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FULL PAPER

Targeting *Bacillus cereus* cells: increasing efficiency of antimicrobials by the bornyl-possessing 2(5*H*)-furanone derivative

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SUMMARY

Among a variety of antimicrobial compounds, the derivatives of 2(5*H*)-furanone exhibit different effects on Firmicutes and Proteobacteria. While inhibiting quorum-dependent biofilm formation and virulence factor expression by Gram-negative bacteria through specific interference with the AI-2 signaling pathways, these compounds demonstrate bactericidal effects against Gram-positive bacteria. Here we report that 3,4-dichloro-5(*S*)-[(1*S*,2*R*,4*S*)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-yloxy]-2(5*H*)-furanone designed as **F123** inhibits growth and biofilm formation by the food-poisoning bacterium *Bacillus cereus* at 8 μg/ml and kills bacteria at 16 μg/ml. While the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus subtilis* were also inhibited at 8-16 μg/ml of **F123**, no bactericidal effect on these strains was observed at concentrations up to 128 μg/ml, suggesting pronounced specificity of **F123** for *B. cereus*. In a checker-board assay **F123** increased the efficacy of amikacin, gentamicin and benzalkonium chloride against *B. cereus* with medians of fractional inhibitory concentration index of 0.38, 0.56 and 0.56, respectively. Moreover, the number of viable *B. cereus* cells in biofilm was reduced by more than 3 orders of magnitude at 64 μg/ml of **F123**, suggesting its chemotype as a promising enhancer for specific treatment of *B. cereus*-associated topical infections, including biofilm-embedded bacteria.

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INTRODUCTION

Infectious diseases caused by Gram-positive bacteria represent a great challenge in medicine, and increasingly widespread multidrug-resistant strains requires the intensive screening of novel antimicrobial agents and therapeutic approaches (Hancock 2005; Munita *et al.,* 2015). *Bacillus cereus*, a ubiquitous spore-forming bacterium surviving in various environments, is a well-known pathogen that causes food poisoning infections, including diarrheal and emetic types (Arnesen *et al.,* 2008). In addition, it may cause wound infections, bacteremia, septicemia and pneumonia, mainly among immunocompromised patients (Kamar *et al.,* 2013). Moreover, *B. cereus* with resistance to conventional antibiotics, such as vancomycin and ciprofloxacin (Torkar *et al.,* 2016), is an underestimated emerging pathogen that can be involved in fatal healthcare-associated infections in premature newborns (Turabelidze *et al.,* 2013; Lotte *et al.,* 2017). Remarkably, hemolytic *B. cereus* was also found as one of the dominant nosocomial pathogens in the skin microflora of contact-sport athletes suggesting high levels of human-to-human transmission and indicating the requirement of effective antiseptic agents for sports hygiene (Martykanova *et al.,* 2017).

In recent decades, a great increase in the worldwide spread of multidrug-resistant strains has been observed, caused by misuse of antibiotics, migration of vast numbers of people, and extensive agricultural use (Harbarth *et al.,* 2015). *B. cereus* resistance is usually associated with beta-lactamase production (Arslan *et al.,* 2014), and some isolates were reported to exhibit decreased susceptibility to chloramphenicol and tetracycline (Glenwright *et al.,* 2017). Besides the genetically determined resistance to antibiotics, the formation of rigid biofilms on natural and artificial surfaces drastically promotes the resistance capabilities of *B. cereus* (Majed *et al.,* 2016), thereby reducing bacterial susceptibility to antimicrobials and making conventional treatment and disinfection inefficient (Simoes *et al.,* 2010).

The combination of antibiotics with bacterial biofilm-modifying agents (preventing or disturbing) seems

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to be a promising strategy (Baidamshina *et al.,* 2017). Among such pharmacophores, derivatives of 2(5*H*)-furanone are known as inhibitors of biofilm formation of Gram-negative bacteria *Pseudomona aeruginosa*, *Escherichia coli* (Ren *et al.,* 2001; Christensen *et al.,* 2012), as well as Gram-positive *Staphylococcus epidermidis*, *Staphylococcus aureus* (Lonn-Stensrud *et al.,* 2009; Sharafutdinov *et al.,* 2017). It has been shown that the introduction of terpene moiety to pharmacophore increases the antimicrobial activity of a compound (Rogers *et al.,* 2010), probably by enhancing the dermal penetration of pharmaceuticals (Kamatou *et al.,* 2013). Moreover, bornyl-containing compounds significantly inhibit the bacterial growth of *Bacilli* (Setzer *et al.,* 1999; Sharifi-Rad *et al.,* 2015). In this work, we demonstrate that 3,4-dichloro-5(*S*)-[(1*S*,2*R*,4*S*)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-yloxy]-2(5*H*)-furanone (**F123**), carrying bornyl moiety specifically kills *B. cereus* cells in both planktonic and biofilm-embedded forms, and exceeds the antimicrobial activity of such conventional antibiotics as cefazolin, ciprofloxacin and vancomycin.

EXPERIMENTAL SECTION

Chemistry

The structures of studied 2(5*H*)-furanone derivatives are presented in *Figure 1*. 3-Chloro-5-hydroxy-4-[(4-methylphenyl)sulfanyl]-2(5*H*)-furanone **F12** (Kurbangalieva *et al.,* 2007), 3-chloro-5-hydroxy-4-[(4-methylphenyl)sulfonyl]-2(5*H*)-furanone **F70** (Latypova *et al.,* 2014), 3-chloro-5(*S*)-[(1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexyloxy]-4-[(4-methylphenyl)sulfanyl]-2(5*H*)-furanone **F104** (Sharafutdinov *et al.,* 2017), 3-chloro-5(*S*)-[(1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexyloxy]-4-[(4-methylphenyl)sulfonyl]-2(5*H*)-furanone **F105** (Sharafutdinov *et al.,* 2017), 3,4-dibromo-5(*S*)-[(1*S*,2*R*,4*S*)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-yloxy]-2(5*H*)-furanone **F122** (Chen and Huang 1994), 3,4-dichloro-5(*S*)-[(1*S*,2*R*,4*S*)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-yloxy]-2(5*H*)-furanone **F123** (Chen and Huang 1993), 3,4-dibromo-5(*S*)-[(1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexyloxy]-2(5*H*)-furanone **F124** (Chen *et al.,* 1995), 3,4-dichloro-5(*S*)-[(1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexyloxy]-2(5*H*)-furanone **F125** (Fenske and Merzweiler 1989; Sharafutdinov *et al.,* 2017) were synthesized according to the previously reported methods. A detailed description of the preparation and characterization of compounds **F122** and **F123** is presented in the Supplementary data.

Stock solutions of **F123** were prepared by diluting powders in pure DMSO (Sigma-Aldrich, Saint-Quentin Fallavier, France) at a concentration of 20 mg/ml. To solubilize the furanone in medium at high concentrations, pluoronic acid **F-127** (Sigma-Aldrich, 10% stock solution in DMSO) was added up to final concentration of 0.1%. The final concentration of DMSO in bacterial growth medium was 5%, which was verified to be non-toxic for the bacterial strains tested. All conventional antibiotics were purchased from Sigma.

Strains and culture conditions

In this study, Gram-positive bacteria: methicillin-sensitive *Staphylococcus aureus* ATCC®29213 (MSSA), *Staphylococcus epidermidis* (clinical isolate), *Micrococcus luteus* (clinical isolate), *Bacillus subtilis* 168 (wild type), *Bacillus cereus* (clinical isolate) and Gram-negative bacteria: *Klebsiela pneumonie* (clinical isolate), *Pseudomonas aeruginosa* (ATCC®27853), *Enterobacter aerogenes* (clinical isolate), *Escherichia coli* (MG1655) were used. The bacterial strains were stored in 10% (V/V) glycerol stocks at -80°C, freshly streaked on Muller-Hinton (MH) agar plates (Sigma), and grown overnight at 35°C before use. Fresh colony material was used to adjust an optical density of 0.5 McFarland (equal to 10^8 cells/mL) in 0.9% NaCl solution, used as a working suspension.

Synergy testing by checkerboard assay

A checkerboard assay was performed as described previously (den Hollander *et al.,* 1998). Briefly, the final concentrations of both compounds ranged from 1/16 to 4×MIC for a furanone derivative and from 1/256 to 4×MIC for the antibiotics. In total, 11 dilution steps of antibiotics and 7 dilution steps of furanones (**F122**, **F123** and **F125**) were analyzed. The microwell plates were incubated at 35°C for 24 hours. Each test was performed in triplicate and included a growth control without addition of any antibiotic or any furanone. The fractional inhib-

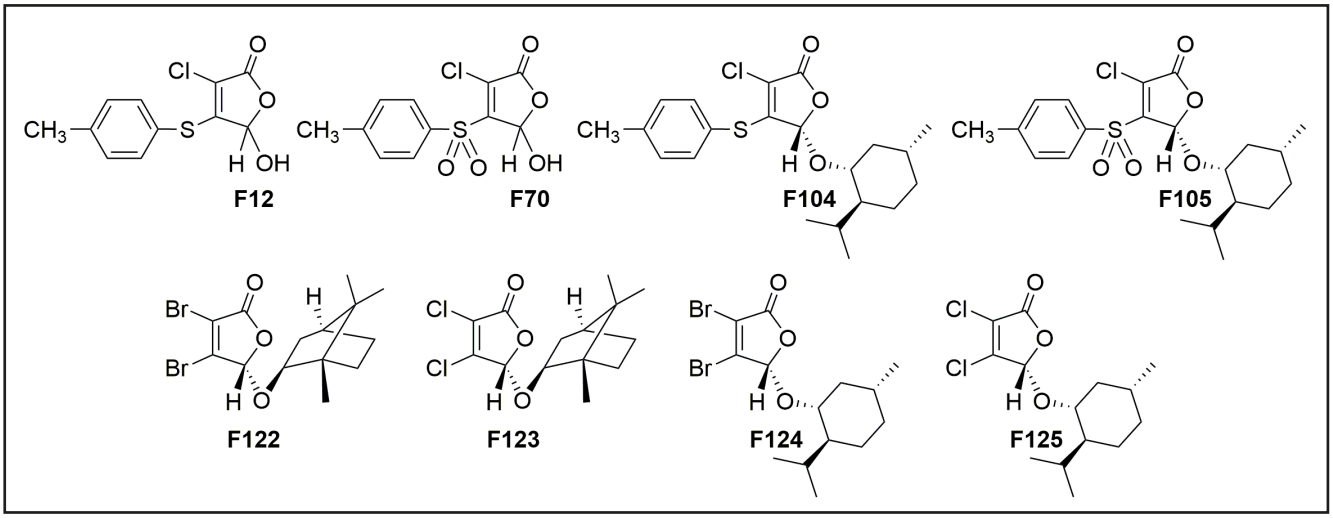

Figure 1 - *Molecular structures of 2(5H)-furanone derivatives.*

itory concentration index (FICI) for each double combination was calculated as follows:

$$FICI_{Antibiotic/Furanone} = \frac{MIC_{Antibiotic\ (combination)}}{MIC_{Antibiotic\ (alone)}} + \frac{MIC_{Furanone\ (combination)}}{MIC_{Furanone\ (alone)}}$$

The FICIs were counted from the concentrations in the first non-turbid well found in each row and column along the turbidity/non-turbidity interface and the lowest FICI value was used to characterize the synergy. For the FICI interpretation we refer to (den Hollander *et al.,* 1998; Odds 2003): FICI<0.5 corresponds to synergy, 0.5<FICI<4 corresponds to either additive effects or indifference, while FICI>4 corresponds to antagonism.

Determination of minimum inhibitory and minimum bactericidal concentrations

The minimum inhibitory concentration (MIC) of antimicrobial compounds was determined by the broth microdilution method in 96-well microtiter plates (Eppendorf) according to the EUCAST rules for antimicrobial susceptibility testing (Leclercq *et al.,* 2013). The concentrations of **F123** ranged from 0.25 to 256 μg/ml. The minimal inhibitory concentration was determined as the lowest concentration of antimicrobial for which no visible bacterial growth could be observed after 24 hours of incubation. Then, to determine a minimum bactericidal concentration, a culture liquid from wells without visible growth was diluted a thousand-fold by fresh medium and incubated for 24 hours growth at 35°C. MBC was assumed as a concentration in which no viable cells were observed (European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical and Infectious 2000).

Testing of resistance development

The development of bacterial resistance was tested by using the serial passages approach as described in (Locher *et al.,* 2014) with modifications. Briefly, sterile 96-well plates were seeded with *B. cereus* cells at different concentrations of a test compound in a liquid medium (similar to the definition of MIC). The plates were incubated for 20 hours at 35°C, then microorganisms from the last well with a visible growth (with sub-lethal concentration of a compound) were transferred to antimicrobial-free agar plate. Cells from the agar surface were resuspended in a liquid medium and used as inoculum for subsequent seeding in a liquid medium with a range of concentrations of antimicrobials. The procedure was repeated to obtain 14 cycles of passages and the MICs of compounds were determined. Then a series of 7 passages on antimicrobial-free agar was done and MICs were again determined.

Influence of antimicrobials on B. cereus biofilm

To determine the biofilm preventing activity (BPC) of antimicrobials, the bacterial culture containing 5×10^5 cells/mL in the MH broth was seeded into 96-well polystyrol culture plates (Eppendorf). Antimicrobials were added in serial dilution to obtain final concentrations in a range of 0.25-256 μg/ml and cells were grown under static conditions for 24 hours at 35°C. Then crystal-violet staining (Sharafutdinov *et al.,* 2017) was applied. Cell-free wells incubated with pure medium subjected to all staining manipulations were used as a control. The BPC was assessed as a concentration providing the complete absence of biofilm.

To determine the minimal biofilm eradicating concentrations (MBECs) of **F123**, cells were grown in 96-well polystyrene culture plates (Eppendorf) for 24 hours. Then **F123** was added to the wells in concentrations in a range of 0.25-256 μg/ml with subsequent cultivation at 35°C. After 24 hours the biofilms were aseptically washed with 0.9% NaCl and the number of CFUs was counted by using the drop plate assay (Herigstad *et al.,* 2001). MBEC was defined as the **F123** concentration reducing a number of viable cells by 2 orders of magnitude.

Time-kill assay

Time-kill assay was performed by detection of a decrease in CFUs number during exposure to **F123**. The bacterial culture adjusted to 10^6 cells/ml in the MH broth was seeded in 96-well polystyrene culture plates (Eppendorf) without and with **F123** at concentrations of 0.25×MIC, 1×MIC and 4×MIC. The bacterial suspension was incubated at 35°C without shaking, and samples were taken after 0, 2, 4, 6, 8, 10 and 24 hours of exposure. The number of viable bacteria was quantified by using the drop plate assay as mentioned above (Herigstad *et al.,* 2001).

*Determination of **F123** cytotoxicity*

F123 cytotoxicity was determined in MTS-assay by using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) using MCF-7 cells. The cells were cultured in DMEM - Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 96-well plates at the density of 3000 cells per well and allowed to attach overnight. Cells were cultured at 37°C and 5% CO_2 in the presence of compounds at concentrations from 1.25 to 160 μg/ml. After 24 hours of cultivation the cells were subjected to MTS-assay as recommended by the manufacturer and colored formazan product which was measured on Tecan Infinite 200Pro at 550 nm. The concentration required to inhibit cellular dehydrogenase activity by 50% (CC_{50} value) was calculated.

RESULTS AND DISCUSSION

Antimicrobial activity

Eight derivatives of 2(5*H*)-furanone possessing different functional groups (halogen atom, sulfanyl or sulfonyl group, *l*-menthol or *l*-borneol moiety, see *Figure 1* for structures) were tested for their ability to repress the growth of various bacteria (*Table 1*). Neither of the compounds tested have any activity against Gram-negative bacteria. Compound **F105**, recently characterized as a promising anti-staphylococcus agent (Sharafutdinov *et al.,* 2017), exhibited comparable activity against all other Gram-positive bacteria. The substitution of sulfonyl with a sulfanyl group completely abrogated the activity, proposing the requirement of sulfonyl group SO_2 for activity. On the other hand, furanone **F12** carrying thioether moiety was inactive. The exclusion of *l*-menthol moiety from **F105** led to the chemotype with no antibacterial activity (**F70**) (Kayumov *et al.,* 2015; Sharafutdinov *et al.,* 2017). Interestingly, compounds possessing only halogen and terpene moieties (**F122**, **F123**, **F125**) restored the activity, suggesting the secondary role of *p*-tolylsulfonyl group in the antibacterial activity of **F105** and the requirement of *l*-menthol moiety for **F105** antibacterial activity (Rogers *et al.,* 2010). Remarkably, both chlorinated compounds **F123** and **F125** exhibited relatively high activity against

Table 1 - *Antibacterial activity of 2(5H)-furanone derivatives with different functional groups (MIC, μg/ml).*

	F12	***F70***	***F104***	***F105***	***F122***	***F123***	***F124***	***F125***
Functional groups	*Cl p-TolS*	*Cl p-TolSO$_2$*	*Cl l-menthol p-TolS*	*Cl l-menthol p-TolSO$_2$*	*Br l-borneol*	*Cl l-borneol*	*Br l-menthol*	*Cl l-menthol*
S. aureus	>128	>128	>128	8	>128	8	>128	>128
S. epidermidis	>128	>128	>128	16	32	8	>128	32
M. luteus	>128	>128	>128	8	16	8	>128	16
B. cereus	32	>128	>128	8	16	8	128	64
B. subtilis	32	>128	>128	16	32	16	>128	32
E. coli	>128	>128	>128	>128	>128	>128	>128	>128
E. aerogenes	>128	>128	>128	>128	>128	>128	>128	>128
K. pneumonie	>128	>128	>128	>128	>128	>128	>128	>128
P. aeruginosa	>128	>128	>128	>128	>128	>128	>128	>128

Gram-positive bacteria, similar to that of **F105**, in contrast to brominated derivatives **F122** and **F124**. During further investigations we found that **F123** has a minimal bactericidal concentration (MBC) of 8 μg/ml against *B. cereus*, while MBCs of **F123** against other bacterial species were more than 128 μg/ml, suggesting the specificity of **F123** to *B. cereus*.

Next, the cytotoxicity of active compounds (**F122**, **F123**, **F125**) was determined using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega). The MCF-7 cells were incubated for 24 hours in the presence of different concentrations of the test compounds and the residual respiratory activity was then determined. Based on the results obtained, the values of CC_{50}-concentration of the substances resulting in a two-fold suppression of respiratory activity were found to be 16 μg/ml for **F122**, 32 μg/ml for **F123**, and 15 μg/ml for **F125** (*Table 2*).

To estimate substance safety, the therapeutic index (TI) values for these compounds were calculated by dividing the CC_{50} values by the MIC values of these substances. **F123** in the case of *B. cereus* exhibited a therapeutic index comparable to that of benzalkonium chloride, widely used for the therapy of external infections. While only compounds with TI values higher than 10 are usually of interest as potential antimicrobials for future investigations (Zapotoczna *et al.,* 2017), in various researches TI of the widely used biocide benzalkonium chloride has been reported to be around 1.0 on different cell lines (Damour *et al.,* 1992; Garipov *et al.,* 2017; Sapozhnikov *et al.,* 2017). Therefore, the chemotype of **F123** can be of interest for the development of topical bactericidal agents.

*Analysis of **F122**, **F123**, **F125** synergy with conventional antibiotics*

It was previously shown that **F105**, possessing *l*-menthol and sulfonyl moieties, exhibits a synergy with aminoglycosides (amikacin and gentamicin) and benzalkonium chloride (Sharafutdinov *et al.,* 2017). Therefore, the antimicrobial efficacy against *B. cereus* cells of **F122**, **F123** and **F125** in combinations with these antibiotics has been characterized by the checkerboard assay. The values of the fractional inhibitory concentrations (FICI) were determined as described in the materials and methods and the median was calculated. For deeper analysis of **F123** synergy with antimicrobials, the combined MICs of the antibiotic/**F123-**mixtures were plotted (as isoboles) and the effective concentrations of **F123** (EC_{50}) leading to the two-fold reduction of antibiotic's MIC were calculated (*Figure S4*). **F122** and **F123** demonstrated marked synergism with amikacin reducing two-fold its MIC in concentrations of 1-2 μg/ml (*Table 3*), which is 10-20 times lower than the respective values of CC_{50} (*Table 2*). To test whether **F123** in combination with amikacin or gentamicin could demonstrate enhanced cytotoxicity, the effect of furanone combinations with antimicrobials on MCF-7 viability was also tested. None of aminoglycosides in concentrations

Table 2 - *CC_{50} and therapeutic indexes of **F122**, **F123**, **F125**.*

Compound	*CC_{50} (μg/ml)*	*CC_{50}/MIC, therapeutic index (TI)*				
		S. aureus	*S. epidermidis*	*M. luteus*	*B. cereus*	*B. subtilis*
Benzalkonium chloride	3.5	1.75	1.75	3.5	1.75	3.5
F122	16	<0.125	0.5	1	1	0.5
F123	32	1	2	2	4	2
F125	15	<0.1	0.5	0.9	0.2	0.5

Table 3 - *FICI and CC_{50} for **F122**, **F123**, **F125** on B. cereus cells.*

	Gentamicin		*Amikacin*		*Benzalkonium chloride*	
	FICI	*EC_{50}*	*FICI*	*EC_{50}*	*FICI*	*EC_{50}*
F122	0.516	1.9	0.375	1.2	0.531	1.7
F123	0.563	1	0.375	0.9	0.563	2.2
F125	0.188	1.8	0.156	5.2	0.328	5.1

of 8×MBC increased the cytotoxic effect of **F123** against mammalian cells in MTS-test, most likely because of their higher selectivity towards bacterial cells (data not shown). We also performed the calculation showing that the application of **F123** at a concentration of 4 μg/ml potentially enhances the effect of amikacin up to 32-fold (*Table S1*).

*Antibacterial properties of **F123***

The antibacterial properties of **F123** are shown in *Table 4*. Minimal inhibitory concentration (MIC) was found to be 8 μg/ml for *S. aureus, S. epidermidis, M. luteus* and *B. cereus* and 16 μg/ml for *B. subtilis*. Interestingly, the minimum bactericidal concentration (MBC) was detected only for *B. cereus* bacteria and was 16 μg/ml. By contrast, *B. subtilis*, the closest relative of *B. cereus,* was tolerant to **F123** even at the concentration of 128 μg/ml as well as all other tested organisms. Since many 2(5*H*)-furanone derivatives demonstrate biofilm-inhibiting properties, the ability of **F123** to repress biofilm formation by various bacteria was determined by crystal violet staining of residual biofilms (see *Table 4*, biofilm preventing concentrations - BPCs). For *S. aureus, S. epidermidis, B. cereus* and *B. subtilis*, BPCs of **F123** were found to correspond to MICs, suggesting no biofilm-preventing properties of **F123**, in contrast to some halogenated furanones which were reported as inhibitors of biofilm formation by Gram-positive bacteria like *Bacilli* and *Staphylococci* (Yujie *et al.,* 2013; Kayumov *et al.,* 2015). Biofilm suppression by **F123** seems to be a consequence of the repressed growth of bacteria. Interestingly, for *M. luteus* the BPC was higher that MIC. We attribute this phenomenon to induction of biofilm formation by the bacteria under sub-lethal concentrations of furanone derivatives, as was reported earlier, for example, for *S. aureus* (Sharafutdinov *et al.,* 2017).

Table 4 - *Antibacterial spectrum of **F123**.*

	MIC	*MBC*	*BPC*	*MBEC*
S. aureus	8	>128	8	>128
S. epidermidis	8	>128	8	>128
M. luteus	8	>128	16	>128
B. cereus	8	8	8	64
B. subtilis	16	>128	16	128
E. coli	>128	>128	>128	>128
E. aerogenes	>128	>128	>128	>128
K. pneumonie	>128	>128	>128	>128
P. aeruginosa	>128	>128	>128	>128

Previously we reported that 2(5*H*)-furanone carrying sulfonyl and *l*-menthol moieties is efficient against biofilm-embedded *S. aureus* (Sharafutdinov *et al.,* 2017). In the case of **F123**, the significant ability to affect the biofilm-embedded cells (minimal biofilm-eradicating concentration, MBEC) was detected only for *Bacillus* species. **F123** reduced the CFUs number of *B. cereus* by 3 orders of magnitude at a concentration of 64 μg/ml (4×MBC), while for *B. subtilis* the concentration was two-fold higher, suggesting the specificity of **F123** for *B. cereus* cells.

The biocidal properties of **F123** were next investigated by time-kill assay on Gram-positive bacteria (*S. aureus, S. epidermidis, M. luteus*, *B. subtilis*, *B. cereus*) and Gram-negative *E. coli* as a negative control. **F123** demonstrated weak bacteriostatic properties at a concentration of 4×MIC against *S. aureus*, *S. epidermidis*, *M. luteus* and *B. subtilis* (*Figure 2*). In agreement with earlier observations, *B. cereus* cells were killed within 8 h at 4×MIC, suggesting that **F123** exhibits a specific bactericidal effect against these bacteria.

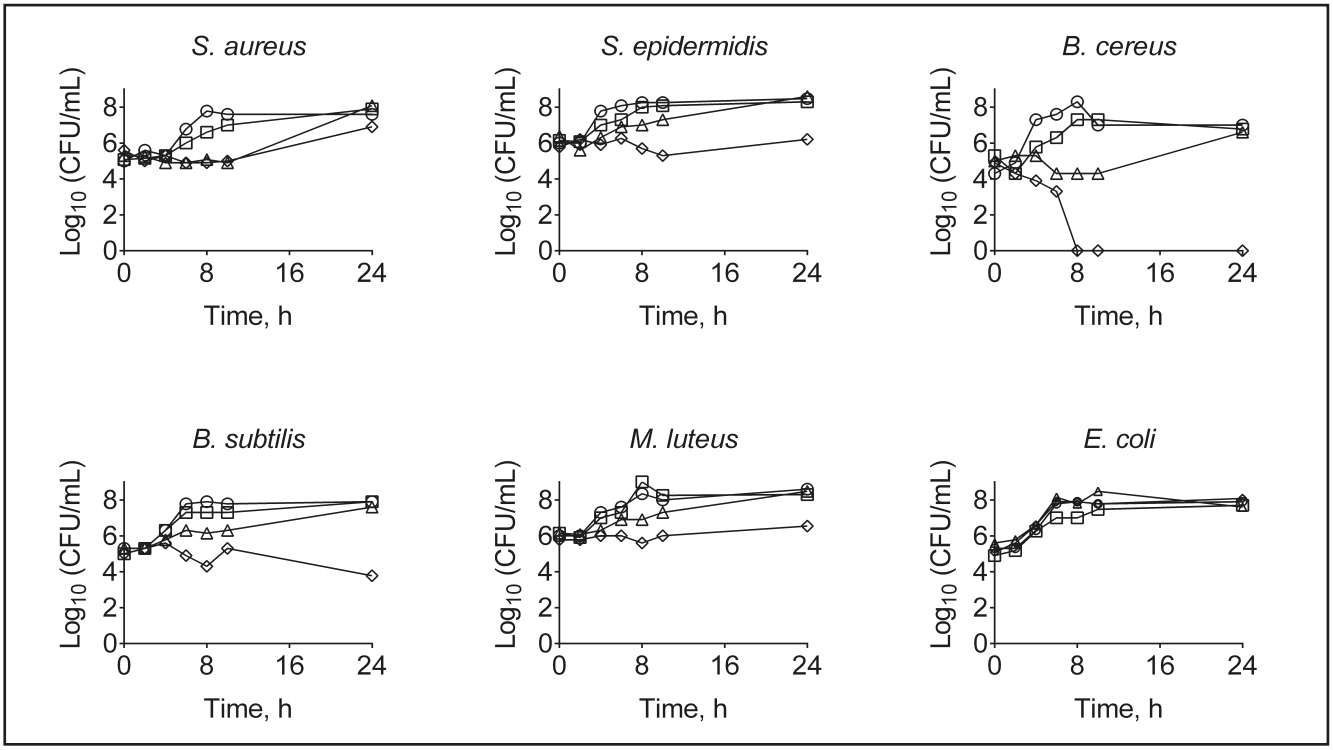

Figure 2 - *Time-kill curves of B. cereus without (circles) and with **F123** at concentrations of 0.25×MIC (squares), 1×MIC (triangles) and 4×MIC (diamonds).*

*B. cereus and development of resistance to **F123***

The development of bacterial resistance to benzalkonium chloride has been reported in some recent papers (Ortiz *et al.,* 2014; Alotaibi *et al.,* 2017; Meier *et al.,* 2017). We tested whether *B. cereus* could also develop resistance to **F123** in a series of passages (*Figure 3*). The MICs of **F123** and benzalkonium chloride as a reference biocide were determined after fourteen passages of *B. cereus* in the presence of sub-lethal concentrations of antimicrobials. There was no significant change in bacterial sensitivity to **F123** after 14 passages, which is an undoubted advantage of this compound, while MIC of benzalkonium chloride increased from 1 to 16 μg/ml and decreased only up to 4 μg/ml after seven passages in the absence of antibacterial pressure.

*Comparison of **F123** and conventional antibiotics activity on mature culture of B. cereus*

When embedded in biofilm or in detached cell clumps, bacteria become unsusceptible to antibiotics because the biofilm matrix is impenetrable for the majority of antimicrobials. Since the biofilm structure and permeability for antimicrobials vary significantly among bacterial species (Sharafutdinov *et al.,* 2016), we tested various antibiotics recommended for *Bacillus spp.* (see *Table S2*) in order to characterize their efficiency against both planktonic and biofilm-embedded cells. *B. cereus* was grown for 24 hours to obtain a rigid biofilm. Then antibiotics in concentrations ranging from 1 to 1024-fold of their respective MBCs were added directly to the 24-hour cell cultures, followed by incubation for another 24 hours. The number of CFUs was counted separately in both the culture liquid and the biofilm layer to distinguish antibiotic efficiency against swimming and biofilm-embedded cells, respectively. The concentration of antibiotic decreasing the number of CFUs in 24 hours old culture liquid or in mature biofilm by at least 3 orders of magnitude was considered as the minimal bactericidal concentration for planktonic cells (MBC-P) or biofilm-embedded cells (MBC-B), respectively (Mah 2014).

Among all antibiotics tested, rifampicin, aminoglycosides (amikacin and gentamycin), tetracycline, and to a lesser extent the macrolides (erythromycin and azithromycin) and chloramphenicol, exhibited promising activity against both detached and biofilm-embedded cells at relatively low concentrations (see MBC-P and MBC-B values, *Table 5*).

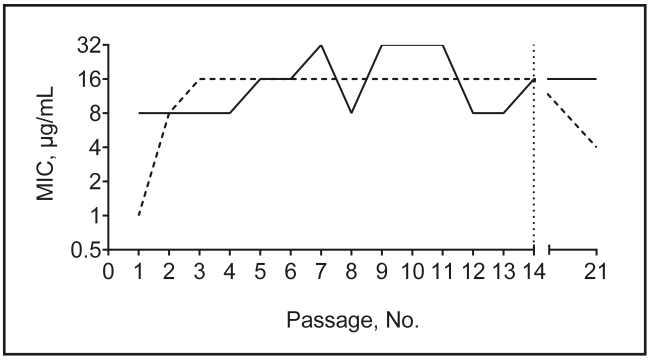

Figure 3 - *Development of resistance by B. cereus to **F123** (solid line) and benzalkonium chloride (broken line). Passages from 1 to 14 were performed in the presence of, and from 14 to 21 without, test compound.*

Table 5 - *Comparative antibacterial activity of **F123** and antibiotics on mature B. cereus culture.*

Antimicrobial	*MIC, μg/ml*	*MBC, μg/ml*	*MBC-P, μg/ml*	*MBC-B, μg/ml*
F123	8	8	32	64
F105	8	16	128	>256
Ampicillin	64	64	65536	>65536
Cefazolin	64	64	1024	65536
Imipenem	0.5	>16	>1024	>16384
Meropenem	0.25	0.25	64	>256
Vancomycin	2	8	128	>8192
Amikacin	4	4	8	32
Gentamycin	0.5	0.5	1	8
Erythromycin	0.125	0.125	0.5	8
Azithromycin	0.25	0.5	2	32
Tetracycline	0.5	4	32	64
Ciprofloxacin	0.064	0.064	8	>64
Moxifloxacin	0.032	0.032	4	>32
Clindamycin	4	64	64	1024
Chloramphenicol	4	16	128	1024
Rifampin	0.25	0.25	2	2

By contrast, while the fluoroquinolones ciprofloxacin and moxifloxacin exhibited the lowest MICs, they were almost ineffective against *B. cereus* mature biofilm, with MBC-P and MBC-B exceeding more than 1000-fold the respective MBCs. Similarly, meropenem with MICs of 0.5 μg/ml and vancomycin with MIC of 2 μg/ml (MBCs are 0.5 and 8 μg/ml, respectively) were also inactive against biofilm-embedded cells: the concentrations required to kill bacteria dramatically increased over than 1000-fold of the respective MBCs, most likely because of the penetration barrier formed by the biofilm matrix. While in this assay chloramphenicol and tetracycline exhibited activity against biofilm-embedded bacteria, some *B. cereus* isolates were reported to be tolerant to these antimicrobials (Agerso *et al.,* 2002; Glenwright *et al.,* 2017). So far, our *in vitro* data demonstrate that only rifampin, aminoglycosides and macrolides seem to be effective to cure *B. cereus* infections, including biofilm-associated forms. At the same time, fluoroquinolones and vancomycin, which are widely used in practice, showed inefficiency when treating biofilm-embedded bacteria *in vitro;* clinical evidence is still missing.

While the efficacy of **F123** in this assay was comparable with azitromycin, amikacin and tetracycline with MBC-B/MIC ratio of 8, its concentration required to affect sessile *B. cereus* cells is nevertheless higher than its CC_{50} value, limiting its application area. By contrast, low risk of resistance development to **F123** allows use of this compound for aseptic treatment of various surfaces in hospitals and industry. Next, although none of the aminoglycosides or macrolides is recommended for treatment of biofilm-associated *B. cereus* infections, they remain attractive therapeutics because of their high availability and low costs in most countries, and a combination of amikacin with **F123** could be a promising approach for the treatment of topical *B. cereus* infections. Further investigation is required.

CONCLUSIONS

When the common mechanism of biofilm prevention in Gram-negative bacteria by furanones is interference with

the quorum signaling AI-2 system, the mechanisms of activity against Gram-positive bacteria are still elusive and species-specific (Sharafutdinov *et al.,* 2017). While some molecular targets have been proposed, such as common stress proteins and global regulators like Spo0A in *Bacillus subtilis* (Ren *et al.,* 2004; Kayumov *et al.,* 2015; Trizna *et al.,* 2015), many investigations indicate that halogen-containing 2(5*H*)-furanone derivatives drastically change bacterial metabolism, suggesting their common toxicity and rather high specificity to molecular targets (Lattmann *et al.,* 2005; Janssens *et al.,* 2008; Sharafutdinov *et al.,* 2017).

As was found, the *l*-borneol moiety is an essential part of the **F123** molecule with respect to demonstrating its antibacterial activity against *B. cereus*. Thus, **F105** which has similar antibacterial activity against planktonic cells of *B. cereus*, was unable to target biofilm-embedded cells (*Table 2*), and **F125** was inefficient with *l*-menthol instead of *l*-borneol moiety. **F123** was found to selectively kill both planktonic and biofilm-embedded cells of *B. cereus* and to a lesser extent *B. subtilis* cells, in contrast to such conventional antibiotics as vancomycin and ciprofloxacin. Since there are only a few antibiotics that are able to directly affect biofilm-embedded cells, **F123** seems to be a promising compound with activity comparable to gentamycin for the treatment of *B. cereus* and, probably, *B. anthracis* infections because of the close properties of these two species (Ivanova *et al.,* 2003; Brezillon *et al.,* 2015). **F123** is of interest for specific topical treatment of *B. cereus* skin infections, recently detected by the metagenomic approach in contact sports athletes (Martykanova *et al.,* 2017), without affecting residential skin microflora. Finally, **F123** could be applicable in appropriate fields where the prevention or eradication of *B. cereus*-specific contaminations is required, for example in the food industry or eye-healthcare (Arnesen *et al.,* 2008; Kivanc *et al.,* 2014).

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Conflict of interest

We declare that there is no conflict of financial/personal interests.

Supplementary Materials

Supplementary material is presented on one data sheet. Synthetic procedures with NMR (nuclear magnetic resonance) and IR (infrared) spectra of compounds **F122** and **F123** are given in *Figures S1-S3*. Supplementary biological data contains *Table S1* (Increase of an antibiotic efficacy in the presence of **F123**), *Table S2* (Minimum inhibitory and bactericidal concentrations of antibiotics against *B. cereus*) and *Figure S4* (EC_{50} values of the compounds **F122**, **F123** and **F125** reducing twice the MICs of gentamicin, amikacin and benzalkonium chloride on *B. cereus* cells).

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