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Cytotoxic Effects of *Rapana venosa* Hemocyanin on Bladder Cancer Permanent Cell Lines

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Abstract: The cytotoxic activities of hemocyanin from *Rapana venosa* and its structural subunits with different oligosaccharide structures are studied *in vitro* on the bladder cancer permanent cell lines CAL-29, T-24, and the normal urothelial cell line HL 10/29. A significant cytotoxic effect against these tumor cells was observed for the functional unit RvH1-c after a treatment in contrast to native RvH and its structural subunits. Cells, treated with RvH1-c, show a mixture of both, necrotic and apoptotic cells. Cytotoxicity of RvH1-c is probably due to specific oligosaccharide structures exposed on the surface of the glycoprotein.

Key words: Cytotoxic activity, bladder cancer, hemocyanin from *Rapana venosa.*

Abbreviation

Dox	Doxorubicin
ESI-MS	Electrospray ionisation mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption ionization-time of flight-mass spectrometry
Hc	Hemocyanin
HIH	*Helix lucorum* hemocyanin
HvH	*Helix vulgaris* hemocyanin
KLH	Keyhole limpet *Megatura crenulata* hemocyanin
RvH	*Rapana venosa* hemocyanin
RvH1, 2	Structural subunits 1 and 2 of *Rapana venosa* hemocyanin
RvH1-c	Functional unit c of *Rapana venosa* hemocyanin

1. Introduction

Hemocyanins (Hcs) are copper-containing glycoproteins acting as oxygen-transporting proteins of arthropods and molluscs. These biomacromolecules have also been applied in cancer therapy as non-specific stimulators of the immune system [1-4].

Keyhole limpet hemocyanin (KLH) is a well-known immunomodulator frequently used in research and clinical trials [5, 6], e.g. for the treatment of patients with bladder [7-9], breast [10], colorectal [11], pancreatic cancer [12] or melanoma [1, 12, 13]. KLH was administered as single substance or in combination with cytokines, polysialic acid, low molecular weight haptens, hormones, dendritic cells, synthetic oligosaccharides, gangliosides, or peptides [14, 15]. The immunological response of KLH has often been attributed to its carbohydrate rather than its protein moiety alone, and its clinical success against bladder cancer is assumed to be based on cross-reaction of the disaccharide epitope Gal (β 1- 3) Gal NAc with an epitope on the bladder tumor cell surface [16, 17].

In 2006, Moltedo et al. [18] detected antitumoral effects of a Hc, isolated from the gastropod

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Concholepas concholepas: in a murine bladder cancer model, the authors observed decreasing tumor growth and prolonged survival after its administration without any toxic effects.

In our previous study, cytotoxic effects and strong activation of the immune system were observed for tumor-bearing mice after their treatment with *Rapana venosa* hemocyanin, and it was suggested that its carbohydrate moieties are involved in its antitumoral activity [19-21]. Recently, different hemocyanins, KLH, *Helix lucorum* (HlH), RvH, and two of its structural subunits were studied for their antitumoral effects on CAL-29 and T-24 bladder cancer permanent cells [22, 23]. Moreover, the mechanism of antitumor activity of HlH on CAL-29 and T-24 bladder cancer cell lines and the genes involved in this mechanism were also suggested [23]. Urothelial bladder cancer is the fifth most common cancer in the Western society and will increase significantly in the future as a result of the increasing world pollution in developing countries [24].

One of the fundamental prerequisites for evaluation of a general principle of Hcs' cytotoxicity is first based on detailed structure determinations of these giant glycoproteins [19-23]. Applying different chromatographic and spectroscopic techniques, we present structural properties and oligosacchiraide profiles of *Rapana venosa* hemocyanin: the native molecule consists of two structural subunits, RvH1 and RvH2, with molecular masses of 400 and 420 kDa respectively [25]. Each of them contains 8 functional units (RvHa - RvHg) with molecular masses of about 45 kDa and different carbohydrate contents [26]. We also determined its oligosaccharide profiles applying MALDI-TOF-MS, ESI-MS, and tandem mass spectrometry in a Q-Trap [26-30] and found novel structural motifs containing unusual N-glycan structures with an internal fucose residue connecting one GalNAc(ß1-2) and one hexuronic acid [28, 30].

Due to our previous data about the antitumor activity of hemocyanins [22, 23] and knowledge on the structure of *R. venosa* hemocyanin [27-30], we decided to use RvH and its structural subunits RvH1, RvH2 and functional units (FUs) as a model to analyze their cytotoxic effects on the bladder cancer permanent cells CAL-29 and T-24.

2. Materials and Methods

2.1 Materials

Rapana venosa Hc was isolated from marine snails living in the Black Sea as described before [25]. Two structural subunits, RvH1 and RvH2, were separated on a Resource Q column using a Fast Protein Liquid Chromatographic (FPLC) system. Functional units were isolated from structural subunit RvH1 as described by Dolashka et al. [25, 26]. The native molecule, two structural subunits, RvH1, RvH2, and functional unit RvH1-c were analysed by SDS-polyacrylamide gel electrophoresis, carried out as described by Laemmli using either 7.5% or 10% gels at pH 8.6. Coomassie Blue R-250 was used to stain the protein bands. All other (bio) chemicals, unless otherwise noted, were purchased from Sigma-Aldrich.

2.2 Amino Acid Sequence Determination

The isolated FU RvH1-c with cytotoxic activity was additionally purified on a HPLC system, equipped with a Nucleosil RP18 column, as described by Dolashka-Angelova et al. [25]. The purified fraction was dried, dissolved in 40% methanol/1% formic acid, and subjected to automated Edman N-terminal sequencing on a pulsed liquid protein sequencer (Applied Biosystems GmbH, Foster City, CA).

Database searches and further analyses were performed using sequence analysis tools (e.g., Blastn and Blastx, Lalign, available at NCBI).

2.3 Effects of Rapana Isoforms on Bladder Cancer Permanent Cell Lines

2.3.1 Cell Culture

Experiments were carried out with two commercially available permanent human tumor cell

lines from different stages of human urinary bladder transitional carcinoma cells (TCC). T-24 cells were established from the primary tumor of an 81-year-old Caucasian woman with urinary bladder cancer (transitional cell carcinoma (TCC), grade III) in 1970 producing a variety of cytokines (e.g. G-CSF, IL-6 and SCF) with a p53 mutation. CAL-29 cells were established from the primary lesion of a fatally invasive, metastatic TCC of the bladder (grade IV, stage T2) from an 80-year-old woman before treatment.

The T-24 and Cal-29 cells were cultured in Dulbecco Modified Eagle's Medium (DMEM, Lonza, Austria), supplemented with 10% fetal bovine serum (Gibco, Austria), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1% non-essential amino acids in 75 cm^3 tissue culture plastic flasks (Falcon). TrypLE Express, EDTA, and PBS were products of Gibco, Austria. Cells were maintained in log growth phase at 37^oC in humidified air containing 5% CO_2.

Normal human urothelial cells were grown to confluence in complete keratinocyte serum-free medium (cKSFM) with human recombinant epithelial growth factor (5 ng/ml), bovine pituitary extract (50 mg/ml; Gibco), supplemented with cholera toxin (30 ng/ml; List Biological Laboratories, Campbell, CA, USA) under standard conditions.

2.3.2 Antiproliferative Activity of the Tested Hemocyanins

The antiproliferative activity of the tested hemocyanins on T-24 and CAL-29 cell lines was determined in 50 mM Tris/HCl buffer, pH 8.0, using WST-1 and BrdU assays (Roche Diagnostics, Germany) as referred by the manufacturer. Cell proliferation reagent WST-1 provides a method to measure cell proliferation or metabolic activity, based on the enzymatic cleavage of the tetrazolium salt WST-1 to a water-soluble formazan dye detectable by its absorbance at 420-480 nm. WST-1 assay reading was performed at 440 nm using an ELISA plate reader (Milenia Kinetic Analyzer, Diagnostic Products Corporation).

The BrdU Cell Proliferation ELISA Kit measures cell proliferation by quantification of BrdU incorporated into the newly synthesized DNA of replicating cells. It offers a nonradioactive alternative to the [3H]-thymidine-based cell proliferation assay with comparable sensitivity.

Cells were washed with 0.1% EDTA/PBS for 5 min, trypsinized with TrypLE Express (Gibco), centrifuged for 5 min at 1500 g, and counted in a hemocytometer. Cells were transferred to a 24-well tissue culture plate to ensure a concentration of 0.5×10^4 cells/cm^2 and incubated overnight in a thermostat at 37℃ in a humidified container to allow cell attachment. The T-24 and CAL-29 tumor cells were treated for 24 h, 48 h, and 72 h with various concentrations (0.1, 0.25 and 1.0 mg/ml) of the test substances, doxorubicin (DOX, 0.1mg/ml positive control), and cultured medium (negative control), respectively. The effects of different concentrations: (0.1, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, and 3.0 mg/ml) of native subunit RvH1 and functional unit RvH1-c of *R. venosa* hemocyanin on cell viability were assessed by the WST-1 assay (Roche Diagnostics, Germany) as referred by the manufacturer and performed 3 times.

The percentage of cell growth inhibition was calculated as follows:

Cell viability (%) = OD_{440} (experimental)/OD_{440} (control) × 100

2.3.3 Pro-apoptotic Activity of Hemocyanins

The analysis of the exposure of phosphatidylserine on the outer leaflet of apoptotic cell membranes was performed using the Annexin-V-FLUOS Kit (Roche, Diagnostics) and propidium iodide (PI) for the differentiation from necrotic cells. Then, the medium was replaced and T-24 and CAL-29 tumor cells were treated with various concentrations of the test substances, RvH1 (1.5 mg/ml) and RvH1-c (1.0 mg/ml), which gave inhibitory effects in previous experiments, cultured for 24 h and compared with doxorubicin for positive control and medium only for negative control. The effect of different hemocyanins

on the induction of apoptosis was assessed by the Annexin-V-FLUOS Kit as referred by the manufacturer. Briefly, the culture medium was removed, and the cells were washed twice with PBS (Gibco) for 5 min and then covered with Annexin V labeling solution. After adding 1 µl of PI solution and 2 µl of fluorescein-annexin solution, the plates were incubated for 15 min at room temperature (15-20^{o}C) and then analyzed by fluorescent microscopy.

The cells were determined based on the following staining: Positive Annexin-V-FLUOS-staining is characteristic for apoptotic cells; positive Annexin-V-FLUOS- and PI-staining is observed for late apoptotic or necrotic cells; specific PI-staining is typical for necrotic cells.

2.4 Statistical Analysis

The data are given as the mean ± standard deviation (SD). Significance testing was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Values of $*P < 0.05$, $** P < 0.01$ and $*** P < 0.001$ were considered to be significant.

3. Results and Discussion

The immunological potential [20] and antitumor properties of molluscan hemocyanins have been studied, combining *in vitro* and *in vivo* methods [19, 21]. In our previous investigation, we found that RvH, its derivatives and conjugates prolong in experimental tumor-bearing animals with ascites tumor of Guerin and Graffi the median survival time compared to non-immunized animals. We also demonstrated that HlH have a direct antiproliferative effect on CAL-29, and T-24 bladder cancer cell lines, and that the antitumor properties of *Helix lucorum* are superior to those of KLH [22]. Moreover, a slight inhibitory effect was observed after a 24 h treatment of CAL-29 cells with the subunits, RvH1 and RvH2 (18.01% and 15.53%, respectively). On contrary, no cytotoxic effects or stimulations were observed with the native molecules RvH and KLH [22, 23].

Similar effects were observed following the treatment of T-24 cells with the same compounds. However, an extremely low cytotoxic effect was detected after 24 h and 48 h incubations with RvH2 (17.61% and 13.98%) with further stimulation at 72h. An opposite tendency was observed subsequently to RvH1 treatment at 24 h, 48 h and 72 h (4.72%, 16.99% and 10.51% cytotoxicity, respectively) [22].

However, only a limited number of hemocyanins were investigated so far and no conclusion about the molecular mechanism is available. Based on these results, *in vitro* experiments were performed to analyze the cytotoxic activities of native RvH compared to its isoforms and to find optimal doses arresting growth of T-24 and CAL-29 cancer cells and normal urothelial cells.

Therefore, the effect of RvH1 was analysed after treatment of T-24 tumor cells with different doses (0.25, 0.5 and 1.0 mg/ml) at 24 h, 48 h, and 72 h. The results obtained from the proliferation WST-1 assay (Fig. 1A) showed a relatively small cytotoxic activity of RvH1 applied at a concentration of 0.50 mg/ml in comparison to Dox (0.1 mg/ml and $P < 0.001$), and the cell viability is reduced to about 76% after a period of 72 h. At a concentration of 0.25 mg/ml, RvH1 has only minor influence on cell viability even after 72 h. The optimal doses arresting T-24 cancer cell growth were determined to be 1.0 mg/ml of RvH1 at 48 h (about 76%) and at 72 h (about 75%). Cellular morphology of T-24 tumor cells, treated with RvH1, was compromised in the presence of 1.0 mg/ml of hemocyanin and 0.1 mg/ml of doxorubicin compared to the control, respectively (Fig. 1B).

To identify if subunit RvH1 and functional unit RvH1-c reduced the viability of T-24 tumor cells *via* apoptosis, cells were cultured in DMEM, maintained at 37^{o}C and 5% CO_2, stained with Annexin-V-Fluos Kit, and co-stained with PI after 24 h incubation with: 1.5 mg/ml of RvH1 and 1.0 mg/ml of RvH1-c, respectively. The fluorescent micrographs of T-24 cells, after 24 h

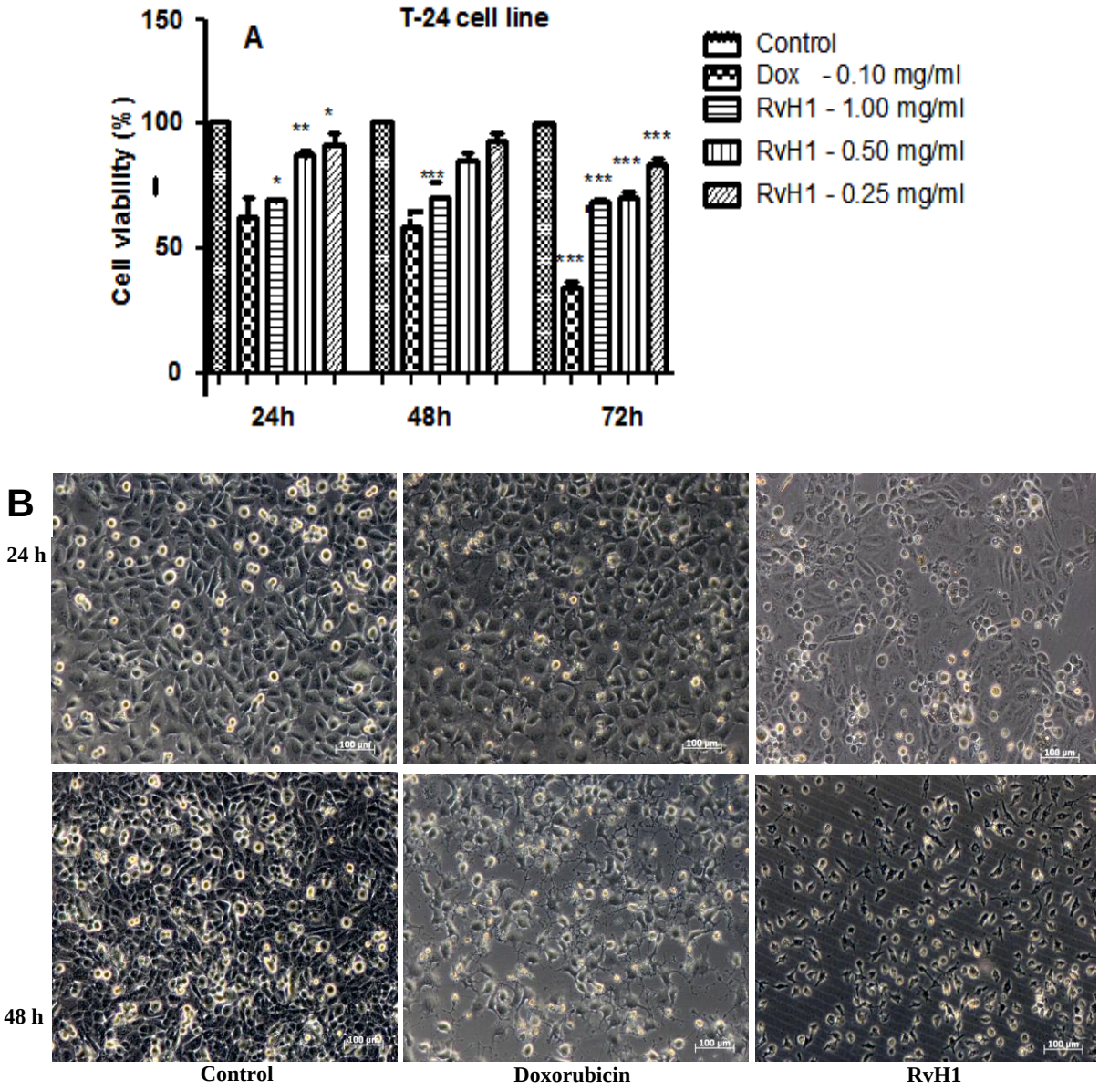

Fig. 1 A) Cell viability (%) of T-24 tumor cells after treatment with different doses of RvH1 (0.25, 0.5, and 1.0 mg/ml) and doxorubicin (Dox, 0.1 mg/ml) for 24, 48, and 72 h. B) Morphological alterations of T-24 tumor cells treated with RvH1 (1.0 mg/ml) and doxorubicin (0.1 mg/ml) for 24 h and 48 h. Control (A, B) refers to untreated cells.

incubation with the hemocyanin derivatives mentioned above, were compared to non-treated cells and doxorubicin-treated ones (date not show). The control cells exerted slight green and very low red signals, characteristic for active cells. The cultures, treated with doxorubicin, exhibit strong green and low red signals. After treatment of T-24 cells with the structural subunit RvH1, both populations are found in the wells, apoptotic cells fluorescing in green only and bright green cells with bright red nuclei. A similar behavior is observed after treatment of the cells with the functional unit RvH1-c.

In continuation of our investigations of cytostatic effects on CAL-29 tumor cells [22], we tested RvH1-c as follow. Fig. 2 shows a strong dose- and time-dependent inhibitory effect of RvH1-c on

CAL-29 tumor cells. After 24 h incubation of the cells, their viability decreases to values between 60 to 70% for different concentrations (0.75, 1.5 and 3.0 mg/ml) of RvH1-c. The effect of doxorubicin is stronger than the effect of RvH1-c after an incubation of 24 h. The inhibitory effects of RvH1-c increase after 48 h and appear to be even stronger than those of doxorubicin after an incubation of 72 h, at all doses tested (Fig. 2A).

Alterations in cell morphology of 24 h- and 48 h-RvH1-c treated cells in comparison to untreated specimens are obvious from Fig. 2B. RvH1-c-treated cells lost adhesion and are found in the medium.

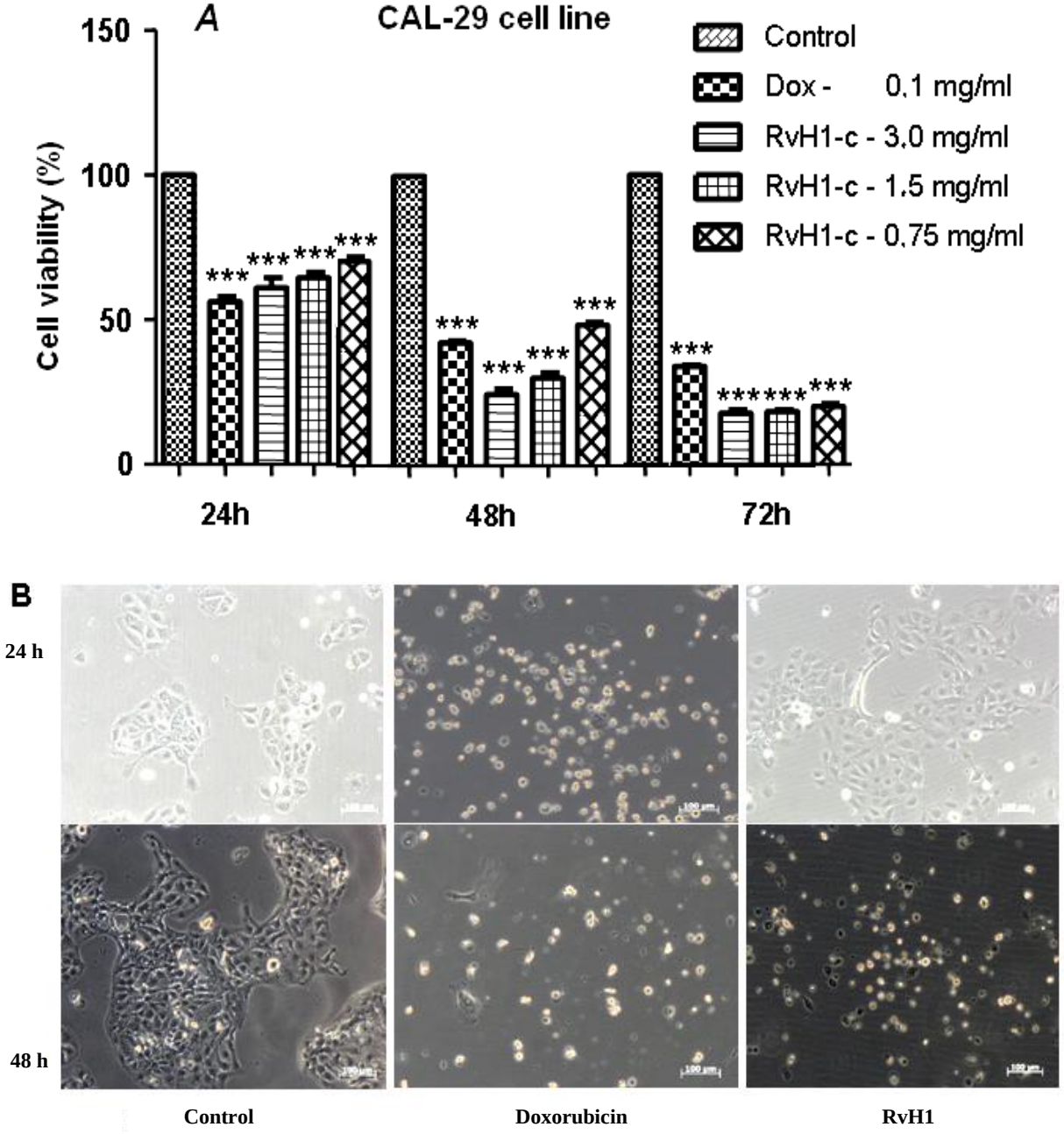

Fig. 2 A) Cell viability (%) of CAL-29 tumor cells after treatment with different doses of RvH1-c (0.75, 1.5, and 3.0 mg/ml) and doxorubicin (Dox, 0.1 mg/ml) for 24 h, 48 h, and 72 h. B) Morphological alterations of CAL-29 tumor cells treated with RvH1-c (3.0 mg/ml) and doxorubicin (0.1 mg/ml) for 24 h and 48 h. Control (A, B) refers to untreated cells.

Fluorescent micrographs of CAL-29 cells after 24 h incubation with medium (control cells), doxorubicin (0.1 mg/ml), subunit RvH1 (1.5 mg/ml), and functional unit RvH1-c (1.0 mg/ml), appear as follows: control cells are shown as intact cells with a slight green and very low red signal, while the doxorubicin-treated cells show a strong green and a low red signal with alterations in their morphology (e.g. swollen, rounded, etc.), they are mainly found in the supernatant medium and include mostly late apoptotic or necrotic cell populations. Treatment of CAL-29 cells with RvH1 produces mostly necrotic and to a lesser extent apoptotic cells. However, both, apoptotic cells fluorescing in green and late apoptotic or necrotic bright green cells with bright red nuclei, are found after treatment with RvH1-c.

To evaluate and compare the effect of *R. venosa* hemocyanin and its isoforms on primary normal urothelial cells (HL 10/29), this culture was treated and handled as described above. The effects of RvH1 (1.5 mg/ml) and RvH1-c (1.0 mg/ml) are shown in Fig. 3A. The morphologies of untreated (Fig. 3Ba) and hemocyanin-treated, RvH1 (Fig. 3Bb) and RvH1-c (Fig. 3Bc) HL 10/29 urothelial cells demonstrated, opposite to the results of WST-1 and BrdU assays, a minor inhibition of metabolic activity and proliferation for RvH1-c.

In conclusion, the functional unit, RvH1-c, shows the most significant inhibitory effect against T-24 and CAL-29 bladder cancer cells in comparison to the native *R. venosa* hemocyanin and its structural subunit RvH1. And the most exciting, the inhibitory effect of

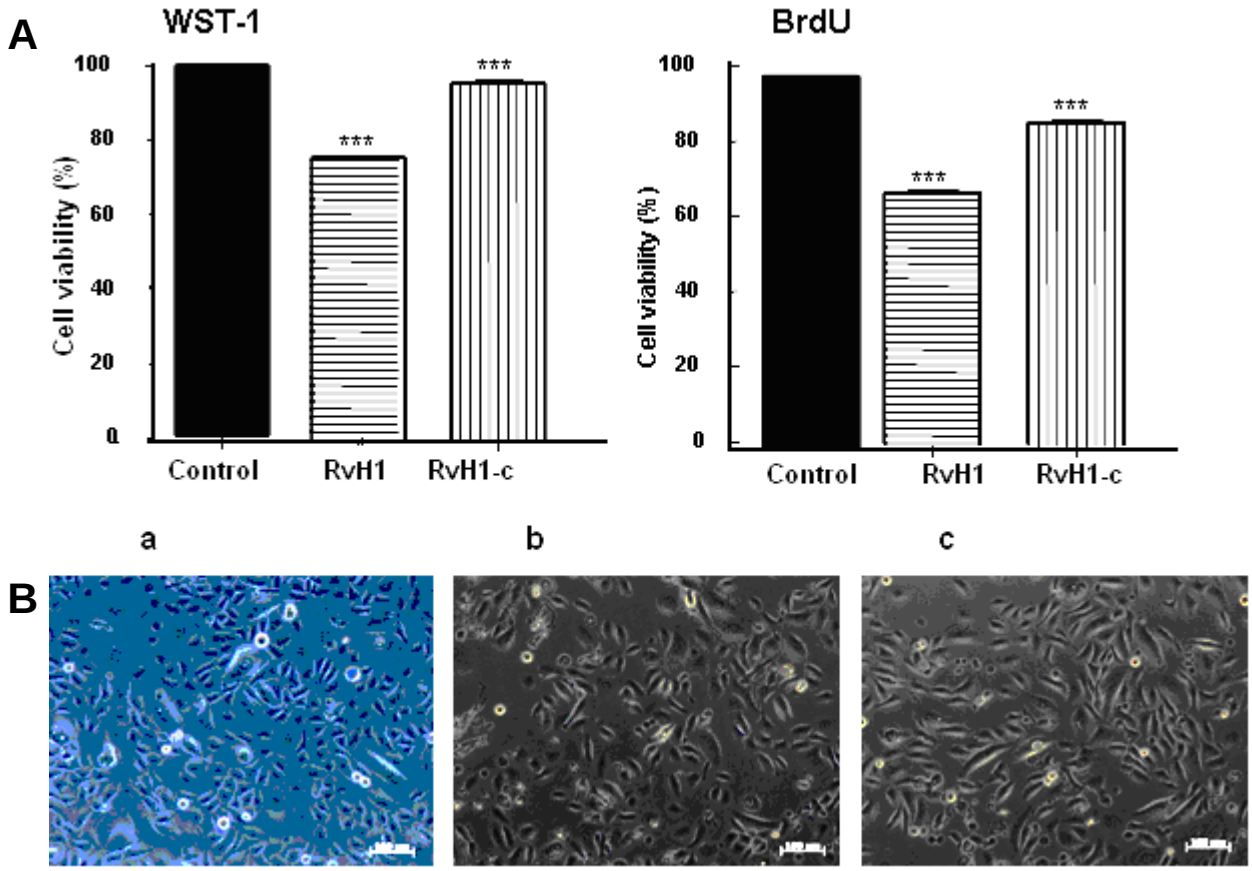

Fig. 3 A) Cell viability (%) of HL 10/29 urothelial cells after treatment with 1.5 mg/ml of RvH1 and 1.0 mg/ml of RvH1-c for 24 h, 48 h, and 72 h. Control refers to untreated cells. B) Morphology of HL 10/29 urothelial cells: (a) untreated cells, (b) cells treated for 24 h with RvH1 (1.5 mg/ml) and (c) cells treated for 24 h with RvH1-c (1.0 mg/ml).

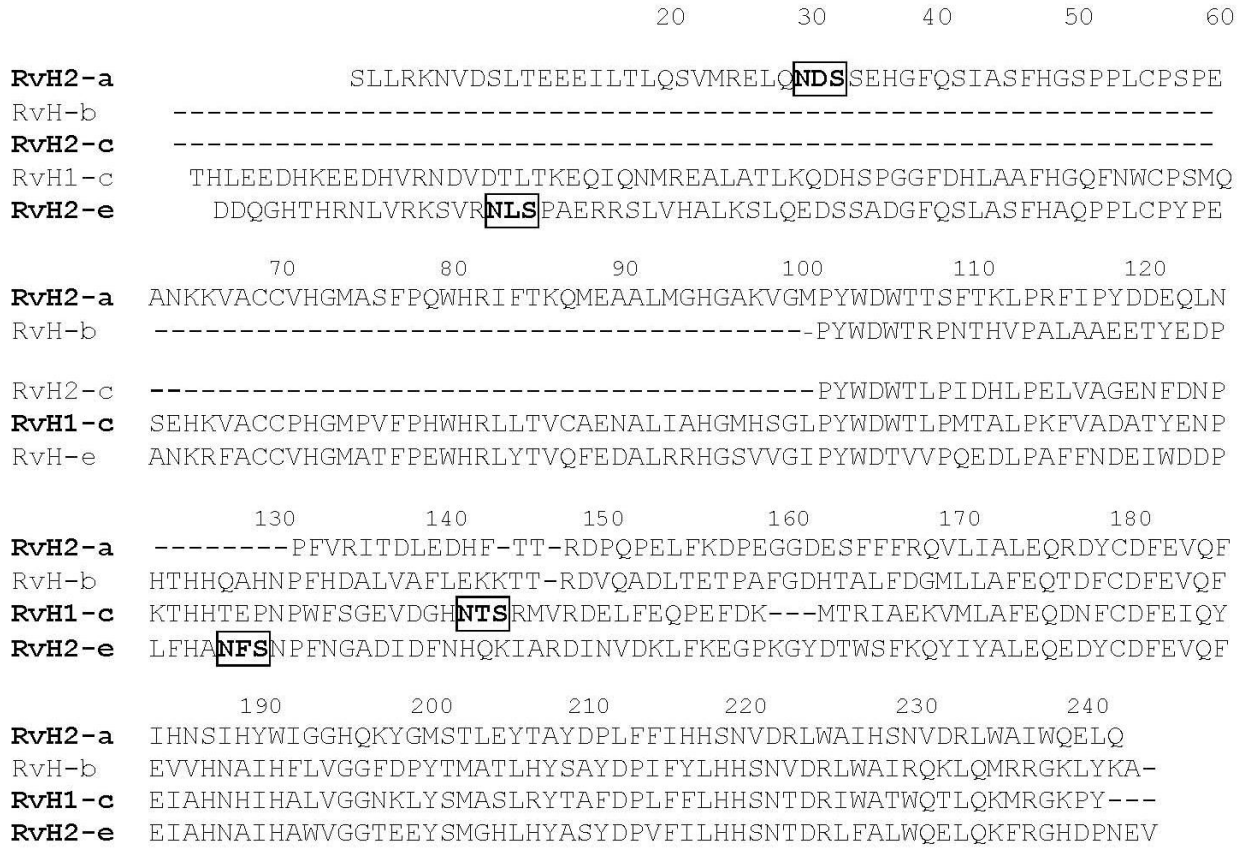

Fig. 4 Alignment of amino acid sequences of RvH2-a, RvH-b, RvH1-c, RvH2-c, and RvH2-e, using sequence analysis tools (e.g., Blastn and Blastx, Lalign, available at NCBI). Putative glycosylated sites are in bold.

RvH1-c is comparable to the effect of doxorubicin, without disturbing the metabolic activity or proliferation of normal HL 10/29 urothelial cells. Treatment with RvH1-c is most effective after a 72 h application of tumor cells and both populations, necrotic and apoptotic cells were observed.

The potent inhibiting activity of the functional unit is probably due to its specific oligosaccharide structures, which are more accessible in the subfractions. The alignment of amino acid sequences of RvH2-a, RvH-b, RvH1-c, RvH2-c, and RvH2-e, shows one putative glycosylated site in RvH1-c (Fig. 4). Our previous analyses of oligosaccharide structures of RvH1 and RvH1-c have shown a highly heterogeneous mixture of glycans and propose that the carbohydrate chain of RvH1-c is exposed on the surface of the functional unit [27].

In our preliminary study we found that the glycosylated functional unit RvH-c exhibits antiviral activity against the respiratory syncytial virus (RSV) and Herpes simplex virus (HSV) virus type 1 [27, 28]. The carbohydrate chains of the FUs are likely to interact with specific regions of glycoproteins of HSV through van der Waals interactions in general or with certain amino acid residues in particular. These glycosylation moieties might be important structural features for the interaction of the hemocyanin molecules with the target cells as was reported for the clinical success of KLH to patients with bladder tumor cells [16, 31]. A similar cytostatic mechanism of action might be assumed for *R. venosa* hemocyanin and its isoforms.

These results support our suggestion about the relation between inhibiting activity of RvH1-c and its specific oligosaccharide structures. This exciting efficacy of a natural glycoprotein needs as next to be confirmed *in vivo* to elucidate the mechanism of action in our pipeline.

Acknowledgments

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