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ANTIMICROBIAL ACTIVITY OF CHOLANIC ACIDS' STEREOISOMERS COMPARED TO CHOLIC ACID ON THE TEST CULTURES OF MICROORGANISMS

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With the emergence of many strains of multidrug-resistant bacteria interest has been renewed in new antimicrobial agents. Cationic peptide antibiotics, which have been isolated from organisms ranging from bacteria to animals, have received considerable attention in part because of their broad spectrum of activity[1]. Generally, the targets of these antibiotics are bacterial membranes [2]. It was recently reported about the activities of series of membrane-active cationic cholic acid derivatives with potent antimicrobial activities that might share mutual aspects of action's mechanism with cationic peptide antibiotics [3,4]. The bactericidal action was shown on the examples of these compounds(cholic acids derivatives) to a broad spectrum of gram-negative and gram-positive organisms. Other cholic acid derivatives were weakly active against gram-negative organisms, but effectively permeabilized the outer membranes and sensitized the bacteria for hydrophobic antibiotics such as erythromycin and rifampicin. To better characterize the cholic acid derivatives, it has been measured their antibacterial activities against multidrug-resistant bacteria, including both gram-negative and gram-positive microorganisms. In addition, it has been measured the abilities of the cholic acid derivatives to sensitize multidrug-resistant gram-negative bacteria. This ability may enhance effect of antibiotics that can be used against these organisms [5].

A feature of membrane-active antimicrobial agents is often display of haemolytic properties that may be an impediment to their systemic use [2]. The rate (extent) of haemolytic properties of the cholic acid derivatives, as it was reported, has ranged from very haemolytic to weakly haemolytic [3]. The analysis of the literature has shown that the study of antimicrobial action of cholic acid derivatives almost wasn't held in these latter days whereas other pharmacological actions of cholic acid derivatives were the subject of study. This work is dedicated to new steriomeric compounds-the derivatives of cholic acid with the weakest haemolytic activity and potentially powerful antimicrobial action[3].

As it was previously reported, bile acids have a suppressive effect on the microflora [1-9], so it was interesting to investigate the activity of α/β-amino bile acids' stereoisomers to determine whether there is a difference in their effects on the microflora. Based on the research concerning the optical structure and pharmacological action of stereoisomers of the same substance it was found that many enzymes, hormones, neurotransmitters and amino acids alter their pharmacological properties depending on the stereo-configuration [10]. Therefore, 2 stereoisomers of cholic acid derivative were taken for this analysis. 3D structures of 3β-amino-7α, 12α-dihydroxy-5β-cholanic acid and 3α-amino-7α, 12α-dihydroxy-5β-cholanic acid are shown in Fig. 1 and Fig. 2 and built using "ChemBioOffice ChemBio3D Ultra 12.0"

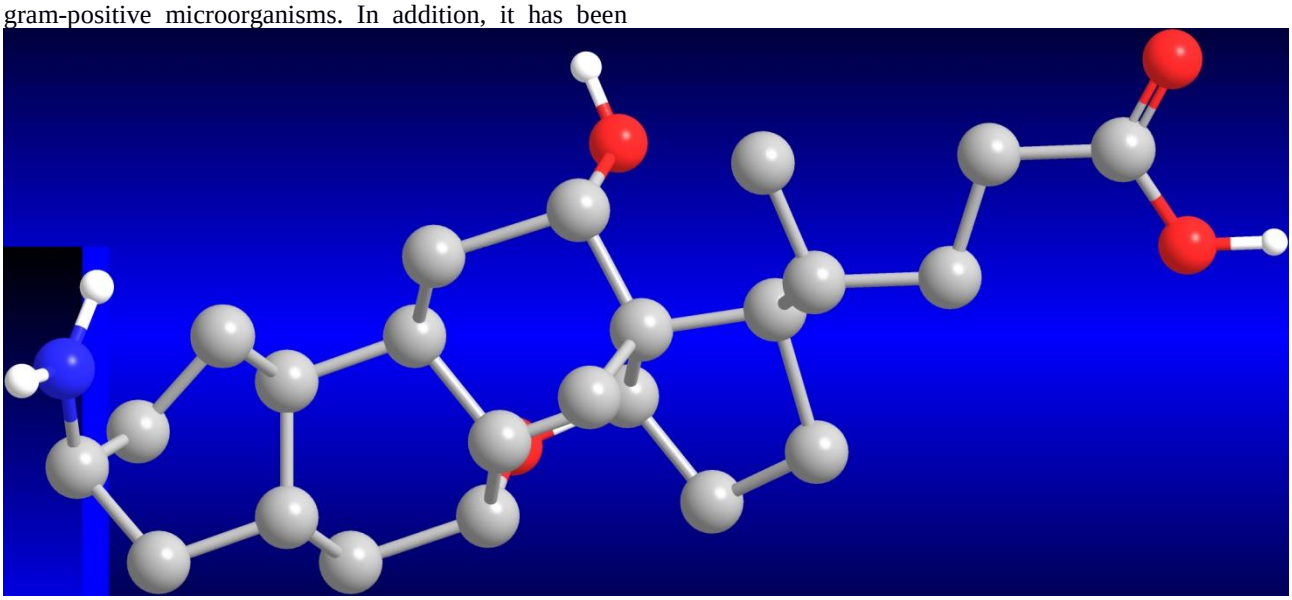

Figure 1. Spatial structure of 3β-amino-7α,12α-dihydroxy-5β-cholanic acid

In the 3D-model 3β-amino-7α,12α-dihydroxy-5β-cholanic acid has a more unfavorable position of the amino group at the 3rd carbon atoms and requires a large amount of energy of activation and stricter conditions of reaction than 3α-isomer is needed, so due to it, it makes possible the blocking of bacterial metabolism.

We assumed that bacteria which used bile acids more intensively in their metabolism were more vulnerable to 3β stereoisomer. It may serve as antagonist-substrate for receptors and enzymes of microorganisms.

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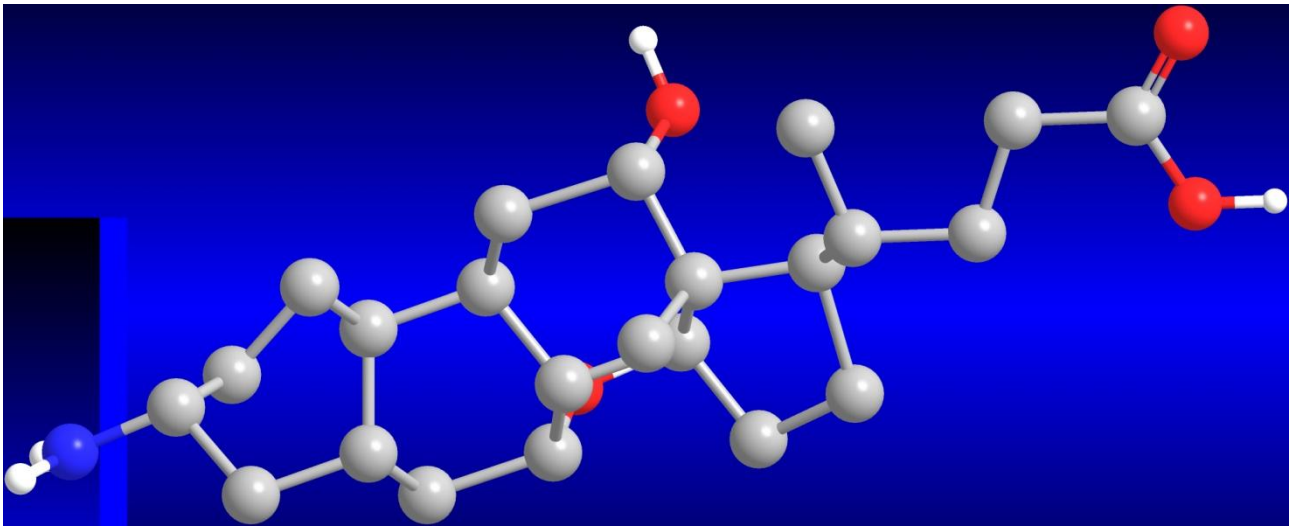

Figure 2. Spatial structure of 3α-amino-7α,12α-dihydroxy-5β-cholanic acid

The source materials (3α-and 3β-amino-7α-12α-dihydroxy-5β- cholanic acid) were obtained by sequential stereochemical synthesis in the laboratory of the National University of Pharmacy based on the isolated from birds'(chickens') bile and purified cholic acid.

Materials and methods

The study of antibacterial properties of compounds was performed by the method of a diffusion in agar in the laboratory of Microbiology in the National University of Pharmacy. As it was recommended by the World Health Organization (WHO) for assessing the activity of drugs we used reference test cultures: *Staphylococcus aureus* ATCC 26923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 885/653, *Proteus vulgaris* ATCC 4636. Microbial load(filling) was equaled to $1{,}5x10^8$ microbial cells per 1 ml of solution and it was determined by standard of (0.5)McFarland(McF). In this paper we use the 18-24 hour culture of microorganisms. Also we used Mueller-Hinton agar (Dagestan NII of nutrient media) for studies. According to method of "wells" we performed the determination of antibacterial activity in two layers of solid medium, poured into Petri dishes. In the bottom layer we used "hunger" non-planted medium (agar-agar, water, salt). The bottom layer was a 10 mm height substrate on which we set(placed) 3-6 thin horizontal cylinders of stainless steel. Their diameter is 8 mm and height 10 mm.

We filled the Petri dish with top layer that consisted of nutrient agar medium, it was melted and cooled to 40 ^{0}C, in which we added an appropriate standard amount of daily test-culture. Before filling the upper layer was mixed well until a homogeneous mass was formed. After hardening cylinders were pulled out of the hole by sterile tweezers, then we placed test substance into hole in according amount to it's size. Diameter of the well for the upper layer was ranged from 14 to 16 mm. Petri dishes were dried for 30-40 min. at room temperature and then they were placed in a thermostat at temperature of 37 º C for 18-24 hours and for the yeast similar fungus *Candida albicans* we placed in a thermostat for 24-48 hours.

The standard of microbial suspension was $1{,}5x10^8$ microbial cells in 1 ml standardized using 0,5 McF standart solution. When evaluating an antimicrobial properties we took into account the following criteria:

- absence of zones of microbial growth delay around the holes, and zones of delay up to 10 mm indicate that the organism is not sensitive to sample placed into the hole;
- zones of microbial growth delay 10-15 mm in diameter indicate low sensitivity of culture to testing concentration of antibacterial substances;
- zones of microbial growth delay 15-25 mm in diameter are characterized as an indicator of the sensitivity of the microorganism to the test sample;
- zones of microbial growth delay which exceeds 25 mm in diameter indicate a high sensitivity of microorganisms to samples.

Quantitative evaluation of antimicrobial activity was evaluated by method of serial dilutions. The method consists of determination of the minimal inhibitory concentration (MIC), which describes the bacteriostatic properties of objects of study. In the first tube we placed the concentration of the substance, which was 1000.0 mcg/ml, then we diluted by the nutrient broth in 2 times. From the first test tube by the rolling over we took 1 ml of diluted investigated substance. The concentration of substances was 1) 500,0 mcg/ml; 2) 250,0 mcg/ml; 3) 125,0 mcg/ml; 4) 65,5mg/ml; 5) 31,2 mcg/ml; 6) 15,6 mcg/ml 7) 7,8 mcg/ml;8) 3,9 mcg/ml; 9) 2,0 mcg/ml; 10) 1,0 mcg/ml. In each tube we placed 0.1 ml of standardized microbial cells of test cultures (*Staphylococcus aureus* ATCC 26923, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 653/885, *Proteus vulgaris* ATCC 4636). We cultivated cultures for 24-48 h., in test tubes, where was no growth (no clouding) we measured MIC. From last 3 tubes in which there were no clouding, we made hanging on nutrient agar and determined the MBC (minimum bactericidal concentration)[11-15].

Results and discussion

The results of antimicrobial activity indicate that the investigated substances have a wide(broad) spectrum

of antibacterial properties regarding cultures of aerobic bacteria and fungi. Results of the study of growth delay are presented in Table. 1

Table 1 Antimicrobial activity of substances regarding aerobic bacteria and fungi

Test cultures	**The diameters of the zones of growth delay of microorganisms, mm**		
	3β-amino-7α,12α-dihydroxy-5β-cholanic acid	**3α-amino-7α,12α-dihydroxy-5β-cholanic acid**	**3α,7α,12α- trihydroxy-5β-cholanic acid(cholic acid)**
Staphylococcus aureus ATCC 26923	26.50±1.10	23.00±0.9	16.00±0.57
Escherichia coli ATCC 25922	25.50±1.2	27.30±1.2	18.20±0.10
Pseudomonas aeruginosa ATCC 27853	9.40±1.1	15.80±0.95	17.00±0.57
Proteus vulgaris ATCC 4636	20.50±1.2	17.60±0.95	6.50±0.76
Bacillus subtilis ATCC 6633	21.70±1.1	16.50±1.05	08.50±0.29
Candida albicans ATCC 653/885	23.40±0.45	26.20±1.15	09.85±0.46

Notice. n=3

Both isomers have showed high antimicrobial activity, the highest it was to the culture of *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Candida albicans*. Areas of growth delay of isomer compounds were comparable and didn't show big difference(4±0.9 mm).

Concerning other cultures isomers showed a moderate effect, but it should be noted that the activity of both compounds was higher than the corresponding activity of cholic acid. Data of antimicrobial action of cholic acid coincided with earlier studies by other researchers [9]. As it was assumed, there was a difference in the effects of different isomers on the microflora: 3β isomer showed the greatest effect to *Proteus vulgaris* and *Bacilus subtilis*, growth delay was observed at lower concentrations than in cultures with the alpha isomer. But 3β-isomer did not cause growth delay *of Pseudomonas aeruginosa*.

The value of MIC (Table 2) describes the bacteriostatic properties of the described compounds regarding the aerobic bacteria and fungi, which is consistent with the literature data about the antimicrobial activity of bile acids derivatives. In our study we succeeded to confirm the literature data about the MIC of cholic acid

Table 2 Determination of the MIC against aerobic bacteria and fungi

Test-cultures	**MIC, mcg/ml**		
	3β-amino-7α,12α-dihydroxy-5β-cholanic acid	**3α-amino-7α,12α-dihydroxy-5β-cholanic acid**	**3α,7α,12α- trihydroxy-5β-cholanic acid(cholic acid)**
Staphylococcus aureus ATCC 26923	300±21.2	350±15.8	550±17.4
Escherichia coli ATCC 25922	350±11.5	300±17.5	350±15.3
Pseudomonas aeruginosa ATCC 27853	>1000	600±19.1	500±18.5
Proteus vulgaris ATCC 4636	450±10.8	800±17.4	>1000
Bacillus subtilis ATCC 6633	400±14.5	600±16.2	>1000
Candida albicans ATCC 653/885	400±18.3	350±21.1	850±13.5

Notice. n=3

According to MIC values for both isomers their antimicrobial action are 2 times higher than the effect of cholic acid. An exception was the effect of 3β-amino-7α, 12α-dihydroxy-5β-cholanic acid on the inhibition of *Pseudomonas aeruginosa* and concentration was significantly higher(>1000mcg/ml) but the exact concentration wasn't established.

Exact mechanism of antimicrobial action is still unclear, perhaps the presence of antimicrobial activity associated with surface activity on membranes, but there is a assumption that bile acids alter the structure of the cell membrane[2] and disrupt normal metabolism on it[7,10]. Finally it should be noted that all substances has showed fungistatic or fungicidal action.

Conclusions

1. All compounds showed wide spectrum of antimicrobial activity and might be used as potential antibiotics.
2. We confirmed our assumption about the activity of different isomers of amino functionalized cholic acid. Also substances are perspective for further study *in vivo*.

References

1. Cationic bactericidal peptides[Text]./Hancock, R. E. W., Falla, T. & Brown, M. // Advances in Microbial Physiology- 1995-.37, 135–75.
2. Shai, Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides[Text]/ Shai, Y // Biochimica et Biophysica Acta-1999- 1462, 55–70.
3. Antimicrobial activities of amine- and guanidine-functionalized cholic acid derivatives[Text] / Li, C., Lewis, M. R., Gilbert, A. B., Noel, M. D., Scoville, D. H., Allman, G. W. et al.// Antimicrobial Agents and Chemotherapy-1999. 43, 1347–9.
4. Incremental conversion of outer-membrane permeabilizers into potent antibiotics for gram-negative bacteria[Text]/ Li, C., Budge, L. P., Driscoll, C. D., Willardson, B. M., Allman, G. W. & Savage, P. B. // Journal of the American Chemical Society -1999- 121, 931–40.
5. Erica J. Schmidt, J. Scott Boswell, Joshua P. Walsh, Matthew M. Schellenberg, Timothy W. Winter, Chunhong Li, Glenn W. Allman, and Paul B. Savage/ Activities of cholic acid-derived antimicrobial agents against multidrug-resistant bacteria[Text].// *J. Antimicrob. Chemother.* -2000- 47 (5): 671-674
6. Barsuk D. O. Preparation and microbiological activity of derivatives of bile acids [Text]/ Barsuk D. O., Stremouhov A. A. // Actual questions of pharmacy, 23-24 Apr. -2009, Kharkiv:NUPh publishing ,p. 27.
7. Silverman J. Bile acids; co-mutagenic activity in the Salmonella/mammalian-microsome mutagenicity test: brief communication[Text] / J. Silverman, A. W. Andrews // J. Natl. Cancer Inst. – 2007. – V. 65. – P. 1557-1559.
8. Antibacterial activity of organometallic complexes of cholic acid.[Text]/Tripathi Kishu, Kumar T. Siva// Digest Journal of Nanomaterials and Biostructures-2010- Vol. 5, No 3, July-September 2010, p. 763-770.
9. Oral bile acids reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhotic rats[Text]/Vicente Lorenzo-Zúñiga, Ramón Bartolí, Ramón Planas, Alan F. Hofmann, Belén Viñado, Lee R. Hagey, José M. Hernández, Josep Mañé, Marco A. Alvarez, Vicente Ausina and Miquel Angel Gassull//, Hepatology, March 2007 - V. 37, Issue 3, p 551–557.
10. V. V. Alekseyev. Optical isomerism and drugs pharmacological activity[Text]/ Alekseyev V. V. // Soros Educ. J. 2002- p.056-61.
11. NCCLS. Performance standards for antimicrobial susceptibility testing; ninth informational supplement// M100-S9.- 1999.- V.19.- N.1.
12. Sidorenko, S.V. Antibiotic susceptibility testing: disc-diffusion method. Results interpretation. [Text] / Sidorenko S.V., Kolupaev E.V.. – M. "Arina". – 1999. –33 p. – 1000 copies. – ISBN 5-93235-005-9.
13. Guidelines for susceptibility testing of Microorganisms to Antibiacterial agents[Text] /Semina N.A., Sidorenko S. V., Rezvan S. P., Grudinina S. A.// Clin mircobiol antimic therapy – 2004-6(4)
14. Methodological guidelines "Determination of the sensitivity of microorganisms to antibiotics"// Ministry of Public Health of Ukraine, Kiev – 2007, № MB 9.9.5-143-2007.
15. Bacteriological control of culture media. //Newsletter Ministry of Public Health of Ukraine № 05.4.1/1670, Kiev, 2001 – 45 p.

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АНТИМІКРОБНА АКТИВНІСТЬ СТЕРЕОІЗОМЕРІВ ХОЛАНОВОЇ КИСЛОТИ В ПОРІВНЯННІ З ХОЛЕВОЙ КИСЛОТОЮ НА ТЕСТ-ШТАМАХ МІКРООРГАНІЗМІВ

Барсук Д. О., Стремоухов О.О., Коваленко С. М.

У статті представлено результати вивчення мікробіологічної активності 3α та 3β аміно жовчних кислот. Виявлена виражена антимікробна активність. Наявність та спектр антимікробної активності виявляли методом серійних розведень у щільному поживному середовищі та показали як бактеріостатичний так і бактеріцидний ефекти.

16. Ключові слова: жовчні кислоти, 3α та 3β стереоізомери 3-аміно-7α,12α-дигідрокси-5β-холанової кислоти, антимікробна активність, мікробіологічний скринінг.

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АНТИМИКРОБНАЯ АКТИВНОСТЬ СТЕРЕОИЗОМЕРОВ ХОЛАНОВОЙ КИСЛОТЫ В СРАВНЕНИИ С ХОЛЕВОЙ КИСЛОТОЙ НА ТЕСТ-ШТАММАХ МИКРООРГАНИЗМОВ

Барсук Д. О. , Стремоухов А.А., Коваленко С.Н.

В статье представлены результаты изучения микробиологической активности 3α и 3 β амино желчных кислот. Выявлена выраженная антимикробная активность. Наличие и спектр антимикробной активности выявляли методом серийных разведений в плотной питательной среде и показали как бактериостатический, так и бактерицидний эффекты.

Ключевые слова: желчные кислоты, 3α- та 3β стереоизомеры 3-амино-7α,12α-дигидрокси-5β-холановой кислоты, антимикробная активность,

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Barsuk D.O., Stremouhov O.O., Kovalenko S.M.

The paper presents the results of a study of microbiological activity 3α and 3β amino bile acids. We revealed high antimicrobial activity. The availability and range of antimicrobial activity were revealed by the method of serial dilutions in a solid nutrient medium and shown as bacteriostatic as bactericidic effects.

Keywords: bile acid, 3α and 3β stereoisomers of 3-amino-7α,12α-dihydroxy-5β-cholanic acids, antimicrobial activity, microbial screening