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Batumin—A Selective Inhibitor of Staphylococci—Reduces Biofilm Formation in Methicillin Resistant *Staphylococcus aureus*

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Abstract

The antibiotic batumin, produced by *Pseudomonas batumici*, has been shown to be highly active against 123 type and reference strains and clinical isolates of 30 *Staphylococcus* species (including MRSA and small colony variants—(SSCVs) of *S. aureus*, *S. epidermidis* and *S. haemolyticus*). Batumin activity against these bacteria did not depend on the species, origin or resistance to other antibiotics and its MIC was 0.0625 - 0.5 μg/ml. Batumin influence on biofilm formation was studied in clinical isolates of *S. aureus*, *S. epidermidis* and *S. intermedius*. Addition of batumin at a concentration of half of the MIC in the broth, *i.e.* 0.125 μg/ml, decreased the biofilm of 16 out of 20 *S. aureus* strains to varying degrees. Batumin was more effective against *Staphylococcus* strains with strong biofilm formation. Using atomic-force microscopy, it could be shown that batumin reduced the number of *S. aureus* ATCC 25923 adherent cells more than fourfold. The adherent cells of staphylococci were visualized as monolayers of separate islets. A detailed study of the surface of bacterial cells treated with batumin allowed to establish significant reduction of their roughness values. Observed values were typical for planktonic *S. aureus* cells. The obtained data explain one of the mechanisms of the antimicrobial activity of batumin, which is based on preventing the formation of *S. aureus* biofilm. As such, batumin could be considered as an agent offering opportunities

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for the treatment of staphylococcal biofilm-associated infections.

Keywords

Batumin, Staphylococci, SSCVs, Biofilm

1. Introduction

Batumin, a polyketide antibiotic, produced by *Pseudomonas batumici*, has high and selective activity against staphylococci [1] [2]. This predetermined its use not only for treatment of infections and nasal carriage of staphylococci but also for diagnosis of staphylococcocal infection, using the "Diastaph" preparation, which consists of batumin-impregnated disks [3]. The high susceptibility of staphylococci to batumin distinguishes them from representatives of other taxons, as was demonstrated using "Diastaph" in several thousands of clinical isolates of bacteria [4].

Bacteria growing as biofilm are highly resistant to antibiotics [5]. However, our previous studies have shown that addition of half the concentration of the batumin MIC (0.125 μg/ml) to the media considerably reduced biofilm formation in 80% of nasal staphylococcal strains, which had initially high levels of biofilm formation [6].

The objective of this work was to broaden our knowledge about batumin activity against *Staphylococcus* species, against atypical forms of this pathogen, *i.e.* small staphylococcal colony variants (SSCVs), and to obtain more detailed insights into the influence of batumin on biofilm formation by using atomic-force microscopy.

2. Materials and Methods

Batumin, obtained by fermentation of *Pseudomonas batumici* and purified by silica gel preparative chromatography to 85% of purity, was used.

Batumin is commercially available from Santa Cruz Biotechnology (Santa Cruz, CA) or Enzo Life Sciences (Antwerp, Belgium).

Type and collection strains of 30 different species belonging to the genus *Staphylococcus* (**Table 1**) and 50 methicillin resistant *S. aureus* (MRSA) strains isolated in the Institute of Traumatology and Orthopedics, Medical Science Academy of Ukraine, from patients with osteomyelitis, were included. 20 *Staphylococcus aureus* strains, 19 *Staphylococcus epidermidis* and 4 *Staphylococcus intermedius* strains, isolated from skin microbial biocenosis and nasal mucous membrane (microbial collection of the Institute of cellular and intracellular symbiosis Ural Branch of Russian Academy of Sciences, Orenburg, Russia) were also studied for their susceptibility to batumin and for its antibiotic effect upon biofilm formation. Thus, in total 123 strains of staphylococci were included.

Identification of MRSA was carried out according to methods described in [7]. Susceptibility of staphylococcal clinical strains to a wide spectrum of antibiotics was tested by the Kirby-Bauer method. NCCLS criteria [8] were used to interpret the susceptibility to antibiotics.

Thirty strains of SSCVs were isolated as subpopulations on Columbia agar with 5% sheep blood, as pinpoint colonies (0.1 - 0.3 mm) after 48 hours of aerobic incubation at 37˚C, among the more numerous colonies (2 - 3 mm) with normal *Staphylococcus* morphology, considered as the parental isolates of the SSCVs. Identification of the staphylococci was carried out according to standard methods [9].

The atypical forms of staphylococci were previously identified by standard methods and using tDNA-PCR analysis and were assigned to three species: *S. aureus*, *S. epidermidis* and *S. haemolyticus*. Their detailed description was presented previously [10].

The minimal inhibitory concentration (MIC) of batumin was studied according to CLSI Standards (2005) in Mueller-Hinton agar or broth [11]. The microbial load of SSCVs was 0.5×10^8 cfu/ml and the Petri dishes were incubated at 37˚C for 48 hours.

Half the MIC of batumin was used to study its effect upon biofilm formation in staphylococci. Biofilm formation was studied by a photometric method determining the bacterial capacity to adhere onto the 96-hole polystyrole plane-table surface with subsequent crystal violet colouring [12]. Optical density measurement was done using a photometer ELx808 (BioTek, USA) at a wavelength of 630 nm. Degree of biofilm formation was ex-

pressed in conditional units (un.) which was the optical density of studied strain (experiment) in relation to the nutrient broth density (the control).

For the study of batumin effect on biofilm formation we used *S. aureus* B-904 (UCM) as test-culture. For biofilm production, glass coverslips were immersed into Luria-Bertani broth with 0.125 μg/ml of batumin and incubated for 48 h at 37˚C.

Visualization of the biofilms was done by atomic force microscopy using the SMM-2000 microscope (Proton-MIET Closed JOINT Stock Company, Russia), in contact mode in air environment [13] [18].

3. Results and Discussion

The susceptibility to batumin of *Staphylococcus* species reference strains of some collection strains and of methicillin-resistant *S. aureus* strains are presented in (**Table 1**). It is of interest to note the high uniformity of the susceptibility to batumin for the different *Staphylococcus* species. Batumin inhibited most of the studied strains and species, including MRSA, at concentrations between 0.25 and 0.5 μg/ml, and rarely 1.0 μg/ml was needed.

Table 1. Susceptibility to batumin of species belonging to different 16S RNA complexes of the genus *Staphylococcus.*

RNA-complex	**Species** (strain number)	MIC **μg/ml**
S. aureus	***S. aureus*** B-918 (ATCC 6538)	0.25
	S. aureus B-4001 (ATCC 6538P)	0.25
	S. aureus B-904 (ATCC 25923)	0.25
	S. aureus B-909 (GISK 906)	0.25
	MRSA (50 strains)	0.25 - 0.5
S. carnosus	***S. carnosus*** B-4005^T (DSM 20501^T)	0.25
	S. piscifermentans B-4028^T (ATCC 51136^T)	0.25
	S. simulans B-4033^T (ATCC 27848^T)	0.25
S. epidermidis	***S. capitis*** B-4002^T (ATCC 27840^T)	0.125
	S. caprae B-4007^T (ATCC 35538^T)	0.125
	S. epidermidis B-4023^T (ATCC 14990^T)	0.0625
	S. epidermidis B-919 (ATCC 12,228)	
S. haemolyticus	***S. devriesei*** B-4022^T (CNS 159^T)	0.125
	S. haemolyticus B-4018^T (ATCC 29970^T)	0.25
	S. hominis B-4019^T (DSM 20328^T)	0.25
S. hyicus-S. intermedius	***S. chromogenes*** B-4003^T (ATCC 43764^T)	0.25
	S. felis B-4016^T (ATCC 49168^T)	0.25
	S. delphini B-4008^T (ATCC 49171^T)	0.25
	S. hyicus B-4020^T (ATCC 11249^T)	0.25
	S. intermedius B-4009^T (ATCC 29663^T)	0.5
	S. schleiferi B-4032^T (ATCC 49545^T)	0.5
	S. pseudointermedius B-4029^T (LMG 22219^T)	0.25
S. lugdunensis	***S. lugdunensis*** B-4025^T (ATCC 43809^T)	0.5
S. warneri	***S. warneri*** B-4013^T (ATCC 27836^T)	0.25
	S. pasteurii B-4026^T (ATCC 51129^T)	0.25
S. sciuri	***S. sciuri*** B-4012^T (ATCC 290762^T)	0.5
	S. pulvereri B-4031^T (ATCC 51698^T)	1.0
	S. lentus B-4024^T (CCM 2598^T)	0.5
	S. lentus B-4010 (ATCC 29,070)	0.5
S. saprophyticus	***S. equorum*** B-4015^T (ATCC 43959^T)	0.5
	S. gallinarum B-4017^T (ATCC 35539^T)	0.5
	S. kloosii B-4021^T (ATCC 43959^T)	1.0
	S. saprophyticus B-4011^T (ATCC 15305^T)	0.25
	S. saprophyticus B-4034 (CLO 059)	0.5
	S. cohnii B-4004^T (ATCC 29974^T)	0.25
	S. xylosus B-4014^T (ATCC 29971^T)	0.25
Macrococcus caseolyticus	***M. caseolyticus*** B-4006^T (ATCC 13548^T)	0.125

The strain numbers of the Ukrainian Collection of Microorganisms (UCM) are presented. Numbers from ATCC or from other collections between brackets.

Our data show that the most susceptible species to batumin were species of the *S. epidermidis* complex (MIC 0.0625 - 0.125 μg/ml). Strains of this complex demonstrated the highest batumin susceptibility also during previous clinical trials (unpublished data).

Figure 1 shows the antibiotic-resistance profile of the studied MRSA strains, indicating that these strains are resistant to all commonly used antibiotics. At the same time, all 50 strains—regardless their origin and their susceptibility to different antibiotics—were inhibited by 0.5 μg/ml of batumin.

Batumin is also effective against macrococci which were set separately from the genus *Staphylococcus* and included in the new genus *Macrococcus*. On the other hand, *Gemella morbillorum* and *G. haemolysans*, isolated from osteomyelitis patients, did not show growth inhibition zones around batumin disks [14], despite their relationship to staphylococci. Batumin was also not effective against bacilli, *Listeria*, planococci and other bacteria which belong to the order of the *Bacillales*, comprising the family of the *Staphylococcaceae* [2] [4].

Data presented here, as well as studies of the past years, demonstrate high activity of batumin against metabolically normal members of the genus *Staphylococcus* [2] [3], as well as against atypical forms of staphylococci, the so-called small colony-variants (SSCVs). SSCVs are formed as a result of mutations and characterized by a complex pleiotropic phenotype which can be largely explained by defective electron transport [15] [16].

All 30 SSCVs included in this study were isolated on solid growth media as a subpopulation among the larger number of colonies with normal for staphylococci morphology. They were characterized by altered colony morphology, delayed growth and lack of pigmentation. Moreover, many of them lacked lecithinase, phosphatase, coagulase and haemolytic activity and the metabolism of carbohydrates such as sucrose, lactose and fructose was altered, which is consistent with the literature and with our previous results [10] [17].

Coming from our previous data on batumin antimicrobial action against SSCVs we first estimated this effect studying the growth inhibiting concentrations.

Most of the SSCVs were inhibited by a concentration of 0.25 μg batumin/ml. Strain *S. aureus* 71 was most resistant, with an MIC of 0.5 μg/ml.

So the data presented in this work and first obtained using strains of practically all known staphylococci species, multiresistant clinical isolates and atypical forms of this pathogen give evidence that batumin is highly active selective inhibitor of representatives of the genus Staphylococcus.

Susceptibility of nasal and skin clinical isolates of staphylococci to batumin was the same as of type and collection strains of different species of the genus *Staphylococcus* studied before [6]. At the same time, the antibiotic effect against biofilm formation was different in different strains and species. Their biofilm formation values varied from 2.6 - 3.1 CU for *S. aureus* to 1.8 - 2.3 for *S. epidermidis* and 1.7 - 1.9 for *S. intermedius* strains (**Table 2**). Presence of half of batumin MIC in the broth reduced the biofilm formation in 85% of studied

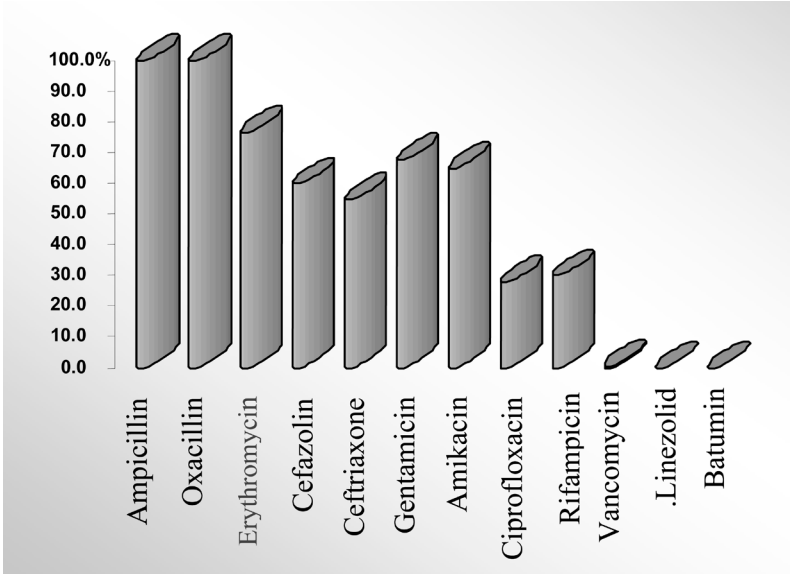

Figure 1. The antibiotic resistance profiles of 50 methicillin-resistant *Staphylococcus aureus* strains. Legend: x-axis: studied antibiotics; y-axis: % of MRSA strains, resistant to these antibiotics.

Table 2. Batumin effect against biofilm formation by staphylococci.

	Strains	Source of isolation	Optical density (OD_{630}) without batumin	CU* without batumin	Optical density (OD_{630}) batumin (0.125 μg/ml)	CU* batumin (0.125 μg/ml)
1		Reference	0.131	2.6	0.095	1.9
2	*S. aureus* 3	Nose	0.157	3.1	0.077	1.5
3	*S. aureus* 17	Nose	0.134	2.7	0.094	1.9
4	*S. aureus* 24	Nose	0.142	2.8	0.104	2.1
5	*S. aureus* 28a	Nose	0.131	2.6	0.091	1.8
6	*S. aureus* 29	Nose	0.130	2.6	0.083	1.7
7	*S. aureus* 35	Skin	0.131	2.6	0.096	1.9
8	*S. aureus* 39	Colon	0.136	2.7	0.074	1.5
9	*S. aureus* 59d	Skin	0.131	2.6	0.132	2.6
10	*S. aureus* 68	Skin	0.132	2.6	0.097	1.9
11	*S. aureus* 75	Nose	0.156	3.1	0.157	3.1
12	*S. aureus* 89-a	Colon	0.147	2.9	0.081	1.6
13	*S. aureus* 104-a	Colon	0.142	2.8	0.105	2.1
14	*S. aureus* 120	Nose	0.156	3.1	0.155	3.1
15	S. aureus 121	Nose	0.131	2.6	0.093	1.9
16	*S. aureus* 132	Nose	0.131	2.6	0.095	1.9
17	*S. aureus* 146-a	Nose	0.130	2.6	0.081	1.6
18	*S. aureus* 144	Skin	0.135	2.7	0.090	1.8
19	*S. aureus* 159	Skin	0.140	2.8	0.084	1.7
20	*S. aureus* 165	Skin	0.130	2.6	0.133	2.6
21	*S. epidermidis* 11	Skin	0.105	2.1	0.085	1.7
22	*S. epidermidis* 47	Skin	0.115	2.3	0.095	1.9
23	*S. epidermidis* 57k	Skin	0.105	2.1	0.105	2.1
24	*S. epidermidis* 61	Skin	0.105	2.1	0.105	2.1
25	*S. epidermidis* 64	Nose	0.115	2.3	0.115	2.3
26	*S. epidermidis* 73	Nose	0.095	1.9	0.08	1.6
27	*S. epidermidis* 99-b	Skin	0.097	1.9	0.095	1.9
28	*S. epidermidis* 124	Nose	0.104	2.1	0.107	2.1
29	*S. epidermidis* 137	Skin	0.105	2.1	0.093	1.9
30	S. epidermidis 140	Skin	0.092	1.8	0.080	1.6
31	*S. epidermidis* 143	Nose	0.094	1.9	0.097	1.9
32	*S. epidermidis*147	Skin	0.111	2.2	0.095	1.9
33	*S. epidermidis* 154	Nose	0.097	1.9	0.096	1.9
34	*S. epidermidis* 155 c	Colon	0.091	1.8	0.09	1.8
35	*S. epidermidis* 172	Nose	0.114	2.3	0.113	2.3
36	*S. epidermidis* 183	Nose	0.115	2.3	0.091	1.8
37	*S. epidermidis* 184	Nose	0.095	1.9	0.084	1.7
38	*S. epidermidis* 215	Colon	0.113	2.3	0.115	2.3
39	*S. epidermidis* 187	Nose	0.11	2.2	0.11	2.2
40	*S. intermedius* 193	Nose	0.083	1.7	0.075	1.5
41	*S. intermedius* 195	Nose	0.095	1.9	0.074	1.5
42	*S. intermedius* 107	Skin	0.09	1.8	0.09	1.8
43	*S. intermedius* 111	Skin	0.086	1.7	0.084	1.7

*The ratio of the optical density of the samples in the experiment and control. Expressed as conventional unit (CU) = OD_{630} in experimental samples/OD_{630} in control samples. Optic density of control samples is 0.05, which is the nutrient broth density.

S. aureus strains. It should be noted that batumin is more effective against *Staphylococcus* strains with strong biofilm formation (CU values between 2.6 and 3.1).

Analysis of experimental data on batumin effect on the stages of biofilm formation in S. aureus demonstrated that the effectiveness of batumin depended on the stage of biofilm formation (**Table 3**). Simultaneous batumin addition with *S. aureus* into culture medium promoted reduction of biofilm formation values in all studied staphylococci strains, at that already formed film was more resistant to studied preparation.

More detailed study of batumin action upon *S. aureus* biofilm formation was carried out using atomic force microscopy. The object of this study was the reference strain *S. aureus* UCM B-904. This strain formed biofilm on the surface of the glass, as can be seen from **Figure 2(a)**. Surface biofilm was formed by exopolymeric matrix with cells of round shape immersed in it (**Figure 2(b)**).

For strain *S. aureus* B-904, the effect of batumin with regard to biofilm formation disturbance corresponded to a more than fourfold decrease of the number of adherent cells. Moreover, in the presence of batumin, the cells of staphylococci were observed in the form of monolayer of separate islets (**Figure 2(c)**). Particles of exopolymeric matrix were determined with average values of thickness of 63 - 62 nm, located between the cells and on their surface (**Figure 2(d)**). In this case the transverse dimension of cells was 0.64 ± 0.08 mm, not significantly different from control values. A detailed study of the surface of bacterial cells treated with batumin allows to establish significant reduction of their roughness values (**Table 4**). Observed values were typical for planktonic *S. aureus* cells [19].

Atomic force microscopy revealed qualitative and quantitative changes in the exopolymeric matrix due to batumin treatment, as well as a significant reduction in the number of cells adhered to the coverslip, preventing formation of *S. aureus* biofilm.

It is known that some biofilms are covered by a surface film composed of lipid components similar to those in bacterial membranes which are a barrier for the penetration of antibiotics [20]. We have previously shown that batumin has significant effects on lipid metabolism of *S. aureus* [21]. Based on the similarity of a biofilm matrix lipid on the one hand and membrane of bacterial cell on the other hand, it can be assumed that batumin penetrates well into staphylococcal biofilm, explaining the significant changes of exopolymeric matrix that can be observed.

4. Conclusions

The data presented in this work show that batumin is a highly active antimicrobial agent, inhibiting all species of staphylococci and macrococci, regardless their origin or antibiotic susceptibility. The MIC-values for batumin

Table 3. Influence of batumin (0.125 μg/ml) on the steps of biofilm formation by S*taphylococcus aureus.*

	Strains	Before batumin		After batumin (simultaneously with inoculation)		After batumin (90 min)		After batumin (24 hours)		After batumin (48 hours)
		Optical density	CU*	Optical density (OD_{630})	CU*	Optical density (OD_{630})	CU*	Optical density (OD_{630})	CU*	Optical density (OD_{630})
1	*S. aureus* 25923 ATCC	0.132	2.6	0.097	1.9	0.081	1.6	0.135	2.7	0.164
2	*S. aureus* 3	0.155	3.1	0.074	1.5	0.153	3.0	0.155	3.1	0.153
3	*S. aureus* 17	0.137	2.7	0.095	1.9	0.099	2.0	0.105	3.1	0.135
4	*S. aureus* 24	0.140	2.8	0.102	2.0	0.140	2.8	0.169	3.4	0.142
5	*S. aureus* 28a	0.133	2.7	0.090	1.8	0.156	3.1	0.161	3.2	0.161
6	*S. aureus* 35	0.130	2.6	0.094	1.9	0.103	2.1	0.130	2.6	0.130
7	*S. aureus* 39	0.138	2.8	0.071	1.4	0.085	1.7	0.084	1.7	0.077
8	*S. aureus* 68	0.132	2.6	0.095	1.9	0.097	1.9	0.097	1.9	0.093
9	*S. aureus* 89-a	0.148	3.0	0.079	1.6	0.097	1.9	0.089	1.8	0.147
10	*S. aureus* 121	0.133	2.7	0.093	1.9	0.101	2.0	0.101	2.0	0.155
11	*S. aureus* 144	0.131	2.6	0.087	1.7	0.094	1.9	0.088	1.8	0.085
12	*S. aureus* 159	0.142	2.8	0.085	1.7	0.141	2.8	0.183	3.7	0.140

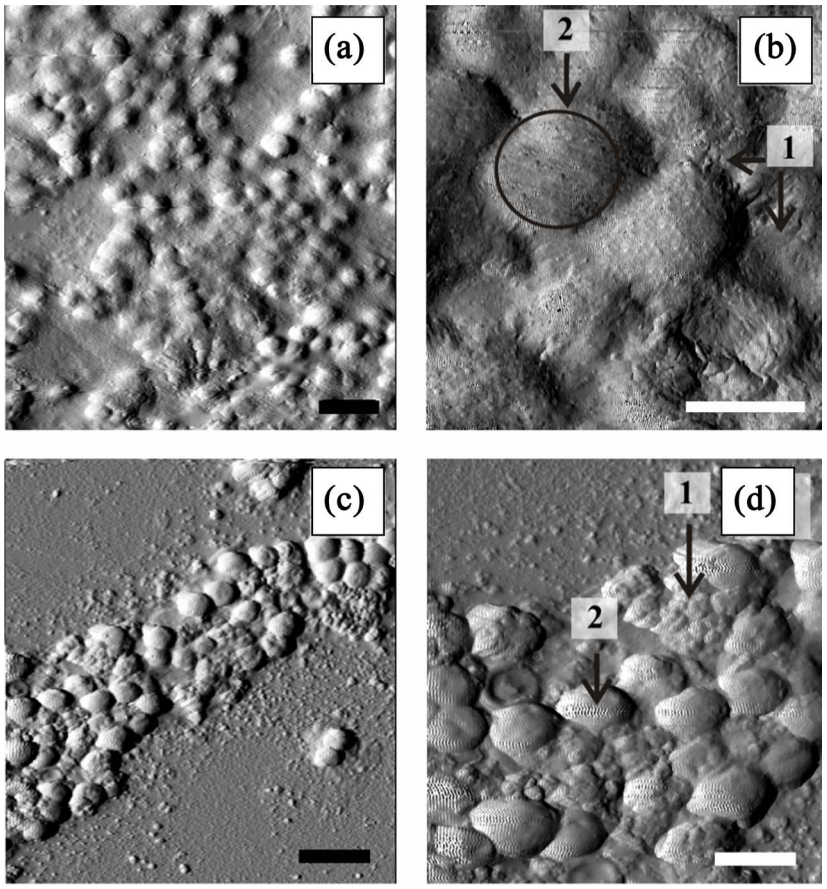

Figure 2. Atomic force microscopy-topography images of *Staphylococcus aureus* B-904. ((a) (b)) *S. aureus* B-904 without batumin; ((c) (d)) *S. aureus* B-904 grown in the presence of 0.125 μg/ml of batumin. Arrows: 1: particles of exopolymeric matrix; 2 bacterial cells; Scale—2 μm ((a) (b)); 1 μm ((c) (d)).

Table 4. Morphological characteristics of cells and adherence to glass of *S. aureus* B-904 in the presence of 0.125 μg/ml batumin.

Conditions of the experiment	Adherent cells, %	Length (μm)	Width (μm)	Height (μm)	Roughness values (nm)
Control (without batumin)	100 ± 11	0.69 ± 0.05	0.64 ± 0.08	N/A**	19.4 ± 5.50
Experiment (half MIC of batumin, *i.e.* 0.125 μg/ml)	24 ± 4*	0.73 ± 0.12	0.64 ± 0.07	0.68 ± 0.08	9.59 ± 1.43*

*$p < 0.05$ (Mann-Whitney U-test). **To determine bacterial cell length and width, cells that were at least immersed into exopolymeric matrix were selected. However, height determination of the cells in this case would have been incorrect and therefore was not implemented.

range between 0.25 and 0.5 μg/ml, rarely reaching 1.0 μg/ml. It should be noted that these results were obtained with 85% pure batumin, whereas, according to [22], the MIC of 95% pure batumin against *S. aureus* was much higher, *i.e.* 0.05 μg/ml.

Probably batumin differs in its mechanism of antimicrobial action from all antibiotic substances used at present in clinical practice, and the nature of its selectivity is connected with some peculiarity of staphylococcal metabolism which sets them apart from other genetically related bacteria. Taking into account, the polyketide nature of batumin and our earlier data about batumin activity upon *Staphylococcus* lipids [21], we may suppose that these distinctions are determined by specific characteristics of the staphylococcal fatty acids metabolism.

The obtained data on atomic force microscopy explain one of the mechanisms of the antimicrobial action of batumin, based on preventing formation of *S. aureus* biofilm that allows considering it as a promising agent in treatment of staphylococcal biofilm-associated infections.

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