Anti-Herpes Effect of Hemocyanin Derived from the Mollusk *Rapana thomasiana*

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The cytotoxicity and the antivirus activity of native hemocyanin, RtH, derived from the Bulgarian marine mollusk *Rapana thomasiana* and its structural isoform, RtH2, against HSV replication was evaluated on three HSV strains – two *wt* strains, TM (HSV 1) and Bja (HSV 2), and one ACV^R mutant with *tk* gene mutation, DD (HSV 2). The experiments were performed on continuous RD 64 cells and three HSV 1 and HSV 2 strains were used, two mutants sensitive to acyclovir and one resistant mutant.

Both compounds were found to be effective inhibitors of *wt* HSV replication. Both compounds did not exhibit any effect on the infectious virus yield on ACV^R mutant. The most promising, active and selective, anti-HSV agent, especially to genital herpes virus, was found to be the functional unit of native hemocyanin – RtH2. RtH2 did not induce apoptosis/necrosis 8 h after virus infection and the target of its action, was found to be the viral but not the host cell DNA.

Key words: Herpes Simplex Virus, *Rapana thomasiana,* Hemocyanin, Resistance

Introduction

The most common infections are those caused by human herpes viruses including the worldwide spread Herpes simplex viruses 1 and 2 (HSV 1 and HSV 2) (Arao *et al*., 1999; Arvin and Prober, 1995). Acyclovir (ACV) is a prodrug and it is the first nucleoside-based therapeutic effective for the treatment of primary and recurrent HSV infections (Elion, 1989; O'Brien and Campoli-Richards, 1989). However, under systematic administration resistant mutants appear with high frequency and their main sources are immune-compromised individuals (Vere Hodge, 1993; De Clercq *et al*., 2001; Kimberlin *et al*., 1995; Crumpacker and Shaeffer, 2002). The most common causes of resistance are mutations in the thymidine kinase (*tk)* gene. The problem for effective treatment of HSV infections is still open, since the resistance to ACV and the cross-resistance to other nucleoside analogues increases with relatively high frequency.

Mollusk hemocyanins have been studied intensively for many years with respect to their structure and function (Van Holde and Miller, 1995; Van Holde *et al.*, 2001). For over 40 years, researchers have been discovering that the mollusk hemocyanin polymers have the ability to cause strong immune responses in mammals due to their xenogenic nature and their big size, which support T and B lymphocyte multi-epitope recognition. Indeed, hemocyanins have been extensively used as carrier proteins for haptens and peptides, as standard antigens in the studies of the immune response, and as nonspecific immunostimulant (Harris and Markl, 1999; Markl *et al*., 2001). The hemocyanin from the mollusk keyhole limpet (*Megathura crenulata*), known as KLH, has been used for the above purposes. Besides these biomedical effects, KHL has been used in the diagnosis and immunotherapy of *Shistosomias* (Li *et al*., 1994), in drug addiction (Ettinger *et al.*, 1997;

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Beike *et al.*, 1997), and as a component of experimental synthetic minimal viral vaccines against AIDS (Naylor *et al.*, 1991) and papilloma virus (Meyer *et al.*, 1998), and exhibit also antitumour activity against different kinds of tumours (Olsson *et al.*, 1974; Lamm *et al.*, 2000; Jurincic-Winkler *et al.*, 2000; Sandmaier *et al.*, 1999; McFadden *et al.*, 2003; Vona-Davis *et al.*, 2004).

All the above-mentioned studies show that the evaluations of the antiviral activity of many mollusk hemocyanins are of interest. This prompted us to look for the anti-herpes viral effect of native blue copper oxygenated respiratory protein RtH – *Rapana thomasiana* hemocyanin, and its structural isoform RtH2 found in the hemolymph of the marine mollusk *Rapana thomasiana* in cultured cells. The source of RtH lives in shallow waters of the Black Sea coast. *Rapana thomasiana* hemocyanin is a mixture of two hemocyanin isoforms, termed RtH1 and RtH2 (Idakieva *et al.*, 2001). This is the first one of a series of laboratory studies directed to the biological activity of *Rapana thomasiana* hemocyanin and its structural isoform.

Materials and Methods

Hemocyanin and chemicals

Rapana thomasiana mollusk specimens were caught at the west coast of the Golden Sands region near Varna, Bulgaria. Hemolymph was collected from animals weighing ~20–25 g. The crude material was filtered on gauze and centrifuged for 30 min at 5000 rpm. The isolation of the hemocyanin was performed as described previously by Boteva *et al.* (1991) using a Spinco ultracentrifuge at 180,000 rpm (Spinco Biotech, Chennai, India). The obtained material was stored at −20 °C in the presence of 20% sucrose until used. DEAE-Sepharose CL-6B was obtained from Fluka AG (Basel, Switzerland). The chemicals and reagents used were of analytical grade.

Isolation of the Rapana thomasiana structural subunits and the functional unit RtH2

Native *R. thomasiana* hemocyanin was dissociated to subunits by dialysis against 0.05 M glycine/NaOH buffer containing 0.02 M EDTA, pH 9.6. The two structural subunits, RtH1 and RtH2, were separated and purified by ion-exchange chromatography on DEAE-Sepharose CL-6B according to the procedure described by Idakieva *et al.* (1993). Each of the two subunits contains eight functional units of ~50 kDa. The functional unit has a single copper-containing site reversibly binding the dioxygen molecule. FU RtH2 is the fifth unit from the amino-terminus of the RtH2 polypeptide chain. It was isolated after treatment of RtH2 with plasmin, separation of the products, and subsequent trypsinolysis of a fragment containing RtH2, as described by Stoeva *et al.* (2002). The FU was purified to homogeneity by FPL chromatography on a Mono Q (HR 10/10) column (Amersham Biosciences, Freiburg, Germany).

Preparation of compounds

Rapana limpet hemocyanin (RtH) and its structural subunit (RtH2) were first dissolved in phosphate buffer, pH 7.4, to concentrations of 21 mg/ml and 11 mg/ml, respectively (stock solutions). The following dilutions were made in minimum essential medium (MEM; Applichem, Darmstadt, Germany) supplemented with 5% FBS (BioWhittaker, Verviers, Belgium) and antibiotics (Sopharma, Sofia, Bulgaria). All compound solutions were stored at 4 °C. The anti-herpes drug ACV was used as control. It was first dissolved in dimethylsulfoxide (DMSO) (Applichem) and diluted in culture medium.

Cells and viruses

Continuous rhabdomyosarcoma cell line, RD 64 (National Centre of Infectious and Parasitic Diseases, Laboratory of Cell Cultures, Sofia, Bulgaria), was used. The cells were grown at 37 °C in MEM medium supplemented with 10% FBS and antibiotics. During the experiments the FBS content was reduced to 5%. Antiviral experiments were done on the following three viruses: two wild strains, (HSV 1) and Bja (HSV 2), and one mutant resistant to ACV, DD (ACV^R, HSV 2) (National Centre of Infectious and Parasitic Diseases, Laboratory of Herpesviruses, Sofia, Bulgaria). Viruses were grown in RD 64 cell monolayers. Cultures were harvested at full cytopathic effect (CPE), freezed, thawed and stored at −70 °C.

Methods of detecting the effect on growth kinetics, cell viability, maximal nontoxic concentration (MNC) and concentration required to inhibit cell viability by 50% (CC_{50})

Confluent monolayer was washed, covered with media containing the test hemocyanins RtH and RtH2 in concentrations from 0.01 μg/ml to 7000 μg/ml, and cultured at 37 °C for 48 h and 72 h. Cells grown in compound-free medium served as a control. The CPE was read by microscopy of an unstained cell monolayer and by the trypan blue exclusion test. The growth kinetics and cell viability were calculated as percentage from the total number of cells per sample. Each experiment was done in triplicate. The MNC and CC_{50} values for each compound were calculated from the dose-response curves. The maximal concentration, which altered neither the morphology of monolayer nor the cell survival rate, was recognized as MNC. Therapeutic efficacy (TE) was calculated as the CC_{50} to MNC ratio.

Assay of antiviral activity

The effect of the compounds tested on HSV replication was evaluated on the basis of their effects on the infectious HSV titer. RD 64 cells grown in 96-well plates were infected, and 1 h later cells were covered with medium modified with the hemocyanin RtH and its structural subunit RtH2 in ten-fold dilutions (starting from MNC). CPEs and virus titers were determined after 48 h (for wild strains) or 72 h (for resistant mutants). Effective concentrations required to inhibit the virus yield by 50% (IC_{50}) were calculated from dose-regression lines. Selectivity indexes (SI) were calculated as CC_{50} to IC_{50} ratios. Data were compared to that of ACV.

Definition of the influence of apoptosis/necrosis on noninfected cells and on cells infected with HSV

The staining methods of one double-chain helix DNA have been used with a 0.1% solution of acridine orange, and for mitochondria a solution of 0.1% of Janus Green B has been used. Both original methods have been suggested for prokaryotic models. As we worked on an eukaryotic model of cells we adapted the following modifications for the purpose of the corresponding system: (i) fixing of cells with methanol not with formaldehyde; (ii) after a standard procedure of staining in view of further conservation of the preparations we introduced a treating with glycerol/PBS (1:1). The experiments were carried out after 8 h of infection in the initial period of active virus morphogenesis. The controls were as follows: 1) cells which were neither infected nor treated with the investigated compounds; 2) cells that were not infected but were treated with compounds; 3) cells infected with HSV which were cultivated in a medium without an inhibitor.

Results and Discussion

Cytotoxic activity of Rapana thomasiana hemocyanin (RtH) and its structural subunit (RtH2)

Both MNC and CC_{50} values were evaluated simultaneously by morphological and by cell survival criteria. When microscopic observation of the morphology of the monolayer was carried out at 24 h, 48 h and 72 h after the treatment with RtH and RtH2 in a concentration range from 10,000–50,000 μg/ml a typical cytopathology characterizing the toxic effect was registered. This typical cytopatology was visualized in a round form of the cells and their grouping in "islands" isolated from the surface of the cells. We found some nuclear morphology changes. When a treatment with concentrations lower than 50,000 μg/ml was performed, no essential change was registered in the monolayer in comparison with the control.

In vitro cytotoxicity data for the tested hemocyanins are summarized in Table I.

Preliminary data presented here show that the tested hemocyanins expressed a different degree of cytotoxicity against the RD 64 cell line. This phenomenon was dose-dependent. Both compounds exhibited lower cytotoxicity than ACV. The MNC ranged from 10–110 μg/ml. On the basis of MNC values at 24 h, 48 h and 72 h after treatment the investigated compounds can be arranged as follows: ACV > RtH > RtH2.

The weakest cytotoxic hemocyanin according to MNC was the structural subunit RtH2, whose MNC values were 5- and 4-times lower than those of the native form. Obviously, the structural subunit decreases the cytotoxicity of native hemocyanin. The data presented by Table I show that the cytotoxicity of both compounds is predetermined by structural specificities.

According to CC_{50} values hemocyanins at 24 h, 48 h and 72 h after treatment can be arranged as follows: ACV > RtH > RtH2.

The CC_{50} ranged from 30–10,000 μg/ml. In addition, the cytotoxicity of RtH increased 3.8-times

Table I. Cytototxic effect of native hemocyanin and its subunit on RD 64 cells, at 24 h, 48 h and 72 h.

Compound	MNC [μg/ml]			CC_{50} [μg/ml]		
	24 h	48 h	72 h	24 h	48 h	72 h
RtH	20.87	23.50	21.86	4 985	3 669	1 320
RtH2	110	110	87	6 950	7 260	10 000
ACV	12.5	12.5	12.5	45.5	45.5	35.5

with the prolonging of action. The cytotoxicity of RtH2 decreased with the prolonging of treatment, the induction for this was the correspondingly 1.4-times higher value of CC_{50} at 72 h compared to those at 24 h of action.

Based on the data from cytotoxicity experiments we calculated the CC_{50} to MNC ratio – therapeutic efficacy (TE). The ratio characterizes the tolerable concentration range in which the particular compound could be applied avoiding significant cell alterations. The data for TE determined at 24 h, 48 h and 72 h after treatment with the tested compounds are presented on Table II.

Table II. Therapeutic efficacy (TE) of native hemocyanin and its functional subunit on RD 64 cells, at 24 h, 48 h and 72 h.

Compound	$TE \cdot 10^3$		
	24 h	48 h	72 h
RtH	0.24	0.16	0.06
RtH2	0.06	0.07	0.11
ACV	0.004	0.004	0.003

On the basis of the obtained data the compounds can be divided into two groups:

Group 1 – substances with TE increase with prolonged time of action. Here was RtH2; its TE at 72 h was 1.8-times higher than the one determined after 24 h of action.

Group 2 – compounds with TE decrease with prolonged time of action. Here were the native compound RtH and clinically used ACV. As it can be seen RtH was in this group with a TE of $0.06 \cdot 10^3 - 0.24 \cdot 10^3$. This TE of native hemocyanin at 24 h was 4-times higher than the one determined after 72 h of action. It has to be noted that after 24 h of treatment the TE of the most toxic compound RtH was 60-times higher than that of ACV, and at 72 h of action the TE of the same compound was 20-times higher than the one of the control drug.

Anti-HSV activity of Rapana thomasiana hemocyanin (RtH) and its structural subunit (RtH2)

The activity of the tested compounds was evaluated against *wt* HSV 1, strain TM, *wt* HSV-2, strain Bja, and one ACV^R mutant with *tk* gene mutation, DD (ACV^R, HSV 2), in cultured cells, and the data were compared to that of ACV (Table III). Both compounds effectively inhibited the growth of *wt* HSV 1 and HSV 2 strains, and the effect was found to be predetermined by compounds and virus specificities. The most effective inhibitor of the *wt* HSV 1 growth was the native hemocyanin RtH, while its structural isoform RtH2 was most sensitive to *wt* HSV 2. Contrary, the growth of the ACV^R virus was not effectively suppressed by the both compounds. The investigated compounds are arranged according to their efficacy against all three HSV strains in the following order:

for *wt* HSV 1, strain TM:
RtH $\geq$ ACV > RtH2;
for *wt* HSV 2, strain Bja:
RtH2 $\geq$ ACV > RtH;
for ACV^R, mutant DD:
ACV >> RtH2 > RtH.

The selectivity of *Rapana thomasiana* hemocyanin, RtH, and its structural subunit, RtH2, is shown in able , and it was found to be predetermined by both complex and virus specificities. Compounds are arranged according to their selectivity in the following order against all three strains:

for *wt* HSV 1, strain TM:
RtH > ACV $\geq$ RtH2;
for *wt* HSV 2, strain Bja:
RtH2 > ACV > RtH;
for ACV^R, mutant DD:
ACV >> RtH2 > RtH.

The structural subunit RtH2 of native hemocyanin was more sensitive to the *wt* HSV 2 strain,

Table III. Selectivity of native hemocyanin and its structural subunit against HSV infection in cultured RD 64 cells.

Compound	IC_{50} [μg/ml]			$SI \cdot 10^3$		
	TM	Bja	DD	TM	Bja	DD
RtH	0.0001	10	12	36 690	0.132	0.11
RtH2	0.1	0.001	5	72.6	10 000	0.5
ACV	0.0002	0.002	0.02	125	10	1

while the native hemocyanin RtH has more selectivity to *wt* HSV 1, strain TM. Both compounds did not exhibit any effect on the infectious virus yield on the ACV^R mutant.

The effect of both hemocyanins on programmed cell death was evaluated in order to study morphologically if they make DNA fragmentation and if it is cell- and/or virus-specific. Using acridine orange and Janus Green B staining apoptosis/necrosis was found neither in *wt* HSV 1 and *wt* HSV 2 nor in mock-infected cells 8 h after the action of structural subunit RtH2. However, some morphological changes were studied in cells infected with *wt* HSV 1 strain and noninfected cells treated with native hemocyanin RtH. It was observed that RtH2 specifically affects HSV replication simultaneously suppressing the expression of the essential virus specific proteins and nonspecific destruction of viral DNA after entering the host cell nucleus. This also explains the fact that the most stable fragments are found in all cleavage mixtures of RtH2 (Idakieva *et al*., 2000).

The experimental data show that the structural isoform subunit RtH2 of native hemocyanin from *Rapana thomasiana* is a promising anti-HSV agent, especially against genital herpes virus. RtH2 decreases the cytotoxicity of native hemocyanin and directs its activity to viral and not to host cell DNA.

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Arao Y., Schmid D., Pellett P., and Inoue N. (1999), Herpesviruses beyond HSV-1 and HSV-2. Clin. Microbiol. Newsl. **21**, 153–159.

Arvin A. and Prober C. (1995), Herpes simplex viruses, 876–883. In: Manual of Clinical Microbiology, 6th ed. (Murray P., Baron E., Pfaller M., Tenover F., and Yolken R., eds.). American Society for Microbiology, Washington, DC.

Beike J., Köhler H., and Blaschke G. (1997), Antibody-mediated clean-up of blood for simultaneous HPLC determination of morphine and morphine glucoronides. Int. J. Legal. Med. **110**, 226–229.

Boteva R., Severov S., Genov N., Beltramini M., Pallhuber M., Tognon G., and Salvato B. (1991), Biochemical and functional characterization of *Rapana thomasiana* hemocyanin. Comp. Biochem. Physiol. **100B**, 493–501.

Crumpacker C. and Shaeffer P. (2002), New anti-HSV therapeutics target the helicase-primase complex. Nat. Med. **8**, 327–328.

De Clercq E., Andrei G., Snoeck R., De Bolle L., Naesens L., Ying C., and Neyts J. (2001), Acyclic/carbocyclic guanosine analogues as anti-herpes virus agents. Proceedings of the XIV. International Round Table "Nucleosides, Nucleotides and their Biological applications", San Francisco, USA, pp. 271–285.

Elion G. (1989), The purine path to chemotherapy (Nobel Lecture). Angew. Chem. Int. Ed. Engl. **28**, 870–878.

Ettinger R., Ettinger W., and Harless W. (1997), Active immunization with cocaine-protein conjugate attenuates cocaine effects. Pharmacol. Biochem. Behav. **58**, 215–220.

Harris J. and Markl J. (1999), Keyhole limper hemocyanin (KLH): a biomedical review. Micron **30**, 597–623.

Idakieva K., Severov S., Svendsen I., Genov N., Stoeva S., Beltramini M., Tognon G., Di Muro P., and Salvato B. (1993), Structural properties of *Rapana thomasiana grosse* hemocyanin: isolation, characterization and *N*-terminal amino acid sequence of two different dissociation products. Comp. Biochem. Physiol. **106B**, 53–59.

Idakieva K., Stoeva S., Parvanova K., Genov N., and Voelter W. (2000), Arrangement of functional units within the *Rapana thomasiana* hemocyanin subunit RtH2. Biochem. Biophys. Acta **1479**, 175–184.

Idakieva K., Schwarz H., Genov N., Voelter W., and Stoeva S. (2001), *Rapana thomasiana* hemocyanin (RtH): dissociation and reassociation behaviour of two isoforms, RtH1 and RtH2. Micron **33**, 7–14.

Jurincic-Winkler C., Metz K., Beuth J., and Klippel K. (2000), Keyhole limpet hemocyanin for carcinoma *in*

situ of the bladder: A long-term follow-up study. Eur. Urol. **37**, 45–49.

Kimberlin D., Crumpacker C., Straus S., Drew W., Biron K., and Hadgen F. (1995), Antiviral resistance in clinical practice. Antivir. Res. **26**, 423–438.

Lamm D., DeHaven J., and Riggs D. (2000), Keyhole limpet hemocyanin immunotherapy of bladder cancer: laboratory and clinical studies. Eur. Urol. **37**, 41–44.

Li Y., Rabello A., Simpson A., and Kats N. (1994), The serological differentiation of acute and chronic *Schistosoma japonicum* infection by ELISA using keyhole limpet hemocyanin as antigen. Trans. R. Soc. Trop. Med. Hyg. **88**, 249–251.

Markl J., Lieb B., Gebauer W., Altenhein B., Meissner U., and Harris J. (2001), Marine tumor vaccine carriers: structure of the molluscan hemocyanins KLH and HtH. J. Cancer Res. Clin. Oncol. **127**, 3–9.

McFadden D., Riggs D., Jackson B., and Vona-Davis L. (2003), Keyhole limpet hemocyanin, a novel immune stimulant with promising anticancer activity in Barrett's esophageal adenocarcinoma. Am. J. Surg. **186**, 552–555.

Meyer D., Anderson D., Gardner M., and Torres J. (1998), Hypervariable epitope constructs representing variability in envelope glycoprotein of SIV induce a broad humoral immune response in rabbits and *Rhesus macaques* AIDS. Res. Hum. Retrov. **14**, 751–760.

Naylor P., Sztein M., Wada S., Maurer S., Holterman D., Kirkley J., Naylor C., Zook B., Hitzelberg R., Gibbs C., Zagury D., Achour A., O'Toole C., Gazzard B., Youle M., Rios A., Sarin P., and Goldstein A. (1991), Preclinical and clinical studies on immunogenicity and safety of the HIV-1 p17-based synthetic peptide AIDS vaccine-HPG-30-KLH. Int. J. Immunopharmacol. **13**, 117–127.

O'Brien J. and Campoli-Richards D. (1989), Acyclovir. An update review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy. Drugs **37**, 233–309.

Olsson C., Chute R., and Rao C. (1974), Immunologic reduction of bladder cancer recurrence rate. J. Urol. **111**, 173–176.

Sandmaier B., Oparin D., Holmberg L., Reddish M., MacLean G., and Longenecker B. (1999), Evidence of a cellular immune response against sialyl-Tn in breast and ovarian cancer patients after high-dose chemotherapy, stem cell rescue, and immunization with theratope STn-KLH cancer vaccine. J. Immunother. **22**, 54–66.

Stoeva S., Idakieva K., Betzel Ch., Genov N., and Voelter W. (2002), Amino acid sequence and glycosylation of functional unit RtH2e from Rapana thomasiana (gastropod) hemocyanin. Arch. Biochem. Biophys. **399**, 149–158.

Van Holde K. and Miller K. (1995), Hemocyanins. Adv. Protein. Chem. **47**, 1–81.

Van Holde K., Miller K., and Decker. (2001), Hemocyanins and invertebrate evolution. J. Biol. Chem. **276**, 15563–15566.

Vere Hodge R. (1993), Long-term suppression of genital herpes. Antiviral. Chem. Chemother. **4**, 67–84.

Vona-Davis L., Vincent T., Zulfigar S., Jackson B., Riggs D., and McFadden D. (2004), Proteomic analysis of SEG-1 human Barrett's-associated esophageal adenocarcinoma cells treated with keyhole limpet hemocyanin. J. Gastrointest. Surg. **8**, 1018–1023.