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Chloroquine fumardiamides as novel *quorum* sensing inhibitors

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A B S T R A C T

Quorum sensing inhibitors (QSIs) that specifically interfere with bacterial cell-to-cell communication are considered as an alternative approach to conventional antibacterial therapy. In our study, a set of twenty-six fumardiamides with a quinoline head-group were evaluated as potential QSIs. Two strains of Gram-negative *Chromobacterium violaceum* (violacein-producing strain ATCC31532 and violacein-negative, mini-Tn5 mutant derivative CV026) were used as QS reporters for testing anti-QS and bactericidal activity of various quinoline fumardiamides. The initial screening of eighteen fumardiamides with primaquine, mefloquine and chloroquine scaffolds identified chloroquine derivatives as the most promising QSIs. Tail-group optimization of chloroquine fumardiamides led to the most active compounds **27**, **29** and **30** bearing aminoethyl or piperidine moieties. At 400 μM concentration, these compounds inhibited the QS of *C. violaceum* strains in a manner similar to

quercetin (the model QSI), while at the 40 µM concentration their inhibitory effect was twice less than that of quercetin. As none of the compounds displayed a bactericidal effect and that the QS inhibition was specific to the CV026 strain, our findings indicate that the structurally optimized chloroquine derivatives could function as *quorum* quenching (QQ) agents with a potential to block the signaling without entering the cell. In conclusion, our finding provides an important step toward the further design of agents targeting cell-to-cell communication.

Most microbial pathogens exploit adherent growth on abiotic/biotic surfaces (i.e., biofilm formation) as a default growth mode to escape the action of antibiotics and host immune cells.[1] A cell-to-cell communication, termed *quorum* sensing (QS), plays a key role in coordinating the biofilm formation, secondary metabolite production, bioluminescence and virulence.[2] QS enables the microbial cells to detect and respond to cell population density via the production and release of a chemical signal molecule called autoinducer (AI). Many bacteria possess several interacting QS modules organized into regulatory hierarchies employing multiple signal molecules from the same or different chemical classes.[3] It is considered that molecules or compounds able to interrupt QS pose less selective pressure on the microbes than the conventional antibiotics.[4] Strategies that interrupt the QS-communication outside the cell, also called *quorum* quenching (QQ), are highly promising since the communication system can be blocked without entering the cell.[5,6] Thus, search for novel anti-QS compounds preventing the development of specific group behavior and causing chemical attenuation of biofilm formation is considered the method of choice to combat biofilm infections without boosting the resistance.[5,7] However, there are currently no approved therapeutic treatment options that target machinery critical to biofilm formation or biofilm maintenance.[8]

Our studies have focused on the derivatization of the quinoline drug primaquine (PQ) with the aim to get novel biologically active agents.[9] A literature survey revealed a number of reports on quinoline derivatives as effective antagonists of PqsR, the receptor of pqs system of the human pathogen *Pseudomonas aeruginosa*, which controls the expression of various virulence factors and is involved in the biofilm formation. Based on the scaffold of the quinolone signal molecule PQS (2-heptyl-3-hydroxy-4(1*H*)-quinolone) and its precursor HHQ (2-heptyl-4-hydroxyquinoline), several classes of quinoline and quinazolinone PqsR antagonists were developed.[10–12] Furthermore, Abouelhassan et al. have reported on quinoline small molecules with potent dispersal activity against methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms.[13]

Based on these facts, we have evaluated the potential of PQ derivatives synthesized in-house as antibiofilm agents. We have shown that ureidoamides with PQ and amino acid moieties exerted strong activity in a biofilm eradication assay involving *Escherichia coli*, *P. aeruginosa* and *Candida*

albicans as the biofilm models.[14] Asymmetric PQ-halogenoaniline fumardiamides **1–6** have also demonstrated a significant antibiofilm activity.[15] Susceptibility of the tested microorganisms towards PQ derivatives showed both a compound- and species-dependent patterns. The most active compounds were fluoro derivatives **1** and **2** (Fig. 1), whereas the most susceptible microorganisms were *Enterococcus faecalis* and *S. aureus*, followed by *E. coli*, *Streptococcus pneumoniae* and *P. aeruginosa*. Our compound library was further enriched with analogous derivatives bearing mefloquine (MQ) (**7**–**12**) or chloroquine (CQ) pharmacophores (**13**–**18**) (Fig. 1).[16,17]

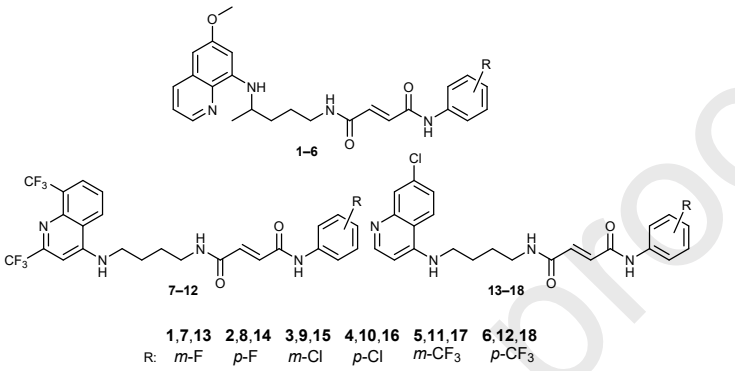
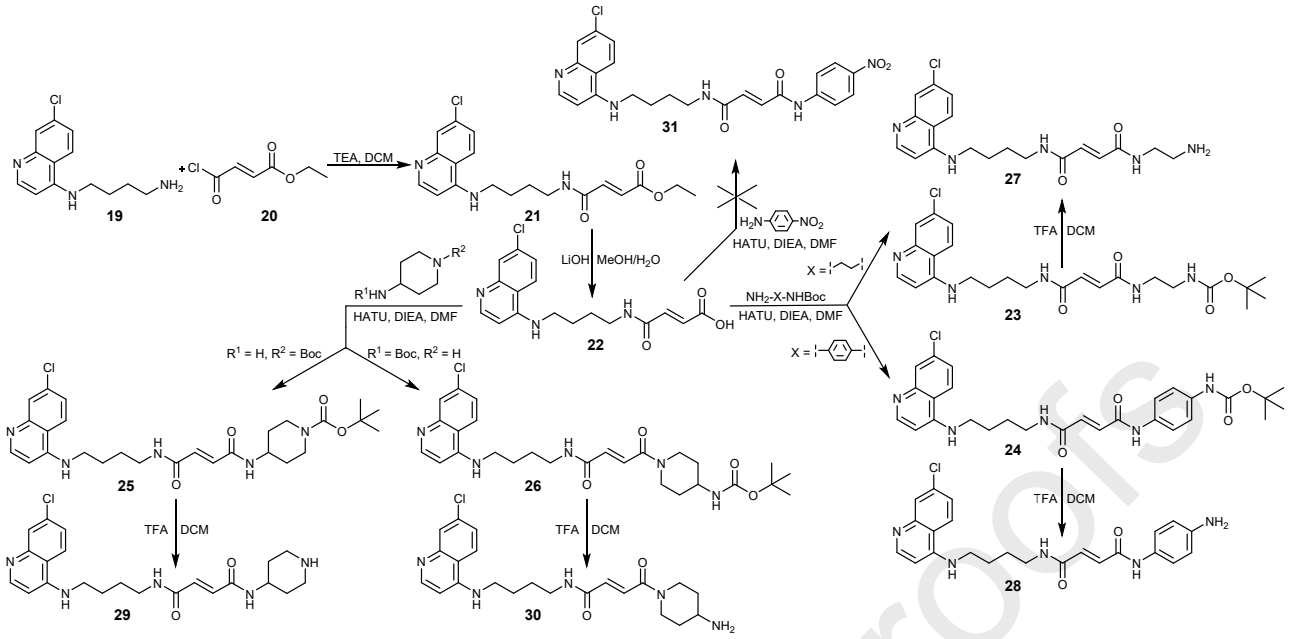


Fig. 1. Chemical structures of primaquine- (**1**–**6**), mefloquine- (**7**–**12**) and chloroquine-based fumardiamides (**13**–**18**).

However, the potential of quinoline derivatives **1**–**18** as *quorum* sensing inhibitors (QSIs) has not been explored until now. *Chromobacterium violaceum* has shown promise in screening compounds with QSI activities, as changes in QS can be directly detected and quantified by monitoring the QS-dependent formation of a colored indicator/pigment, termed violacein.[18] We have previously established a miniaturized 96-well based screening platform based on the *C. violaceum* reporter strains (ATCC31532 and CV026), which allow the distinction of QSIs from QQs that sequester/inhibit the AI signal (C6-HSL), as well as the exclusion of compounds having bactericidal effects.[19,20] Here, the further-improved screening method, in which the growth conditions were optimized to avoid bottlenecks interfering the maximal synthesis of violacein, was used to screen the synthesized quinoline derivatives **1**–**18** and four newly prepared CQ derivatives **27**–**30**, designed after the initial screenings.

PQ, MQ and CQ derivatives **1**–**18** were prepared according to procedures reported in our previous papers.[15–17] The initial screening of these eighteen quinoline fumardiamides pointed chloroquine derivatives as the most promising QS inhibitors. Based on the obtained results, we have designed and prepared four novel CQ derivatives **27**–**30**, in which halogenoaniline moiety was replaced by scaffolds bearing primary or secondary amino group as the key feature (Scheme 1). The reaction pathway leading to the title compounds included several synthetic steps: *i*) preparation of N^1-

(7-chloroquinolin-4-yl)butane-1,4-diamine (**19**) from 4,7-dichloroquinoline and 1,4-diaminobutane; *ii*) synthesis of (*E*)-ethyl 4-chloro-4-oxobut-2-enoate (monoethyl fumaroyl chloride, **20**) from monoethyl fumarate and thionyl chloride; *iii*) reaction of compounds **19** and **20** to obtain amido-ester **21**; *iv*) basic hydrolysis of product **21** to amido-carboxylic acid **22**; *v*) coupling reaction between **22** and the corresponding *N*-protected diamine in the presence of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) and *N,N*-diisopropylethylamine (DIEA); *vi*) the deprotection step: removing of *t*-butyloxycarbonyl group (Boc) from intermediates **23**–**26**. Compounds **23** and **24** were prepared using Boc-monoprotected 1,4-diaminoethane or 1,4-benzenediamine in the coupling step, whereas compounds **25** and **26** were obtained from two related 4-aminopiperidines, one bearing Boc protecting group on piperidine nitrogen atom (4-amino-1-Boc-piperidine or *t*-butyl-4-aminopiperidine-1-carboxylate) and the second one with Boc-protected primary amino group, i.e. 4-(*N*-Boc-amino)piperidine or *t*-butyl piperidin-4-ylcarbamate. It is worth to mention that the coupling reaction between compound **22** and *p*-nitroaniline failed in our hands, so fumardiamide **31** bearing CQ scaffold on one side and *p*-nitroaniline motif on the other side was not obtained. We have also tried to prepare compound **31** in the opposite direction: first to achieve the amide bond between monoethyl fumaroyl chloride and *p*-nitroaniline and then the amide bond between CQ derivative, but this experiment was also unsuccessful. The final CQ-based fumardiamides **27**–**30** were obtained by treating of precursors **23**–**26** with trifluoroacetic acid (TFA). The following compounds were prepared: N^1-(2-aminoethyl)-N^4-[4-[(7-chloroquinolin-4-yl)amino]butyl]fumaramide (**27**), N^1-(4-aminophenyl)-N^4-[4-[(7-chloroquinolin-4-yl)amino]butyl]fumaramide (**28**), N^1-[4-[(7-chloroquinolin-4-yl)amino]butyl]-N^4-(piperidin-4-yl)fumaramide (**29**) and (*E*)-4-(4-aminopiperidin-1-yl)-*N*-[4-[(7-chloroquinolin-4-yl)amino]butyl]-4-oxobut-2-enamide (**30**).



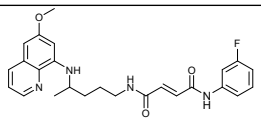
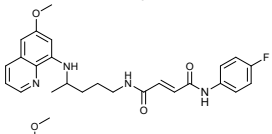
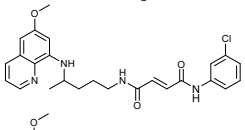
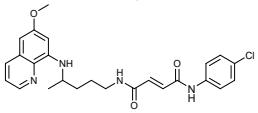
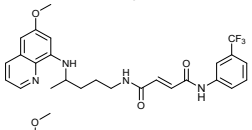
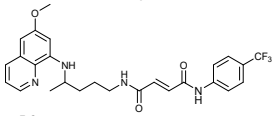
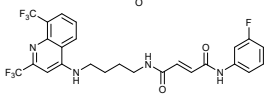
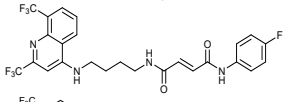
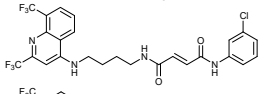
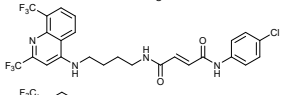
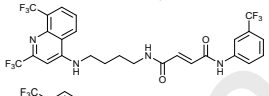
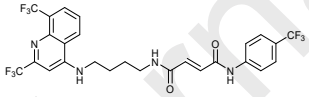
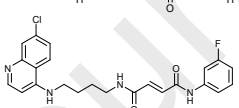
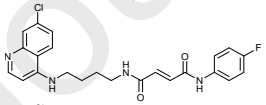
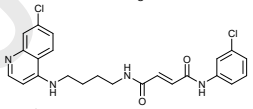
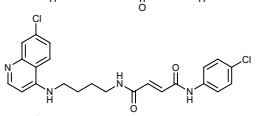
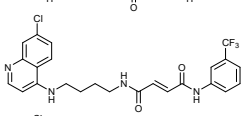
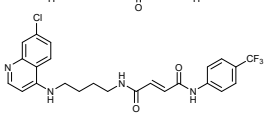
Scheme 1. Synthesis of novel chloroquine-based fumardiamides **27**–**30**.

The *C. violaceum* ATCC31532 and CV026 strains were used as QS reporters for testing anti-QS and bactericidal activities of the indicated quinoline fumardiamides, using quercetin (Q) as the positive control for QS and azithromycin (AZ) as the positive control for bactericidal activity (cell viability). Parallel assays monitoring both the violacein production (indication of induced QS signaling) and the reduction of resazurin to resorufin (indication of viable cells) in both reporter strains grown with/without the compound allow screening of a genuine anti-QS agent from compounds with bactericidal effect. The initial evaluation was performed for 18 compounds: six PQ derivatives (**1**–**6**), six MQ derivatives (**7**–**12**) and six CQ derivatives (**13**–**18**) (Table 1, Fig. 2). Inhibitory effects were tested at 400 μM concentration and for the most potent QSIs also at 40 μM. Five tested compounds, namely **13**–**16** and **18**, all bearing the CQ scaffold, were able to reduce the violacein production in ATCC31532 from 75 to 87%. Compounds **13**–**16** decreased cell viability of the same strain from 27 to 57% in comparison to cells with 2% DSMO (solvent used in sample preparation). These five compounds were further tested at 40 μM concentration both on the wild-type and the mutant cells, and the results indicated that the compounds were able to inhibit QS only at a higher concentration (400 μM). None of the tested compounds displayed QQ activity.

Table 1

Quorum sensing inhibition of quinoline fumardiamides **1**–**18** at 400 μM concentration.

Cmpd.	Chemical structure	*Chromobacterium violaceum* strain	
		ATCC31532	CV026

		QSI (%)	Bactericidal effect (%)	QQ (%)	Bactericidal effect (%)
1		ne	ne	nt	nt
2		ne	24.2 ± 0.3	nt	nt
3		ne	17.9 ± 4.1	nt	nt
4		ne	ne	nt	nt
5		ne	16.4 ± 0.7	nt	nt
6		ne	31.2 ± 6.7	nt	nt
7		16.9 ± 16.6	ne	nt	nt
8		29.1 ± 11.5	ne	nt	nt
9		22.3 ± 6.1	22.3 ± 0.9	nt	nt
10		29.9 ± 12.7	ne	nt	nt
11		22.3 ± 6.1	13.8 ± 0.1	nt	nt
12		ne	ne	nt	nt
13		79.4 ± 0.4	27.8 ± 12.2	ne	ne
14		87.9 ± 1.8	39.7 ± 7.7	ne	ne
15		75.3 ± 2.8	45.8 ± 8.2	ne	ne
16		85.0 ± 1.3	56.9 ± 10.1	ne	ne
17		ne	ne	nt	nt
18		85.7 ± 2.2	27.7 ± 5.4	ne	ne

PQ	ne	ne	ne	ne
Q	77.8 ± 10.1	ne	71.7 ± 6.6	ne
AZ	ne	94.8 ± 3.6	94.4 ± 0.4	67.2 ± 1.5

QSI – *quorum* sensing inhibition; QQ – *quorum* quenching; PQ – primaquine; Q – quercetin; AZ – azithromycin; nt – not tested; ne – no effect

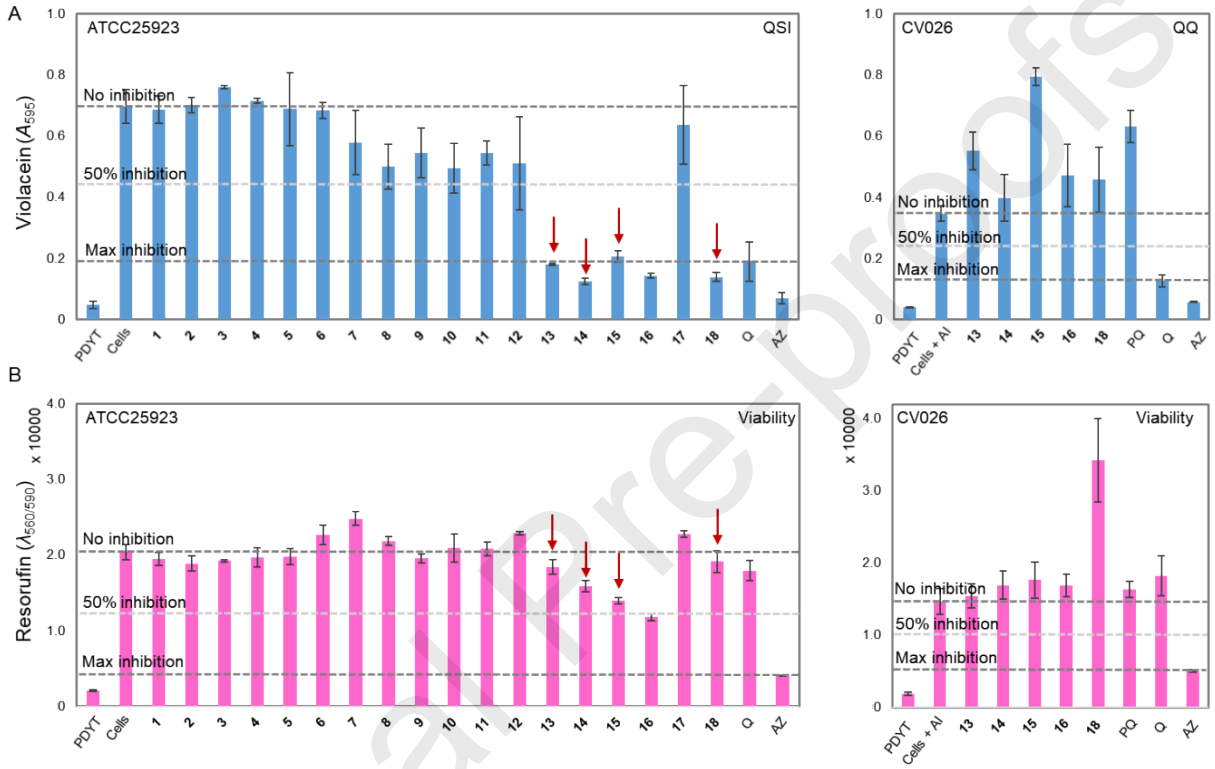


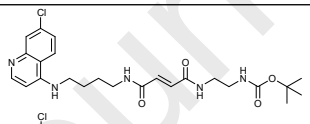
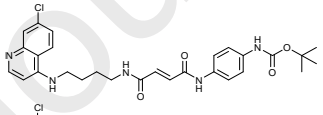
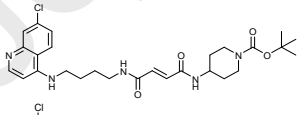
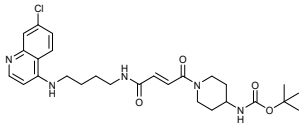
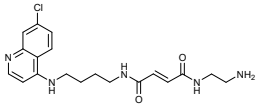
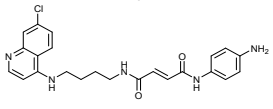
Fig. 2. Comparison of violacein production (A) and cell viability of (B) ATCC31532 (left panel) and CV026 (right panel) cultures with and without the indicated compounds. PDYT, wells containing only the culture medium; Cells, cultures containing 2% DMSO (ATCC31532) or 2% DMSO and 0.5 µM C6-HSL (CV026). Error bars, ± SD (n = 3–4). No inhibition, cells with maximal violacein production or maximal viability; Max inhibition, inhibition achieved with quercetin (Q) or with bactericidal drug azithromycin (A). Red arrows indicate the most potent non-bactericidal compounds with QSI activity.

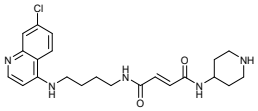
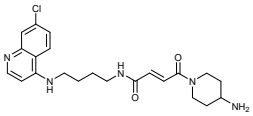
After the initial head-group studies, CQ moiety was viewed as an attractive platform for the synthesis of a focused small molecule library. We have designed CQ-fumardiamides with a primary amino group attached to a flexible carbon chain (**27**), benzene ring (**28**) or piperidine heterocycle (**30**), and one piperidine derivative with a secondary amino group (**29**). The amino motif was chosen based on literature reports, which pointed out amine-containing small molecules as compounds most likely to cross cellular membranes of Gram-negative pathogens and accumulate in

the cell cytoplasm,[21] whereas replacing amine with other functional groups (hydroxyl, carboxyl, amide, ester, azide, nitrile) or alkylation resulted in markedly reduced accumulation of the compound. It is also accepted that promising Gram-negative antibacterial agents are amphiphilic, rigid, less than 600 Da in size, and have low globularity.[21,22] With their higher polarity and higher molecular weight, they have been considered as an exception to the Lipinski's rule of five.[22]

We have evaluated the effectiveness of amino CQ-fumardiamides **27**–**30**, their chemical precursors **23**–**26** with Boc-protected amino groups and the parent drug CQ as potential QSIs. Our results revealed that compound **27** with the amino group attached to a short aliphatic chain and piperidine derivatives **29** and **30**, inhibited QS by approx. 46% in both the wild-type and the mutant strains in comparison to the same strains grown in the presence of 2% DMSO, a solvent used in dissolving the compounds. These compounds were almost as effective as quercetin that inhibited the violacein production by 56% in comparison to violacein production in the wild-type with 2% DMSO (Table 2, Fig. 3A). The cell viability staining indicated that among the series **23**–**30**, only two compounds, **24** and **26**, displayed some bactericidal activity. On the other hand, the remaining six compounds, including those with the highest QS inhibition, showed a negligible bactericidal effect in both strains ranging from 6.5 to 16.2% compared to the same strains with 2% DMSO (Table 2, Fig. 3B).

Table 2
Quorum sensing inhibition of chloroquine fumardiamides **23**–**30** at 400 μM concentration.

Cmpd.	Chemical structure	*Chromobacterium violaceum* strains			
		ATCC31532		CV026	
		QSI (%)	Bactericidal effect (%)	QQ (%)	Bactericidal effect (%)
23		23.0 ± 1.7	10.7 ± 0.4	nt	nt
24		ne	43.7 ± 4.0	nt	nt
25		ne	8.3 ± 0.6	nt	nt
26		9.1 ± 0.3	44.6 ± 3.0	nt	nt
27		46.3 ± 0.9	12.1 ± 0.5	53.3 ± 4.7	10.3 ± 0.2
28		4.7 ± 0.6	17.5 ± 1.5	nt	nt

29		45.9 ± 6.7	6.5 ± 0.6	61.6 ± 6.7	10.3 ± 0.3
30		47.2 ± 5.0	16.2 ± 1.5	62.6 ± 7.5	13.4 ± 0.2
CQ		13.2 ± 1.8	10.1 ± 0.9	nt	nt
Q		55.8 ± 7.4	14.7 ± 0.9	66.8 ± 1.4	17.1 ± 0.3
AZ		90.6 ± 10.6	85.6 ± 7.7	86.3 ± 12.6	85.9 ± 9.7

QSI – *quorum* sensing inhibition; QQ – *quorum* quenching; CQ – chloroquine; Q – quercetin; AZ – azithromycin; nt – not tested; ne – no effect

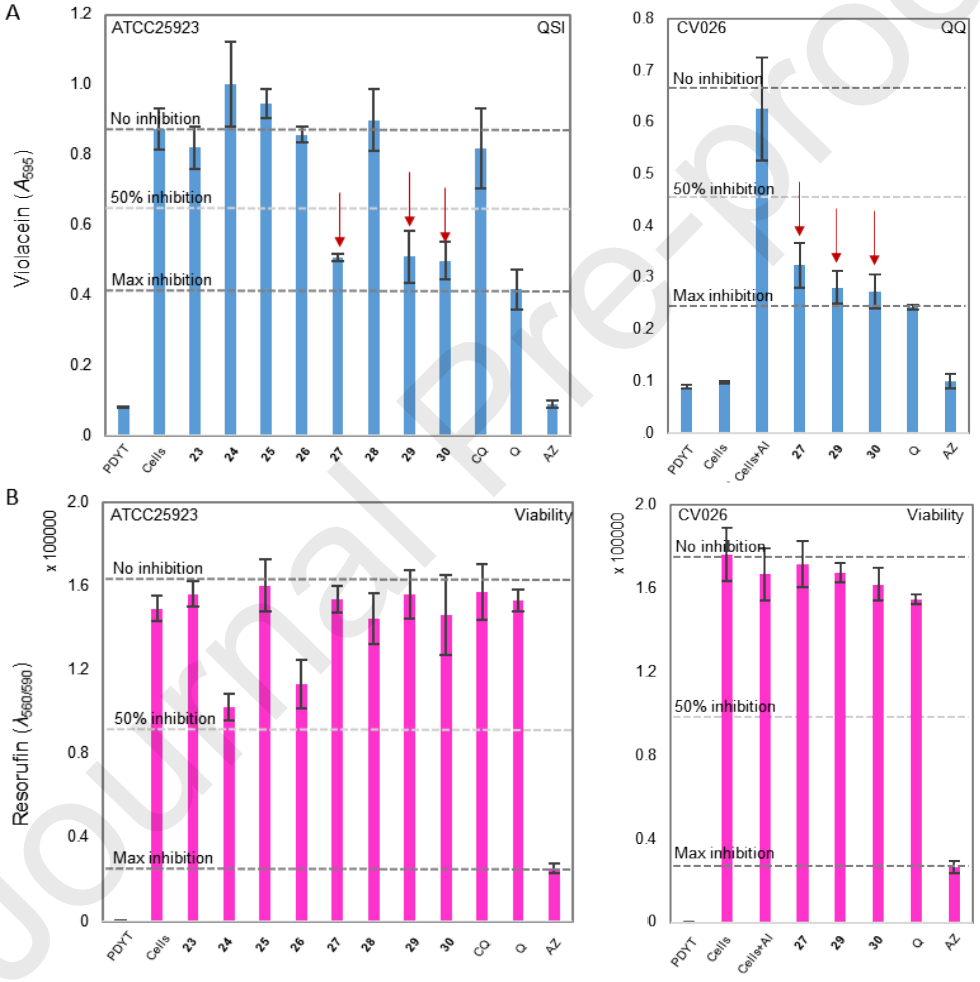


Fig. 3. Comparison of violacein production (A) and cell viability (B) of ATCC31532 (left panel) and CV026 (right panel) cultures with and without the indicated compounds. PDYT, wells containing only the culture medium; Cells, cultures containing 2% DMSO (ATCC31532) or 2% DMSO and 0.5 µM C6-HSL. Error bars, ± SD (n = 3–4). Violacein was detected at 595 nm and the reduction of resazurin to resorufin using λ_{ex} = 560 nm and λ_{em} = 590 nm. No inhibition, cells with maximal violacein production or viability. Max inhibition, QS inhibition achieved with quercetin (Q) or inhibition of viability with bactericidal drug azithromycin (AZ). Red arrows indicate the most efficient QSIs/QQs.

The QS inhibition of the most potent compounds **27**, **29** and **30** was further evaluated against both reporter strains at 10-fold lower concentration (40 μM). The results showed that all three compounds displayed a higher QS inhibition in CV026 than in ATCC31532 strain, with no detectable bactericidal effects (Table 3, Fig. 4). In CV026 strain, compounds **27** and **29** showed more than 50% of quercetin's inhibitory activity, while compound **30** was less effective (approx. 30%). Due to significant differences in QS inhibition profiles between the two reporters, we suggest that none of the compounds interacted with CviR. This is supported by the finding that the final violacein levels reached by both strains without the compound are comparable (Fig. 4A), which also suggests similar CviR levels in both strains. In view of this, we propose two alternative mechanisms of action. In the first proposal, the active compounds bind the externally added AHL molecules (C6-HSL), hamper their diffusion into the cells and prevent the induction of QS (Fig. 5A). The tested compounds might change the membrane permeability for AHLs as well. In the second proposal, the formed hybrid molecules reach the cytoplasm, but due to steric hindrances are not able to interact with the CviR receptor (Fig. 5B). Thus, the interaction between the compound and the AHL occurs outside the *C. violaceum* cells, which suggests that compounds **27**, **29** and **30** act as QQs.

Table 3
Quorum sensing inhibition of chloroquine fumardiamides **27**, **29** and **30** at 40 μM concentrations.

Cmpd.	QSI in *C. violaceum* strains ATCC31532 (%)	QQ in *C. violaceum* strain CV026 (%)
27	ne	52.5 ± 3.2
29	5.4 ± 3.8	42.8 ± 1.9
30	7.0 ± 0.1	39.9 ± 0.6
Q	55.8 ± 7.4	66.8 ± 1,41
AZ	90.6 ± 10.6	86.3 ± 12.6

[a] quercetin concentration *c* = 400 μM; QSI – *quorum* sensing inhibition;
QQ – *quorum* quenching; CQ – chloroquine; Q – quercetin; AZ – azithromycin

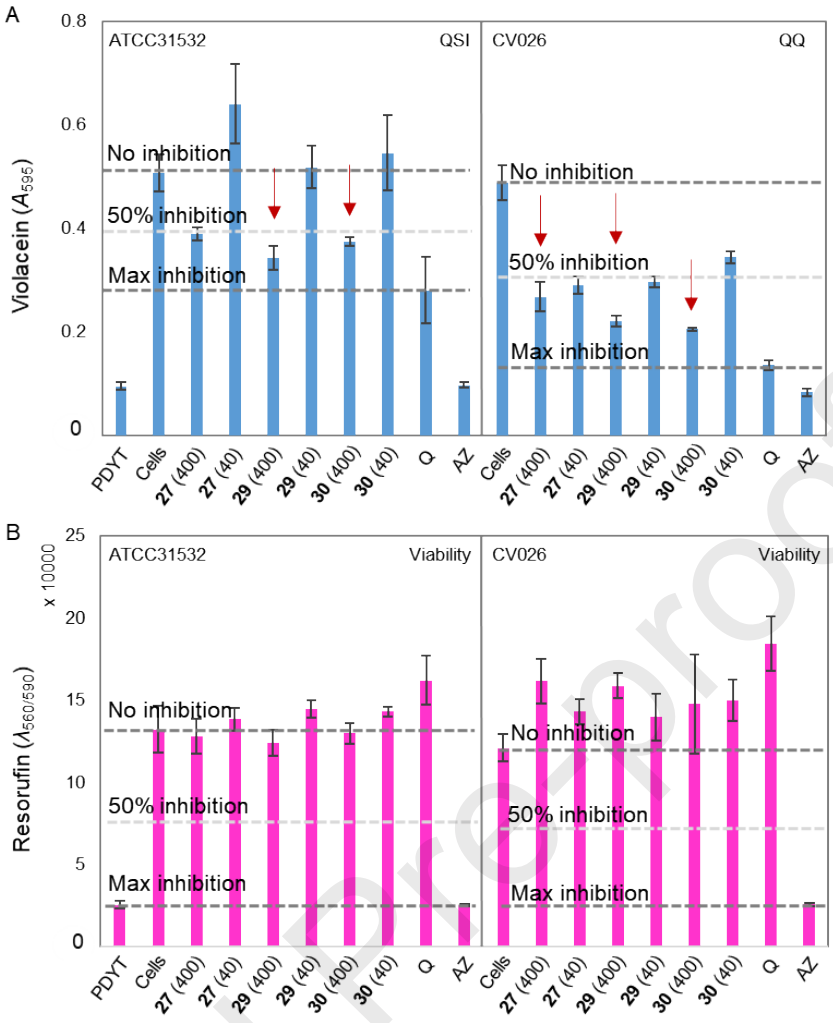


Fig. 4. Comparison of violacein production (A) and cell viability (B) of ATCC31532 (left panel) and CV026 (right panel) cultures with and without the indicated compounds at 400 and 40 µM concentration. PDYT, wells containing only the culture medium; Cells, cultures containing 2% DMSO (ATCC31532) or 2% DMSO and 0.5 µM C6-HSL. Error bars, ± SD (n = 3–4). No inhibition, cells with maximal violacein production or maximal viability. Max inhibition, QS inhibition achieved with quercetin (Q) or inhibition of viability with bactericidal drug azithromycin (AZ). Red arrows indicate the most efficient QSIs/QQs.

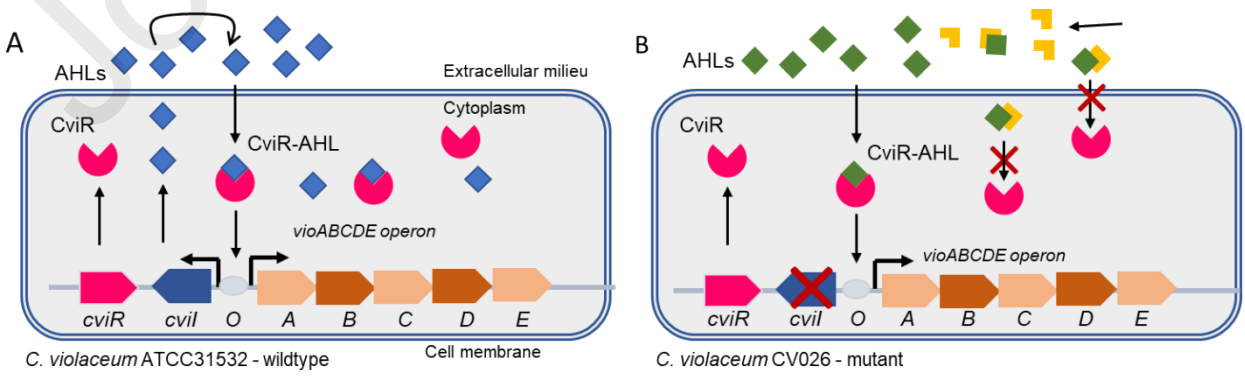


Fig. 5. Proposed mechanisms-of-actions behind the **27-**, **29-**, **30**-mediated inhibition of QS in *C. violaceum*. (A) In *C. violaceum* ATCC 31532, the *cviI* synthase gene produces the AHL (C6-HSL) molecule that is recognized by the cytoplasmic receptor CviR. When bound to AHL, CviR dimerizes and binds DNA, leading to the expression of QS-regulated genes, including the *vioABCDE* operon genes coding for the colored pigment, violacein. (B) In CV026, the QS is activated by the addition of AHL (C6-HSL) that after diffusion into the cells and interaction with the CviR leads to the activation of the *vio* operon genes. Compounds **27**, **29** and **30** (yellow objects) are likely to bind the AHL, which either impairs the diffusion of the hybrid-molecule into the cell or prevents the interaction with CviR. O, operator sequence harboring the CviR binding site.

Calculation of the physicochemical parameters of compounds **23**–**30** perfectly suits with the biological findings and the above-mentioned requirements for Gram-negative bacteria. The most potent compounds **27**, **29** and **30** are very polar with low log *P* (0.409 or 1.05) and negative distribution coefficient at pH 7.4 (log $D_{7.4}$ –1.38 and –1.73, respectively), whereas the inactive aromatic amino compound **28** and *N*-protected compounds **23**–**26** are less polar (log *P* = 2.101–4.335 and log $D_{7.4}$ = 1.86–4.09) and less basic (Table 4).

Table 4
Properties of novel compounds **23**–**30** calculated with Chemicalize.org program.[23]

Cmpd.[a]	Molecular formula	Number of atoms	MW	log *P*	log $D_{7.4}$	HBD	HBA	Lipinski score[b]	MR (cm^3/mol)	TPSA (Å^2)
23	$C_{24}H_{32}ClN_5O_4$	66	489.21	2.101	1.86	4	5	4	133.33	121.45
24	$C_{28}H_{32}ClN_5O_4$	70	537.21	4.335	4.09	4	6	3	152.41	121.45
25	$C_{27}H_{36}ClN_5O_4$	73	529.25	2.333	2.09	3	5	3	145.72	112.66
26	$C_{27}H_{36}ClN_5O_4$	73	529.25	2.333	2.09	3	5	3	145.72	112.66
27	$C_{19}H_{24}ClN_5O_2$	51	389.88	0.409	–1.38	4	5	4	108.51	109.14
28	$C_{23}H_{24}ClN_5O_2$	55	437.16	2.595	2.35	4	5	4	126.82	109.14
29	$C_{22}H_{28}ClN_5O_2$	58	429.19	1.05	–1.73	4	5	4	120.72	95.15
30	$C_{22}H_{28}ClN_5O_2$	58	429.19	1.05	–1.73	4	5	4	120.72	95.15

[a] Physicochemical data of compounds **1**–**18** are reported previously.[15–17] MW – molecular weight; log *P* – partition coefficient; log $D_{7.4}$ – distribution coefficient at pH 7.4; HBD – number of H-bond donors; HBA – number of H-bond acceptors; MR – molecular refractivity; TPSA – topological polar surface area; [b] out of four.

We demonstrated that CQ-fumardiamides **13**–**16** and **18** are promising QSIs. Optimization of their tail domain led to more effective QS inhibition in the *C. violaceum* reporter strains. At 400 μM concentrations, three compounds, namely N^1-(2-aminoethyl)-N^4-[4-[(7-chloroquinolin-4-yl)amino]butyl]fumaramide (**27**), N^1-[4-[(7-chloroquinolin-4-yl)amino]butyl]-N^4-(piperidin-4-yl)fumaramide (**29**) and (*E*)-4-(4-aminopiperidin-1-yl)-*N*-[4-[(7-chloroquinolin-4-yl)amino]butyl]-4-oxobut-2-enamide (**30**), i.e. fumardiamides bearing CQ head-group and aminoethyl or piperidine motifs, demonstrated excellent QS inhibition on both *C. violaceum* reporter strains. At 40 μM concentrations, the differences in the QS inhibition profiles between the wild-type and the mutant

reporters occurred, indicating that QS communication might be interrupted outside the cells. Since compounds **27**, **29** and **30** had no bactericidal effect on the *C. violaceum* cells, we considered them as new QQs able to block QS without inducing natural selection pressure as traditional antimicrobial agents. Our results could help in further design of agents targeting cell-to-cell communication and may open new avenues for the combat against drug-resistant bacteria.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chloroquine fumardiamides as novel *quorum* sensing inhibitors

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