In vitro Antiproliferative Effect of *Helix aspersa* Hemocyanin on Multiple Malignant Cell Lines

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As an extension of our studies on the antitumour properties of various hemocyanins, we sought to compare the antiproliferative effects of hemocyanins derived from two snail species: *Helix lucorum* (HlH) and *Helix aspersa* (HaH). This is the first report on the antitumour effects of HaH. We hypothesized that HaH has antitumour effects not only against bladder cancer, as previously shown with other hemocyanins, but also on other cancer cell lines. The antiproliferative properties of the mentioned hemocyanins were investigated *in vitro* on the following human cell lines: bladder cancer (CAL-29 and T-24), ovarian cancer (FraWü), acute monocytic leukemia (THP-1), prostate cancer (DU-145), glioma cancer (LN-18), and Burkitt's lymphoma (Daudi). The properties of HaH were compared to those of HlH, keyhole limpet hemocyanin (KLH), and two positive controls (doxorubicin and mitomycin C).

An antiproliferative effect of the total molecule and one structural subunit of HaH, βc-HaH, against both bladder cancer cell lines, T-24 and CAL-29, was observed. The cytotoxic effect of HaH ranged between 15 % and 60 % among the other tested cell lines. The endotoxin contamination did not affect the efficacy of HaH. Therefore, HlH and HaH could be appropriate for more detailed investigations of their use as antitumour agents for the studied cancers.

Key words: Hemocyanin, Bladder Cancer, Cell Line, Therapy

Introduction

Malignant diseases are a major public health problem. Although great advances have been made in research and treatment, cancer remains a key health concern. The growing cancer burden is mostly due to lung, breast, colon, prostate, stomach, and bladder cancers (Botteman *et al.*, 2003; Ferlay *et al.*, 2010). In the past few years, the therapeutic properties of substances called "hemocyanins" have been intensively studied. These are large, multisubunit, high-molecular weight, oxygen-carrying metalloproteins found in the hemolymph of arthropods and molluscs. Such hemocyanins are the keyhole limpet hemocyanin (KLH), as well as hemocyanins isolated from *Concholepas concholepas* (CCH), *Rapana venosa* (RvH), *Haliotis tuberculata* (HtH), and *Helix lucorum* (HlH). The best studied amongst them is KLH – a highly antigenic respiratory protein of the giant keyhole limpet *Megathura crenulata*. It has been used in clinical trials either as a drug for the treatment of bladder cancer (US National Institutes of Healths, 2013a) or as an adjuvant in melanoma, prostatic, and ovarian cancer treatment (Cancer Research Institute – NY, 2013; US National Institutes of Healths, 2013b, c). The presumed mechanism of their action is the immune response activation

due to the presence of cross-reacting epitopes, such as the Thomson-Friederich antigen [Gal(β1-3)-*N*-acetyl epitope] and T helper type-1 immunity enhancement (Wirguin *et al.*, 1995; Moltedo *et al.*, 2006).

Riggs *et al.* (2002, 2005) reported an *in vitro* growth-inhibiting effect of KLH on multiple cancer cell lines including breast (MCF-7 and ZR75-1), pancreatic (PANC-1, MIAPaCa), prostate (DU-145), and Barrett's esophageal adenocarcinoma cancer cell lines.

The antitumour effects of hemocyanins other than KLH have also been studied in the last years. It was found that the hemocyanin from the Chilean mollusc *Concholepas concholepas* (CCH) and its subunits CCHA and CCHB are reliable alternative candidates to KLH either as protein carrier or for providing a safe and effective immunotherapy for human superficial bladder cancer (Moltedo *et al.*, 2006; Becker *et al.*, 2009; Del Campo *et al.*, 2011). Combining *in vivo* and *in vitro* methods, Toshkova *et al.* (2009) also proved the immunological and antitumour potential of HlH and RvH against Guerin ascites tumour, as well as progressing myeloid Graffi tumours. It was also demonstrated that HlH is superior to KLH in its antitumour activity on T-24 and CAL-29 bladder cancer cell lines (Boyanova *et al.*, 2013).

Therefore, we investigated in the present study the antiproliferative effects of the hemocyanins from two garden snail species with a structure markedly different from those of KLH and RvH: *Helix lucorum* (HlH) and *Helix aspersa* (HaH). Additionally, HaH was studied on cell lines other than bladder cancer. We hypothesized that the antiproliferative effect of HaH is not tumour-specific. We were also searching for new areas of application of this hemocyanin. We employed not only bladder, prostate, and ovarian cancer cell lines, which are already known to be sensitive to other hemocyanins, but cell lines of various origins as well (glyoblastoma, lymphoma, leukemia).

The aim of this study was to demonstrate that the antiproliferative effect of HaH is firstly not bladder cancer-specific, and secondly to search for new areas of application of the hemocyanin.

Materials and Methods

Animals

H. aspersa and *H. lucorum* are species of land snails. *H. aspersa* was formerly named *Cornu aspersum* and was placed in the genus *Helix* in all sources between 1774 and 1988 (Giusti *et al.*, 1995). *H. lucorum* was named by Linnaeus in 1758 (Cameron and Carter, 1979; Staikou and Lazaridou-Dimitriadou, 1989).

Specimens of *Helix lucorum* were collected from a garden in Sofia, while those of *Helix aspersa* were obtained from a Bulgarian snail breeding farm. The weight of an individual snail was between 28 and 30 g.

The whole complexes of HlH and HaH and the structural subunit of HaH, βc-HaH were used.

Isolation of native HlH

HlH was isolated from the hemolymph of *H. lucorum* as described by Velkova *et al.* (2010).

Isolation of native HaH

H. aspersa hemolymph was collected from the excised feet of 100 snails, solubilized in 1 l 50 mM sodium acetate buffer (pH 5.8), and centrifuged at $15{,}000 \times g$ for 15 min to remove the hemocytes. Total hemocyanin was sedimented at $40{,}000 \times g$ in an Beckman Optima L-80 ultracentrifuge (Beckman Coulter, Brea, CA, USA) for 4 h at 5 °C. The resulting pellet of native hemocyanin HaH was resuspended in 100 ml 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM $CaCl_2$ and 10 mM $MgCl_2$.

Isolation of structural subunit βc-HaH

After overnight dialysis of HaH against 0.13 M glycine buffer (pH 9.0) at 4 °C, the βc-hemocyanin fraction was purified by anion-exchange chromatography on a DEAE-Sepharose CL-6B column (GE Healthcare Bio-Sciences, Uppsala, Sweden). Elution was performed using a gradient of 0 – 1 M NaCl in 50 mM Tris-HCl buffer (pH 8.0) at 1.5 ml/min. Finally, the isolated components were concentrated by ultrafiltration (100 kDa Amicon® PM membranes; Merck Millipore, Billerica, MA, USA) and further purified by gel filtration chromatography on a Sephacryl S 300 column (GE Healthcare Bio-Sciences). HaH was identified by its blue colour, and its N-terminal amino acid sequence was determined by Edman degradation with a Procise 494A pulsed liquid protein sequencer (Applied Biosystems, Weiterstadt, Germany).

Materials and assays

Seven cancer cell lines were used in this study: CAL-29 and T-24 (human bladder carcinoma), DU-145 (human prostate carcinoma), LN-18 (human ma-

lignant glioma), FraWü (ovarian carcinoma), Daudi (human Burkitt's lymphoma), and THP-1 (human acute monocytic leukemia), which were all obtained from the Interfaculty Institute for Cell Biology, Department of Immunology, University of Tübingen, Tübingen, Germany.

The antibiotics mitomycin C (MIT-C) and doxorubicin (DOX), LPS (*E. coli* serotype 0111: B4), KLH, and Bradford reagent were purchased from Sigma-Aldrich (Taufkirchen, Germany). The WST-1 cell proliferation assay kit was purchased from Roche Diagnostics (Mannheim, Germany), while the Limulus amebocyte lysate (LAL) assay kit was purchased from Lonza (Verviers, Belgium).

Cell culture

Cell lines CAL-29, T-24, DU-145, LN-18, and FraWü were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM; Lonza) or RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (P/S) (Gibco Invitrogen, Karlsruhe, Germany) at 37 °C in a humidified atmosphere with 5% CO_2 until 80% confluence. Cells were harvested by trypsin/EDTA (Lonza) and counted using a hemocytometer.

Cell lines Daudi and THP-1 were maintained in suspension culture at densities between $2 \cdot 10^5$ and $1.5 \cdot 10^6$ cells/ml in RPMI-1640 medium and DMEM, respectively, supplemented with 10% FCS and 1% P/S. Cell cultures were diluted with fresh medium every 2 – 3 d.

Preparation of test substances

The hemocyanins 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 hemocyanin solutions was determined spectrophotometrically with Bradford reagent. KLH at 5.1 mg/ml was used as the standard (Sigma-Aldrich). Optical density (OD) at 595 nm was read using an ELISA reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA, USA).

In vitro cytotoxicity assay

Cell viability was determined using a standard WST-1 cell proliferation assay. The cell lines mentioned above were seeded into 96-well plates (20,000 cells/well). HlH and HaH in concentrations of 0.5 μg/ml to 500 μg/ml were added to the solution after 12 – 18 h. Lipopolysaccharide (LPS) was used in concentrations ranging from 0.5 ng/ml to 500 ng/ml. DOX and MIT-C were used as positive controls at concentrations of 10 μg/ml and 1 μM, respectively. Medium alone (cells without treatment) was used as negative control. After incubation for 24, 48, and 72 h, 20 μl ready-to-use WST-1 reagent were added to each well, and culture continued for another 2 h, after which cell viability was determined.

LAL assay

All working procedures were done under sterile conditions with pyrogen-free material. To rule out an accidental endotoxin contamination that could interfere with the experiments, we tested the CAL-29 and T-24 cell line supernatant as well as the HaH at a concentration of 500 μg/ml for endotoxin (LPS) content by means of the LAL assay (Obayashi *et al.*, 1985, Lindsay *et al.*, 1989) according to the manufacturer's protocol.

Electron microscopy

Electron micrographs were taken with a Philips® CM10 transmission electron microscope with a 30 mm objective aperture. HaH samples were adsorbed for 60 s to a glow-discharged pistoform/carbon-coated support film, washed three times with droplets of distilled water to remove buffer salts, and then negatively stained with 1% uranyl acetate. Electron micrographs were routinely recorded at an instrumental magnification of 45,000.

Statistical analysis

The data were processed with Exel and GraphPad Prism 5 and are presented as means with standard deviation (SD). Significance was tested using one-way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference (shown in the figures as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in comparison to a negative control, and $\hat{}P < 0.05$, $\hat{}\hat{}P < 0.01$, and $\hat{}\hat{}\hat{}P < 0.001$ in comparison to a positive control). The experiments were performed in triplicate and repeated at least three times.

Results

Isolation of HaH and its structural subunits

The *H. aspersa* hemocyanin is present in the hemolymph as α-HaH and β-HaH isoforms. β-HaH is composed of only one subunit type (βc subunit), and α-HaH is composed of two types of subunits (α_D-HaHc and α_N-HaHc). βc-HaH was isolated by anion-exchange chromatography on a DEAE Sepharose CL-6B column (Fig. 1A). Analysis of the isolated fraction 1 (isoform β-HaH) by Edman degradation revealed an N-terminal amino acid sequence different from that of the β-HlH isoforms (data not shown). The alignment of their N-terminal sequences revealed about 50 – 67 % identity. The molecular masses of the native complex and the isolated isoform of HaH were determined by native polyacrylamide gel electrophoresis (PAGE) (Fig. 1B). A single band of about 450 kDa was revealed for the structural subunit βc-HaH, which correlates very well to the masses of ferritin and the subunits of HlH.

The absorption spectrum of the isolated isoform of βc-HaH was similar to that of other hemocyanins and showed three peaks at 278, 344, and 550 nm, corresponding to aromatic residues, Cu^{2+}-O^{2-}, and Cu^{2+}-histidine coordination centres, respectively (data not shown). Moreover, electron micrographs of the negatively stained native hemocyanin revealed top and side views of complexes. The native HaH was mostly present as didecamers and few decamers (Fig. 1C) typical for all gastropod hemocyanins.

Choice of time point for comparison of the test agents

The antiproliferative effect of βc-HaH was studied on T-24 and CAL-29 cell lines in comparison to KLH and HlH, based on the established protocol. The effect of the tested hemocyanins (KLH, HlH, and βc-HaH) in a concentration of 500 μg/ml on T-24 bladder cancer cells is presented in Fig. 2. Cell viabilities measured via the WST-1 assay after 24, 48, and 72 h in the presence of KLH were 107, 77, and 69 %, respectively. Cell viabilities of approximately the same magnitude were observed for the treatment with βc-HaH at 24, 48, and 72 h (109, 101, and 65 %, respectively). Lowest viabilities were observed for the HlH treatment (72, 66, and 44 %).

The respective data for the CAL-29 cell line are shown in Fig. 3. As with the T-24 cell line, the results with KLH (cell viabilities of 114, 99, and 85 %) and βc-HaH (102, 105, and 87 %) are quite similar. The

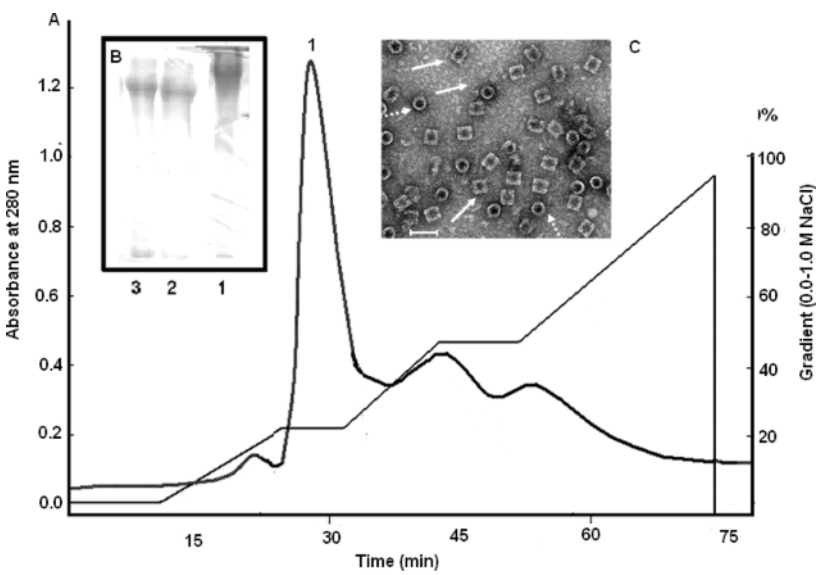

Fig. 1. (A) Purification of the βc subunit of *Helix aspersa* hemocyanin (HaH) by anion-exchange chromatography on a DEAE-Sepharose CL-6B column using 50 mM Tris-HCl buffer, pH 8.0. Elution was performed with a stepwise gradient of 0 – 1 M NaCl, with a rate of 1.5 ml/min. (B) 7.5 %-PAG-electrophoresis of the native HaH (1), structural subunit βc-HaH (2), and ferritin (3). (C) Electron micrograph of the native HaH, showing didecamers in side view and top view and decamers (dashed arrows). The scale bar indicates 100 nm.

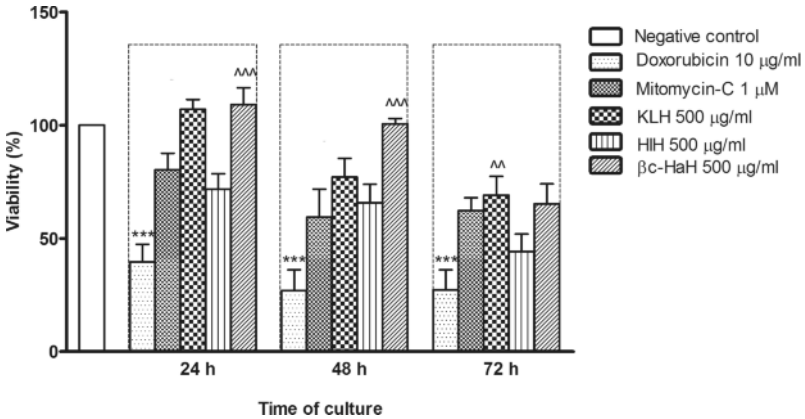

Fig. 2. Effect of native hemocyanin from *Helix lucorum* (HlH) and the *Helix aspersa* hemocyanin (HaH) structural subunit βc-HaH at a concentration of 500 μg/ml on the human tumour cell line T-24, after 24, 48, and 72 h of incubation, in comparison to keyhole limpet hemocyanin (KLH) and negative and positive controls [doxorubicin hydrochloride (DOX), mitomycin C]. $^{***}P < 0.001$ in comparison to the negative control; $^{\wedge\wedge}P < 0.01$ and $^{\wedge\wedge\wedge}P < 0.001$ in comparison to DOX.

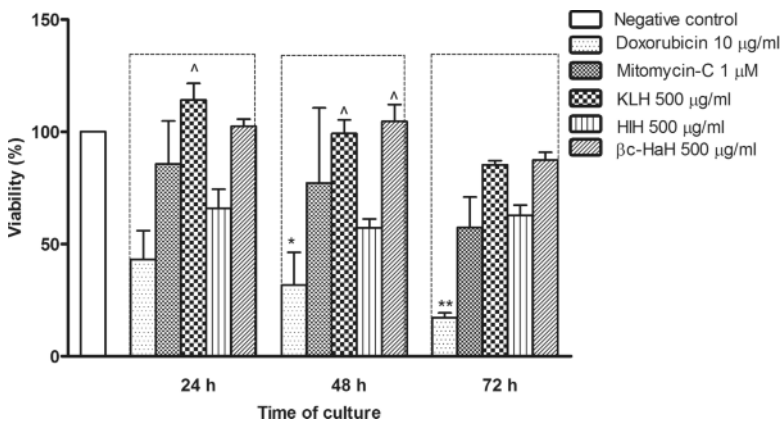

Fig. 3. Effect of the native *Helix lucorum* hemocyanin (HlH) and the *Helix aspersa* hemocyanin (HaH) structural subunit βc-HaH at a concentration of 500 μg/ml on the human tumour cell line CAL-29, after 24, 48, and 72 h of incubation, in comparison to keyhole limpet hemocyanin (KLH) and negative and positive controls [doxorubicin hydrochloride (DOX), mitomycin C]. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison to the negative control; $^{\wedge}P < 0.05$ in comparison to DOX.

highest growth-inhibiting effect was again observed with native HlH (66, 57, and 63 %, respectively).

As growth inhibition was strongest after 72 h, cell viability in all subsequent experiments was determined at this time point.

Comparison between the antiproliferative effects of the whole complexes of HaH and HlH

The cytotoxic effects of the whole complexes of HlH and HaH on the T-24 and CAL-29 cell lines were analysed at 100 μg/ml and 500 μg/ml. As can be seen in Fig. 4, the two substances produced similar effects after 72 h at both concentrations. However, a higher inhibition was observed after treatment of the CAL-29 cell line with 500 μg/ml of HaH. The viabilities of the T-24 and CAL-29 cell lines after incubation with 500 μg/ml HlH were determined to be 49 and 70 % and with 500 μg/ml HaH 52 and 54 %, respectively.

Effects of the whole HaH complex on multiple malignant cell lines

To determine whether the cytotoxic effect of HaH is specific only to bladder cancer, we performed additional experiments with five cancer cell lines of dif-

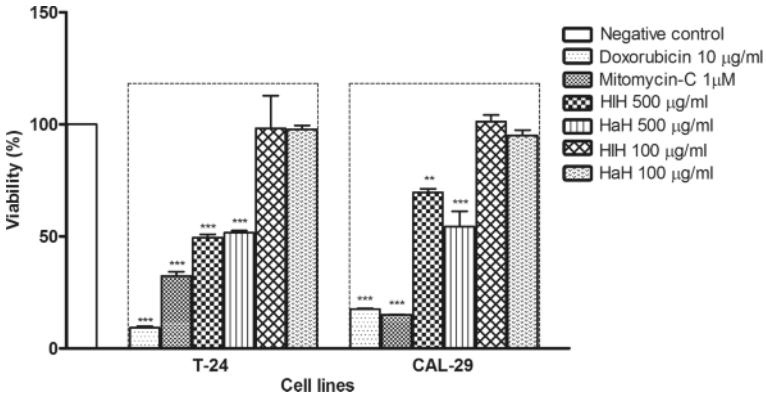

Fig. 4. Effect of the native *Helix lucorum* hemocyanin (HlH) and the *Helix aspersa* hemocyanin (HaH) at concentrations of 100 μg/ml and 500 μg/ml on the CAL-29 and T-24 bladder cancer cell lines, after 72 h of incubation, in comparison to negative and positive controls [doxorubicin hydrochloride, mitomycin C]. $^{**}P < 0.01$ and $^{***}P < 0.001$ in comparison to the negative control.

ferent origin: FraWü (ovarian cancer cell line), THP-1 (human acute monocytic leukemia cell line), DU-145 (prostate cancer cell line), LN-18 (human malignant glioma cancer cell line), and Daudi (human Burkitt's lymphoma cell line).

The antiproliferative effects of 500 μg/ml HaH were evaluated in parallel on these five cancer cell lines and on the already tested bladder cancer cell lines CAL-29 and T-24 in comparison to the positive (DOX) and negative controls (Fig. 5).

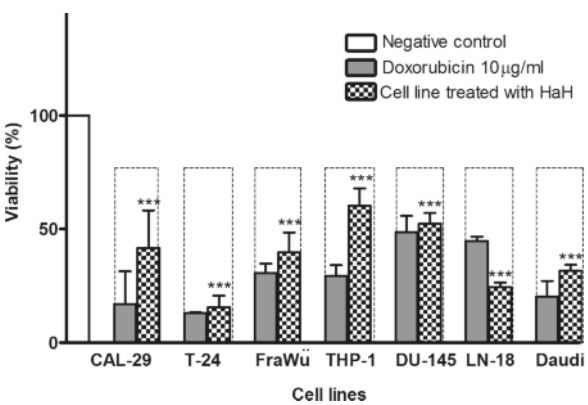

Fig. 5. Effect of the native *Helix aspersa* hemocyanin (HaH) at a concentration of 500 μg/ml on the human tumour bladder cancer (CAL-29 and T-24), ovarian carcinoma (FraWü), human acute monocytic leukemia (THP-1), prostate carcinoma (DU-145), human malignant glioma (LN-18), and human Burkitt's lymphoma (Daudi) cell lines, after 72 h of incubation, in comparison to negative and positive controls (doxorubicin hydrochloride). $^{***}P < 0.001$ in comparison to the negative control.

A strong antiproliferative effect was observed upon treatment of all tested cell lines with the native HaH complex. Cell viabilities, shown in Fig. 5, ranged from 16% for the T-24 bladder cancer cell line to 60% for the THP-1 human acute monocytic leukemia cell line (24% for the LN-18 human malignant glioma cell line, 32% for the Daudi lymphoma cell line, 40% for the FraWü ovarian cancer cell line, 42% for the CAL-29 bladder cancer cell line, and 52% for the DU-145 prostate cancer cell line).

Comparison of the effect of HaH and E. coli [serotype 0111:B4] LPS

The endotoxin contents of the CAL-29 and T-24 cell line supernatants were less than 0.2 EU/ml. In the HaH solution (at 500 μg/ml) the endotoxin content was equivalent to that of *E. coli* LPS at 500 ng/ml.

To be sure that the cytotoxic effect of HaH was not due to LPS (Lamm *et al.*, 2000; Lamm, 2003) a series of titration experiments were carried out. The two bladder cancer cell lines, T-24 and CAL-29, were used to compare the effects of doxorubicin, LPS, and HaH, the positive control doxorubicin being titrated from 10 μg/ml to 0.01 μg/ml, LPS from 500 ng/ml to 0.5 ng/ml, and HaH from 500 μg/ml to 500 ng/ml. The LPS concentration used was comparable to that found for HAH using the LAL assay.

Both bladder cancer cell lines cultured in the presence of LPS proliferated well, regardless of the concentration used. In the cells incubated with 500 ng/ml LPS, proliferation reached 115% for T-24 and 105% for CAL-29 cells. On the contrary, the bladder can-

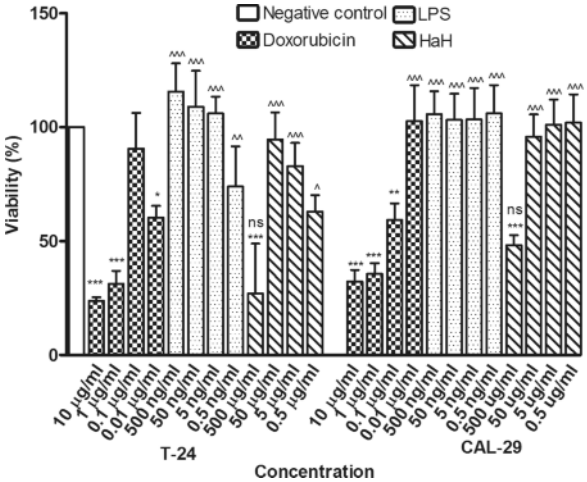

Fig. 6. Effect of the native *Helix aspersa* hemocyanin (HaH) at concentrations varying from 500 μg/ml to 500 ng/ml on the human tumour cell lines T-24 and CAL-29, after 72 h of incubation, and LPS at a concentration ranging from 500 ng/ml to 0.5 ng/ml in the presence of the negative and positive controls [doxorubicin hydrochloride (DOX) at a concentration from 10 μg/ml to 0.01 μg/ml]. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in comparison to the negative control; $^{\wedge\wedge\wedge}P < 0.001$, $^{\wedge\wedge}P < 0.01$, $^{\wedge}P < 0.05$, ns, non-significant in comparison to DOX at 10 μg/ml.

cer cell lines cultured with HaH manifested an antiproliferative effect in a concentration-dependent manner, as well as the positive control (Fig. 6). The cell viability measured after the incubation with HaH was around 48 % for CAL-29 and 27 % for T-24 cells. Consequently, the cytotoxicity in the CAL-29 and T-24 cell lines was 52 % and 73 %, respectively. These results confirmed that the observed antiproliferative effect is due exclusively to the whole HaH complex.

Discussion

Hemocyanins are substances the function, structure, and application of which in medicine have been actively investigated since Olsson *et al.* (1974) reported the immunological potential of KLH and an anticancer effect predominantly against superficial bladder carcinoma. In these studies, KLH was used in subcutaneous immunization or as an intravesical instillation into the bladder. The reported antitumour effect is thought to result from a stimulation of the immune system, because production of protective antibodies against the carbohydrate residues of the KLH was observed along with a cytotoxic T-cell response. Following KLH immunization, patients generate IgG antibodies against KLH (Hortobagyi *et al.*, 1981). There is a proven cross-reactivity between the epitopes of bladder cancer, *Schistosoma mansoni* larval schistosomes, and the carbohydrate epitopes of the hemocyanins (Burke *et al.*, 1977; Wirguin *et al.*, 1995). Antibodies generated against such hemocyanins are probably responsible for the anticancer immune response.

Strong immunogenicity was also shown by *Concholepas concholepas* hemocyanin (CCH) in the murine bladder cancer model (Moltedo *et al.*, 2006) and by *Rapona venosa* hemocyanin (RvH) in hamsters with myeloid Graffi tumours (Toshkova *et al.*, 2009). Arancibia *et al.* (2014) reported that the hemocyanin from the limpet *Fissurella latimarginata* (FLH) induces a stronger humoral immune response, has a stronger antitumour activity, and is superior to KLH and CCH in delaying tumour growth as well as in increasing the survival of mice challenged with B16F10 melanoma cells.

Reports have been published on the *in vitro* effect of KLH against breast, pancreas, prostate, and esophageal adenocarcinoma cancer cell lines: the growth inhibition after 72 h ranged between 6 and 43 % (Riggs *et al.*, 2002, 2005; Mc Fadden *et al.*, 2003). Sarker and Zhong (2014) demonstrated that proliferation of Meth A cells was inhibited markedly by KLH in a dose-dependent manner. The experiments with HlH, RvH, RvH1, and RvH2 showed that HlH has a direct growth-inhibiting effect on bladder cancer cell lines superior to that of KLH at the same concentration (Boyanova *et al.*, 2013).

Therefore, in this study, a previously not tested hemocyanin and one of its two structural subunits, βc-HaH, were isolated from the garden snail *Helix aspersa*, and their anticancer properties were investigated under *in vitro* condition in comparison to HlH. Both whole hemocyanin complexes, HlH and HaH, exhibited a measurable effect on the tested bladder cancer cell lines.

A comparison of the effects of βc-HaH, KLH, and HlH on the CAL-29 and T-24 bladder cancer cell lines showed that the cell viability in the presence of βc-HaH was equivalent to that in the presence of KLH. The lowest cell line viability, *i. e.* the highest antiproliferative effect, was seen with the HlH treatment (Figs. 2 and 3). These findings are in agreement with the previously published results from our group (Boyanova *et al.*, 2013).

The most important observation was that the cytotoxic effect of HaH was not limited to bladder cancer cell lines only, but extended to human prostate

and ovarian carcinoma, malignant glioma, Burkitt's lymphoma, and acute monocytic leukemia as well. The achieved growth inhibition of approximately 48 % with HaH in a concentration of 500 μg/ml against the prostate cancer cell line DU-145 was better than the growth inhibition reported by Riggs *et al.* (2002), *i. e.* 19 to 55 %, with KLH at twice higher concentration.

The mechanism of the *in vitro* action of the hemocyanins has not yet been determined. Mc Fadden *et al.* (2003) reported different apoptotic responses of the SEG-1 and BIC-1 esophageal adenocarcinoma cells to KLH. SEG-1 cells (without the p53 mutation) exhibited an increased apoptotic activity, whereas apoptosis in BIC-1 cells (with the p53 mutation) was not increased, despite significant cell growth inhibition. Riggs *et al.* (2005) demonstrated early and late apoptotic activity of the MCF-7 breast cancer cell line in response to KLH, while no apoptotic activity was induced by KLH in the pancreas cancer cell line (PANC-1), which exhibited only growth inhibition. According to Presicce *et al.* (2008), the stimulation by KLH of the activation and maturation of human monocyte-derived dendritic cells is partially mediated by its interaction with mannose receptors. KLH is known to be rich in mannose and fucose that are the natural ligands of mannose receptors (Stoeva *et al.*, 1999).

In this study, we did not investigate by which mechanism HlH and HaH cause inhibition of tumour cell proliferation *in vitro*. We have evidence that their mechanism of action involves apoptosis (unpublished data) and is related to their carbohydrate residues.

Lamm (2003) reported that a crude KLH preparation had a higher antitumour activity against the MBT-2 murine bladder cancer in C3H/HeJ (endotoxin-resistant) mice than the same KLH after removal of LPS. Pre-immunization and treatment with crude KLH resulted in 100 % survival of the mice, while only 90 % of the animals treated with LPS-free KLH survived. The murine bladder tumour volume was lowest in the treatment with KLH low in LPS, followed by KLH high in LPS, then LPS-free KLH, and was highest in the treatment with LPS alone. Therefore, to show that the observed pronounced cytotoxic effect was due to HaH rather than LPS, we performed an experiment that demonstrated that the endotoxin content did not affect the efficacy of HaH upon the CAL-29 and T-24 bladder cancer cell lines (Fig. 6). On the contrary, the bladder cancer cell lines, when cultured with HaH, manifested an antiproliferative effect in a concentration-dependent manner up to 48 % cell viability for the CAL-29 and 27 % for the T-24 cell line, respectively (Fig. 6). One of the limitations of our study is the use of the *E. coli* endotoxin (LPS) (the same as the *E. coli* endotoxin standard used in the LAL assay for LPS determination) as a positive control. We can therefore not completely exclude the possibility that the antiproliferative effects of the hemocyanins could be due to a particular LPS present in some species-specific microorganisms living in the respective molluscs, but we consider such alternative explanation unlikely.

In conclusion, we have demonstrated that the cytotoxic effects of βc-HaH and the whole KLH complex were similar and lower than those of HlH and HaH. The effect of HaH is not limited to bladder cancer and is not due to endotoxin contamination. Moreover, LPS alone did not inhibit the proliferation of the tested cell lines. Because the whole *H. lucorum* and *H. aspersa* hemocyanins inhibited proliferation of a number of cancer cell lines, we consider HlH and HaH appropriate for further investigations as antitumour agents for different superficial cancers like bladder, oral, esophageal, and skin cancer.

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