Acta Scientific Pharmaceutical Sciences (ISSN: 2581-5423)

Volume 7 Issue 6 June 2023

Research Article

Recombinant Apolipoprotein A-I Is Able Bind and Transport Doxorubicin into Ehrlich Carcinoma Cells

Roman A Knyazev[1]*, Alexander V Ryabchenko[1], Wu Naishi[2], Maria V Kotova, Natalia V Trifonova[1], Lev M Polyakov[1] and Mikhail I Voevoda[1]

[1]*Federal Research Center for Fundamental and Translational Medicine, Novosibirsk, Russia*

[2]*The Department of Cardiovascular Surgery, Tianjin Medical University General Hospital, China*

***Corresponding Author:** Roman A Knyazev, Federal Research Center for Fundamental and Translational Medicine, Novosibirsk, Russia.

DOI:10.31080/ASPS.2023.07.0962

Received: May 02, 2023

Published: May 26, 2023

© All rights are reserved by **Roman A Knyazev., *et al.***

Abstract

The study tries to solve the problem related to the development of new transport forms of anticancer drugs.

The methods of fluorescence quenching and gel filtration have shown that the recombinant apolipoprotein A-I obtained by us has the ability to form a stable complex with doxorubicin. The calculated value of the association constant of the complex confirms the possibility of using the recombinant apolipoprotein A-I as a transport form into tumor cells. The number of molecules of the anticancer drug bound per protein molecule represents a wide range of dosage adjustment. Proven ability of recombinant apolipoprotein A-I c doxorubicin penetrate into cytoplasm and nucleus of Ehrlich ascites carcinoma cells.

The results obtained make it possible to consider the real possibility of using recombinant apolipoprotein A-I as a transport form of doxorubicin.

Keywords: Recombinant Apolipoprotein A-I; Transport form of Anticancer Drugs; Ehrlich's Ascites Carcinoma; Chemotherapy; Doxorubicin

Abbreviations

apoA-I (apoA): Apolipoprotein A-I; LP: Lipoproteins; HDL: High Density Lipoproteins; LDL: Low Density Lipoprotein; VLDL: Very Low Density Lipoproteins; PAAG: Polyacrylamide Gel; IPTG: Isopropyl-β-D-1-thiogalactopyranoside; PAAG: Polyacrylamide Gel; rapoA-I is a mature form of human apolipoprotein A-I, obtained from a recombinant polypeptide by cleavage of a chimeric leader sequence

Introduction

The creation of new highly effective transport forms of drugs is an urgent task. This is especially in demand in the treatment of oncological diseases, where the achievement of the necessary therapeutic effect is always associated with the use of high doses of drugs and, as a result, a toxic effect on organs and functional systems of the body [1-3]. The choice of the transport system is of great importance, since it significantly affects the pharmacokinetics and pharmacodynamics of drugs [4,5].

Various types of transport forms are already widely used in experimental and clinical research. Among them, one can distinguish polymer nanoparticles, liposomes, viral nanoparticles, carriers based on organometallic compounds [6-8]. One of the solutions to the problem of targeted delivery of antitumor drugs

can be constructs based on ligands grafted to the surface of nanocarriers that allow active targeting by binding to receptors overexpressed by cancer cells or angiogenic endothelial cells [8,9].

Apolipoprotein A-I (apoA-I) is the main protein component of the formation of high-density lipoprotein (HDL), it is the main mediator of the reverse cholesterol transport pathway (RTC). It is also known for its anti-atherogenic effect and provides cardio protection [10,11]. Analysis of the state of research in this area indicates the use of apolipoprotein A-I (apoA-I), as a promising carrier of biologically active compounds and drugs. Due to its amphiphilic properties, apoA-I is able to bind and transport both fat-soluble [12] and water-soluble compounds [13]. In the works of Kreuter J., *et al*. and Wohlfart S., *et al*. shows the ability of apoA-I in the nanoparticle composition containing drugs pass through blood-brain barrier (BBB). The mechanism of absorption of these particles is associated with the presence of scavenger receptors class B type I (SR-BI) on the surface of brain endothelial cells [14].

The Scavenger Class B type I receptor (SR-BI), or as it is also called the "scavenger receptor", was the first cell surface protein to be characterized as both a physiologically and molecularly corresponding HDL receptor. Since apoA-I is the main structural component of HDL, this fact can be used to create new therapeutic agents in anticancer therapy [15,16].

SR-B1 is known to promote cancer progression. High expression of SR-B1 has been demonstrated in various types of tumors in the experiment, as well as in patient tumor samples [17,18]. Clinical studies have shown that overexpression of SR-B1 correlates with a more negative prognosis for the survival of cancer patients [19]. The mechanism of this action is associated with the use of SR-B1 as a carrier of tumor cell building materials (lipids, cholesterol in the composition of lipoprotein particles (LP), including apoA-I containing LP), thereby contributing to its nutrition and aggressive growth [19,20].

Thus, apoA-I can be used as a "Trojan horse", which, when it enters the bloodstream, will be used in the synthesis of new HDL or circulate in isolation and, through receptor-mediated endocytosis, will transport the antitumor drug to tumor cells and trigger its death.

Previously, we have shown the ability of apolipoprotein A-I obtained from human blood plasma to form stable complexes with anticancer drugs actinomycin D, vinblastine. Calculated the qualitative and quantitative characteristics of this interaction. It was demonstrated possibility of drugs as part of complexes to penetrate into tumor cells [21]. To date, various lipoprotein delivery systems are known for doxorubicin. Some of the bottom contain APOA-1 mimetics. However, the use of isolated protein for the delivery of doxorubicin has little studied [22,23].

The aim of this work is to study the ability of wild-type recombinant apolipoprotein A-I to act as a vehicle for the anticancer drug doxorubicin.

Materials and Methods

Preparation of recombinant apolipoprotein A-I. Earlier, in the laboratory of medical biotechnology, Research Institute of Biochemistry, Federal Research Center for Fundamental and Translational Medicine, we obtained a producer of the full-length human apolipoprotein A-I protein with a high level of gene expression and protein yield and developed a method for its isolation from producer cells [24]. The resulting protein (rapoA-I) with a molecular weight of ~ 28 kDa does not have two amino acid residues at the N-terminus (aspartic and glutamic acid); otherwise, in terms of its primary structure, it is identical to the mature human apolipoprotein A-I protein.

The production of recombinant apolipoprotein A-I (wild type) (rapoA-I) was carried out according to the method described in detail in our work [24,25]. For the synthesis of recombinant apolipoprotein A-I, E. coli strain - BL21(DE3) cells were used as host cells, into which the mutant gene of the mature form of human apoA-I was inserted as part of the expression vector pET36b (+). To grow E. coli, we used the classical Luria-Bertani (LB) medium containing 1% trypton, 1% sodium chloride, 0.5% yeast extract, medium pH 7.0. If necessary, a solid medium was prepared by adding microbiological agar to 2%. Cells were grown at 370C to the required optical density and induced at 300C. The optical density of cells was measured spectrophotometrically at a wavelength of 600 nm. Induced culture in the logarithmic phase of growth, corresponding to 0.6-1.0 p.u., by adding IPTG to 0.2 mm, followed by incubation over night.

To isolate recombinant proteins, we used the method of isolating proteins under denaturing conditions from the manufacturer of affinity sorbenate Qiagen (USA) after additional cell lysis by ultrasound. Ni-NTASuperflow resin was used as a sorbent. Chromatography was carried out on a column by conventional methods, the results of optical density were recorded on a recorder in the form of a chromatographic pattern. Cleavage and removal of the leader sequence of chimeric recombinant proteins was carried out using acid hydrolysis for 4 hours in the presence of 20-25% formic acid, followed by acid dialysis and transfer of the protein to saline. At the final stage, the protein solution was desalted by dialysis in PBS and sterilized by filtration through a filter with a pore size of 0.22 μm Syringe-DivenFilters (JetBiofilm, Korea). Sterile pure protein was poured into sterile vials for lyophilization, the protein concentration in the solution was ~1 mg/mL. The protein solution was poisoned for lyophilization by freezing and vacuum dehydration on a Virtis EL 25L freeze dryer (USA). The lyophilizates were stored at 5°C. If necessary, the drug was diluted with pharmaceutical sterile water for injection in proportion to the dehydrated volume and used for 5 days.

Figure 1 shows an electrophoretogram of the analysis of the stages of obtaining rapoA-I. Lanes: 1 and 2 - lysate of cells of the producers of chimeric recombinant apoA-I without the addition of an inducer and with the addition of an inducer, respectively. Lane 3 is the product of chimeric apoA-I (33 kDa). 4 - Products of acid hydrolysis of chimeric apoA-I; 5 - products of acid hydrolysis after extraction of pApo-I, a sample of purified chimeric recombinant apoA-I (~ 33 kDa). 6 - a sample of the purified protein rapoA-I of the mature form (~ 28 kDa) with a cleaved "leader sequence"; 7 - sample of purified apoA-I from human blood plasma (~ 28 kDa); 8 - marker proteins (20, 25, 30, 40, 50, 60, 80 kDa "Thermo Scientific").

Figure 1: Electrophoretogram of analysis of fractions for obtaining recombinant protein rapoA-I (wild type) from E. coli cells in 12% polyacrylamide gel.

The obtained protein in terms of electrophoretic mobility corresponded to 28 kDa, which corresponds to native apoA-I. In the last step was obtained lyophilisate rapoA-I. After lyophilization, its solubility was 3.8-4.0 mg/ml in a physiologically acceptable solvent (water for injection).

Study of the absorption spectra of the compounds in the optical region of the electromagnetic radiation carried Evolution 300 spectrophotometer (Thermo Fisher Scientific, USA).

We used 1 mM stock solutions of doxorubicin (Dox) (AppliChem, Germany). The interaction of apoA-I with anticancer drugs studied by spectrofluorimetry at an excitation wavelength of 285 nm and emission in the range from 300 to 450 nm (Shimatzu) [26]. Titration was performed by adding 10 μl aliquots of the anticancer drug to 2 ml of rapoA-I. The association constant was calculated by the method of Attala and Lata [26,27].

To confirm the possibility of rapoA-I complex with doxorubicin entering tumor cells, we used a fluorescent marker - fluorescein isothiocyanate (FITC). rApoA-I was labeled with 1% FITC solution in 0.1 M Na2HP04. The conjugation process was carried out at 4 °C for 18 h in 0.1 M bicarbonate buffer (pH 9.0) and in a protein/dye molar ratio of 10/1 [28]. Unbound label was removed by gel filtration.

The experiments were carried out on Ehrlich ascites carcinoma cells. Isolation of cells from peritoneal exudate was performed on day 10 after transplantation. Under light ether anesthesia, the ascitic fluid was taken with a sterile needle. Peritoneal exudate was centrifuged for 10 min at 150g to precipitate cellular elements. The cell pellet was washed three times with large volumes of cold Ringer-Krebs solution. Cells were counted in a Goryaev chamber. Cell viability was assessed by excluding trypan blue [29].

Experiments on laboratory animals were carried out in accordance with the "Rules of work with the use of experimental animals" (order of the Ministry of Health of the USSR dated 12.08.1977 No. 755 and annex to order No. 565 dated 04.10.1977), in compliance with the principles of the Declaration of Helsinki of the World Medical Association (2000). The animals were kept on a standard diet and had free access to water. The Bioethics Committee of the Research Institute of Biochemistry, FRC FTM, approved the presented work.

The data obtained were subjected to statistical analysis using the StatPlus 2009 Professional 5.8.4 program (USA). The distribution normality was determined according to Kolmogorov-Smirnov. The statistical significance of the results obtained was assessed using the Student's t-test at a significance level of $p < 0.05$.

Results and Discussion

Tryptophan fluorescence quenching is a type of fluorescence spectroscopy used for binding assays. The assay relies on the ability to quench the intrinsic fluorescence of tryptophan residues within a protein that results from changes in the local environment polarity experienced by the tryptophan(s) upon the addition of a binding partner or ligand. The quenching can arise from local changes near the interaction site or from binding-induced conformational changes. [28,30]. Analysis of the spectra of apolipoprotein A-I (apoA-I) with the test substances showed a decrease in tryptophan fluorescence for doxorubicin (Dox) by 90%. The decrease in fluorescence can be characterized as a result of the formation of a complex apoA-I-pro-tumor drug [30]. Figure 2 shows the quenching curves for the Dox complex.

Figure 2: Changes in the tryptophan fluorescence spectra of rapoA-I during the formation of a complex with doxorubicin.

Note:

10 – rApoA spectrum

1 – Spectrum of doxorubicin without protein

2-9 – Adding aliquots of Dox to rapoA-I.

The figure shows the quenching curves of rapoA-I itself, as well as a decrease in the fluorescence intensity when adding equal aliquots of Dox. The study of the time dependence of the quenching of fluorescence upon the simultaneous addition of saturating amounts of drugs showed, that complete binding of the complexing regions of $_{r}$apoA-I was observed within 15 min from the start of titration. Based on the fluorescence quenching curves, the association constant for the interaction of rapoA-I with doxorubicin was calculated. The value was 6.3 ± 0.2 x 10^5 M^{-1}. Also, on the basis of these data, we determined that up to 25 molecules of doxorubicin are bound per 1 molecule of $_{r}$apoA-I.

To confirm the complexation process, as well as to assess the stability of the apoA-I-cytostatic complex, we used the gel filtration method on Sephadex G-25. Previously, we scanned the investigated anticancer drugs on a spectrophotometer to measure the absorption spectra of these compounds in the optical region of electromagnetic radiation. The concentration of the drug was selected experimentally, taking into account the resolution of the device and amounted to 10 μg/ml. For doxorubicin, two absorption maxima were determined at wavelengths 288nm and 447nm (Figure 3).

Figure 3: Scanning spectrophotometry data. Absorption spectra of doxorubicin.

To investigate the possibility of complexation, we used peak absorption characteristics that are maximally distant from apoA-I. For a protein molecule, the absorption maximum corresponds to 280 nm, therefore, to obtain more objective data, we took a value of 477 nm for doxorubicin.

In figure 4 Data of gel filtration of apoA-I complex with doxorubicin are presented. At 280 nm, we got a high peak, which indicates the presence of both protein and doxorubicin in the sample. This is the first peak - 6-9 ml and the second peak - 15.5-17.5 ml line - 1. When performing elution at a wavelength of 477 nm, we also got two peaks: one coincided with the sample in which

apoA-I came out - this is the first peak (6-9 ml) line - 2. An excess of unbound drug is also seen - second peak (15.5-17.5 ml) line - 2.

Figure 4: Chromatographic elution profile of apoA-I with doxorubicin. 1- wavelength 280 nm; 2- wavelength 477 nm.

On the cell culture of Ehrlich's ascites carcinoma, we investigated the possibility of penetration of the apoA-I complex with doxorubicin into the cytoplasm and nuclei of tumor cells. For this, we obtained a rpoA-I conjugate with a fluorescent label with fluorescein isothiocyanate (FITC). Then a complex of rapoA-I-Dox labeled with FITC was obtained. Fig. 5 shows a culture of tumor cells to which apoA-I without marker was added to the incubation medium (A). In figure 5 (B) FITC-labeled apoA-I complex with doxorubicin.

Figure 5: Fluorescence microscopy (magnification 200). A - Ehrlich carcinoma cells with the addition of apoA-I without marker (FITC). B - Ehrlich carcinoma cells in the presence of a FITC-labeled apoA-I complex with doxorubicin. Incubation time 3 hours.

A bright green glow in the cytoplasm and cell nuclei, Figure 4 (B), confirms the possibility of penetration of apoA-I complexes with Dox into tumor cells [31]. At the same time, the number of fluorescent cells after 1.5 hours of incubation reached about 23-31%, and after three hours this value increased to 68-85%, the calculation was made per one hundred visualized tumor cells.

Conclusion

The studies carried out indicate that the recombinant apolipoprotein A-I obtained by us has the ability to form a stable complex with the anticancer drug doxorubicin. The calculated binding constant indicates that the protein-ligand interaction cannot be called highly specific, but this may turn out to be a positive moment when the drug is released in target cells. The number of molecules of anticancer drugs associated per 1 molecule of a complexing agent represents a wide range of drug dosage regulation.

The fundamental possibility of penetration of the investigated complex into the cytoplasm and nuclei of Ehrlich's ascites carcinoma cells has been shown. The results obtained allow us to consider a real possibility of using the recombinant apolipoprotein apoA-I as a transporter molecule for the targeted delivery of doxorubicin to the tumor

Acknowledgements

The work was performed within the framework of the state task of the Ministry of Science and Higher Education of the Russian Federation (registration number 1021050400914-1-1.6.4) using the equipment of the Spectrometric Measurements Center for Collective Use and the Proteomny Analysis Center for Collective Use, supported by funding from the Russian Ministry of Education and Science (agreement No. 075-15 -2021-691).

Conflict of Interest

The authors declare no conflict of interest.

Bibliography

1. Naz S., *et al*. "Advances in Therapeutic Implications of Inorganic Drug Delivery Nano-Platforms for Cancer". *International Journal of Molecular Sciences* 20.4 (2019): 965.

2. Remesh Ambili. "Toxicities of anticancer drugs and its management". *International Journal of Basic & Clinical Pharmacology* 1.1 (2012): 2-12.

3. Basak Debasish., *et al*. “Comparison of Anticancer Drug Toxicities: Paradigm Shift in Adverse Effect Profile”. *Life* 12.1 (2021): 48.
4. Park JH., *et al*. “Targeted delivery of low molecular drugs using chitosan and its derivatives”. *Advanced Drug Delivery Reviews* 62.1 (2010): 28-41.
5. Yihan Yao., *et al*. “Nanoparticle-based drug delivery in cancer therapy and its role in overcoming drug resistance”. *Frontiers in Molecular Biosciences* (2020): 7.
6. Amreddy N., *et al*. “Recent Advances in Nanoparticle-Based Cancer Drug and Gene Delivery”. *Advances in Cancer Research* 137 (2018): 115-170.
7. Mitchell MJ., *et al*. “Engineering precision nanoparticles for drug delivery”. *Nature Reviews Drug Discovery* 20 (2021)101-124.
8. Hirsjarvi Samuli., *et al*. “Passive and active tumour targeting with nanocarriers”. *Current Drug Discovery Technologies* 8.3 (2011): 188-196.
9. Manhas Daljeet Singh. “Passive and active tumor targeting of nano carriers for anti-cancer drug delivery-a review”. *Indo American Journal of Pharmaceutical Sciences* 3.11 (2016): 1345-1349.
10. Pourmousa Mohsen., *et al*. “Tertiary structure of apolipoprotein AI in nascent high-density lipoproteins”. *Proceedings of the National Academy of Sciences* 115.20 (2018): 5163-5168.
11. DC Chan., *et al*. “Apolipoproteins as markers and managers of coronary risk”. *QJM: An International Journal of Medicine* 99.5 (2006): 277-287.
12. Polyakov LM and Panin LYe. “High-density lipoproteins and apolipoprotein A-I: regulatory role and new therapeutic strategies for the treatment of atherosclerosis”. *Atherosclerosis (Russia*) 1 (2013): 42-53.
13. Torchilin VP. “Passive and Active Drug Targeting: Drug Delivery to Tumors as an Example”. *Drug Delivery* 197 (2010)3-53.
14. Wohlfart S., *et al*. “Transport of drugs across the blood–brain barrier by nanoparticles”. *Journal of Controlled Release* 161.2 (2012): 264-273.
15. Leiva A., *et al*. “Mechanisms regulating hepatic SR-BI expression and their impact on HDL metabolism”. *Atherosclerosis* 217.2 (2011): 299-307.
16. Carballo-Jane Ester., *et al*. “ApoA-I mimetic peptides promote pre-β HDL formation in vivo causing remodeling of HDL and triglyceride accumulation at higher dose”. *Bioorganic and Medicinal Chemistry* 18.24 (2010): 8669-8678.
17. Vasquez Marcos., *et al*. “Exploiting scavenger receptors in cancer immunotherapy: Lessons from CD5 and SR-B1”. *European journal of immunology* 47.7 (2017): 1108-1118.
18. Yuan B., *et al*. “High scavenger receptor class B type I expression is related to tumor aggressiveness and poor prognosis in breast cancer”. *Tumour Biology* 37 (2016): 3581-3588.
19. Schorghofer D., *et al*. “The HDL receptor SR-BI is associated with human prostate cancer progression and plays a possible role in establishing androgen independence”. *Reproductive Biology and Endocrinology* 13.1 (2015): 1-10.
20. Gusev EYu., *et al*. “Physiological and pathogenetic role scavenger receptors in humans”. *Medical Immunology (Russia)* 22.1 (2020): 7-48.
21. Knyazev RA., *et al*. “Study of the ability of high density lipoproteins and apolipoprotein A-I to bind and transport anticancer drugs into the cells of Ehrlich’s ascites carcinoma”. *International Journal of Applied and Fundamental Research* 11 (2015): 538-542.
22. Makwana Vivek., *et al*. “Liposomal doxorubicin as targeted delivery platform: Current trends in surface functionalization”. *International Journal of Pharmaceutics* 593 (2021): 120117.
23. Maryam Zamanian-Daryoush and Joseph A DiDonato. “Apolipoprotein A-I and Cancer”. *Experimental Pharmacology and Drug Discovery* 6 (2015): 265.
24. Ryabchenko AV., *et al*. “Production and analysis of biological properties of recombinant human apolipoprotein A-I”. *Bulletin of Experimental Biology and Medicine* 160.1 (2015): 129-133.
25. Ryabchenko AV., *et al*. “The use of modified variants of recombinant apolipoprotein A-I as conveyors of small interface RNA into tumor cells”. *Siberian Scientific Medical Journal* 38.6 (2018): 29-34.
26. Ghisaidoobe ABT and Chung SJ. “Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on Förster resonance energy transfer techniques”. *International Journal of Molecular Sciences* 15.12 (2014): 22518-22538.

27. Polyakov LM., *et al*. "The analysis of interaction lipoproteins and steroid hormones". *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry* 4 (2010): 362-365.
28. Lakowicz JR. "Principles of Fluorescence Spectroscopy". 3rd ed./Spinger: Berlin/Heidelberg, Germany, (2006): 964.
29. Strober W. "Trypan blue exclusion test of cell viability". *Current Protocols in Immunology* 111.1 (2015): A3-B.
30. Lommen J., *et al*. "Release kinetics of the model protein FITC-BSA from different polymer-coated bovine bone substitutes". *Head Face Medicine* 15 (2019): 1-3.
31. Yammine A., *et al*. "Tryptophan Fluorescence Quenching Assays for Measuring Protein-ligand Binding Affinities: Principles and a Practical Guide". *Bio-protocol* 9.11 (2019): e3253.