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1 Antimicrobial activity of the quinoline derivative HT61 against *Staphylococcus aureus*
2 biofilms

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20

21 **Short Title**

22 Response of *S. aureus* biofilms to HT61

23

24 **Word count**

25 1687 words

26

27 **Abstract (73 words)**

28 *Staphylococcus aureus* biofilms are a significant problem in healthcare settings, in
29 part, owing to the presence of a non-dividing, antibiotic tolerant sub-population. Here
30 we evaluated treatment of *S. aureus* UAMS-1 biofilms with HT61, a quinoline
31 derivative shown to be effective against non-dividing *Staphylococcal spp.* HT61 was
32 effective in reducing biofilm viability, associated with increased expression of cell
33 wall stress and division proteins, confirming its potential as a treatment for *S. aureus*
34 biofilm infections.

35

36 **Keywords**

37 *Staphylococcus aureus*, biofilm, HT61, proteomics, antimicrobial tolerance

38

39 Antimicrobial tolerant *Staphylococcus aureus* biofilms are commonly associated with
40 chronic infections, particularly of the skin and soft tissue (1, 2). Biofilms are highly
41 heterogeneous, containing cellular sub-populations that are non-dividing and/or are
42 metabolically inactive. As a large proportion of clinically administered antimicrobials
43 target actively dividing cells this adopted quiescent state renders these
44 antimicrobials ineffective, thus allowing biofilm bacteria to survive therapeutic
45 intervention and contribute to chronic disease (3). Ineffective treatment can also
46 promote the evolution of resistance mechanisms within bacterial populations. In *S.*
47 *aureus*, commonly evolved resistance mechanisms can render β -lactams such as
48 penicillin, and glycopeptides such as vancomycin ineffective (MRSA and VRSA,
49 respectively) (4, 5). The combination of biofilm tolerance and evolved resistance
50 mechanisms means that the development of novel antimicrobials targeting biofilm
51 bacteria is highly desirable.

52

53 HT61 is quinoline derivative that has demonstrated efficacy against both dividing and
54 non-dividing planktonic cultures of *Staphylococcal spp.* (6–8). HT61 preferentially
55 binds to anionic staphylococcal membrane components, causing structural instability
56 within the membrane and cell depolarisation (6, 8). Given its effectiveness against
57 non-dividing cells, HT61 represents an ideal candidate for targeting the dormant sub-
58 populations present in *S. aureus* biofilms.

59

60 In this study, we investigated the efficacy of HT61 against established *in vitro* *S.*
61 *aureus* biofilms. We also utilised a quantitative label-free proteomic approach to
62 identify changes in protein expression following treatment of planktonic and biofilm
63 cultures with sub-inhibitory and inhibitory concentrations of HT61, to further elucidate
64 cellular processes linked to HT61's mechanism of action. Understanding its
65 mechanism of action further could provide insight into effective treatments for biofilm-
66 associated chronic infections.

67

68 *S. aureus* UAMS-1, a methicillin sensitive osteomyelitis isolate (9), was used in all
69 experiments. Susceptibility of planktonic and biofilm cultures of *S. aureus* to a range
70 of HT61 (Helperby Therapeutics) and vancomycin (Hospira Inc) concentrations (0.5
71 to 128 mg/L) was compared. HT61 is being developed as a topical agent and
72 vancomycin has been used extensively as a successful topical treatment for chronic
73 wounds and acute surgical site infections (10–12). All experiments were performed
74 in tryptic soy broth, (TSB, Oxoid), using a starting inoculum of 10^5 cells ml^{-1} , diluted
75 from an overnight culture. All cultures were performed at 37 °C, with agitation
76 (planktonic: 120 rpm, biofilm: 50 rpm).

77
78 Planktonic minimum inhibitory concentrations (MIC, minimum concentration to inhibit
79 growth) were obtained using the broth microdilution method (7) and minimum
80 bactericidal concentrations (MBC, concentration to elicit a 99.9% reduction in
81 viability) were obtained after subsequent plating and colony forming unit (cfu)
82 enumeration on tryptic soy agar (TSA). Biofilm MBCs were calculated as per Howlin
83 *et al* (2015) (13). Briefly, biofilms were cultured in Nunc-coated 6 well plates,
84 (Thermo-Fisher, UK), for 72 hours, with media replacements every 24 hours prior to
85 antibiotic treatment. Following 72 hours, spent media was replaced with TSB
86 containing the appropriate antibiotic dilution. Biofilms were incubated for a further 24
87 hours. The media was then removed, the biofilms rinsed twice with HBSS to remove
88 non-adhered cells, and the biofilms detached and suspended in 1 ml HBSS using a
89 cell scraper. Suspensions were serially diluted, plated onto TSA and cfus were
90 enumerated following a final 24 hour incubation.
91
92 The planktonic MIC and MBC values for HT61 were 16 mg/L and 32 mg/L
93 respectively in comparison to 4 mg/L for both the vancomycin MIC and MBC.
94 Towards biofilms, HT61 presented with improved killing of *S. aureus* UAMS-1
95 biofilms compared to vancomycin, demonstrated by a biofilm MBC half that of
96 vancomycin (32 mg/L compared to 64 mg/L). At the maximum concentration tested
97 (128 mg/L), HT61 caused a further 1.3 log reduction in CFUs compared to
98 vancomycin utilised at the same concentration (Figure 1). The mechanism of action
99 for vancomycin necessitates active cell wall turnover (14) so it is possible that its
100 reduced biofilm efficacy can be attributed to the presence of a dormant cell
101 subpopulation. As HT61 was equally effective against biofilms and planktonic

102 cultures, this may suggest that its activity against non-dividing cells, as per
103 references (6–8), confers an advantage against the biofilm phenotype.

104

105 The cellular response of planktonic and biofilm cultures following treatment with 0, 4
106 or 16 mg/L HT61 was then investigated using liquid chromatography mass
107 spectrometry^{Elevated Energy}, (UPLC/MS_E). These HT61 concentrations were chosen as
108 they were below the calculated planktonic and biofilm MBCs. Use of higher
109 concentrations would have been highly bactericidal and led to the accumulation of
110 dead cells and unwanted noise within the proteome datasets. Full details of the
111 proteomic methods, including the method of protein isolation and instrument settings
112 utilised, can be found in the supplementary methods. Briefly, planktonic cultures
113 were grown in TSB for 12 hours at 37 °C with the appropriate HT61 concentrations.
114 Biofilms were cultured for 72 hours as described, prior to replacement of the used
115 media with TSB supplemented with HT61 at the same concentrations. Biofilms were
116 then incubated for a further 12 hours before being harvested and suspended into 1
117 ml HBSS. Following mechanical lysis of the cells, proteins were extracted, purified
118 and normalised to a final concentration of 0.25 µg/µL in 3% acetonitrile, 0.1 % formic
119 acid (v/v).

120

121 Prepared samples were analysed using a Waters Synapt G2Si high definition mass
122 spectrometer coupled to a nanoAcquity UPLC system using 4 µl of peptide extract.
123 Processed data were searched against the Uniprot *S. aureus* MN8 reference
124 database (accessed 25/01/2018) and further analysed using a combination of uniprot
125 database searches (www.uniprot.org, accessed between 01/05/18 and 07/07/18)
126 and gene ontology analysis using GeoPANTHER(15). Each data set was normalised

127 to the top 200 most abundant proteins (per ng) and proteins were suitable for
128 quantitative analysis if the following inclusion criteria were met; present in all 3
129 biological replicates, false discovery rate (FDR) \leq 1%, sequence coverage \geq 5%.
130 Differential expression was defined as an expression fold-change of \geq 1.5 and \leq
131 0.667 with $p \leq$ 0.05, calculated using a one-tailed student t-test.

132

133 A total of 1,448 proteins were identified across planktonic and biofilm cultures. For
134 HT61 treated planktonic cultures, 568 (4 mg/L) and 495 (16 mg/L) proteins met the
135 inclusion criteria for quantitative analysis. For HT61 treated biofilm cultures, 461 (4
136 mg/L) and 498 (16 mg/L) proteins met the inclusion criteria (Table 1). HT61
137 treatment resulted in the differential expression of proteins involved in a variety of
138 functions including cell wall biosynthesis, DNA synthesis, and metabolism. (see
139 Tables S1 and S2). Interestingly, metabolic processes were generally decreased
140 which may be an attempt by the cell to limit HT61 damage, similar to the proteomic
141 response of MSSA to oxacillin (16).

142

143 Treatment of planktonic cultures with sub-MIC HT61 (4 mg/L), revealed the
144 upregulation of MurD and Murl, two cell wall biosynthesis associated proteins
145 required for the incorporation of D-glutamate into cell wall peptidoglycan (17) (Table
146 2). Increasing the concentration of HT61 from 4 mg/L to 16 mg/L led to upregulation
147 of 93% (14/15) of proteins associated with cell wall biosynthesis, including 6
148 components of the mur ligase pathway (MurACDEFI, 2.63 mean fold increase),
149 FemA-like protein and FemB, which are required for peptidoglycan crosslinking (2.53
150 mean fold increase) and a 2.19 fold upregulation of VraR, the regulator of the cell
151 wall stress (CWS) stimulon, which is activated following stress to the cell envelope

152 (18). Proteins associated with DNA synthesis were also affected by HT61 treatment
153 (Table 2). Sub-inhibitory treatment of planktonic cultures led to increased expression
154 of DnaA and DnaX, indicating a general rise in DNA synthesis (mean 1.84 fold
155 increase). Cell cycle associated proteins, FtsA and Obg were also upregulated
156 (mean 2.35 fold increase) and four downregulated (GpsB, GroL, Tig and DivIVA
157 domain protein, mean 0.28 fold decrease). Treatment with 16 mg/L HT61 led to the
158 increased expression of proteins associated with DNA maintenance, including three
159 protein with helicase activity (PcrA, GyrA and ParE).

160

161 Biofilms treated with HT61 presented with a similar, albeit more muted response
162 (Table 1). Notably, when treated with HT61 at 16 mg/L, increased expression was
163 observed for both MurD (1.59 fold) and PcrA (2.13 fold), similar to planktonic cultures
164 (Table 2). It is possible that the response across both planktonic and biofilm cultures
165 is a result of SOS response activation. The SOS response is activated upon DNA
166 damage and due to its quinolone-like structure, it is possible that HT61 is
167 moonlighting as a DNA gyrase inhibitor, or other SOS-response inducer, leading to a
168 cellular response much like that induced by quinolone antimicrobials, such as
169 ciprofloxacin (19–21).

170

171 As well as being part of the CWS stimulon, a number of the differentially expressed
172 cell wall biosynthesis components, DNA synthesis/maintenance genes and cell cycle
173 components comprise a segment of the division cell wall, *dcw* cluster, a family of
174 genes that are vital for maintaining cell shape and integrity (22, 23). Previous studies
175 have shown that HT61 preferentially binds to anionic phospholipids in the *S. aureus*
176 cell membrane, in a manner similar to the lipopeptide antimicrobial, daptomycin (8,

177 24, 25). Daptomycin inserts into the cell membrane, leading to alterations in
178 membrane curvature, potassium efflux and membrane depolarisation (24, 25), with
179 membrane curvature shown to impair cell wall synthesis by affecting the cell wall
180 biosynthesis protein, MurG (26). In addition, transcriptional profiling has also shown
181 that daptomycin upregulates components of the cell wall stimulon, suggesting a
182 secondary mechanism of action and/or interactions with the associated components
183 (27). Altered expression of the *dcw* cluster has also been documented in biofilms of
184 *Haemophilus influenzae* following D-methionine treatment, contributing to altered cell
185 morphology (22). It is possible that HT61 functions in a similar manner to these
186 examples, either by directly interfering with cell wall biosynthesis machinery or
187 placing stress directly on the cell membrane, interfering with the cell wall machinery.

188

189 To conclude, we have demonstrated that HT61 is more effective than vancomycin at
190 treating *in vitro* biofilms of *S. aureus*, although whether this translates to efficacy *in*
191 *vivo* needs to be determined. Furthermore, the safety and tolerated dose of HT61 will
192 need to be evaluated in order to determine whether it is a superior therapy to
193 vancomycin in a clinical setting. We have also shown that HT61 influences the
194 expression of the CWS stimulon, *dcw* cluster, in line with its predicted mechanism of
195 action. Similar to other quinoline-like compounds it may also stimulate the SOS
196 response.

197 **Acknowledgements**

198 Proteomic data is available at the following: <https://doi.org/10.5258/SOTON/D0619>
199 Statistical analyses were performed using R version 3.6.0 and figures were plotted
200 using ggplot2 and cowplot(28–30).

201

202 **Source(s) of Support**

203 This work was funded by a Biotechnology and Biological Sciences Research Council
204 CASE Studentship award in partnership with Helperby Therapeutics,
205 (BB/L016877/1). Instrumentation in the Centre for Proteomic Research is supported
206 by the BBSRC (BM/M012387/1) and the Wessex Medical Trust.

207

208 **Declarations of Interest**

209 YH and ARMC are shareholders in Helperby Therapeutics Group plc. YH is the
210 Director of Research and ARMC is a company founder and the Chief Scientific
211 Officer.

212

213 **Ethical Approval**

214 Not required.

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327

Table 1: Summary of differential protein expression between untreated, sub-MIC (4 mg/L), and MIC (16 mg/L) treated *S. aureus* planktonic and biofilm cultures. Inclusion criteria for quantitative analysis and comparison was set at 3 peptide matches, false discovery rate (FDR) $\leq 1\%$, sequence coverage $\geq 5\%$, with $p \leq 0.05$.

Planktonic				
HT61 Concentration	Unchanged	Up Regulated	Down Regulated	Total
4 mg/L	540 (88.7%)	39 (6.9%)	25 (4.4%)	568
16 mg/L	270 (54.5%)	103 (20.8%)	122 (24.6%)	495

Biofilm				
HT61 Concentration	Unchanged	Up	Down	Total
4 mg/L	436 (94.6%)	3 (0.7%)	20 (4.3%)	461
16 mg/L	472 (94.8%)	9 (1.8%)	17 (3.4%)	498

Table 2: Differentially expressed proteins associated with the *dcw* and cell wall stimulon in *S. aureus* following treatment of planktonic cultures with HT61. Expression ratios reflect changes in expression between untreated cultures and those treated with either sub-MIC (4 mg/L) or MIC (16 mg/L) concentrations of HT61. Differential expression in biofilms indicated in brackets. Differential expression is defined as a fold change ≥ 1.5 for upregulation (green cells) and ≤ 0.667 for down regulation (red cells). Grey cells indicate no change in expression. Empty cells – proteins not identified.

	Accession Number	Protein Name	Gene	Expression Ratio	
				Sub-MIC	MIC
Cell Cycle	A0A0E1X830_STAAU	Cell division protein FtsA	<i>ftsA</i>	1.38	1.66
	A0A0E1X718_STAAU	GTPase Obg	<i>cgtA</i>	1.30	3.04
	A0A0E1X5J2_STAAU	Cell cycle protein GpsB	<i>gpsB</i>	1.10	0.20
	A0A0E1XAY0_STAAU	60 kDa chaperonin	<i>groL</i>	1.13	0.29
	A0A0E1XGT1_STAAU	DivIVA domain protein	HMPREF0769_12587	1.05	0.29
	A0A0E1X4P6_STAAU	Trigger factor	<i>tig</i>	1.01	0.34
Cell Wall Biosynthesis	A0A0E1XH19_STAAU	DltD central region	<i>dltD</i>	1.78	2.51
	A0A0E1X5R6_STAAU	FemAB family protein (FemA)	HMPREF0769_12373 (<i>femA</i>)	1.05	1.82
	A0A0E1XIT0_STAAU	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	<i>murA1</i>	0.98	2.05
	A0A0E1XAN0_STAAU	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	<i>murA2</i>	1.12	2.83
	A0A0E1X4D8_STAAU	UDP-N-acetylmuramate--L-alanine ligase	<i>murC</i>		2.40
	A0A0E1X8P8_STAAU	UDP-N-acetylmuramoylalanine--D-glutamate ligase	<i>murD</i>	1.84	3.43 (1.59 Biofilm)
	A0A0E1X6V3_STAAU	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--L-lysine ligase	<i>murE</i>	1.05	1.76
	A0A0E1XIV1_STAAU	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	<i>murF</i>	1.33	2.31
	A0A0E1X8U4_STAAU	Glutamate racemase	<i>murI</i>	1.52	3.62
	A0A0E1XKB3_STAAU	Ribulose-5-phosphate reductase	<i>tarJ</i>	1.12	2.58
	A0A0E1XJG3_STAAU	Response regulator protein VraR	<i>vraR</i>		2.19
	A0A0E1X974_STAAU	Mur ligase middle domain protein	HMPREF0769_11280	1.32	2.67
	A0A0E1X785_STAAU	D-alanine--D-alanyl carrier protein ligase	<i>dltA</i>	1.15	1.92

	A0A0E1XG48_STAAU	Aminoacyltransferase FemB	<i>femB</i>	0.99	3.24
	A0A0E1X6S7_STAAU	Mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase	HMPREF0769_12730	0.89	0.63
DNA Maintenance/Synthesis	A0A0E1XAS7_STAAU	ATP-dependent DNA helicase	<i>pcrA</i>	1.31	3.07 (2.13 Biofilm)
	A0A0E1X928_STAAU	DNA ligase	<i>ligA</i>	1.29	1.73
	A0A0E1XAK8_STAAU	Chromosomal replication initiator protein DnaA	<i>dnaA</i>	2.07	2.90
	A0A0E1XB29_STAAU	DNA polymerase III subunit gamma/tau	<i>dnaX</i>	1.60	2.07
	A0A0E1X6I5_STAAU	DNA polymerase I	<i>polA</i>	1.37	1.51
	A0A0E1XAK2_STAAU	DNA gyrase subunit A	<i>gyrA</i>	1.12	1.55
	A0A0E1X7H6_STAAU	DNA topoisomerase 4 subunit B	<i>parE</i>	1.30	3.34
	A0A0E1XFV3_STAAU	DNA-binding protein HU	<i>hup</i>	0.91	0.33
	A0A0E1X9G8_STAAU	Nucleoid-associated protein HMPREF0769_10004	HMPREF0769_10004		0.15

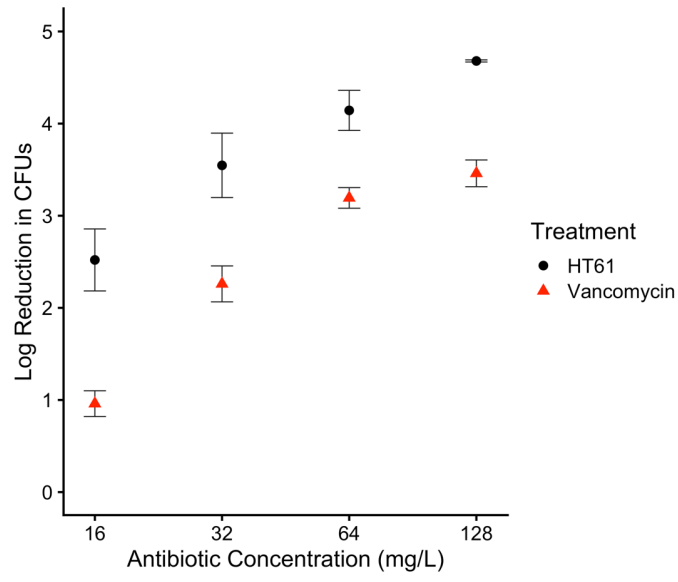


Figure 1: Log Reduction in *S. aureus* UAMS-1 viable counts of an established 72 hour biofilm following treatment with HT61 and vancomycin. HT61 consistently elicited a greater log reduction in CFU counts than vancomycin, demonstrating its potential as an antibiofilm agent. A higher value indicates a greater log reduction in CFUs. $n = 3$. Error bars indicate standard deviation. Statistical analyses were performed using R version 3.6.0 and figures were plotted using ggplot2 and cowplot [25–27]