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Antiviral Activity of *Rapana thomasiana* Hemocyanin against Bovine Rotavirus

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Abstract

The aim of this study was to investigate the antiviral activity of hemocyanin isolated from natural marine snail *Rapana thomasiana* (RtH) and its structural subunit RtH-1 against Bovine rotavirus (BRV). *R. thomasiana* is widespread in the Black Sea, Mediterranean Sea and Marmara Sea, and hemocyanins have received extensive research in recent years due to their antitumor, antibacterial, antiviral and antifungal activities.

We examined RtH from *R. thomasiana* and its subunit (RtH-1) for its effect on the replication of Bovine rotavirus (BRV), Negovan strain, in Madin-Darby bovine kidney (MDBK) cells. The experiments were performed at non-toxic concentrations of both products - lower than the maximum tolerable concentration (MTC) (10 mg/mL). The anti-BRV activity of RtH and RtH-1 was determined in two time intervals - at 48 hours and 120 hours of exposure. At 48 hours, the activity of both products was significant, showing dependence on the concentration of the applied substance. In RtH-1 the most noticeable decrease in viral titer with $\Delta lg = 2.25$ was observed at concentrations higher than 1 mg/mL, and in RtH the same effect was observed at a concentration of 3.2 mg/mL. In the second time interval (120 hours), the activity of both products decreased significantly.

It could be concluded that conformational changes in proteins are the most likely reason for the clear antiviral activity at 48 hours, followed by a decrease in activity as time progresses to 120 hours. On the other hand, virus molecules can also undergo conformational changes over time.

Keywords: bovine rotavirus, viral replication, hemocyanins, *Rapana thomasiana*, antiviral activity, antiviral therapy

Резюме

Целта на това проучване е да се изследва антивирусната активност на хемоцианина, изолиран от морския охлюв *Rapana thomasiana* (RtH) и неговата структурна субединица RtH-1 срещу говеждия ротавирус (BRV). *R. thomasiana* е широко разпространен в Черно, Средиземно и Мраморно море, а хемоцианините се изследват интензивно през последните години поради техните активности - антибактериална, антитуморна, антивирусна, антигъбична и пр.

Ние изследвахме ефекта на хемоцианина от *R. thomasiana* (RtH) и неговата субединица (RtH-1) върху репликацията на говеждия ротавирус (BRV), щам Negovan в клетки Madin-Darby bovine kidney (MDBK). Експериментите бяха проведени при нетоксични концентрации и за двата тестирани продукта, а именно по-ниски от максимално допустимата концентрация (MTC) (10 mg/mL). Анти- BRV активността на RtH и RtH-1 беше определена в два времеви интервала - на 48-ия час и 120-ия час от въздействието. След 48-ия час активността на двата продукта е отчетлива, като показва зависимост от концентрацията на прилаганото вещество. При RtH-1 най-забележимо намаляване на вирусния титър с $\Delta lg = 2.25$ се наблюдава при концентрации по-високи от 1 mg/mL, а при RtH същият ефект се наблюдава при концентрация от 3.2 mg/mL. На втория изследван времеви интервал (120-ия час) активността и на двата продукта значително намалява.

В заключение, най-вероятно причина за отчетливата антивирусна активност на 48-ия час, последвано от намаляване на активността с напредването на времето до 120 часа са конформационни

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промени в протеините. От друга страна, вирусните молекули също могат да претърпят конформационни промени с течение на времето.

Introduction

Bovine rotavirus (BRV) belongs to the *Reoviridae* family, genus *Rotavirus* (Swiatek *et al*., 2010). BRV is the causative agent of severe gastroenteritis in newborn calves. The infection mainly affects calves aged between 15 and 45 days and has been associated with high mortality rates, stunted growth and increased susceptibility to other infections (Makwana *et al*., 2020). The development of potential rotavirus inhibitors is of prime interest, especially given that the efficacy and safety of available live, lyophilized and liquid vaccines are currently questioned (Anil *et al*., 2018). Several *in vitro* and *in vivo* studies have been carried out in order to investigate the anti-BRV activity of different compounds. A number of N-acetylneuraminic acid-based compounds studied as potential inhibitors of rotavirus (Kiefel *et al*., 1996) and a number of nucleoside and nucleoside triphosphate analogues (Pizarro *et al*., 1991) are among the compounds with proven activity against BRV *in vitro*. A synthetic peptide corresponding to bovine rotavirus C486 (BRV) VP4 amino acid sequence 232-255 (VP4-peptide) protects subjects *in vivo* from infection with virulent rotavirus (Ijaz *et al*., 1998).

Hemocyanins (Hcs) are large multisubunit copper proteins composed of different subunit types and found freely dissolved in the haemolymph of arthropods and molluscs (van Holde *et al*., 1995). Their primary biological function is the transport of oxygen. Inaddition, there are data on their antimicrobial (Qin *et al*., 2018; Yang *et al*., 2018) and antifungal activity (Destoumieux-Garzón *et al*., 2001). The antitumor effect of Hcs isolated from the sea snail *R. venosa* (RvH) and garden snails *Helix lucorum* (HlH) and *Helix aspersa* (HaH) associated with the induction of apoptosis in tumor cells has also been demonstrated (Gesheva *et al*., 2017; Georgieva *et al*., 2020). The antiviral activities of insect haemolymph (Chernysh, 2002) have been described. Several physicochemical properties of Hcs are very similar to those of phenoloxidases. The plasma phenoloxidase of the tobacco budworm *Heliothis virescens* exibits antiviral activity against several vertebrate viruses (Ourth *et al.,* 1993). Zhang *et al*. (2004) have investigated the antiviral properties of Hc isolated from the shrimp *Penaeus monodon* by using fish virus cell culture systems. Study on the cytotoxicity and the antiviral activity of native Hc isolated from the marine mollusc *Rapana thomasiana* (RtH) and its structural subunit RtH-2 has shown that both compounds are active against herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) (Genova-Kalou *et al*., 2008). Dolashka *et al. (*2010) have also reported on the antiviral activity of the structural subunit RvH-2 of the RtH against HSV-1 and respiratory syncytial virus (RSV). The activity of Hcs isolated from different biological species influencing different stages of viral replication of the herpes simplex viruses, Epstein-Barr virus, respiratory syncytial virus, human adenovirus, simian rotavirus and others is also described (Dang *et al*., 2015). The application and mechanism of action of various types of molluscan Hcs as antiviral agents have been recently discussed in a review by Zanjani *et al*. (2018).

The aim of the present work was to investigate the activity of native Hc isolated from marine snails *R. thomasiana* and its structural subunit RtH-1 against the BRV infection *in vitro*.

Material and Methods

Cells

Madin-Darby bovine kidney (MDBK) cells were obtained from the National Bank for Industrial Microorganisms and Cell Cultures, Sofia. The cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), supplemented with 10 mM HEPES buffer and antibiotics (penicillin 100 IU/mL, streptomycin 100 μg/mL).

Virus

Bovine rotavirus, Negovan strain, was replicated in monolayer MDBK cells in a maintenance solution DMEM plus 0.5% FBS and 5 μg/mL trypsin. The stock virus was titrated by the limited dilution method in 96-well plates.

Test substance

Hemocyanin (whole molecule - RtH and subunit 1 - RtH-1). The native RtH was purified from the hemolymph collected from marine snails *R. thomasiana*, as previously described by Idakieva *et al*. (2009). The structural subunit RtH-1 was isolated by anion exchange chromatography of dissociated RtH (by dialysis against 50 mM glycine/NaOH buffer, pH 9.6) on DEAE-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden), as described by Idakieva *et al*. (1993). Protein concentration was determined spectrophotometrically using the absorption coefficient $A_{278}^{0.1\%} = 1.36$ mL.mg^{-1}.cm^{-1} (Idakieva *et al*., 1993).

Cytotoxicity assay

The *in vitro* cytotoxic effects of the RtH and RtH-l were examined using MDBK cells. Confluent monolayer in a 96-well plate was treated with culture medium modified with increased concentrations ranging from 0.01 mg/mL to 10 mg/mL of the compounds. The cells were incubated at 37ºC and 5% CO_2. After microscopic evaluation the treated monolayers were compared to those of the controls - untreated cells cultured in equal experimental conditions. The maximum tolerable concentration (MTC) of a substance in the highest concentration of the compound is that which does not cause death to treated cells.

Antiviral activity assay

Confluent cell monolayer in 96-well plates were infected with ten - fold dilutions of viral suspension (0.1 mL per well). The compounds were applied in concentrations from 0.01 mg/mL to 3.2 mg/mL. The infectious viral titre was determined at 48 - 120 h.p.i. (hours post infection) and compared to that in the viral controls (with no compound in the medium). The antiviral activity was expressed as difference in the viral titre between experimental and control wells (Δlog).

Results and Discussion

Isolation and cultivation of rotaviruses were problematic until it was found that treatment with proteolytic enzymes enhances viral replication. Our experience showed that the maximal cytopathic effect (CPE) (++++) was achieved after five adaptive passages. The viral titre at 96-120 h.p.i. reached 5.58 log10 $CCID_{50}$ (cell culture infectious dose 50%) /0.1 mL. RtH and RtH-1were not toxic for MDBK cells in the concentration range tested and the MTC was determined to be 10 mg/mL.

The effect of RtH and RtH-1 on BRV replication was reported by determining the degree of viral titer reduction and calculating Δlg. The degree of anti-BRV activity was monitored for two time intervals - at 48 h.p.i. and at 120 h.p.i. The results presented in Fig. 1 show that at 48 h.p.i. both RtH and RtH-1 did not show significant activity at concentrations lower than 0.032 mg/mL.

At a concentration of 0.1 mg/mL, RtH showed significant inhibition on BRV replication by Δlg = 1.75, while RtH-1 at the same concentration had a weaker effect Δlg = 1.52. The activity of the two studied products showed a dose-dependent effect on the replication of BRV - with the increase in the concentration of the substance (in the non-toxic range) its antiviral effect increased.

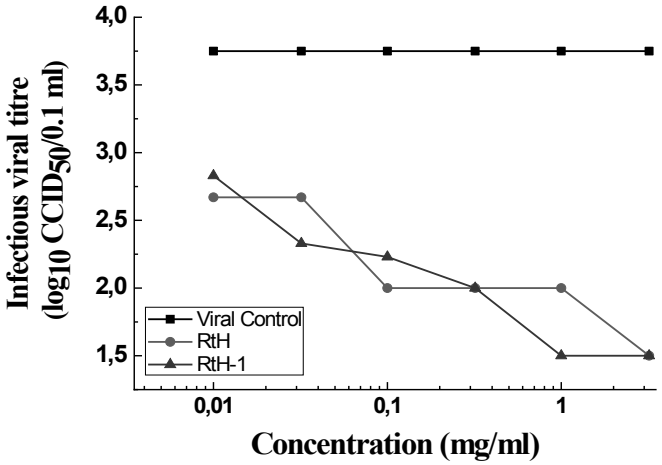

Fig. 1. Effect of RtH and RtH-1 on bovine rotavirus replication *in vitro* (at 48h)

For RtH-1, remarkable activity with Δlg = 2.25 was observed at concentrations above 1 mg/mL, and for RtH with a concentration of 3.2 mg/mL.

In the second study interval of exposure (120 h.p.i.), the activity of both test substances was significantly reduced. RtH showed maximum activity at concentrations greater than 1 mg/mL, with the effect not exceeding Δlg = 1.33 (Fig. 2).

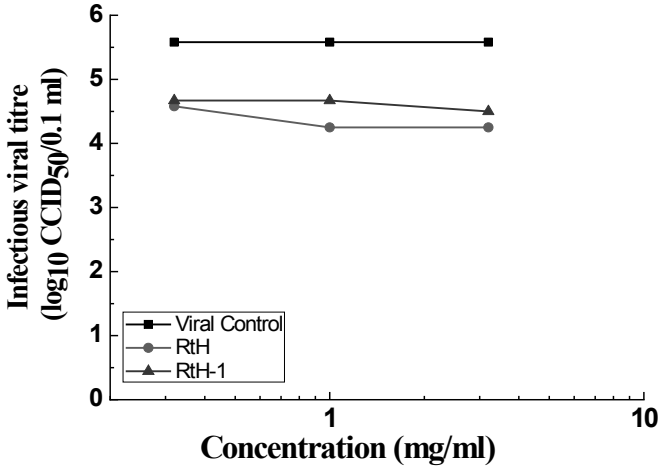

Fig. 2. Effect of RtH and RtH-1 on bovine rotavirus replication *in vitro* (at 120h)

Despite the numerous biological activities that Hcs possess and the wide application they receive in experimental virology, immunology and clinical medicine, there is little information on the stability of their molecules. However, the existing liquid forms of these giant copper-containing glycoproteins dispersed in the hemolymph of most molluscs and arthropods have been found to have a short availability life and activity of the molecules (Zanjani *et al*., 2014), which is a limiting factor in the characterization, development and use of these products.

Various methods have been developed for the preparation of a dry solid form using lyophilization, which usually increases the shelf life of proteins (Wang, 2000; Han *et al*., 2007), as well as the addition of various stabilizers to protect molecules from freezing and dehydration (Wang, 2000; Zanjani *et al*., 2014).

The two products studied were isolated and applied in their natural form in buffer solution. From data described by other teams on the stability of Hc molecules, it could be concluded that conformational changes in proteins are the most likely reason for the clear antiviral activity at 48 h.p.i., followed by a decrease in activity as time progresses to 120 h.p.i. On the other hand, virus molecules can also undergo conformational changes over time.

A marked decrease in viral titers up to the 48th h of exposure indicates that the RtH and RtH-1 inhibit one or more stages of viral reproduction. The anti-BRV activity of Hcs has not been well studied. There is evidence that these proteins may inhibit viral attachment of simian rotavirus to the cell surface (Dang *et al*., 2015). According to other authors, Hcs have the ability to affect the intracellular replication cycle of viruses from other families by inhibiting specific viral proteins required for viral replication or by inhibiting viral nucleic acid reproduction (Genova-Kalou *et al*., 2008; Dang *et al.,* 2015). Probably RtH and RtH-1 have a similar inhibitory effect on BRV.

Conclusion

Hcs are natural copper - containing glycoproteins with a variety of biological activities including antiviral. The anti-BRV activity of the Hc from *R. thomasiana* and its structural subunit RtH-1 is pronounced. This activity is dose - and time-dependent, and with the increase in the exposure time it decreases. It could be concluded that conformational changes in proteins are the most likely reason for the clear antiviral activity for 48 h.p.i., followed by a decrease in activity as time progresses to 120 h.p.i. On the other hand, virus molecules can also undergo conformational changes over time. The experiments conducted in this study revealed that Hcs can be used as promising antiviral drugs when administered in appropriate concentrations and well-designed application regimens to keep their bioavailability for a longer period of time.

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