JBUON 2015; 20(1): 180-187
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Changes in the gene expression profile of the bladder cancer cell lines after treatment with *Helix lucorum* and *Rapana venosa* hemocyanin

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Summary

Purpose: *The purpose of this study was to elucidate the mechanism of action of the Helix lucorum hemocyanin (HlH), b-HlH-h, and RvH2-g hemocyanins as potential agents against bladder cancer.*

Methods: *We evaluated the viability of 647-V, T-24, and CAL-29 bladder cancer cell lines after treatment with the tested hemocyanins. The cell viability was measured at 72 hrs with MTT and WST-1 assays. Acridine orange/propidium iodide double staining was used to discriminate between apoptotic and necrotic cells. Gene expression profiling of the 168 genes from human inflammatory cytokines and signal transduction pathways were performed on the tumor cells before and after hemocyanins' treatment.*

Results: *The results showed decreased survival of cancer cells in the presence of HlH and two functional units: b-HlH-h and RvH2-g. Acridine orange/propidium iodide double staining revealed that the decreased viability was due to apoptosis. The gene expression data showed up-regulation of genes involved in the apoptosis as well as of the immune system activation, and downregulation of the CCL2, CCL17, CCL21, CXCL1, and ABCF1 genes.*

Conclusions: *The present study is the first to report gene expression in human cells under the influence of hemocyanins. The mechanism of antitumor activity of the HlH, b-HlH-h, and RvH2-g hemocyanins includes induction of apoptosis. In addition to the antiproliferative effect, down-regulation of the genes with metastatic potential was observed. Together with the already known immunogenic effect, these findings support further studies on hemocyanins as potential therapeutic agents against bladder cancer.*

Key words: *apoptosis, bladder, cancer, expression, gene, hemocyanin*

Introduction

The interest in the hemocyanins – large, multisubunit, oxygen-carrying metalloproteinases found in the hemolymph of arthropods and molluscs - dates over 40 years. Many clinical trials as well as a number of experiments showing the properties of the hemocyanins in the treatment of malignant diseases have been reported [1-7].

The proposed mechanism of action of the best studied hemocyanin-keyhole limpet hemocyanin (KLH) involves immune activation due to the presence of cross-reacting epitopes, such as the Thomson–Friederich antigen and N-linked oligosaccharides carbohydrates motifs, as well as T helper type-1 immunity enhancement [7,8]. KLH has been studied in clinical trials as a drug against bladder cancer [9].

Recently, the potential antitumor properties of hemocyanins other than KLH (HlH, Rapana venosa (RvH), and Concholepas concholepas) were demonstrated [7,10,11]. Using in vitro and in vivo methods, Toshkova et al. showed the immunological and antitumor potential of HlH and RvH against myeloid Graffi tumor, and Guerin ascites tumor [12]. In our previous study we also demon-

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Received: 24/06/2014; Accepted: 07/07/2014

strated that HlH and RvH have a direct antiproliferative effect on CAL-29, and T-24 bladder cancer cell lines, and that the antitumor properties of HlH are superior to those of KLH [13]. Although the structure of RvH and HlH hemocyanin has been elucidated [14,15], the mechanism of their action is not clear yet.

The aim of the present study was to elucidate the mechanism of HlH and RvH action on the cell level. Therefore, we performed acridine orange/propidium iodide double staining, and gene expression profiling on the bladder cancer cell lines cultured with and withouth HlH and RvH hemocyanins.

Methods

Test substances - hemocyanins

The test substances in this study were the whole molecules of HlH and RvH hemocyanins, as well as the structural subunits of RvH1 and RvH2, β-HlH-h and α_{N+D}--HlH), and the functional units RvH2-g and HlH-6. These were isolated from the garden snail *H.lucorum* and the marine snail *R.venosa* [14,15], as was described by Dolashka-Angelova et al. [14], and Velkova et al. [15].

All the hemocyanins were filtered through 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, Munich, Germany). Optical density (OD) at 595 nm was read using an ELISA reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA, USA).

Cell culture and viability assay

The bladder tumor cell lines 647-V (grade II), CAL 29 (grade III), and T 24 (grade IV) were obtained from the Interfaculty Institute for Cell Biology, Department of Immunology, University of Tübingen, Germany. They were cultured as a monolayer in Dulbecco's modified Eagle medium (DMEM) (Lonza, Verviers, Belgium), supplemented with 10% foetal 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.

The 647-V, CAL-29, and T-24 bladder cancer cell lines were seeded into 96 well plates (20,000 cells/well). The hemocyanins were added after 18-20 hrs of incubation. The native molecule (RvH), the structural subunits (RvH1, RvH2, β-HlH, and α_{N+D}- -HlH), and the functional units (RvH2-g, and β–HlH-h), at concentration 100 μg/well, were added to the 647-V cell culture. The native molecule of HIH at concentration 500 μg/ml was added to the CAL-29 and T-24 cell line cultures. Doxorubicin (DOX) and mitomycin-C (MIT C) were used as positive controls at concentration of 10 μg/ml and 1 μM/L, respectively. Untreated cell lines were used as negative control. The cell viability was determined by MTT and WST 1 cell proliferation assays (Roche Diagnostics, Mannheim, Germany) at 24, 48, and 72 hrs of incubation.

Acridine orange/propidium iodide double staining

Acridine orange/propidium iodide staining was used to explore the morphological differences in the 647-V cell line treated with β-HlH-h for 72 hrs in comparison to untreated 647-V cells. Thermanox™ coverslips with attached 647-V cells were immediately stained with fluorescent dyes containing acridine orange (10 μg/ml) and propidium iodide (10 μg/ml), mounted onto glass slides and processed for fluorescent microscopy (Leika DM 500B, Wetzlar, Germany).

Acridine orange is permeable to viable cells and can stain the DNA directly. It emits a green fluorescence. Propidium iodide is a dye impermeable to viable cells. It can bind to DNA only when the cells are dead, and emits a red-orange fluorecence.

RNA processing and DNA array

RNA was extracted from the 647-V cell line treated with β-HlH-h and RvH2-g, CAL-29, and T-24 cell lines treated with HlH, and the control cell lines, after 72 hrs of incubation. RNA was isolated using TriFast ready to use reagent (PEQLAB Biotechnologie, Erlangen, Germany). The total RNA (2 μg) was used for cDNA preparation. Double-stranded cDNA was prepared using the RT2 First Strand Kit for cDNA synthesis, Qiagen (sGp Biodynamics LTd, Sofia, Bulgaria). RNA yield and purity were determined using NanoDrop® ND-2000c Thermoscientific (BioMed Future Ltd, Sofia, Bulgaria).

The relative mRNA expression of genes involved in the regulation of apoptosis, cell cycle progression, cellular stress, toxicity, and inflammatory responses were analysed with Human Inflammatory Cytokines & Receptors (PAHS-011) and Human Signal Transduction Pathway Finder (PAHS-014) RT^2 Profiler PCR Arrays (sGp Biodynamics LTd, Sofia, Bulgaria).

Statistics

The data were presented as means with standard deviations. One way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons was used. The level of statistical significance was $p<0.05$.

Differential gene expression was calculated by means of the ΔΔCt method with RPL13A as housekeeping gene. The 3.0-fold change was used as the cut-off threshold to determine up- or downregulation. Statistical analysis was done by the web based SuperArray software -RT^2 Profiler PCR Arrays Data Analysis version 3.5 and g:Profiler web interface [16].

Results

647-V-cell line

MTT assay

The antiproliferative effect of RvH, RvH1, RvH2, RvH2-g, HlH-6, β-HlH-h, and α_{N+D}-HlH was studied on 647-V cell line in comparison to KLH and doxorubicin, based on the established protocol. The lowest cell viability was achieved at 72 hrs of incubation with β-HlH-h and RvH2-g (Figure 1A). The measured viability of the 647-V cells after 24, 48, and 72 hrs of incubation was 67.98%, 22.55%, and 20.84% (β-HlH-h), respectively, as well as 73.51%, 65.79%, and 65.58% (RvH2-g), respectively. This was lower than the viability of the 647-V cell line after culturing with KLH – 92.41%, 88.56% ($p<0.05$) and 75.28% ($p<0.001$) at 24, 48 and 72hrs, respectively.

The viability of the 647-V cells cultured with RvH, RvH II, and HlH-6 was higher than those cultured with KLH. The cell viability measured with RvH-I and α_{N+D}-HlH had approximately the same magnitude with cells cultured with KLH (Figure 1A; $p<0.001$).

Acridine orange/propidium iodide double staining

MTT assay results showed that the β-HlH-h and RvH2-g exhibited the strongest antiproliferative effect on the 647-V cells. As the β-HlH-h showed the strongest action, it was used for an additional analysis with acridine orange/propidium iodide double staining to assess the apoptotic effect of the β-HlH-h on 647-V cells.

From acridine orange/propidium iodide double staining untreated 647-V cells displayed light green nucleus, bright yellow nucleolus, and perinuclear accumulation of orange granules, which pattern of staining was missing in the treated cells (Figure 1 Ba). From the cell line cultured with the β-HlH-h, early and late apoptotic cells

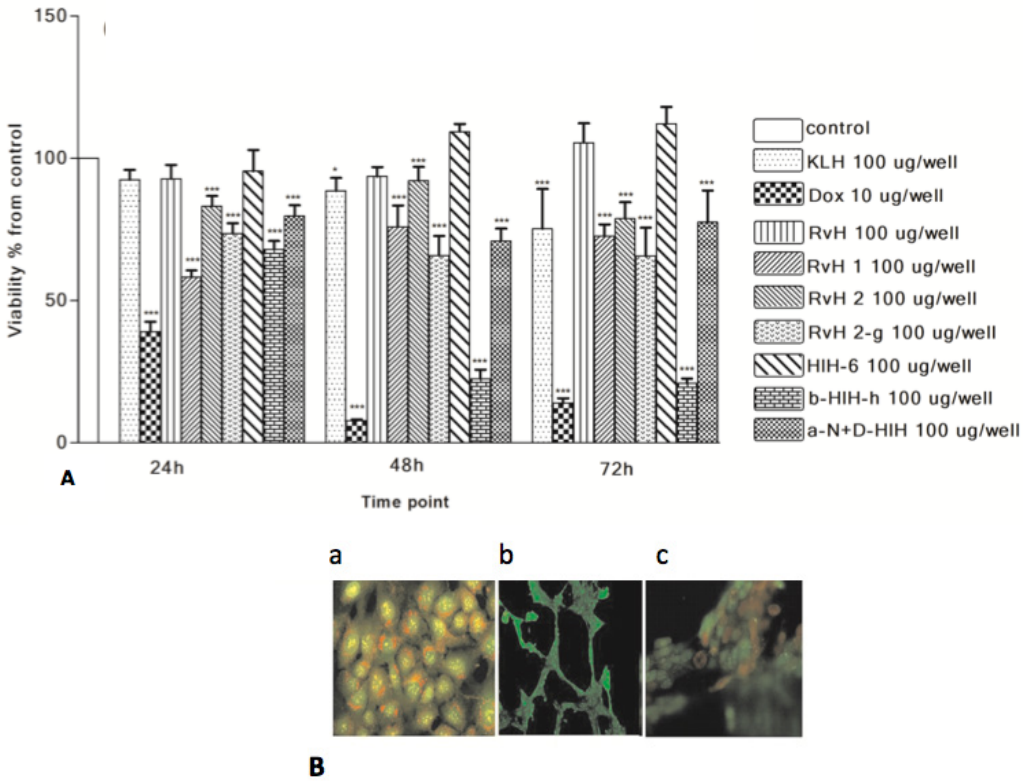

Figure 1. A. Cell line viability of the 647-V human bladder cancer cell line after 24, 48, and 72 hrs of incubation with the native molecule of Rapana venosa Hemocyanin (RvH), and subunits RvH 1, RvH 2, RvH2-g as well as Helix lucorum Hemocyanin subunits HlH-6, ß-HlH-h, and alpha N+D at 100 μg/well concentration in the presence of negative and positive controls [doxorubicin hydrochloride (DOX) and Keyhole Limpet Hemocyanin (KLH)], * $p<0.05$; *** $p<0.001$. **B.** Acridine orange/propidium iodide double staining for determination of apoptotic cells after 72 hrs of ß-HlH-h treatment. **a:** Untreated 647-V cell line (negative control). The cells are presented with light green nucleus, bright yellow nucleolus, and perinuclear accumulation of orange granules that are missing in the treated cells. **b:** Early apoptotic 647-V cells in the presence of ß- HlH-h. These cells are presented with bright green nucleus and chromatin condensation (solid green areas). **c:** Late apoptotic 647-V cells in the presence of ß- HlH-h. These cells are presented with condensation of chromatin and orange stained nucleus (x100).

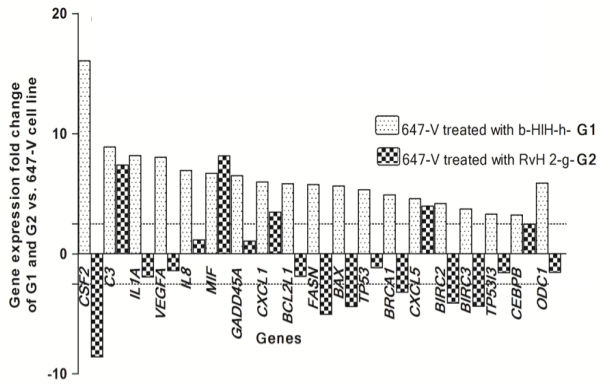

Figure 2. Relative gene expression level of the genes from Human Inflammatory Cytokines & Receptors (PAHS-011) and Human Signal Transduction Pathway Finder (PAHS-014) macroarray gene panels of the treated 647-V cell line, compared to untreated 647-V cell line. 647-V cells treated with ß-HlH-h are indicated as Group 1 **(G1)** and 647-V cells treated with RvH2-g hemocyanins are indicated as Group 2 **(G2)**. The 3.0-fold change in gene expression was used as the cut-off threshold to determine up- or down-regulation.

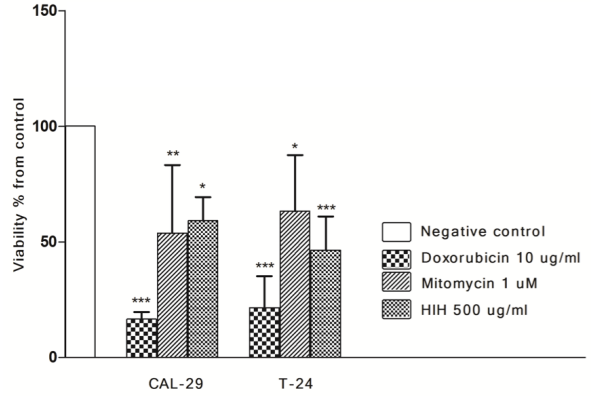

Figure 3. Cell line viability of the bladder human tumor cell lines CAL-29 and T-24 after 72 hrs of incubation with the native molecule of HlH at 500 µg/ml concentration. Negative control and positive controls (doxorubicin hydrochloride and mitomycin-C) were used; * p<0.05, ** p<0.01, *** p<0.001.

were observed. Early apoptotic cells were demonstrated with bright green nucleus and chromatin condensation (solid green areas) (Figure 1 Bb). Late apoptotic cells showed chromatin condensation and orange stained nucleus (Figure 1Bc). A change in the shape and size of the treated cells was also observed.

Gene expression profiling of the 647-V cell line

Because the strongest antiproliferative effect on 647-V cells was achieved with the β-HlH-h and RvH2-g test substances at 72 hrs, further experiments for gene expression evaluation were carried out with these two functional units. The gene expression analyses of the 647-V cells treated with β-HlH-h (Group 1) are shown on Figure 2, and demonstrate upregulation of genes coding for proteins involved in PI3K-Akt (*VEGFA, BCL2L1, TP53, IL4R, FN1, GYS1, BRCA1, CCND1, CDK2, ATF2*) and p53 signalling pathways (*GADD45A, TP53, TP53I3, BAX, CCND1, CDK2, CDKN2A*), apoptosis (*IL1A, BCL2L1, TP53, BAX, BIRC2, BIRC3*), TNF signalling (*CXCL1, CXCL5, CEBPB, CCL20, CSF2, BIRC2, BIRC3, ATF2*), cell surface receptor signalling (*C3, MIF, CXCL1, IL1A, VEGFA, IL8, BCL2L1, TP53, IL4R, PMEPA1, CSF2, BAX, LEF1, CCND1, BIRC2, BIRC3, HSPB1*), leukocyte activation (*MIF, IL8, TP53, IL4R, CSF2, BAX, LEF1, CDKN2A*), and cell cycle regulation (*TP53, CCND1, CDK2, CDKN2A*).

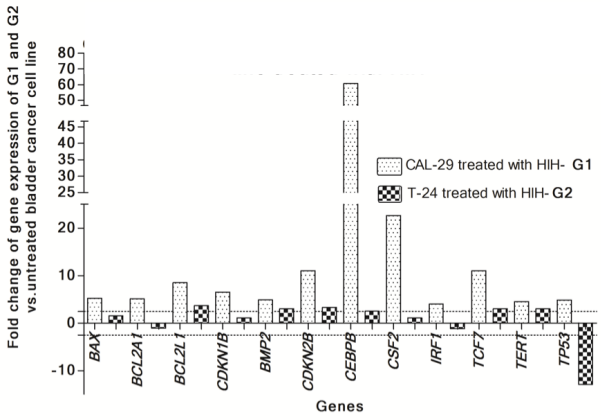

Figure 4. Relative expression level of the genes involved in the programmed cell death on the CAL-29 and T-24 bladder cancer cell lines measured after 72 hrs incubation with the native molecule of HlH at 500 µg/ml concentration. The 3.0-fold change in gene expression was used as the cut-off threshold to determine up- or down-regulation.

In Group 2- 647-V cell line treated with RvH2-g, the genes *C3, MIF, CXCL1, CXCL5*, and *CEPB* were overexpressed compared to untreated 647-V cells (Figure 2).

In both groups *TOLLIP, CCL2, IL1RN, TNF, CCL21, CCL17, IL36RN, IL10*, and *IL9* gene were under-regulated compared to untreated cell line.

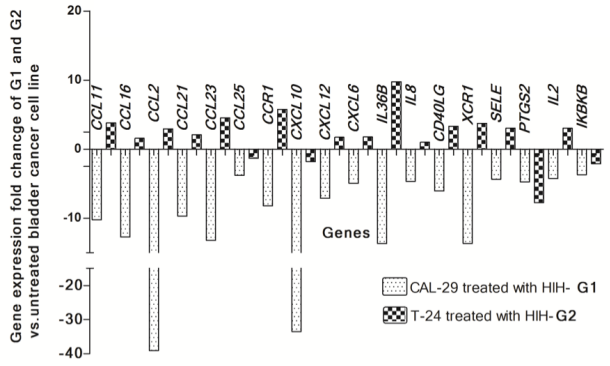

Figure 5. Relative expression level of the genes associated with response to angiogenesis, lipopolysaccharide, and wounding on the CAL-29 and T-24 bladder cancer cell lines after 72 hrs incubation with total molecule HlH at 500µg/m concentration l. The 3.0-fold change in gene expression was used as the cut-off threshold to determine up- or down-regulation.

CAL-29 and T-24 cell lines

WST-1 assay

The cell line viability measured at 72 hrs of incubation with HlH in a concentration of 500 µg/ml was 59.2% for CAL-29 ($p<0.05$) and 46.36% for T-24 ($p<0.001$) cell line, respectively (Figure 3).

Gene expression profiling of the CAL-29 and T-24 cell line

A number of the up-regulated genes for both cell lines treated with HlH were associated with immune system activation, transcriptional misregulation and programmed cell death (Figure 4). The downregulated genes were associated with response to wounding, lipopolysaccharide, and angiogenesis (Figure 5).

For the CAL-29 cell line we found upregulation of protein coding genes involved in the immune processes (*CXCR1, CEBPB, CSF2, IL36A, CDKN2A, IL9, MIF, BCL2L1, IL37, CCR4, CDKN1B, CCL13, CCR2, CX3CR1, IL10, BAX, TP53, IL5RA, CXCL13, CCR8, IRF1, SELPLG, IL1RN*), response to external stimulus (*CXCR1 ,CCND1, MIF, CCR4, CCL13, CCR2, CX3CR1, IL10, TP53, CXCL13, CCR8, IRF1*), response to other organism (*CEBPB, CSF2, MIF, BCL2L1, CX3CR1, IL10, CXCL13, IRF1*), leukocyte activation (*CSF2, CDKN2A, MIF, CCR2, CX3CR1, IL10, BAX, TP53, IRF1, SELPLG*), cell surface receptor signalling pathway (*CXCR1, CCND1, CSF2, MIF, BCL2L1, CCR4, CDKN1B, CCR2, CX3CR1, BAX, BMP2, TP53, IL5RA, TERT, CXCL13, CCR8, IRF1, IL1RN*), Jack-STAT signalling pathway (*CCND1, CSF2, IL9, BCL2L1, IL10, IL5RA*), chemokine signalling pathway (*CXCR1, CCR4, CCL13, CCR2, CX3CR1, CXCL13, CCR8*), and apoptosis-related processes (*CEBPB, CSF2, CDKN2A, MIF, BCL2L1, CARD18, CDKN1B, CX3CR1, IL10, BAX, BCL2A1, BMP2, TP53, TERT, IRF1, IL1RN*). Among the latter, genes coding for *CEBPB, CDKN2A, BCL2L1, CDKN1B, IL10, BAX, BMP2, TP53,* and *IRF1* were related to positive regulation of programmed cell death, and *CEBPB, CDKN2A, CDKN1B, BAX, TP53,* and *IRF1* genes were responsible for induction of programmed cell death.

Downregulated genes took part in cell proliferation (*BCL6, CCL11, CCL2, CCL23, CXCL1, CXCL10, CXCL12, IL1A, IL1B, IL8, CD40LG, VCAM1, VEGFA, PTGS2, JUN, IL2*), response to lipopolysaccharide (*CCL2, CXCL10, IL1B, IL8, SELE, PTGS2, JUN*), and wounding (*BCL6, C3, CCL11, CCL16, CCL2, CCL21, CCL23, CCL25, CCR1, CXCL1, CXCL10, CXCL6, IL10RB, IL1A, IL1B, IL36B, IL8, CD40LG, XCR1, VCAM1, VEGFA, SELE, PTGS2, JUN, IKBKB, IL2*), cell migration (*CCL11, CCL2, CCL21, CCL23, CCL25, CCR1, CCR6, CXCL10, CXCL12, IL1B, IL8, VCAM1, VEGFA, SELE, PTGS2, MMP10*), and angiogenesis (*C3, CCL11, CCL2, CXCL10, CXCL12, IL1A, IL1B, IL8, VEGFA, PTGS2, JUN*).

In the T-24 cells cultured with HlH, we found upregulation for genes related to almost the same pathways like in CAL-29 cell line: immune processes (*IFNA2, CXCR1, IL37, LTB4R, IL1B, IL5, IL10RB, CCL2, AIMP1, IL36B, CCR6, CXCL2, CCR7, CXCR2, CCL8, IL5RA, IL36A, CCR1, CCL4, CCL23, IL9, CXCL14, MYC, C5, CXCL9, IL10, CCR9, CCL11, BCL2L1, CCL3, CCL7, CD40LG, CDKN2B, SPP1, CXCL11, BCL6, BCL2*), response to external stimulus (*CXCR1, IL1B, CCL2, AIMP1, CCR6, CXCL2, CCR7, CXCR2, CCL8, CCR1, CCL4, CCL23, CXCL14, MYC, C5, CXCL9, IL10, CCR9, CCL11, XCR1, CCND1, CCL3, CCL7, CDKN2B, HOXA1, SPP1, CXCL11, BCL6*), and other organism (*IFNA2 ,IL1B, CCL2, CXCL2, CCR7, CCL8, CCL4, CXCL9, IL10, CCL11, BCL2L1, CCL3*), leukocyte activation (*IFNA2, IL1B, IL5, CCL2, CCR7, CXCR2, IL10, CCL3, CD40LG, BCL6*), programmed cell death (*IFNA2, IL1B, CCL2, AIMP1, CCR7, CXCR2, MYC, C5, IL10, BCL2L1, CCL3, CD40LG, BCL6, BCL2, BMP2*), among them- positive regulation of programmed cell death (*IFNA2, MYC, C5, IL10, BCL2L1, CCL3, CD40LG, BCL6, BMP2*), cell surface receptor signalling (*IFNA2, CXCR1, LTB4R, IL1B, IL5, CCL2, CCR6, CCR7, CXCR2, IL5RA, CCR1, IL9R, CCL23, MYC, C5, CXCL9, CCR9, XCR1, BCL2L1, CCND1,*

CCL3, CDKN2B, BMP2), Jack-STAT signalling (*IFNA2, IL22, IL5, IL10RB, IL10RA, IL5RA, IL9R, IL9, MYC, IL10, BCL2L1, CCND1*), and chemokine signalling pathway (*CXCR1, CCL2, CCR6, CXCL2, CCR7, CXCR2, CCL8, CCR1, CCL4, CCL23, CXCL14, CXCL9, CCR9, CCL11, XCR1, CCL3, CCL7, CXCL11*).

The downregulated genes in the T-24 cell line treated with HlH were related to regulation of cell proliferation (*NOS2, PPARG, CXCL1, ODC1, PTGS2, VEGFA, TP53*), negative regulation of transcription from RNA polymerase II promoter (*NRIP1, NFKB1, PPARG, VEGFA, TP53*), and response to wounding (*NOS2, NFKB1, BIRC3, PPARG, CXCL1, PTGS2, VEGFA, TP53*).

Discussion

The present study is the first to report gene expression in human cells under the influence of hemocyanins. We investigated the changes in gene expression of the 647-V (grade I), T-24 (grade II), and CAL-29 (grade III) bladder cancer cell lines under the tested hemocyanins, and offered new insights into their molecular mechanism of action.

First we showed a growth inhibitory effect of RvH2-g and β-HlH-h hemocyanins on 647-V cells (Figure 1a). This *in vitro* effect was significantly stronger than that of the KLH, used as a drug against bladder cancer in clinical practice [3,17,18]. These results correlate with our previous study where we demonstrate that the total molecule of HlH has a superior growth inhibiting effect than KLH on CAL-29 and T-24 bladder cancer cell lines [13].

The 647-V cell line cultured with β-HlH-h showed up-regulation of genes involved in the p53, TNF signalling pathways, and apoptosis. These findings are consistent with the experiments, where we showed that the reduced 647-V cells viability is due to apoptosis (Figure 1).

Among the downregulated genes in the 647-V cell line, treated both with β-HlH-h (Group 1 on Figure 2) and RvH2-g (Group 2 on Figure 2) are the genes *CCL2* (for monocyte chemotactic protein-1, MCP-1), *CCL17*, and *CCL21*. Levels of MCP-1 correlate with the clinical stage and tumor grade in patients with bladder cancer [19]. The expression of chemokine ligands CCL17 and CCL21 is associated with tumor metastasis and serves as a prognostic factor in gastric cancer patients [20].

The viability of T-24 and CAL-29 bladder cancer cell lines under the HlH action was found to be similar to our previous results where we demonstrated the viability under HlH of the same cell lines to be 49.34% and 62.73%, respectively [13].

The overexpressed genes *CEBPB, CDKN2A, BCL2L1, CDKN1B, IL10, BAX, BMP2*, and *TP53* for CAL-29 cell line and *IFNA2, MYC, C5, IL10, BCL2L1, CCL3, CD40LG, BCL6, and BMP2* for T-24 cell line are related to positive regulation of apoptosis, and *CEBPB, CDKN2A, CDKN1B, BAX, TP53,* and *IRF1* genes are responsible for induction of programmed cell death [16]. As with the 647-V line, the finding of apoptosis in the WST-1 assay is consistent with the up-regulation of genes inducing apoptosis in the genetic analysis.

Unlike the case with the genes related to apoptosis, we had no relevant reference assay (such as the MTT and WST-1 assays) to account for an explanation for the observed changes in the expression of the other tested genes and to show if such changes are at all translated into altered cell function. We have nevertheless tried to give possible speculative suggestions for a potential significance of the observed changes in the gene expression across diverse pathways. For other pathways, such as the JAK/STAT pathway, the pathways related to external stimuli or the cell surface receptor pathways, there is a very broad range of possible functions to allow for a specific suggestion why the observed change in the gene expression could be relevant. For example, the changed expression of some genes involved in the JAK-STAT signalling pathway and cell surface receptor signalling pathway in the HlH treated cell lines could be attributed to the regulation of immune effector cells, leading to controlled inflammatory responses [21].

The activation of the JAK/STAT pathway leads in general to the transcription of genes associated with cell survival [22,23]. Additionally, STAT3 is required in many aspects of tumorigenesis, including differentiation, proliferation, apoptosis, increased sensitivity to cytotoxic agents, angiogenesis, recruitment of immune cells, and metastasis [24].

The downregulated genes of both cell lines treated with HlH compared to the untreated cell line are related to response to lipopolysaccharide and wounding, cell proliferation and angiogenesis. Similar findings are reported for intravesically applied BCG, where they are interpreted as an expression of the effect of BCG to inhibit neovascularization [25,26].

In both cell lines (CAL-29 and T-24) cultured with HIH, the Chemokine C-X-C motif ligand 1 (*CXCL1*) and *ABCF1* genes, which are associated

with the metastatic potential in cancer, showed decreased expression compared to the untreated control. An aberrant higher expression of CXCL1 is associated with the growth and progression of certain tumors and higher pathologic stages in bladder cancer *in vivo*. Urinary CXCL1 levels are significantly higher in patients with invasive bladder cancer (pT1-4) than those with non-invasive pTa tumors [27,28]. *ABCF1* gene is involved in the processes of cell migration, cell proliferation, and malignant transformation [29].

Our findings of upregulation of genes involved in immune processes and leukocyte activation correspond to previous studies on the hemocyanin immunogenic properties [8,10,30,31]. It was reported that in vivo KLH induces a protective antibody production against the carbohydrate sequence along with a cytotoxic T-cell response [30] and development of delayed-type hypersensitivity responses (DTH) [31]. The specific TFN-gamma production was demonstrated in splenocyte cultures of mice from HvH (HlH) and RvH hapten carriers. For HvH (HlH) this production lasted longer than for KLH and RvH [10].

In conclusion, the mechanism of antitumor activity of the tested hemocyanins includes the induction of apoptosis. In addition to the decreased cell viability, a downregulation of the genes with metastatic potential is observed. In the combination with their already known systemic (immunogenic) effect, these findings support further studies of *Helix lucorum* hemocyanins as potential therapeutic agents against bladder cancer.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinstchaft (DFG-STE 1819/5-1/2012), Germany and Bulgarian Ministry of Education, projects ТК01-496/2009 and ДМУ03/48, 12.12.2011.

References

1. Stellar Biotechnologies. KLH in Clinical Trials. 2013; Available from: http://www.klhsite.org/klh-clinical-trials/category/cancer-bladder.Access date 28.10.2013.
2. Olsson C, Chute R, Rao C. Immunologic Reduction for Bladder Cancer Recurrence Rates. J Urol 1974;111:173-176.
3. Jurincic C, Engelmann U, Gasch J, Klippel K. Immunotherapy in bladder cancer with keyhole-limpet hemocyanin: a randomized study. J Urol 1988;139:723-726.
4. Lamm D,Morales A, Grossman H et al. Keyhole limpet hemocyanin (KLH) immunotherapy of papillary and in situ transitional cell carcinoma of the bladder. A multicenter phase I-II clinical trial. J Urol 1996; 155:1405-1412.
5. Riggs DR, Jackson B, Vona-Davis L, Mc Fadden D. In Vitro Anticancer Effects of a Novel Immunostimulant: Keyhole Limpet Hemocyanin. J Surg Res 2002;108:279-284.
6. Riggs DR, Jackson BJ, Vona-Davis L, Nigam A, Mc Fadden DW. In vitro effects of keyhole limpet hemocyanin in breast and pancreatic cancer in regards to cell growth, cytokine production, and apoptosis. Am J Surg 2005;189:680-684.
7. Moltedo B, Faunes F, Haussmann D et al. Immunotherapeutic Effect of Concholepas Hemocyanin in the Murine Bladder Cancer Model: Evidence for Conserved Antitumor Properties Among Hemocyanins. J Urol 2006;176:2690-2695.
8. Kurokawa T, Wuhreh M,Lochnit G, Geyer H, Markl J, Geyer R. Hemocyanin from the keyhole limpet Megathura crenulata (KLH) carries a novel type of N-glycans with Gal(beta1-6)Man-motifs. Eur J Biochem 2002;269:5459-5473.
9. US National Institutes of Health. Keyhole Limpet Hemocyanin Compared With Doxorubicin in Treating Patients With Bladder Cancer. 2013; Available from: http://www.clinicaltrials.gov/ ct2/show/ NCT00006034?term=KLH+bladder&rank=1. Access date 06.02.2013.
10. Dolashka-Angelova P, Stefanova T, Livaniou E et al. Immunological potential of Helix vulgaris and Rapana venosa hemocyanins. Immunol Invest 2008;37:822-40
11. Iliev I, Toshkova R, Dolashka-Angelova P et al. Haemocyanins from Rapana venosa and Helix vulgaris display an antitumour activity via specific activation of spleen lymphocytes. Compt Rend Acad Bulg Sci 2008;61:203-210.
12. Toshkova R, Ivanova E, Hristova R, Voelter W, Dolashka-Angelova P. Effect of Rapana venosa Hemocyanin on Antibody-Dependent Cell Cytotoxicicity (ADCC) and Mitogen Responsibility of Lymphocytes from Hamsters with Progressing Myeloid Tumors. World J Med Sci 2009;4:135-142.
13. Boyanova O, Dolashka P, Toncheva D, Rammensee H-G, Stevanovic S. In vitro effect of molluscan hemocyanins on CAL-29 and T-24 bladder cancer cell lines. Biomed Rep 2013;1:235-238.
14. Dolashka P, Genov N, Pervanova K, Voelter W, Geiger M, Stoeva S. Rapana thomasiana grosse (gastropoda) haemocyanin: spectroscopic studies of the structure in solution and the conformational stability of the

native protein and its structural subunits. Biochem J 1996;315:139-144.

15. Velkova L, Dimitrov I, Schwarz H et al. Structure of hemocyanin from garden snail Helix lucorum. Comp Biochem Physiol 2010;157:16-25.
16. Reimand J, Kull M, Peterson H, .Hansen J, Vilo J. g:Profiler-a web-based toolset for functional profiling of gene lists from large-scale experiments. Nucleic Acids Res 2007;35:W193-200.
17. Lamm DL. Laboratory and Clinical Experience with Keyhole limpet hemocyanin (Immucothel ®) in Superficial Bladder Cancer. J Urol Urogynäkol 2003;2:18-21.
18. Jurincic-Winkler CD, Metz KA, Beuth J, Klippel KF. Keyhole Limpet Hemocyanin for Carcinoma in situ of the Bladder: A Long-Term Follow-Up Study. Eur Urol 2000;37:45-49.
19. Amann B, Perabo FG, Wirger A, Hugenschmidt H, Schultze-Seemann W. Urinary levels of monocyte chemo-attractant protein-1 correlate with tumour stage and grade in patients with bladder cancer. Br J Urol 1998;82:118-121.
20. Hwang TL, Lee LY, Wang CC, Liang Y, Huang SF, Wu CM. CCL7 and CCL21 overexpression in gastric cancer is associated with lymph node metastasis and poor prognosis. World J Gastroenterol 2012;18:1249-1256.
21. Zhang H, Nguyen-Jackson H, Panopoulos AD, Li HS, Murray PJ, Watowich SS. STAT3 controls myeloid progenitor growth during emergency granulopoiesis. Blood 2010;116:2462-2471.
22. Henson ES, Gibson SB. Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. Cell Signal 2006;18:2089-2097.
23. Kisseleva T, Bhattacharya S, Braustein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene 2002;285:1-24.
24. Yu H,Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 2009;9:798-809.
25. Pavlovich CP, Kraling BM,Stewart RJ et al. BCG-induced urinary cytokines inhibit microvascular endothelial cell proliferation. J Urol 2000;163:2014-2021.
26. Morgan BE, Salup R, Morgan MB. Differential C-erbB-2 and VEGF expression following BCG immunotherapy in superficial papillary transitional cell carcinoma of the bladder. Urol Oncol 2002;7:67-72.
27. Miyake M, Lawton A, Goodison S et al. Chemokine (C-X-C) ligand 1 (CXCL1) protein expression is increased in aggressive bladder cancers. BMC Cancer 2013;13:322.
28. Kawanishi H, Matsui Y, Ito M et al. Secreted CXCL1 is a potential mediator and marker of the tumor invasion of bladder cancer. Clin Cancer Res 2008;14:2579-2587.
29. Cunha IW, Carvalho KC, Martins WK et al. Identification of genes associated with local aggressiveness and metastatic behavior in soft tissue tumors. Transl Oncol 2010;3:23-32.
30. Burke GP, Smith KA, Stocking R, Ferm M, Mc Intyre OR. Anti-keyhole limpet hemocyanin antibody in normal unsensitized individuals. J Allergy Clin Immunol 1977;59:309-313.
31. Hortobagyi GN, Smith TL, Swenerton KD et al. Prognostic value of prechemotherapy skin tests in patients with metastatic breast carcinoma. Cancer 1981;47:1369–1376.