

## NECESSITY OF TRANSLOCATION DOMAIN FOR REALISATION OF CYTOSTATIC EFFECT OF NON-TOXIC DERIVATIVES OF DIPHTHERIA TOXIN

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The aim of the work was to evaluate *in vitro* the cytostatic effect of recombinant fragments of the non-toxic point mutant of diphtheria toxin — CRM197, which was suggested as a potent medication for treatment of triple negative breast cancer. For this purpose, non-toxic recombinant derivatives of diphtheria toxin — CRM197, subunit B SbB and receptor domain Rd had been isolated by Ni-NTA agarose affinity chromatography and their effect on the growth of individual colonies of triple negative breast cancer MDA-MB-231 cells were characterized by such parameters as average colony area, perimeter and circularity index. According to the obtained results, CRM197 and SbB, whose molecules contain the translocation domain Td, exhibited the same cytostatic effect against MDA-MB-231 cells, reducing the area and perimeter of individual colonies. Rd protein did not affect the last two parameters that characterize the size of the colonies, but changed the form of the margin of colonies, as evidenced by an increase in the circularity index.

It is supposed that Td may be involved in the implementation of cytostatic action due to its inherent pore-forming activity in relation to lipid membranes. It is concluded that Rd and Td, unlike the catalytic domain of diphtheria toxin, play important roles in the implementation of the cytotoxic properties of CRM197, while SbB consisting of Rd and Td is the structural DT fragment of smallest molecular weight that can be used as the analog of CRM197.

**Key words:** CRM197, diphtheria toxin, HB-EGF, toxoid, triple negative breast cancer.

CRM197 protein (cross-reacting material 197) is a non-toxic point mutant of diphtheria toxin (DT) produced by *C7 Corynebacterium diphtheriae* strain which was lysogenically transformed by a mutated coryneophage  $\beta$ , containing the altered  $tox^+$  gene. Mutated phage named  $\beta 197^{tox^-}$  was obtained by nitrosoguanidine treatment of toxigenic *C7*( $\beta$ ) *C. diphtheriae*, containing prophage  $\beta$ , during the lytic phase induced by UV-light exposure. Then *C7*(-) *C. diphtheriae* cells were transformed by the resulted phage mutants and obtained lysogenic clones were tested for production of non-toxic material that was cross-reacting with anti-DT polyclonal antibodies [1]. Clone 197 which produced crossreacting with DT protein, was found to synthesize a DT polypeptide chain with a single Gly52Glu mutation [2] which leads to an almost complete loss of the catalytic activity of A-subunit of DT. Apart from this single mutation, protein

CRM197 structure is absolutely the same as that of the native toxin: its molecule contains subunit A with a non-active catalytic or C-domain and subunit B which comprises functional and entire receptor or R-domain (Rd) and translocation or T-domain (Td).

It should be noted that the ability of native DT to inhibit the growth of malignant cells in resistant to toxin mice has been already known for a relatively long time [3]. The non-toxic to DT-sensitive species, CRM197 turned out to be a promising agent in applying to humans. It has been demonstrated that this toxoid effectively inhibits the growth of human malignant cells *in vivo* in nude mice model [4–6] and increases survival of patients with progressive cancer [7–9]. There are a lot of evidence that CRM197 is effective in suppressing the cancer of breast [6, 10, 11], oral cavity [12], stomach [13], immune cells [14] and ovaries [4, 5].

Nowadays, CRM197 is already undergoing clinical trials for introduction in the therapeutic practice of human cancer treatment [8]. Production of CRM197 in *Escherichia coli* greatly facilitated the large-scale manufacturing of this protein [15–17].

However, as a medicine for intraperitoneal administration, CRM197 possess essential disadvantages, as it preserves almost the full immunogenicity of the native DT. This means, that after the first few administrations, like native DT, toxoid CRM197 will provoke a strong immune response and its molecules will be eliminated fast from the bloodstream. Besides, some parts of the CRM197 molecule may be functionally superfluous, as they do not participate in the implementation of its cytostatic effect and may have potential side effects. We suppose that subunit A of CRM197 may be of a low necessity in the realization of its anticancer properties.

Recombinant fragments of CRM197 with smaller molecular weight can be less immunogenic than full molecule. Besides, these fragments could be constructed to contain only functionally beneficial structural parts. The anticancer potentials of different structural parts of the CRM197 molecule have not yet been studied. The aim of the present work was to evaluate the cytostatic effect of recombinant non-toxic fragments of DT molecule for finding a compound of a smaller molecular size, which preserve the most of the anticancer properties of entire CRM197.

The effect of CRM197 on tumors is implemented by the interaction of this protein with growth factor HB-EGF. It was demonstrated that HB-EGF is often overexpressed in the transformed cells and that soluble form of HB-EGF (sHB-EGF) promotes the development of a malignant phenotype. Today, the gene of HB-EGF is considered to be strongly responsible for chemotherapy resistance [18]. Production of HB-EGF promotes cell surveillance and development of signs of oncogenic transformation. It is generally accepted that treatment with CRM197 leads to decrease in cell proliferation, because when sHB-EGF is bound to CRM197, it is unable to interact with its natural cell receptor EGFR [13,18,19].

There are some findings that sHB-EGF and its transmembrane precursor can be especially important targets for CRM197 in breast cancer therapy [10]. In the case of triple-negative breast cancer, there is no increase in expression of estrogen, progesterone and HER2 receptors in cells, which are targeted by conventional

hormonal therapy (such as tamoxifen or aromatase inhibitors) or therapies that target HER2 receptors (Herceptin). However, there is an evidence that triple-negative breast cancer can be effectively suppressed by CRM197 [6, 11, 19]. HB-EGF-targeted therapy for triple negative breast cancer is of a high relevance, as it can sufficiently increase surveillance in patients with this type of oncology. Thus, in the present study, it was decided to analyze the effects of DT toxoids in relation to triple negative MDA-MB-231 cell line, derived from the human organism.

Previously in the department of molecular immunology at Palladin Institute of Biochemistry of the NAS of Ukraine was created a set of *pET24(a)*-based plasmid genetic constructions for production of different non-toxic structural DT derivatives in *BL21 Rosetta (DE3) E. coli*: CRM197 – the product of site-specific mutagenesis of native *tox*<sup>+</sup> gene from PW8 *C. diphtheriae* strain [20]; SbB – subunit B of DT which contain the entire Td and Rd of DT [21]; Rd – entire receptor domain of toxin possessing no other structural parts. All of the above listed recombinant DT toxoids retain the ability to bind proHB-EGF on the surface of mammalian cells [22–25] and have been used in present work to evaluate the cytostatic effect of toxin recombinant derivatives produced and purified from *E. coli* on malignant cells.

## Materials and Methods

**Materials and reagents.** In present work there were used: acrylamide, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine, N,N'-methylenebisacrylamide, phenylmethylsulfonyl fluoride (AppliChem GmbH, Germany); chloramphenicol, kanamycin (Arterium Co., Ukraine); eukaryotic cell culture Petri dishes (Greiner Bio One, Great Britain); centrifugal filters with 10 kDa nominal molecular weight limit (Merk, Germany); KCl, KH<sub>2</sub>PO<sub>4</sub>, NaCl, Na<sub>2</sub>HPO<sub>4</sub>, NaH<sub>2</sub>PO<sub>4</sub>, NaOH, β-mercaptoethanole (β-ME) (“Miranda-C”, Ukraine); crystal violet, imidazol, sodium deoxycholate (Shanghai Synnad, China); amphotericin B, foetal bovine serum (FBS), lysozyme from chicken egg, paraformaldehyde, penicillin G, RPMI-1640 media, streptomycin, LB media, tricine, tris-hydroxymethyl aminomethane, Triton X-100, urea (Sigma Aldrich, USA); human recombinant DNase I, isopropyl β-D-1-thiogalactopyranoside, molecular weight markers for protein gel electrophoresis nickel-

nitrilotriacetic acid agarose (Ni-NTA) (Thermo Fisher Scientific, USA).

*Production of CRM197, SbB and Rd in E. coli cells.* Creation of the *pET24a(+)*-based genetic constructs for expression of CRM197 and SbB was described in [20,21]. Creation of *pET24a(+)-Rd* construct for expression of Rd was carried out as described in [20]. *E. coli BL21 Rosetta (DE3)* cells containing the appropriate expression vectors were cultivated on LB media with kanamycin and chloramphenicol at 37 °C under rotation (250 rpm). Expression was induced by isopropyl  $\beta$ -D-1-thiogalactopyranoside and conducted at 30°C under rotation (250 rpm).

Bacterial peptidoglycan cell walls were digested by addition of 10 mg/ml lysozyme to bacteria suspension in 50 mM TrisHCl, 1 mM EDTA, 100 mM NaCl, 0.01 M phenylmethylsulfonyl fluoride, pH = 8.0 at 4 °C. Then concentrations of MgCl<sub>2</sub> and CaCl<sub>2</sub> in resulted suspensions were brought to 25 mM and 55 mM respectively, and protoplast membranes were destroyed by addition of 40 mg/ml of sodium deoxycholate. Bacterial DNA was digested by DNase I at room temperature until suspension become non-viscous. Then the insoluble fraction of cell lysate containing inclusion bodies was collected by centrifugation and washed by resuspension in the initial cell solubilizing buffer solution containing 0,5% Triton X-100.

Proteins CRM197, SbB and Rd were extracted from the water-insoluble fraction of bacterial cell lysate by 8 M urea in 250 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 M NaCl, 10 mM imidazole and 10 mM  $\beta$ -ME, pH = 8.0. Purification by imidazole elution gradient and refolding by decreasing urea and  $\beta$ -ME concentration were carried out on Ni-NTA agarose. Eluted samples were dialyzed against phosphate buffered saline (PBS), 0.01 M Na<sub>2</sub>HPO<sub>4</sub>, 0.002 M KH<sub>2</sub>PO<sub>4</sub>, 0.137 M NaCl, 0.003 M KCl, pH 7.4, and concentrated. The purity and concentration of the target proteins in resulted samples were estimated on tricine SDS-PAGE [26] electrophoregrams by gel densitometry using Fiji software.

*Cultivation of cancer cells.* MDA-MB-231 cells were obtained from the Bank of cell lines from human and animal tissues of Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine. Cells were maintained at 37 °C and under 5% CO<sub>2</sub> on RPMI-1640 media with 10% FBS, 0.3 g/l Lglutamine, and antibiotics: 100 mg/l streptomycin 10 000 U/l penicillin G and 250 mg/l amphotericin B.

*Colony assay.* To obtain individual colonies,  $5.5 \times 10^3$  of MDA-MB-231 cells were seeded on plastic 60 mm Petri dishes on RPMI-1640 with 10% FBS. Next day, when individual cells were already attached to the plastic surface, the medium was changed to RPMI-1640 with 5% FBS and  $0.254 \times 10^{-6}$  M of a recombinant DT derivative of interest. Equal volumes of filtrates from the same protein samples, obtained by gravity concentration on the semi-permeable membrane, were used as controls. The absence of protein in the obtained controls was also demonstrated by SDS-PAGE. Cultivation in presence of DT recombinant derivatives was carried out during 7 days until colonies visible to the naked eye were formed. The medium was changed to fresh every 2–3 days. After 7 days, cells were fixed by 5% of paraformaldehyde in PBS for 40 min at room temperature and stained with 0.2% crystal violet solution for 3 h at 37 °C under rotation (60 rpm). Excess of crystal violet dye was removed by washing 3 times in deionized H<sub>2</sub>O under 37 °C and rotation (60 rpm). Petri dishes were dried and scanned at 720 dpi, 48-bit color (Fig. 2). Size, perimeter and circularity of colonies on resulted pictures were analyzed by Fiji software.

Area of colonies was calculated in pixels, perimeter — in arbitrary units. Shape descriptor of Fiji, that calculates object circularity, uses the formula:

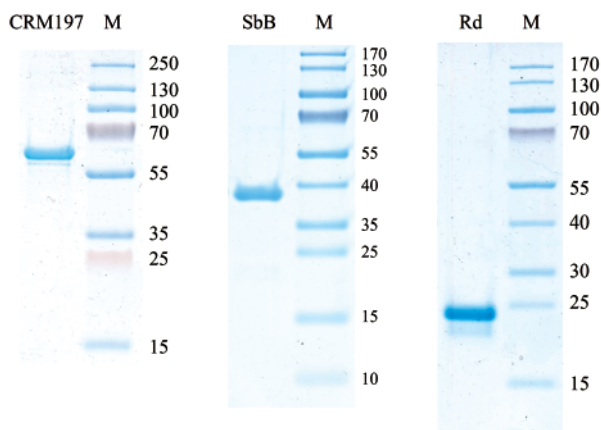
$$\text{Circularity} = 4 * \pi * (\text{Area} / \text{Perimeter}^2),$$

that allows differentiating objects with same values of area and perimeter, but with different shapes. A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon.

*Statistical data analysis.* The mean values were calculated on the basis of 3 independent experiments in each case. Error bars represent the standard deviations or respective mean values. The *t*-test for experimental and control groups with a significance level of 0.05 was carried by Origin9 software.

## Results and Discussion

*Characterisation of resulted protein samples by gel electrophoresis.* After the lysis of *E. coli* cells, proteins CRM197, SbB and Rd were found in the insoluble fraction of the lysate, which suggest the accumulation of studied proteins in inclusion bodies. The effectiveness of *in vitro* renaturation (refolding, [22–28]) of fluorescently labeled diphtheria toxoids after urea extraction from the inactive water-



**Fig. 1. SDSPAGE electrophoregram of CRM197, SbB and Rd samples:**  
M — molecular weight markers

insoluble state was demonstrated previously in [22–25]. SDS-PAGE showed the high purity and concentration of targeted proteins in resulted samples (Fig. 1).

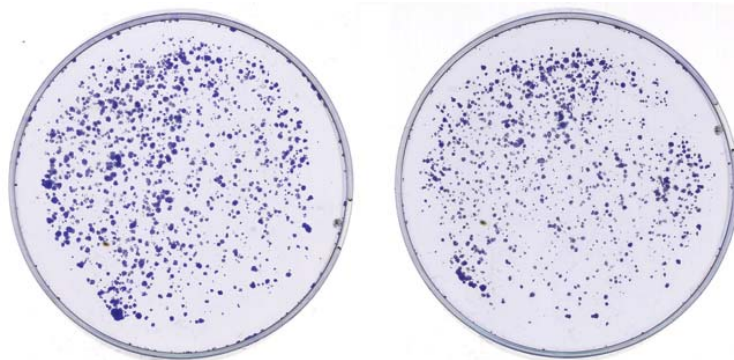
*The growth of single MDA-MB-231 colonies in presence of DT recombinant derivatives.* Previously, it has been shown that recombinant SbB at a concentration of  $1.28 \times 10^{-6}$  M exhibits a sufficient cytotoxic effect on human histiocytic lymphoma cell line U937, which expresses a large amount of sHB-EGF [26–29]. In the present study, we decided to compare cytotoxicity of different DT derivatives which retain the receptor-binding ability in relation to HB-EGF. It was shown that presence of  $0.254 \times 10^{-6}$  M of recombinant CRM197, SbB and Rd in the culture media allow the single MDA-MB-231 cells to grow into colonies. Studied DT derivatives affected size and shape of MDA-MB-231 colonies arising from the single cells on the flat surface.

It was found that toxin derivatives which contain Td (CRM197 and SbB) are

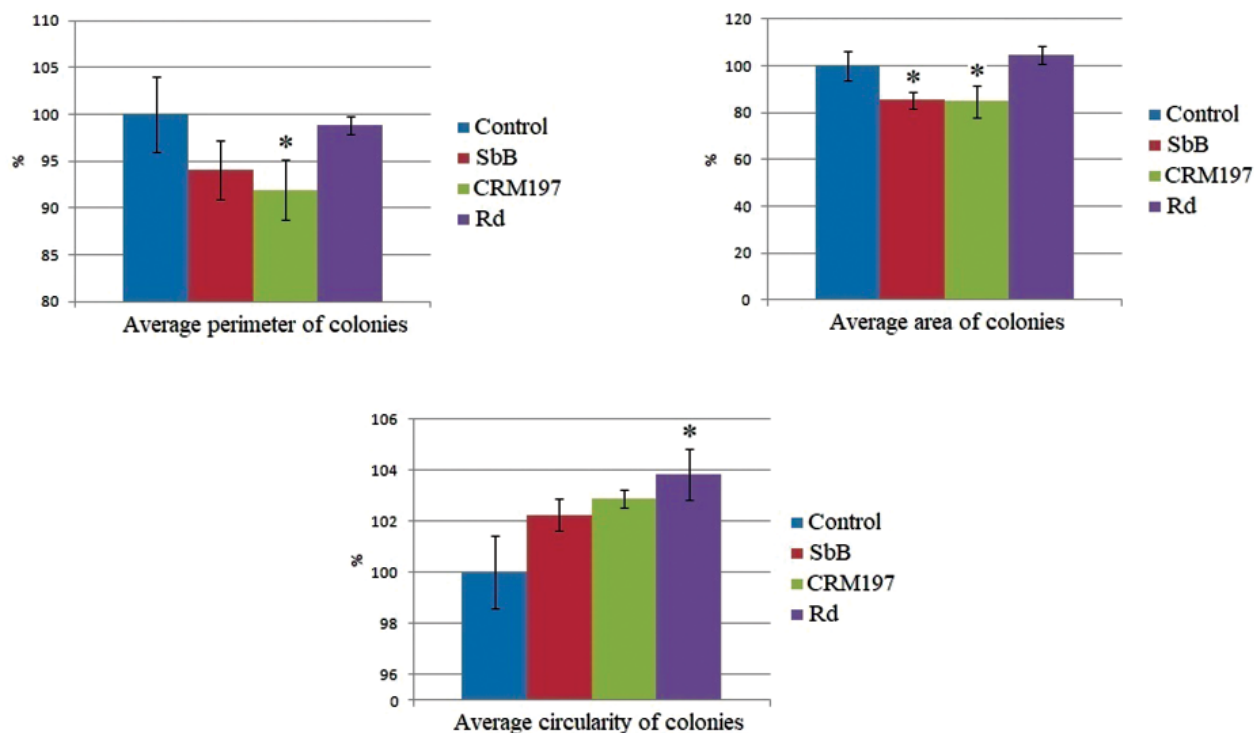
more effective in inhibiting the growth of MDAMB231 colonies than toxin derivatives which do not contain Td (Rd). Parameter of colony area which characterize the growth of cancer cells, reduced significantly compared to controls in the presence of proteins CRM197 and SbB (Fig. 3). The perimeter changes were generally in accordance with the area of the colonies. Compared to each other, CRM197 and SbB have approximately the same inhibitory effect on colony area and perimeter. In relation to controls, Rd did not affect the size and perimeter of the resulted MDA-MB-231 colonies. Nevertheless, Rd has still exerted a significant influence on cells which resulted in the change of the colony shape.

To characterize the shape changes under the influence of recombinant DT derivatives, we use the parameter of circularity. Circularity — is a value that shows how the shape of a particular object approaches the shape of an ideal circle. In the case of colonies of eukaryotic cells, circularity characterizes the asymmetry and boarder of colonies which possibly may reflect the intercellular organization and interactions on the surface in 2D. Recombinant Rd significantly increased the circularity of growing MDA-MB-231 colonies.

Decrease in circularity of colonies can be a sign of a malignant phenotype since more disorganized cells on the surface should also have the increased ability to migrate and reduced capacity to form and maintain the intercellular contacts. It is naturally to assume that cells, forming colonies with a smaller circularity, can exhibit an increased ability of metastatic invasion *in vivo*. Thus, it was decided that the increase in the circularity of MDA-MB-231 colonies in 2D culture in presence of Rd may be an indicator of reduced expression of malignancy in Rd-treated cells.



**Fig. 2. MDAMB231 colonies, obtained from the single cells attached to the plastic surface and formed under: the presence of 10 mkg/ml (0.254 mkM) of SbB (right) and the equal volume of PBS (left) after 7 days of culturing. Fixation with paraformaldehyde and crystal violet staining**



**Fig. 3. The average size, perimeter and circularity of MDA-MB-231 colonies formed under:** the influence of equimolar concentrations of CRM197, SbB and Rd. Asterisks indicate when the  $p$ -values of  $t$ -test for control and experimental groups are less than 0.05

The most common hypothesis about the CRM197 antitumor effect is that this toxoid blocks the ability of sHB-EGF to bind its natural receptor EGFR. Besides, the affinity of the CRM197–HB-EGF interaction is of a relatively high value, which makes the mentioned protein complex stable from the reverse dissociation [10,13]. Inactivated by a toxoid, sHB-EGF is removed from the intercellular space, which leads to a weakening of the sHB-EGF-dependent paracrine and autocrine stimulation of cell proliferation. The role of the CRM197 interaction with the transmembrane proHB-EGF in the realization of its cytostatic effect has not been sufficiently investigated.

According to our results, it is suggested that Td is sufficiently involved in the realization of the total cytostatic effect of CRM197. The influence of Td on cell proliferation may suggest that recombinant DT derivatives can affect cells by their Td due to the inherent ion-conductive and pore-forming properties of this domain regarding lipid membranes [30, 31]. The data on Td-influence on cell growth which was obtained in present work is in a good agreement with the previous our data on the characterization of ion-conductive properties of CRM197 and SbB

[32, 33], which strongly suggest that function of Td in *E. coli*-derived and *in vitro* renaturated recombinant products is well reproduced.

Td pores are permeable to  $K^+$ ,  $Na^+$  and  $H^+$  [34]. The ionic conductivity caused by Td may interfere with the balance of ions and protons between cytoplasm and endosome lumen [24, 25]. As a result, enzymes that require highly acidic environment are prevented from the activation, endosome maturation slows down [25] and degradative endocytic pathway in cells becomes disrupted. The greater amount of proHB-EGF receptor is located on the surface of cancer cells, the more endosome pathway of cells is affected. Disorder in general physiological cellular condition due to the influence of Td may ultimately result in proliferation slowdown.

Thus, our results support the idea that CRM197 and other DT toxoids can realize their tumor suppressive effects by two interdependent mechanisms: 1. sHB-EGF-dependent, when cells affected indirectly by depletion of proliferative soluble signal in intercellular space; and 2. proHB-EGF-dependent, when cells affected directly by functional pore-forming Td of DT.

Obtained results suggest that non-active subunit A of CRM197 is not required for

cytostatic action of this toxoid and Rd alone is not so effective in suppression of cancer cell proliferation as DT fragments that contain the Td. Thus, we recommend the use of SbB produced by *E. coli* for cancer therapy, as the closest functional analog of CRM197 that contains no functionally superfluous structural parts with potential side effects.

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**НЕОБХІДНІСТЬ  
ТРАНСЛОКАЦІЙНОГО ДОМЕНУ  
ДЛЯ РЕАЛІЗАЦІЇ ЦИТОСТАТИЧНОГО  
ЕФЕКТУ НЕТОКСИЧНИХ ПОХІДНИХ  
ДИФТЕРІЙНОГО ТОКСИНУ**

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Метою роботи було оцінити *in vitro* цитостатичну дію рекомбінантних фрагментів нетоксичного точкового мутанту дифтерійного токсину — CRM197, який було запропоновано як потенційний препарат для лікування трійчасто-негативного раку грудної залози. Із цією метою з використанням методу металоафінної хроматографії на Ni-NTA агарозі виділили рекомбінантні похідні дифтерійного токсину — CRM197, SbB (субодиницю B) і рецепторний домен Rd та дослідили їх вплив на ріст поодиноких колоній клітин трійчасто-негативного раку грудної залози людини MDA-MB-231 за такими показниками, як площа, периметр та індекс циркулярності. Одержані результати показали, що CRM197 і SbB, які містили у складі молекули транслокаційний домен Td, однаковою мірою виявляли цитостатичний ефект стосовно клітин MDA-MB-231, зменшуючи площу та периметр поодиноких колоній. Протеїн Rd не впливав на останні два параметри, які характеризують розмір колоній, однак змінював форму їхнього краю, про що свідчить підвищення індексу циркулярності. Ймовірно, що Td може брати участь у реалізації цитостатичної дії через притаманну йому пороутворювальну активність щодо ліпідних мембран. Зроблено висновок, що Rd і Td, на відміну від каталітичного домену дифтерійного токсину, відіграють важливу роль у реалізації цитотоксичних властивостей CRM197, а SbB, яка складається з Rd і Td, є структурним фрагментом дифтерійного токсину з найменшою молекулярною масою, і її можна використовувати як аналог CRM197.

**Ключові слова:** CRM197, дифтерійний токсин, HB-EGF, токсод, трійчасто-негативний рак грудної залози.

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Целью работы была оценка *in vitro* цитостатического действия рекомбинантных фрагментов нетоксичного точечного мутанта дифтерийного токсина — CRM197, который был предложен как потенциальный препарат для лечения тройчато-негативного рака грудной железы. С этой целью с использованием метода металлоафинной хроматографии на Ni-NTA агарозе выделены рекомбинантные производные дифтерийного токсина — CRM197, субъединица B SbB, рецепторный домен Rd и исследовано их влияние на рост одиночных колоний клеток тройчато-негативного рака молочной железы человека MDA-MB-231 по таким показателям, как площадь, периметр и индекс циркулярности. Полученные результаты показали, что CRM197 и SbB, которые содержали в составе молекулы транслокационный домен Td, в равной степени проявляли цитостатический эффект по отношению к клеткам MDA-MB-231, уменьшая площадь и периметр отдельных колоний. Протеин Rd не влиял на два последних параметра, которые характеризуют размер колоний, однако изменял форму их края, о чем свидетельствует повышение индекса циркулярности. Вероятно, Td может принимать участие в реализации цитостатического действия за счет присущей ему порообразующей активности по отношению к липидным мембранам. Сделан вывод, что Rd и Td, в отличие от каталитического домена дифтерийного токсина, играют важную роль в реализации цитотоксических свойств CRM197, а SbB, состоящая из Rd и Td, является структурным фрагментом дифтерийного токсина с наименьшей молекулярной массой, и ее можно использовать в качестве аналога CRM197.

**Ключевые слова:** CRM197, дифтерийный токсин, HB-EGF, токсод, трійчато-негативний рак грудної залози.