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Hemocyanin from *Rapana thomasiana* – structure and anti-breast cancer activity in a presence of cholinium amino acids

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The focus of the present research is a hemocyanin, isolated from the hemolymph of marine snails *Rapana thomasiana* (RtH), and its interactions with ionic liquids, based on a cholinium cation and a non-polar amino acid as an anion [Chol][AA]. Six RtH-[Chol][AA] complexes have been obtained. Fragmentation and structural changes in the protein, even at low protein to ionic liquid ratios, were observed using static light scattering and fluorescence spectroscopy. For the first time the cytotoxicity of RtH, [Chol][AA] and their complexes on adenoma breast cancer MDA-MB-231cells was tested. All protein-ionic liquid complexes reduced moderately the cell viability of MDA-MB-231 cells, however, the effect was weaker than those estimated for the pure RtH or [Chol][AA].

Keywords: hemocyanin, ionic liquids, conformational changes, anti-breast cancer activity.

INTRODUCTION

Development of new types of human therapeutics is a fast growing field. Recent trends in the drug development are biotechnological drugs; incl. protein drugs [1]. It is noteworthy to be mentioned that the therapeutic activity of proteins is highly dependent on their conformational structure and maintaining a correct (specific) three-dimensional structure is a key to its biological activity [2].

In the recent years hemocyanins, large respiratory proteins that are found in the hemolymph of mollusks and arthropods, are intensively studied in view of their potential against various socially significant diseases like tumors, viral infections, etc., or of their possible application as vaccine adjuvants [3, 4].

The marine snail *Rapana thomasiana* is an invasive marine species in the Black Sea, Marmara Sea and Mediterranean Sea [5]. The hemocyanin isolated from the hemolymph of this mollusk has been extensively studied and very well structurally characterized [6-12]. During the last decade, a great attention has been paid on its biomedical application. For example, there are reports that the hemocyanin from *Rapana thomasiana* (RtH) possesses an anti-viral activity against herpes and Epstein-Barr viruses [13, 14]. On the other hand, RtH exhibits a radioprotective effect upon radiation-induced injuries and facilitates post-radiation recovery in gamma induced acute radiation syndrome in male albino mice [15]. Elsewhere it was reported that RtH, tested in vitro, has shown a good cytotoxic effect against two human bladder cancer cell lines: CAL‑29 and T‑24 [16]. In addition, in vivo RtH expresses strong anti-cancer and anti-proliferative effects in the mice challenged with colon carcinoma [17]. Furthermore, in vivo RtH and its subunits act as a non-specific immunostimulators i.e. induce a strong humoral immune response and can serve as vaccine-carriers [18]. In recent studies, we have demonstrated that the modification of RtH with ionic liquids (ILs) containing imidazolium or choline cation and amino acid anions alter or enhance its cytotoxicity against hormone-dependent breast cancer cells (MCF-7) [19, 20].

There is no literature data on the effect of RtH on the highly invasive MDA-MB-231 breast cancer cells. This cell phenotype is known as triple negative breast cancer (TNBC) because these breast cancer cells do not express on their surface estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2) [21]. TNBC is aggressive, hard to be treated because it does not respond to normal hormone therapy and chemotherapeutics that target HER2, thus they have a poor prognosis [22].

In the view of the above-mentioned, the aim of this study is to assess the effect of RtH and its complexes with choline amino acids on MDA-MB-231 cell viability. In addition, we are seeking a correlation between the IL-induced changes in the protein structure and activity.

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EXPERIMENTAL

Materials

The hemocyanin was isolated from marine snails *Rapana thomasiana* as described earlier [6]. The ionic liquids: (2-hydroxyethyl)trimethylammonium glycinate [Chol][Gly], (2-hydroxyethyl)trimethylammonium L-valinate [Chol][Val], (2-hydroxyethyl)trimethylammonium L-leucinate [Chol][Leu], (2-hydroxyethyl)-trimethylammonium L-isoleucinate [Chol][Ile], (2-hydroxyethyl)trimethylammonium L-methionate [Chol][Met], (2-hydroxyethyl)trimethylammonium L-tryptophanate [Chol][Trp] were synthesized following the previously published procedure [19]. Human breast cancer (MDA-MB-231) cell line was purchased from American Type Culture Collection (ATCC). Thiazolyl Blue Tetrazolium Bromide (MTT) (98%) was purchased from Sigma. RPMI-1640, DMEM high glucose media, L-glutamine, and sodium bicarbonate were purchased from PAN-Biotech GmbH, Aidenbach, Germany.

Scattering experiments

The scattering measurements were performed on a fiber optics specifically elaborated spectrophotometer on the basis of Ocean Optics QE 65000 spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) with Spectra Suite Software (Ocean Optics Inc., Dunedin, USA) set-up as it is described in [23] and with excitation at 660 nm. The data were analyzed and graphically represented by means of computer programme Origin 8.0 (Microcal Software, Inc., Northampton, MA. USA).

According to the theory, if the size of a spherical particle is much less than the wavelength of light in the surrounding medium and if the refractive index of the sphere divided by the refractive index of the surrounding medium, $m = \frac{n_{sph}}{n_{md}}$, is not too large, the calculation can be simplified and scattering in such conditions is widely referred to as Rayleigh scattering [24].

In such conditions, if r is the radius of the sphere and λ is the free-space wavelength and πr^2 is the effective scattered area, then the cross sections forabsorption and scattering are [25, 26]

$$C_{abs} = (\pi r^2).4x\,\mathrm{Im}(|\frac{m^2-1}{m^2+2}|) = (\pi r^2).Q_{abs} \quad (1)$$ and

$$C_{scat} = (\pi r^2).(\frac{8}{3}).x^4|\frac{(m^2-1)}{(m^2+2)}|^2 = (\pi r^2).Q_{scat}, \quad (2)$$

where x is the size parameter and equals to $\frac{2\pi r n_{med}}{\lambda}$ and Q is scattering or absorption efficacy where is the particle radius. Q_{scat} compares the scattering cross section to the physical cross sectional area $\pi r^2$of the particle. Q_{scat} is actually not an efficiency (because it can have values greater than 1), but that is the terminology used in light-scattering theory. For values of Q_{scat} a greater than 1, the particle is able to scatter photons which are outside the physical cross-sectional area of the particle. If the sample is excited far from absorption spectral maximum, then the intensity of scattered light (I_{scat}) is given by:

$$I_{scat} \approx (C_{scat}.(1+\cos 2\theta) + C_{abs}).I_0 \quad (3),$$

where I_0 is the intensity of incident monochromatic light, and θ is angle between excited light beam and direction at which the intensity of scattered light is detected.

On the basis of literature data for the size and shape of a protein, by means of static light scattering measurements it is enough to determine scattered intensities at same conditions for investigated protein and for his products due the investigated processes. On the base of dependence on scattered intensity Iscat, due to N molecules in a scattered volume (V) and environment the equivalent radius of sphere for scattering at θ = 900 will be:

$$R_x = R_0.\frac{Is_x}{Is_o}.\frac{1}{2}K_{1/2} \quad (4)$$

$$K = \frac{(Q_{0scat} + Q_{oabs})}{(Q_{xscat} + Q_{xabs})} \quad (5)$$

where Ro and Iso are the effective radius of known initial compound and measured intensity of scattered light in solution with this compound, Rx, Isx – respectively for product obtain at corresponding process. In (4) the term K depends on many parameters, inherent for light scattering process as it can be seen from equation (1) and (2). According to the definition of Q_{scat} and Q_{abs} and (5) it can be expected that the value of square root of K will be near one.

Absorption spectroscopy

Evolution™ 300 UV-Vis spectrophotometer (Thermo Electron Corporation), equipped with a Peltier temperature control accessory with the highest resolution (1 nm), was used to monitor the formation of the protein-IL

complexes. The changes in the intensities of the absorption bands typical for the aromatic amino acid residues (λ_{max} at 280 nm) and the copper active site (λ_{max} at 345 nm) were followed [27]. RtH concentration was kept constant–1.52 mg/mL (0.17 μM), while the concentration of [Chol][AA] was varied in the range 0.01–0.4 M.

Fluorescence spectroscopy

Steady-state fluorescence measurements were conducted on Perkin Elmer LS-5 spectrofluorometer equipped with a data station model 3600. All fluorescence spectra were collected using 1 cm quartz cuvette with excitation and emission band-width set at 2.5 and 20 nm, respectively. The emission spectra were recorded over the range 310–450 nm at a fixed excitation wavelength of 295 nm. The instrument was calibrated using 10 μM N-acetyl-L-tryptophanamide dissolved in MilliQ water. Solution of RtH (11 nM) and [Chol][AA] (5 mM) in PBS (25 mM, pH 7.2) were mixed and incubated for 60 min at room temperature prior to measurements.

Cell culture

The human adenocarcinoma breast cancer cell line, MDA-MB-231 was cultured in DMEM high glucose medium containing L-glutamine, penicillin, streptomycin, amphotericin B, and 10% fetal bovine serum, and maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2.

Cell viability assay

The viability of MDA MB-231 cells after their incubation with RtH, [Chol][AA] and RtH-[Chol][AA] complexes was assessed in MTT colorimetric test [28].

Briefly, 1x 10^4 cells were seeded in each well of a 96-well plate and maintained for 24 h at 37°C and 5% CO_2. Then, the cells were incubated for additional 24 h with RtH, pure [Chol][AA] or RtH-[Chol][AA] complexes, at concentrations ranging from 100–700 μg/mL and 0.67–4.7 mM for the protein complexes and pure ILs, respectively.

Finally, 20 μL of MTT was added directly to all 96-wells plates with the adherent cells. They were incubated for 3 h at 37°C and 5% CO_2, and the absorbance at 570 nm that is due to the formation of a purple blue formazan product was recorded with a 96-well plate reader Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg). The quantity of formazan is directly proportional to the number of viable cells.

Blank experiments containing only reaction medium as well as control experiments with not-treated MDA MB-231 cells were performed. The data from two independent experiments were used.

The viability of the MDA MB-231 cells that are treated with the tested compounds is presented in percentages from the control.

Data were analyzed using Graph Pad Prism software (Graph Pad Software Inc., San Diego, USA). Statistical test one-way ANOVA and Tukey–Kramer post-hoc test was conducted for pairwise comparison. P-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Absorption spectroscopy

The absorption spectrum of the oxy-form of RtH is characterized with two maxima. The first one is centered at 280 nm and is due mainly to the side-chains of Tyr, Trp and Phe comprising the protein molecule, while the absorption band at 345 nm is due to the copper active side and is assigned to $O^{2-}{}_2$ (π_σ*) Cu(II) charge transfer transition [29]. The absorbance ratio of A_{345}/A_{280} = 0.25 is a common characteristic for the fully oxygenated RtH [27]. In presence of [Chol][AA], we observed a decrease in the intensity of the two absorption bands of RtH in a concentration-dependent manner (Fig. 1). The effect is simultaneous and of the same order of magnitude for the two absorption bands, which suggests that in spite of the observed IL-induced conformational changes in the RtH, the active site remains preserved.

Interestingly, the strongest protein-IL interactions were observed for the choline glycinate.

The further structural and biological studies were performed with protein-IL complexes prepared at a molar ratio of 1:6000.

Fluorescence spectroscopy

Tryptophyl (Trp) fluorescence was used to monitor the conformational changes or unfolding of the RtH molecules in various media. Fig. 2 shows the emission spectra of RtH in a native state (25 mM PBS buffer, pH 7.2) in the absence and in presence of choline amino acids. Interestingly, the intensity of Trp fluorescence increased in all media containing ILs. Red-shift in the wavelength maximum of about 3 to 5 nm was observed in presence of [Chol][Val], [Chol][Met] and [Chol][Ile], while in [Chol][Gly] and [Chol][Leu] no shift was detected. In comparison to the emission spectra of the native RtH, the emission spectra of RtH-[Chol][Trp] characterizes with marked red-shift of about 31 nm.

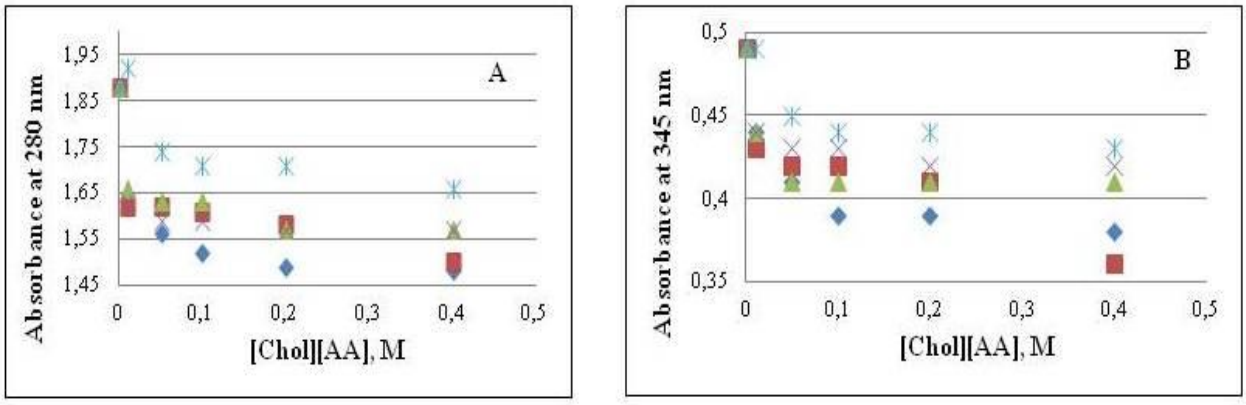

Fig. 1. Changes in the absorbance spectra at 280 nm (A) and 345 nm (B) of RtH (1.5 mg/mL) as a function of the [Chol][AA] concentration.

◆RtH-[Chol][Gly] ■RtH-[Chol][Val] ▲RtH-[Chol][Leu] ×RtH-[Chol][Ile] ✳RtH-[Chol][Met]

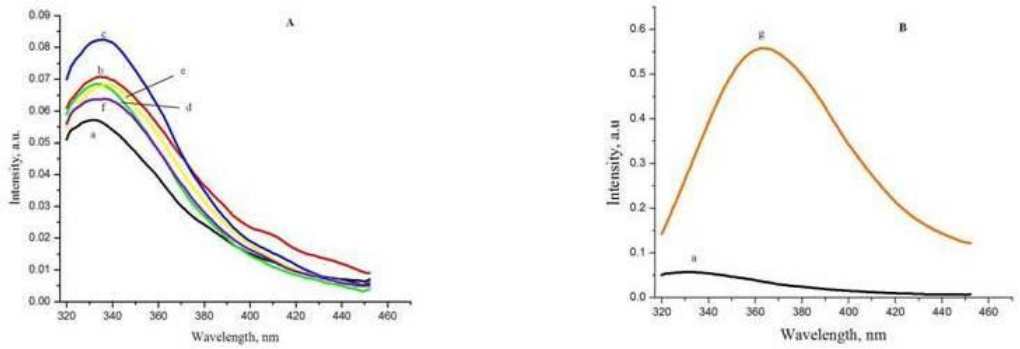

Fig. 2. Fluorescence emission spectra of RtH (11nM) in 25 mM PBS buffer (pH 7.2) (a) and in presence of 5mM [Chol][AA] (b-g). λex = 295 nm, λem 310–460 nm. A) RtH (a), RtH-[Chol][Gly] (b), RtH-[Chol][Val] (c), RtH-[Chol][Leu] (d), RtH-[Chol][Ile] (e), RtH-[Chol][Met] (f); B) RtH (a), RtH-[Chol][Trp] (g).

To evaluate the cytotoxic effect of the ILs on MDA-MB-231 cells we carried out an experiment with [Chol][AA] in concentrations of 0.67 mM and 4.7 mM, corresponding to the lowest and the highest amount of ILs introduced into the system with the RtH-complexes. As can be seen in Fig. 4, most of the tested ILs had moderate cytotoxic effects. However, a strong cell growth inhibition was observed in presence of [Chol][Trp]. The observed effect is time- and dose-dependent. It is noteworthy to be mention that in presence of 4.7 mM of [Chol][Trp] the reduction of cell viability for 48 h exceeded to 90 %.

This may be due to changes in electronic transitions in RtH molecules as a result of electrostatic interactions between ILs and protein molecules. Typically, the red-shift in Trp fluorescence is associated with a presence of positively charged residues near the benzene ring and/or negatively charged residues near the pyrrole end of the Trp [30]. The increase in fluorescence intensity and the red-shift in the λmax can be also a consequence of IL-induced protein unfolding, which makes Trp residues more exposed to the polar solvent (water). Reports for a red-shift of the Trp peak of cytochrome C in presence of 1-butyl-1-methyl imidazolium chloride and an increase in its fluorescence intensity in presence of alkyl ammonium formates can be found in the literature [31]. In contrast, some authors observed a blue shift in the Tyr emission spectra and decrease in fluorescence intensity for insulin in presence of 1, 3- dialkyl-imidazolium salts [32]. The Trp emission spectra of monellin in presence of 1,1-dialkyl pyrrolidinium based ILs is also blue-shifted [33]. In addition, Fan et al reported a decrease in Trp fluorescence intensity of trypsin in respect to the concentration of the added 1-methyl-3-alkyl imidazolium salts [34].

Static light scattering

SLS experiments were performed in order to assess the effect of the tested ILs on the oligomeric state of RtH. The hydrodynamic radii value for oxy-RtH (23.6 nm) [11] in Tris/HCl buffer (pH 7.0) was taken from the literature and was used as a

basis for estimation of the parameters for protein-IL complexes. The calculated effective radius (R_x) of RtH in presence of [Chol][AA] are given in Table 1.

Table 1. The effective radius (R_x) of RtH at presence of [Chol][AA].

RtH in 0.01M Tris/HCl buffer, pH 7.0	R_x, nm
IL-free medium	23.6[11]
[Chol][Gly]	10.1
[Chol][Val]	6.0
[Chol][Leu]	6.0
[Chol][Ile]	6.0
[Chol][Met]	4.1
[Chol][Trp]	6.0

In all SLS experiments the amount of RtH (0.1 mg/mL; 1nM) and ILs (5mM) were kept constant.

All tested ILs caused changes in the protein supramolecular assembly. The estimated decrease in R_x of RtH is two- to six-fold in comparison to the value set for the protein dissolved in the IL-free medium. We assume that due to strong electrostatic interactions between [Chol][AA] and RtH, the protein molecules undergo fragmentation (dissociation). The result correlates with the observed increase in Trp fluorescence i.e. more Trp residues become exposed to the protein surface. We did not find, however, a linear dependence between the two effects – decrease in the R_x and the increase in the Trp fluorescence. Probably, along with the dissociation of the RtH in presence of ILs, a rearrangement in the protein molecules occurs. This conclusion is in agreement with the earlier reported distinct differences in RtH calorimetric profiles in presence of [Chol][AA], which we ascribed to the presence in the hemocyanin molecule of more than one structural domains undergoing denaturation more or less independently of each other [19].

Cytotoxicity of RtH, [Chol][AA] and their complexes

The effects of RtH on MDA-MB-231, hormone-independent breast cancer, cell growth were assessed using concentrations ranging from 100 to 700 µg/mL after 24 and 48 h. RtH exhibited moderate cytotoxic effect on the tested cancerous cells as compared to the control consisting of non-treated with RtH cells (Fig. 3). The observed effect is concentration independent. Our earlier studies have shown that RtH stimulates the proliferation of fibroblasts and in presence of 700 µg/mL hemocyanin, we estimated an increase in cell viability up to 40% in comparison to the control (100%) [19]. In addition, we have shown that RtH does not affect significantly MCF-7 cells (hormone dependent breast cancer). This means that RtH is highly selective and inhibit only the more resistant to various chemotherapies triple negative breast cancer cell line.

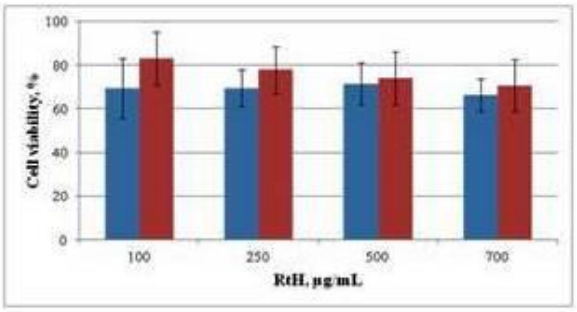

Fig. 3. Viability of MDA-MB-231 cells incubated for 24 and 48 h with various concentrations of RtH. The statistic was performed by ANOVA test. **- p<0.01, ***- p<0.001. 24 h 48 h

To evaluate the cytotoxic effect of the ILs on MDA-MB-231 cells we carried out an experiment with [Chol][AA] in concentrations of 0.67 mM and 4.7 mM, corresponding to the lowest and the highest amount of ILs introduced into the system with the RtH-complexes. As can be seen in Fig. 4, most of the tested ILs had moderate cytotoxic effects. However, a strong cell growth inhibition was observed in presence of [Chol][Trp]. The observed effect is time- and dose-dependent. It is noteworthy to be mention that in presence of 4.7 mM of [Chol][Trp] the reduction of cell viability for 48 h exceeded to 90 %.

The RtH-[Chol][Trp] complex is more cytotoxic to MDA-MB-231 cells than the non-modified native RtH (Fig. 5). RtH's potential to inhibit the cell growth of this hormone independent breast cancer cells, however, is not influenced from the modified with the rest of the tested ILs protein. On the other hand, comparing these results with our previous findings it is interesting to note that RtH-[Chol][AA] complexes are two times more effective against MCF-7 than MDA-MB-231 cells [19].

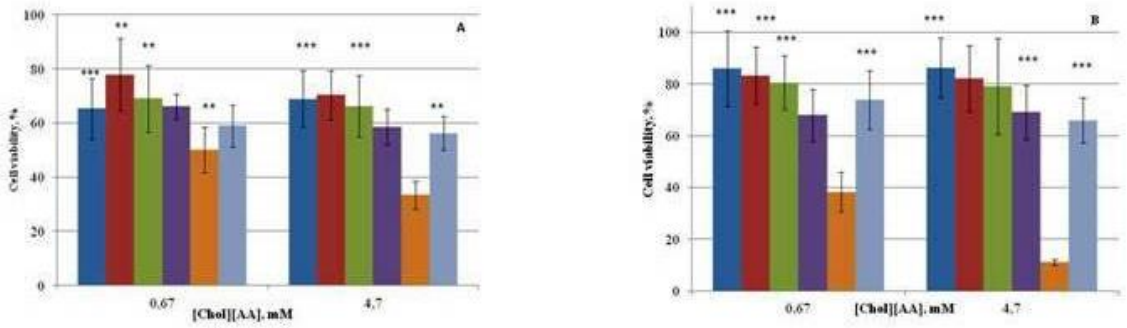

Fig. 4. Viability of MDA-MB-231 cell line incubated for 24 h(A) and 48 h (B) with 0.67 and 4.7 mM [Chol][AA]. Data are means±SD of eigth replicates. The statistic was performed by ANOVA test. **- p<0.01, ***- p<0.001.

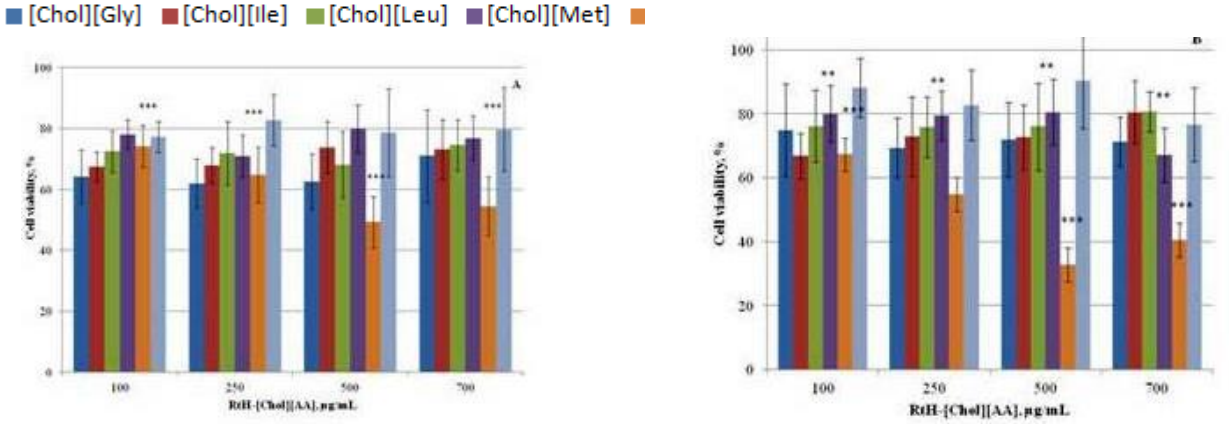

Fig. 5. Viability of MDA-MB-231 cells after incubation with various concentrations of RtH-[Chol][AA] complexes for 24 h (A) and 48 h (B). Data are means ± SD of eigth replicates. The statistic was performed by ANOVA test. **- p<0.01, ***- p<0.001.

CONCLUSION

In this study, we showed that RtH-[Chol][AA] complexes had reduced cytotoxic effect on MDA-MB-231 cells as compared with separate applications of the pure RtH or ILs when the latter were applied at the same concentrations. Probably, the induced by [Chol][AA] conformational changes in the secondary structure of RtH hinders the effective binding of the protein on the cell surface. On the other hand, in a previous study, we have shown that the growth of MCF-7 cells was significantly reduced by RtH-[Chol][AA], which may be an indication for a different mechanism of anti-tumor activity of these complexes. The obtained results testified the cell specific effect of RtH which could be explored in treatment of the different tumors.

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СТРУКТУРА И АНТИТУМОРНА АКТИВНОСТ НА ХЕМОЦИАНИН ОТ *Rapana thomasiana* В ПРИСЪСТВИЕ НА ЙОННИ ТЕЧНОСТИ НА ОСНОВАТА НА ХОЛИН И АМИНОКИСЕЛИНИ

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(Резюме)

Във фокуса на настоящето изследване са взаимодействията на хемоцианин, изолиран от хемолимфата на морски охлюв от вида *Rapana thomasiana* (RtH) с йонни течности, съдържащи катион и анион, съответно холин и неполярна аминокиселина [Chol][AA]. Бяха получени шест комплекса на протеина с йонните течности [RtH-[Chol][AA]]. За изследвания концентрационен интервал, с помощта на флуоресцентна спектроскопия и светоразейване беше установено, че RtH претърпява конформационни промени и се наблюдава фрагментация на протеиновата молекулата при добавянето на [Chol][AA]. За първи път е изследвана цитотоксичността на нативния RtH, чистите [Chol][AA] и на техните комплекси спрямо клетки на хормон-независим рак на млечната жлеза (MDA-MB-231). Всички RtH-[Chol][AA] комплекси инхибират умерено клетъчния растеж на MDA-MB-231. Ефектът, обаче е по-слаб от този, който е отчетен при третиране на клетките с немодифициран RtH и с разтвори само на йонните течности.