activity against *Staphylococcus aureus* SH1000 (MIC 8 mg/L) and against a panel of clinical *S. aureus* isolates (MIC_{90} 16 mg/L). TBBQ at 4× MIC caused a >4 $\log_{10}$ drop in cell viability within 6 h without lysis, and eradicated staphylococcal biofilms at 8× MIC. TBBQ did not display preferential inhibition of any single macromolecular synthetic pathway, but caused loss of staphylococcal membrane integrity without haemolytic activity.

Conclusions: TBBQ is responsible for the antibacterial activity previously ascribed to TBHQ. TBBQ prompts loss of staphylococcal membrane integrity; it is rapidly and extensively bactericidal, but is non-lytic. In view of the potent and selective bactericidal activity of TBBQ, this compound warrants further investigation as a candidate antistaphylococcal agent.

Keywords: *Staphylococcus aureus*, antioxidants, membrane integrity, haemolysis, biofilms

Introduction

The lipophilic antioxidant *tert*-butylhydroquinone (TBHQ) (Figure 1a) was approved for use in the USA as a food preservative in 1972.[1–3] It has predominantly been employed for the stabilization and preservation of fats and foods with high fat content owing to its radical-scavenging activity.[1,4] However, TBHQ has also been reported to have antibacterial properties, exhibiting a spectrum of activity that encompasses bacterial species responsible for human disease, including *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus mutans* and *Staphylococcus aureus*.[1,3,5,6] In view of its history of safe use in foods and its antibacterial activity, TBHQ has been under consideration for use in healthcare products by Evocutis plc.[7] This study therefore sought to characterize the antibacterial activity of TBHQ, with a particular emphasis on its antistaphylococcal activity, and to determine its mode of action (MOA).

An important consideration when seeking to characterize the biological properties of antioxidants is their stability in solution, since they can undergo oxidation to form other chemical species. Because the biological properties of an antioxidant and its breakdown products may differ, care must be taken to relate observed properties to the chemical species present. As TBHQ has most often been used as an additive for vegetable oils,[4] previous studies have examined its stability at temperatures reached in the frying process. At such temperatures (175–185°C) TBHQ is oxidized primarily to *tert*-butylbenzoquinone (TBBQ; Figure 1a and b), although smaller quantities of a number of other compounds have been detected, including dimerized TBHQ[8,9] and free radical species that decompose to alternate

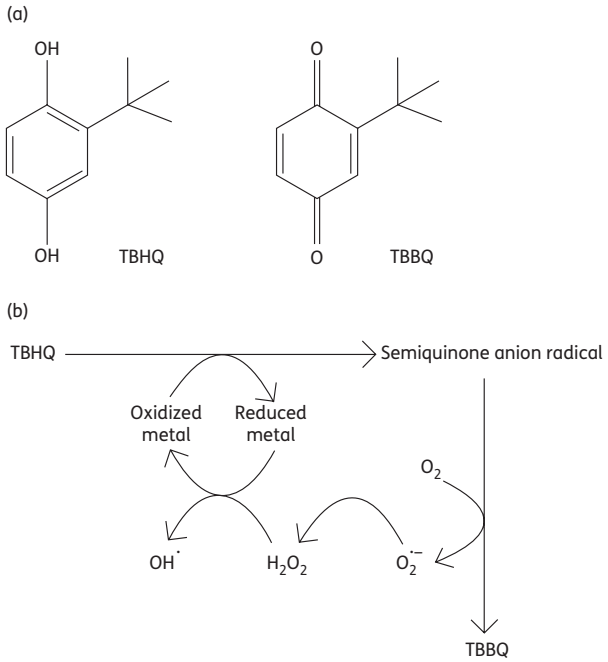

Figure 1. Chemical structures of TBHQ and TBBQ, and oxidative conversion of the former into the latter. (a) Chemical structures of TBHQ and TBBQ. (b) A schematic for the transition metal-assisted oxidation of TBHQ to TBBQ with the associated production of superoxide, hydrogen peroxide and hydroxyl radicals.[10]

oxidation products (Figure 1b).[8] However, the stability of TBHQ has not been examined at temperatures relevant to the study of its antibacterial activity. Consequently, we initiated this work with an investigation into the stability of TBHQ in solution at 37°C. We then progressed to a more comprehensive study to examine the microbiological activity and MOA of TBHQ/TBBQ, with a view to determining whether TBHQ might have potential as an agent for use in human healthcare.

Materials and methods

Bacterial strains, growth media and chemicals

Antibiotics and chemicals were from Sigma-Aldrich (Poole, UK), with the exception of daptomycin (Cubist Pharmaceuticals, Lexington, MA, USA), triclosan (LG Life sciences, Seoul, South Korea), ciprofloxacin (Bayer, Leverkusen, Germany), vancomycin (Duchefa Biochemie, Haarlem, The Netherlands), SEP 155342 (Sunovion Pharmaceuticals Inc., Marlborough, MA, USA), D-luciferin (Melford, Ipswich, Suffolk, UK), ethanol (Fisher Scientific Ltd, Loughborough, UK) and the radiolabelled chemicals [*methyl*-^{3}H]thymidine (70–95 Ci/mmol), [5,6-^{3}H]uridine (31–56 Ci/mmol) and L-[G-^{3}H]glutamine (20–50 Ci/mmol) (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The Live/Dead *Bac*Light™ kit was from Invitrogen (Paisley, UK).

Laboratory strains of bacteria used in this study are listed in Table 1. Clinical *S. aureus* isolates used for susceptibility testing were part of a culture collection belonging to the Antimicrobial Research Centre, University of Leeds, UK. Staphylococci were propagated in Mueller Hinton broth (MHB) and on Mueller Hinton agar (MHA) (Oxoid Ltd, Cambridge, UK); for studies with daptomycin and valinomycin, culture media were supplemented with calcium (50 mg/L, in the form of $CaCl_2$) and potassium chloride (0.1 M), respectively. Strains of *E. coli* and *Bacillus subtilis* were grown in Luria-Bertani broth and on Luria-Bertani agar (Oxoid).

Table 1. Bacterial strains used in this study

Strain	Comments	Reference(s)
S. aureus		
SH1000	standard laboratory strain	29,30
ATCC 23723	antibiotic susceptibility testing strain	31
ATCC 29213	antibiotic susceptibility testing strain	31
E. coli		
1411	K-12 strain	23
SM1411	1411Δ*acrAB*	23
AB734	K-12 strain	24
ES100	AB734Δ*tolC*	24
B. subtilis		
1S34 (pS77)	DNA synthesis biosensor	19
1S34 (pS63)	RNA synthesis biosensor	19
1S34 (pS72)	protein synthesis biosensor	19
1S34 (pS107)	cell wall synthesis biosensor	19
1S34 (pNS14)	fatty acid synthesis biosensor	32

Stability studies with TBHQ and TBBQ

Extinction coefficients for TBHQ (2945.1 M^{-1} cm^{-1}) and TBBQ (16274.3 M^{-1} cm^{-1}) were determined at their absorbance maxima (290 and 252 nm, respectively)[10] using known concentrations of both compounds in PBS. Stability studies were performed at 37°C with shaking, using starting concentrations of TBHQ and TBBQ for which absorbance readings could be taken without requiring dilution of the solution (absorbance values of <0.8); for TBHQ, the concentration used was 385 μM (64 mg/L), and for TBBQ, 97 μM (16 mg/L). The Beer–Lambert law was used to calculate the concentrations of TBHQ and TBBQ present in solution over time.

Antibacterial activity testing

MICs were determined by exposing bacteria to serial dilutions of antibacterial agents in MHB according to CLSI broth microdilution guidelines.[11] MBCs were determined by enumerating bacteria surviving challenge with concentrations of antibacterial agents above the MIC; bacterial colonies were counted after incubation at 37°C for 18–24 h on MHA, and the MBC was defined as the minimum concentration of antibacterial agent that caused a 99.9% kill.[12] Biofilm MICs (bMICs) and minimum biofilm eradication concentrations (MBECs) were determined using *S. aureus* SH1000 biofilms grown using the Calgary Biofilm Device (Nunc, Roskilde, Denmark), as previously described.[13,14]

Time–kill assays were performed using a method adapted from Oliva *et al.*[15] Briefly, bacteria were cultured to early exponential phase (OD_{600} of 0.2) in MHB and challenged with antibacterial agents at 4× MIC. In some instances, bacteria were cultured to early exponential phase, washed and resuspended in PBS pre-warmed to 37°C, before exposure to antibacterial agents. Bacterial viability was monitored by plating cultures onto MHA, and enumerating colonies after incubation at 37°C for 18–24 h. To detect bacterial lysis following challenge with antibacterial agents, the culture turbidity of early exponential phase cultures was monitored by absorbance measurements at 600 nm at 37°C.[15]

Antibacterial MOA studies

The effect of TBBQ on biosynthesis of DNA, RNA and protein was monitored in early exponential phase cultures of *S. aureus* SH1000 (OD_{600} of 0.2) by following the incorporation of the radiolabelled precursors [*methyl*-^{3}H]thymidine, [5,6-^{3}H]uridine and L-[G-^{3}H]glutamine into macromolecules.[16,17] The action of TBBQ was also investigated using a suite of pathway-specific biosensor strains of *B. subtilis* that have previously been described for use in MOA studies.[18,19]

The effect of antibacterial compounds on the integrity of the staphylococcal membrane after 10 min exposure was assessed using the *Bac*Light™ assay.[17,20] The ability of compounds to damage mammalian membranes was examined by measuring the haemolysis of equine erythrocytes.[15] Briefly, lithium heparin-treated whole equine blood (Matrix Biologicals Ltd, Hull, UK) was centrifuged at 1000 **g** for 10 min at 4°C, and the supernatant and buffy coat discarded. The erythrocyte pellet was washed and resuspended to 5% v/v in 10 mM Tris-HCl buffer containing 0.9% NaCl (pH 7.4) at 4°C. Erythrocytes were further diluted 25-fold in buffer, and pre-incubated at 37°C for 15 min prior to use. Erythrocytes were exposed to a concentration of antibacterial agent equivalent to 4× MIC for *S. aureus* SH1000 for 1 h at 37°C. Mixtures were centrifuged at 3000 **g** for 5 min; the extent of haemoglobin leakage was measured at OD_{540} and expressed as a percentage of that recorded for the positive control (5% SDS).

Results

Stability studies with TBHQ and TBBQ

TBHQ maintained in PBS at 37°C was found to be unstable, degrading in a pH- and oxygen-dependent manner (Figure 2).

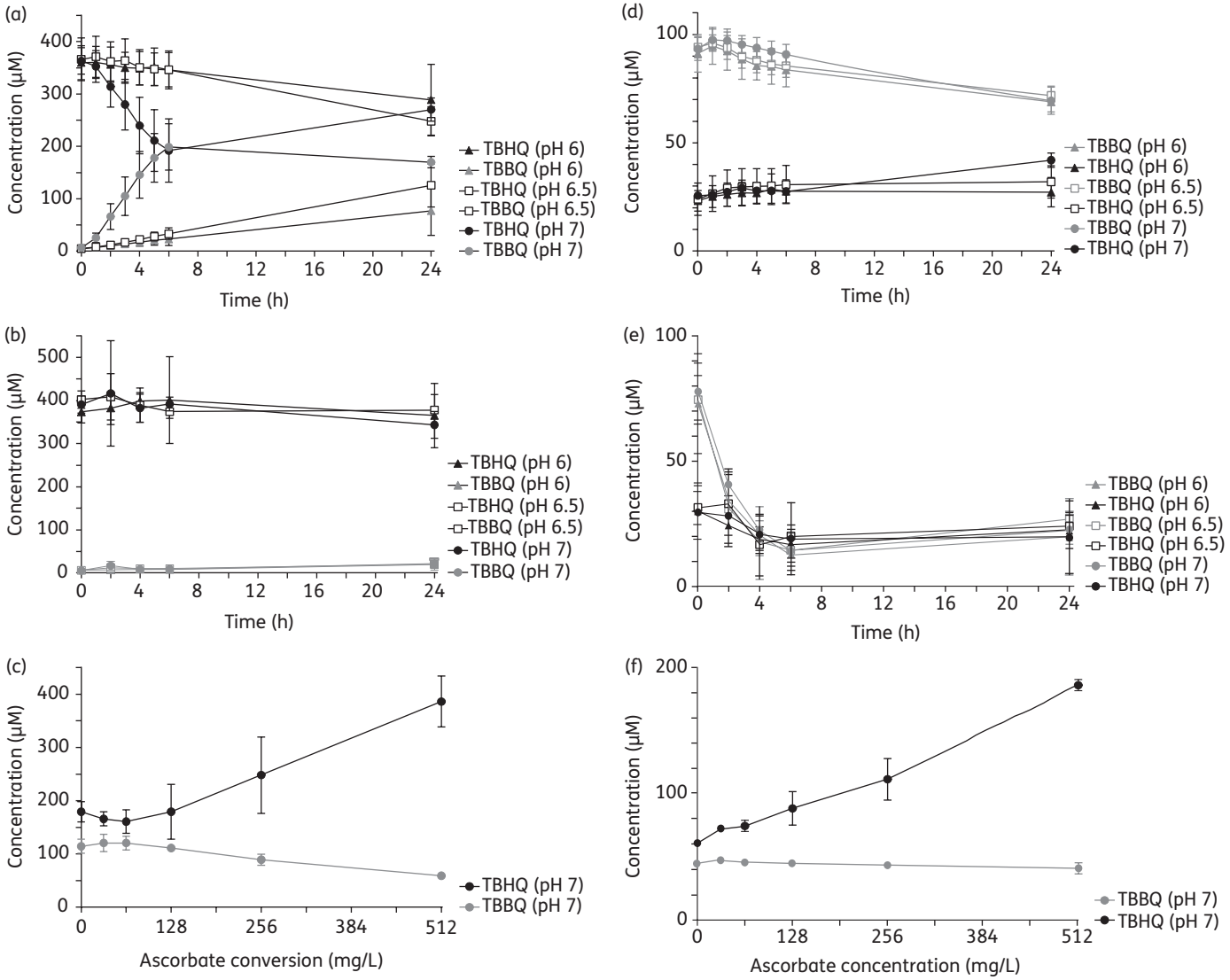

Figure 2. Stability of TBHQ and TBBQ in solution. Stability of TBHQ at 385 μM (64 mg/L) in PBS (a–c) and stability of TBBQ at 97 μM (16 mg/L) in PBS (d–f). (a) and (d) Aerobic stability. (b) and (e) Anaerobic stability. (c) and (f) Stability following a 24 h incubation in the presence of ascorbate (pH 7). Concentration of the compound in PBS is plotted against the *y*-axis (means of at least three independent replicates; error bars show standard deviations derived from replicates).

At neutral pH, this degradation proceeded rapidly, with only ~50% remaining after 6 h (Figure 2a). Concentrations of TBHQ decreased and TBBQ increased concurrently, indicating that TBHQ was primarily being oxidized to form TBBQ. The rate of degradation of TBHQ was slower at more acidic pH (Figure 2a), and degradation did not occur under anaerobic conditions (Figure 2b). Addition of the antioxidant ascorbate, which has previously been shown to inhibit the decomposition of TBHQ at 100 mg/L at room temperature,[21] was able to inhibit the degradation of TBHQ at pH 7 in a concentration-dependent manner (Figure 2c).

Since TBHQ undergoes rapid oxidation to TBBQ in aqueous solutions, experiments were performed to assess whether this oxidation product is stable in solution. Under aerobic conditions at 37°C, TBBQ was found to degrade only slowly and in a pH-independent manner, with ~90% of TBBQ remaining after 6 h (Figure 2d). However, and in contrast to TBHQ, TBBQ was unstable under anaerobic conditions, and its degradation could not be prevented by concentrations of ≤512 mg/L of ascorbate (Figure 2e and f). The reduction in TBBQ concentration was not accompanied by an increase in TBHQ, indicating that TBBQ must have been undergoing conversion into an alternative product. Since the addition of ascorbate to this product prompted regeneration of TBHQ (Figure 2f), the TBBQ breakdown product is likely to be the intermediary semiquinone anion radical (Figure 1b).

Antibacterial activity of TBHQ and TBBQ

TBHQ and TBBQ MICs and MBCs were determined for the *S. aureus* laboratory strain SH1000, and two commonly used *S. aureus* susceptibility testing strains (ATCC 23723 and 29213). TBHQ exhibited an MIC and MBC of 8 and 8–16 mg/L, respectively, for all three strains. TBBQ had comparable activity, exhibiting an MIC and MBC of 4–8 mg/L and 8–16 mg/L, respectively. The MIC_{90} of TBHQ and TBBQ for 71 clinical *S. aureus* isolates (which included methicillin-resistant and vancomycin-intermediate strains) was 16 mg/L, with MICs of TBHQ and TBBQ ranging from 8 to 32 mg/L and from 4 to 16 mg/L, respectively. TBHQ and TBBQ displayed activity against *S. aureus* biofilms, both inhibiting the shedding of cells from the biofilm (bMICs of 64 mg/L) and achieving complete eradication of biofilms (MBECs of 128 and 64 mg/L, respectively).

Our studies on the stability of TBHQ implied that this agent would undergo extensive conversion during the course of a 24 h MIC determination (Figure 2a), with potential impact on its antibacterial activity. Therefore, we investigated the possibility of stabilizing TBHQ in solution. Since both the absence of oxygen and the addition of ascorbate inhibit degradation of TBHQ,[22] MICs were determined anaerobically and following the addition of ascorbate to the culture media. Under anaerobic conditions, there was an 8-fold increase in TBHQ MIC for *S. aureus* SH1000, from 8 to 64 mg/L. TBHQ MICs also increased dramatically in the presence of ascorbate, reaching 512 mg/L (a 64-fold increase) upon addition of ascorbate at 1024 mg/L. Therefore, TBHQ lost useful antibacterial activity when spontaneous oxidation of TBHQ to TBBQ was prevented. In contrast, TBBQ retained activity in the absence of oxygen, with only a 2-fold increase in TBBQ MIC observed, and the addition of 1024 mg/mL of ascorbate caused only an 8-fold increase in MIC.

The antibacterial activity of a solution containing the breakdown product of TBBQ was also assessed against *S. aureus* SH1000, and gave an MIC of 128 mg/L. However, this activity was attributed to the TBBQ remaining in the solution; ~10% of the starting TBBQ remained, a concentration of TBBQ that would provide this level of antibacterial activity. Thus, the final breakdown product of TBBQ appears to have little or no antibacterial activity.

TBHQ has been reported to display limited activity against *E. coli*,[1] and indeed we saw activity only at high concentrations (MICs of 512 mg/L) for TBHQ and TBBQ against *E. coli* 1411. To explore the reason for the poor activity of this compound against Gram-negative bacteria, we performed susceptibility testing with TBHQ and TBBQ against *E. coli* in the presence of an outer membrane permeabilizing agent [polymyxin B nonapeptide (PMBN), at a concentration of 4 mg/L] and against efflux pump-deficient strains.[23,24] The antibacterial activity of TBHQ and TBBQ increased against *E. coli* strains lacking AcrAB or TolC (256 and 128 mg/L for strains SM1411 and ES100, respectively) and reached a level of activity comparable to that observed against *S. aureus* (16 mg/L) in the presence of PMBN (MICs of 32 and 16 mg/L for SM1411 and ES100, respectively). The results suggest that the limited activity of the compounds against *E. coli* is both the result of limited ingress across the outer membrane, and a consequence of active efflux from the cell by AcrAB–TolC.

Evaluation of bacterial killing and lysis by TBHQ and TBBQ

In order to further evaluate the bactericidal activity of TBHQ/TBBQ, the killing kinetics of these compounds were assessed alongside established antibiotics. Daptomycin caused a reduction in cell viability of ~3 $\log_{10}$ cfu/mL after 6 h in PBS, while tetracycline caused only bacteriostasis (Figure 3a). TBHQ had no effect on bacterial viability for the first 120 min of the experiment, but killing was observed thereafter (Figure 3a). The initiation of bacterial killing coincided with the commencement of conversion of TBHQ into TBBQ (Figure 3b), and the extent of kill increased with rising TBBQ concentration, ultimately leading to a 4 $\log_{10}$ drop in cell number after 6 h (Figure 3b). TBBQ showed no initial lag in killing activity and was rapidly and extensively bactericidal, causing a >5 $\log_{10}$ drop in cell number within 3 h, effectively sterilizing the culture within 6 h. Over this time period, TBBQ degradation was minimal (Figure 3c).

In nutrient-rich culture medium (MHB), TBHQ/TBBQ displayed reduced activity against *S. aureus* SH1000 compared with that seen in PBS. Nonetheless, TBBQ retained bactericidal activity in MHB, causing >4 $\log_{10}$ reduction in viable cell number within 6 h (Figure 4a). At TBHQ/TBBQ concentrations of up to 256 mg/L, there was little or no reduction in culture turbidity over a 6 h period (Figure 4b), indicating that bacterial killing by TBBQ occurred without concomitant cell lysis.[25]

MOA studies on TBHQ and TBBQ

Since MOA studies are an essential part of the pre-clinical evaluation of candidate antibacterial agents, we sought to investigate the MOA of TBHQ and TBBQ using a variety of approaches. Antibacterial agents commonly exert their activity through inhibition

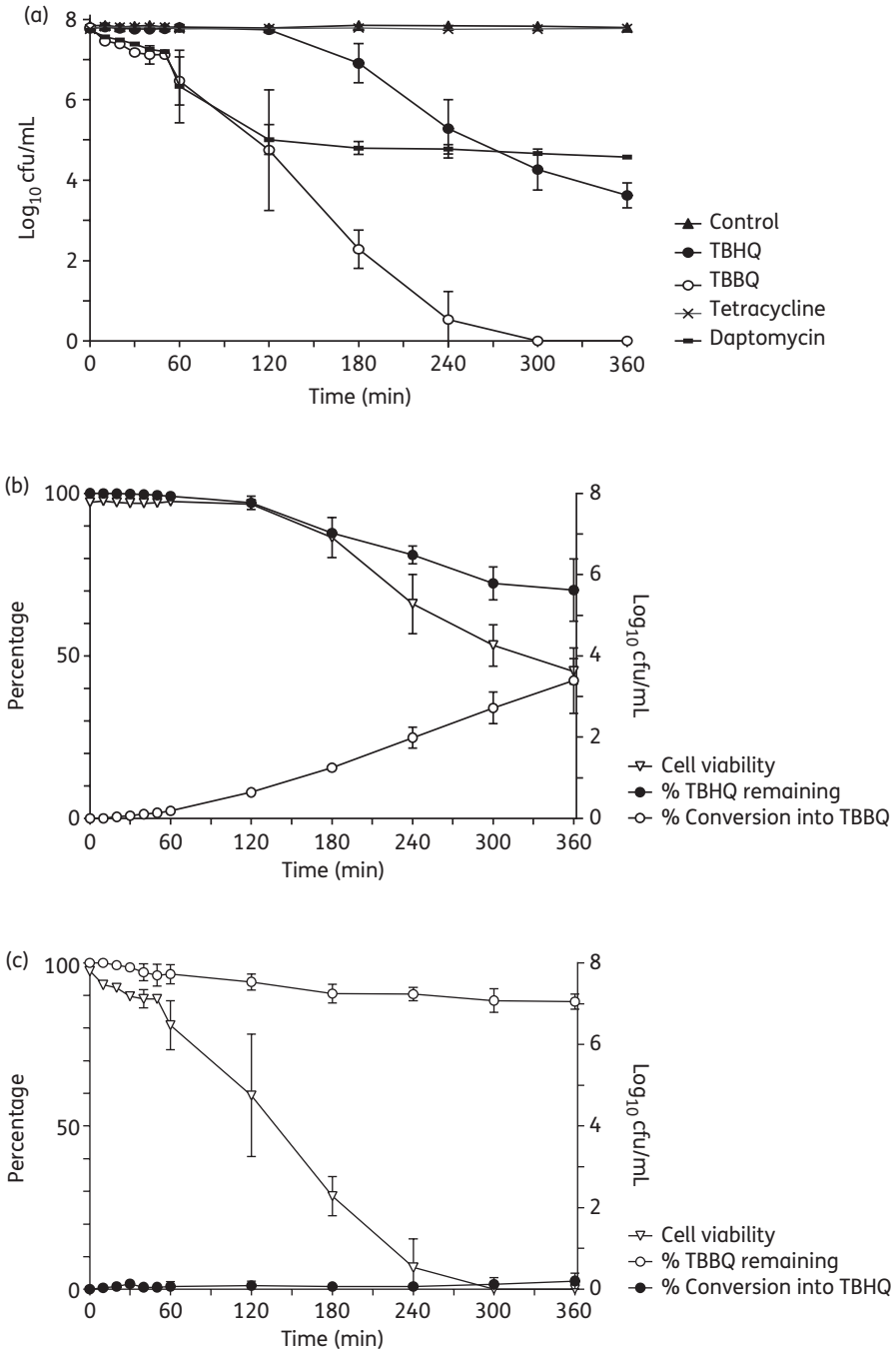

Figure 3. Effect of antibacterial agents at 4× MIC on *S. aureus* SH1000 suspended in PBS. (a) Killing kinetics of TBHQ, TBBQ and comparators. (b) Stability and killing kinetics of TBHQ. (c) Stability and killing kinetics of TBBQ. For (b) and (c) the percentage of the starting concentration of compound that remains or the percentage that is converted is plotted against the left *y*-axis. Viable count is plotted against the right *y*-axis (means of at least three independent replicates; error bars show standard deviations derived from replicates).

of macromolecular biosynthesis pathways.[25] Therefore, we initially examined the effects of TBHQ/TBBQ using a suite of *B. subtilis* biosensors responsive to the inhibition of specific macromolecular biosynthesis pathways (DNA, RNA, protein, cell wall and fatty acid).[18,19] No induction was observed with any of the biosensors for TBHQ/TBBQ in the concentration range 0.005–20 mg/L (data not shown). The effect of TBHQ/TBBQ on the incorporation of radiolabelled precursors into staphylococcal macromolecules was compared with known inhibitors of DNA, RNA and protein synthesis (ciprofloxacin, rifampicin and

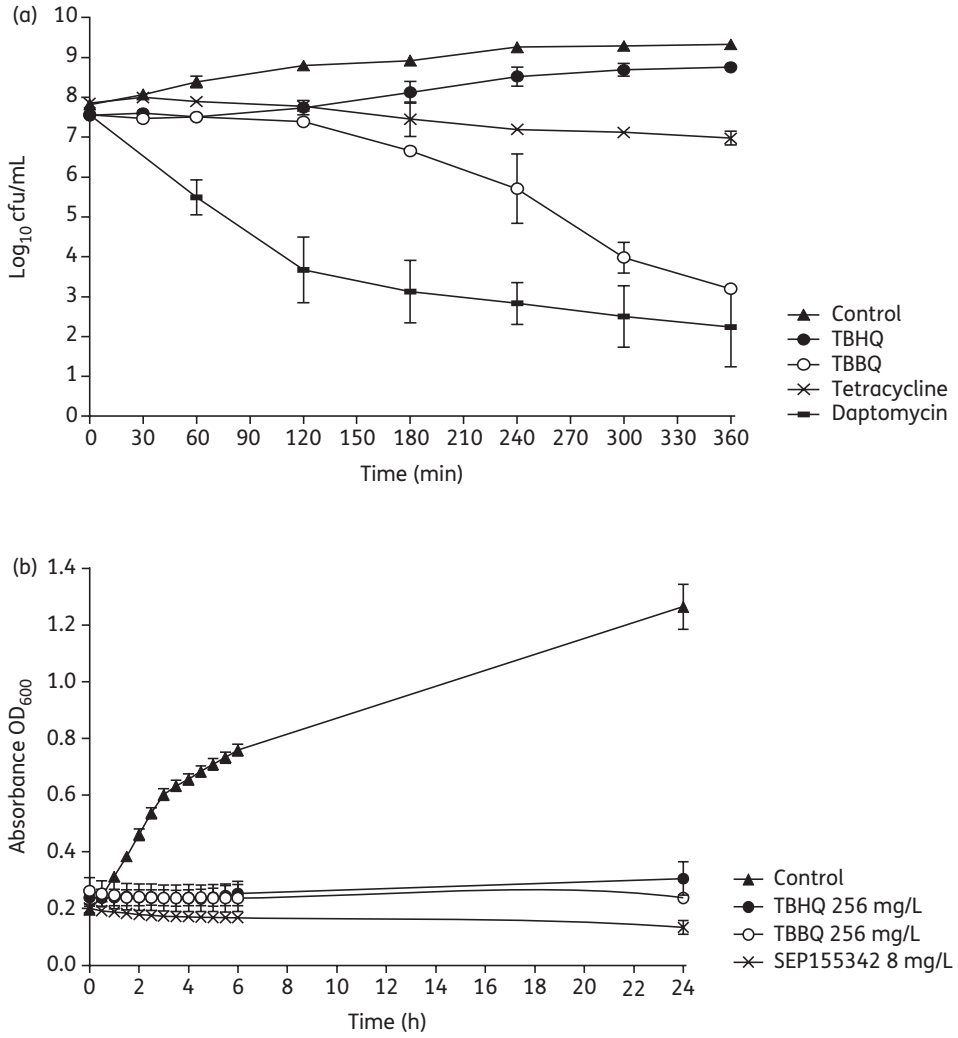

Figure 4. Evaluation of killing and lytic action of TBHQ/TBBQ and comparator agents against *S. aureus* SH1000 in MHB. (a) Killing kinetics of compounds at 4× MIC. (b) Absorbance at 600 nm of cultures exposed to TBHQ and TBBQ at 256 mg/L, or the positive control agent SEP155342 at 4× MIC[15] (means of at least three independent replicates; error bars show standard deviations derived from replicates).

tetracycline, respectively) at 4× MIC. Both TBHQ and TBBQ inhibited all three macromolecular synthetic pathways within 10 min, with no evidence of preferential inhibition of a single biosynthetic process (Figure 5).

Rapid bactericidal activity, failure to induce pathway-specific biosensors, and non-specific inhibition of macromolecular synthesis are features common to antibacterial agents that act on bacteria by compromising the integrity of the cytoplasmic membrane (CM).[17] To examine whether TBHQ/TBBQ act on bacterial membranes, we employed the *Bac*Light™ assay. *Staphylococcus aureus* cells exposed to vancomycin, tetracycline, daptomycin or TBHQ for 10 min retained between 90% and 100% of their membrane integrity, while TBBQ and the known membrane disruptors valinomycin and cetyltrimethylammonium bromide (CTAB) significantly reduced membrane integrity over the same time period (Table 2). Since antibacterial compounds that compromise the bacterial membrane may show comparable effects on mammalian membranes, it is important to establish the prokaryotic selectivity of antibacterial candidates with this MOA.[26] Therefore, TBBQ was tested for its ability to cause haemolysis of mammalian erythrocytes. Non-membrane-damaging agents caused <5% loss in erythrocyte integrity, while the known membrane-disruptors valinomycin and CTAB induced haemolysis (Table 2). However, TBBQ had little effect on erythrocyte integrity, suggesting that the interaction with cell membranes is prokaryotic-specific (Table 2).

Discussion

TBHQ and TBBQ both demonstrated potent bactericidal activity against planktonic cells and biofilms of *S. aureus*. However, several lines of evidence indicate that TBBQ is responsible for

the antibacterial activity of TBHQ. Halting the conversion of TBHQ into TBBQ (via the addition of ascorbate or incubation under anaerobic conditions) dramatically reduced antibacterial activity, i.e. TBHQ had little intrinsic antibacterial activity when prevented from undergoing oxidation. This suggests that the antibacterial effect associated with TBHQ either results from the process of conversion of TBHQ into TBBQ (e.g. through the production of reactive intermediates) or is intrinsic to TBBQ. As TBHQ and TBBQ are equally effective at inhibiting bacterial growth (an MIC of 8 mg/L for SH1000 in both cases), the antibacterial activity of TBHQ cannot be attributed to an intermediate species arising from conversion of TBHQ into TBBQ, and must be a property of TBBQ.

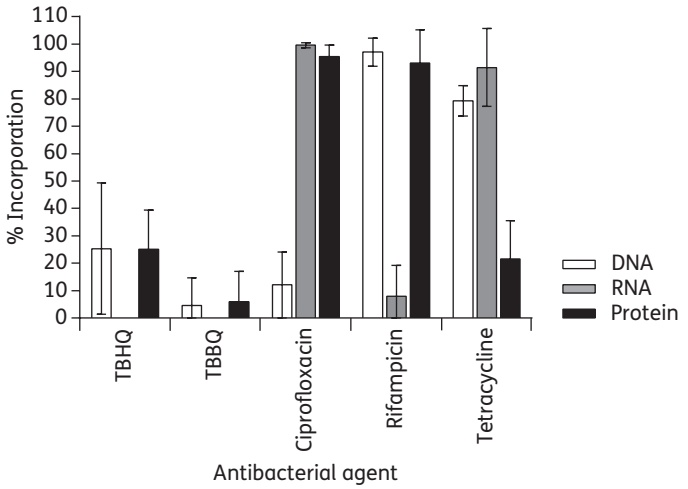

Figure 5. Effect of antibacterial agents on macromolecular synthesis in *S. aureus* SH1000. Percentage incorporation of ^{3}H thymidine, uridine and glutamine into DNA, RNA and protein, respectively (means of at least three independent replicates; error bars show standard deviations).

Table 2. Effect of TBHQ, TBBQ and comparator agents at 4× MIC on cellular membranes; results are the means of at least three independent determinations

Antimicrobial compound	Percentage bacterial membrane integrity (±SD)	Percentage erythrocyte integrity (±SD)
None	100±0	100±0
CTAB	0.5±0.6	0±0
Daptomycin	94.7±14.1	97.9±1.4
5% SDS	0±0	0±0
TBBQ	66.1±8.7	93.5±3.5
TBHQ	97.5±5.6	95.4±2.5
Tetracycline	90.9±22.2	99.2±0.7
Vancomycin	100±0	96.3±6.4
Valinomycin	27.4±8.5	49.1±6.7

Further support for this idea is provided by the time–kill studies. In contrast to TBBQ, TBHQ at 4× MIC does not immediately trigger bacterial killing, but only begins to do so after ∼2 h (Figure 3b). This initial lag in killing activity corresponds to the time required for the conversion of sufficient quantities of TBHQ into TBBQ for bacterial killing to occur. Levels of conversion into TBBQ and bacterial cell death increased concurrently over a 6 h period, reaching ∼40% conversion of TBHQ into TBBQ and more than a 4 $\log_{10}$ drop in cell number. By contrast, TBBQ initiated bacterial killing immediately and was more active than all comparator antibiotics, leading to >5 $\log_{10}$ drop in viability within 3 h (Figure 3a). Degradation of TBBQ was limited during this period, indicating that the process of conversion of TBBQ, or a breakdown product of TBBQ, is not responsible for the lethal effect. Furthermore, the primary breakdown product of TBBQ was shown in separate experiments to possess no or only limited antibacterial activity. In conclusion, TBHQ exhibits antibacterial activity upon degradation and formation of TBBQ; this compound possesses intrinsic antibacterial activity that is then lost upon degradation (Figure 6).

The MOA of TBBQ appears to involve compromising the integrity of the bacterial CM, without causing cell lysis. The observed lack of activity for TBBQ against *E. coli* suggests that it is unable to damage the outer membrane of Gram-negative bacteria. Nevertheless, since permeabilization of the outer membrane renders *E. coli* susceptible to TBBQ, the CM of Gram-negative bacteria is susceptible to its action. Importantly, TBBQ appears to display selective action against the bacterial CM, as it did not induce haemolysis of erythrocytes. The fact that TBBQ acts to compromise the integrity of the bacterial membrane may explain why this compound has the ability to eradicate staphylococcal biofilms; potent antibiofilm activity is a property that is often associated with membrane-perturbing agents.[27,28] In addition to antibiofilm activity, the membrane-perturbing MOA of TBBQ may also be advantageous in respect of restricting the selection of bacterial resistance, as there is evidence to suggest that bacteria do not readily acquire resistance to membrane-active agents.[17,27]

In summary, TBBQ is a potent, bactericidal, and apparently selective antistaphylococcal compound that demonstrates the ability to eradicate *S. aureus* biofilms *in vitro*; it therefore warrants further investigation as a candidate antistaphylococcal agent for use in the healthcare setting.

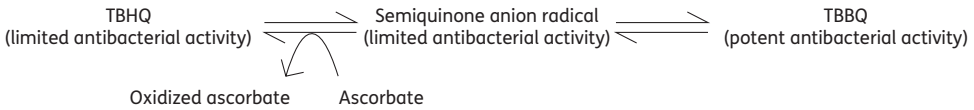

Figure 6. Proposed reaction scheme for the degradation of TBHQ under bacterial culture conditions.

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Transparency declarations

J. C. and A. E. are former employees and R. B. is a current employee of Evocutis plc (formerly known as Syntopix Group plc.). J. C. and R. B. hold shares in Evocutis plc. N. O. received a travel grant from the BSAC to attend an international scientific meeting. I. C. and A. J. O.: none to declare.

References

1 Fung DYC, Lin CCS, Gailani MB. Effect of phenolic antioxidants on microbial growth. *Crit Rev Microbiol* 1985; **12**: 153–83.

2 Przybylski R, Lee YC, Eskin NAM. Antioxidant and radical scavenging activities of buckwheat seed components. *J Am Oil Chem Soc* 1998; **75**: 1595–601.

3 Raccach M, Henningsen EC. Antibacterial effect of tertiary butylhydroquinone against 2 genera of Gram-positive cocci. *J Food Sci* 1982; **47**: 106–9.

4 Kashanian S, Dolatabadi JEN. DNA binding studies of 2-*tert*-butylhydroquinone (TBHQ) food additive. *Food Chem* 2009; **116**: 743–7.

5 Kupp LI, Rosen S, Beck FM. Effect of anti-oxidants on growth and lactic acid production by *Streptococcus mutans*. *J Dent Res* 1985; **64**: 1016–8.

6 Ogunrinola OA, Fung DYC, Jeon IJ. *Escherichia coli* O157:H7 growth in laboratory media as affected by phenolic antioxidants. *J Food Sci* 1996; **61**: 1017–21.

7 Jones SP, Fitzgerald DJ, Read NE *et al*. The use of benzoquinones and hydroquinones as antibacterial agents in the treatment of periodontal diseases. Evocutis plc. UK patent GB2455625, 2008.

8 Hamama AA, Nawar WW. Thermal decomposition of some phenolic antioxidants. *J Agric Food Chem* 1991; **39**: 1063–9.

9 Kim CM, Pratt DE. Degradation products of 2-*tert*-butlyhydroquinone at frying temperature. *J Food Sci* 1990; **55**: 847–53.

10 Li YB, Seacat A, Kuppusamy P *et al*. Copper redox-dependent activation of 2-*tert*-butyl(1,4)hydroquinone: formation of reactive oxygen species and induction of oxidative DNA damage in isolated DNA and cultured rat hepatocytes. *Mutat Res* 2002; **518**: 123–33.

11 Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically — Ninth Edition: Approved Standard M07-A9*. CLSI, Wayne, PA, USA, 2012.

12 National Committee for Clinical Laboratory Standards. *Methods for Determining Bactericidal Activity of Antimicrobial Agents: Approved Guideline M26-A*. NCCLS, Wayne, PA, USA, 1999.

13 Miller K, Storey C, Stubbings WJ *et al*. Antistaphylococcal activity of the novel cephalosporin CB-181963 (CAB-175). *J Antimicrob Chemother* 2005; **55**: 579–82.

14 Ooi N, Miller K, Randall C *et al*. XF-70 and XF-73, Novel antibacterial agents active against slow-growing and non-dividing cultures of *Staphylococcus aureus* including biofilms. *J Antimicrob Chemother* 2010; **65**: 72–8.

15 Oliva B, Miller K, Caggiano N *et al*. Biological properties of novel antistaphylococcal quinoline-indole agents. *Antimicrob Agents Chemother* 2003; **47**: 458–66.

16 Wilson JM, Oliva B, Cassels R *et al*. SB 205952, A novel semisynthetic monic acid analog with at least two modes of action. *Antimicrob Agents Chemother* 1995; **39**: 1925–33.

17 Ooi N, Miller K, Hobbs J *et al*. XF-73, a novel antistaphylococcal membrane-active agent with rapid bactericidal activity. *J Antimicrob Chemother* 2009; **64**: 735–40.

18 Mariner KR, Ooi N, Roebuck D *et al*. Further characterization of *Bacillus subtilis* antibiotic biosensors and their use for antibacterial mode-of-action studies. *Antimicrob Agents Chemother* 2011; **55**: 1784–6.

19 Urban A, Eckermann S, Fast B *et al*. Novel whole-cell antibiotic biosensors for compound discovery. *Appl Environ Microbiol* 2007; **73**: 6436–43.

20 Hilliard JJ, Goldschmidt RM, Licata L *et al*. Multiple mechanisms of action for inhibitors of histidine protein kinases from bacterial two-component systems. *Antimicrob Agents Chemother* 1999; **43**: 1693–9.

21 Tsuji S, Nakano M, Terada H *et al*. Determination and confirmation of five phenolic antioxidants in foods by LC/MS and GC/MS. *Shokuhin Eiseigaku Zasshi* 2005; **46**: 63–71.

22 Roginsky VA, Barsukova TK, Stegmann HB. Kinetics of redox interaction between substituted quinones and ascorbate under aerobic conditions. *Chem Biol Interact* 1999; **121**: 177–97.

23 O'Neill AJ, Bostock JM, Moita AM *et al*. Antimicrobial activity and mechanisms of resistance to cephalosporin P1, an antibiotic related to fusidic acid. *J Antimicrob Chemother* 2002; **50**: 839–48.

24 Shapiro E, Baneyx F. Stress-based identification and classification of antibacterial agents: second-generation *Escherichia coli* reporter strains and optimization of detection. *Antimicrob Agents Chemother* 2002; **46**: 2490–7.

25 O'Neill AJ, Chopra I. Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. *Expert Opin Investig Drugs* 2004; **13**: 1045–63.

26 Payne DJ, Gwynn NM, Holmes DJ *et al*. Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat Rev Drug Discov* 2007; **6**: 29–40.

27 Hurdle JG, O'Neill AJ, Chopra I *et al*. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat Rev Microbiol* 2011; **9**: 62–75.

28 O'Neill AJ. Bacterial phenotypes refractory to antibiotic-mediated killing: mechanisms and mitigation. In: Miller AA, Miller PF, eds. *Emerging Trends in Antibacterial Discovery: Answering the Call to Arms*. Wymondham: Caister Academic Press, 2011.

29 Horsburgh MJ, Aish JL, White IJ *et al*. σ^B modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325–4. *J Bacteriol* 2002; **184**: 5457–67.

30 O'Neill AJ. *Staphylococcus aureus* SH1000 and 8325–4: comparative genome sequences of key laboratory strains in staphylococcal research. *Lett Appl Microbiol* 2010; **58**: 358–61.

31 Report of the Working Party on Antibiotic Sensitivity Testing of the British Society for Antimicrobial Chemotherapy. *A guide to sensitivity testing. J Antimicrob Chemother* 1991; **27** Suppl D: 1–50.

32 Fischer HP, Brunner NA, Wieland B *et al*. Identification of antibiotic stress-inducible promoters: a systematic approach to novel pathway-specific reporter assays for antibacterial drug discovery. *Genome Res* 2004; **14**: 90–8.