International Immunopharmacology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Interaction of holothurian triterpene glycoside with biomembranes of mouse immune cells

E.A. Pislyagin [a,*], R.V. Gladkikh [a], I.I. Kapustina [a], N.Yu. Kim [a], V.P. Shevchenko [b], I.Yu. Nagaev [b], S.A. Avilov [a], D.L. Aminin [a]

[a] G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far East Division of the Russian Academy of Sciences, Vladivostok, 690022, Russia
[b] Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, 123182, Russia

ARTICLE INFO

Article history:
Received 20 March 2012
Received in revised form 21 May 2012
Accepted 26 May 2012
Available online xxxx

Keywords:
Holothurians
Triterpene glycosides
Biomembranes
Immune cells

ABSTRACT

The *in vitro* interactions between triterpene glycoside, cucumarioside A_2-2, isolated from the Far-Eastern holothurian *Cucumaria japonica*, and mouse splenocyte and peritoneal macrophage biomembranes were studied. Multiple experimental approaches were employed, including determination of biomembrane microviscosity, membrane potential and Ca^{2+} signaling, and radioligand binding assays. Cucumarioside A_2-2 exhibited strong cytotoxic effect in the micromolar range of concentrations and showed pronounced immunomodulatory activity in the nanomolar concentration range. It was established that the cucumarioside A_2-2 effectively interacted with immune cells and increased the cellular biomembrane microviscosity. This interaction led to a dose-dependent reversible shift in cellular membrane potential and temporary biomembrane depolarization; and an increase in $[Ca^{2+}]_i$ in the cytoplasm. It is suggested that there are at least two binding sites for [^{3}H]-cucumarioside A_2-2 on cellular membranes corresponding to different biomembrane components: a low affinity site match to membrane cholesterol that is responsible for the cytotoxic properties, and a high affinity site corresponding to a hypothetical receptor that is responsible for immunostimulation.

1. Introduction

The triterpene glycosides are composed of a carbohydrate chain and triterpene aglycone and are widely distributed in sea cucumbers or holothurians (*Holothurioidea*, *Echinodermata*) and some sponges. Most aglycones have 18(20)-lactones and belong to the holostan type. Carbohydrate chains of sea cucumber glycosides have two to six monosaccharide residues including xylose, quinovose, glucose and 3-O-methylglucose and sometimes 3-O-methylxylose, 3-O-methylquinovose, 3-O-methylglucuronic acid and 6-O-acetylglucose. They may contain one, two or three sulfate groups [1–4].

At the milli- and micromolar concentrations sea cucumber glycosides show hemolytic, cytotoxic, antifungal and other biological activities caused by membranotropic action. The basis of membranotropic action is the ability to attach to cell biomembranes and form nonselective ion-conducting complexes with 5(6)-nonsaturated sterol components of cell membranes, preferably with cholesterol. Such sterol/saponin interactions result in an efflux of some ions, nucleotides and peptides, disrupting ion homeostasis and osmolarity followed by lysis and cell death [5–7]. The cytotoxic activity of sea cucumber glycosides against various cells and cell lines including human tumor cell lines has been studied for many years. These studies have shown that the triterpene glycosides may inhibit growth of pathogenic fungal microflora, block egg cleavage and development of sea urchin embryos, and suppress the proliferation of different types of human tumor cells [8–10]. Recently it was reported that some of the sea cucumber glycosides exhibited a remarkable pro-apoptotic effect *in vitro* and a pronounced antitumor activity against different forms of experimental tumors *in vivo* [11–14].

Some sea cucumber glycosides show an immunostimulatory effect at sub-toxic nanomolar concentrations. Incubation of immune cells with the glycosides induces their activation resulting in an increase in immune cell adhesion on an extracellular matrix, enhanced cell spreading and motility, increased macrophage lysosomal activity, ROS formation and phagocytic activity [15–18]. Injection of sub-toxic doses of some glycosides induces an increase in the number of antibody-producing plaque-forming cells in mouse spleens, an increase in the number, size and acidity of lysosomes of peritoneal macrophages, and increased phagocytic index, resulting in a significant overall increase in the resistance of animals against bacterial infections [15–20]. Proteomic methods have demonstrated that the mechanism of immunomodulatory action of cucumarioside A_2-2 from *Cucumaria japonica* and frondoside A from *C. frondosa* on mouse splenocytes includes regulation of the expression of some proteins participating in formation of the cellular immune response. These glycosides similarly regulate the expression of such proteins

* Corresponding author at: G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far East Division of the Russian Academy of Sciences, Prospect 100 let Vladivostoky, 159, Vladivostok 690022, Russia. Tel.: +7 4232 31 99 32; fax: +7 4232 31 40 50.
E-mail address: pislyagin@hotmail.com (EA. Pislyagin).

1567-5769/$ – see front matter © 2012 Published by Elsevier B.V.
doi:10.1016/j.intimp.2012.05.020

as NSFL1, cofactor p47 and hnRNP K (down-regulated), as well as Septin-2, NADH dehydrogenase [ubiquinone] iron–sulfur protein 3, and GRB2-related adaptor protein 2 (up-regulated), which are involved in the processes of lysosome maturation, activation and merging, phagocytosis, cytoskeletal reorganization, cell adhesion, mobility and proliferation of immune cells [21]. A new immunomodulatory compound Cumaside has been created based on the triterpene glycosides isolated from Far Eastern sea cucumber *C. japonica*, that is a complex of monosulfated glycosides (mainly cucumarioside A_2-2) with cholesterol in an approximate molar ratio of 1:2 [22]. Cumaside at nanomolar concentrations has no hemolytic activity and moderately stimulates lysosomal activity of lymphocytes, ROS formation in human neutrophyles, induces production of some cytokines (IL-6, INF-γ, TNF-α), restores the level of some CD-markers of human lymphocytes (CD3, CD4 and CD8) after preincubation of the cells with hydrocortisone, increases phagocytosis and bactericidal activity of human blood leucocytes and induces a significant increase in mouse resistance to lethal doses of some pathogenic microorganisms such as *Staphylococcus aureus* and *Yersinia pseudotuberculosis* [23]. However, the molecular mechanism(s) of triterpene glycoside immunomodulatory action and the biomembrane targets of the glycoside for interaction with immune cells remain unclear.

This article reports an investigation of the triterpene glycoside cucumarioside A_2-2, the main glycoside isolated from the Far-Eastern edible holothurian *Cucumaria japonica*. It describes a study of membranotropic activity of cucumarioside A_2-2 at sub-toxic concentrations *in vitro* and presents the experimental results of the glycoside interactions with mouse splenocyte and peritoneal macrophage membranes using various approaches including biomembrane microviscosity, membrane potential and Ca^{2+} signaling study, and radioligand binding assay.

2. Materials and methods

2.1. Triterpene glycoside isolation

Triterpene glycoside cucumarioside A_2-2 or 3β-O-{[3-O-methyl-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)]-[β-D-xylopyranosyl-(1→2)]-β-D-quinovopyranosyl-(1→2)-4-O-sodium sulfate-β-D-xylopyranosyl}-holosta-7,25-diene-16-one was isolated from an ethanol extract of Far-Eastern holothurian *Cucumaria japonica* using hydrophobic chromatography on polytetrafluoroethylene powder Polychrom-1 (Biolar, Latvia) followed by chromatography on a Si gel column and HPLC as described previously [24]. The purity of the compound was checked by ^{13}C NMR and compared with published data. The chemical structure of cucumarioside A_2-2 is presented in Fig. 1.

Aqueous solutions of cucumarioside A_2-2 at different concentrations were used in all experiments. The solution having no cucumarioside A_2-2 was applied as a negative control.

2.2. Animals

Female BALB/c mice weighing 18–20 g were purchased from the nursery RAMS «Stolbovaya» (Russia), and kept at an animal facility under standard conditions. All experiments were conducted in compliance with all rules and international recommendations of the European Convention for the Protection of Vertebrate Animals used for experimental studies.

2.3. Splenocyte and peritoneal macrophage isolation

Mice were sacrificed by cervical dislocation. Spleens were surgically removed within 10 min after death. Cells were isolated from the spleen using a cell strainer and transferred to 15 ml plastic tubes, washed three times in 10 ml PBS buffer and centrifuged for 5 min at 1500 rpm. Cells were resuspended in RPMI-1640 cell culture medium with 2 mM glutamine and counted with a hemocytometer to adjust the concentration to $1–2 \times 10^6$ cells/ml.

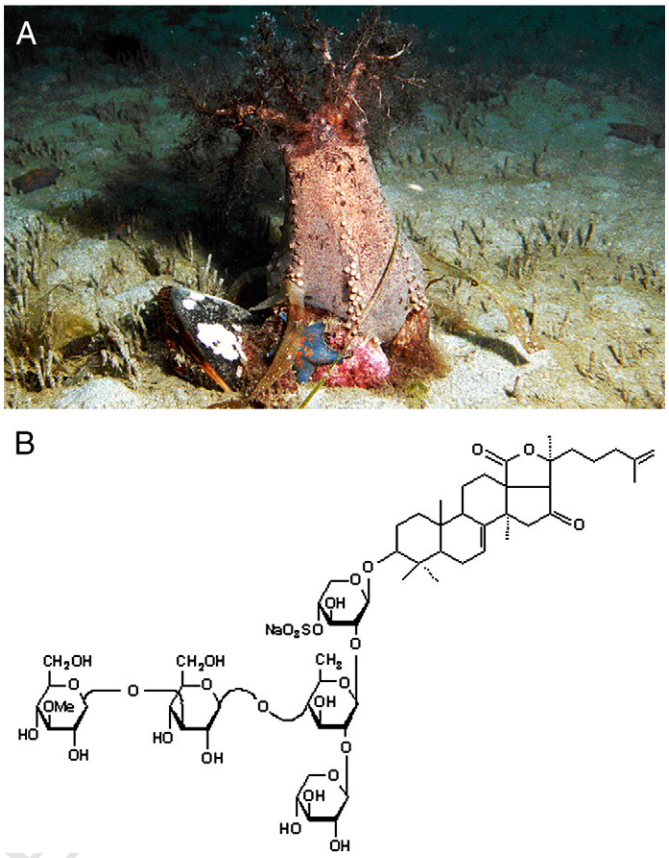

Fig. 1. Far-Eastern holothurian *Cucumaria japonica* (A) and chemical structure of cucumarioside A_2-2 (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Peritoneal macrophages were isolated using standard procedures. For this purpose 1 ml of PBS (pH 7.4) was immediately injected into the peritoneal cavity and the body intensively palpated for 1–2 min. Then the peritoneal fluid was aspirated with a syringe and transferred to plastic Petri dishes. Petri dishes with the fluid were incubated at 37 °C for 1 h to facilitate attachment of peritoneal macrophages to the dish. Then a cell monolayer was triply flushed with PBS (pH 7.4) for deleting attendant lymphocytes, fibroblasts and erythrocytes. Further macrophages were removed from the surface of the dishes with a scraper and flow of a saline solution, and placed on an ice bath until use. The working concentration of cells was usually 2×10^6 cells/ml.

2.4. Cell viability assay

BALB/c mouse peritoneal macrophage suspension was placed in 24-well microplates and incubated at 37 °C for 1 hr for cell adhesion, after which the medium in each well was replaced with medium containing different concentrations of glycoside. The plates were shaken and incubated at 37 °C for an additional hour. Cell viability was determined with trypan blue (Flow, USA) staining followed by microscopy. The number of trypan blue stained cells (dead) and unstained cells (living) were counted using the computer program AxioVision 2.0 (Zeiss, Germany), and the percentage of dead cells was determined.

2.5. Membrane microviscosity assay

To investigate membrane fluidity, we used the optical method described by Galla and Sackmann [25]. The method is based on the

Please cite this article as: Pislyagin EA, et al, Interaction of holothurian triterpene glycoside with biomembranes of mouse immune cells, Int Immunopharmacol (2012), doi:10.1016/j.intimp.2012.05.020