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PECULIARITIES OF ANTIBIOTIC BATUMIN ACTION ON BIOFILM FORMATION BY *Staphylococcus aureus* AND *Pseudomonas batumici*

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The aim of the work was the research of antibiotic batumin action on the biofilm formed by staphylococci and on the process of the biofilm formation by strain-producer *Pseudomonas batumici*. The minimal inhibiting concentration (MIC) of batumin was studied according to The Clinical and Laboratory Standards Institute standards — CLSI. Biofilm formation was studied using photometric O'Toole method with flatbed photometer at wavelength 540 nm. The batumin penetrated into biofilms of staphylococci and reduced the biomass of the formed biofilm of *Staphylococcus aureus* and *S. epidermidis* at the antibiotic concentration of 0.5 μg/ml, which was only twice higher than MIC of batumin for planktonic cells. The degree of biofilm changes in the presence of batumin differed for different staphylococcus strains. However, these differences did not depend on strains sensitivity or their resistance to the antibiotic.

The ability of batumin strain-producer to form the biofilm and the ability of antibiotic to stimulate this process in concentrations of 1 and 10 μg/ml was established for the first time. In addition, it was found that in the *Pseudomonas batumici* B-321 planktonic culture in the presence of 10 μg/ml of batumin, the increase in length of producer cells on average by 15% was noted.

The obtained data shed the light on batumin antimicrobic action on the staphylococci biofilm. It can be argued that is a perspective for treatment of staphylococcal infections. It enabled to consider batumin as a promising drug for the staphylococcal infections treatment.

Key words: batumin, *staphylococci*, biofilm.

The polyketide antibiotic batumin synthesized by the producer strain *Pseudomonas batumici* has a high selective activity against staphylococci [1,2], which determines the prospects for its medical application in the treatment of staphylococcal infections and the control of the nasal carriage of this pathogen [3]. Data obtained in recent years indicate that 95 to 99% of microorganisms in natural habitats exist in the form of biofilms. It is known that bacterial biofilms are involved in the pathogenesis of chronic infections such as osteomyelitis, rhinosinusitis, etc. [4, 5]. At the same time, it is known that bacteria structured in biofilm are more resistant to antibiotics [5], which is one of the problems of successful chemotherapy.

Our preliminary studies on the effect of batumin on biofilm formation in representatives of the genus *Staphylococcus* showed that the presence in the medium of 0.125 μg/ml of batumin (half of growth inhibiting concentration) significantly reduced the formation of biofilm in 85% of the studied nasal strains of staphylococci with an initially high level of biofilm formation [6]. The use of atomic force electron microscopy showed that the batumin reduced several times the staphylococci adhesion to the surface, thus inhibiting the early stages of biofilm formation [7].

On the other hand, the role of antimicrobial substances for cells of producer strains in nature is far from being exhausted by their antibiotic activity. Strains of *P. batumici* are isolated from the soil and are rhizosphere

bacteria that interact with plants and compete with different microflora. The formation of biofilm by such bacteria ensures the nutrients exchange with the plant, the synthesis of antimicrobial substances that protect against phytopathogens and many other effects [8].

In connection with the foregoing, the aim of this work was to study the effect of batumin on the biofilm formed by staphylococci, and to study the influence of batumin on the process of biofilm formation by the producer strain *P. batumici*.

Materials and Methods

The object of the study were 16 strains of *Staphylococcus aureus* and *S. epidermidis*, including 5 methicillin-resistant strains (MRSA) isolated at the Institute of Orthopedics and Traumatology of the Academy of Medical Sciences of Ukraine from patients with osteomyelitis. The strains of MRSA are identified by the method suggested by Boutiba-Ben Boubaker [9].

Another object was a typical antibiotic batumin producing strain *Pseudomonas batumici* UCM B-321 from the Ukrainian Collection of Microorganisms (Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine).

Antibiotic batumin was obtained by fermentation of *B. batumici* strain UCM B-321 and purified by preparative chromatography (90% purification degree) [10].

The sensitivity of clinical strains of staphylococci to a broad spectrum of antibiotics was determined using the Kirby-Bauer method, CLSI criteria were used to interpret antibiotic sensitivity. Discs impregnated with the appropriate antibiotics were used: amoxiclav, erythromycin, clindamycin, rifampicin, ciprofloxacin, doxycycline, vancomycin, teicoplanin, amikacin, linezolid, lincomycin (Himedia, India). The minimal inhibiting concentration of the batumin was studied in accordance with CLSI standards on Müller-Hinton agar and Müller-Hinton broth [11].

The biofilm formation study was carried out according to the O'Toole method [12]. In studying the activity of batumin against biofilms formed by *S. aureus* and *S. epidermidis* cultures, biofilms were grown on the bottom of plastic 96-well plates (Thermo Scientific, USA). 0,2 ml of staphylococci broth culture was introduced into the wells. The initial concentration of cells was $5 \cdot 10^7$ cells/ml, cultivation was carried out for 24 hours at t = 37 °C, further the batumin was added in concentrations of 0.1; 0.5 and 5.0 μg/ml, followed by incubation for 24 hours. After that, the liquid contents of the wells were removed, washed three times with phosphate buffer (pH 7.2), dried and stained. The percentage of biofilm inhibition (BI) was calculated as follows [5]:

$$BI\ (\%) = 100 - [\ OD_{batumin}/OD_{control}],$$

where $OD_{batumin}$ is optical density in the holes with the batumin, $OD_{control}$ is optical density in wells without batumin (control).

To study the biofilm formation by *P. batumici* strain UCM B-321, it was grown at t = 28 °C for 48 h in 96-well plates on Luria-Bertani medium (Becton, USA). 0.25 ml of the cell culture suspension ($5 \cdot 10^7$ cells/ml) was introduced into the wells in a nutrient medium, as well as various (1 and 10 μg/ml) concentrations of batumin.

A 0.1% solution of gentian violet ColorGram 2-F (bioMerieux, France) was used as the biofilm dye, and 0.1 ml was added to each culture well. After thirty minutes of contact at room temperature, the dye was washed three times with distilled water and the ink was dissolved with 96% ethyl alcohol. The results were recorded using a Multiskan FC (Thermo Scientific, USA) flatbed photometer at a wavelength of 540 nm.

Results and Discussion

Table 1 shows the results of studying the sensitivity of clinical staphylococcus strains to widely used in medical practice antibiotics.

The obtained data suggest the high resistance of MRSA strains to the studied antibiotics (the growth retardation zones were only 6 mm), which agrees with the literature data [13, 14]. At the same time, all investigated staphylococci strains, including MRSA, regardless of the source of their isolation and sensitivity to different antibiotics, were sensitive to the batumin.

The next stage of our work was to study the ability of clinical isolates of *S. aureus* and *S. epidermidis* to form biofilms (Fig. 1).

All studied strains of staphylococci formed a biofilm with different biomass values from 0.20 to 0.68 units of optical density. Strains that did not synthesize biofilm were not detected.

Further, we determined the MIC of the batumin with its action on staphylococcus cells, both in planktonic culture and in the composition of biofilms (Table 2).

Table 1. **Sensitivity of clinical *Staphylococcus* strains to antibiotics**

Strains	Zones of growth retardation, mm											
	Amoxiclav	Erythromycin	Clindamycin	Rifampicin	Ciprofloxacin	Doxycycline	Vancomycin	Teicoplanin	Amikacin	Linezolid	Lincomycin	Batumin
S. aureus 75(MRSA)	6	6	6	18	6	22	19	17	16	6	6	25
S. aureus 76 (MRSA)	6	6	6	6	6	20	18	20	21	6	26	30
S. aureus 77 (MRSA)	6	6	22	6	6	6	17	20	17	6	20	32
S. aureus 1463(MRSA)	6	6	6	25	20	20	16	19	16	24	28	28
S. aureus 1813(MRSA)	6	6	6	6	6	24	18	22	22	25	6	35
S. aureus 80	22	6	20	31	24	28	18	24	22	25	24	26
S. aureus 175	6	18	22	35	26	6	15	18	Ч	19	30	32
S. aureus 1306	25	10	25	25	28	6	20	18	26	26	26	30
S. aureus 1310	30	16	18	24	22	6	19	22	25	28	22	28
S. aureus 1316	30	30	22	24	6	20	18	22	18	25	22	32
S. aureus 1358	20	26	18	30	6	22	17	22	19	25	20	32
S. aureus 1440	20	6	20	6	30	15	17	18	24	21	28	35
S. aureus 1749	25	28	24	28	30	26	20	20	22	22	25	35
S. epidermidis 151	6	20	28	6	24	17	22	20	22	24	20	30
S. epidermidis 618	6	32	22	22	19	19	20	24	20	20	22	32
S. epidermidis 1803	6	6	26	22	20	17	24	26	25	6	18	28

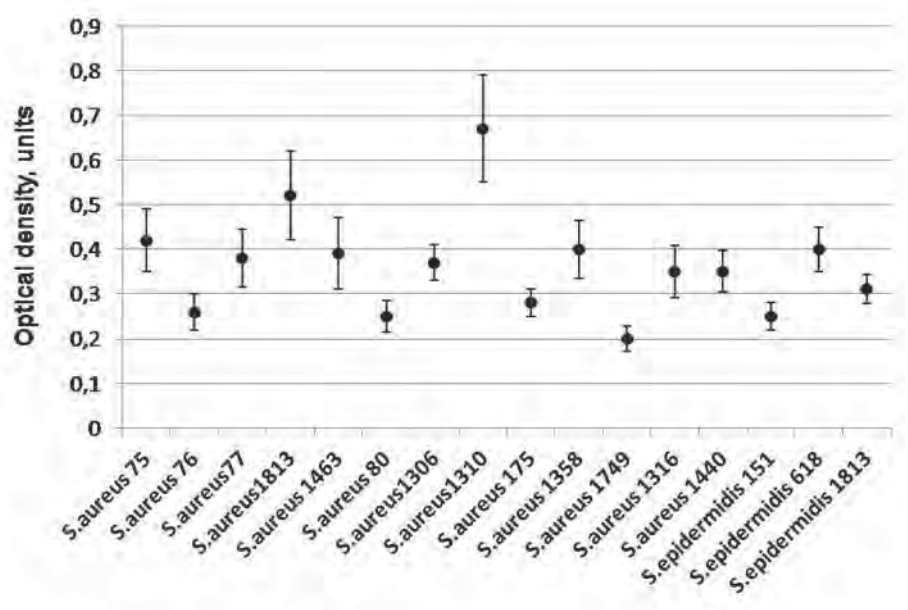

Fig. 1. **Biofilm formation *in vitro* in strains *S. aureus* and *S. epidermidis***

Table 2. **The minimal inhibitory concentration of batumin for plankton cells and *Staphylococcus* biofilms**

Strains	Batumin concentration, μg/ml	
	Plankton cells	Biofilm
S. aureus 75 (MRSA)	0.25	0.5
S. aureus 76 (MRSA)	0.25	0.5
S. aureus 77 (MRSA)	0.25	0.5
S. aureus 1463 (MRSA)	0.25	0.5
S. aureus 1813(MRSA)	0.25	0.5
S. aureus 80	0.25	0.5
S. aureus 175	0.25	0.5
S. aureus 375	0.25	0.5
S. aureus 1306	0.25	0.1
S. aureus 1310	0.25	0.5
S. aureus 1316	0.25	0.5
S. aureus 1358	0.25	0.5
S. aureus 1440	0.25	0.5
S. aureus 1749	0.25	0.5
S. epidermidis 151	0.25	0.1
S. epidermidis 618	0.25	0.5
S. epidermidis 1803	0.25	0.5

For planktonic staphylococcus cells, the MIC of the batumin was 0.25 μg/ml, the MIC for the biofilms of each studied strain was 0.5 μg/ml, except for the strain of *S. aureus* 1306 (MIC = 0.1 μg/ml).

In the course of the work, it has been found that in the presence of batumin the significant changes occurred in the biomass of the microbial community of staphylococci in the formed biofilm (Table 3).

From the results obtained, it can be seen that the decrease in the biomass of the formed biofilm depended on the concentration of the antibiotic, and the intensity of the effect was different in the studied strains. Thus, the batumin concentration of 0.5 μg/ml, which was only twice as high as MIC, caused a decrease in biomass of biofilm from 34.7 to 77.0%. A further increase in the concentration of antibiotic to 5 μg/ml only slightly changed the mass of the biofilm.

According to the literature, the addition of antibiotics such as ampicillin, cefatoxime, levofloxacin, rifampicin in concentrations 50 times higher than MICs causes a decrease in the mass of the formed biofilm up to 30% maximum [15]. Highly active against staphylococci antibiotic mupirocin isolated from bacteria of the genus *Pseudomonas*, reduced biomass of the formed biofilm by 90% in all investigated isolates of *S. aureus* at concentrations 250 times higher than MIC [5].

According to Moreno M.G. et al. [16] the MICs of phosphomycin, rifampicin and levofloxacin when they act on plankton cells were 1–16 μg/ml, and on biofilms — more than 1024 μg/ml. The exception was the antibiotic gentamicin, the MIC of which, when it acts on plankton cells was 4–8 μg/ml, on biofilms — 8–16 μg/ml. Allison et al. [17] found that gentamicin kills persistent cells of *Escherichia coli* and *Staphylococcus aureus*. Persistent cells are a subpopulation in biofilm and are tolerant to antibiotics, thus remain viable when exposed to pharmaceuticals. The author suggests that rifampicin and benzylpenicillin kill most of the cells in the

Table 3. **Batumin effect on formed biofilms biomass of staphylococci**

Strains	Batumin concentration, μg/ml		
	0.1	0.5	5.0
	Decrease in relation to control,%		
S. aureus 75 (MRSA)	47.7	57.2	57.4
S. aureus 76 (MRSA)	31.1	34.7	34.1
S. aureus 77 (MRSA)	57.9	63.2	60.3
S. aureus 1463 (MRSA)	67.7	69.3	68. 2
S. aureus 1813 (MRSA)	75.2	77.0	77.0
S. aureus 80	36.0	40.1	40.0
S. aureus 175	39.3	50.0	49.3
S. aureus 375	38.1	52.4	51.0
S. aureus 1306	56.8	56.8	54.0
S. aureus 1310	64.0	74.3	74.2
S. aureus 1316	25.8	37.2	34.3
S. aureus 1358	65.0	67.5	68.5
*S. aureus*1440	57.2	65.8	62.9
*S. aureus*1749	35.0	60.0	60.1
S. epidermidis 151	48.4	51.5	54.8
S. epidermidis 618	40.2	44.1	44.0
S. epidermidis 1803	50.0	60.3	60.0

biofilm, except persisters, and gentamycin, selectively acting, leads to their complete eradication. Earlier, we showed that the batumin also has a high activity against SSCVs persisters (*S. aureus* small colony variants) [18]. Thus, by the mechanism of action on staphylococcus biofilms the batumin is probably similar to gentamicin.

The obtained results indicate that the batumin penetrates into the formed biofilms of clinical strains of *S. aureus* and *S. epidermidis*, and after 24 hours of action causes a decrease in the biofilm formation process. It should also be noted that the antibiotic significantly reduces the biomass of the biofilm formed in strains with an initially high level of biofilm formation (Table 3). We obtained similar results when studying the action of antibiotic batumin on biofilm developing. The efficacy of batumin at a concentration of 0.125 μg/ml (half of its MIC) was higher for strains with a high biofilm weight [6].

It is known that matrix components play an important role in the process of antibiotics penetration into biofilms. The lipids of the biofilm matrix and bacterial membranes are identical in qualitative composition [19]. When studying the mechanism of batumin action on staphylococci, we found that in this pathogen under the action of antibiotic the significant changes in the fatty acid profile, which leads to an increase in fluidity of membrane lipids and permeability of the membrane in *S. aureus* take place [20]. Based on the similarity of the composition of the biofilm matrix lipids on one side, and the membrane of the bacterial cell on the other side, it can be assumed that the batumin penetrates well into the biofilm of staphylococci and causes significant changes in the biofilm formed, which we observed.

Attention is drawn to the fact that the intensity of changes in biofilms in the presence of batumin differs in different strains of staphylococci, however, these differences do not depend on the sensitivity or resistance of strains to the studied antibiotics (Tables 1 and 3).

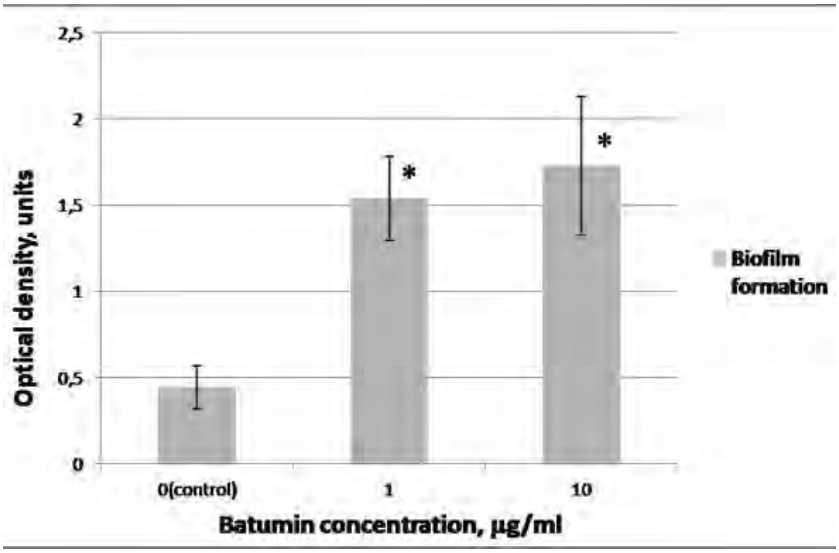

Fig. 2. **Batumin effect on the biofilm formation by strain *P. batumici* UCM B-321:** marked columns (*) are significantly differ from the control at $P \leq 0{,}05$

Apparently, the changes we have detected in the biofilms of staphylococci under the influence of batumin make them unstable, incapable of persistence and dissemination in the human body.

Another stage of our work was the study of the batumin influence on the processes of biofilm formation by the strain-producer *P. batumici* (Fig. 2).

Batumin in concentrations of 1 and 10 µg/ml increased biofilm formation by a culture from 0.442 ± 0.126 units of optical density in the control without antibiotic up to 1.539 ± 0.246 and 1.728 ± 0.402, respectively. It was also found that in the planktonic culture of *P. batumici* B-321 in the presence of 10 µg/ml of batumin, an increase in cell length of the producer by an average of 15% was noted. In control (without batumin), the cell length was 2.34 ± 0.22 × 0.65 ± 0.02 µm; at a concentration of 10 µg/ml of batumen it was 2.69 ± 0.23 × 0.75 ± 0.09 µm. Such an increase in cell length in pseudomonads may be associated with a decrease in the frequency of cell division [21].

In the literature, information is provided on the role of antibiotics, in particular phenazines synthesized by *Pseudomonas* species, in the biofilms formation by pseudomonads. In this case, phenazine antibiotics are able to change the expression of genes associated with cell adhesion and the subsequent formation of biofilms [22, 23]. It is possible that the batumin also plays a certain regulatory and functional role in the processes of biofilm formation by *P. batumici* strain.

Thus, the results of the studies of the batumin influence on biofilm formation processes by *P. batumici* strain suggest that along with high antimicrobial activity, the positive influence of batumin on the biofilm formation enhances the competitiveness of the producer in relation to other inhabitants of the rhizosphere and attest to the importance of this compound for life-support processes in *P. batumici*.

Studies of the batumin effect on the formed biofilm of staphylococci showed a significant decrease in its biomass in *S. aureus* and *S. epidermidis* at antibiotic concentrations of 0.5 µg/ml, which is only twice as high as the MIC of batumin when it acts on plankton cells. The obtained data for the batumin effect on the formed biofilm of staphylococci shed light on the mechanisms of batumin antimicrobial action, based both on the prevention of the formation of *S. aureus* and *S. epidermidis* biofilm, and on the functioning of the biofilm already formed, which makes it possible to consider it as a promising tool in the treatment of infections caused by staphylococci.

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ОСОБЛИВОСТІ ДІЇ АНТИБІОТИКА БАТУМІНУ НА ФОРМУВАННЯ БІОПЛІВКИ У *Staphylococcus aureus* І *Pseudomonas batumici*

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Метою роботи було дослідити дію антибіотика батуміну на сформовану стафілококами біоплівку, а також на процес біоплівкоутворення штамом-продуцентом *Pseudomonas batumici*. Мінімальну інгібувальну концентрацію (МІК) батуміну встановлено згідно зі стандартами CLSI Інституту клінічних та лабораторних стандартів. Формування біоплівки вивчали фотометричним методом O'Toole за допомогою планшетного фотометра за довжини хвилі 540 нм. З'ясовано, що батумін проникав у біоплівки стафілококів і знижував біомасу сформованої біоплівки у *Staphylococcus aureus* і *S. epidermidis* за концентрації антибіотика 0,5 мкг/мл, яка лише у два рази перевищує МІК батуміну за дії на планктонні клітини. Зміни біоплівки у присутності батуміну різняться у різних штамів стафілококів, однак ці відмінності не залежать від чутливості або резистентності штамів до антибіотика.

Уперше встановлено здатність штаму-продуцента батуміну до утворення біоплівки і стимуляції цього процесу самим антибіотиком у концентраціях 1 і 10 мкг/мл. Також відзначено, що у планктонній культурі *P. batumici* B-321 за присутності 10 мкг/мл батуміну довжина клітин продуцента збільшувалась у середньому на 15%.

Отримані дані відкривають нові аспекти антимікробної дії батуміну на біоплівку стафілококів, що полягають як у запобіганні формуванню, так і в інгібуванні вже сформованої біоплівки. Це дає підстави розглядати батумін як перспективний засіб лікування стафілококових інфекцій.

Ключові слова: батумін, *staphylococci*, біоплівка.

ОСОБЕННОСТИ ДЕЙСТВИЯ АНТИБИОТИКА БАТУМИНА НА ФОРМИРОВАНИЕ БИОПЛЕНКИ У *Staphylococcus aureus* И *Pseudomonas batumici*

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Целью работы было исследование действия антибиотика батумина на сформированную стафилококками биопленку, а также на процесс биопленкообразования штаммом-продуцентом *Pseudomonas batumici*. Минимальная ингибирующая концентрация (МИК) батумина определена в соответствии со стандартами CLSI Института клинических и лабораторных стандартов. Формирование биопленки изучали фотометрическим методом O'Toole с помощью планшетного фотометра при длине волны 540 нм. Установлено, что батумин проникал в биопленки стафилококков и снижал биомассу сформированной биопленки у *Staphylococcus aureus u S. epidermidis* при концентрации антибиотика 0,5 мкг/мл, которая лишь в два раза превышала МИК батумина при действии на планктонные клетки. Изменения биопленки в присутствии батумина различаются у разных штаммов стафилококков, однако эти различия не зависят от чувствительности или резистентности штаммов к антибиотику.

Впервые установлена способность штамма-продуцента батумина к образованию биопленки и стимуляции этого процесса самим антибиотиком в концентрациях 1 и 10 мкг/мл. Также было отмечено, что в планктонной культуре *P. batumici* B-321 в присутствии 10 мкг/мл батумина длина клеток продуцента увеличилась в среднем на 15%.

Полученные данные открывают новые аспекты антимикробного действия батумина на биопленку стафилококков, которые состоят как в предотвращении формирования биопленки, так и в ингибировании уже сформировавшейся биопленки. Это дает основания рассматривать батумин как перспективное средство лечения стафилококковых инфекций.

Ключевые слова: батумин, *staphylococci*, биопленка.