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ANTI-ADHESION PROPERTIES OF AMINOPROPANOL DERIVATIVE WITH N-ALKYLARYL RADICAL KVM-194 AGAINST *PSEUDOMONAS AERUGINOSA*

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Key words: *Pseudomonas aeruginosa, hydrophobicity, motility, adhesion, biofilms.*

Introduction. *The present study assessed Pseudomonas aeruginosa surface characteristics, motility and adhesion properties under the influence of 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride (KVM-194).*

Material and methods. *The clinical strain P. aeruginosa 449 was used in the study. The cell surface hydrophobicity (CSH) was evaluated by adhesion to solvent (MATS test). Swimming, swarming and twitching motility of P. aeruginosa were studied by standard methods in media with different agar contents. Cells ability to adhere to polystyrene was assessed by the Christensen method. The effect of KVM-194, meropenem and ciprofloxacin on hydrophobicity and motility was evaluated both at 0.5 or 2.0 minimal inhibitory concentrations (MIC), while on adhesion abilities – only 0.5×MIC.*

Results. *It was shown that 0.5× MIC KVM-194 reduced CSH of P. aeruginosa (by 16%, p<0.05), affected swimming motility, and decreased its adhesion to polystyrene. The most pronounced changes in adhesion properties were recorded after 3-5 hours of pre-treatment with this compound. Moreover, it was proven that sub-MICs of meropenem and ciprofloxacin did not alter bacterial cells hydrophobicity and had no significant influence on P. aeruginosa motility and adhesion properties.*

Conclusions. *The present study suggested that KVM-194 affected the initial steps of P. aeruginosa biofilm formation and thus had tremendous potential for new antibiofilm agents' development.*

Cuvinte cheie: *Pseudomonas aeruginosa, hidrofobicitate, mobilitate, aderență, biofilme.*

PROPRIETĂȚI ANTI-ADERENTE A DERIVATULUI AMINOPROPANOL CU RADICAL N-ALCHILARIL KVM-194 ÎMPOTRIVA *PSEUDOMONAS AERUGINOSA*

Introducere. *Prezentul studiu a evaluat caracteristicile suprafeței, mobilitatea și proprietățile de aderență a Pseudomonas aeruginosa, sub influența clorurii de 1-[4-(1,1,3,3-tetrametil-butil) fenoxi]-3-(N-benzil-hexametilenimin)-propan-2-ol (KVM-194).*

Material si metode. *În studiu au fost utilizate 449 de tulpini clinice de P. aeruginosa. Hidrofobicitatea suprafeței celulare (CSH) a fost evaluată prin aderență la solvent (testul MATS). Mobilitatea, roirea și mișcarea bacteriilor P. aeruginosa au fost studiate prin metode standard în medii cu conținut diferit de agar. Capacitatea celulelor de a adera la polistiren a fost evaluată prin metoda Christensen. Efectul KVM-194, meropenemului și ciprofloxacinei asupra hidrofobicității și mobilității a fost evaluat atât la concentrații inhibitorii minime de 0,5 sau 2,0 (CMI), cât și asupra abilităților de aderență – doar 0,5×CMI.*

Rezultate. *S-a demonstrat că 0,5×CMI KVM-194 a redus CSH-ul P. aeruginosa (cu 16%, %, p<0,05), a afectat mobilitatea și a redus aderența la polistiren. Cele mai pronunțate modificări ale proprietăților de aderență au fost înregistrate după 3-5 ore de pre-tratament cu acest compus. De asemenea, s-a arătat că sub-CMI-urile meropenemului și ciprofloxacinei nu au modificat hidrofobicitatea celulelor bacteriene, nu au avut nicio influență semnificativă asupra mobilității și proprietăților de aderență ale P. aeruginosa.*

Concluzii. *Prezentul studiu sugerează că KVM-194 acționează la etapele inițiale de formare a biofilmului de către P. aeruginosa și, prin urmare, are un potențial enorm pentru dezvoltarea de noi preparate antibiofilm.*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen, characterized by resistance to a wide range of antibacterial drugs and the ability to form biofilms. Microbial community is irreversibly associated with biotic (tissue, wounds) and abiotic surfaces (intravascular, urethral catheters, various metal grafts, contact lenses) (1). Biofilm formation leads to an increase both in the duration of hospitalisation and in the persistence of infectious disease. It could also cause fatal complications of catheter-associated infections. Adhesions, proliferation, formation of microcolonies are conditions required for effective colonization of favourable surface. Adhesion is provided by various motility modes (2). The bacterial cell surface hydrophobicity (CSH) is one of the factors that determines the strength of adhesion (3, 4).

The migration and dispersion provide *P. aeruginosa* with potential advantages while searching for sources of nutrients, avoiding negative environmental factors, access to optimal colonization and distribution sites in the environment, etc. (5). Bacteria of the genus *Pseudomonas* can move via several motility modes on environments with various viscosities. Swimming motility is derived by flagella that allows bacteria to disperse in liquid environments (6, 7). Type IV pili are involves twitching motility over the top of solid surfaces (2). Flagella and pili, as well as production of rhamnolipids, provide swarming motility in viscous environment of the lungs with cystic fibrosis, which is as closely as possible mimicked *in vitro* by semi-solid agar (5, 8, 9). It is remarkable that, in the same conditions, cells can also exhibit a sliding movement that does not require pili or flagella (8).

Some antibiotics at subinhibitory concentrations (sub-MICs) can affect the colonization of surfaces by microorganisms, via disturbing the biofilm formation processes. Thus, β-lactam antibiotics such as penicillins and cephalosporins reduce the cell hydrophobicity of gram-positive (*S. aureus*) and gram-negative (*P. aeruginosa*) bacteria (3, 10). At the same time carbapenems, other β-lactam antibiotics show no effect on CSH, motility and adhesion properties (10). Sub-MICs of tobramycin cause no changes in swarming motility of *P. aeruginosa* (11) but reduce this parameter in *E. coli* (7). The macrolide antibiotic azithromycin also inhibits this mode of motility due to impaired synthesis of rhamnolipids, which are surfactants (12).

Investigation of the mechanisms of various surfaces colonized by bacteria, as well as evaluation of antimicrobials effect on the initial stages of biofilm formation, are essential for further deve-lopment of preventive strategies against biofilm formation, as well as for solving the problems of ineffective antimicrobial therapy for infections caused by microbial communities.

Previously, we had found that 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride (KVM-194) showed a broad spectrum of antibacterial activity (13), among which – *in vitro* activity against biofilms of gram-negative and gram-positive bacteria (14, 15, 16). As KVM-194 possessed membranotropic effects and affected the composition of cell walls of gram-negative bacteria (17), we hypothesized that this compound could affect bacterial cell surface properties and adhesion to polystyrene.

The purpose of this present paper was to study the influence of 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride on adhesion properties of *Pseudomonas aeruginosa*.

MATERIAL AND METHODS

Bacterial strains and subculture conditions

The bacterial strain used in the present study was *Pseudomonas aeruginosa* 449 isolated from pus. The test strain showed resistance to cefepime and tetracycline, intermediate susceptibility to ceftriaxone, cefotaxime and meropenem, susceptibility to aztreonam, cefoperazone, ciprofloxacin, amikacin, and gentamicin. The strain was subcultured at 37°C on Tryptone Soya Agar plates.

Antimicrobials, chemicals, and media

The 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride (KVM-194) used in the present study was synthesised within the Institute of Organic Chemistry of NAS of Ukraine. KVM-194 was dissolved in 10% dimethyl sulfoxide; the stock solution concentration was 1 mg/mL. All other chemicals were obtained from commercial sources.

Ciprofloxacin and meropenem were purchased in the pharmacy under the trade name Ciprinol (CIP, solution for infusion, manufactured by KRKA, Slovenia) and Meronem (MER, powder for solution for injection, manufactured by AstraZeneca UK Limited, United Kingdom) were used as comparator agents. The following media were used in the

present study: Luria-Bertani broth (Conda, Spain), Luria-Bertani agar and Tryptone Soya Broth (TSB, HiMedia, India).

Bacterial surface hydrophobicity assay

P. aeruginosa surface hydrophobicity was measured using previously described microbial adhesion to solvents (MATS) method with modifications (18). The affinity to ethyl acetate that is a monopolar and basic solvent was studied. An overnight culture in TSB medium was 10-fold diluted with fresh TSB medium. The hydrophobicity properties were estimated by growing strain in TSB (optical density OD_{600} 0.3) with or without KVM-194 (25 μg mL^{-1}), MER (1.0 μg mL^{-1} or 4.0 μg mL^{-1}) or CIP (0.125 μg mL^{-1} or 0.5 μg mL^{-1}) at 37°C for 90 min. After incubation, bacteria were washed twice in 0.9% NaCl solution by centrifugation for 15 min at 3000 rpm and were resuspended in same solution to OD_{600}0.18-0.22 (A_0). Afterwards, ethyl acetate (0.5 mL) was added to the bacterial suspensions (3.0 mL), which were then kept at room temperature (RT) for 10 min to saturate. Each sample was then mixed by vortexing (model V-3, ELMI, Latvia) for 2 min and then allowing the mixture to stand for 15 min at room temperature for phase separation. The aqueous phase was collected and the OD_{600} was measured (A). The results were expressed as the percentage decreased in the OD of the aqueous phase (A) compared with the OD of the initial cell suspension (A_0): $100\times[1-(A/A_0)]$. Each assay was repeated three times in duplicate.

Motility assay

The swarming, swimming, and twitching motilities of *P. aeruginosa* were investigated using the following media: (I) swim plates [1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.3% agar], (II) swarm plates [1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% agar, 1M $MgSO_4$, 0.5% glucose], and (III) twitch plates [1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.0% agar]. An overnight cell culture in TSB medium was incubated 30-45 min with 0.5 or 2.0×MIC KVM-194 (25.0 μg mL^{-1} and 100 μg mL^{-1} respectively), meropenem (1.0 μg mL^{-1} and 4.0 μg mL^{-1} respectively), ciprofloxacin (0.125 μg mL^{-1} and 0.5 μg mL^{-1} respectively). Control cultures contained no antimicrobials. Each assay was repeated three times in duplicate.

For the *swimming motility assay*, the plates were inoculated in the centre with a sterile toothpick and incubated for 16-20 h at RT (10). Motility was assessed by observation of the circular turbid zone formed by bacteria migrating away from the inoculation point.

For the *swarming motility assay*, the bacterial cells were gently inoculated by micropipette (2 μL) into the top of semisolid agar, and the plates were incubated at 37°C for 16-24 h (19).

For the *twitching motility assay*, the cells were stabinoculated with a sterile toothpick through an agar layer to the bottom of the Petri dish. After incubation for 24-48 h at 37°C, a hazy zone of growth at the interface between the agar medium and the glass surface was observed. The ability of bacteria to twitch strongly on the glass surface was examined by removing the agar, washing out the untouched cells and staining the attached cells by a crystal violet solution (10).

Adhesion assay

The adhesion of *P. aeruginosa* was estimated by the method by Christensen (20). An overnight culture in TSB medium was grown at 37°C in the presence or absence of 0.5× MIC KVM-194 (25 μg mL^{-1}), meropenem (1.0 μg mL^{-1}) or ciprofloxacin (0.125 μg mL^{-1}). After 1, 3, 5 or 7 h, the bacterial cells were diluted 100-fold with fresh TSB medi-um; cell suspension (100 μL) was transferred into individual wells of sterile, polystyrene, 96-well plate and incubated at 37°C. After a 24-hour incu-bation, the TSB medium was discarded, and the wells were washed thrice with distillate water to remove nonadherent bacteria. Adherent cells were fixed in place for 15 min with 96% ethanol, dried and then stained for 5 min with 0.1% crystal violet. Excess stain was rinsed off. After drying, the optical densities (OD) of stained adherent bacterial films were measured using Absorbance Microplate Reader (model ELx800, BioTek, USA) at 630 nm. Adherence measurements were re-peated at least three times in quadruplicate; the values were then averaged.

The adherence capability of the test strain was classified into four categories: non-adherent, slightly adherent, moderately adherent, or strongly adherent, based upon the OD of bacterial films. The cut-off optical density (ODc) was defined as three standard deviations above the mean OD of the negative control. The strength of adhesion was calculated by the following formula: $OD \leq ODc$ – non-adherent; $ODc < OD \leq 2\times ODc$ – slightly adherent; $2\times ODc < OD \leq 4\times ODc$ – moderately adherent; $4\times ODc < OD$ – strongly adherent.

Statistical Analysis

The obtained data were expressed as means±standard deviation (SD). The nonparametric Kruskal-Wallis H-test was used to compare the continuous variables. A p-value of <0.05 was considered as significant. STATISTICA, version 10 (StatSoft, USA) was used for the data analysis.

RESULTS

Effect of KVM-194 on Pseudomonas aeruginosa surface hydrophobicity

The cell surface hydrophobicity is an important physical factor at the stage of surface attachment, which determined the strength of adhesion. Particularly, bacteria with a hydrophobic surface adhere better than with a hydrophilic one (3, 4), which allow them to colonize the tissues and surfaces of medical devices, form biofilms and exacerbate the course of the infection process.

The present study investigated the influence of KVM-194, meropenem and ciprofloxacin on the hydrophobic properties of *P. aeruginosa*. The data reported in Table 1 showed that KVM-194 at 0.5× minimal inhibitory concentration (MIC) reduced bacterial hydrophobic properties by 16% compared to the intact control ($p < 0.05$). High turbidity of medium was observed in the presence of KVM-194 at 2.0×MIC, which caused erroneous results (data not shown).

Table 1. The effect of KVM-194, meropenem or ciprofloxacin on MATS of *P. aeruginosa* depending on concentration.

Antimicrobials	Solvent affinity (%, mean±SD) to ethyl acetate	
	0.5×MIC	2.0×MIC
KVM-194	52.77±2.492*	N/A
Meropenem	60.23±3.499	60.86±1.196
Ciprofloxacin	61.05±0.316	58.55±0.971
Control (without antimicrobials)	62.64±2.905	

** in comparison with control (bacterial growth without antimicrobials) p<0.05.*

Both Meropenem concentrations did not alter *P. aeruginosa* 449 cells hydrophobic properties. The same results were obtained for ciprofloxacin.

Effect of KVM-194 on Pseudomonas aeruginosa motility

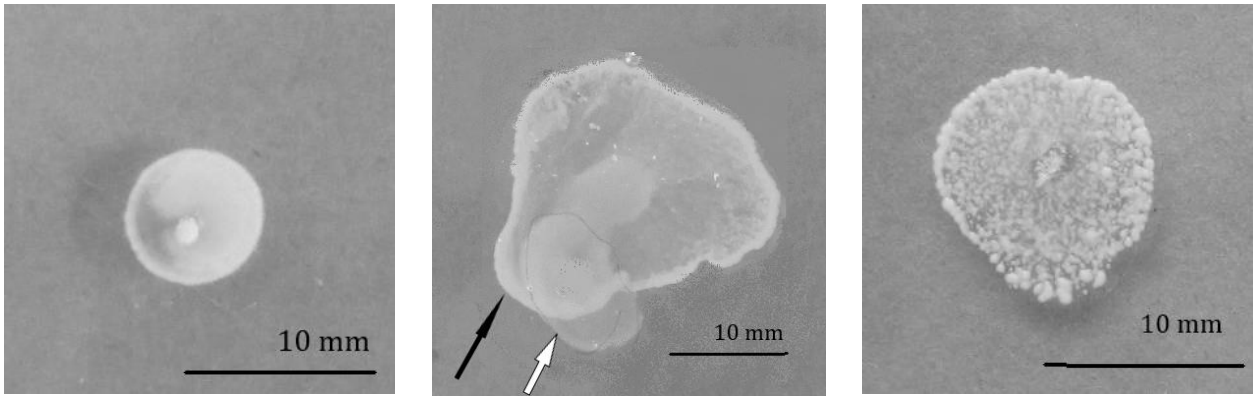
Single gram-negative bacteria cells swim in a liquid environment using flagella, which allow *P. aeruginosa* to respond to attractants and repellents. For solid surfaces or human body tissues, colonization pili are required (2), which are also responsible for intercellular aggregation (21). Type IV pili allow cells to move on the top of solid surface by extension and retraction of filaments (2).

Flagella and type IV pili are essential for swarming; these structures are involved in colonization of the mucus. Swarming is initiated when cell density reached a certain threshold (8, 22). Strains of *P. aeruginosa* with altered swarming motility are defective in biofilm formation that indicates its important role during early stages of biofilm development. Conversely, strains with a swarming phenotype were more resistant to antibiotics (ciprofloxacin, gentamicin, polymyxin) (11). In general, changes in *P. aeruginosa* motility properties are correlated with impairment in biofilm formation (23).

The present study assessed the effect of KVM-194, meropenem, and ciprofloxacin on the movement of *P. aeruginosa* within or on the top of media surface. It was found that cell movement was dependent on the presence of flagella (swimming), type IV pili (twitching), as well as their combination (swarming): the diameters of corresponding motility zones were 7.0±0.05 mm, 9.0± 2.28 mm and 10.7±1.03 mm (fig. 1).

In subsequent experiments, the effect of different concentrations (0.5×and 2.0×MIC) of KVM-194, meropenem, and ciprofloxacin on different modes of *P. aeruginosa* motility was studied (tab. 2).

KVM-194 sub-MIC affected swimming. The diameter of motility zone was reduced by 46% compared to the control ($p<0.05$). The higher concentration of KVM-194 (2.0×MIC) completely inhibited the motility. Meropenem reduced swimming zone of the culture by 14% compared to the control ($p<0.05$), the effect was not dose-dependent. Ciprofloxacin did not affect swimming of *P. aeruginosa*.



A B C

Figure 1. Swimming (A), twitching (B) and swarming (C) motility in *P. aeruginosa*.
Colonies of strain P. aeruginosa 449 from a TSB were inoculated, as described in Material and Methods. The black arrow shows the edge of colony on the agar surface; the white arrow shows the edge of colony between the agar and glass surface.

Table 2. The effect of KVM-194, meropenem or ciprofloxacin on motility of *P. aeruginosa* depending on concentration.

Antimicrobials	Motility (mm, mean±SD)					
	Swimming		Twitching		Swarming	
	0.5×MIC	2.0×MIC	0.5×MIC	2.0×MIC	0.5×MIC	2.0×MIC
KVM-194	3.8±0.98 #, **	ND	12.5±9.09	ND	7.0±1.79 **	ND
Meropenem	6.0±0.05 #	6.0±0.05 #	12.2±7.22	ND	11.5±4.14	ND
Ciprofloxacin	6.7±0.52	6.7±0.52	14.2±13.12	ND	14.7±3.88	ND
Control (without antimicrobials)	7.0±0.05		9.0±2.28		10.7±1.03	

ND – non-detected, complete inhibition of growth
 # – in comparison with control (bacterial growth without antimicrobials) p<0.05.
*** – in comparison with the same concentration of ciprofloxacin p<0.05.*

Complete inhibition of cell growth and twitching motility was observed under the influence of 2.0×MIC of KVM-194 or antibiotics. With sub-MICs, the twitching zones of the culture acquired a more elongated shape compared to the control samples; however, no significant differences were found (p>0.05).

KVM-194 2.0×MIC completely inhibited the swarming motility. The motility zones diameter reduction of *P. aeruginosa* under the influence of KVM-194 sub-MIC was also registered, however, the changes were not significant compared to control samples (p>0.05). Meropenem and ciprofloxacin also inhibited the motility and growth of *P. aeruginosa* at a concentration of 2.0×MIC. Pre-treatment with sub-MIC of antibiotics led to swarming zones induction, however, there was no statistically significant difference compared to control samples (p>0.05).

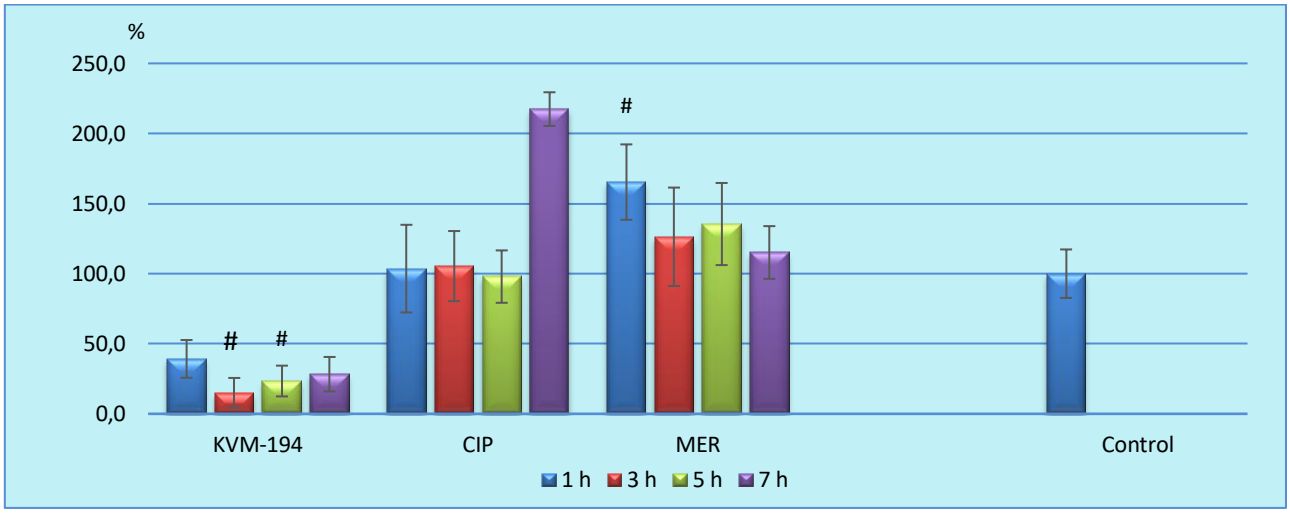
Thus, KVM-194 at subinhibitory concentration reduced the swimming motility of *P. aeruginosa*; at higher concentration (2.0×MIC); inhibition of all motility patterns was observed. It could be assumed that KVM-194 largely affected the motility of *P. aeruginosa* due to flagella rather than to type IV pili. Meropenem and ciprofloxacin at concentration of 2.0×MIC inhibited the growth, twitching and swarming motility of *P. aeruginosa*, however, both antibiotics showed no effects on motility under the influence of sub-MIC. The reduction in the swimming zone was noted under the influence of meropenem. Ciprofloxacin at a subinhibitory concentration did not affect this bacterial motility mechanism.

Effect of KVM-194 on adhesion of Pseudomonas aeruginosa

P. aeruginosa 449 was a slightly adherent strain. The influence of time of pre-treatment with KVM-194, meropenem and ciprofloxacin on adhesion ability of *P. aeruginosa* was compared with the intact control sample (100%) (fig. 2). The experiments showed that KVM-194 decreased adhesion of *P. aeruginosa* cells by 60.8–85.0%, the most pronounced changes occurring after 3 and 5 h of incubation.

Meropenem and ciprofloxacin did not decrease

the adhesion of *P. aeruginosa* cells; moreover, there was a significant increase in adhesion of bacteria after 1 hour of incubation in the presence meropenem compared to the control sample (p<0.05).



 # - in comparison with control (bacterial growth without antimicrobials) p<0.05.

Figure 2. Influence of 0.5×MIC of KVM-194, meropenem (MER) or ciprofloxacin (CIP) on adhesion (%) of *P. aeruginosa* grown until exponential phase.

DISCUSSION

Short-term contacts of *P. aeruginosa* with the surfaces of tissue cells or medical devices lead to the bacterial attachment and biofilm production. An important physical factor at this stage is the hydrophobicity of the surface of bacterial cells, which determines the strength of adhesion, especially when the surface of the substrate shows a marked hydrophobic property (3, 4).

The present study showed that pre-treatment of *P. aeruginosa* cells with KVM-194 (0.5×MIC) decreased CSH that might cause reduction of cell adhesion to the surface. According to other authors' data, penicillins and cephalosporins at subinhibitory concentrations also decreased CSH of *P. aeruginosa* (3, 10).

Ciprofloxacin and meropenem showed no influence on CSH. These results were confirmed by another study, which described no *P. aeruginosa* hydrophobicity changes induced by sub-MIC imipenem (10).

The ability to colonize surfaces could lead to biofilm formation (23). *P. aeruginosa* cell generates a single polar flagellum, which helps in moving through liquid environments (8). Flagellum and chemoreceptors respond to attractants and repellents, which allows bacteria to detect the appropriate substrate for colonization. On the top of solid surfaces, microorganisms can move by twitching, powered by the extension and retraction of type IV pili (2, 6, 7). Pili are also important for attachment to epithelial cells and abiotic surfaces (2).

When cells transited from swimming to swarming due to environmental viscosity increase, *P. aeruginosa* may produce two polar flagella. Swarming is initiated when cell density reaches a certain threshold (8, 22). This motility pattern is regulated by Las and Rhl-systems of intercellular communication (*Quorum sensing*) (5). This movement pattern is also associated with the regulation of virulence factor genes, particularly, *lasB* (elastase activity) and *pvdQ* (pyoverdine biosynthesis) (11). Additionally, swarming plays an important role during early stages of biofilm formation, because the non-motile strains are defective in biofilm formation (11).

The obtained data suggested that KVM-194 largely affected the motility of *P. aeruginosa* due to flagella (than type IV pili). Both KVM-194 concentrations caused considerable changes in swimming motility. According to other researchers, inhibition of swimming and twitching motility was also common to piperacillin/tazobactam (10). Another motility pattern was affected by azithromycin, which inhibited the swarming motility of *P. aeruginosa* via impairment of rhamnolipids synthesis (12).

The obtained data suggested that KVM-194 largely affected the motility of *P. aeruginosa* due to flagella (than type IV pili). Both KVM-194 concentrations caused considerable changes in swimming motility. According to other researchers, inhibition of swimming and twitching motility was also common to piperacillin/tazobactam (10). Another motility pattern was affected by azithromycin, which inhibited the swarming motility of *P. aeruginosa* via impairment of rhamnolipids synthesis (12).

It is known that aminoglycoside antibiotics at subinhibitory concentrations increase the expression of regulators for various motility genes (24), and strains with a swarming phenotype are characterized by a decrease in sensitivity to ciprofloxacin, gentamicin, and polymyxin. However, the effect of subinhibitory concentrations of gentamicin and tobramycin on swarming motility of *P. aeruginosa* has not been established (11). Carbapenems (imipenem) do not affect swimming and twitching motility (10). According to our results, meropenem and ciprofloxacin at subinhibitory concentration had practically no effect on the examined motility patterns of *P. aeruginosa*.

A decrease in the hydrophobic properties of the cell surface and inhibition of motility in the presence of piperacillin/tazobactam led to a disruption of surface attachment (10). Similar results were obtained when studying the KVM-194. It was also found that carbapenems (meropenem) and fluoroquinolones (ciprofloxacin) in sub-MICs caused no changes in the cell surface hydrophobicity ans had no significant impact on the motility and adhesion capacity of *P. aeruginosa.*

CONCLUSIONS

1. The present study showed that 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride is a promising candidate for further development of new agents for preventing biofilm formation.

2. The KVM-194 at subinhibitory concentration (0.5×MIC) decreased the cell surface hydrophobicity of *P. aeruginosa* (by 16%, $p<0.05$) and its motility via flagellum (swimming), which led to reduction of *P. aeruginosa* attachment to polystyrene. The most remarkable changes in adhesion properties were recorded after 3 to 5 hours of pre-treatment with this compound.

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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