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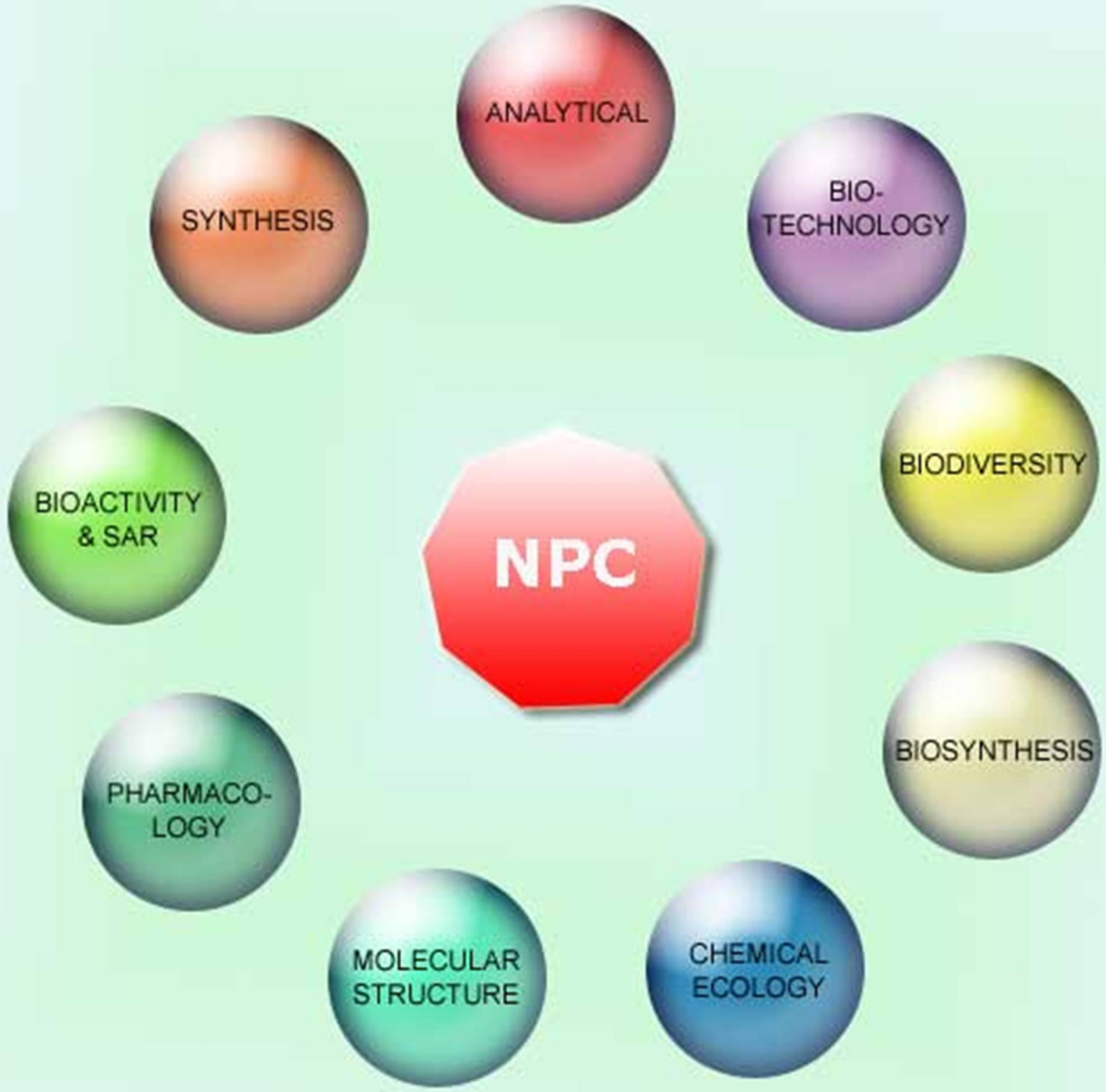

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Immunomodulatory Action of Monosulfated Triterpene Glycosides from the Sea Cucumber *Cucumaria okhotensis*: Stimulation of Activity of Mouse Peritoneal Macrophages

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Six monosulfated triterpene glycosides, frondoside A_1 (**1**), okhotoside B_1 (**2**), okhotoside A_1-1 (**3**), frondoside A (**4**), okhotoside A_2-1 (**5**) and cucumarioside A_2-5 (**6**), isolated from *Cucumaria okhotensis* Levin et Stepanov, stimulate spreading and lysosomal activity of mouse macrophages and ROS-formation in the macrophages. The highest macrophage spreading and stimulation of their lysosomal activity was induced by glycosides **1**, **4** and **6.** All glycosides similarly stimulate ROS formation in macrophages, but glycoside **2** caused minimal stimulation.

Keywords: triterpene glycosides, *Cucumaria okhotensis*, macrophages, ROS formation, spreading, lysosomal activity.

Triterpene glycosides of sea cucumbers possess a wide spectrum of biological activity caused by their membranolytic action [1]. During recent decades their immonomodulatory activity in subtoxic doses has attracted attention. Monosulfated glycosides from the sea cucumbers *Cucumaria japonica* [2–5] and *C. frondosa* [6] stimulate mammalian cell immunity while their activity is effective in doses significantly less than the cytotoxic ones against immune cells [7].

Recently it was found that populations of *C. japonica* in the northern parts of the Sea of Japan and Sea of Okhotsk, including the shallow waters of the Kamchatka Peninsula and North Kuril Islands, really are a series of endemic species that differ from *C. japonica* by several features, including composition of triterpene glycosides [8,9]. Because monosulfated glycosides from *C. japonica* are used for the preparation of immunostimulants [5], it is of interest to investigate the immunostimulatory activity of the glycosides isolated recently from the newly described *C. okhotensis* Levin et Stepanov, found near the south-western shore of Kamchatka Peninsula [10].

The structures of the studied monosulfated glycosides from *C. okhotensis* are presented in Figure 1. Glycosides **1** and **4** were also isolated from *C. frondosa* [11,12], and glycoside **6** from *C. conicospermium* [13]. Because the immunomodulatory action of frondoside A (**4**) has been studied previously [6], we used it as a positive control. To study immunomodulatory activity we chose spreading and lysosomal activity of mouse macrophages, and the formation of reactive oxygen species (ROS) in them.

Macrophage adhesion onto an extracellular matrix, followed by their spreading, is necessary to follow their functional activity. These indexes reflect the initial stage of phagocytosis and macrophage ability for phagocytosis. It is accompanied by changes in macrophage geometric parameters, including cell area and perimeter. The cell may elongate and change shape because of the formation of lamellae and fillopodia [14,15].

All the studied glycosides induced an increase of all these indexes on the fourth day after intraperitoneal injection. Macrophages of mice administrated with frondoside A (**4**) had larger geometrical parameters and their shape was more differentiated because of an increase in spreading, elongation, and formation of lamellae and fillopodia compared with macrophages of mice from the control group.

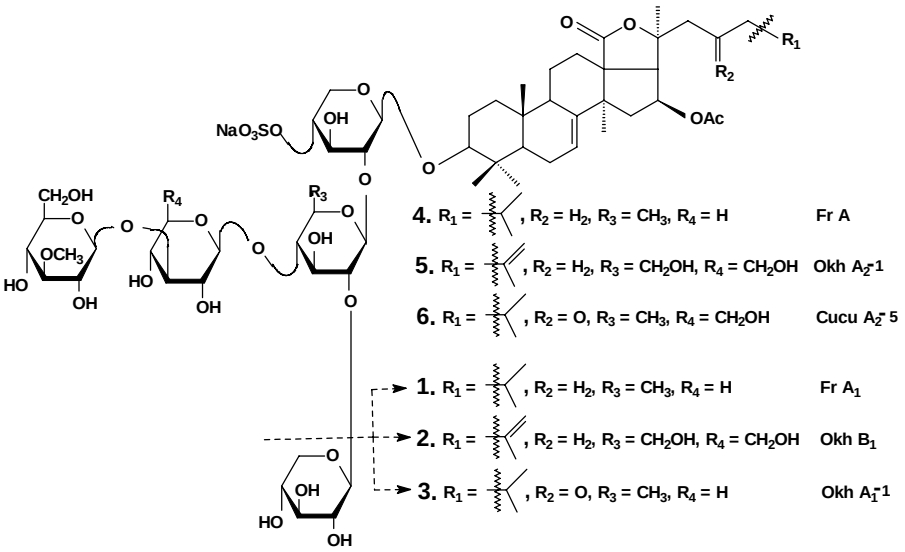

Figure 1: Chemical structures of monosulfated triterpene glycosides from *Cucumaria okhotensis*: 1 – frondoside A_1 [9,11]; 2 – okhotoside B_1 [9]; 3 – okhotoside A_1-1 [8]; 4 – frondoside A [9,12]; 5 – okhotoside A_2-1 [8]; 6 – cucumarioside A_2-5 [9,13].

Table 1: Effect of triterpene glycosides on peritoneal macrophage spreading. The glycosides were administrated by intraperitoneal injection of 0.5 mL at a dose of 0.2 μg/mouse. On the fourth day the mice were killed by pervisceral dislocation followed by isolation of macrophages that were then stained with the fluorescent probe 5-carboxyfluorescein diacetate and analyzed. The data are presented as m ± se (n=100); *– $p < 0.05$.

Substance	Geometric parameters of macrophages, pixels			
	Cell area	Perimeter	Maximal chord	Shape
Control	92.163 ± 13.111	80.698 ± 9.484	11.050 ± 1.094	0.189 ± 0.016
Fr A_1 (**1**)	239.315 ± 25.444*	104.849 ±7.578*	21.284 ± 1.465*	0.245 ± 0.012*
Okh B_1 (**2**)	107.989 ± 15.989	120.333 ± 8.621*	14.774 ±0.863*	0.075 ± 0.003*
Okh A_1-1 (**3**)	154.146 ± 18.282*	142.343 ± 12.40*	19.214 ± 1.452*	0.106 ± 0.007*
Fr A (**4**)	250.125 ± 33.284*	128.659 ± 12.240*	22.472 ± 1.611*	0.210 ± 0.011*
Okh A_2-1 (**5**)	157.138 ± 22.937*	88.400 ± 8.956	17.493 ± 1.064*	0.285 ± 0.011*
Cucu A_2-5 (**6**)	229.608 ± 39.828*	125.411 ± 16.941*	22.592 ± 2.453*	0.206 ± 0.014

Almost all the studied glycosides induced statistically significant increases in cell area, perimeter, maximal chord and integral index of cell shape at an injection dose of 0.2 μg/mouse. Quantitative estimation of the influence of the glycosides on macrophage spreading is presented in Table 1. The most effective glycosides were frondoside A_1 (**1**), frondoside A (**4**) and cucumarioside A_2-5 (**6**). These substances activate spreading 2–2.5 fold greater than the control. Frondoside A was more active than frondoside A_1 and cucumarioside A_2-5.

Lysomal activity is one of the important markers of physiological and biochemical macrophage status. Almost all the studied glycosides induced a statistically significant increase in lysosomal activity of peritoneal macrophages on the fourth day after a single intraperitoneal injection of 0.2 μg/mouse. The most effective glycosides were frondoside A_1 (**1**), frondoside A (**4**) and cucumarioside A_2-5 (**6**). These compounds activated lysosomal activity approximately1.5–2.5 times greater than that of control cells (Figure 2). The most active glycoside was frondoside A, whereas the activity of glycoside **6** was similar to that of glycoside **1**.

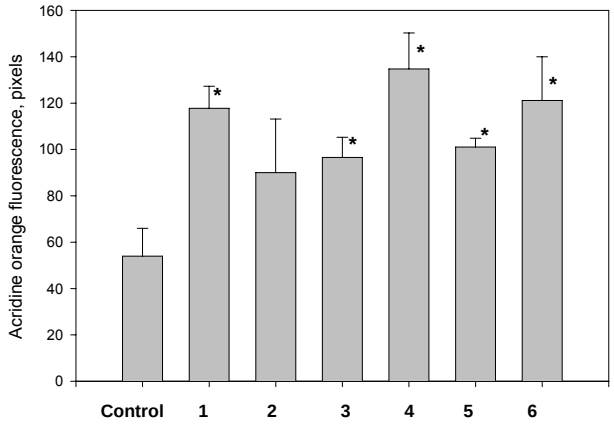

Figure 2: Influence of triterpene glycosides on lysosomal activity of mouse peritoneal macrophages. Glycosides were administrated by intraperitoneal injection of 0.5 mL at a dose 0.2 μg/mouse. On the fourth day the mice were killed by pervisceral dislocation followed by isolation of macrophages. The lysosomes were stained with acridine orange and analyzed. The data are presented as m ± se (n=100); *– $p < 0.05$.

The ability to generate ROS, singlet oxygen, hydrogen peroxide and the products of their interaction with each other, and NO, the so called "oxidative burst", is

important for the ability of macrophages to kill ingested microorganisms.

Almost all the studied glycosides induced statistically significant increases of ROS formation in peritoneal macrophages isolated from mice on the fourth day after a single intraperitoneal injection at a dose of 0.2 μg/mouse (Figure 3). All the glycosides activated lysosomal activity 1.3–1.8 folds greater than control cells. Okhotoside B_1 (**2**) showed minimal activity.

Hence all the studied monosulfated glycosides isolated from *Cucumaria okhotensis* showed immunostimulatory activity of mouse macrophages, as indicated by an increase in macrophage spreading, their lysosomal activity and ROS-formation. This indicates that *C. okhotensis* can be used as a source for immunostimulant preparations.

The activity of the studied glycosides was changed depending on their structure, but not significantly. Glycosides **4** and **5** having a fifth terminal monosaccharide unit (xylose) were more active than their corresponding tetrasaccharide analogs **1** and **2**.

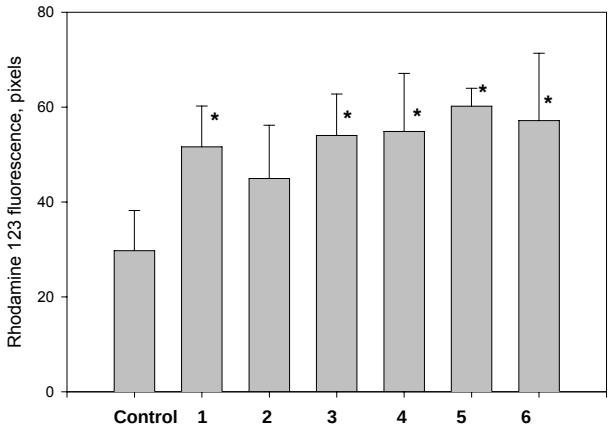

Figure 3: Influence of triterpene glycosides on the ROS-formation in peritoneal mouse macrophages. The glycosides were administrated by intraperitoneal injection of 0.5 mL at a dose of 0.2 μg/mouse. On the fourth day the mice were killed by pervisceral dislocation followed by isolation of macrophages. The cells were stained with dihydrorhodamine 123 probe and analyzed. The data are presented as m ± se (n=100); *– $p < 0.05$.

Experimental

Triterpene glycosides: The glycosides were isolated from *Cucumaria okhotensis* using common procedures of hydrophobic chromatography of the extracts on teflon powder Polichrom-1, followed by chromatography on a Si gel column and HPLC [8,9]. Structures and purity of individual isolated frondoside A_1 (**1**), okhotoside B_1 (**2**), okhotoside A_1-1 (**3**), frondoside A (**4**), okhotoside A_2-1 (**5**) and cucumarioside A_2-5 (**6**) were confirmed by ^{13}C NMR spectra [8,9]. Individual substances were dissolved in sterile distilled water at a concentration of 1 mg/mL (initial concentration). Forty μL initial glycoside solution were added to 960 μL of distilled water to produce a solution of 40 μg/mL. Ten μL of this solution was added to 990 μL of distilled water to obtain the final concentration of 0.4 μg /mL. The final concentration was used for injection into mice.

Determination of immunomodulatory activity: CBA mice (female, 20 g) were used for *in vivo* testing. The glycosides were intraperitoneally injected (0.5 mL of a glycoside solution in distilled water) at a final glycoside dose of 0.2 μg/mouse. The control mice were injected with distilled water. On the fourth day after injection the mice were killed by pervisceral dislocation and peritoneal macrophages were isolated by standard procedures. The estimation of immunomodulatory activity was carried out by staining and localization of lysosome in macrohages, determination of reactive oxygen species (ROS) formation in macrophages, and determination of macrophage spreading on an extracellular matrix. Molecular fluorescent probes followed by cell imaging analysis were used. Mice peritoneal liquor (250 μL) containing macrophages was placed onto a microscope cover slip and incubated for 1 h at 37°C. After macrophage adhesion, the cover slip was washed twice with phosphate buffered saline (PBS, pH 7.5). Then 250 μL of a solution of the fluorescent probe was added to the cell monolayer in drops and the cells were incubated at 37°C. To determine macrophage lysosomal activity, a solution of acridine orange (Calbiochem, 100 μg/mL in PBS) was applied and the cells were incubated for 30 min. To estimate ROS formation, a solution of dihydrorhodamine 123 (Sigma, 100 ηg/mL in PBS containing 0.5 mM of sodium azide) was used. The cells were incubated for 10 min. To estimate macrophage spreading, a solution of 5-carboxyfluorescein diacetate (Molecular Probes, 50 μg/mL in PBS) was applied. The cells were incubated for 60 min. The cell monolayer was washed twice with PBS. Cover slips were placed on the stage of a fluorescent scanning device composed on the base of an inverted microscope Axiovert 200 (Zeiss, Germany). A luminiscent 75 W Optosource xenon arc lamp and a DAC-controlled monochromator Optoscan (Cairn Research Ltd., UK) were used as light sources for inducing fluorescence at λex = 488 ηm; a HQ FITC filter-block (Chroma Technology Corp., USA) and Fluar 40×/1.3 oil objective lens (Zeiss, Germany) were used to visualize cell fluorescence. Cell fluorescence images were recorded using a Hamamatsu Orca-ER C4742-95 digital monochrome video camera (Hamamatsu Photonics K.K., Japan) and an IBM-compatible computer having a Fireware data interface.

The intensity of fluorescence of 100 randomly chosen cell images was estimated using an AQM Advance 6 computer program (Kinetic Imaging Ltd., UK) and calculated along with average intensity of fluorescence for each cell, in pixels. Geometric cell parameters (area, perimeter, maximal chord and shape) were also estimated. Each experiment was repeated in triplicate. Average value, standard error, standard deviation and p-values were calculated and plotted on the chart using a SigmaPlot 3.02 computer program (Jandel Scientific, USA).

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