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Bioactive compounds isolated from garden snails

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ABSTRACT

The recent appearance of a growing number of resistant to conventional antibiotics, has become a serious medical problem. To overcome this resistance, the development of new compounds is encouraged. Hemolymph and mucus of *Helix lucorum* and *Helix aspersa* garden snails and *Rapana venosa* marine snail are a complex mixture of biochemically and pharmacologically active components. Glycoprotein 'hemocyanin' and antimicrobial peptides from the hemolymph and mucus are important components of the innate immunity. Some isoforms and peptides serve as effector molecules of the defense system, providing an efficient initial effect against infectious pathogens. The *in vitro* antitumor activity of Helix and Rapana hemocyanins and their isoforms with different oligosaccharide structures was established on the bladder carcinoma permanent cell lines T-24. This is probably due to the specific oligosaccharide structures of hemocyanins which are exposed on the surface of the molecule.

Key words: Antibacterial activity, antitumor activity, hemocyanins, *Helix lucorum*, *Helix aspersa*, peptides

Introduction

The phylum Mollusca is probably the third most important animal group after the arthropods and vertebrates, forming a major part of the world fauna. Although most natural medicines are derived from plants, marine invertebrate phyla, including the Mollusca, are of increasing interest as a source of novel bioactive compounds (Andrejko et al. 2009; Badiu et al. 2008; Coates et al. 2014; Dang et al. 2015). Molluscs are currently used for a range of therapeutic applications, with purified or synthesised bioactive compounds developed as pharmaceuticals and crude or semi-purified extracts as nutraceuticals (Dwek et al. 2001; Dolashka-Angelova et al. 2008).

Snails belong to the class Gastropoda and land snails are one of the most numerous with almost 35,000 described species of the world. The marine snail *Rapana venosa* and garden snails *Helix aspersa* and *Helix lucorum*, from the family Helicidae are very well known species of gastropod mollusk. The hemolymph from snails contain bioactive compounds as glycans, peptides, glycopeptides, proteins. Many of them have been discovered in recent years (De Smet et al. 2011; Dolashka-Angelova et al. 2003; Velkova et al. 2010; Gabriel et al. 2011).

Several glycoproteins as hemocyanins and lectines were isolated from the hemolymph of marine and garden snails and analysed using different methods and techniques. Recently, we identified that two structural subunits, RvH1 and RvH2, with molecular masses of 400 - 450 kDa aggregate into didecamers for *R. venosa* hemocyanin and three subunits (□-

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HIH, α_D-HIH and α_N-HIH) for *H. lucorum* and *H. aspersa* hemocyanins. Each structural subunit is composed of eight FUs of masses of ~ 50 kDa, which can be isolated from the structural subunits of RvH, HlH and HaH (De Smet et al. 2011; Dolashka-Angelova et al. 2003; Velkova et al. 2010; Dolashka et al. 2012).

Moreover, we identified several novel proline-rich antimicrobial peptides with molecular masses between 3000 and 9500 Da from the hemolymph of *R. venosa* snails and garden snails *H. lucorum* showing strong structural subunits antimicrobial activities against the Gram-positive (Gram+) and the Gram-negative bacteria (Dolashka-Angelova et al. 2008; Dolashka et al. 2011). The antibacterial activity of hemolymph from *Galleria mellonella* infected with entomopathogenic strain of *Pseudomonas aeruginosa* and non-pathogenic bacterium *Escherichia coli* was also established (Andrejko et al. 2009). *In vivo*, the antimicrobial activity induced by *E. coli* sustained on the high level until 48 h after infection, while the maximum level for *P. aeruginosa* reached the at 18 h postinjection.

Recently, a series of active peptides and glycoproteins with different physiological functions were also extracted from snail mucus. The amount of mucin isolated from *H.pomatia* species is higher than mucin isolated from *H. aspersa*. However, the amount of mucoproteins isolated from *H. aspersa* species after shaken overnight at 4^0C is higher than mucuproteins isolated from *H. pomatia* (Gabriel et al. 2011).

In recent past, people used to eat alive snails to ease heartburn: when snails reach stomach they produce mucus, contrasting acidity. Considering the role of snail mucus in repairing ulcers and thinking about the role of human mucus to prevent or fight acidity, it has been developed syrups against stomach acidity and gastric-esophagus reflux.

Several scientific researchers have demonstrated that some of these bioactive compounds-derivated drugs can be used in a large variety of therapies, as in creams to ease skin abrasions and scars, to cure respiratory diseases, heartburn and at last scientists discovered unexpected and previously unknown properties (Ortega et al. 2006; Benkendorff et al. 2015; Dang et al. 2015).

The present study dealt with the analysis of the extracts of marine snail *R. venosa* and two garden snail *H. aspersa* and *H. lucorum* and their putative application.

Materials and Methods

Isolation of hemocyanins

Helix and *R. venosa* hemolymphs were isolated from the leg of collected snails, solubilized in 50 mM sodium acetate buffer, pH 5.8, and the hemocyanin was sedimented as described by (Dolashka-Angelova et al. 2003; Velkova et al. 2010; Gabriel et al. 2011). After removal of the blue native hemocyanin pellet, the supernatant was lyophilized.

Isolation of peptides from the mucus

Collected mucus from the snails were lyophilized and separated using Milipore filters (3 and 10 kDa). The lyophilized supernatant from the hemolymph was also separated using the same filters. Three fractions were obtained: Fraction A (masses between 0-3 kDa), Fraction B (masses between 3-10 kDa), and Fraction C (masses above 10 kDa).

Fraction B was lyophilized and then applied on a Nucleosil C18 column, equilibrated with 0.10% trifluoroacetic acid (TFA, v/v) (solution A). Elution was performed with a linear gradient formed by solutions A (0.1% TFA/water) and B (80% acetonitrile in 0.1% TFA (v/v)) at a flow rate of 1.5 ml/min, over 60 min. Ultraviolet absorption was monitored at 214 nm. The eluted fractions were collected and lyophilized. The fractions were reconstituted in Milli Q water containing 0.10% TFA (v/v). The molecular masses of isolated fractions were measured by an Autoflex™III, High-Performance MALDI-TOF & TOF/TOF System (Bruker Daltonics).

Amino acid analisys of mucus

Approx. 1-2 mg of sample B was weighed accurately in a hydrolysis vial, solved in 800µL 6N HCl, closed under vacuum (< 10mbar) and was hydrolysed for 24 hours at 110°C. The HCl was evaporated and the sample was solved in sample dilution buffer to obtain a concentration of approx. 1 mg sample per ml buffer.

Antibacterial assays of the hemocyanins and their isoforms

The antimicrobial activities of the isolated fraction from the mucus of *H. aspersa*, were tested against two Gram+ strains, *Propionibacterium acnes* (strain 266 (IA) and *Propionibacterium acnes* KPA171202) and two Gram- bacteria (*E.coli* NBIMCC and *Helicobacter pylori*). The samples were qualitatively tested according to the growth inhibition assay. Antimicrobial assays of isolated Fractions

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were obtained on agar plates containing the likewise Gram+ and the Gram- bacteria. Each fraction was spread on agar medium with two different amounts (5 and 20 µl of the sample solutions). The incubation was for 24-36 h at 37°C.

Antiproliferative activity of the tested hemocyanins

Experiments were carried out with one commercially available permanent human tumor cell line from different stages of human urinary bladder transitional carcinoma cells (TCC) :

T-24 cells were established from the primary tumor of an 81-year-old Caucasian woman with urinary bladder cancer (transitional cell carcinoma (TCC), grade III) in 1970 producing a variety of cytokines (e.g. G-CSF, IL-6 and SCF) with a p53 mutation.

The T-24 cells were cultured in Dulbecco Modified Eagle's Medium (DMEM, Lonza, Austria), supplemented with 10% fetal bovine serum (Gibco, Austria), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1% non-essential amino acids in 75 cm^3 tissue culture plastic flasks (Falcon).

The T-24 tumor cells were treated for 24,48, and 72h, respectively, with various concentrations (0.25 and 1.0 mg/ml) of the test substances, doxorubicin (DOX, 0.1mg/ml positive control), and cultured medium (negative control). The antiproliferative activity of the tested hemocyanins on T-24 cell line of native molecule of RvH and HlH and their isoforms, subunits and functional units, on cell viability were assessed in 50 mM Tris/HCl buffer, pH 8.0, using the WST-1 and BrdU ELISA assays (Roche Diagnostics, Germany).

Results

In the recent years, the extracts from marine and garden snails were analysed and was found that there are very rich sources of bioactive compounds. Several relatively small antimicrobial peptides and the much larger protein hemocyanins are isolated from the hemolymph of molluscs (Coates et al. 2014; Dwek et al. 2001; Zhuang et al 2015) We investigated the structures and properties of hemocyanins isolated from the marine snail *Rapana venosa* (RvH) and the garden snail *H. lucorum* and *H. aspersa*, and studied their structural and functional units (FUs) using different techniques [13-16]. In this study we represent the properties and antitumore, and antimicrobial activities of bioactive compounds isolated from hemolymph and mucus of garden snail *H. aspersa* (Velkova et al. 2010; Dolashka-Angelova et al. 2003).

Purification of bioactive compounds

After collection and purification the mucus from garden snail *H. aspersa* was subdivide into three fractions: Fraction A (masses between 0-3 kDa), Fraction B (masses between 3-10 kDa), and Fraction C (masses above 10 kDa), using Millipore filters with a cut-off of 3 and 10 kDa, respectively.

Upon testing their antimicrobial activity on an agar medium after incubation for 24-36 h at 37°C, only Fraction B (masses between 3-10 kDa), appeared to generate a zone of inhibition of bacterial strains of *Propionibacterium acnes* (strain 266 (IA), and KPA171202), *Helicobacter pylori* and *Echerishie coli* NBIMCC 3486 (not illustrated). Therefore, Fraction B was purified and the structures of some compounds were analyzed.

High concentrations of Asp, Glu, Gly, Leu, Pro and Lys were calculated by the amino acid analyses of Fraction B (Table 1).

Table 1. *Amino acid composition of Fraction B from the mucus of garden snail Helix aspersa.*

Amino acid	**µg/mg**	**Amino acid**	**µg/mg**
Asp	52.611	**Met**	7.350
Thr	23.271	**Ile + allo-Ile**	21.995
Ser	21.237	**Leu**	36.921
Glu	63.277	**Tyr**	14.372
Pro	25.108	**Phe**	21.396
Gly	38.474	**His**	15.317
Ala	27.497	**Lys**	29.882
Cys(O_3H) + Cys +Cys_2	2.758	**Trp + deg. prod. Trp**	0.000
Val	25.532	**Arg**	21.915
		TOTAL:	**448.913**

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Fraction B was applied on a Nucleosil 7 C18 column (Figure 1) and fourteen fractions were eluted by reversed-phase column chromatoghy.

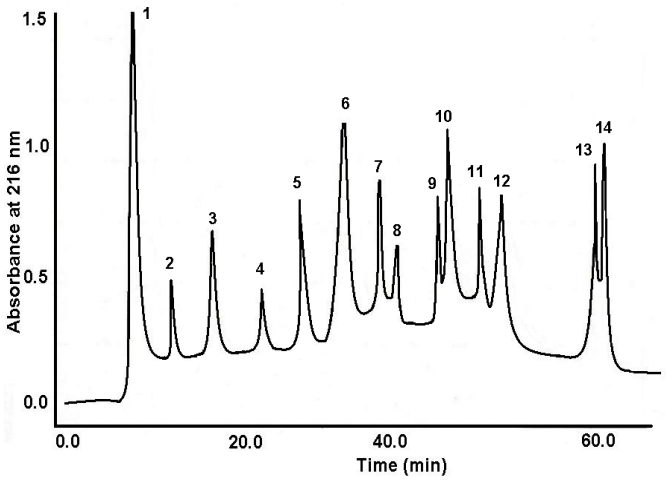

Figure 1. *HPLC purification of peptides fromFraction B of the mucus of garden snail Helix aspersa on a Nucleosil 7 C18 column (250x10 mm; Machery–Nagel, Duren, Germany) using the following conditions: eluent A, 0.1% trifluoroacetic acid; eluent B, 80% acetonitrile in A; gradient program, 15% B for 5, followed by 15–100% B in 55 min at a flow rate of 1 ml/min.*

They were additionally purified on the same column and analysed by orcinol–sulphuric test. As is shown on Figure 2 (spot 3) no brown colour was observed for the hemolymph of crab *Eriphia verrucosa*.

However, the acid test shows that peptides eluted as Fraction 5 (spot 7) and Fraction 6 (spot 8) by HPLC change the colour in brown on spots 7 and 8 on a silica-gel plate. No brown colour was observed for the other fractions isolated by HPLC. Therefore, Fraction 5 was analysed by MALDI-TOF. A mass spectrum of Fraction 5, containing peptides with masses between 2 and 10 kDa, is shown in Figure 3.

The molecular masses of the isolated peptides were determined by MALDI/MS. Two main ions were identified on MS spectrum on Fraction 5, revealed a main ion at m/z 4021.04 $(M+H)^+$ and ion at m/z 6403.73 $(M+H)^+$ (Figure 3).

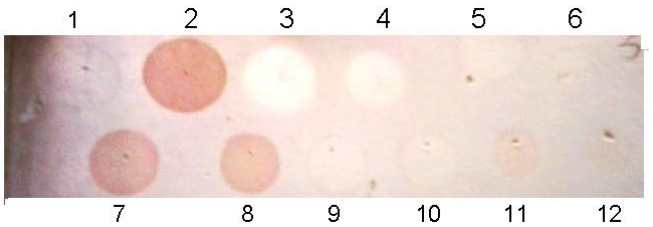

Figure 2. *Orcinol–sulphuric acid test of peptides eluted by HPLC and applied on to a silica-gel plate. The spots on positions: 1). Whater; 2). Glucose 3); Eriphia verrucosa Hc < 10 kDa; 4). Fraction 2 of mucus < 10 kDa; 5). Fraction 3 of mucus < 10 kDa; 6). Fraction 4 < 10 kDa; 7). Fraction 5 < 10 kDa; 8). Fraction 6 < 10 kDa; 9). Fraction 7 < 10 kDa; 10). Fraction 8 < 10 kDa; >10 kDa; 11). Fraction 9 < 10 kDa; 12). Fraction 10 < 10 kDa.*

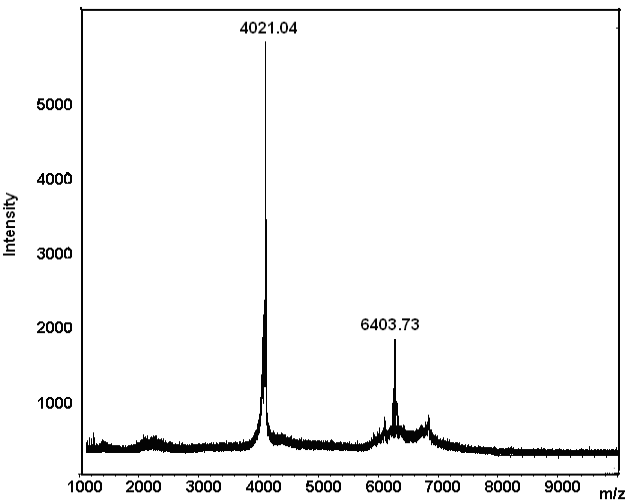

Figure 3. *MALDI spectrum of Fraction 5 from the mucus of garden snail Helix aspersa purification on a Nucleosil RP C18 column (Figure 2). The sample was measured by MALDI-TOF Ultraflex II (Bruker Daltonics, Bremen, Germany).*

Antibacterial activity of peptides from the mucus of garden snail Helix aspersa

In vivo, the antimicrobial activity of fractions isolated from the mucus of garden snail *H. aspersa* were tested against different species of Gram+ (*Propionibacterium acnes* strain 266 (IA) and *Propionibacterium acnes* KPA171202) and two Gram- bacterium (*E.coli* NBIMCC and *Helicobacter pylori*). The organisms were chosen because they are human pathogenic bacteria and commonly used in antimicrobial tests. The results show that Fraction 2 exhibited inhibition

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effect on growth of the bacteria *Propionibacterium acnes* strain 266 (IA) and *Helicobacter pylori* (Figure 4 A,B).

To explain the observed effects of the mucus against various bacteria, the peptides and glycopeptides of mucus were purified by high-performance liquid chromatogram (Figure 1), and the antibacterial effect of the resulting pure fractions was also tested against *Propionibacterium acnes* KPA171202 and *E.coli* NBIMCC 3486. Figure 4 C and D shows the antibacterial effect of the two peptides with masses of 4021.04 and 6403.73 Da, isolated from the mucus of the snail, which to varying degrees affect the *Propionibacterium acnes* KPA171202 and *E.coli* NBIMCC 3486.

Antitumore activity of hemocyanins isolated from the hemolymph of snails

After purification of the native hemocyanin from *R. venosa*, *H. aspersa* and *H. lucorum* hemolymphs and dissociation against 0.13 M Glycine buffer, pH 9.0, three isoforms were identified by electrophoresis (data not shown).

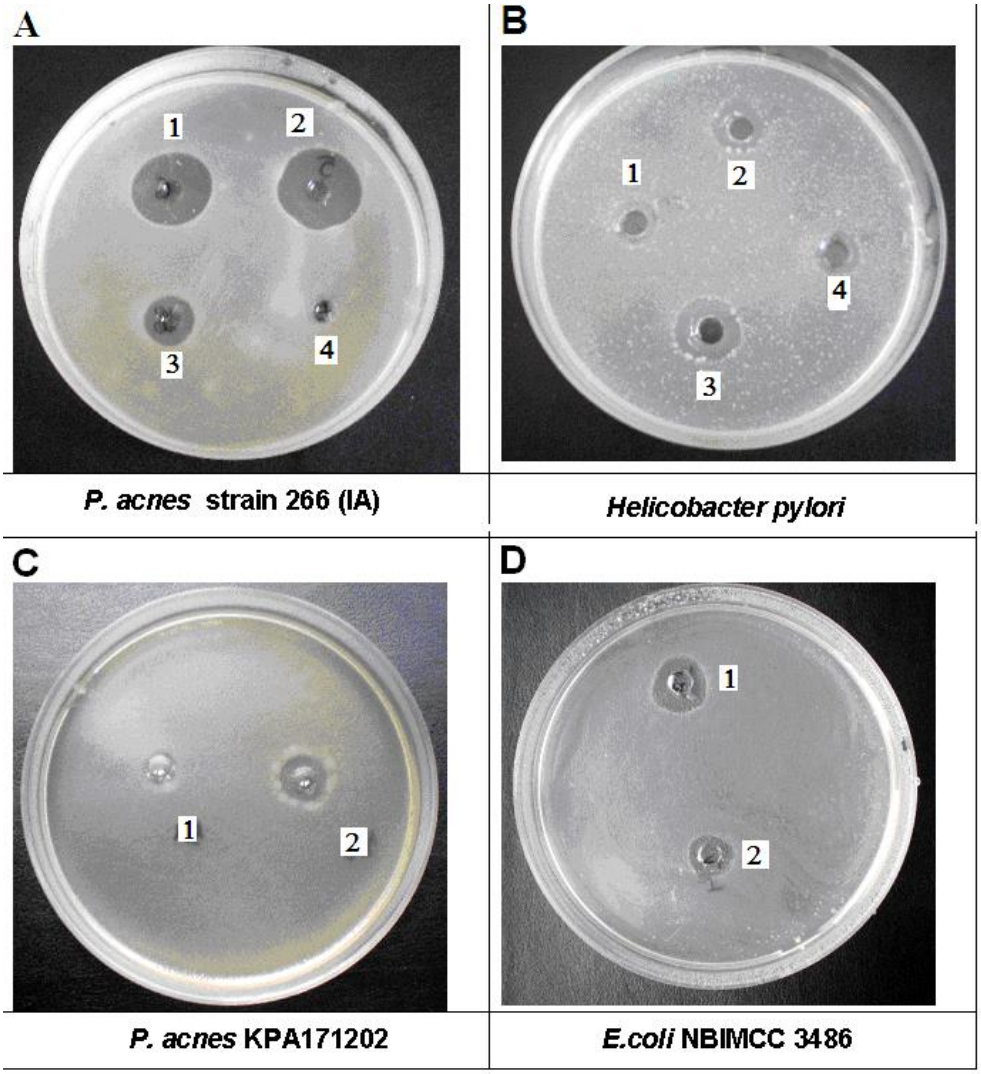

Figure 4*. Antibacterial effect of different fractions: position1)Fraction A; position 2. Fraction B; position 3) Fraction C; position 4) Water) of mucus from the snail Helix aspersa against: A) Propionibacterium acnes (strain 266 (IA) and B) Helicobacter pylori. Antibacterial activity of (1) Fraction 5 and (2) Fraction 6 against C) Propionibacterium acnes KPA171202 and D) E.coli NBIMCC 3486.*

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The direct *in vitro* effect of the isolated hemocyanins with concentration of 500 μg/ml on bladder cancer cell lines T-24 were evaluated in a number of experiments lasting 24h, 48h and 72h. The effects of the native molecule of molluscan Rapana, Helix and keyhole limpet hemocyanins, structural subunits and functional units on cell line T-24 are presented in Figure 5.

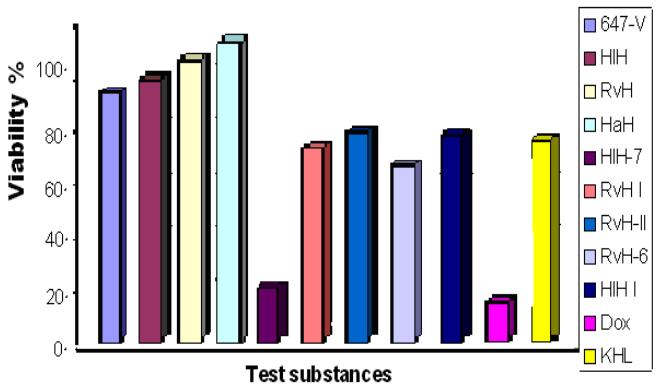

Figure 5. *Effect on the human tumor cell lines T-24 after 72h of incubation with native molecule of RvH, HaH and HlH, structural subunits and functional units with concentration of 500 μg/ml in the presence of negative control and positive controls (Doxorubicin hydrochlorid and KLH).*

From the Figure 5 it is clearly visible that only HlH showed a cytotoxic effect after 72 h of incubation compared to the native molecules of others Hcs. Very slight inhibition effect was observed after 72 h of treatment of T-24 cells with subunits RvH I, RvH II and HlH I. In the opposite, the lack of cytotoxic effect, even stimulation was measured with the native molecule of RvH, HaH and KLH. However, two functional units exhibited very high inhibition effect, FU RvH-6 and HiH-7. A cytotoxic effect after 72 h of incubation of HlH-7 was similar to Doxorubicin hydrochlorid (21%).

We have observed an antiproliferative effect of the functional units isolated from RvH and HlH against bladder cancer cell line T-24. The effect was found to be dose- and time-dependent and similar to the effects of doxirubicin. Therefore, the effect of this FU was studied additional.

To identify, if the subunit RvH1 and FUs of RvH1 induced reduction in viability of T-24 tumor cells *via* apoptosis, they were incubated with the cells and stained using Annexin-V-Fluos Kit and co-stained with PI. Figure 6 shows fluorescent micrographs of T-24 cells after 24 h incubation with the hemocyanins tested. The left side shows micrographs with necrotic cells (PI fluorescence, red), in the center are merged images of the red and green fluorescence and day light and the right panel demonstrates apoptotic cells (Annexin-V-FLUOS fluorescence, green). The results was compared with the control (non-treated cells) and doxorubicin-treated cells (Figure 6A,B).

After treatment of T-24 cells with the structural subunit RvH1 (Figure 6C), both populations were found in the wells – apoptotic cells fluorescing in green only and bright green cells with bright red nuclei. These cells could be late apoptotic or necrotic as well. The same behavior was observed after treatment of the cells only with one FU RvH1-c, identified, as described below (Figure 6D).

Discussion

Recently, a series of active peptides and glycopeptides with different physiological functions were extracted from marine molluscs (Coates et al. 2013, Rong et al. 2013, Gabriel et al. 2011). Somel peptides/proteins from the hemolymph of molluscs and arthropods were also found to exhibit a broad-spectrum of microbial activity against Gram-positive (Gram+) and Gram-negative (Gram-) bacteria and yeast.

We have isolated and analysed several bioactive compounds, peptides, glycopeptides, hemocyanins, from marine and garden snails ((De Smet et al. 2011; Dolashka-Angelova et al. 2003; Velkova et al. 2010; Dolashka et al. 2012). Biochemically and pharmacologically active peptides in the hemolymph of garden snail *Helix lucorum* and marine snails *R. venosa* were analysed (Dolashka et al. 2014; Dolashka et al. 2011). Some of them, rich in Cys, Pro, Ser or Gly residues showed high antimicrobial activity against *S. aureus* and low activity against *Klebsiella pneumoniae* (Dolashka et al., 2011).

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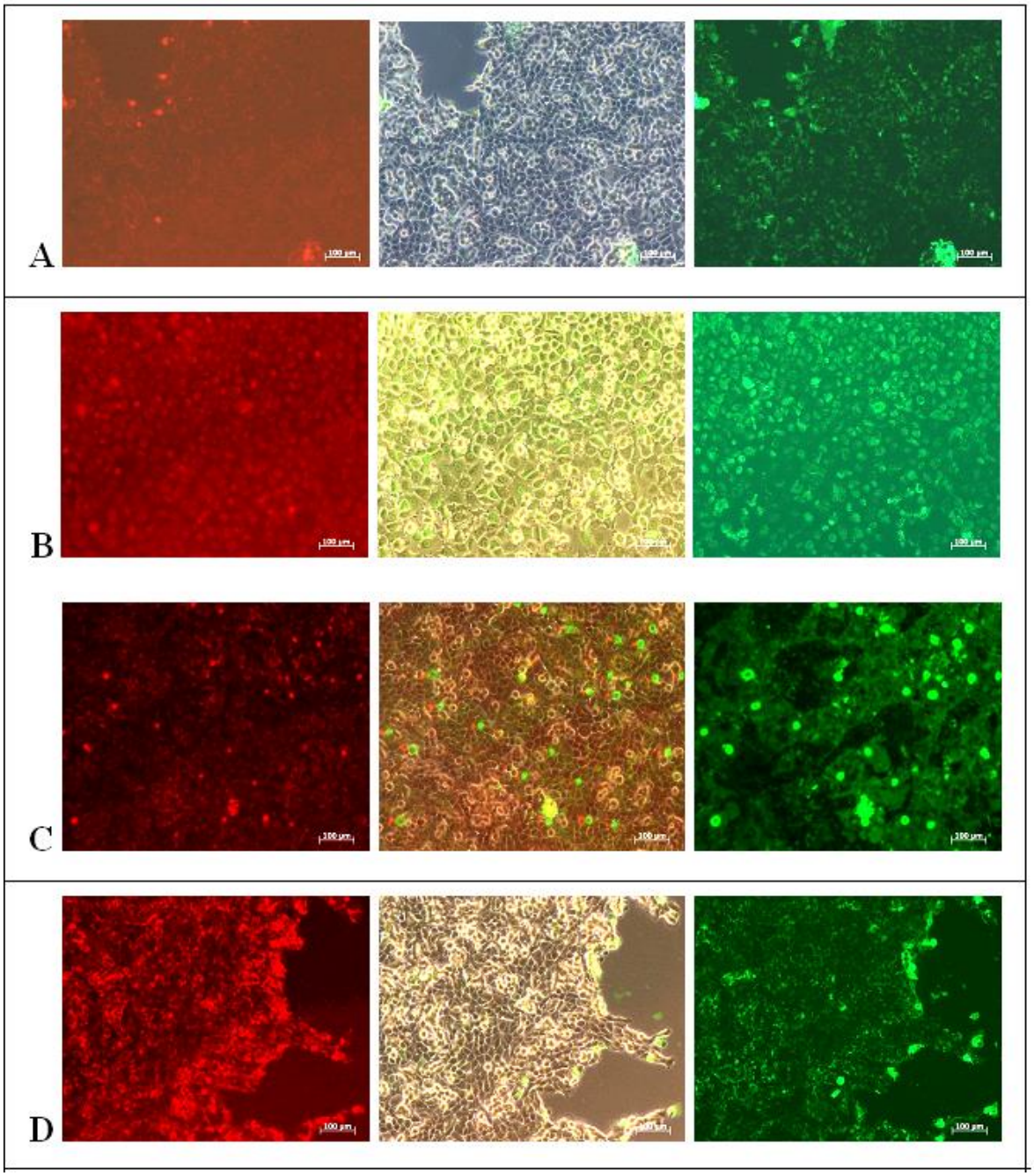

Figure 6. *Fluorescence of T-24 cancer cells treated with Annexin-V-Fluos Kit and PI and incubated for 24 h with 1,5 mg/ml of RvH1 and 1.0 mg/ml of RvH1-c. Cells were cultured in DMEM and maintained at 37o C and 5% CO2: (A) Fluorescence microscope photographs of T-24 control cells without treatment. (B) T-24 cells: treated with mg/ml of doxorubicin, (C) with RvH1, (D) with RvH1-6. Left side micrographs, red filter (PI fluorescence, red); center: merged images of red and green fluorescence and day light; right side micrographs, green filter (Annexin-V-FLUOS fluorescence, green).*

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Here we represent the peptides isolated from the mucus of garden snail *H. aspersa*. It is known that mucus has lots of active compounds, many of them have been discovered even in early history and in recent years scientific researchers have demonstrated that mucus-derivated drugs can be used in a large variety of therapies (Dang et al. 2015; Bonnemain 2005).

Here we represent two glycosilated peptides with mass of 4021.04 and 6403.73 Da, isolated from Fraction B (2-10 kDa) on a Nucleosil column. Antibacterial test on these peptides and Fraction B against Gram+ (*Propionibacterium acnes* strain 266 (IA) and *Propionibacterium acnes* KPA171202) and two Gram- bacterium (*E.coli* NBIMCC and *Helicobacter pylori*) showed the inhibition activity of Fraction 2 on growth of the bacteria *Propionibacterium acnes* strain 266 (IA) and *Helicobacter pylori* and the activity of two peptides on *Propionibacterium acnes* KPA171202 and *E.coli* NBIMCC 3486

It is possible that the Gly- and Pro-content in peptides plays a structural role in the activity against this bacteria (Ortega et al. 2006). Understanding the function and mechanism of action of the new antibacterial peptides from the mucus of *H. laspersa* may contribute to the potential of this compound in anti-infection therapeutics.

Glycoproteins with antitumore activity

Hemocyanins from mollusks are very well known as immunostimulators, and possess an antimicrobial, an antifungal, an antiviral and an antitumore activities (Boyanova et al. 2012; Coates et al. 2014; Dwek et al. 2001; Rong et al. 2013; Zhuang et al 2015).

In vitro experiments were performed to compare the anti-tumor activities of the native molecules of *R. venosa*, *H. lucorum* and *H. aspersa* and some isoforms, and optimal doses that arrest T-24 acancer cell and benign urothelial cell growth. Alterations in the cell morphology of the treated and untreated cells were observed. Comparison of the actions of the structural and the functional units RvH, HlH and HaH on T-24 tumor lines and benign urothelial cells show that treatment with the functional unit RvH1-c is most effective after the 72 h application on tumor cells without disturbing the metabolism and proliferation of normal cells. The potent inhibiting activity of the functional unit is probably due to its specific oligosaccharide structures. Functional units, RvH1-c, shows the most significant inhibitory effect against T-24 bladder carcinoma cells, which is comparable to the antitumoral activity of doxorubicin without disturbing the metabolic activity or proliferation of normal HL 10/29 urothelial cells. Cells treated with RvH1-c showed mostly apoptotic and less necrotic cell populations, and lots of cells were observed in the medium which lost adherence. These cells could be late apoptotic or necrotic as well, but there is no accurate fluorescence test which could identify the difference between these two types of cell deaths at this stage.

This exciting efficacy of a natural glycoprotein needs, as next, to be confirmed by animal trials, and experiments to elucidate the mechanism of action which are in our pipeline.

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