Comparative Target Analysis of Chlorinated Biphenyl Antimicrobials Highlights

MenG as Molecular Target of Triclocarban

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Abstract

Triclocarban (TCC), a formerly used disinfectant, kills bacteria via an unknown mechanism of action. A structural hallmark is its \textit{N,N'}-diaryl urea motif which is also present in other antibiotics including the recently reported small molecule PK150. We here show that, like PK150, TCC exhibits an inhibitory effect on \textit{Staphylococcus aureus} menaquinone metabolism via inhibition of the biosynthesis protein MenG. However, the activity spectrum (MIC\textsubscript{90}) of TCC across a broad range of multi-drug resistant...
staphylococci and enterococci strains was much narrower compared to PK150. Accordingly, TCC did not cause an over-activation of signal peptidase SpsB, a hallmark of the PK150 mode of action. Furthermore, we were able to rule out inhibition of FabI, a confirmed target of the diaryl ether antibiotic triclosan (TCS). Differences in the target profile of TCC and TCS were further investigated by proteomic analysis, showing complex, but rather distinct changes in the protein expression profile of S. aureus. Downregulation of the arginine deiminase pathway provided additional evidence for an effect on bacterial energy metabolism by TCC.

**Importance**

TCC’s widespread use as an antimicrobial agent has made it a ubiquitous environmental pollutant despite its withdrawal due to ecological and toxicological concerns. With its antibacterial mechanism of action still being unknown, we undertook a comparative target analysis between TCC, PK150, a recently discovered antibacterial compound with structural reminiscence to TCC, and TCS, another widely employed chlorinated biphenyl antimicrobial, in the bacterium *Staphylococcus aureus*. We show that there are distinct differences in each compound’s mode of action, but also identify a shared target between TCC and PK150, which is interference with menaquinone metabolism by inhibition of MenG. The prevailing differences, however, which also manifest in a remarkably better broad spectrum activity of PK150, suggest that even high levels of TCC or TCS resistance observed by continuous environmental exposure may not affect the potential of PK150 or related *N,N*'-diaryl urea compounds as new antibiotic drug candidates against multi-drug resistant infections.
Introduction

Triclocarban (TCC) is a broad-spectrum anti-infective agent that has been used in personal care products such as soaps and lotions since the 1960s (1). In 2017, however, the Food and Drug Administration (FDA) banned the use of TCC together with triclosan (TCS), another halogenated biphenyl antimicrobial agent (Fig. 1), because of safety concerns. TCC and TCS can be absorbed by the body (2,3) and were found to interfere with several mammalian off-targets: both act as weak endocrine disruptors (4); TCC was additionally found to exhibit anti-inflammatory effects by inhibiting soluble epoxide hydrolase and to alter cardiac function by interference with fatty acid metabolism in murine mouse models (3, 5, 6). TCS, in contrast, was demonstrated to depolarize the inner mitochondrial membrane and uncouple oxidative phosphorylation (7–9). Adding to these health-related concerns are environmental problems: due to their multiple chlorination sites, these compounds are persistent to biodegradation, and were thus shown to accumulate in the environment and cause potentially toxic effects in soil and water organisms (10–15).

In contrast to the very extensive studies on its impact on health and environment, surprisingly little is known about the antibacterial mode of action of TCC. While TCS is known to inhibit enoyl-acyl-carrier-protein reductase FabI (16–18) and proposed to further disturb the lipid bilayer non-specifically at higher concentrations (19), the corresponding mechanisms for TCC are rather unclear and studies into their elucidation are scarce: one study investigated the production of membrane leakage by several compounds, but found no such effect for TCC (20). Comparison of TCC and TCS by molecular dynamics simulations, though, suggested that at high concentrations both...
compounds might destabilize the lipid bilayer membrane in a similar non-specific manner (21). Finally, a study which aimed at generating improved derivatives of TCC investigated the mode of action by fluorescence staining and microscopy, and found TCC to induce cell lysis and membrane deformation (22). However, a specific protein target as in the case of TCS has not been identified so far.

Recently, we published the development of a novel antibacterial compound named PK150, which was derived from the anti-cancer drug sorafenib (SFN, Fig 1) (23). Extensive mode of action analysis validated two independent protein targets in *Staphylococcus aureus*, which involved the over-activation of signal peptidase Ib (SpsB), a key enzyme in the bacterial secretory pathway, and inhibition of demethylmenaquinone methyltransferase (MenG), the last step in the biosynthesis of the essential electron carrier menaquinone. Given the structural similarity between PK150 and TCC, including the characteristic *N,N'*-diaryl urea motif, it is well conceivable that some of these mechanistic features identified for PK150 might also apply to TCC. This is corroborated by some similarities which both compounds exert on target cells including membrane deformation and cell lysis (23). Additionally, both compounds are only active in Gram-positive bacteria and development of resistance has not yet been observed under laboratory conditions (23, 24).

In this work we show that the target profile of TCC exhibits similar but also distinct features compared to PK150. While MenG is inhibited by both compounds, we rule out a contribution of SpsB to TCC’s mode of action. Furthermore, TCC does not inhibit FabI and also shows distinct differences to TCS in proteomic profiling. As such, this work underlines the versatility of *N,N'*-diaryl urea and diaryl ether antibiotics and proposes the contribution of a specific target to the overall mode of action of TCC.
Results

PK150 and TCC differ in their spectrum of activity against multi-drug resistant staphylococci and enterococci. We first analyzed whether the two \textit{N,N'-diaryl ureas} PK150 and TCC exert a comparable activity profile against a variety of multi-drug resistant clinical strains, among them bacteria resistant to last resort antibiotics such as linezolid, vancomycin or daptomycin. We determined the minimal inhibitory concentration (MIC) of both compounds in 100 different staphylococci strains, mainly \textit{S. aureus}, and 100 enterococci strains, mainly \textit{E. faecium} and \textit{E. faecalis}, to define the compound concentration at which the growth of at least 90\% of strains is inhibited (MIC$_{90}$, Fig. 2 and Supplementary Data 1). We found the activity profile of both compounds to be very different: PK150 exhibited activity against all tested strains, with an MIC$_{90}$ of 0.78 µM against staphylococci and an MIC$_{90}$ of 3.13 µM against enterococci. In contrast, TCC showed a remarkably broad MIC distribution and was inactive against 17\% of staphylococci and 89\% of enterococci (MIC$_{90}$ of >25 µM in both cases). This not only demonstrates that the antibacterial properties of PK150 are superior to TCC, but also suggests fundamental differences in their mode of action. We further looked into 40 staphylococci strains exhibiting intermediate or high level resistance against TCC (MIC $\geq$ 6.25 µM), but could not observe any differences regarding their general resistance pattern compared to the full strain selection (Supplementary Data 1). However, we noted that none of those 40 strains belonged to the clonal complex subtype 5 ST225 (spa type t003; “Rhine-Hesse Epidemic strain”), which is one of the predominant hospital-associated MRSA in Germany, although 9\% of our collection comprised this subtype (25, 26).
TCC and TCS show little to no effect on bacterial membrane integrity. We next investigated the mechanistic reasons for these observed differences. First, we looked into the effect of TCC on the general integrity of the bacterial cell membrane. As mentioned above, evidence for induced membrane leakage was not found in previous experiments, but TCC is predicted to perturb the membrane at higher concentrations (20, 21). For our assay, we analyzed the interaction of the dye propidium iodide with bacterial DNA, which is only accessible if the cell membrane is permeabilized. As a positive control, the detergent benzalkonium chloride (27) was used and evoked an immediate and drastic increase in fluorescence (Fig. 3A). PK150 was previously shown to induce an increase in dye fluorescence, likely a consequence of autolysin induced cell lysis (23), and a moderate, concentration-dependent effect was also observed here (Fig. 3A). Of note, the strain tested herein differs from the strain used in the previous study, which could account for differences in total fluorescence increase. In contrast, TCC induced a much weaker effect than PK150 and TCS shows no change compared to the DMSO control at all (Fig. 3A). This confirms that both TCC and TCS do not act via membrane perturbation at concentrations close to their MIC and further suggests the existence of specific targets for TCC and TCS (where FabI is a known target).

TCC does not overactivate PK150’s target SpsB. Given their structural similarity, we next examined whether TCC interacts with the known targets of PK150. One unprecedented property of PK150 is its capability to overactivate the signal peptidase SpsB. This can be measured in a FRET-based peptidase assay using an artificial fluorescent substrate and endogenous SpsB embedded into native membranes of S. aureus (23). We performed this assay at three different concentrations of both TCC and TCS and also added PK150 as positive control for an SpsB activator (Fig 3B). TCC
did not exhibit a stimulatory effect on SpsB activity, indicating an important mechanistic
difference to PK150. Interestingly, TCS induced a moderate activation of the enzyme,
despite its structure being more distant to PK150 compared to TCC.

**TCC inhibits PK150’s other target MenG.** Regarding the second confirmed
cellular mechanism of PK150, inhibition of MenG, we examined whether addition of
menaquinone-4 (MK-4) rescued bacteria from TCC and TCS treatment. Interestingly, a
moderate shift of MIC up to about 6-fold, dependent on MK-4 concentration, was
observed (Fig. 4A) for both compounds. To exclude that unspecific effects, such as
aggregation of the compounds by high concentrations of MK-4 or a general fitness
increase of the cells (due to the additional vitamin supply), interfere with the assay and
contribute to these results, we performed further validation experiments: First, we
quantified endogenous levels of menaquinone-8 (MK-8) in live *S. aureus* cells treated
with TCC, TCS or ciprofloxacin as control. MK-8 levels were reduced in a concentration-
dependent manner by up to 47% by TCC and up to 29% by TCS on average, which was
not observed by ciprofloxacin treatment (Fig. 4B). Second, we measured MenG
inhibition directly by methylation of the artificial substrate demethylmenaquinone-2
(DMK-2) in lysates of *S. aureus* cells overexpressing MenG, extraction and subsequent
LC-MS analysis. Again, TCC showed stronger effects and reduced MenG activity by
23% at 5 µM compound concentration and up to 73% at 50 µM, while TCS inhibited
MenG only at a concentration of 50 µM and to an extent of 41% (Fig. 4C). PK150, in
comparison, had been shown to diminish endogenous MK-8 by up to 33% and to reduce
MenG activity by 70% at the same respective concentrations (23).

**Neither TCC nor PK150 inhibit TCS’s target FabI.** Given the similarity between
TCS and TCC with regard to human off-targets and environmental behavior and the fact
that FabI is a confirmed target of TCS, we next investigated whether TCC might interact
with FabI in a similar manner. We cloned and purified *S. aureus* FabI and assayed its
activity on reducing trans-2-octenoyl *N*-acetylcysteamine thioester (t-o-NAC thioester), a
common artificial substrate for this enzyme (28). We were able to reproduce the
inhibition caused by TCS, while neither TCC nor PK150 exhibited a significant inhibitory
effect (Fig. 5).

Full proteome analysis reveals different patterns for TCC and TCS
treatment. In order to analyze the effects evoked by the compounds on a global level,
we performed whole proteome analysis of *S. aureus* cells treated with sub-inhibitory
concentrations (0.5 x MIC) of TCC or TCS (Fig. 6A,B, Supplementary Data 2). Bacterial
proteins were digested with trypsin and subjected to analysis by liquid chromatography-
tandem mass spectrometry following label free quantification of identified peptides (29).
Analysis of proteins whose expression levels were significantly altered between DMSO
and compound-treated condition (|log(protein ratio)| > 1 & -log(P-value) > 1) revealed
only one overlapping protein between TCC and TCS treatment (PTS system EIIBC
component, Q2G1G5), supporting the view that the underlying mechanisms of action
are rather distinct from each other (Fig. 6C). We further tried to identify particular
pathways that might be up- or downregulated by network analysis with STRING (Fig.
6A,B,D, Supplementary Data 2) (30). Most strikingly, a significant downregulation of the
entire arginine deiminase pathway (arginine deiminase (*arcA*, Q2FUX7), carbamate
kinase 1 (*arcC1*, Q2FZA9), carbamate kinase 2 (*arcC2*, Q7X2S2), and two ornithine
carbamoyltransferases (*arcB/argF*, Q2FUX8, and *argF*, Q2FZB0)) was observed upon
TCC treatment (Fig. 6D) (31). Regarding upregulated proteins, two proteins were found
to be generally associated with dicarboxylic acid metabolism by GO (32, 33) annotation.
(Supplementary Data 2), but no significant interaction network was detected. For TCS treatment, there was no significant pathway enrichment among upregulated proteins and only a few downregulated proteins were found to be involved in functional enrichment, which are associated with carbohydrate phosphotransferase activity or transmembrane transport by GO (32, 33) and Pfam (34) annotation, but do not give rise to a comprehensive pathway (for details see Supplementary Data 2).

Discussion

Overactivation of SpsB has been identified as a key feature of the PK150 scaffold, leading to increased secretion of autolytic enzymes and subsequent cell lysis, but, despite its structural similarity, we could not confirm the same activity for TCC. In addition, TCC was inactive against most tested enterococci and also a good proportion of staphylococci. Although the reasons for this observation might be complex and further work is required for their exact elucidation, it seems plausible that the differences in SpsB activation could contribute to these diverging effects. Furthermore, it indicates that the mechanisms leading to TCC resistance do not affect susceptibility towards PK150, suggesting that environmental exposure of bacteria to TCC is unlikely to evoke cross-resistance against PK150 or related \(N,N'\)-diaryl ureas. However, the shift of MIC in the presence of MK-4, the reduction of endogenous menaquinone levels in live cells and the direct inhibition of MenG activity in cellular lysate are comparable traits of both compounds (23). Based on these findings, it is well conceivable that MenG inhibition is involved in TCC’s mode of action, which points
towards it being the first ever identified molecular target of TCC. Of course, this does not exclude the existence of additional target mechanisms, which can include other proteins and in principle also non-specific effects such as membrane disruption. However, our finding that TCC barely affects membrane permeability, which is in accordance with its failure to induce potassium leakage in an earlier study (20), is contradictory to unspecific membrane damage and supports the view of a more specific mechanism.

Nevertheless, the proteomic data deliver a complex picture of the processes underlying TCC treatment. Of note, no effect on menaquinone metabolism was observed. Instead, the most significant finding was a downregulation of the arginine deiminase pathway. Interestingly, this pathway was found to be upregulated in the proteomes of *S. aureus* resistant to the PK150 parent compound SFN (23). The arginine deiminase pathway catabolizes arginine to carbamoyl phosphate, which is further converted to ammonia, carbon dioxide and ATP (31). It has been associated with enhanced fitness and virulence as the MRSA strain USA300 carries a mobile genetic element containing an additional arginine deiminase operon, which serves as an epidemiological marker (35). Importantly, it represents a way to generate energy which is independent of menaquinone (31). Thus, while its upregulation makes sense for cells resistant to a disruptor of menaquinone biosynthesis, it is rather surprising that the exact opposite is found in the case of TCC and non-resistant cells. Such a response would actually aggravate the detrimental consequences of MenG inhibition. Hence, although the exact mechanisms inducing these changes remain unclear, they appear to strengthen the antibiotic effect of TCC and also provide an independent link for alterations in bacterial energy metabolism.
The proteomic data further revealed a completely different picture in the case of TCS treatment. Unfortunately, the pattern of up- and downregulated proteins does not give rise to a clearly targeted pathway. Nevertheless, together with the finding that TCC does not inhibit FabI, these data suggest that both compounds act by different mechanisms.

An interesting finding is that TCS exhibits a stimulation of SpsB activity as well as an inhibitory effect on menaquinone biosynthesis. Both effects are only weak to moderate and comparable to what was observed with SFN, which is about ten times less potent than TCS (23). Of note, mutations in FabI, which are commonly referred to as conferring TCS resistance, do not produce fully resistant strains, but merely decrease the activity of TCS of about an order of magnitude (18, 36). In addition, similarly TCS-resistant bacteria have also been isolated which show no alterations in the fabI gene (36). It is thus not surprising that the involvement of other, unknown targets for the mode of action of TCS has been proposed before (37). Although further experiments are needed for validation, our experiments point to the possibility that SpsB and MenG could represent such additional targets, particularly at higher TCS concentrations.

Taken together, we identified MenG as a putative molecular target of TCC, reminiscent to structurally related PK150, but also established distinct differences between the two compounds as well as to TCS. Hence, our findings shed some light on the obscure mechanism of TCC and underline menaquinone biosynthesis inhibition as an important antibiotic target pathway. In addition, our work highlights the novelty of the PK150 structure as a promising entity with potential against multi-drug resistant pathogens, which is unlikely to be affected by environmentally acquired TCC resistance.
Materials and Methods

Materials and strains. TCC and TCS were purchased from Sigma Aldrich. MK-4 and MK-9 were purchased from Santa Cruz Biotechnology and Carbosynth, respectively. PK150 and DMK-2 were synthesized as reported previously (23). Unless otherwise stated, S. aureus NCTC 8325 (Institute Pasteur, France) was used for laboratory experiments and cultured in B medium (10 g/L casein peptone, 5 g/L NaCl, 5 g/L yeast extract, 1 g/L K₂HPO₄, pH 7.5). The collection of staphylococci and enterococci for MIC₉₀ determination was provided by the National Reference Centre for Staphylococci and Enterococci (Robert Koch Institute, Wernigerode, Germany). Further details on the specific strains can be found in Supplementary Data 1.

MIC determination, MIC₉₀, and MK-4 shift assay. Staphylococcus aureus cultures were grown at 37°C with gentle shaking (200 rpm) in B medium from overnight cultures until OD₆₀₀~0.4–0.6, and diluted into B medium to give a final a concentration of 10⁵ colony forming units (CFU)/mL. Compounds at various concentrations were added to diluted bacterial cultures (100 µL/well final volume; final concentration of DMSO from compound stocks 1%) in triplicates. A growth control containing DMSO only and a sterile control containing medium were included. After incubation (37°C, 200 rpm, 24 h), the dilution series was analyzed for microbial growth as indicated by turbidity. The lowest concentration in the dilution series at which no growth of bacteria could be observed by eye was defined as the minimum inhibitory concentration (MIC) of the compound. MIC values were determined by three independent experiments. Note that for the case of TCS the MIC in S. aureus NCTC 8325 was found to vary between 0.3 µM and 0.5 µM. For experiments relating to this MIC, a value was determined beforehand and this same...
value taken for the subsequent respective experiment. These values were: 0.3 μM for metabolic menaquinone profiling (Fig. 4B) and full proteome analysis (Fig. 6), 0.39 μM for membrane integrity assay (Fig. 3A), and 0.5 μM for MIC shift assay (Fig. 4A).

For MIC₉₀ determination, clinical isolates were grown on Mueller-Hinton Broth (MHB) culture plates and 3 to 4 single colonies were picked and used to inoculate 5 mL MHB. Cultures were grown until they reached an optical density corresponding to 0.5 McFarland units. These cultures were diluted 1:50 in 0.85% (w/v) NaCl solution to prepare the final inoculum. 1 μL of a 3 mM stock solution in DMSO of the respective test compounds was placed in the first column of a round-bottom 96-well plate. The wells of this column were filled up with 99 μL of MHB. Columns 2 to 12 were filled with 50 μL MHB and two-fold serial dilutions of the test compounds were made by transferring 50 μL from column 1 to column 2 and so forth until column 10. Column 11 served as growth control and column 12 as sterile control. The plate was inoculated by adding 10 μL of the freshly prepared inoculum to the wells of column 1 through 11. Bacteria were grown at 37 °C for 20 h without agitation. The MIC was determined as the lowest compound concentration with no observable bacterial growth after visual inspection. All MIC raw data and information on the characteristics of the tested strains have been uploaded to the supplementary information as an Excel file (Supplementary Data 1).

For MIC shift assays in the presence of exogenous menaquinone-4 (MK-4), the growth medium was additionally supplemented with different concentrations of MK-4 before addition of the bacteria, giving a total DMSO concentration in the final assay of 1.5%.

**Membrane Integrity assay.** To assess cell wall permeability upon treatment with compounds, cells (S. aureus NCTC 8325) were grown (37°C, 200 rpm) from a
respective over-night culture until they reached an OD$_{600}$ value of 0.4–0.8. The cells were then harvested (6000 x g, 4°C, 5 min), washed with 5 mM HEPES-NaOH buffer (pH 7.2 supplemented with 5 mM glucose) and then resuspended in the same buffer to an OD$_{600}$ of 0.4. The assay was conducted in 96-well plates (Nunc flat-bottom transparent 96-well plates, Thermo Fischer Scientific) with 99 µL cell-suspension per well. The assay was started by adding 1 µL of 1 mM propidium iodide (Sigma-Aldrich) in DMF to each well (10 µM final concentration) and incubating at 37°C in the TECAN Infinite M200 Pro microplate reader to allow the propidium iodide to be integrated into the membranes. During incubation, fluorescence was measured (535 nm excitation and 617 nm emission). After 15 min, 1 µL of the respective compound or control was added from DMSO stocks with the appropriate concentrations to each well. The fluorescence was measured for 60 min at 37°C. As a positive control, 16 µg/mL (8-fold MIC) of the detergent benzalkonium chloride (BAC) was added to the cell suspension, while 1 µL DMSO was added for a baseline fluorescence control.

**FRET-based SpsB activity assay.** SpsB activities were measured using membranes of *S. aureus* and a Förster Resonance Energy Transfer (FRET) assay as described previously (23, 38). Briefly, *S. aureus* NCTC 8325 membranes containing native SpsB (0.2 mg/mL total protein concentration) were pretreated with different concentrations of compound (TCC, TCS, and positive control PK150) or DMSO for 5 min at 37°C. A synthetic peptide substrate based on SceD modified by 4-(4-dimethylaminophenylazo)benzoic (DABCYL) acid and 5-((2-aminoethyl)amino)-1-naphthalenesulfonic acid (EDANS) was added (DABCYL-AGHDAHASET-EDANS, AnaSpec) to give 10 µM final substrate concentration (1:100 from DMF stock) and fluorescence was monitored by a Tecan Infinite 200Pro plate reader for 1 h at 37°C.
(345 nm excitation and 510 nm emission wavelength). SpsB activity was determined by the slope of the fluorescence increase and normalized to the DMSO control.

**Metabolic profiling of endogenous MK-8.** The experiments were performed as described previously (23). Mid-exponential phase cultures of S. aureus NCTC 8325 were diluted into B medium to give a final concentration of 10⁵ CFU/mL. TCC, TCS and control ciprofloxacin (Cipro) were added to various final concentrations (TCC, TCS: 0.1, 0.33 and 0.5 x MIC; Cipro: 0.1, 0.5 x MIC) and bacteria were incubated for 24 h (or longer if OD₆₀₀ was << 6) at 37°C, 200 rpm. Equivalent amounts of cells (equal to 4 mL OD₆₀₀ = 6) were harvested (6,000 x g, 4°C, 10 min), washed with PBS and either stored at -80°C for later use or directly resuspended in 10 mL 2-propanol 70% (v/v), supplemented with 500 ng/mL menaquinone-9 (MK-9) as internal standard, and sonicated (3 x 30s, 80% intensity, Sonopuls HD 2070 ultrasonic rod, Bandelin electronic GmbH) for extraction. Cellular debris was removed by centrifugation (12,000 x g, 30 min, 4°C) and the supernatant was loaded onto a Chromabond® C18 solid phase extraction column (3 mL, 500 mg, Macherey Nagel), preconditioned with 6 mL methanol and 6 mL water. Columns were washed with 3 mL water, then 3 mL 2-propanol 70%, and eluted with 10 mL methanol/2-propanol 3:1 (v/v). The eluate was concentrated to dryness and resolved in 200 μL acetonitrile. LC-MS analysis was performed on a Thermo Fisher Scientific LTQ-FT Ultra (FT-ICR-MS) coupled with an UltiMate 3000 HPLC system (Thermo Fisher Scientific) using atmospheric pressure chemical ionization (APCI). 10 μL of sample was injected into an XBridge BEH300 C4 3.5 μm 2.1 x 150 mm column (Waters) and eluted with a gradient of 54% acetonitrile/water, 0.1% FA to 90% acetonitrile/water, 0.1% FA over 1 h at a flow rate of 0.2 mL/min. Single ion monitoring
(SIM) of MK-8 ([M+H], 717.56) and MK-9 ([M+H], 785.62) was performed in parallel and the respective area under the curve (AUC) was determined for quantification using XCalibur software (Thermo Fisher Scientific). AUCs for MK-8 were first normalized to the internal standard MK-9 and then normalized to the DMSO-treated controls.

**MenG activity assay.** Cellular lysates containing overexpressed MenG were prepared from *S. aureus* pRMC2-MenG as described previously (23). Briefly, cells were grown in B medium with chloramphenicol (10 μg/mL) until OD$_{600}$~0.5–0.6, induced with anhydrotetracycline (400 ng/mL) and incubated over night. Cells were harvested, washed with PBS, resuspended in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, then lysed and adjusted to a final protein concentration of 50 mg/mL.

For the assay, 500 μL of solution containing 100 mM Tris-HCl pH 8, 10 mM MgCl$_2$, 10 mM DTT, 100 μM S-adenosyl methionine, 25 μM DMK-2, 200 μL MenG lysate and compounds (TCC, TCS) at different concentrations (5, 25, 50 μM) were incubated at 37°C, 200 rpm, for 80 min. After that, 7 mL 2-propanol 70%, supplemented with 500 ng/mL MK-4 as internal standard, were added and extraction and quantification of menaquinone was performed as for metabolic profiling of MK-8, with the following alterations: 3 mL H$_2$O was added to the centrifuged supernatant prior to loading onto the solid phase extraction columns and washing of the columns was only conducted with 3 mL H$_2$O. The gradient of the LC-MS analysis ranged from 18% acetonitrile/water, 0.1% FA to 90% acetonitrile/water, 0.1% FA over 1 h and single ion monitoring was performed for DMK-2 ([M+H], 295.17), MK-2 ([M+H], 309.18) and MK-4 ([M+H], 445.31) in parallel. Resulting data were acquired by three independent experiments.
**FabI cloning and activity assay.** FabI was amplified from genomic S. aureus DNA using a forward (ggggacaagttgtacaaaaaagcaggctttATGTTAAATCTTGAAAAC-AAAACA; capital letters indicate overlap with the fabI gene) and reverse (ggggaccactttgacaagaaagctgggtgTTATTTAATTGCGTGGAATCC) primer that introduced AttB sites for Gateway® cloning. The PCR product was cloned into donor vector pDONR201 (*Invitrogen*) by BP reaction, transformed in *E. coli* XL1 blue, reisolated, and a subsequent LR reaction was performed to clone the sequence into pET300/NT-Dest destination vector (*Invitrogen*), giving a FabI construct with an N-terminal His tag. This construct was first transformed into *E. coli* XL1 blue, then into *E. coli* BL21(DE3) for overexpression. Integrity of the construct was confirmed by Sanger sequencing (*Genewiz*). For expression and purification, mid-exponential phase cultures were induced with 1 mM IPTG and incubated for 4 h at 37 °C, 200 rpm. Cells were harvested, washed with PBS, resuspended in lysis buffer (50 mM Tris pH 8, 300 mM NaCl, 5 mM imidazole), and lysed by sonication (4 x 5 min, 80% intensity, Sonopuls HD 2070 ultrasonic rod, *Bandelin electronic GmbH*). Debris was removed by centrifugation (22000 x g, 4°C, 30 min) and the lysate was purified by affinity chromatography on a Ni-NTA agarose column (5 mL column volume, *GE Healthcare*) on an Äkta protein purification system (*GE Healthcare*), using the buffer described above with 20 mM imidazole for washing and 500 mM for elution. After buffer exchange to 50 mM Tris pH 8.5, 200 mM NaCl, 2 mM DTT by dialysis, a size exclusion chromatography with a Superdex 200 10/300 GL (120 mL column volume, *GE Healthcare*) was performed for final purification. Protein purity was checked by SDS-PAGE.

The substrate t-o-NAC thioester was synthesized as described in the literature (28). Conditions for the FabI assay were adapted and modified from the procedure described
therein. Briefly, 1.4 μM FabI, 0.8 mM NADPH and compounds at various concentrations (0.1 μM to 100 μM) or DMSO were incubated in 50 mM sodium acetate, pH 6.5, for 10 min at 30°C, then l-o-NAC thioester (1.6 mM final concentration) was added and the decrease in absorbance at 340 nm was monitored for 30 min at 30°C. Inhibition was determined by the slope of the absorbance curve at t = 0, relative to the DMSO-treated control (100% activity) and the heat control (0% activity, after incubation at 95 °C for 10 min). IC₅₀ values were determined by fitting to a sigmoidal equation.

**Full proteome analysis.** S. aureus NCTC 8325 overnight cultures were diluted into B medium to give a final OD₆₀₀ of 0.1 (40 mL per biological replicate) and then grown (37°C, 200 rpm) for 5 h. Cells were harvested (6,000 x g, 4°C, 10 min), washed with PBS and resuspended in B medium to give a final cell density of 1.5 x 10⁹ CFU/mL. 10 mL portions were aliquoted into polypropylene plastic tubes and incubated with TCC (0.5 μM, 0.5 x MIC), TCS (0.15 μM, 0.5 x MIC) or DMSO at 37°C, 200 rpm for 1.5 h. Cells were pelleted by centrifugation (6,000 x g, 4°C, 10 min) and washed with PBS. Cells were stored at -80°C for at least 16 h and then lysed. For cell lysis, bacterial cell pellets were resuspended in 200 μL 100 mM Tris pH 7.4 and lysed by sonication (5 x 20 s, 65% int. with 30 s rest on ice in between, Sonopuls HD 2070 ultrasonic rod, Bandelin electronic GmbH). 80 μL of 10% (w/v) SDS and 1.25% (w/v) sodium deoxycholate was added and samples heated for 10 min at 90°C. Lysates were sonicated for 10 s at 10% intensity to shear nucleic acids and centrifuged (13000 x g, RT, 10 min) to pellet debris. Protein concentrations were measured using the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Pierce Biotechnology) and full proteome samples were adjusted to equal protein amounts. Subsequently, proteins were precipitated with ice-cold acetone...
(4 volumes) at -20°C overnight, centrifuged (16,900 x g, 4°C, 15 min), and the pellets washed twice with ice-cold methanol (1 mL). Protein pellets were air dried and dissolved in denaturation buffer (7 M urea, 2 M thiourea in 20 mM pH 7.5 HEPES buffer), then reduced with TCEP (10 mM; 37°C, 1,200 rpm, 1 h) and subsequently alkylated with iodoacetamide (12.5 mM; 25°C, 1,200 rpm, 30 min). Alkylation was quenched by the addition of dithiothreitol (12.5 mM) and subsequent incubation (RT, 1,200 rpm, 30 min). For digestion, LysC (1:200 enzyme:protein ratio; 0.5 μg/μL) was added to each sample and the samples incubated at RT with shaking at 700 rpm for 2 h. Following dilution with 50 mM TEAB buffer (1:5), samples were further digested with trypsin (1:100 enzyme:protein ratio; 0.5 μg/μL) at 37°C overnight. The reaction was stopped by adding FA to a final pH of 2–3. Peptides were desalted on-column using 50 mg SepPak C18 Vac cartridges (Waters): SepPak C18 cartridges were equilibrated with ACN (1 mL), elution buffer (1 mL; 80% ACN, 0.5% FA) and three times with aqueous 0.5% FA solution (1 mL). Subsequently, the samples were loaded by gravity flow, washed three times with aqueous 0.5% FA solution (1 mL), eluted with elution buffer (0.5 mL, 80% ACN, 0.5% FA) and lyophilized using a vacuum centrifuge.

Prior to mass spectrometry peptides were reconstituted in 0.5% (v/v) FA and filtered through 0.22 μm PVDF filters (Millipore). Samples were analyzed via HPLC-MS/MS using an UltiMate 3000 nano HPLC system (Dionex) equipped with Acclaim C18 PepMap100 75 μm ID x 2 cm trap and Acclaim C18 PepMap RSLC, 75 μm ID x 50 cm separation columns coupled to a Q Exactive Plus (Thermo Fisher Scientific). Peptides were loaded on the trap column at a flow rate of 5 μL/min with aqueous 0.1% TFA and then transferred onto the separation column at 0.4 μL/min. Buffers for the nanochromatography pump were aqueous 0.1% FA (buffer A) and 0.1% FA in ACN (buffer
B). Samples were separated using a gradient raising buffer B from 5 to 22% in 112 min, followed by a buffer B increase to 32% within 10 min. Buffer B content was further raised to 90% within the next 10 min and held another 10 min at 90%. Subsequently buffer B was decreased to 5% and held until end of the run (total: 152 min). The MS instrument was operated in a TOP12 data dependent mode. MS full scans were performed at a resolution of 140,000 in the orbitrap and the scan range was set from 300 to 1,500 m/z. The AGC target was set to 3.0e6, the maximum ion injection time was 50 ms and internal calibration was performed using the lock mass option. Monoisotopic precursor selection as well as dynamic exclusion (exclusion duration: 60 s) was enabled. Precursors with charge states of >1 and intensities greater than 1.6e4 were selected for fragmentation. Isolation was performed in the quadrupole using a window of 1.6 m/z and an AGC target to 1.0e5 with a maximum injection time of 50 ms. Fragments were generated using higher-energy collisional dissociation (HCD, normalized collision energy: 27%) and detected in the orbitrap.

Peptide and protein identifications were performed using MaxQuant (version 1.6.0.1) with Andromeda (39) as search engine using following parameters: Carbamidomethylation of cysteines as fixed and oxidation of methionine as dynamic modifications, trypsin/P as the proteolytic enzyme, 4.5 ppm for precursor mass tolerance (main search ppm) and 0.5 Da for fragment mass tolerance (ITMS MS/MS tolerance). Searches were performed against the Uniprot database for S. aureus NCTC 8325 (taxon identifier: 93061, downloaded on 19.08.2019). Quantification was performed using MaxQuant’s LFQ algorithm (29). The “I = L”, “requantify” and “match between runs” (default settings) options were used. Identification was done with at least 2 unique peptides and quantification only with unique peptides. The mass spectrometry
proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (40) partner repository with the dataset identifier PXD018347.

For statistics with Perseus (version 1.6.0.0) (41), four biological replicates were analyzed. Putative contaminants, reverse hits and proteins, identified by side only, were removed. LFQ intensities were log₂(x) transformed and filtered to contain minimum three valid values in at least one condition. Missing values were imputed on the basis of a normal distribution (width = 0.3, down-shift = 1.8). P-values were obtained by a two-sided two sample t-test over the four biological replicates.

For pathway enrichment analysis using STRING (30), all proteins that showed an log₂(protein enrichment) ratio of compound vs. DMSO-treated control of more than 1 or less than -1 and a log₁₀(P-value) > 1 were entered into the database (https://string-db.org/, multiple proteins analysis) and resulting enriched pathways were inspected further.

The processed tables for protein group analysis in Perseus and the results of the STRING analysis have been uploaded to the supplementary information as an Excel file (Supplementary Data 2).
Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (40) partner repository with the dataset identifier PXD018347.

Acknowledgements

R.M. was supported by a doctoral fellowship of the Boehringer Ingelheim Fonds. S.A.S. was funded by the Center for Integrated Protein Science Munich (CIPSM) and Deutsche Forschungsgemeinschaft (DFG). M.W.H. and C.F. were funded by the Federal Ministry for Education and Research (BMBF) under the framework programme 'VIP+' – project 'aBacter'. We thank Philipp Le for critical discussions, Sabrina Schönberger for excellent experimental support, and Mona Wolff, Katja Bäuml, Katja Gliesche and Franziska Erdmann for excellent technical support.

Supplementary Information

The manuscript is supported by two Excel files as supplementary information.

Supplementary Data 1: MIC\textsubscript{90} raw data and information on tested strains.

(Tab 1) Table of contents of Supplementary Data 1.

(Tab 2) Origin, antibiotic resistance pattern (interpretation according to EUCAST guidelines) and molecular typing results of the clinical Staphylococcus strains used in this study.
Origin, antibiotic resistance pattern and molecular typing results of those clinical *Staphylococcus* strains with intermediate or high level resistance to TCC (MIC ≥ 6.25 μM).

(Tab 4) MIC raw data for all tested *Staphylococcus* strains. Data relate to Fig. 2A.

(Tab 5) Characteristics of the clinical *Enterococcus* strains used in this study.

(Tab 6) MIC raw data for all tested *Enterococcus* strains. Data relate to Fig. 2B.

**Supplementary Data 2:** Proteomic data table and STRING analysis.

(Tab 1) Table of contents of Supplementary Data 2.

(Tab 2) List of proteins found in the full proteome of *S. aureus* NCTC 8325 after treatment of bacterial cells with DMSO or TCC (0.5 μM), respectively. Average LFQ log₂ fold-change ratios and −log₁₀ P-values (t-test) are given. Data relate to Fig. 6A.

(Tab 3) List of proteins found in the full proteome of *S. aureus* NCTC 8325 after treatment of bacterial cells with DMSO or TCS (0.15 μM), respectively. Average LFQ log₂ fold-change ratios and −log₁₀ P-values (t-test) are given. Data relate to Fig. 6B.

(Tab 4) List of proteins that were significantly up- or downregulated (|log₂(protein ratio)| > 1 & -log(P-value) > 1) by either TCC or TCS treatment. Overlap between both treatments is indicated in yellow. Data relate to Fig. 6C.

(Tab 5) STRING network enrichment analysis for TCC treatment. Proteins that were significantly up- or downregulated (log₂(protein ratio) > 1 or < -1; -log(P-value) > 1) by proteomic analysis (see Tab 1 and Tab 3) were entered into the “multiple proteins analysis” tab of the online tool (https://string-db.org/) and the resulting significantly enriched pathways including the network map posted for up- and downregulation separately.
(Tab 6) STRING network enrichment analysis for TCS treatment. Proteins that were significantly up- or downregulated ($\log_2$ (protein ratio) $> 1$ or $< -1$; $-\log(P$-value) $> 1$) by proteomic analysis (see Tab 2 and Tab 3) were entered into the "multiple proteins analysis" tab of the online tool (https://string-db.org/) and the resulting significantly enriched pathways including the network map posted for up- and downregulation separately.

References


5. Liu JY, Qiu H, Morisseau C, Hwang SH, Tsai HJ, Ulu A, Chiamvimonvat N, Hammock BD. 2011. Inhibition of soluble epoxide hydrolase contributes to the anti-


Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet 367:731–739.


Figures

Figure 1 – Structures of the antibacterial agents triclocarban (TCC) and triclosan (TCS), structurally related PK150, and sorafenib (SFN). Their respective activities (minimal inhibitory concentrations) in S. aureus NCTC 8325 are displayed below.

Figure 2 – TCC exhibits a different activity spectrum compared to PK150. MIC determination of PK150 and TCC against a panel of 100 strains of staphylococci (A) and enterococci (B). MICs were tested up to a concentration of 25 μM and the distribution plotted for each compound. Dotted lines indicate the respective MIC₉₀ value, i.e. the concentration at which the growth of 90% of tested strains was inhibited. Experiments were performed in two biologically independent replicates, and for cases in which the MIC differed between the replicates the higher value was taken as resulting MIC. See Supplementary Data 1 for further details.

Figure 3 – Effects on membrane integrity and SpsB activation potential by TCC, TCS and PK150. (A) Membrane integrity assay analyzing the permeability of S. aureus NCTC 8325 cells upon treatment with TCC, TCS, PK150 or positive control benzalkonium chloride (BAC). Interaction of propidium iodide with DNA was measured over time. Data are representative for three biological replicates. (B) FRET-based SpsB activity assay with membrane-bound endogenous SpsB from S. aureus NCTC 8325 (200 μg/mL total protein concentration) and different concentrations of TCC, TCS, and PK150 (positive control). Substrate cleavage rates are normalized to DMSO-treated
samples. Data represent mean values ± s.d. of averaged triplicates of n = 4 biologically independent experiments.

**Figure 4 – Interference with menaquinone biosynthesis and inhibition of MenG by TCC and TCS.**

**(A)** MIC shift by addition of menaquinone-4 (MK-4) to the bacterial growth medium. MICs of TCC and TCS were determined in the presence of various concentrations of MK-4 as indicated. The data represent results from three biologically independent experiments performed in triplicates. Where the MIC varied between replicates, the MIC is given as a range as displayed by the extension of the data point.

**(B)** Metabolic profiling of endogenous menaquinone levels in *S. aureus* NCTC 8325 cells on compound treatment. Bacteria were treated with sub-inhibitory concentrations (0.1-fold to 0.5-fold of the respective MIC) of TCC, TCS, or ciprofloxacin (Cipro) and menaquinone-8 (MK-8) was extracted and quantified by LC–MS. MK-8 levels are normalized to DMSO-treated samples. Each color represents an individual, independent extraction experiment, where differently shaped symbols (circles, rectangles, triangles and diamonds) indicate independent biological samples. Error bars denote mean values ± s.d.

**(C)** Enzymatic assay monitoring the methylation of DMK-2 by cellular lysate of MenG-overexpressing *S. aureus* pRMC2-MenG (20 mg/ml total protein concentration). Production of MK-2 was quantified by LC–MS and normalized to the respective DMSO-treated samples. Data represent the averaged values ± s.d. from three independent experiments.

**Figure 5 – Inhibition of FabI by TCC, TCS and PK150.** Recombinant FabI was pre-incubated with NADPH and compounds at different concentrations, then the substrate t-o-NAC thioester was added and the decrease in absorbance at 340 nm was recorded.
Enzyme activity was determined by the slope at $t = 0$ and relative activity was calculated by normalization to the DMSO-treated control (100% activity) and the heat-inactivated negative control (0% activity). The data were subsequently fitted to a sigmoidal equation. For TCS the $IC_{50}$ was calculated to be $3.76 \pm 0.11 \mu M$. Data represent mean values ± s.d. of averaged triplicates from three independent experiments.

**Figure 6** – Full proteome analysis of *S. aureus* NCTC 8325 upon TCC and TCS treatment. (A,B) Volcano plots showing the log$_2$-fold change of protein levels in the full proteome of *S. aureus* treated with sub-inhibitory concentrations (0.5 x MIC) of TCC (A) or TCS (B). Colors denote pathways that were significantly enriched among downregulated proteins ($\log_2$(protein ratio) $< -1$ & $-\log(P$-value) $> 1$) by STRING (30) analysis. Blue dots represent the proteins associated with the arginine deiminase (ADI) pathway (see below) which were affected by TCC treatment. Green and brown dots represent proteins associated with the EIIB/EIIC phosphotransferase system and ABC transporter transmembrane domains, respectively. These terms were found to be enriched in the STRING analysis of TCS treatment. Pink circles represent all proteins – regardless of $P$-value – which were up- or downregulated ($|\log_2$(protein ratio)$| > 1$) by both TCC and TCS treatment. The only protein also meeting the significance cut-off ($-\log(P$-value) $> 1$) is shown in green (PTS system EIIBC component, Q2G1G5). The data represent average values and the $P$-values were calculated using a two-sided two-sample $t$-test; $n = 4$ independent experiments per group. See Supplementary Data 2 for further details. (C) Venn diagram showing overlap in protein up- or downregulation between TCC and TCS treatment. The respective circles indicate the total number of proteins for each stated case ($|\log_2$(protein ratio)$| > 1$ & $-\log(P$-value) $> 1$) (D) Table
identifying proteins associated with the ADI pathway (31) in the volcano plots in panels (A) and (B). All proteins are annotated within the KEGG pathway "arginine biosynthesis". (1), (3), and (4) are directly annotated as elements of the ADI pathway in S. aureus NCTC 8325 by GO (33). (2) is located in the same gene cluster as (1) and its homologs in related S. aureus strains (such as COL, MW2 or USA300) are annotated as part of the ADI pathway in Uniprot. (5) catalyzes the same reaction as (2), but according to its annotation seems to be involved in arginine biosynthesis rather than catabolism.
Triclocarban (TCC)  
Triclosan (TCS)  
PK150  
Sorafenib (SFN)  

MIC in *S. aureus* NCTC 8325  
1 μM  
0.3-0.5 μM  
0.3 μM  
3 μM
### Gene Expression Analysis

#### Comparison between TCC and DMSO conditions

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#### Bar Chart Analysis

- **A**: Scatter plot showing log2(protein ratio TCC/DMSO) vs. -log10(P-value).
- **B**: Scatter plot showing log2(protein ratio TCS/DMSO) vs. -log10(P-value).
- **C**: Venn diagram illustrating upregulation and downregulation:
  - TCC upregulation: 13
  - TCS upregulation: 7
  - TCC downregulation: 10
  - TCS downregulation: 41