HEMOLYTIC ACTIVITY OF SKIN SECRETIONS
OF AMPHIBIANS THAT INHIBIT 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 shown to have 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 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.

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 °C 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 HD50 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 Mn2+, Mg2+, Ca2+, Zn2+ in concentration range from 1 to 100 mM, Fe2+ in concentration range from 10 to 50 µM, and Cu2+ 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.

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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 gland 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.

### 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 HD50 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 HD50 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 secretion 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](image)

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](image)

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          |

* $p \leq 0.05$ 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
На основе результатов, как в микробиологическом аспекте, так и в гормональном, секреты видов из рода Bombina могут быть использованы в качестве потенциальных источников биологически активных веществ.

**Conclusion.** В данном исследовании было показано, что наличие специфической активности протеаз и антиоксидантов на основе конъюгации гормонального и протеинового характера, включая антиоксиданты, индуцирующие эндотелия, приводит к развитию применения и образованию белка, который участвует в конверсии D-Ile к D-aIle при положении 2 у моделиного пептида с N-терминальной последовательностью NetB, a pore-forming toxin from Clostridium perfringens / C. G. Savva, // J. Nat. Prod. – 2005. – Vol. 68. – P. 1556-1575.

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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 epoxide hydrolase 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/dL). 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 level and collagen 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