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COMPLEXATION OF CURCUMIN WITH BOVINE SERUM ALBUMIN AND DIPHTHERIA TOXOID CRM197

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Aim. The goal of the study is to demonstrate the binding sites for curcumin on the protein carriers — bovine serum albumin and diphtheria toxoid CRM197. BSA was chosen as a potential non-specific protein carrier because of its widely used in medicine as a drug carrier.

Methods. In the investigation, both spectrophotometric and molecular docking methods were used.

Results. Two stable binding sites were demonstrated for BSA to bind curcumin. CRM197 was taken as a well-studied carrier protein with its own antitumor activity and has been investigated as a specific carrier with a high affinity for cancer cells with overexpression of epidermal growth factor receptor.

Our results showed one possible curcumin binding site, making CRM197 an ideal specific curcumin delivery platform that provides at least an additive effect in anticancer therapies.

Conclusions. In conclusion, both studied proteins form stable complexes with curcumin that can lay in base of the commercial drug application.

Key words: curcumin; blood proteins; BSA; toxoid; CRM197; complex formation; macromolecular complexes; nanocomplex; protein structure; molecular docking.

Cancer is still a major factor threatening human life around the world, and anticancer drugs remain a huge unmet clinical need [1]. New approaches, use of synthetic molecules [2], cell therapy techniques [3], application of peptides [4] or nanocomplexes [5] are still under consideration.

The object of our study was CRM197 — a recombinant non-toxic diphtheria toxin derivative that differs from the native toxin by only one substitution in the amino acid sequence of the catalytic domain. CRM197 is obtained by replacing glycine in position 52 of the DT gene with glutamic acid [6]. This replacement leads to the loss of catalytic activity by the C-domain and the loss of cytotoxicity by the entire CRM 197 molecule [7]. Despite the loss of toxicity, the CRM 197 molecule retains all the structural components characteristic of the DT molecule, including the structures responsible for binding the R domain to the EGFR demonstrated an overexpression on the surface of the row of the cancer cells [8].

A non-toxic recombinant derivative of diphtheria toxin has antitumor effects in several types of tumor cells, in particular, CRM197 has been shown to block proliferation and angiogenesis and induce apoptosis in human SW-13 and H295R adenocarcinoma cells in culture and in mouse xenografts [9]. In addition, CRM197 has been shown to be a promising carrier for many drugs, such as paclitaxel [10], doxyrubicin [11], cisplatin [12].

All this makes CRM197 a promising possible carrier for curcumin, which can not only increase the bioavailability of anticancer drugs, but also have a synergistic effect on tumor cells.

We tested BSA as a vector for nonspecific delivery of curcumin to evaluate the difference between specific and nonspecific protein carriers. BSA was chosen because of its ability to bind a variety of chemicals such as paclitaxel, metal ions etc [4, 5].

In present study, we focused of the possible complexation of CRM197 with curcumin that is known low-molecular weight compound with prominent anticancer activity [13]. The aim of a present study was to explore the mechanism of complex formation between CRM197 and curcumin and to compare it to the complex formed by BSA and curcumin.

Materials and Methods

BSA (bovine serum albumin), tablets for buffer preparation, PVDF membrane, SDS acrylamide and Curcumin (5 mg/ml stock in 96% ethanol) were purchased from Sigma-Aldrich, USA.

The recombinant protein CRM197 was expressed in *E.coli* BL21(DE3) Rosetta (Sigma-Aldrich, USA) (0.5 mM IPTG (Sigma, USA), 5 hours at 30 °C) with a His-tag at the C-terminal. It was purified using His-Trap affinity column as per the manufacturer's protocol, and the residual imidazole was removed by dialysis against phosphate-buffered saline (PBS, pH 7.4).

SDS-PAGE (sodium dodecyl sulfate — polyacrylamide gel electrophoresis) was used for the characterization of obtained protein. SDS-PAGE was performed at 60 V, 20 min and 120 V, 60 min. Coomassie Brilliant Blue 40 was used as the protein dye. Obtained gels were scanned and protein concentrations were calculated by was purchased from Sigma-Aldrich, USA. PBS.

Formation of Curcumin-Protein Complexes. The curcumin stock solution (5 mg/mL) was prepared in 96% ethanol. A required amount of curcumin was added to the calculated amount of protein (CRM197 or BSA) in PBS solution (pH 7.4) to achieve desired molar ratios. Curcumin in PBS at the required concentration and solvent ratio was used as the control. Formed complexes were dialysated in PBS using PVDF dialysis tubing membrane (Sigma-Aldrich, USA)

Spectrophotometric characteristics. The absorption spectrum of complexes was obtained by spectrophotometry using Optizen-POP (Optizen, Korea). The measurement was provided against PBS solution (pH 7.4). Curcumin dissolved in PBS was used as a control when we examined the spectral properties of curcumin complexes both with BSA or CRM197.

Molecular docking. The protein structures of CRM197 (5I82) and BSA (2VUE) were acquired from Protein Data Bank (http://www.rcsb.org/) and prepared in Chimera software. Ligand structures were built by Marvin Sketch software. Ligands were then protonated and generated in the low-energy conformations (MarvinSketch version 21.16.0, ChemAxon (https://www.chemaxon.com)). A molecular docking simulation of protein and ligand was performed using SwissDock web server, which uses the protein–ligand docking program EADock DSS v3 [14]. A search space of 20×20×20 was used with a grid box centered on the binding sites. Additionally, an accurate docking type was selected with default parameters.

Results and Discussion

Expression of recombinant protein CRM197. Recombinant CRM197 molecule is presented on the Fig. 1. It was obtained from *E. coli* cell lysate by refolding of protein from inclusion bodies on affinity column (Ni-NTA-agarose). The purified protein was further characterized by SDS-PAGE (Fig. 2). The molecular weight of matured protein CRM197, purified from *E. coli* cell lysate, was approximately 60 kDa that coincides with literature data [15].

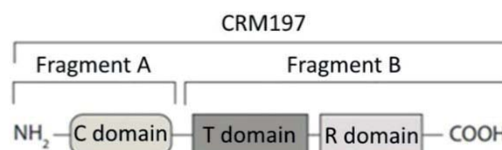

Fig. 1. **Schematic structure of nontoxic derivative od diphtheria toxin CRM197**

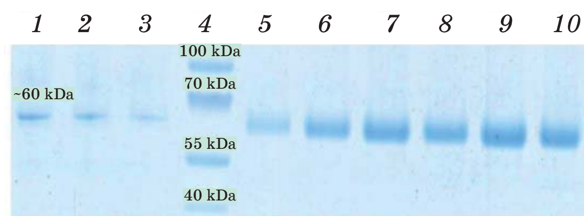

Fig. 2. **SDS-PAGE analysis of purified protein CRM197**

1–3 samples of CRM197 fractions; *4* — Molecular Mass Marker (PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, ThermoFisher); *5–10* — gradient of BSA concentration: *5* — 0.3 mkg; *6* — 0.84 mkg; *7* — 1.38 mkg; *8* — 1.92 mkg; *9* — 2.46 mkg; *10* — 3.0 mkg

Spectrometric characteristic of curcumin complexes with BSA or CRM. CRM197 is a well-known protein with a high affinity to HB-EGFR. Row of cancer cell lines demonstrate of overexpression of these receptors that makes CRM197 is a possible specific curcumin deliver agent. We also used BSA as a widely used protein carrier of chemicals in medicine [16]. Both BSA and CRM197 were considered as the perspective non-specific carrier of curcumin in our investigation.

The absorption spectrum of curcumin was a broad band with maximum absorbance peak at a wavelength ~ 425 nm. However, complexes of curcumin with proteins demonstrated different spectrums with three peaks in the case of CRM197 (Fig. 3) and two peaks in the presence of BSA (Fig. 4).

Both tested proteins showed increasing of absorption rate with increasing of curcumin concentration. When ratio of saturation of proteins by curcumin was achieved there is no further increasing of absorption rate that is represented as a plato on the graph. Based on the data the calculated molar ratio was 1:2,5 and 1:3 for BSA and curcumin and CRM and curcumin respectively.

This allowed us to conclude that the spectral behavior of complexes can be evidence of the stoichiometry of complexes of curcumin with BSA or CRM197. We can speculate that more than one molecule of curcumin can interact with one protein. To prove this hypothesis, we used the molecular docking of BSA or CRM197 with curcumin using SwissDock.

Molecular modeling. The next stage of our work was research *in silico* of the possible binding sites of the curcumin molecule with the CRM197 or BSA molecules. The molecular docking method using the SwissDock software allowed predicting the possible binding modes for the formation of stable complexes.

Study of complexation of BSA and curcumin allowed to obtain two possible binding sites for such interactions (Fig. 5, *A*, *B*). According to the first binding site curcumin forms hydrogen bonds with Tyr138, Tyr161 and His146 of BSA molecule (Fig. 5, *A*).

Second binding mode of curcumin was characterized by the hydrogen bonds between Ser202, Ile290 and Pi-Pi interactions with Trp214 (Fig. 5, *B*).

Complex of CRM197 with curcumin were stabilized by formation of hydrogen bonds with Lys20, Gly18 and Pi-Pi interactions between aromatic moieties of curcumin and His17 and Tyr61 of CRM197 (Fig. 6).

BSA and CRM197, a non-toxic derivative of diphtheria toxin, are promising carriers due to their binding capabilities and potential anticancer properties. The present study was dedicated to investigation of protein carriers for curcumin as drug-delivery platforms. Two promising protein carriers were evaluated such as BSA and CRM197, non-toxic derivatives of diphtheria toxin. BSA was chosen as a non-specific protein carrier because of its ability to bind different substances and deliver them to the cells [12, 18, 19]. Two stable binding sites were demonstrated for BSA to bind curcumin, which allow it serving as a promising carrier for anticancer therapy. CRM197 is a well-studied carrier protein [10, 20,] with own intrinsic anticancer activity [10] was investigated as a specific carrier with high

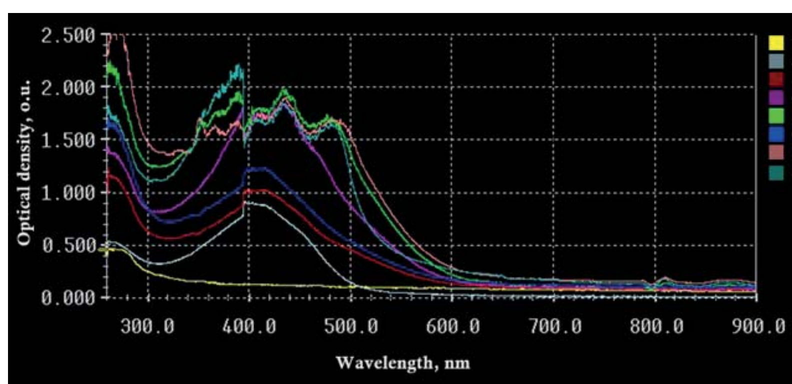

Fig. 3. **The absorption spectrum of curcumin and its complexes with CRM197**
1 — Solution of CRM197 (0.05%); *2* — solution of curcumin in PBS (50:1); *3* — 0.05% solution of CRM197 and curcumin (50:1); *4* — 0.05% solution of CRM197 and curcumin (25:1); *5* — 0.05% solution of CRM197 and curcumin (17:1); *6* — 0.05% solution of CRM197 and curcumin (33:1); *7* — 0.05% solution of CRM197 and curcumin (12:1); *8* — 0.05% solution of CRM197 and curcumin (8:1). All curcumin-containing solutions were prepared in the presence of 0.5% ethanol

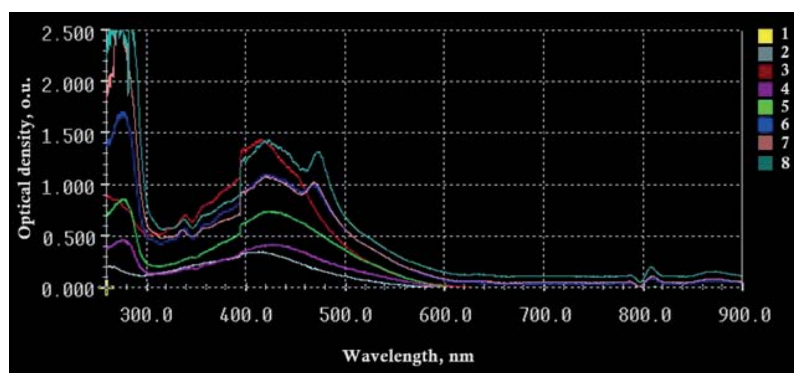

Fig. 4. **The absorption spectrum of curcumin and its complexes with BSA**
1 — PBS; *2* — solution of curcumin in PBS (50:1); *3* — 0.05% solution of BSA and curcumin (50:1); *4* — 0.05% solution of BSA and curcumin (25:1); *5* — 0.05% solution of BSA and curcumin (17:1); *6* — 0.05% solution of BSA and curcumin (33:1); *7* — 0.05% solution of BSA and curcumin (12:1); *8* — 0.05% solution of BSA and curcumin (8:1). All curcumin-containing solutions were prepared in the presence of 0.5% ethanol

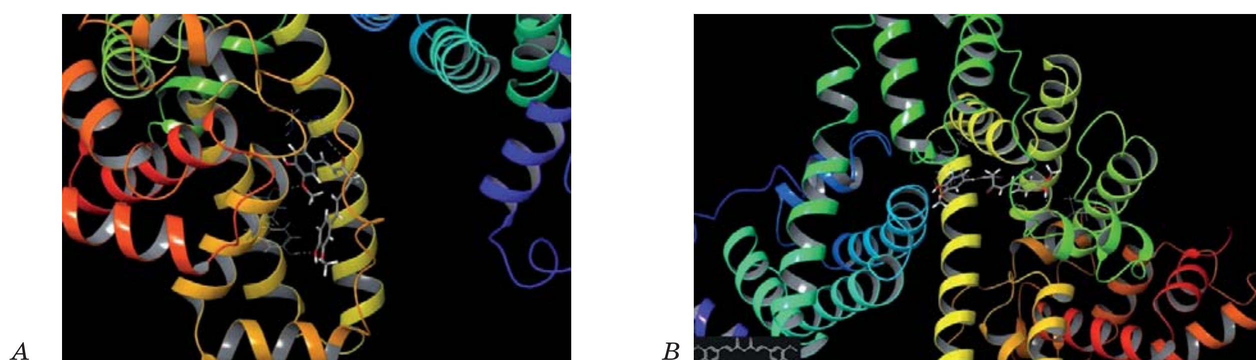

Fig. 5. **Binding modes of BSA and curcumin**
Yellow dashed lines — hydrogen bonds; blue dashed lines — Pi-Pi interactions

affinity to cancer cells with overexpression of marker proHB-EGF like A431 (human adenocarcinoma) or MDA-MB (human breast cancer) cell lines. These results showed one possible binding site for curcumin for CRM197 that makes it perfect specific drug-delivery platforms.

Conclusions

Two sites of curcumin binding with BSA were detected. This was confirmed by the spectral analysis and also by the docking in SwissDock. As for CRM197 *in silico* studies allowed us to detect only one binding site that contradict the spectrometry data. The domains of CRM197 are highly flexible and the availability of only one crystallographic structure did not allow us to take into consideration all conformational changes that can result in the formation of curcumin binding sites.

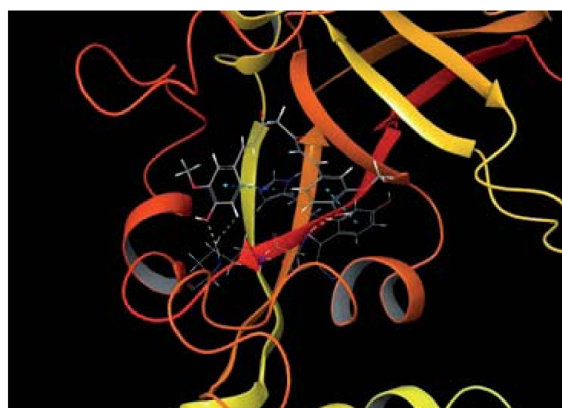

Fig. 6. **Binding modes of CRM197 and curcumin**
Yellow dashed lines — hydrogen bonds; blue dashed lines — Pi-Pi interactions

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КОМПЛЕКСОУТВОРЕННЯ КУРКУМІНУ З BSA ТА CRM197

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Мета. Метою дослідження було довести наявність сайтів зв'язування куркуміну з протеїновими носіями — сироватковим альбуміном великої рогатої худоби та дифтерійним токсоїдом CRM197. БСА був обраний як потенційний неспецифічний протеїновий носій через його широке застосування в медицині як носія багатьох лікарських засобів.

Методи. У дослідженні було використано як спектрофотометричний метод, так і метод молекулярного докінгу.

Результати. Було продемонстровано два стабільних сайта зв'язування БСА з куркуміном. CRM197 був обраний як добре вивчений протеїн з власною протипухлинною активністю, що широко застосовують у медичній практиці щодо терапії пухлинних клітин з підвищеним рівнем експресії EGFR. Наші результати показали один можливий сайт зв'язування для молекули куркуміну, що робить CRM197 ідеальною платформою специфічної доставки куркуміну, яка забезпечує принаймні адитивний ефект в протипухлинній терапії.

Висновки. Підсумовуючи, обидва досліджені протеїни утворюють стабільні комплекси з куркуміном, що може лягти в основу комерційного застосування ліків.

Ключові слова: кумин; протеїни крові; BSA; анатоксин; CRM197; комплексоутворення; високомолекулярні комплекси; нанокомплекс; структура протеїну; молекулярний докінг.