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

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The cytotoxicity and the antiviral 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 *Shistosomiasis* (Li et al., 1994), in drug addiction (Ettinger et al., 1997;
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 Botheva 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 (Sofarma, 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<sub>R</sub>, 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), freeze-dried, 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<sub>50</sub>)
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 CC50 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 CC50 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% (IC50) were calculated from dose-regression lines. Selectivity indexes (SI) were calculated as CC50 to IC50 ratios. Data were compared to that of ACV.

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/H11022 > RtH/H11022 > 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.

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According to CC50 values hemocyanins at 24 h, 48 h and 72 h after treatment can be arranged as follows: ACV > RtH > RtH2.

The CC50 ranged from 30–10,000 µg/ml. In addition, the cytotoxicity of RtH increased 3.8-times 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 thomasi ana hemocyanin (RtH) and its structural subunit (RtH2)

Both MNC and CC50 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 cytopathology 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.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>MNC [μg/ml]</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; [μg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>RtH</td>
<td>20.87</td>
<td>23.50</td>
</tr>
<tr>
<td>RtH2</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>ACV</td>
<td>12.5</td>
<td>12.5</td>
</tr>
</tbody>
</table>

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<sub>50</sub> at 72 h compared to those at 24 h of action.

Based on the data from cytotoxicity experiments we calculated the CC<sub>50</sub> 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TE · 10&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>RtH</td>
<td>0.24</td>
</tr>
<tr>
<td>RtH2</td>
<td>0.06</td>
</tr>
<tr>
<td>ACV</td>
<td>0.004</td>
</tr>
</tbody>
</table>

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 · 10<sup>3</sup> – 0.24 · 10<sup>3</sup>. 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 ACVR mutant with tk gene mutation, DD (ACVR, 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 ACVR 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 ≅ ACV > RtH2;

for wt HSV 2, strain Bja:  
RtH2 ≅ ACV > RtH;

for ACVR, 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 ≅ RtH2;

for wt HSV 2, strain Bja:  
RtH2 > ACV > RtH;

for ACVR, 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ [µg/ml]</th>
<th>SI · 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TM</td>
<td>Bja</td>
</tr>
<tr>
<td>RtH</td>
<td>0.0001</td>
<td>10</td>
</tr>
<tr>
<td>RtH²</td>
<td>0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>ACV</td>
<td>0.0002</td>
<td>0.002</td>
</tr>
</tbody>
</table>

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™ 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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