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HEMOLYTIC ACTIVITY OF SKIN SECRETIONS OF AMPHIBIANS THAT INHABIT THE UKRAINE TERRITORY

Secretions derived from amphibian skin glands serve as a potential reservoir of various valuable active molecules. Currently, the multiple substances with diverse therapeutic activities among the components of glandular secretions of different species of amphibians have been found. It has been proven that they have antibacterial, antifungal, antiprotozoal, antidiabetic, antineoplastic, analgesic, and sleep-inducing properties. Taking this into consideration, to get the basic knowledge about the properties of the components of skin secretions of some Anura species that inhabit the territory of Ukraine is crucial for further investigation of the most potential ones. The red blood cell hemolysis assay is a prevalent test to study the cytotoxicity of studied samples. The aim of the present study was to analyze the hemolytic activity of skin secretions of Bombina bombina, Bombina variegata, Bufotes viridis, Rana temporaria, Pelophylax ridibundus, and Pelobates fuscus, and to obtain the primary data on the possible mechanism of their toxicological action on the blood cells membranes. The skin secretions of six amphibian species mentioned above were incubated with erythrocyte suspension in different concentrations. Eminently active B.variegata skin secretions, having the HD HD50 value at 0.5 μg/ml, were taken for the subsequent researches, where the effects of osmotic protectants, divalent cations, antioxidants, chelating agent, and serine protease inhibitor on the cell lysis ability of B. variegata skin secretions was studied. All studied cations inhibited the hemolytic activity of B. variegata secretions in a dose-depend manner. While the serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), markedly decreased the hemolytic activity of studied skin secretions. We can assume that the bioactive peptides in these skin secretions have an enzymatic mechanism of action.

Keywords: biologically active molecules, amphibians, hemolytic activity, skin gland secretions.

Introduction. The Amphibians skin provides a rich source of bioactive molecules (peptides, proteins, steroids, alkaloids, opiods) that have some effective therapeutic potencies, such as: antibacterial, antifungal, antiprotozoal, antidiabetic, antineoplastic, analgesic and sleep-inducing properties [1]. Isolation and identification of novel metabolites from amphibian skin secretions could be a promising course to create efficient drugs with valuable therapeutic and pharmaceutical potential [2, 3, 4]. Nevertheless, the composition and the mechanism of action of biologically active compounds from amphibian skin secretions are not fully investigated by this time.

Toxicity of an active molecule is a key factor during drug design, and the hemolytic activity represents a useful starting point in this regard. It provides the primary information on the interaction between molecules and biological entities at cellular level. Hemolytic activity of any compounds is an indicator of general cytotoxicity towards normal healthy cells. On the other hand, some proteins that affect the biological membranes might induce the formation of pores or channels in natural and model bilayer lipid membranes [5, 6]. Thus, hemolytic activity that is induced by these protein toxins could be used as a sensitive toxicological tool to investigate the targeting and attachment of proteins to the cell membranes.

Thus, the aim of this work was to study the hemolytic activity of skin secretions of amphibians prevalent on the territory of Ukraine, such as *Bombinabombina, Bombinavariegata, Bufotesviridis, Rana temporaria, Pelophylaxridibundus, and Pelobatesfuscus*, and to get the basic information on the possible mechanism of their toxicological action on the blood cells membranes.

Materials and Methods. Adult specimens (both sexes) of *B. bombina, B. variegata, B. viridis, R. temporaria, P. ridibundus* and *P. fuscus* were collected and authenticated by the Department of Zoology and Ecology of Taras Shevchenko National University of Kyiv, Ukraine. All animal procedures followed the European Directive 2010/63/EU (EC, 2010) on protecting animals used for experimental and other scientific purposes. All manipulations were approved by the Ethical Committee of Educational and Scientific Centre "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv, Ukraine.

The crude skin secretions were collected after a short-term mechanical irritation of amphibian skin. The secret was washed with a small amount of distilled water, filtrated, lyophilized (Testar Lyo Quest, Spain) and stored at 4 °C until use. Before each experiment dried material was dissolved in phosphate-buffered saline (PBS), pH 7.2, that contained 137 mM NaCl, 1.5 mM KH2PO4, 2.7 mM KCl and 8.1 mM Na2HPO4. Further, the suspension was centrifuged at 2500 g for 10 min and the supernatant was collected and used in the study. Bradford method [7] was used to measure the concentration of total protein in the samples.

Rabbit blood was collected from the ear artery in the tubes containing 3.8 % sodium citrate in ratio of 9:1 to prevent coagulation, and centrifuged at 500 g for 10 min at 4 °C. Plasma was removed carefully and the erythrocytes were washed for additional three times in 5 volumes of PBS, pH 7.2. Washed erythrocytes were stored at 4 °C and used within 6 h for the hemolysis assay. For the experiment 2 % (v/v) erythrocyte suspension was prepared by mixing 0.1 ml of packed red blood cells with 4.9 ml of PBS, pH 7.2.

For the preliminary study of hemolytic activity of the skin secretions of six studied amphibian species, erythrocyte suspension was incubated with various concentrations of these secretions (the final concentrations of total protein were 0.5, 5 and 50 μg per 1 ml of erythrocyte suspension) at 37 ºC for 30 min and then centrifuged at 2500 g for 6 min. The absorbance of supernatant was measured at 541 nm to establish the amount of hemoglobin released due to erythrocytes lysis. Two controls were prepared (both without frog secretions): negative control contained only PBS, and positive control – 1 % Triton X-100 that was taken as 100 % cell lysis. The most active *B. variegata* skin secretion, which had HD_{50} value 0.5 μg/ml, was used for further research to establish basic information on its toxicological properties. For this purpose we investigated the effects of different factors on the skin secretion hemolytic activity, including osmotic protectants – 25 mM D-glucose and 25 mM D-lactose; divalent cations, such as Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+} in concentration range from 1 to 100 mM, Fe^{2+} in concentration range from 10 to 50 μM, and Cu^{2+} in concentration range from 1 to 100 μM; antioxidants – 2 mM ascorbic acid and 2 mM cysteine; chelating agent – 2 mM EDTA; serine protease inhibitor – 2 mM PMSF.

Each of these was added to erythrocytes suspension followed by addition of *B. variegata* skin secretion (the final concentration of total protein in all experiments was 0.5 μg per 1 ml of erythrocytes). After incubation at 37 °C for 30 min, the hemolytic activity was determined as described above.

Data analysis was performed using Student's t test to evaluate the statistical significance of experimental groups. The results were recorded as means M±SD. p≤0.05

Results and Discussion. Granular glands (also called serous glands or poison glands) of amphibian skin produce a large variety of bioactive substances, including antimicrobial peptides [8], neurotoxic peptides [9], gastric disturbance peptides [10], and alkaloids [11]. In the middle of the grand the granules with active peptides are located. When the animal is injured or alarmed, the content is released through skin secretions [12]. Despite the immense richness of wild amphibians in Ukraine, current knowledge about the presence of bioactive molecules and mechanisms of their action is limited to only few species. The literature data on the biological activity of peptides isolated from amphibians indicates that skin secretions could be an attractive source of new therapeutic candidates. Although, the therapeutic potential of many active molecules from amphibian skin secretions is limited due to their high hemolytic activity against human erythrocytes.

Taking into account that skin secretions, which are used for isolation and purification of bioactive molecules, have to be evaluated for their potential hemolytic activity, in this study we examined the effect of skin secretions from six amphibian species widespread on Ukrainian territory – *B. bombina, B. variegata, R. temporaria, B. viridis, P. fuscus* and *P. ridibundus* on the red cell membrane integrity.

The hemolytic activity of the amphibian crud skin secretions was tested at three protein concentrations 0.5, 5 and 50 μg per 1 ml of erythrocytes suspension (Table 1). According to the obtained results, the skin secretions of *R. temporaria* and *B. viridis* had no effect on the erythrocytes integrity even at the highest level of tested concentrations (50 μg/mL), while skin secretions of other studied species with various intensity induced the erythrocyte lysis. As follows, the skin secretion of *P. ridibundus* showed significant hemolytic values (76 %) only at the concentration of 50 μg/mL, while at the lower concentration no negative influence on the red blood cells was observed. *B. bombina, P. fuscus* and *B. variegata* skin secretions showed strong hemolytic effect in a concentration-depend manner. As demonstrated in the Table 1, the most harmful effect on the erythrocytes was observed while studying the *B. variegata* skin secretion, which caused 50 % of cell lysis even at the lowest concentration – 0.5 μg/ml. The skin secretion of this species therefore has been chosen for further experiment to investigate the possible mechanism of its action.

***Table* 1. The degree of erythrocyte lysis (%) caused by skin secretions of studied amphibian species**

Species	Total protein concentration (μg per 1 ml of erythrocyte suspension)		
	0.5	5	50
B. variegata	48 ± 5*	72 ± 7*	76 ± 6*
P. fuscus	17 ± 3*	42 ± 5*	66 ± 6*
B. bombina	5 ± 3	18 ± 3*	63 ± 5*
P. ridibundus	4 ± 2	5 ± 3	76 ± 4*
B. viridis	3 ± 2	4 ± 3	9 ± 4
R. temporaria	3 ± 2	4 ± 2	6 ± 4

*p ≤ 0.05 the difference is comparable to the effect of PBS (negative control)

To evaluate the HD_{50} value – the concentration of secretion causing 50 % hemolysis of red blood cells – erythrocyte suspension was incubated with various concentrations of *B.variegata* skin secretion (the final concentrations of total protein were 0.25, 0.5, 1, 2.5, 5, 10, 25 μg per 1 ml). The HD_{50} value of *B. variegata* skin secretion was found to be 0.5 μg/mL (Fig. 1). Thus, the degree of hemolysis caused by this concentration of *B. variegata* skin secretions was used as the reference value in our following experiments, which were carried out to investigate the effects of different factors, such as osmotic protectants, divalent cations, antioxidants, EDTA, and serine protease inhibitor (PMSF) on skin secretion hemolytic activity.

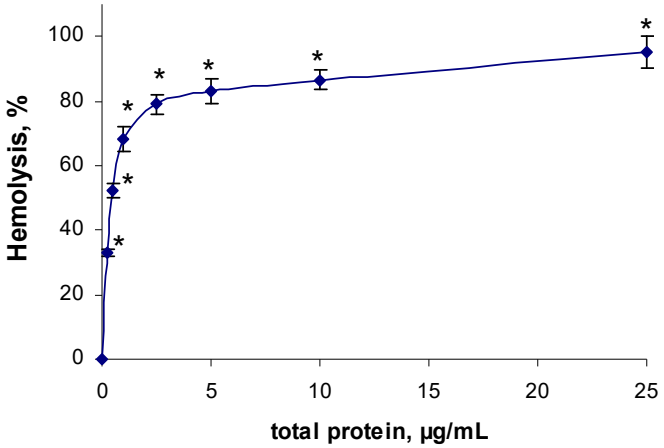

Fig. 1. Dose-response curve of the *B. variegata* skin secretion hemolytic activity

All the results are expressed as the mean ± SD (n = 3);
*p ≤ 0.05 the difference is comparable to the effect of PBS (negative control)

The influence of cations on the hemolytic activity of *B. variegata* skin secretion was determined using divalent cations Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} at different concentrations. Cations were incubated separately with erythrocyte suspension and skin secretion at the final concentration of total protein of 0.5 μg/mL for 30 minutes, centrifuged and the absorbance of supernatant was measured at 541 nm.

As shown in Fig. 2, all cations decreased the hemolytic activity of *B. variegata* skin secretion in dose-depend manner, but with different intensity. The lowest effect had Mg^{2+} which at its highest concentration decreased the hemolytic effect only to 29±2 %. Ca^{2+} at low concentrations (1–10 mM) enhanced the cell lysis to 69±7 %, but inhibited it at highest studied concentration (100 mM) to 26±3 %. Mn^{2+} showed full inhibition effect on hemolytic activity at concentration of 50 mM, while Cu^{2+} and Fe^{2+} had the same effect at concentration of 50 μM. Zn^{2+} at concentration ranging from 1 to 100 mM completely inhibited the hemolytic activity of *B. variegata* skin secretion (Fig. 2).

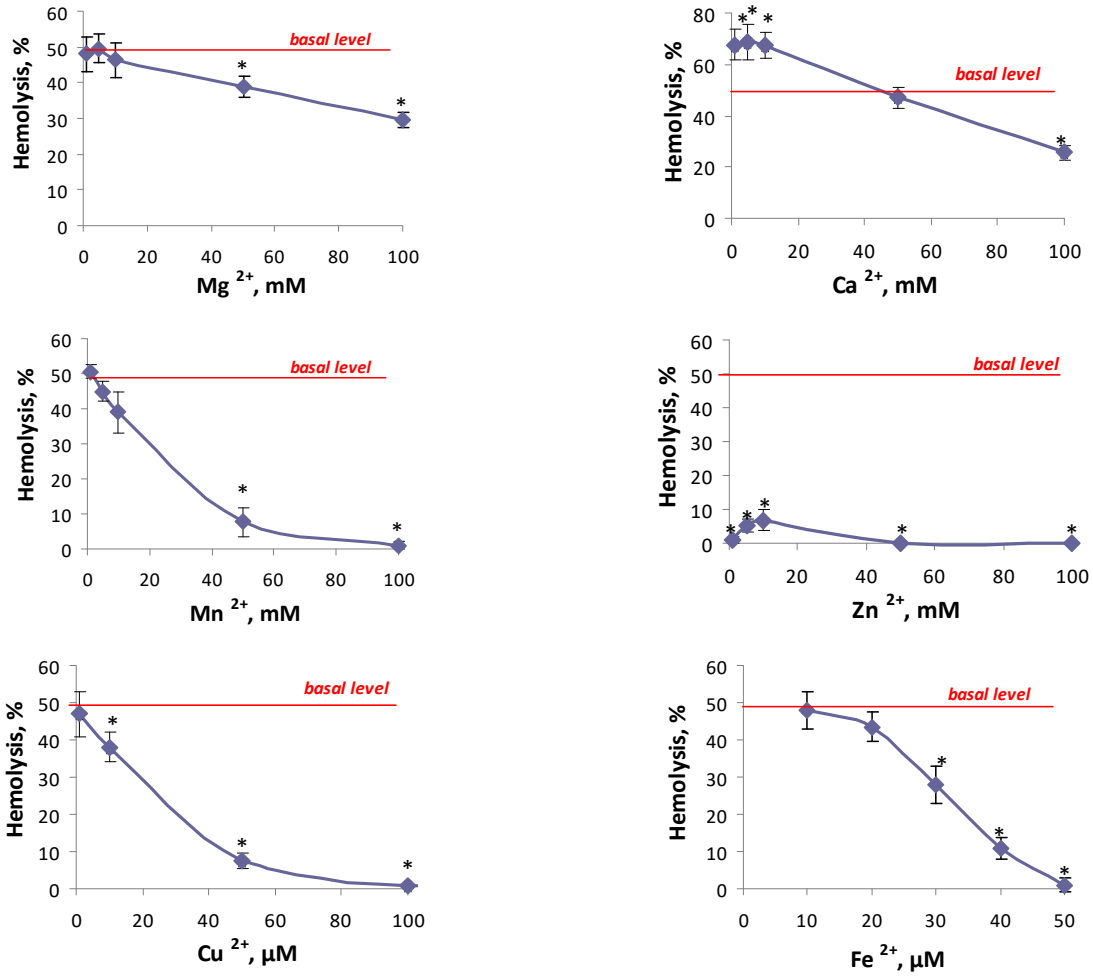

Fig. 2. Effect of six metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}) on the hemolytic activity of *B. variegata* skin secretions

All the results are expressed as the mean ± SD (n = 3);
*p ≤ 0.05 the difference is comparable to the basal level which is equal to the degree of erythrocyte lysis (%) caused by *B. variegata*skin secretion at the concentration of 0.5 μg/mL

The effects of osmotic protectors, antioxidants, EDTA and PMSF on hemolytic activity of *B. variegata* skin secretion are summarizes in Table 2. The degree of erythrocyte lysis (50±3 %) caused by *B. variegata* skin secretion at the concentration of 0.5 μg/mL was taken as the reference value for this experiment (control). The percentage of inhibition was calculated having regard to the control (without agent) which was defined as "100 %".

Glucose and lactose had no significant effect on hemolytic activity of *B. variegata* skin secretion. The antioxidants ascorbic acid and cysteine, as well as EDTA, also showed no remarkable outcome. In contrast, a serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), substantially inhibited hemolytic activity of frog's skin secretion (Table 2).

***Table* 2. Effect of different agents on the hemolytic activity of *B. variegata* skin secretion**

Agent	Hemolysis (%)	Inhibition (%)
control (without agent)	50 ± 3	no effect
25mM D-glucose	49 ± 3	no effect
25mM D-lactose	58 ± 5	no effect
2mM ascorbic acid	51 ± 4	no effect
2mM cysteine	51 ± 2	no effect
2mM EDTA	50 ± 3	no effect
2mM PMSF	3 ± 2*	~ 95

*p ≤ 0.05 the difference is comparable to the basal level which mean the degree of erythrocyte lysis (%) caused by *B. variegata* skin secretion at the concentration of 0.5 μg/mL

Based on our results, as the hemolytic activity of *B.variegata* secretions was inhibited by PMSF, we can suggest, that the mode of action of bioactive peptides in this secret was enzymatic. Our results are proved by other literature data. Thuswise, in the late 1980s and early 1990s, certain studies described the peptidases discovered in the cutaneous secretion of *Xenopus laevis* [13, 14]. The recent study showed, that the skin secretions of several amphibians contained a wide variety of proteins (molecular weight from 8 to 150 kDa), which exhibited proteolytic activity with selective effects on different substrates [15]. In addition, Jilek et al. found, that skin secretion of *B. variegata* contains the protein, which catalyzes the conversion of L-Ile to D-alle at position 2 of a model peptide with the N-terminal sequence of bombinin H. It approves the fact, that amphibian skin secretions have various enzymes in their content [16]. However, further investigations are needed to better characterize the active peptides of this secretion and to clarify their biochemical functions.

Conclusion. In this study, we examined the effects of osmotic protectants, cations, and antioxidants on erythrocyte hemolysis stimulated by the skin secretions from the frog *B. variegata*. We observed that serine protease inhibitor – PMSF – completely inhibited the hemolytic activity of studied secterion. Consequently, an assumption was made, that this skin secretion induced erythrocytes lysis by catalytic way. This hypothesis should be explored in more depth in future studies.

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ГЕМОЛІТИЧНА АКТИВНІСТЬ СЕКРЕТІВ ШКІРНИХ ЗАЛОЗ АМФІБІЙ, ПОШИРЕНИХ НА ТЕРИТОРІЇ УКРАЇНИ

Виділення шкірних залоз амфібій є потенційним джерелом різноманітних цінних біоактивних молекул. На сьогодні серед компонентів шкірних секретів різних видів роду Amphibia знайдено велику кількість речовин, які володіють різноманітними терапевтичними активностями. Показано, що вони володіють антибактеріальними, протигрибковими, антипротозойними, антидіабетичними, антинеопластичними, знеболюючими та снодійними властивостями. Ураховуючи це, отримати основну інформацію про властивості компонентів шкірних секретів деяких видів земноводних, які населяють територію України, є передовим завданням сучасної біохімії для подальшого дослідження найбільш перспективних із них. Аналіз гемолізу еритроцитів є поширеним методом перевірки цитотоксичності досліджуваних речовин. Ліофілізовані шкірні виділення, що складаються із різноманітних біоактивних речовин, були перевірені на токсичність. Метою цього дослідження було проаналізувати гемолітичну активність шкірних виділень Bombina bombina, Bombina variegata, Bufotes viridis, Rana temporaria, Pelophylax ridibundus та Pelobates fuscus і отримати дані про можливий механізм їхньої токсикологічної дії на мембрани клітин крові. Секрети шести видів амфібій, зазначених вище, інкубували із суспензією еритроци-

тів у різних концентраціях. Шкірні виділення B. variegata, які характеризувалися найвищою активністю (HD50 = 0,5 мкг/мл), були використані для подальших досліджень, де визначали вплив осмотичних протекторів, двовалентних катіонів, антиоксидантів, хелатуючого агента та інгібітора серинових протеаз на здатність досліджуваного секрету до лізису клітин. Усі досліджені катіони інгібували гемолітичну активність секрету B. variegata залежно від дози, тоді як інгібітор серинової протеази, фенілметилсульфонілфторид (PMSF), помітно знизив гемолітичну активність досліджуваного шкірного секрету. Можна припустити, що біоактивні пептиди секрету шкірних залоз B. variegata мають ферментативний механізм дії.

Ключові слова: біологічно активні молекули, амфібії, гемолітична активність, секрети шкірних залоз.

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ГЕМОЛИТИЧЕСКАЯ АКТИВНОСТЬ СЕКРЕТОВ КОЖНЫХ ЖЕЛЕЗ АМФИБИЙ, РАСПРОСТРАНЕННЫХ НА ТЕРРИТОРИИ УКРАИНЫ

Выделения кожных желез амфибий являются потенциальным источником разнообразных ценных биоактивных молекул. На сегодня среди компонентов кожных секретов различных видов рода Amphibia найдено большое количество веществ, обладающих различными терапевтическими активностями. Показано, что они обладают антибактериальными, противогрибковыми, антипротозойными, антидиабетическими, антинеопластическими, обезболивающими и снотворными свойствами. Учитывая это, получить основную информацию о свойствах компонентов кожных секретов некоторых видов земноводных, населяющих территорию Украины, является передовым заданием современной биохимии для дальнейшего исследования наиболее перспективных из них. Анализ гемолиза эритроцитов является распространенным методом проверки цитотоксичности исследуемых веществ. Лиофилизированные кожные выделения, состоящие из различных биоактивных веществ, были проверены на их токсичность. Целью этого исследования было проанализировать гемолитическую активность кожных выделений Bombina bombina, Bombina variegata, Bufotes viridis, Rana temporaria, Pelophylax ridibundus и Pelobates fuscus и получить данные о возможном механизме их токсикологического действия на мембраны клеток крови. Секреты шести видов амфибий, указанных выше, инкубировали с суспензией эритроцитов в различных концентрациях. Кожные выделения B. variegata, которые характеризовались высокой активностью (HD50 = 0,5 мкг/мл), были использованы для дальнейших исследований, где определяли влияние осмотических протекторов, двухвалентных катионов, антиоксидантов, хелатирующего агента и ингибитора серинових протеаз на способность исследуемого секрета к лизису клеток. Все исследованные катионы ингибировали гемолитическую активность секрета B. variegata в зависимости от дозы, тогда как ингибитор сериновых протеаз, фенилметилсульфонилфторид (PMSF), заметно снизил гемолитическую активность исследуемого кожного секрета. Можно предположить, что биоактивные пептиды секрета кожных желез B. variegata имеют ферментативный механизм действия.

Ключевые слова: биологически активные молекулы, амфибии, гемолитическая активность, секреты кожных желез.

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THE EFFECT OF COMPOUND DM509 ON KIDNEY FIBROSIS IN THE CONDITIONS OF THE EXPERIMENTAL MODEL

Kidney fibrosis is a key event in the development of chronic kidney disease, leading to end-stage renal failure. Unfortunately, there are now few drugs capable of preventing fibrosis in the kidneys, which is accompanied by the progression of chronic kidney disease in the terminal stage of renal failure. The results show the effectiveness of the use of a new dual-acting agent DM509 in the prevention of renal fibrosis using a model of unilateral obstruction of the ureter in mice. DM509 is both a farnesoid X-receptor agonist and a soluble epoxyhydrolase inhibitor. In this study, there were 8-12 week old C57BL/6J males undergoing surgery, which led to the development of unilateral ureteral obstruction and a control group. Mice received DM509 (10 mg/kg/day) or DM509-free solution together with drinking water for 10 days the day before surgery. Samples of kidney and blood tissues were collected at the end of the experiment. In the unilateral ureteral obstruction group, kidney dysfunction was detected, which was accompanied by increased urea nitrogen content in the blood compared to the control group (63 ± 7 vs. 34 ± 6 mg/d). The reduction of urea nitrogen in the blood by 36 % in mice with unilateral ureteral obstruction treated with DM509 is shown compared to mice with this pathology without treatment, which in turn proved the effectiveness of DM509 in preventing renal dysfunction. In mice with unilateral ureteral obstruction, which did not receive DM509, the development of kidney fibrosis with a high content of hydroxyproline in the kidneys and also increased collagen content in histological sections of the kidneys were detected. In the DM509 group, the renal and collagen hydroxyproline content were 34-66 % lower, indicating the effectiveness of this agent in the treatment of renal fibrosis. Thus, we have shown that the new DM509 is effective in preventing renal dysfunction and renal fibrosis using a murine model of unilateral ureteral obstruction.

Keywords: soluble epoxide hydrolase inhibitor, farnesoid x receptor agonist, kidney fibrosis.

Introduction. Renal fibrosis is considered as critical pathophysiological event in the development and progression of chronic kidney disease (CKD). Progressive CKD results in end-stage renal disease (ESRD), which is the common clinical end point for all progressive renal diseases [3]. The common CKD etiologies and the consequent ESRD include diabetes, hypertension, glomerulonephritis, acute kidney injury, and chronic pyelonephritis. ESRD is a major burden to the health care system and a large percentage of the patients are inevitably placed on dialysis and ultimately require transplantation [3, 16]. The ESRD burden on health care is caused largely due to the lack of an effective anti-fibrotic agents that can target CKD.

Indeed, little success has been made over the past decade in developing agents or therapies that can prevent renal fibrosis to slow the progression of CKD to ESRD [23]. Currently, angiotensin-converting enzyme