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1 The use of quorum sensing inhibitors to interfere with biofilm formation and development in
2 *Burkholderia multivorans* and *Burkholderia cenocepacia.*
3
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Abstract:

Burkholderia cepacia complex strains are opportunistic pathogens, causing life-threatening infections in CF patients. *Burkholderia cepacia* complex strains are resistant to many antimicrobial agents and commonly produce biofilms *in vitro* and *in vivo*. This contributes to their virulence and makes *Burkholderia* infections difficult to treat. Recently, the quorum sensing system of *Burkholderia* spp. has been found to affect their biofilm forming ability, making it an attractive target for antimicrobial therapy. However, detailed information about the antibiofilm effect of these compounds is still lacking. In the present study we evaluated the anti-biofilm effect of several known quorum sensing inhibitors. The effect on *Burkholderia* spp. biofilm formation was examined using crystal violet, resazurin and SYTO9 staining, confocal laser scanning microscopy as well as plating. Used in sub-inhibitory concentrations, several compounds interfered with biofilm formation by *Burkholderia* spp. Our results suggest that the quorum sensing inhibitors affect later stages of biofilm formation and detachment.

Keywords: *Burkholderia cepacia* complex; biofilms; quorum sensing

1 **1. Introduction**

2 *The Burkholderia cepacia* complex (Bcc) is a group of closely related Gram-negative bacteria
3 [6]. Bcc species are opportunistic pathogens causing severe infections in patients with
4 immune suppression, chronic granulomatous disease and cystic fibrosis (CF) [21]. Although
5 all Bcc species are found in CF patients, overall *Burkholderia multivorans* and *Burkholderia*
6 *cenocepacia* predominate [20; 38]. Bcc species are capable of forming biofilms *in vitro* and *in*
7 *vivo* [8], which probably plays an important role in virulence [35]. In addition, bacteria
8 growing in biofilms are less susceptible to many antibacterial agents [5]. For these reasons,
9 novel approaches to treat these biofilm-related infections are needed. A possible novel target
10 is the bacterial communication system [37]. Bacteria monitor their population density through
11 the production and sensing of small signal molecules called autoinducers [3]. This
12 communication system, known as quorum sensing (QS), consists of three components: signal
13 molecules, signal synthases and signal receptors. At low population density only basal
14 amounts of diffusible signal molecules are produced, not provoking an effect. However, when
15 the population density is sufficiently high, signal molecules will bind to a receptor and this
16 signal/receptor complex will then induce or repress the transcription of QS regulated genes.
17 Several N-acyl-homoserine-lactone (AHL)-dependent QS systems have been reported in
18 *Burkholderia* species [37]. In most Bcc species, CepI catalyzes the production of two AHL's,
19 N-octanoyl-homoserine lactone (C8-HSL) (in high amounts) and N-hexanoyl-homoserine
20 lactone (C6-HSL) (in lower amounts). *B. cenocepacia* epidemic strains contain a second QS
21 system, CciIR [2]. The major signal molecule involved in the latter is C6-HSL [22]. In some
22 *Burkholderia* spp. a quinolone dependent QS system can also be found [42]. This type of
23 communication system was previously described in *Pseudomonas aeruginosa* and makes use
24 of hydroxy-alkylquinolines (HAQ) as signal molecules [30]. It has recently been discovered
25 that HAQs produced by *Burkholderia* spp. predominantly contain an unsaturated aliphatic

side chain and are typically methylated [42]. In several *Burkholderia* spp., the QS system is presumably involved in biofilm formation [8; 14; 41]. Hence, QS inhibitors have been proposed as potential novel antibiofilm agents [37]. Since these QS inhibitors are typically used in concentrations below the minimal inhibitory concentration (MIC), it is less likely that they would impose a selective pressure for the development of resistance. Many studies have been conducted to find natural and synthetic QS inhibitors [9-10; 15-16; 18; 26; 31-32; 34; 40; 44]. However, detailed information about the antibiofilm effect of these compounds is still lacking. In the present study, we examined the antibiofilm effect of several established QS inhibitors in *B. multivorans* and *B. cenocepacia*, the two Bcc species most often responsible for infections in CF patients.

1 **2. Materials and Methods**

2 *2.1. QS inhibitors*

3 Cinnamaldehyde [26], resveratrol [43], L-canavanine [16], 4-nitropyridine N-oxide, p-
4 benzoquinon and indole [31], azithromycin [40], ceftazidime hydrate and tobramycin [10],
5 farnesol [9], (-)- epigallocatechin gallate and (+)- catechin hydrate [15], 2-amino-4-
6 chlorobenzoic acid (4CABA) , 2-amino-6-chlorobenzoic acid (6CABA) and 2-amino-6-
7 fluorobenzoic acid (6FABA) [18], curcumin [34], baicalein, baicalin hydrate and esculin
8 hydrate [44] were purchased from Sigma-Aldrich (Bornem, Belgium). Esculetin [44] and
9 N'3-(2-thienylcarbonyl)-4-bromo-1,5-dimethyl-1H-pyrazole-3-carbohydrazide ("compound
10 1") [32] were purchased from Acros Organics (Geel, Belgium). N'-(6-tert-butyl-2,3-dihydro-
11 2-methylpyridazin-4-yl)-5-chlorothiophene-2-carbohydrazide ("compound 3") was
12 synthesized as previously described [32]. All compounds were diluted in 0.5 % DMSO. A
13 control solution (CS) containing only DMSO was made in MilliQ water. The stock solutions
14 were stored at -20°C.

15

16 *2.2. Strains and culture conditions*

17 *Chromobacterium violaceum* CV026 [24] and *Escherichia coli* JB523 [1] were grown
18 aerobically on Luria Bertani (LB) agar (BD, Sparks, MD, USA) containing 20 µg/ml
19 kanamycin or 100 µg/ml tetracycline, respectively. For *E. coli* JB523 the medium contained 4
20 % NaCl. The QS-inhibition (QSI) selector *P. aeruginosa* QSIS2 [31] was cultured aerobically
21 in ABT minimal medium (AB medium, containing 2.5 mg/l of thiamine) supplemented with
22 0.5 % glucose, 0.5 % casamino acids and 80 µg/ml gentamycin. *B. cenocepacia* strains
23 LMG18828 and LMG16656 (containing a CepI/R and CciI/R QS system) and *B. multivorans*
24 strains LMG13010 and LMG17588 (containing the CepI/R QS system) were cultured
25 aerobically on Tryptone Soya Agar (Oxoid, Hampshire, UK) or in Scientific Cystic Fibrosis

1 Medium (SCFM) [28]. All strains were grown at 37°C, with the exception of *E. coli* JB523
2 (30°C) and *C. violaceum* CV026 (27°C).
3
4 *2.3. Determination of the minimal inhibitory concentration (MIC)*
5 MIC's were determined using a modified microdilution assay in 96-well microtiter plates
6 (MTP), using the media mentioned above [13]. The inoculum, prepared from a 24 h old
7 culture contained approximately 10^6 CFU/ml in double concentrated medium. The
8 compounds stock solutions were diluted in MilliQ water in a series of twelve 2-fold dilutions
9 in the wells of a 96 well microtiter plate (MTP) (TPP, Trasadingen, Switzerland). Two rows
10 of dilutions were made for each compound and the volume of each sample was 100 µl. To the
11 wells of one row of each compound, 100 µl inoculum was added. To the other row, 100 µl
12 double concentrated medium was added as a negative control. The plates were incubated for
13 24 h and the absorbance at 590 nm was measured using a Victor Wallac^2 multilabel counter
14 (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). The absorbances of the
15 negative controls were subtracted from those of the corresponding inoculated wells to give net
16 absorbances. The plates were incubated for 24 h. For all further experiments, a single sub-
17 MIC concentration was selected for each compound, being the highest concentration that did
18 not affect growth in any of the strains included in the present study.
19
20 *2.4. QS inhibition (QSI) assays*
21 *C. violaceum* CV026 is a mini-Tn*5* mutant of ATCC 31532 allowing the detection of
22 violacein in response to C6-HSL QS activation [24]. The *C. violaceum* CV026 QSI assay was
23 conducted as previously described [23]. In brief, an overnight culture of the reporter strain
24 was diluted in fresh sterile LB medium to an OD_{590nm} = 0.1 and 100 µl of this cell suspension
25 was added to the wells of a 96-well MTP (Perkin Elmer). Kanamycin was added to a final

1 concentration of 20 µg/ml. Activation of QS by AHL was tested by addition of C6-HSL (2.5
2 µM) (Sigma). Sterile MilliQ water served as a negative control. To study inhibition using *C.*
3 *violaceum* CV026, test compounds were added in selected sub-MIC concentrations. The MTP
4 was then incubated for 48 h at 27°C to allow induction of violacein formation. The plates
5 were then dried at 60°C until all medium had evaporated (6 h). The violacein was
6 resolubilized by adding 100 µl of DMSO to each well and the plates incubated on a lab shaker
7 for two hours. The absorbance was measured at 590 nm using a Victor Wallac² multilabel
8 counter (Perkin Elmer). *E. coli* JB523 contains the pJBA130 plasmid (expressing the green
9 fluorescent protein GFP in response to C6-HSL) [1]. The *E. coli* JB523 QSI assay was carried
10 out in a 96-well MTP. In brief, an overnight culture of the reporter strain was diluted in fresh
11 sterile LB medium containing 4 % (w/v) NaCl to an OD_{590nm} = 0.1 and 100 µl of this cell
12 suspension was added to the wells of a black 96-well MTP (Perkin Elmer). Tetracycline was
13 added to a final concentration of 100 µg/ml. Activation of QS by AHL was tested by addition
14 of C6-HSL (5 µM) (Sigma). Sterile MilliQ water served as a negative control. To study
15 inhibition using *E. coli* JB523, test compounds were added in selected sub-MIC
16 concentrations. The MTP was then incubated for 24 h at 30°C and fluorescence from GFP
17 expression was measured at λ_{ex} 475 nm and λ_{em} 515 nm using a Victor Wallac² multilabel
18 counter (Perkin Elmer). The QSI selector strain *QSIS2,* previously developed by Rasmussen
19 et al. (2005) is a *P. aeruginosa lasI rhlI* double mutant harbouring pLasB-SacB1 encoding an
20 AHL-induced killing system. A *QSIS2* QSI assay was developed in microtiter format based
21 on the plate assay described by Rasmussen et al. (2005). An overnight culture of the reporter
22 strain was diluted in fresh sterile ABT medium to an OD_{590nm}= 0.1 and 50 µl of this cell
23 suspension was added to the wells of a 96-well MTP. Fifty µl LB supplemented with sucrose
24 (56 mg/ml) was added to each well. Gentamycin was added in a concentration of 80 µg/ml.
25 Activation of QS by AHL's was tested by addition of 3-oxo-C12-HSL and C4-HSL (Sigma)

1 (200 nM each) to the wells. Sterile MilliQ water served as a negative control. To study
2 inhibition using *QSIS2*, test compounds were added in selected concentrations. The MTP was
3 then incubated for 10 h at 37°C and the absorbance was measured at 450 nm using a Victor
4 Wallac² multilabel counter. The difference in growth of the negative control (without addition
5 of compound and without AHL) and that of the positive control (without addition of
6 compound and with AHL) was set at 100 %. Each compound was tested six times in each
7 assay and each assay was repeated at least six times ($n \geq 36$).
8
9 *2.5. Biofilm formation in 96-well MTP*
10 Biofilms were formed as previously described [4]. In brief, the Bcc strains were grown
11 overnight in SCFM, centrifuged, resuspendend in double concentrated SCFM (2xSCFM) and
12 diluted to an $OD_{590nm} = 0.1$ in 2xSCFM. Fifty µl of the diluted bacterial suspension was
13 transferred to the wells of a round-bottomed 96-well microtiter plate (TPP). Negative controls
14 received 50 µl of CS. Positive controls received 50 µl of the test compound. Bacteria were
15 allowed to adhere and grow without agitation for 4 h at 37°C. After 4 h, plates were emptied
16 and washed with sterile physiological saline (PS). After this washing step, negative control
17 wells were filled with 50 µL 2xSCFM and 50 µl CS. Other wells were filled with 50 µl
18 2xSCFM and 50 µl compound solution and the plate was incubated for 24 h at 37°C. The
19 biomass was quantified by crystal violet (CV) staining, as described previously [29]. In brief,
20 plates were rinsed with sterile PS, biofilms were fixed by adding 100 µl 99 % methanol for 15
21 min, after which the methanol was removed and plates were air-dried. Biofilms were then
22 stained with 100 µl CV (Pro-lab Diagnostics, Richmond Hill, ON, Canada). After 20 min, CV
23 was removed and wells were filled with 150 µl 33 % acetic acid (Sigma-Aldrich). The
24 absorbance was measured at 590 nm using a Wallac Victor² multilabel counter.
25 Quantification of the number of metabolically active (i.e. living) cells in the biofilm was done

1 using resazurin assay (CellTiter-Blue CTB) [29]. In brief, wells were rinsed after 24 h biofilm
2 formation and 100 µl PS was added, followed by addition of 20 µl CellTiter-Blue (CTB)
3 (Promega, Leiden, The Netherlands) solution. After 60 min, fluorescent (excitation and
4 emission filters of 486 nm and 535 nm) was measured using a Wallac Victor^2 multilabel
5 counter. Each compound was tested six times in each assay and each assay was repeated at
6 least four times ($n \geq 24$).
7
8 *2.6. Biofilm formation on silicone disks and evaluation of the effect on biofilms*
9 For the quantification of the number of adhered cells and number of detached cells, *B.*
10 *cenocepacia* LMG16656 was allowed to form biofilms on silicone disks (Q7-4735; Dow
11 Corning) in a 24-well plate in the presence and absence of test compounds. In brief, 500 µL of
12 the diluted bacterial suspension was transferred to the wells of a 24-well plate (TPP,
13 Trasadingen, Switzerland) containing a sterile silicone disk. Subsequently, 500 µl of the test
14 compound was added. To control wells we added 500 µl CS. Bacteria were allowed to adhere
15 and grow without agitation for 4 h at 37°C. After 4 h, the growth medium was removed, the
16 disks were rinsed with 0.9 % (w/v) NaCl and 500 µl 2xSCFM and 500 µl test compound was
17 added. The plate was further incubated for 24 h at 37°C. The number of culturable sessile
18 cells and the total number of cells present on the silicone disks were determined after 4 h and
19 24 h by plating as previously described [7]. In brief, each disk was transferred to 10 ml 0.9 %
20 (w/v) NaCl. The tubes were subjected three times to 30 s of sonication (Branson 3510,
21 42 kHz, 100 W; Branson Ultrasonics Corp., Danbury, CT) and 30 s of vortex mixing to
22 remove the biofilm cells from the disks. Using this procedure, all cells are removed from the
23 disks and clumps of cells were broken apart (data not shown). The number of sessile
24 *Burkholderia cenocepacia* LMG16656 cells was quantified by plating on TSA. All plates
25 were incubated at 37°C for 24 h and the number of CFU per disk was calculated by counting

colonies on the plates. To quantify the number of bacteria released from the biofilms on the silicone disks and the number of cells adhering to the disk, the number of cells in the medium and the rinsing solution after 4 and 24 h was determined by plating. Each compound was tested on three disks in each assay and each assay was repeated at least three times ($n \geq 9$).

2.7. Confocal laser scanning microscopy (CLSM)

Biofilms of *Burkholderia cenocepacia* LMG16656 were formed on silicone disks as described above. The effect of “compound 3” on biofilm formation was evaluated after 4 h of adhesion and after 24 h of biofilm formation. For CLSM, the biofilm-covered disks were placed in the wells of a 24-well plate (Greiner bio-one, Longwood, FL, USA) and covered with 1 ml of PS containing 3 µl of SYTO9. The plates were incubated for 15 min at room temperature and the biofilm was visualised with a Nikon C1 confocal laser scanning module attached to a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels, Belgium) equipped with a Plan Apo VC 60x 1.4 NA oil immersion objective and suitable optical elements to obtain fluorescent and differential interference contrast (DIC) transmission images. SYTO9 was excited with the Argon ion 488 nm laser line and emission light was collected using a 500-530 nm band pass filter and Z-stacks were recorded. Tests were performed on at least two disks for each situation and representative images are shown.

2.8. Statistics

The normal distribution of the data was checked using the Shapiro-Wilk test. Normally distributed data were analyzed using an Independent samples T-tests. Non-normally distributed data were analyzed using the Mann-Whitney U test. Statistics were performed using the SPSS software, version 15.0 (SPSS, Chicago, IL, USA).

1 **3. Results**

2 *3.1. AHL-QSI*

3 The MICs for all compounds were determined (data not shown). In all further experiments,
4 the compounds were used in concentrations that were below this MIC. In order to confirm
5 QSI, we retested the 22 QS inhibitors in the three QSI detection systems (Table 1). Maximum
6 levels of inhibition in the three assays were found for the following compounds: esculetin (55
7 ± 23 %) (*C. violaceum* CV026 QSI assay), esculetin (50 ± 20 %), baicalin hydrate (50 ± 7 %)
8 and p-benzoquinon (41 ± 17 %) (*E. coli* JB523 QSI assay), esculetin (67 ± 2 %), “compound
9 3” (58 ± 4 %), “compound 1” (38 ± 17 %) and esculin hydrate (29 ± 8 %) (*P. aeruginosa*
10 QSIS2 QSI assay).

11

12 *3.2. Quantification of the effect of QSI on biofilm formation in 96-well MTPs*

13 There was a significant decrease in biofilm formation relative to the control when the
14 different strains were grown in the presence of various QS inhibitory compounds (Table 2).
15 The use of “compound 3” (500 µM), baicalin hydrate (500 µM) and farnesol (2500 µM)
16 resulted in a reduction of biofilm biomass in all strains tested, as quantified by CV staining.
17 The biofilm biomass in any of the four strains was not reduced by 2-amino-6-chlorobenzoic
18 acid, 2-amino-4-chlorobenzoic acid and (+)- catechin. The 16 other compounds affected the
19 biofilm biomass in at least one of the strains tested. Using CTB-staining, nine out of the 22
20 compounds were found to reduce the number of metabolically active biofilm cells in all four
21 strains tested. In addition, all 22 compounds significantly affected the number of
22 metabolically active cells in at least one strain. In contrast to what was observed for the other
23 compounds, the use of tobramycin (2 µM) resulted in a moderate but significant increase in
24 biomass and numbers of metabolically active cells in *B. multivorans* LMG17588 and *B.*
25 *cenocepacia* LMG18828.

1

2 *3.3. Effect of QS inhibitors on sessile cell numbers and detachment*

3 After 4 h, the number of cells present on the disks and in the growth medium did not
4 significantly differ between the treated and untreated disks (Table 3). However, significantly
5 more cells were removed during the rinsing step after 4 h from the disks treated with
6 azithromycin, cinnamaldehyde, farnesol and indole. After 24 h, significantly fewer cells were
7 present on the silicone disks treated with QS inhibitory compounds compared to the control
8 (Table 3). In contrast, after 24 h a significantly higher number of colony forming units (CFU)
9 was present in the growth medium of the biofilms treated with azithromycin, ceftazidime,
10 "compound 3", farnesol and indole compared to the growth medium of the control biofilm
11 (Table 3). The number of cells removed during the final rinsing step was significantly higher
12 for the biofilms treated with azithromycin, cinnamaldehyde and farnesol in comparison to the
13 control (Table 3). Total numbers of CFU (both sessile and planktonic) were not significantly
14 different after 4 h and 24 h of growth.

15

16 *3.4. Confocal scanning laser microscopy*

17 Consistent with the results obtained by plating, no differences between the treated biofilm and
18 the untreated control were observed after 4 h of adhesion (Fig 1A). However, after 24 h
19 CLSM revealed clear differences in *B. cenocepacia* LMG16656 biofilm structure between
20 biofilms treated with "compound 3" (500 µM) and the untreated control (Fig 1B), as the
21 former biofilm was less structured and contained less cells.

22

23

24

25

1 **4. Discussion**

2

3 *4.1. AHL-QSI*

4 Since the compounds used in this study were previously reported to affect QS in at least one
5 QS reporter, we expected that they would exhibit an effect in at least one of the QSI detection
6 systems used in this study. However, we often observed only a moderate QS-inhibitory effect.
7 This may be due to the lower concentrations used in the present study in order to perform
8 experiments with sub-MIC concentrations for all test strains. A second possible explanation
9 concerns the mechanism by which the compounds affect the QS system. For example,
10 azithromycin reportedly affects several QS regulated phenotypes in *P. aeruginosa* [12; 40] by
11 decreasing LasI and RhlI gene expression. However, since the biosensor strains used in the
12 present study are unable to produce signal molecules themselves, the latter were externally
13 added so that no effect at the level of the biosynthesis of signal molecules can be observed.
14 Recently, Skindersoe et al. (2008) found that several antibiotics, including azithromycin and
15 ceftazidime influenced QS in *P. aeruginosa* by changing membrane permeability, thereby
16 influencing the uptake of signal molecules. However, this effect could not be confirmed in the
17 present study. A third explanation for the inability of some compounds to interfere with QS in
18 these detection systems could lie in the specificity of the detection system and/or of the
19 compound. For example, cinnamaldehyde seems to have no effect on *P. aeruginosa* QS, using
20 the *QSIS2* detection system, while moderate effects were observed in the other two QS
21 detection systems. Niu et al. (2006) previously showed that cinnamaldehyde interfered with
22 the binding of short chain signal molecules (like C6-HSL used as a signal molecule in *E. coli*
23 JB523 and *C. violaceum* CV026 detection system), but that it failed to substantially reduce the
24 binding of longer chain signal molecules like 3-oxo-C12-HSL. In addition, farnesol and
25 various halogenated benzoic acids were previously found to influence PQS related QS

1 systems in *P. aeruginosa*, but the systems used here only detect a direct effect on the
2 interaction between AHL signal molecules and the AHL-receptor. However, it should be
3 noted that 2-amino-6-chlorobenzoic acid affect QS in the *E. coli* JB523 QSI detection system
4 and that 2-amino-6-fluorobenzoic acid inhibited QS in both the *E. coli* JB523 and *C.*
5 *violaceum* CV026 QSI systems.
6
7 *4.2. Effect of QS inhibitors on biofilms*
8 Results from several reports suggest that targeting QS could be a new strategy to fight biofilm
9 infections [25]. However, only few QS inhibitory compounds have been evaluated in sub-
10 MIC concentrations for their anti-biofilm effect against Bcc biofilms. Several QS inhibiting
11 polyphenols and "compound 3" influenced biofilm formation of *B. cenocepacia* H111 [15;
12 32]. For the other QS inhibitory compounds the effect on biofilms has not been evaluated at
13 all or only on non-Bcc species [12; 17; 34; 39; 44]. For this reason we wanted to explore
14 whether QS inhibitory compounds (when used in sub-MIC concentrations) could affect Bcc
15 biofilm formation in this study. To this end we selected 22 established QS inhibitory
16 compounds and evaluated their antibiofilm effect against the two Bcc species most often
17 involved in CF infections. As can be seen in Table 2, 21 out of 22 components tested resulted
18 in a reduction in biofilm formation in at least one strain. Interestingly, several compounds
19 were shown to affect biofilm formation in all the strains tested, while others did so in one or a
20 few strains only. Most of the compounds reduced the total biofilm biomass (as quantified by
21 CV staining), as well as the number of metabolically active cells (as quantified by CTB
22 staining). However, some compounds that yielded in a reduction of the number of living cells
23 failed to reduce the total biomass (e.g. (+)-catechin). The use of tobramycin (2 µM) resulted
24 in an increase in biofilm biomass and in the number of metabolically active cells in two
25 strains, i.e. *B. multivorans* LMG17588 and *B. cenocepacia* LMG18828. This increase was

1 previously also observed for *P. aeruginosa* [11]. Since all compounds were tested in sub-MIC
2 concentrations, the reduction in metabolically active cells can not be explained by lethal
3 effects. Indeed, when using SYTO9 (a DNA-binding dye that stains both living and dead
4 cells) a reduction in fluorescence was observed in treated biofilms. As the fluorescence of
5 dead cells is approximately equal to that of living cells, this reduction suggests that treatment
6 with QS inhibitors results in lower cell numbers through reduced attachment and/or increased
7 detachment. This was investigated by determining the number of CFU present on the disks
8 (after 4 and 24 h of growth), by determining the number of CFU present in the surrounding
9 growth medium and in the rinsing solution (after 4 h and 24 h) and by visualizing the biofilms
10 with CSLM (after 4 and 24 h of growth). Significantly lower amounts of cells were indeed
11 present on the disks treated with the compound after 24 h, while no differences were observed
12 after 4 h. In addition, more detached cells were found in the growth medium and rinsing
13 solution after 24 h. These data suggest that the QS inhibitors do not exert their effect during
14 initial stages of attachment, but rather promote detachment at later stages. This is in
15 agreement with previously published data. Huber et al. (2001) found no difference in initial
16 attachment for a *B. cenocepacia* H111 CepIR mutant compared to the wild type but suggested
17 that a functional QS system was required for maturation of the biofilm. In addition,
18 biosurfactant production, which was previously found to be regulated by QS in several
19 bacteria [19; 27] possibly influences detachment from surfaces [33]. However, whether these
20 QS inhibitory compounds indeed exert their effect through a change in biosurfactant
21 production in Bcc biofilms remains to be determined.
22 Whether these compounds, either alone or in combination with conventional antimicrobial
23 agents, will ever be useful as anti-biofilm agents remains to be determined in future studies.
24 However, there are indications that there may be a role for these compounds, even though
25 their anti-biofilm effect is only moderate. For example, Tomlin et al. [41] showed that, despite

the fact that the changes observed with their biofilm model were not biologically relevant, disrupting the QS system made sessile *B. cenocepacia* cells more susceptible to antibiotics. This supports the idea that a decrease in the overall structural stability of the biofilm, although it may not be biologically relevant at first sight, may allow for more efficacious removal of bacteria by other means.

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1 Figure 1: Representative confocal images of *B. cenocepacia* LMG16656 biofilms treated with
2 “compound 3” (500 µM) or an untreated control. Biofilms were stained using SYTO9 and
3 CLSM pictures were taken after 4 h adhesion (A) and after 24 h of biofilm growth (B) (at
4 both time points following the rinsing step). The larger central plots are fluorescence
5 projections. Shown in the right and lower frames are vertical sections through the biofilms.
6 The scale bar represents 10 µm.

1 Table 1 **:** QS inhibitory effects of several QS inhibitors in the three QSI detection systems. QSI is expressed as % inhibition (± standard deviation).

2 NS: not significant ($p > 0.05$; Independent Sample T-test).

Compound	Conc (µM)	% inhibition (± SD)		
		C. violaceum CV026	*E. coli* JB523	*P. aeruginosa* QSIS2
4CABA	50	NS	NS	NS
6CABA	50	NS	22 ± 23	NS
6FABA	50	11 ± 11	32 ± 22	NS
Azithromycin	2	NS	22 ± 12	NS
Baicaleïn	1	15 ± 12	31 ± 28	NS
Baicalin hydrate	100	25 ± 2	50 ± 7	7 ± 11
p-Benzoquinon	20	11 ± 12	41 ± 17	15 ± 8
L-canavanine	62.5	14 ± 4	9 ± 3	NS
(+)- Catechin	312	11 ± 3	8 ± 3	NS
Ceftazidime hydrate	0.075	9 ± 5	11 ± 15	NS
Cinnamaldehyde	100	16 ± 15	15 ± 7	NS
“Compound 1”	500	29 ± 13	22 ± 18	38 ± 17
“Compound 3”	500	9 ± 4	NS	58 ± 4
Curcumin	500	18 ± 12	37 ± 14	NS
(-)- Epigallocatechin gallate	50	16 ± 12	26 ± 4	NS
Esculetin	500	55 ± 23	50 ± 20	67 ± 2
Esculin hydrate	500	25 ± 13	19 ± 13	29 ± 8
Farnesol	500	NS	NS	NS
Indole	312	13 ± 4	19 ± 14	14 ± 14
4-nitropyridine oxide	10	NS	NS	21 ± 4
Resveratrol	25	14 ± 3	19 ± 14	NS
Tobramycin	0.5	14 ± 1	16 ± 4	NS

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1 Table 2: The effect of QSI on biofilm biomass (CV) and amount of metabolically active cells (CTB) after 24 h of biofilm formation. The effect is

2 expressed as % inhibition (± SD). NS: not significant ($p > 0.05$; Independent Sample T-test).

Compound (conc)	percentage inhibition CV signal (± SD)				percentage inhibition CTB signal (± SD)			
	B. multivorans		*B. cenocepacia*		*B. multivorans*		*B. cenocepacia*	
	LMG13010	LMG17588	LMG18828	LMG16656	LMG13010	LMG17588	LMG18828	LMG16656
4CABA (50 µM)	NS	NS	NS	NS	NS	NS	NS	25 ± 2
6CABA (50 µM)	NS	NS	NS	NS	NS	NS	NS	19 ± 9
6FABA (50 µM)	NS	25 ± 8	NS	NS	NS	28 ± 13	NS	29 ± 4
Azithromycin (2 µM)	NS	NS	NS	15 ± 16	25 ± 10	55 ± 3	32 ± 5	40 ± 11
Baicalein (1 µM)	NS	13 ± 7	NS	NS	NS	28 ± 8	NS	16 ± 3
Bacalin hydrate (100 µM)	48 ± 7	37 ± 9	62 ± 2	11 ± 19	46 ± 5	28 ± 12	54 ± 8	16 ± 4
p-benzoquinon (100 µM)	NS	NS	21 ± 8	31 ± 36	NS	NS	18 ± 6	18 ± 24
L-Canavanine (20 µM)	NS	19 ± 7	14 ± 8	43 ± 25	11 ± 13	29 ± 9	10 ± 4	26 ± 20
(+)- Catechin (1000 µM)	NS	NS	NS	NS	26 ± 14	35 ± 11	30 ± 6	48 ± 17
Ceftazidime hydrate (1 µM)	8 ± 12	29 ± 5	NS	41 ± 21	28 ± 11	54 ± 12	37 ± 9	22 ± 15
Cinnamaldehyde (250 µM)	NS	11 ± 10	12 ± 15	31 ± 29	NS	11 ± 12	22 ± 4	12 ± 16
"Compound 1" (500µM)	17 ± 24	39 ± 9	NS	53 ± 18	20 ± 9	22 ± 8	NS	25 ± 14
"Compound 3" (500 µM)	37 ±10	26 ± 14	19 ± 10	54 ± 8	22 ± 9	35 ± 10	NS	45 ± 9
Curcumin (500 µM)	23 ± 12	24 ± 6	NS	NS	21 ± 10	14 ± 4	NS	NS
(-)- Epigallocatechin gallate (0.4 µM)	NS	16 ± 6	24 ± 9	10 ± 14	NS	40 ± 7	20 ± 15	26 ± 8
Esculetin (500 µM)	25 ± 10	26 ± 13	NS	10 ± 15	19 ± 15	11 ± 6	11 ± 6	10 ± 5
Esculin hydrate (500 µM)	22 ± 13	46 ± 4	NS	9 ± 13	17 ± 10	9 ± 9	NS	NS
Farnesol (2500 µM)	42 ± 10	38 ± 5	22 ± 10	50 ± 13	26 ± 8	41 ± 14	41 ± 3	58 ± 11
Indole (312 µM)	NS	NS	NS	23 ± 22	12 ± 8	19 ± 13	28 ± 5	19 ± 11
4-nitropyridine N-oxide (8 µM)	NS	14 ± 6	NS	26 ± 22	NS	27 ± 6	NS	21 ± 3
Resveratrol (25 µM)	NS	12 ± 8	9 ± 13	38 ± 18	17 ± 17	25 ± 11	25 ± 3	30 ± 16
Tobramycin (2 µM)	NS	-16 ± 9	-15 ± 11	NS	NS	-12 ± 18	-9 ± 6	NS

1

1 Table 3: Numbers of cells present after 4 h and 24 h in the growth medium (M), rinsing solution (RS) and on the disks as well as total cell

2 numbers (TCN) present in the wells. *: $p < 0.05$ (Mann-Withney U)

Compound	Numbers of cells present after 4 h			
	M (CFU/ml) ($x10^8$)(±SD)	**RS (CFU/ml) ($x10^8$) (±SD)**	**Disks (CFU/ Disk) ($x10^8$)(±SD)**	**TCN (CFU/well) ($x10^8$)(±SD)**
CTRL	1.01 ± 0.17	0.48 ± 0.10	1.34 ± 0.37	2.83 ± 0.42
Azithromycin (2µM)	0.89 ± 0.28	0.71 ± 0.21*	1.15 ± 0.37	2.76 ± 0.44
Ceftazidime hydrate (1 µM)	0.99 ± 0.48	0.54 ± 0.23	1.28 ± 0.62	2.81 ± 0.78
Cinnamaldehyde (250 µM)	1.04 ± 0.05	0.74 ± 0.13*	1.12 ± 0.37	2.97 ± 0.41
“Compound 3” (500 µM)	1.00 ± 0.04	0.52 ± 0.13	1.14 ± 0.14	2.65 ± 0.24
Farnesol (2500 µM)	0.94 ± 0.06	0.80 ± 0.27*	1.15 ± 0.45	3.18 ± 0.53
Indole (312 µM)	0.82 ± 0.49	0.71 ± 0.13*	1.34 ± 0.11	2.96 ± 0.60

Compound	Numbers of cells present after 24 h			
	M (CFU/ml) ($x10^8$)(±SD)	**RS (CFU/ml) ($x10^8$)((±SD)**	**Disks (CFU/ Disk) ($x10^8$)(±SD)**	**TCN (CFU/well) ($x10^8$)(±SD)**
CTRL	1.36 ± 0.12	0.92 ± 0.24	1.99 ± 0.29	4.27 ± 0.40
Azithromycin (2µM)	2.33 ± 0.33*	1.63 ± 0.55*	0.71 ± 0.12*	4.68 ± 0.66
Ceftazidime hydrate (1 µM)	2.15 ± 0.07*	0.95 ± 0.07	1.30 ± 0.04*	4.40 ± 0.11
Cinnamaldehyde (250 µM)	1.84 ± 0.25*	1.58 ± 0.08*	0.89 ± 0.14*	4.30 ± 0.30
“Compound 3” (500 µM)	2.46 ± 0.34*	1.18 ± 0.23	0.85 ± 0.19*	4.49 ± 0.46
Farnesol (2500 µM)	2.44 ± 0.79*	1.29 ± 0.10*	0.49 ± 0.31*	4.22 ± 0.85
Indole (312 µM)	1.79 ± 0.01*	0.96 ± 0.09	1.32 ± 0.11*	4.15 ± 0.15

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1 Figure 1:

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