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Antineoplastic immuno-modulating properties of hemocyanins

M. Nikolova[1]*, S. Konstantinov[1], P. Dolashka[2]

[1] *Medical University Sofia, Faculty of Pharmacy, Department of Pharmacology, Pharmacotherapy and Toxicology, Bulgaria, Dunav Str, 1000 Sofia, Bulgaria*
[2] *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. Bl. 9, 1113 Sofia, Bulgaria*

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In this review, we summarize most of the available scientific information about the antineoplastic and immuno-modulating properties of hemocyanins. The key points here are the functional structure of hemocyanins, hemocyanins antitumor and immuno-modulating properties, and their possible application as anticancer vaccine carriers and adjuvants.

Key words: hemocyanins; hemocyanins structure; antitumor effect; immuno-modulating effect; vaccine carrier; vaccine adjuvant

INTRODUCTION

Nowadays, malignant diseases are one of the major public health problems. Cancer is characterized by abnormal cell growth with the potential to invade or spread to other parts of the body. Traditional types of cancer treatment (surgery, radiation therapy and chemotherapy) are still not effective enough against the potentially fatal illness. This is the reason why prevalent parts of the scientists are searching for new antineoplastic compounds and methods against different types of tumors, especially those with possible local controllability, e.g. skin cancers and non-invasive urinary bladder cancer.

New plant and animal bioactive compounds raise hopes for a successful fight against cancer. Hemocyanins are a good example of glycoproteins with a potential antitumor effect. They were described for the first time in 1878 by Leon Frederic, while he was studying the physiology of *Octopus vulgaris* [1]. The name of hemocyanins is coming from the Greek *haima (*blood) and *kyanos (*blue pigment) [2]. These huge glycoproteins were isolated from mollusc species and they have demonstrated significant antineoplastic activity. Moreover, there is plenty of information about hemocyanin antitumor effects based on its immuno-modulating properties. Recently, some structure-activity relationship details were found thus underlining the possibilities of these molecules to play different biological roles because of their very complex protein structure. On the one hand hemocyanins main function is to transport oxygen to all molluscan tissues, but on the other hand, they could be beneficial for future innovative anticancer therapies.

Functional structure of HCS

Molluscan hemocyanins are cylindrical multimeric glycoproteins that are found freely dissolved in the hemolymphatic fluid. Their main function is to transport oxygen to all of the molluscan tissues. Hemocyanins are among the largest known proteins, with molecular masses varying approximately from 3.3 to 13.5 MDa. They are composed of subunits that form cylindrical decamers, didecamers or multi-decamers [3]. Each subunit comprises several paralogous functional units (FUs) which are classified into eight groups (FU: a, b, c, d, e, f, g and h) and their homologous FUs (FU-d*,f1, -f2, -f3, -f4, -f5, and -f6). FUs (except FU-h) have a similar structure and composition. Two domains are responsible for their folding: the N-terminal core domain where the active site is located in a four-alpha-helix bundle and the C-terminal domain dominated by a six-stranded β-sandwich and shields the entrance to the active site [3]. The molecular mass of FU-h is larger than that of other FUs due to the presence of an extra cupredoxin-like domain at the C-terminus. All FUs possess a type-3 copper center at their respiratory active site located in the N-terminal core domain. Between two copper atoms are bound two oxygen atoms to form a Cu_2O_2 cluster and the geometry around the Cu_2O_2 -binding site is conserved in all known FUs [3].

Molluscan hemocyanins can be classified into four major groups according to the FU composition

* To whom all correspondence should be sent:
E-mail: *maria.nikolova.sofia@abv.bg*

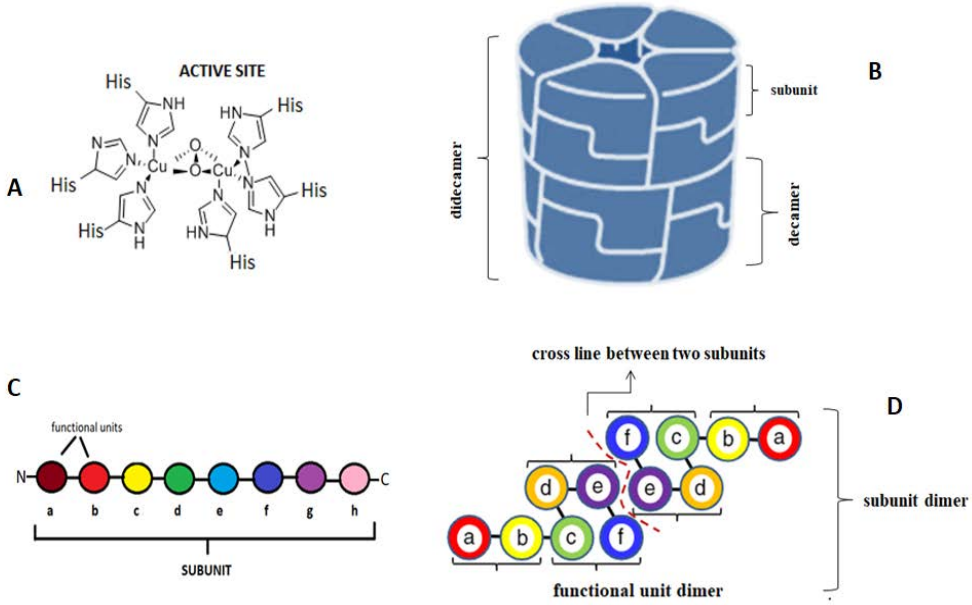

Fig. 1. Schematic illustration of molluscan hemocyanin structure. (A) Scheme of functional unit active site. At the active site, two oxygen atoms are bound between two copper atoms forming a Cu_2O_2 cluster. The copper ions are coordinated by six histidine residues. The active site is located at the N-terminal core domain and the C-terminal β-sandwich domain shields the entrance to the active site. (B) Schematic didecamer hemocyanin structure. Each decamer contains ten subunits and each subunit is composed of functional units. (C) Scheme of a molluscan hemocyanin subunit with eight different functional units (as in many gastropods). N, N-terminus; C, C-terminus; (D) Schematic illustration of subunit dimmer. Five subunit dimmers compose the hemocyanin wall region. One subunit dimmer contains six functional unit dimers (2 a-b dimers, 2 c-f dimers, 2 d-e dimers).

of the subunit: (1) gastropods type, (2) mega-hemocyanin type, (3) nautilus and octopus – type and (4) squid-type [3].

Keyhole limpet belongs to gastropods, and their hemocyanins possess additional FU, namely h. The gastropod hemocyanin type has a hollow cylindrical didecameric structure and molecular size of 8 MDa. The molecule has a wall and inner collar regions. The wall region comprises of FUs: -a, -b, -c, -d, -e, and –f and the inner collar region contains FUs –g and –h. Additionally, it is confirmed by Swerdlow et al. [2], that Keyhole limpet hemocyanin (KLH) has two isoforms (KLH-A and KLH-B) which are composed of distinct subunits. Mega-hemocyanin type can be observed in cerithioid snails. Type -2 hemocyanin consists of two subunits: mega-subunit (550 kDa) and typical-subunit (400 kDa). They form tridecameric structure with a molecular size of 13.5 MDa. The mega subunit comprises of 12 FUs (FU-a, -b, -c, -d, -e, -f, -f1, -f2, -f3, -f4, -f5 and -f6) and the typical-subunit is similar to the Keyhole limpet subunit structure [3]. Nautilus and octopus hemocyanins (type 3) don't have a FU –h and they exist as decamers with a molecular size of 3.5 MDa (FU-a, -b, -c, -d, -e, -f and –g). Type 4 (squid type) is a decamer with a molecular size 0f 3.8 MDa. The subunit contain following FUs: -a, -b, -c, -d, -d*, -e, -f and –g (FU-h is not observed in type 4) [3]. From the hemocyanin classification, it is obvious, that only glycoproteins with the FU – h can form didecamers and multi-decamers [3]. The wall region of hemocyanin molecules has a complex general architecture. The functional units -a, -b, -c, -d, -e and –f compose FU-dimers (a-b, c-f, d-e), which stack to form the plate-like subunit dimer. Five subunits dimers assemble to form the wall region with 5-fold symmetry resulting in a D_5 symmetrical cylinder [3].

It is known that hemocyanins have a high carbohydrate content. Hemocyanin N-glycosylation motifs are conserved near active sites and in between binding sites of their subunits. Glycosylation is a posttranslational process that increases protein solubility and prevents its denaturation or aggregation. Salazar *et al.* [4] demonstrated that N-glycans contribute to the quaternary structure of KLH, *Concholepas concholepas* hemocyanin (CCH) and *Fissurela latimarginata* hemocyanin (FLH). Furthermore, their study has proved that N-deglycosylation didn't change the secondary structure of hemocyanins but altered their refolding mechanisms. Hemocyanin glycans are highly

heterogeneous and primarily composed of mannose-rich N-glycans, N-mixed carbohydrates with fucose, galactose, N-acetylglucosamine and glycosylation branches that are not found in mammals [4]. Furthermore, hemocyanins contain highly immunogenic glycan patterns which were observed in the human parasite *Schistosoma mansoni* [5]. Also, galactose Gal(β1–6) moieties have been found in some O-specific side chains of *Salmonella* lipopolysaccharides and capsular polysaccharides of *Klebsiella pneumoniae* [4]. Additionally, KLH contains Thomsen-Friedenreich antigen (or T-antigen) which is expressed on the cell surface of T-cell lymphomas and most human carcinomas [6]. There are evidences demonstrating how hemocyanin N-glycans promote proinflammatory cytokine secretion, humoral changes and antitumor effects.

Stimulation of the immune system by hemocyanins and mode of action

Cancer immunotherapy is an innovative treatment modality aiming to stop tumor growth, to eliminate tumor cells and prevent the metastatic process by activating the immune surveillance to detect and kill tumor cells. By multiple mechanisms of innate and adaptive immunity can be promote anticancer effect. For instance, T-cells produce cytokines such as tumor necrosis factor (TNF), which induce tumor cell lysis and enhance other antitumor cell effector responses. Macrophages contribute to anticancer defence by their antigen presenting properties and their ability to produce various cytokines. Also, natural killer cells (NK-cells) can lyse target cells, including tumor cells, unrestricted by the expression of antigen on the target cell [7]. There are two types of cancer immunotherapy: active and passive [8]. Passive therapy relies on the repeated application of large quantities of tumor antigen-specific antibodies e.g. Trastuzumab (Herceptin) - monoclonal antibody targeting HER-2/neu antigen presented in breast cancer cells. Passive immunotherapy is effective against cancer diseases with generalized immune dysfunction such as chronic lymphocytic leukemia (CLL), Hodgkin's disease (HD) and non-Hodgkin lymphoma(NHL). Alentuzumab is a recombinant humanized monoclonal antibody of murine origin, which has been approved for CLL therapy. It targets the CD52 antigen expressed on malignant B and T lymphocytes. Rituximab, which is a chimeric murine/human monoclonal antibody, has a long story as registered and approved therapy for B cell NHLs. Rituximab targets the CD20 antigen expressed in B lymphocytes and activates lysis of the respective malignant B-cells. Furthermore, some antibodies can interrupt the interaction between important growth factors and their receptors on the cancer cell surface. For instance, Cetuximab is specific for the epidermal growth factor receptor and it was approved for colorectal cancer treatment [8]. Active immunotherapy aims to generate tumor–specific immune responses by vaccination [8]. The immune response comprises of: presentation of tumor-associated antigens by the antigen presenting cells (APCs), antigen uptake by APCs, epitope presentation to CD4+T cells, cytokine release and B-cell activation. The immune response against cancer cells can be enhanced by unspecific immune stimulators, also called adjuvants. An ideal cancer vaccine should provoke a long-lasting effect by combining T-cell responses and humoral responses. Moreover, it is necessary to be safe without strong side effects [8]. Related to the above mentioned information about cancer immunotherapy, there are plenty of studies and evidences demonstrating the anticancer hemocyanin properties because of their immunomodulating and vaccine enhancing adjuvant functions.

In preliminary experiments, it was proved that hemocyanin glycans interact with several innate immune receptors on murine APCs (carbohydrate-recognizing C-type lectin receptors and Toll-like receptors). Also CCH, FLH and KLH bind *in vitro* to human mannose receptor (MR) and dendritic cell-specific intercellular adhesion molecule – 3-grabbing non-integrin (DC- SIGN) [4]. The main function of these receptors is to recognize pathogen glycosylated structures and to promote endocytosis, pro-inflammatory responses and antigen presentation to T-lymphocytes. APCs incorporate CCH, FLH and KLH by macro-pinocytosis and receptor-mediated endocytosis. Thus, hemocyanins undergo prolonged antigen presentation to T or B lymphocytes, promoting a Th1 immune response and antitumor effect [9]. According to Zhong *et al.* [9], hemocyanins are phagocytized by macrophage cells and slowly processed into smaller peptide fragments. Also, hemocyanins induce M1–polarization of macrophages and downregulate M2 cytokine genes. It is proved that M1 macrophages activate a tumor-killing mechanism and antagonize the suppressive activity of M2 macrophages, which promote tumor growth and metastasis [10].

Otherwise, KLH has been reported as causing a cross-reaction with the Thomsen-Friedenreich (or T-antigen) antigen by Wirguin *et al*. [11].

Antibodies that may recognize T-antigens were determined after rat immunization with KLH. The T-antigen is a simple mucin-type disaccharide presented on the outer cell surface of T-cell lymphomas and most human carcinomas (e.g. superficial non-invasive urinary bladder cancer, breast and prostate cancer). Thomsen-Friedenreich antigen has a significant role for tumor cell adhesion and metastasis formation. Moreover, the T-antigen is a specific tumor marker and a potential target for passive and active immunotherapy [12]. Immunotherapeutic effects of KLH in non-invasive urothelial cancer may be a consequence of a cross-reaction with the T-antigen and activation of the immune response against cancer cells [11].

Scientists are trying to find out more about hemocyanins' mechanism of action and how these glycoproteins stimulate the immune system. Accumulation of CD8+ T cells and activation of CD4+ T-cell response after mice immunization with KLH was demonstrated by Doyle *et al.* [13]. Moreover, mixed interleukin-4 (IL-4)/IFN- γ production profile was observed, too. Also, Salazar *et al.* [4] showed how N-glycosylation of mollusk hemocyanins contributes to their structural stability and immunomodulatory properties in mammals thus indicating their immunogenic potential.

Furthermore, there is research [14] showing how hemocyanins from *Helix lucorum* and *Rapana venosa* change gene expression profile of urothelial cancer cell lines. Significant upregulation of genes involved in the apoptosis and immune system activation were observed. Also, there is a downregulation of CCL2 (for monocyte chemotactic protein -1), CCL17 and CCL21 genes. The expression of chemokine ligands CCL17 and CCL21 is associated with the initiation of tumor metastasis and therefore it serves as a prognostic factor in gastric cancer patients [23].

It can be summarized, that hemocyanins activate immune responses because of their xenogenic structure and N-glycan chains. Plenty of trials demonstrated, that the use of hemocyanins as vaccine carriers and adjuvants could be of clinical relevance. Furthermore, they exert a strong anticancer effect and this could be used for future antineoplastic combination therapy.

Hemocyanin complex structures as vaccine carriers and vaccine adjuvants

Hemocyanins have a very complex structure, containing unique polypeptides with immunogenic properties thus enabling their use as vaccine carriers and adjuvants. Adjuvants (from the Latin *adjuvare* – help or aid) are substances that increase the immunogenicity of a vaccine formulation by enhancing the strength of the antigen-specific immune responses[15,16]. Currently, KLH is used as a carrier protein and adjuvant to produce polyclonal and monoclonal antibodies, because of its structural stability and immuno-stimulating properties. An example of that is the clinical trial of the Sialyl-TN (STn) - KLH Vaccine [17]. Therapeutic cancer vaccines are being studied in the treatment of drug-resistant breast cancer because they induce humoral and cell-mediated immunity to tumor cells. Usually, these vaccines contain tumor lysates or defined tumor antigens. Sialyl-Tn-antigen (STn) is a carbohydrate epitope found on a variety of glycoproteins, including cancer-associated mucins. STn expression is associated with poor prognosis in metastatic colorectal, gastric, ovarian, and breast cancer diseases. Although the trial didn’t show significant results against metastatic breast cancer, STn-KLH vaccine was well tolerated by the patients included. Additionally, KLH has been used as a carrier for N-propionylated polysialic acid (NP-polySA) [18]. Polysialic acid is a polymer weakly expressed on the cell surface of embryonal and adult brain tissues. Interestingly, it is frequently detectable in significant amounts in small cell lung cancer. The main function of polysialic acid is to inhibit cell adhesion and to promote metastasis formation. Patients vaccinated with NP-polySA-KLH vaccine were found to produce IgM antibodies against NP-polySA.

Musseli *et al.* [19], estimated the combination "antigen – KLH carrier –adjuvant" as a successful method for boosting the immune response against cancer cells. QS-21 (a purified saponin fraction separated from the bark of *Quillaja saponaria)* was used as an adjuvant. This substance activates APCs, B-cells and T-cells response. KLH was used as a carrier for the following tumor antigens:

- GM2 – sphingolipid monosialoganglioside and tumor-associated antigen.
- GD3 – acidic glycosphingolipid.
- Fucosyl GM1 – sphingolipid mono-sialoganglioside and tumor-associated antigen.
- Globo H – globohexaosylceramide, glycol-sphingolipid antigen.
- Lewis Y – difucosylated oligosaccharide with two fucoses carried by glycoconjugates (glycolproteins and glycolipids) on the cell surface.

- Tn – monosaccharide structure N-acetyl-galactosamine (GalNAc) linked to serine or threonine by a glycosidic bond.
- sTn – sialyl Tn antigen.
- Thomsen–Friedenreich antigen – disaccharide formed by additional galactose monossacharid to Tn – antigen: (Gal(b1-3)GalNAc).
- MUC1 – mucin-1 glycoprotein; inhibits access of chemotherapeutic drugs to the cancer cell.
- KSA – glycoprotein, a marker for tumors with epithelial origin.
- Polysialic acid – an inhibitor of neural cell and tumor cell adhesion.

The summary of serological results in vaccinated patients showed significant immunoglobulin response to sTn, GM2 and Fucosyl GM1 antigens. Furthermore, Musselli *et al.* [19]. confirmed that KLH in these conjugate vaccines (antigen – KLH carrier – QS-21 carrier) induces Th1 – cell response.

As a continuation, in a pilot trial, 11 patients with ovarian cancer were treated with heptavalent antigen-keyhole limpet hemocyanin vaccine plus QS21 [20]. The vaccine included carbohydrate epitopes, such as GM2, Globo-H, Lewis Y, sTn, Tn, Thomsen-Friedenreich antigen and Tn-MUC-1. As a result, the vaccine safely induced antibody responses against five of seven antigens (there was no significant response to GM2 and Lewis Y antigens). In addition, the heptavalent vaccine administration was well tolerated without any strong side effects. The most frequent unwanted reactions were fatigue, fever, myalgia and local injection site reactions.

Hemocyanins have been used not only for an antigen carrier but also as a vaccine carrier. Tumor antigen-presenting cell vaccine was co-injected with the CCH as an adjuvant in castration-resistant prostate cancer patients. In this study, CCH was able to induce an immune memory response followed by a delayed-type hypersensitivity skin test [21].

Furthermore, CCH and FLH have showed immunological properties based on their glycoprotein structure and ability to be recognized by immune cells. It was demonstrated that CCH and FLH are useful carriers of carbohydrate mimotopes such as P10, a mimetic peptide of GD2 (the major ganglioside constituent of neuroectodermal tumors). This trial indicates possibilities for future cancer vaccine research [22].

According to Dolashka *et al.* [23] hemocyanins, isolated from *Helix lucorum* and *Rapana venosa* could be a serious alternative of KLH as single inductors of nonspecific, cell-mediated immune response and to propose it as a component of non-specific non-conjugated anti-tumor vaccines. It was demonstrated the resistance of the experimental animals against the progressive development of Guerin ascites tumor after treatment with *Rapana venosa* hemocyanin (RvH), *Helix lucorum* hemocyanin (b-HlH) and KLH in correlation to their specific carbohydrate constituents. b-HlH and its conjugate with tumor antigen exhibited stronger immunogenicity probably because of the specific carbohydrate structures (HlH has a heterogeneously glycosylated structure carrying mostly methylated high mannose-type moieties).

Antineoplastic activity of hemocyanins

There is a variety of evidences proving hemocyanins' antitumor activity based on their immune-modulating properties. Chilean gastropod *Concholepas concholepas* was shown to exert cytotoxic activity in murine bladder cancer model. This fact underlines, that not only KLH may have anticancer properties, but this could be a common feature of all hemocyanins regardless of their origin [24]. Moreover, FLH showed more potent anti-tumor activity and stronger humoral immune response in the B16F10 mouse melanoma model, rather than CCH and KLH. *In vitro* assays with FLH demonstrated stimulation of rapid pro-inflammatory cytokines which can explain stronger immunological activity [25].

Additionally, it was observed that *Helix aspersa* hemocyanin (HaH) has cytotoxic effects on a bladder cancer cells, human prostate and ovarian carcinoma, malignant glioma, Burkitt's lymphoma and acute monocytic leukemia tumor cells [26]. Noteworthy, an extract from *Helix aspersa* demonstrated antitumor activity against triple-negative breast cancer cells Hs578T and it was found to be a potent stimulator for TNF induced signal transduction changes, accompanied by beneficial NF-κB inhibition as well [27]. Hemocyanins isolated from *Rapana thomasiana* and *Helix pomatia* showed strong *in vivo* anti-cancer effect in a murine model of colon carcinoma. Moreover, *Helix pomatia* hemocyanin (Hph) *and Rapna thomasiana* hemocyanin (RtH) were described to induce apoptosis in C-26 carcinoma cells *in vitro* [28]. Described experiments of Guncheva *et al.* [29] have shown that preparations of RtH-FO (*Rapana thomasiana* hemocyanins conjugated with folic acid), RtH-FE (ferulic acid), HlH-FO (*Helix lucorum* hemocyanins conjugated with folic acid) and HlH-

FE are not cytotoxic to human fibroblasts (BJ cells) and they exhibit an excellent cytotoxic effect to hormone-dependent MCF-7 and hormone-independent triple-negative MDA-MB-231 breast cancer cells.

CONCLUSION

There is a rising amount of published information linked to the immune-modulating properties of hemocyanins. These molluscan biomolecules are giant glycoproteins acting not only as oxygen-transporting proteins in invertebrates, but they could play a significant role in future immune therapies. The hemocyanins' complex structure is the basis of their multifunctionality. These molecules might be used as anti-cancer vaccine carriers and adjuvants. Moreover, there are evidence data about triggering anticancer immunity. Therefore, we assume that future experiments with hemocyanins (regardless of their origin) could be beneficial for developing anticancer immune therapies and believe in the expectation, that hemocyanins may play a key role in innovative anti-tumor therapies.

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