Antiviral activity of hemocyanins

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Abstract
Hemocyanins are giant biological macromolecules acting as oxygen-transporting glycoproteins. Most of them are respiratory proteins of arthropods and mollusks, but besides they also exhibit protecting effects against bacterial, fungal and viral invasions. As discovered by 2-DGE proteomics analyses, several proteins including hemocyanins of hemocytes from virus-infected arthropods increased upon infection, confirming hemocyanin’s role as part of the organism’s defence system. Based on the structural analyses of molluscan Hcs it is suggested that the carbohydrate chains of the glycoproteins seem to interact with surface-exposed amino acid or carbohydrate residues of the viruses through van der Waals interactions.

Key Words: functional units; hemocyanin; mollusks; arthropods; viruses

Introduction
The persistence of the virus in the organism leads to the development of lymphoproliferative disease, the formation of various carcinomas and to the affection of the peripheral and central nervous system. Antiviral drugs may be divided into acyclic nucleoside analogues (aciclovir, ganciclovir, penciclovir), acyclic nucleotide analogues (cidofovir and adefovir), and substances of natural origin (De Clercq, 2004). Most of the submitted drugs are potent inhibitors of viral reproduction, but not all of them are promising for clinical application, since they are very different in terms of toxicity.

Recently it was found that hemocyanins (Hcs), the oxygen-transporting glycoproteins of many arthropods and mollusks, could be also potential inhibitors of some virus infections (Chongsatja et al., 2007; Flegel et al., 2011). These giant glycoproteins which differ in molecular mass, structure, carbohydrate content, and monosaccharide composition have recently received increasing interest due to their significant immunostimulatory, antitumor, and antiviral properties (Dolashka-Angelova et al., 2009; Dolashka et al., 2010; Mičetić et al., 2010; De Smet et al., 2011; Rehm et al., 2012; Markl et al., 2013). Both species, molluscan and arthropodan Hcs, contain copper-containing active sites in which the Cu(I,I) is oxidized to the Cu(II,II) state, thus accounting for their distinctive deep blue color. The biosynthesis of hemocyanins from e.g. Concholepas concholepas (CcH) takes place in the hepatopancreas (Manubens et al., 2010) but Megathura crenulata’s Hcs originate from rhogocytes (Martin et al., 2011).

Investigations on several molluscan hemocyanins, e.g. from Haliotis tuberculata (HH, Abalon) (Markl et al., 2001); Helix lucorum (HH, garden snail), Rapana venosa (RvH, Black sea murex) (Dolashka-Angelova et al., 2003, 2009, 2011; iliev et al., 2008) or C. concholepas (CCH, Loco) from the Pacific Chilean coast (Moltedo et al., 2006, Arancibia et al., 2012) demonstrated their remarkable immunostimulatory properties in experimental animal model and clinical studies. Moreover, hemocyanins have been extensively used as carriers to generate antibodies against diverse hapten molecules and peptides to induce antigen-specific CD8+ and CD4+ T cell responses (Minozzi et al., 2007, 2011; Arancibia et al., 2012). Recently, a vaccine potential of Oncomelania hupensis Hc against Schistosoma Japonicum parasite was also established (Guo et al., 2011).

Probably, due to their high carbohydrate content and specific monosaccharide composition, Hcs were also found to be active against viruses (Dolashka-Angelova et al., 2009, 2010). Proteomic analysis of differentially-expressed proteins in arthropodan Penaeus vannamei hemocytes upon
**Taura syndrome virus** (TSV) infection showed increased expression of hemocyanin (Rattanarojpong et al., 2009). The efficacy, against white spot syndrome virus (WSSV) and Singapore grouper iridovirus (SGIV) is also assigned to a protein of the arthropod *Penaeus monodon* (Zhang et al., 2004).

Antiviral properties of molluscan Hcs were reported for the first time describing the inhibition effect of hemocyanins from gastropod *R. venosa* (RvH) against *Respiratory syncytial virus* (RSV) and *Herpes simplex virus* type-1, strain Vic (HSV-1) (Dolashka et al., 2010; Dolashka-Angelova et al., 2011; Nesterova et al., 2011). As this property seems to be associated with the glycosylation of functional units, the oligosaccharide structures of *R. venosa* and related molluscan and arthropodan hemocyanins were determined using different analytical physicochemical techniques, (Sandra et al., 2007; Dolashka-Angelova et al., 2009; Dolashka et al., 2010).

In this short review, current knowledge about structural and biochemical aspects and eventual potential future clinical applications of arthropodan and molluscan hemocyanins as antiviral drugs is presented, and biochemical mechanisms causing the antiviral activity are suggested.

**Arthropodan hemocyanins**

Structure of arthropodan hemocyanins

Hemocyanins evolved early in the arthropod stem lineage from phenoloxidases, O$_2$-consuming enzymes involved in the melanin pathway. They form hexamers or oligo-hexamers of identical or related subunits with a molecular mass of about 75 kDa (Fig. 1) (Dolashka-Angelova et al., 2001; Mičetić et al., 2010; Jaenicke et al., 2012). Each subunit contains three domains and O$_2$-binding is mediated by two Cu$^+$ ions which are coordinated by six histidine residues (“type III” copper binding site). Subunit interactions within the multisubunit hemocyanin complex lead to diverse allosteric effects such as the highest cooperativity for oxygen binding found in nature. Based on biochemical, immunochemical and molecular phylogenetic analyses, distinct hemocyanin subunit types have been identified in chelicerata, myriapoda, crustacea and hexapoda (Huang et al., 2008; Rehm et al., 2012). The crystal structure of a native hemocyanin oligomer larger than a hexameric substructure was published for the first time for the 24-meric hemocyanin (MW = 1.8 MDa) from emperor scorpion (*Pandinus imperator*) (Fig. 1) (Jaenicke et al., 2012).

Antiviral activity of arthropodan hemocyanins

Several reports on antiviral effects of arthropodan Hcs appeared in the literature, e.g. Hcs from shrimp *Penaeus japonicus* (PjH) and *P. vannamei* (PvH), against white spot syndrome virus (WSSV), *Taura syndrome virus* (TSV), *yellow head virus* (YHV) (Lei et al., 2008; Chongsatja et al., 2007; Rattanarojpong et al., 2007; Nesterova et al., 2011). Hcs-based antiviral therapies are also reported (Lin, 2005; Balzarini et al., 2007).

One of these viruses, the white spot syndrome virus (WSSV) caused billions of dollars of losses for
shrimp farmers. To reduce shrimp production losses, stimulated since 2000 a plethora of research programmes to test shrimp’s responses against viral pathogens at the molecular level. Humoral responses, binding studies between shrimp and viral structural proteins including intracellular responses, viral persistence co-interactions as well as viral sequence determination of the shrimp genome were subject of these investigations. Based on these results, a novel practical method for improved disease control was developed (Flegel et al., 2011).

Studies on shrimp *P. japonicus* (PjH) showed that its hemocyanin could delay the infection of white spot syndrome virus (WSSV) *in vivo* and its subunits have different behavior in anti-WSSV defense (Table 1). During WSSV infection a strong induced one also cloned subunits, PjHcL was observed in contrast to subunit Pj-HcY. These findings suggest a possible discrepancy between the two subunits in shrimp innate immunity (Lei et al., 2008).

To understand the molecular responses of crustacean hemocytes to virus infection, a two-dimensional electrophoresis (2-DE) proteomics approach was applied to determine altered proteins in hemocytes of *P. vannamei* during *Taura syndrome* virus (TSV) and yellow head virus (YHV) infections (Rattanarojpong et al., 2007; Chongsatat et al., 2007).

Proteomic analysis of *P. vannamei* hemocytes (PvH) upon YHV and TSV infection show the differentially-expressed proteins from the hemocytes. In the proteomic analysis of gills from yellow head virus-infected *P. vannamei*, 13 spots with up-regulated protein expression levels and five spots with down-regulated levels were identified. LC-nano-ESI-MS/MS indicated that the up-regulated proteins included enzymes in the glycolytic pathway, the tricarboxylic acid cycle and amino acid metabolism. The other upregulated proteins were arginine kinase, imaginal disk growth factor (IDGF) and a Ras-like GTP protein. These results provide preliminary data, however, it was not able to assign specific YHV-response status to any of the up-regulated or down-regulated genes identified after YHV challenge (Rattanarojpong et al., 2007).

Proteomic analysis was also applied to explain the antiviral effect of *P. vannamei* hemocyanin (PvH) against *Taura syndrome* virus (TSV) (Chongsatat et al., 2007). At 24 h post infection of PvH with *Taura syndrome* virus (TSV), quantitative
These findings demonstrate for the first time that the ERK1/2 signaling pathway may play an important role in molecular immune response of *P. vannamei* upon TSV infection through its interaction with the C-terminal hemocyanin (Havanapan et al., 2009).

### Table 1 Antiviral activity of several molluscan and arthropodan hemocyanins against different viruses

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<th>Efficacy</th>
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<td><em>P. vannamei</em> (PvH), <em>PvHcY</em>, <em>PvHcL</em></td>
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<td><em>P. monodon</em> (PmH1)</td>
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<td><em>P. monodon</em> (PmH2)</td>
<td>Singapore grouper iridovirus (SGIV)</td>
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<td><strong>Molluscan Hcs</strong></td>
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<td><em>Rapana venosa</em></td>
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<td>Dolashka-Angelova 2009</td>
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<td><em>R. venosa</em></td>
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**Molluscan hemocyanins**

*Structure of molluscan hemocyanins*

Molluscan hemocyanins (Hcs) are giant biological macromolecules acting as oxygen-transporting glycoproteins. They have recently received particular interest due to their immunostimulatory and antitumoral properties. Most of them are glycoproteins with molecular mass around 9000 kDa organized as decamers or didecamers (Fig. 2) (Lieb et al., 2008; Markl, 2013). The native hemocyanins contain one or two structural subunits with molecular masses around 350 - 450 kDa. Seven or eighth functional units with molecular masses of 50 kDa organize one structural subunits (Fig. 2) (Dolashka-Angelova et al., 2003a; Lieb et al., 2008; De Smet et al., 2011).

Molluscan hemocyanins possess certain inhibitory properties against tumor cells and viruses (Iliev et al., 2008; Toshkova et al., 2009; Dolashka et al., 2011), as also found for the molluscan hemocyanins of *R. venosa* (RvH) and *H. lucorum* (HlH) against different viruses (Dolashka-Angelova et al., 2008, 2010; Nesterova et al., 2010, 2011; Zagorodnya et al., 2011; Velikova et al., 2011).

*Antiviral activity of molluscan hemocyanins*

The antiviral effect of RvH and its isoforms was studied against polio virus type 1 (LSc-2ab), coxsackie virus B1 (CV-B1), respiratory syncytial virus (RSV) and Epstein Barr virus (EBV). After treatment of the viruses with the native RvH, the non-glycosylated and glycosylated functional units RvH-b and RvH-c, the quantitative virucidal suspension test did not show any effect against polio virus type 1 (LSc-2ab), coxsackie virus B1 (CV-B1). Also no antiviral effects of native RvH against RSV was observed. However, the results presented in Table 1 show that only the glycosylated.
FU, RvH-c, possesses some antiviral effect against the replication of RSV, at both viral doses tested (Dolashka-Angelova et al., 2009). In contrast, non-glycosylated FU RvH-b does not reveal any antiviral activity against the replication of RSV, poliovirus type 1 (LSc-2ab) and CV-B1. This is the first report of the fact that molluscan hemocyanin functional units with specific glycosylation possess antiviral activity (Dolashka-Angelova et al., 2009).

The properties of native hemocyanins from R. venosa and Helix vulgaris (HvH) snails and their structural subunits RvH1, RvH2, HvH1, and HvH2 have been studied in vitro as substances with feasible anti-EBV activity (Velkova et al., 2009). Epstein Barr virus (family Herpesveridae) is one of the etiologic agents that cause Burkitt’s lymphoma and nasopharyngeal carcinoma, but drugs for anti-EBV treatment are limited. There are few approaches for the treatment of this viral infection, namely, the use of effective antiviral chemotherapy, serotherapy or seroprevention which supposes the use of specific human immunoglobulins and vaccines (Pagano and Gershburg, 2005). The influence of R. venosa hemocyanin on viability and proliferative activity of lymphoblastoid cells was characterized by cytomorphological and colorimetric methods and an antiviral effect was observed after treatment with FUs RvH1-a and RvH2-e (Nesterova et al., 2010).

Antiviral effects on R. venosa and H. vulgaris (HvH) hemocyanins, their structural subunits, the glycosylated functional unit RvH2-e and the non-glycosylated unit RvH2-c were also investigated against HSV virus type 1: only the glycosylated FU RvH2-e exhibited an antiviral activity, which probably is due to its high carbohydrate content and specific monosaccharide composition (Zagorodnya et al., 2011; Velkova et al., 2011).

Based on the obtained results, we suggested for the first time that the complete native RvH molecule lacks any antiviral activity because the carbohydrate chains are buried in between the structural subunits of the global protein and therefore, are unable to interact with the viral surface glycoproteins. To support this hypothesis, we found that two putative glycosylated sites in FUs βcHlH-g are exposed on the surface of the molecule (Fig. 3) (Kostadinova et al., 2013) and that FU RvH1-a, exhibits antiviral activity and bears two N-glycosidic residues attached to Asn262 and Asn401.
Glycosylation of hemocyanins

The relevance of specific glycosylation patterns as a differentiating factor for immunostimulatory properties has already been raised in studies on the hemocyanin isoforms KLH1 and KLH2 of *M. crenulata* and *R. venosa* (Geyer et al., 2005; Sandra et al., 2007). Molluscan hemocyanin genes contain several potential N-glycosylation sites, and some of those have been already effectively demonstrated to be glycosylated using a series of analytical strategies and MALDI-TOF-MS and tandem mass spectrometry (Lieb et al., 2008; Dolashka-Angelova et al., 2009; Dolashka et al., 2010; De Smet et al., 2011). The identification of the oligosaccharide structure of several molluscan hemocyanins revealed a highly heterogeneous mixture of glycans of the compositions Hex0–9 HexNAc2–4 Hex0–3 Pent0–3 Fuc0–3. A novel type of N-glycan with an internal fucose residue connecting one GalNAc(b1-2) and one hexuronic acid, was detected which also occurs in subunits RvH1 and RvH2 (Sandra et al., 2007; Dolashka-Angelova et al., 2009; Dolashka et al., 2010).

Based on the obtained information about carbohydrate structure of hemocyanins the antiviral properties of hemocyanins against Herpes simplex virus type 1, was suggested. One potential site for N-glycosylation in FU RvH2-e at Asn-127 was shown to be glycosylated and this carbohydrate chain is likely to interact with specific regions of glycoproteins of HSV, through van der Waals interactions in general or with certain amino acid residues in particular. As is shown in figure 4 several groups of these residues can be identified on the surface of RvH2-e which may interact with a carbohydrate chains of glycoprotein gp120 of HSV. The surface of glycoprotein gp120 was very well analysed (Sander et al., 2002).

Development and identification of polyclonal antibodies against viruses

Hemocyanins are commonly used to develop polyclonal antibodies e.g. also against predicted B cell epitopes in HIV-1 accessory protein Vpr. The synthesized B-cell peptide epitopes were subsequently conjugated with keyhole limpet hemocyanin (KLH) and then used to immunize rabbits. The antibody titer and specificities were determined by ELISA, Western-blotting, and immunoprecipitation analyses, respectively. Two B
cell epitopes of Vpr were successfully predicted by bio-informatical methods and polyclonal antibodies against those peptides could be successfully prepared (Sun et al., 2012).

KLH was used to analyse the protective potential of polyclonal IgG antibodies specific to the ectodomain (eM2) of M2 protein of influenza A virus (IAV) against lethal influenza infection of mice. Two fractions, KLH alone and the ectodomain eM2, conjugated to KLH, were administered with Freund's adjuvant intraperitoneally (i.p.) to BALB/c mice. Analysis of the preparation of anti-KLH-eM2 IgGs by ELISA revealed that it contained about 25% of anti-eM2 IgGs and 75% of anti-KLH IgGs and after infection with 3 LD50 IAV, a 100% survival of mice was observed with 320 µg anti-eM2 IgGs (Király et al., 2011).

Recently, molluscan hemocyanins receive increasing interest in vaccine development. Several carrier proteins were used to prepare vaccines against highly variable HIV-1. Two synthesized peptides, JY1 (V3 region of HIV-1 gp120, subtype D) and JY1-MAP8, were coupled to carrier proteins such as KLH, hepatitis B surface antigen (HBsAg) and meningococcal P64k protein and used for immunization in mice. It was found that JY1-MAP8 conjugates were more immunogenic than JY1-MAP8 alone. Furthermore, conjugates to HBsAg and KLH were more immunogenic than those to P64k. The analysis showed that conjugate-based immunogens are more prompt to elicit immunogenity and cross-reactivity and hemocyanins are promising candidates the development of HIV vaccines (Cruz et al., 2011).

We found that hemocyanins are a new class of natural antiviral compounds against different viruses. However, arthropodan hemocyanins are active against those own viruses of arthropods, such as WSSV and TSV, while the molluscan hemocyanins are active against viruses of human, such as RSV and EBV. Maybe the mechanisms of antiviral activity of both species are different and therefore, we consider them as an appropriate for further investigation as antiviral agents.

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