Specific Antitumor Effect of the Combined Action of Algal Heteropolysaccharide and Electroporation

Lyudmila Kabaivanova[1*], Juliana Ivanova[2], Viktoria Pehlivanova[3], Biliana Nikolova[3]

[1]*Department of Applied Microbiology*
The "Stephan Angeloff" Institute of Microbiology, Bulgarian Academy of Sciences
26 Acad. G. Bonchev Str., Sofia 1113, Bulgaria
E-mail: lkabaivanova@yahoo.com

[2]*Department of Experimental Algology*
Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences
21 Acad. G. Bonchev Str., Sofia 1113, Bulgaria
E-mail: juivanova@yahoo.com

[3]*Department of Electroinduced and Adhesive properties*
Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences
21 Acad. G. Bonchev Str., Sofia 1113, Bulgaria
E-mails: vik_84peh@abv.bg, nikolova@bio21.bas.bg

[*]*Corresponding author*

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Abstract: *Marine organisms are potentially prolific sources of highly bioactive secondary metabolites that might represent useful leads in the development of new pharmaceutical agents. In this study the biological effect of the freeze-dried heteropolysaccharide, isolated and purified from the red microalga Rhodella reticulata strain using electroporation was evaluated. Two different types of cells – tumor and non-tumor were treated with the heteropolysaccharide alone or together with the application of electroporation. The effect of the treatment was evaluated in parallel: with proliferation test for estimating cell viability and with immunofluorescent cytoskeleton staining to establish changes in morphology. Evidence for cell line specific viability reduction (70% from the control in case of cancer cell line treatment and only 30% in non-tumor cells) in a dose dependent manner was presented. These findings will arouse further interest in heteropolysaccharide as a new anticancer drug suitable for clinical trials.*

Keywords: *Antitumor effect, Rhodella reticulata heteropolysaccharide, Electroporation, Immunostaining.*

Introduction

Marine resources have attracted worldwide attention in many research fields [8-10]. The focus is in the search for bioactive substances for development of new drugs, because of their relatively low toxicity and high bioactivity [24]. Cancer is one of the most lethal diseases that threaten human life. Therefore, it is more than important to find novel, effective and nontoxic compounds from natural sources to be used together with the generally used chemotherapy. The great potential of marine microalgal polysaccharides to be used as anti-inflammatory, antiallergic, analgetic, antitumor agents was recently reviewed [6, 19, 21]. Red algal heteropolysaccharides (hPSHs) have attracted much attention in the biochemical and medical areas because of their anti-cancer effects. The mechanism of the anti-cancer activity is related to the ability of hPSHs to inhibit the growth of cancer cells (cytotoxic or cytostatic effect), to stimulate the immune response, to inhibit tumor angiogenesis and to induce apoptosis

[13, 25]. There are certain compounds isolated from marine *Cyanobacterium* that can be used for a new type of anticancer drugs that act as antimitotic agents (IC50 values in three cell line ranging from 7 to 200 nM) that inhibits microtubule assembly and binding of cholchicine to tubulin [7]. The composition and heterogeneity of the cell-wall polysaccharide of the red alga *Rhodella reticulata* was found to contain xylose, 3-O-methyl pentose, glucose, galactose, rhamnose, ribose, arabinose, 4-O-methylgalactose, mannose and glucuronic acid in the approximate proportions of 12.4:6.7:1.0:2.2:4:0.06:0.4:2.4:0.4:1.6, and traces of 2,4-dimethyl hexose [5], together with 9% sulphates and 3% protein. Glucuronic acid and semi-esterified sulphate groups that determine the acidic properties of the polymer can also be found.

Electroporation is a physical method that involves the application of high voltage electrical pulses to cells or tissue causing temporary permeabilization of the plasma membrane. This technique facilitates cellular gene and drug delivery for agents that have no or limited transmembrane transport [14-16]. Electroporation is used in cell and molecular biology, biotechnology and recently in medicine to potentiate the cytotoxic effect of anticancer drugs (electrochemotherapy). The electrochemotherapy has a high antitumor effectiveness in vitro and now provides a great promise for treatment of accessible human tumors [17, 18, 23]. Its impact is due to the increased uptake and accumulation of chemotherapeutic drugs into cancer cells. However the electric field effects are still under active investigations at the cellular level. The cytoskeleton provides the essential functions of viable cells, as shape maintaining, cell-matrix and cell-cell interactions. The application of external electrical pulses can alter the cytoskeleton reorganization, thus affecting the cell adhesion. For instance, changes in the cytoskeletal structure have been observed during electroporation [11, 26] as well as electro transfer [20]. There are three major types of cytoskeletal structures: microtubules, actin filaments and intermediate filaments.

The aim of this study was to estimate the antitumor effect of red algal hPSH (in different concentrations) applied alone or in combination with electroporation on the cell viability and cytoskeleton integrity of tumor and non-tumor cell lines.

Materials and methods

Strain

The culture of the red alga *Rhodella reticulata* (Rhodophyta, strain UTEX LB2320 from the algal collection of the Department of Botany, University of Austin, Texas, USA) was used in the experiments. *Rhodella reticulata* was intensively cultivated in special glass vessels (Fig. 1) for 96 h in Brody Emerson medium at light intensity of 260 μE/m^{2}s and temperature 27 °C. The cell suspension was aerated with 1% CO_2.

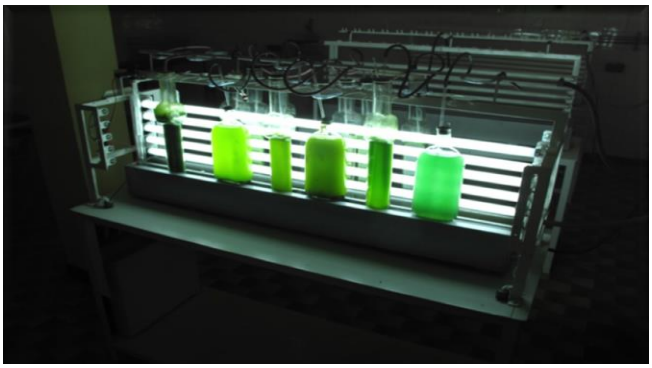

Fig. 1 Installation for microalgae intensive cultivation

Polysaccharide

After the intensive cultivation of the algae, the suspension was subjected to centrifugation for 30 min at 6000 g. Cells were removed and the supernatant was precipitated with ethyl alcohol in a ratio of 1:2. After this operation, the precipitated hPSH was collected. Dissolving in distilled water on a magnetic stirrer was the next step. After that, dialysis was performed for 24 h at 4 °C, followed by freeze drying of the hPSH. Different concentrations (10 μg/ml, 50 μg/ml or 100 μg/ml) of it were tested.

Cell lines

A549 human lung adenocarcinoma cells were grown in RPMI 1640 medium (Lonza) supplied with penicillin-streptomycin in standard concentrations and 10% fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY). MDCK non-tumor epithelial cells were grown in complete Eagle's minimal essential medium with Earle's salts (MEM), penicillin-streptomycin at standard concentrations and 10% FCS (Gibco Laboratories, Grand Island, NY). The cell cultures were maintained in a 37 °C incubator with humidified atmosphere and 5% CO_2. Cultures were routinely passage at confluence using 0.25% trypsin/2mM EDTA (Lonza) for cell detachment.

Electrotreatment protocol

For electrotreatment a new electroporator (Chemopulse IV developed in the Institute of Biophysics and Biomedical Engineering), which generates bipolar pulses was used. The electrotreatment was done by 16 biphasic pulses [4]. In each experiment, electrodes with an inter-electrode distance of 1 cm were used. The intensity of applied electric fields was 200 V/cm. One hundred μl with 1.5×10^5 cells were seeded 24 h prior to electroporation. Immediately before pulse delivery hPSH at different concentrations was added. After the electrical treatment, 900 μl MEM, supplemented with 10% FCS, was added to each sample. The controls were treated under the same conditions but without electrical pulse application and/or hPSH. For immunofluorescence staining experiments, cells were cultivated on cover glasses.

MTS test for cell survival and proliferation

To analyze the cytotoxic effect of the hPSH on A549 cell line and non-cancer MDCK cells, the MTS test (Promega) was performed for cell survival and proliferation as previously described [3]. Briefly, the adherent cells were treated as described above and incubated additionally for 24 h. Then, 50 μl of MTS reagent was added directly to the adherent cells. They were incubated for 2 h at 37 °C and the absorbance at 490 nm was recorded with a 96-well plate reader Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg). Three independent experiments were performed for each treatment.

Actin staining

The cell lines at density of 1.5×10^5 cells/ml were cultivated on cover glasses (18/18 mm) that had been placed in 6 well plates. After 24-hour incubation, the cells were treated with electroporation alone or in combination with different concentrations of hPSH (10 μg/ml, 50 μg/ml or 100 μg/ml) in a basal cell medium and were cultivated for an additional 24 hours in full cell medium. After the incubation period, non-adherent cells were removed by rinsing three times with phosphate-buffered saline (PBS), pH 7.4. The adherent cells were fixed with 1 ml 3% solution of paraformaldehyde (PFA) for 15 minutes at room temperature. The fixed cells were permeabilized using 1 ml 0.5% solution of Triton X-100 for 5 minutes and then incubated with 1 ml 1% solution of bovine serum albumin (BSA) for 15 minutes. The samples

were washed three times with PBS, pH 7.4 and then incubated for 30 minutes at room temperature with BODIPY 558/568 phalloidin. Again, the samples were washed three times with PBS and once with distilled water. Then they were installed on objective glasses by Mowiol. Preparations were analyzed using an inverted fluorescent microscope (Leica DMI3000 B, Leica Microsystems GmbH, Germany) with object HCX PL FLUOTAR 63x/1.25 oil objective.

Results and discussion

In our study the influence of the algal hPSH on the cell viability and reorganization of the actin cytoskeleton of two different types of cell lines was compared: malignant cell line A549 and MDCK – normal cell line. Both lines were from one and the same cytological type – epithelial.

MTS assay

The effect of studied hPSH on the cell line viability was assessed by measuring the number of viable cells using MTS test. 24 h after treatment viability of cells (A549) was reduced (Fig. 2). The investigations of A549 cell line proved that viability was reduced in a dose dependent manner. By increasing of the concentration of the hPSH, statistically significant reduction was obtained. The results showed that even the lowest tested concentration (10 µg/ml) could induce reduction of cell viability of 34% without electroporation and 63% with electroporation. Applying electroporation the percent of viable cells additionally decreased. The highest concentration (100 µg/ml) led to about 70% viability decrease. Additional insignificant decrease of about 2% was measured after the combined treatment with electroporation (200 V/cm).

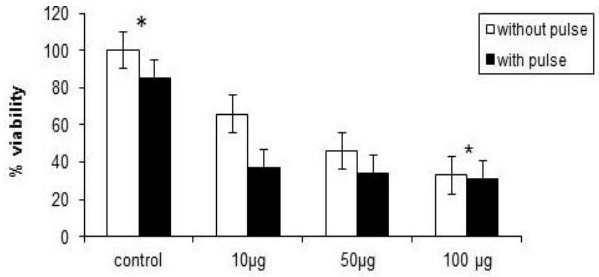

Fig. 2 A549 cell line viability after treatment with hPSH alone (white bars) and in combination with 200 V/cm (black bars), bars – SD (Standart Deviation); *$p < 0.05$.

Importantly, the effect of the hPSH on the viability of normal cells MCDK was significantly lower (Fig. 3).

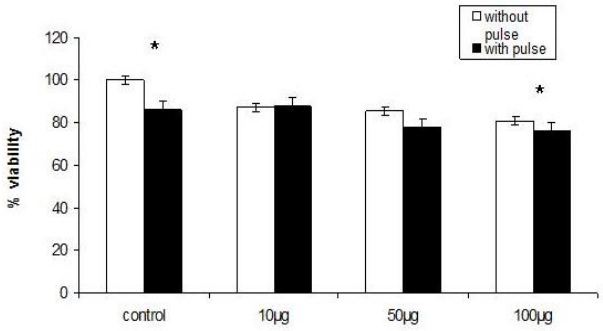

Fig. 3 MCDK cell line viability after treatment with hPSH alone (white bars) and in combination with 200 V/cm (black bars), bars – SD (Standart Deviation); *$p < 0.05$.

Even treatment with concentration of 100 μg/ml resulted in slight inhibition of cell growth by only 20%. Additional 10% reduction was registered at the combined treatment with 200 V/cm electrical pulses and 100 μg/ml hPSH.

Immunofluorescence of actin following solo or combined treatment with hPSH and electrical pulses

The role of the actin microfilaments in maintaining cell viability and functions is well known. In order to determine whether treatment with hPSH and/or electroporation at the above conditions is related with actin reorganization, the two cell lines were stained with phalloidin. In untreated cells intact actin filaments with a lot of stress fibers and typical cell shape was observed (Fig. 4A). After application of 10 μg/ml polysaccharide, thinner stress fibers and localization of actin microfilaments near to the cell membrane were observed (Fig. 4B). By increasing concentrations of applied hPSH to 50 μg/ml and 100 μg/ml, additional reorganization of the actin filaments and losing of the cells polarization were documented (Fig. 4C and D). A good correlation between cell viability test results and immunostaining was proved.

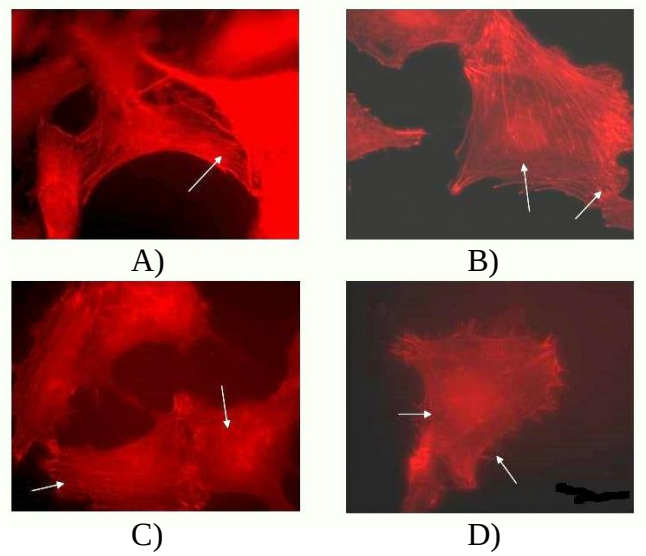

Fig. 4 A549 cell line immunofluorescence of actin:
A) control and after a single treatment with hPSH; B) application of 10 μg;
C) application of 50 μg; D) application of 100 μg.
The arrows point at actin fibers. Magnification 63x.

In response to the combined treatment with 200 V/cm and even 10 μg/ml polysaccharide, the organization of actin filaments could no longer be detected. The overall cell morphology (Fig. 5B, C, D) was altered and cell shape was rounded in contrast with a control treated with electrical pulses only.

In our study, MDCK – normal epithelial cell line was used as a control to A549 cancer epithelial cells. Treatment with 10-50 μg/ml hPSH cannot influence the organization of the actin cytoskeleton as well as the cell viability (Figs. 3 and 6). At application of 100 μg/ml of the hPSH, (Fig. 6D) a slight redistribution of actin filaments was observed. As we can see from Fig. 6A, B, C, D, the polarization and the number of cells were retained.

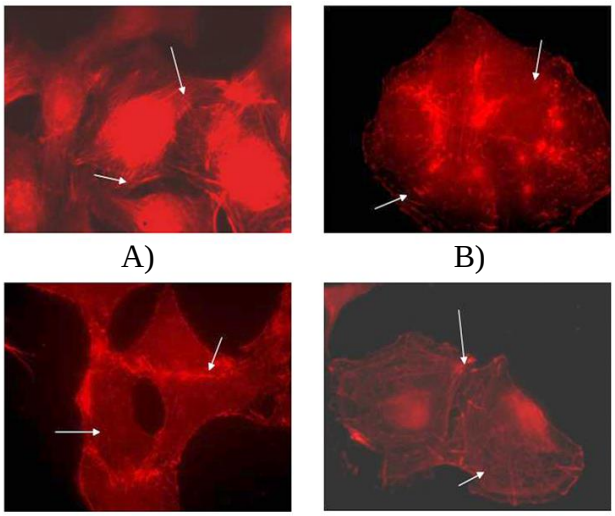

Fig. 5 A549 cell line immunofluorescence of actin:
A) control and 200 V/cm, after combined treatment; B) application of 10 μg;
C) application of 50 μg; D) application of 100 μg.
The arrows point at actin fibers. Magnification 63x.

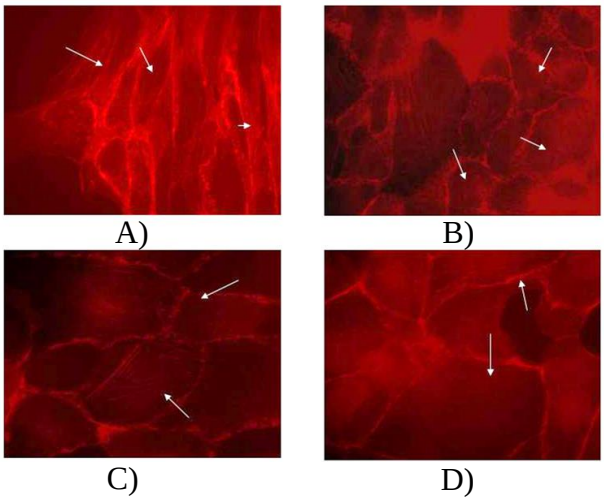

Fig. 6 MDCK cell line immunofluorescence of actin:
A) control and after a single treatment with hPSH; B) application of 10 μg;
C) application of 50 μg; D) application of 100 μg.
The arrows point at actin fibers. Magnification 63x.

Despite additional application of electrical pulses and hPSH with increased concentrations no significant difference between combined and solo treatment was observed in case of treatment of non-tumor MDCK cell line, with almost no morphological changes observed (Fig. 7).

Morphological observations were in good correlation with our functional MTS test results.

Considering the effect against cancer cells, compounds that exhibit cytotoxic activity show valuable effects in chemotherapy only when their toxicity is higher for cancer cells than for non-cancerous cells. This selectivity against specific cancer cell lines is one of the requirements for the development of new drugs in order to lower the extent of side-effects. Despite the intensive investigations in this field in recent years, the mechanism of action of the different polysaccharides from various natural origins on a cell level had not been fully

elucidated [2]. In most cases they were described as possessing an immunomodulating activity [27]. One possible way of direct anti-cancer activity, could be the induction of tumor cell apoptosis [2]. In our study two levels of assessment: physiological-cell viability and morphological-cytoskeleton organization were examined. By immunofluorescence imaging, the reorganization of actin cytoskeleton, leading to reduction in tumor cell viability was visualized. The obtained results proved the selective effect of the hPSH on A549 human lung adenocarcinoma cell lines. The results of our studies, however, indicate that the process of polysaccharide-induced tumor cell death happened after 24 h exposure in our case, which generally depends on the type of cells and type of treatment of tumor cells tested. There is evidence for other sulphated heteropolysaccharides from algae that showed antiproliferative activity in HeLa cells as well as a cytotoxic effect towards human cancer cell lines such as MT-4 [1, 12, 22].

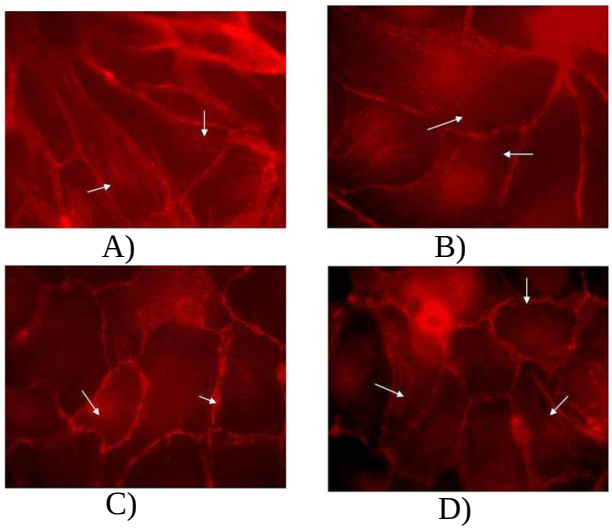

Fig. 7 MDCK cell line immunofluorescence of actin:
A) control and 200 V/cm and after combined treatment with hPSH and 200 V/cm;
B) application of 10 μg; C) application of 50 μg; D) application of 100 μg.
The arrows point at actin fibers. Magnification 63x.

From the results of this study we could conclude that the application of electroporation technique must be an important tool to reduce the applied concentration of hPSH. At a combined treatment (electroporation and hPSH), the effect of the lowest applied concentration of hPSH (10 μg/ml) was comparable to the effect of the highest concentration of polysaccharide (100 μg/ml) without electroporation. In that way optimal results could be reached with only 10 μg/ml treatment.

Conclusion

In conclusion it was evident that low hPSH concentration affected the tumor cells, while the non-tumor cells remained viable even at the highest concentration applied. This important fact could be an opportunity to use very low concentrations of the active substances in combination with electrical pulses aiming to reach the maximal specific antitumor effect on the tumor cells without any damage to the non-tumor cells. These findings will arouse further interest in algal hPSH as a new anticancer drug suitable for clinical trials.

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Assoc. Prof. Lyudmila Kabaivanova, Ph.D.
E-mail: lkabaivanova@yahoo.com

Lyudmila Kabaivanova graduated from Sofia University "St. Kliment Ohridski", Faculty of Biology, with a M.Sc. degree in Biochemistry and Microbiology. She received her Ph.D. degree in Microbiology at The "Stephan Angeloff" Institute of Microbiology, Bulgarian Academy of Sciences. She is currently an Associate Professor at the same institute.

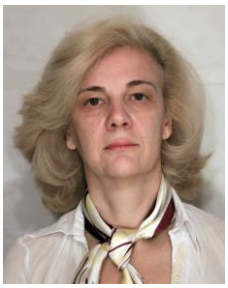
Assist. Prof. Juliana Ivanova, Ph.D.
E-mail: juivanova@yahoo.com

Juliana Ivanova graduated from Sofia University "St. Kliment Ohridski", Faculty of Biology, with a M.Sc. degree in Biotechnology. She received her Ph.D. degree in Plant Physiology at the Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences. She is currently an Assistant Professor at the same institute.

Viktoria Pehlivanova, Ph.D.
E-mail: vik_84peh@abv.bg

Viktoria Pehlivanova graduated Sofia University "St. Kliment Ohridski", Faculty of Biology, specialization Molecular Biology – B.Sc. degree in 2007 and M.Sc. degree in Biophysics in 2009. She received her Ph.D. degree in 2013 in the Institute of Biophysics and Biomedical Engineering. Her scientific topics are anticancer drugs, electroporation, fluorescent imaging, cell adhesion, nanoparticles and drug delivery.

Assoc. Prof. Biliana Nikolova, Ph.D.
E-mail: nikolova@bio21.bas.bg



Biliana Nikolova graduated Sofia University "St. Kliment Ohridski", faculty of Biology, specialization Biochemistry and Microbiology in 1992. She received her Ph.D. degree in 2001 in the Institute of Biophysics and Biomedical Engineering. Since 2013 she is an Associate Professor at the Institute of Biophysics and Biomedical Engineering. She works in the field of electroporation, electroloading, drug delivery systems, *in vivo* imaging and electrochemotherapy.