In vitro effect of molluscan hemocyanins on CAL-29 and T-24 bladder cancer cell lines

OLGA BOYANOVA[1], PAVLINA DOLASHKA[2], DRAGA TONCHEVA[1], HANS-GEORG RAMMENSEE[3] and STEFAN STEVANOVIĆ[3]

[1]Department of Medical Genetics, Medical University of Sofia, Sofia 1431;
[2]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria;
[3]Institute for Cell Biology, Department of Immunology, University of Tübingen, D-72076 Tübingen, Germany

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Abstract. The aim of this study was to investigate the antitumor effects of molluscan hemocyanins (Hcs) isolated from the marine snail *Rapana venosa* (RvH) and the garden snail *Helix lucorum* (HlH) on human bladder cancer cell lines. The antitumor effect of the native molecules of the above-mentioned Hcs and their subunits were examined in comparison to keyhole limpet hemocyanin (KLH), which is the most thoroughly studied Hc. The experiments were conducted using 2 human bladder cancer cell lines: CAL-29 and T-24. Doxorubicin hydrochloride (DOX) and mitomycin-C (MIT-C), which are routinely used in clinical practice to treat bladder cancer, were used for comparison. The viability of the 2 bladder cancer cell lines, used at a concentration of 20,000 cells/well, was measured by WST-1 assay at 24, 48 and 72 h after treatment with the above-mentioned Hcs and their isoforms at a concentration ranging from 0.8 to 500 μg/ml. A direct growth inhibitory effect on the tumor cells was observed mainly after treatment with the native molecule of HlH and the structural subunit, RvH1, at a concentration of 500 μg/ml. The native molecule of RvH exhibited an efficacy similar to that of KLH. However, the observed growth inhibitory effect of HlH was superior to that observed for KLH and RvH, when used at the same concentration. These findings demonstrate the antitumor effect of other Hcs, apart from KLH. Our data suggest that the native molecule of HlH and the subunit, RvH1, are alternative candidates for the treatment of human superficial bladder cancer.

Introduction

Cancer of the bladder is one of the most common types of cancer, while urothelial cancer is the most common histological type of transitional cell carcinoma (TCC), accounting for approximately 90% of cases and it has a poor prognosis (1).

According to the guidelines on bladder cancer, the treatment options are: surgery, radiation treatment, chemotherapy and immunotherapy, depending on the staging and histological type (2). One of the therapeutic approaches with promising results in clinical trials is the non-specific immunostimulant, keyhole limpet hemocyanin (KLH) *Megathura crenulata* (3). Hemocyanins (Hcs) are copper-containing respiratory proteins found in arthropods and mollusks (4,5). Due to their high molecular weight, structural heterogeneity and xenogeneic nature, they are known as some of the strongest antigens. The mechanism of action is immune response activation due to the presence of cross-reacting epitopes, such as the Thomson-Friederich antigen [Gal(b1-3) *N*-acetyl epitope] cross-reactive with an equivalent epitope on the bladder tumor cell surface (6) and a carbohydrate epitope on the surface of *Schistosoma mansoni* larval schistosomes (7). *In vivo* it induces a protective antibody production against this carbohydrate sequence along with a cytotoxic T-cell response. Subsequent to KLH immunization, patients generate IgG antibodies against KLH (8). Apart from KLH, the antitumor activities of other Hcs have also been observed. A previous study on the therapeutic properties of Hcs, isolated from the garden snail *Helix lucorum (*HlH) and the marine snail *Rapana venosa* (RvH), has shown their activity against Guerin ascites tumor (9).

We hypothesized that HlH and RvH may also have a therapeutic effect on bladder cancer. Therefore, the effect of these Hcs on CAL-29 and T-24 bladder cancer cell lines was examined *in vitro,* in comparison with KLH, doxorubicin hydrochloride (DOX) and mitomycin-C (MIT-C) (used for bladder cancer chemotherapy).

Materials and methods

Materials and assays. Two bladder cancer cell lines were used in this study: T-24 (TCC, grade III) and CAL-29 (grade IV, stage T2) obtained from the Interfaculty Institute for Cell Biology, Department of Immunology, University of Tübingen, Tübingen, Germany.

The antibiotics, MIT-C and DOX, KLH and Bradford reagent were purchased from Sigma-Aldrich Chemie Gmbh

Correspondence to: Professor Pavlina Dolashka, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, G. Bonchev 9, Sofia 1113, Bulgaria
E-mail: pda54@abv.bg

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(Eschenstrasse, Germany). The WST-1 cell proliferation assay kit was purchased from Roche Diagnostics Deutschland GmbH (Mannheim, Germany), while the Limulus amebocyte lisate (LAL) assay from (Lonza Verviers Sprl, Verviers, Belgium).

Cell culture. The CAL-29 and T-24 cells were cultured as a monolayer in Dulbecco's modified Eagle medium (DMEM, Lonza) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (P/S) (Gibco Invitrogen GmbH, Karlsruhe, Germany) at 37˚C in a humidified atmosphere with 5% CO_2 until 80% confluent. Cells were harvested using trypsin/EDTA (Lonza) and counted using a hemocytometer.

Test substance preparation. In the current experiments whole molecules of HlH and RvH and 2 structural subunits, RvH1 and RvHI2, were used. Hcs were isolated from the hemolymph of the garden snail HlH and the marine snail RvH as described by Dolashka *et al* (10) and Velkova *et al* (11). The 2 structural subunits, RvH1 and RvH2, were purified after dissociation of the RvH.

The tested substances were filtered using a bacterial filter with a pore size of 0,2 μm (Corning® Incorporated Life Sciences, St. Lowell, MA, USA) under sterile conditions. The concentration of the Hc solutions was determined spectrophotometricaly with Bradford reagent. KLH was used for standard curve preparation, (C=5, 1 mg/ml; Sigma-Aldrich). Optical density (OD) was read on an ELISA reader (SpectraMax 340), λ=595 nm.

In vitro cytotoxicity assay. Cell viability was determined using a standard WST-1 cell proliferation assay. Briefly, the cell lines mentioned above were seeded in 96-well plates (20,000 cells/well). Different concentrations of Hcs ranging from 0.8 to 500 μg/ml were added to the solution after 12-18 h. KLH (Sigma-Aldrich) was used as the positive control at the same concentration as Hcs, while DOX and MIT-C were used at concentrations of 10 μg/ml and 1 μM, respectively. Medium alone (cells without treatment) was used as the negative control. After incubation for 24, 48 and 72 h, 20 μl ready-to-use WST-1 reagent was added to each well and cultured for another 2 h and the cell viability was determined.

LAL assay. The working procedures were completed under sterile conditions, with pyrogen-free material and the solutions that came into contact with the cells were assayed at <200 EU/ml endotoxin using the LAL test.

Statistical analysis. The data are presented as the means with standard deviation (SD). Significance testing was performed using one-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference (shown as $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ in the figures). The experiments were performed in triplicate and at least 3 times. Most of the experiments were performed 5 times.

Results

Based on the published information regarding the antitumor effect of Hcs, the antitumor properties of 2 molluscan Hcs extracted from the Bulgarian species, RvH and HlH, were examined in comparison with KLH. The structure of HlH and RvH has been studied thoroughly and their organization was found to differ markedly from the structure of KLH. RvH, HlH and KLH are composed of several oligosaccharide residues exhibiting various tertiary constitutions. In RvH and KLH 2 structural subunits have been identified, whereas 3 isoforms have been isolated from HlH.

To date, many different protocols have been used to study the antitumor effect of potential pharmacological substances on cancer cell lines (12,13). Two main steps were applied to analyze the antitumor properties of KLH, HlH and RvH Hcs and their isoforms. The first step was to determine the appropriate (working) concentration of Hcs. RvH and HlH were then tested for their effectiveness as antitumor agents on the 2 bladder cancer cell lines. The second step may help us to determine whether Hcs and their isoforms have the potential to be used as an alternative treatment for TCC.

Determination of the appropriate concentrations of Hcs. To determine the working concentration of the Hcs (KLH, RvH and HlH and the isoforms, RvH1 and RvH2) 5 different concentrations were used: 500, 100, 20, 4 and 0.8 μg/ml. Each substance was added to the medium of the bladder cancer cell lines, CAL-29 and T-24. Cell viability was analyzed using a standard WST-1 cell proliferation assay after 24, 48 and 72 h of incubation. The obtained results were compared with the positive control KLH (500, 100, 20, 4 and 0.8 μg/ml), DOX (10 μg/ml) and MIT-C (1 μM) and the negative control cells in medium without treatment. The effect of the tested substances (HlH, RvH, RvH1 and RvH2) at 24 and 48 h was more profound on the CAL-29compared to the T-24 cell line. The greatest cytotoxic effect was observed at a concentration of 500 μg/ml of Hcs; therefore, it was chosen as a working concentration to be used in further experiments. Given the higher effect observed subsequent to the incubation of CAL-29 cells with a lower concentration of RvH (100 μg/ml), additional experiments were conducted. The effectiveness of the Hcs was compared simultaneously at the concentrations of 100 and 500 μg/ml in both cell lines in order to determine the suitable concentration. As shown in Fig. 1, Hcs at a concentration equal to 500 μg/ml exhibited a greater growth inhibitory effect. This concentration was determined as the working concentration and was used in subsequent experiments to further determine the concentration of Hcs with the best killing effect on the CAL-29 and T-24 bladder cancer cell lines.

Determination of the test Hc with the best cytotoxic effect. The direct *in vitro* effect of the tested Hcs on the CAL-29 and T-24 bladder cancer cell lines was evaluated in a number of experiments lasting for 24, 48 and 72 h. The Hc concentration used in each experiment was the determined working concentration mentioned above (500 μg/ml).

The effects of native Hc molecules of KLH, molluscan RvH and HlH and 2 structural subunits, RvH1 and RvH2, on the CAL-29 bladder cancer cell line are presented in Fig. 2. The viability measured at 24, 48 and 72 h subsequent to the incubation with the native molecule of KLH was 111.58, 98.14 and 85.23%, respectively. At the same time-points, the cell viability of CAL-29 cells measured subsequent to RvH treatment was

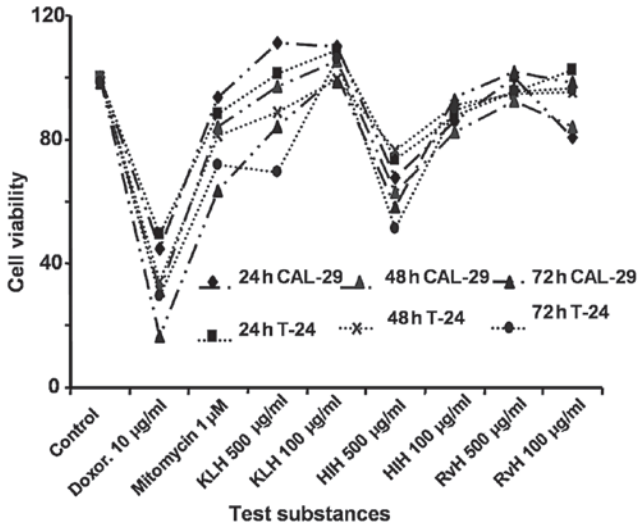

Figure 1. Cell lines viability after 24, 48 and 72 h of incubation with *Rapana venosa* (RvH), *Helix lucorum (*HlH) and keyhole limpet hemocyanin (KLH) is shown. The best growth inhibitory effect was detected at the concentration of 500 μg/ml.

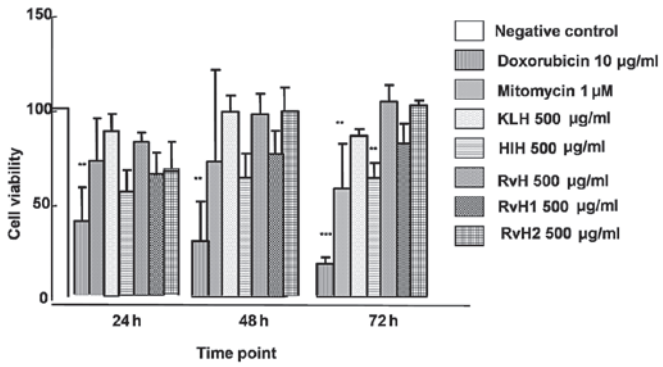

Figure 2. Effect on the human tumor cell line, CAL-29, after 24, 48 and 72 h of incubation with the native molecule of *Rapana venosa* (RvH) and *Helix lucorum (*HlH), and the structural subunits, RvH1 and RvH2, at a concentration of 500 μg/ml are shown. The negative and positive controls used were doxorubicin hydrochloride and mitomycin-C (MIT-C), and keyhole limpet hemocyanin (KLH), respectively. **P<0.01, ***P<0.001.

103.64, 96.81 and 103.23%, respectively. The lowest viability achieved with HlH was 69.99, 63.06 and 62.73%, respectively. As shown in Fig. 2 only HlH of the native Hcs molecules showed a cytotoxic effect above 30% (% cytotoxicity = 100% - % viability) after 24 h of incubation. A slight inhibitory effect was observed after 24 h of treatment of the CAL-29 cells with the subunits, RvH1 and RvH2 (18.01 and 15.53%, respectively). On the contrary, no cytotoxic effects, and stimulation were observed with the native molecule of RvH and KLH. The cell viability of the CAL-29 cell line after 72 h of incubation with the native molecule of HlH was the lowest (62.73%) and a growth inhibition of 37.3% was achieved (at 72 h).

Similar effects were observed following the treatment of the T-24 cell line with the native molecules, RvH, KLH, HlH, and 2 structural subunits, RvH1 and RvH2. As shown in Fig. 3, cell viability after incubation with the native molecule of KLH at 24, 48, and 72 h was 107.01, 77.03 and 73.22%, respectively. Cell viability measured subsequent to incubation with RvH for

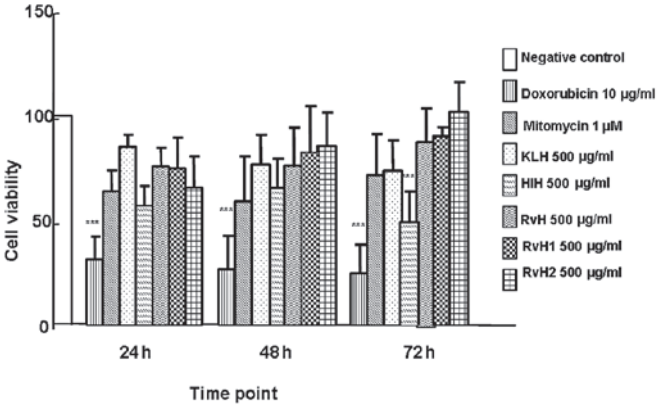

Figure 3. Effect on the human tumor cell line, T-24, after 24, 48 and 72 h of incubation with the native molecule of *Rapana venosa* (RvH) and *Helix lucorum (*HlH), and the structural subunits, RvH1 and RvH2, at a concentration of 500 μg/ml in the presence of the negative and positive controls [doxorubicin hydrochloride (DOX), mitomycin-C (MIT-C) and keyhole limpet hemocyanin (KLH)]. ***P<0.001.

the same time period was 95.28, 76.53 and 87.52%, respectively. The cell viability of the T-24 cells treated with HlH was determined at 71.70, 65.75 and 49.34% at 24, 48 and 72 h respectively. The cell viability of the T-24 cells decreased from 71.7% after 24 h of incubation to 49.34% after 72 h of HlH culturing. The highest growth inhibitory effect (cytotoxic effect) among the Hcs was 50.66%, observed at 72 h of incubation of the T-24 cells with HlH. An extremely low cytotoxic effect was detected after 24 and 48 h of incubation with RvH2 (17.61 and 13.98%). At 72 h, a stimulation with RvH2 was observed. The opposite tendency was observed subsequent to RvH1 treatment at 24, 48 and 72 h (4.72, 16.99 and 10.51% cytotoxicity, respectively).

Discussion

Hcs are high molecular weight substances, with a xenogenic nature, carbohydrate content and a complicated quaternary structure. This explains their strong immunogenicity in mammals as well as their adjuvanticity *in vivo*. Their structure, biological function and potential usage in medicine have been extensively studied. One of these Hcs is the molluscan, KLH, a highly antigenic respiratory protein (5,9). It has been used in phase II clinical trials as a drug against bladder cancer (14-20). Moreover, a growth inhibitory *in vitro* effect of KLH against multiple cancer cell lines, including estrogen-dependent (MCF-7) and -independent breast (ZR75-1), pancreatic (PANC-1, MIAPaCa), prostate (DU145) and Barrett's esophageal adenocarcinoma cancer cell lines, has also been reported (14-16).

Recently, the effect of the application of RvH and KLH on antibody-dependent cellular cytotoxicity (ADCC) and mitogen activity of spleen lymphocytes in hamsters with progressing myeloid Graffi tumors was found subsequent to tumor transplantation (18). The antitumor properties of both RvH and HlH as well as their immunological potential, combining *in vitro* and *in vivo* methods, was also analyzed (9,19). Strong activation of the immune system of tumor-bearing animals following treatment with RvH was demonstrated. Additionally, the immunostimulatant Hcs were found to induce antitumor activity.

KLH, RvH and HlH and their isoforms differ markedly in their structures. Their antitumor effects on 2 human bladder cancer cell lines, CAL-29 and T-24 were studied, in comparison with products used in clinical practice for chemotherapy, such as DOX and MIT-C.

In the present study, we demonstrate the growth inhibitory effect of Hcs *in vitro*. This effect was measured in the presence of a negative and 2 positive controls. The growth inhibitory effect at a range of concentrations between 0.8 and 500 μg/ml was shown in a series of experiments with 24, 48 and 72 h of incubation.

The growth inhibitory effect of common HlH was shown to be superior to that shown by KLH; 80% cell was observed viability following KLH incubation for 72 h, and approximately 55% cell viability was observed following incubation with HlH (for the CAL-29 and T-24 bladder cancer cell lines). The percentage cytotoxicity was calculated according to the cell the viability (% cytotoxicity = 100% - % viability). The KLH cytotoxicity in our experiments (20%) differs from the one reported by Riggs *et al* (16) (6-43%). The difference may be due to the differences in the tumor cell lines tested, the cell density per well and the method used for viability detection. Further studies are required to compare the growth inhibitory effect of structural units and functional subunits isolated from HlH, which may possibly have an even more pronounced effect. In the present study, the growth inhibitory effect of structural subunits isolated from RvH was examined. The effect of the whole molecule of RvH and the structural subunits (RvH1 and RvH2) measured 72 h after incubation was found to be similar or lower compared that of KLH.

These findings are consistent with findings from our previous studies (9,11,18) on the immuno-adjuvant properties of these Hcs, their derivatives and conjugates. Those studies investigated the cell-mediated immunity in experimental tumor-bearing animals with Guerin and Graffi ascites tumor (19,20). It was suggested that Hcs carbohydrate moieties are associated with the antitumor potential of different Hcs. High-mannose type glycans, as observed in most molluscan Hcs, were also identified in Hcs from RvH, HlH and KLH.

In the present study on molluscan Hcs isolated from the marine snail RvH and the garden snail HlH and the CAL-29 and T-24 bladder cancer cell lines, a growth inhibitory effect of the native molecule of HlH Hc and RvH1 subunit *in vitro* was demonstrated. Moreover, HlH was much more effective compared to KLH and RvH on the human bladder cancer cell lines.

In conclusion, of the Hcs tested only HlH showed a higher efficacy compared to KLH investigated in clinical studies as a potential therapy for bladder cancer. Therefore HlH may be considered for further investigation, as an intravesical therapy for bladder cancer.

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