UDC [577.112.083/616.931]:[615(331+371+375)+616-006.04]
https://doi.org/10.15407/biotech11.03.027

BIOLOGICAL PROPERTIES AND MEDICAL APPLICATION OF DIPHTHERIA TOXIN DERIVATIVES

K. Y. MANOILOV

Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, Kyiv, Ukraine

E-mail: manoilov.inbox@gmail.com

Received 02.02.2018

The aim of the review was to analyze the literature data related to the application of a variety of diphtheria toxin derivatives. Although the studies interaction with sensitive and resistant mammalian cells have been held for a relatively long time, there are still some unresolved issues concerning the molecular mechanisms of diphtheria toxin functioning. Native diphtheria toxin and parts of its molecule which preserve toxicity are used as instruments in the newest biotechnological methods for specific cell subtype ablation in multicellular organisms. New recombinant derivatives of diphtheria toxin are periodically obtained in the laboratories throughout the world. Most of these analogs of DT are used in biological studies as the convenient tools for analysis of the functions of natural toxin. A non-toxic analog of diphtheria toxin, protein CRM197, is used in clinical practice as a component of vaccines and as an anticancer agent. Diphtheria toxin — based targeted toxin therapy is another perspective trend for cancer treatment. Therefore, studying of diphtheria toxin derivatives is of a great relevance for biotechnology and medicine.

Key words: cell ablation, CRM197, diphtheria toxin, immunogenicity, targeted toxin therapy, toxoid.

Over the past few decades, a lot of the new information related to diphtheria toxin (DT) have appeared. However, in the most cases, not the DT itself was in the spotlight, but rather its derivatives. There are many different derivatives of DT obtained in cells of a natural producent, such as *Corynebacterium diphtheriae*. However, for several reasons, considerable attention is paid to recombinant analogs of DT. For the first, natural DT possesses one of the lowest values of semi-lethal dose ($LD_{50\%}$) for sensitive cells among the other bacterial exotoxins [1]. The non-toxic DT mutants allow carrying out the research work in a much more safe and convenient way, as they do not pose a threat to laboratory personnel and do not require the implementation of multiple biosafety means. On the other hand, recombinant derivatives of DT are much easier to obtain in the laboratory than native toxin and its fragments. Moreover, nowadays the methods of genetic engineering allow altering the molecules of studied proteins in a desired way. The most commonly used recombinant DNA approaches include introduction of desired mutations and additional amino acid sequences like a fused fluorescent label or a specific affinity tag, deletion of undesired amino acids, construction of the chimeric molecules which combine the necessary functions, etc. Sometimes, in order to study the function of individual structural parts of the whole protein, it is necessary to obtain some certain separate parts of its molecule. The wide possibilities and convenience of the modern recombinant DNA technology led to the almost complete replacement of DT natural mutants and fragments obtained by proteolytic cleavage by corresponding recombinant products. Most of these derivatives are used in the biological studies of native toxin functions and interaction with cells.

Derivatives of DT are important tools for biomedical research, as well as for the most advanced biotechnological methods. For

example, a combination of the catalytic and translocation domain of DT is used for the creation of the targeted toxins, which are mainly used in cancer therapy. DT and its subunit A (SbA) are used for the specific ablation of the desired cell subtypes in multicellular organisms.

In medicine, the most common application of the nontoxic derivatives of DT — is production of vaccines. For example, formalinized diphtheria toxoid (anatoxin) is a standard component of acellular vaccines against diphtheria infection. Besides this straightforward application of diphtheria toxoid, the non-toxic point mutant of DT, protein CRM197 is used as a carrier in conjugate vaccines, as this derivative like the native DT is highly immunogenic. But an even more surprising application of CRM197 is the therapy of oncological diseases — recently, such a medication as BK-UM [2–4] has been successfully introduced in cancer therapy.

There are many other peculiar applications of DT derivatives, as well as outstanding questions relating to the biological functions of respective DT structural parts. The purpose of the present review was to summarize the current literary data on the variety of derivatives of DT molecule produced by the *C. diphtheriae* or either in heterologous systems, to analyze the main features, advantages and problems related to practical application of DT derivatives and provide a description of their current use in the fields of biology and medicine.

Structure and functions of the native DT molecule. DT is produced by the gram-positive cells of *C. diphtheriae* and some other *Corynebacterium* species [5]. It is known that the tox^+ gene which encodes DT [6] is not a native part of *Corynebacterium* genome. The *tox* locus is present in the genomes of several bacteriophages [1]. Most often, this gene is introduced in *C. diphtheriae* with a corynephage β during lysogenic transformation [7, 8]. It is interesting that the synthesis of this foreign to bacteria gene is regulated by the system of bacterial host cells in response to environmental iron concentrations [9, 10].

The precursor of DT [11], is synthesized on polyribosomes in the form of a single-chain polypeptide with the approximate M_r of 68 kDa. This precursor has a signal peptide on its N-terminus, that guides the toxin for cotranslational secretion in the extracellular environment by a bacterial Sec translocation system [12]. After the cleavage of the signal peptide during the process of translocon transfer, a mature DT is already formed (Fig. 1).

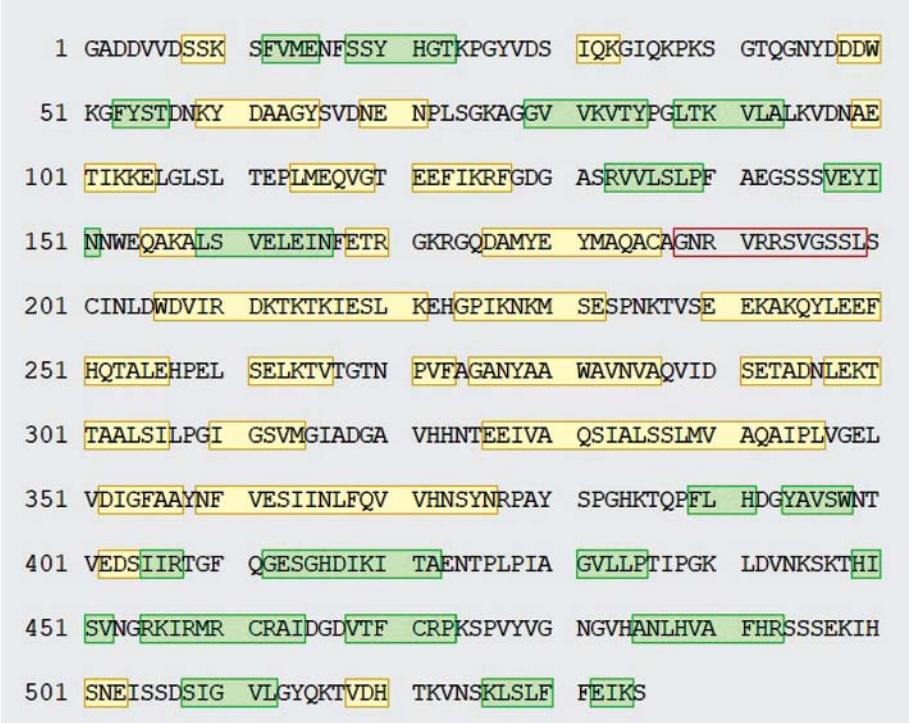

Fig. 1. **The elements of the secondary structure of secreted form of DT superimposed on the amino acid sequence in a single-letter code:**
PDB code — 1GSK, according to [15] by UCSF Chimera software: α-helices are highlighted by yellow, β-strands — by green, non-structured regions are not highlighted , the area of the hinge loop which was not visible on the electronic density maps is marked by a red frame

Mature DT is a single-chain protein of 535 amino acid residues with the SDS-PAGE-estimated M_r of 62 kDa (58.342 kDa according to the theoretical calculations based on the gene sequence). This toxin contains no unusual amino acids and no non-protein moieties [13]. DT belongs to the A-B group of bacterial exotoxins, because the molecule of DT is traditionally divided into two subunits: A and B (SbB). Among the toxins of this group, DT was a first characterized member [1]. Division of DT molecule on subunits emerged historically, because during the proteolysis under mild conditions and in the presence of a reducing agent, the original molecule of toxin breaks up into these two parts. According to SDS-PAGE, SbA possesses the M_r of 24 kDa and SbB — 38 kDa. It should be mentioned that unlike SbB, SbA is characterized by an increased thermostability [14].

At the level of the tertiary structure, DT consists of a C-terminal receptor-binding or R-domain (residues 385–535), a central translocation or T-domain (residues 201–384), and an N-terminal catalytic or C-domain (residues 1–191) [15]. SbA is represented only by the C-domain while SbB includes two domains: T- and R-. The fine structure of particular domains of DT molecule was investigated by X-ray diffraction in protein crystals (Fig. 1): the C-domain contains α-helices and β-strands, T-domain is entirely α-helical and R-domain is a flattened β-barrel with a jelly-roll-like topology, similar to that of the immunoglobulin variable domain [16].

DT contains four cysteine residues, which form two disulfide bonds: Cys186 – Cys201, and Cys461 – Cys471 [17]. The hinge loop that is formed by the disulfide bridge between Cys186 and Cys201 combines the C- and T-domains together. The second disulfide bond is located inside the R-domain.

DT binding to its receptor on a plasma membrane triggers internalization of DT::receptor complex through the clathrin-dependent endocytosis [18].

After DT binding to its receptor, transmembrane furin proteases at the surface of sensitive cells, cleave the peptide bonds that follows after residues Tyr190, Ala192 or Gln193 inside the hinge loop after DT binding. However, after such cleavage, C-domain is still remaining covalently tethered to B-subunit by a respective disulfide bond (the "nicked" or proteolytically cleaved toxin). For cytotoxicity, the mentioned disulfide bridge should be reduced to release the C-domain in the cell cytosol where it can implement its cytotoxic action. It is believed that this reduction occurs due to the glutathione GSH of cytosol [13].

When C-domain is released from the rest of the DT molecule, it is able to catalyze ADP-ribosylation of eukaryotic translation elongation factor 2 (eEF-2). The ADP-ribosyl group from NAD^+ is transferred to the diphthamide residue (post-translationally modified histidine which is found in eEF2). This leads to an almost complete arrest of protein synthesis and cell death. It should be mentioned here, that entire DT which was not cleaved and treated with a reduction agent, is incapable of ribosyltransferase activity in cell lysates [14]. SbA is toxic for cells only in the presence of SbB, which is required for the binding to DT receptor, consequent uptake into endosomes, and translocation of fragment A into the cytosol [19].

Molecular mechanism of SbA translocation through the lipid bilayer is still unknown but it is obvious that T-domain which forms pores in lipid bilayers [20,21] is crucial at this step.

Characterization of the DT receptor. DT receptor is the precursor of heparin-binding epidermal growth factor-like growth factor, proHB-EGF [22]. ProHB-EGF is a single-chain transmembrane glycoprotein of 208 amino acid residues [22, 23]. Significant amounts of this protein can be found on the surface of epithelial, endothelial, smooth muscle cells, fibroblasts, macrophages, etc [24]. ProHB-EGF contains heparin-binding, EGF-like, transmembrane and cytoplasmic domains [25].

R-domain of the DT binds to the EGF-like domain of proHB-EGF. Binding of DT to proHB-EGF is highly specific — the K_b of DT::HB-EGF interaction was estimated to be 10^{-8}–10^{-9} M [26, 27]. The presence of proHB-EGF on the cell surface causes cellular sensitivity to DT. Cells that do not express the proHB-EGF on plasma membrane are not sensitive to DT.

It is known that proHB-EGF forms complexes with some other membrane proteins, such as integrin α3β1, heparan sulfate-containing proteoglycans and CD9 [28,29]. Tetraspanin CD9 is known to sufficiently enhance the DT binding activity of proHB-EGF [30–32].

It was found that mice and rats can tolerate relatively high doses of DT that are enough to kill susceptible animals (dogs) which are much larger in size and weight and with no necrosis occurred at the seat of inoculation [33, 34]. In cell culture experiments it was demonstrated that DT dose which reduces the rate of protein

synthesis by 50% is 10^5–10^6 times bigger for murine L929 cells than for human HeLa and KB-S cells [35].

Not all the rodents possess resistance to DT. Chinese hamsters and especially guinea pigs are sensitive to DT. Information on the resistance of other members of the mammalian class, as well as on the proHB-EGF polymorphism in various taxonomical groups of mammals is limited.

The DT receptor from resistant and sensitive organisms possess a different primary structure due to the amino acid substitutions. It is obvious that differences in the amino acid sequence of proHB-EGF are the main reason for DT resistance in mammals, as murine cells which express human proHB-EGF also become highly sensitive [26]. However, there is no a definite opinion regarding how these differences in receptor structure alter the processes of DT binding and internalization by resistant cells compared to sensitive.

According to one point of view, the receptors of insensitive cells are unable to bind DT [26, 35–39] which is the only reason for DT resistance. As to another opinion, DT binds proHB-EGF from insensitive cells and internalized by endocytosis [40–42]. According to the authors who found the endocytosis of DT by cells of resistant organisms, unsusceptibility to DT is due to the lack of the SbA translocation in the cytosol of resistant cells that may be caused by several factors: a low binding constant of DT to the HB-EGF receptor under low pH of endosomes [43, 44], high activity of endosomal proteases [40], etc.

On the cell surface, proHB-EGF can undergo splitting by metalloproteases to form a soluble growth factor HB-EGF [45], which carries only heparin-binding and EGF-like domains (residues 106–147 of the proHB-EGF primary translation product with signal and pro-peptides [24]). HB-EGF is a natural ligand for the EGF receptor and HER4 [46]. Soluble HB-EGF is a potential mitogen and chemoattractant for various cell types, including smooth muscle cells, fibroblasts and keratinocytes [47, 48]. This factor is involved in many physiological and pathological processes, which include the eyelid closure [49], wound healing [50–52], retinoid skin hyperplasia [53], cardiac hypertrophy [54], hyperplasia of the smooth muscle cells [55], collecting duct morphogenesis [56], blastocyst implantation [57], pulmonary hypertension [58] and oncogenic transformation [59].

Since the binding of DT to its receptor is very effective and highly specific (with the affinity that is close to that of an antigen-antibody interaction), labeled DT derivatives are very perspective for detecting of this receptor in different biological samples and studies of internalization and intracellular transport of proBH-EGF [60].

Classification of DT derivatives. Now, when we have considered the structure of DT and its receptor, it's time to get closer to a variety of its derivatives, which have some differences compared to the original toxin. Derivatives may be ranked according to their structural similarity to the natural toxin and in order of decreasing of their M_r. Compounds with practically the same M_r may differ in the number of amino acid substitutions. Derivatives may be classified by the presence of additional amino acid sequences and tags that are absent in the native toxin.

Besides, all the derivatives can be divided according to some kind of their function: the presence or absence of toxicity, receptor binding, internalization, etc.

I suppose that it is also necessary to distinguish between the DT derivatives that were obtained in the cells of the natural producer *C. diphtheria* and those derivatives that were synthesized in the foreign host cells. Such a division can be useful for systematizing the historical information on the obtaining of certain recombinant derivatives of DT, as at first DT derivatives were produced exclusively in *C. diphtheria* strains and lately — in heterologous systems based on *Escherichia coli* and other producents.

Structure and functions of DT derivatives produced in C. diphtheria. According to [14], the lytic cycle of corynephage β — was induced in *C. diphtheriae C7(β)* strain by UV-light exposure and then nitrosoguanidine was added. The surviving phage particles produced in presence of the mutagen were plated on *C. diphtheriae C7(–)* cells, that does not contain prophage β. Lysogenised corynebacteria from turbid plaques were spotted on agar and their toxinogeny was firstly estimated by the rabbit intradermal test.

By this method, a number of non-toxigenic *C. diphtheriae* clones were found [14]. Obtained mutants produced the non-toxic proteins serologically related to DT. These toxoids were called "crossreacting materials" that contained single or multiple mutations in the tox^+ gene that resulted in deletions or substitutions of individual amino acids in the polypeptide chain of DT.

Among the obtained mutants, protein CRM197 [61] become the best-studied non-

toxic DT analog of same M_r. Substitution of Gly 52 to Glu in this toxoid leads to an almost complete loss of SbA activity, however, there are also some data that mild activity of mutated SbA in CRM197 is preserved [62–64]. Despite the presence of a single mutation, there is a strong evidence that CRM197 has significant functional differences compared to the native DT [65–69].

Another DT mutants — CRM176 and CRM228 with same M_r as that of the native toxin, as well as truncated CRM45 (M_r of 45 kDa) and CRM30 (30 kDa) were created together with CRM197.

CRM45 includes residues 1–386 [70] that appeared as a result of the "TAA" termination signal introduced by the (C to T) point mutation in the "CAA" codon for Gln387 which causes early termination at Thr386 and therefore — the C-terminal lost of 149 amino acid residues (M_r is 16.530 kDa). CRM30 appeared similarly — by a transition of a sense codon to a stop codon. The C-terminal residue of this CRM is probably Ala280 [71], however unknown exactly.

SbA of CRM228 has no transferase activity while the respective activity of CRM176 was approximately 2.6 times less than that of SbA from DT. Besides, CRM228 was also much less effective (10–15% of that of CRM197) in binding to the cell receptor, which indicates multiple mutations. The gene of CRM228 was sequenced [72] and 8 mutations in the mature form of CRM228 were revealed.

Among all the mutants described in work [14], CRMs 197 and 176 turned out to be the closest structural analogs of native DT, as they contain the single point substitutions — Gly to Gln at position 52 [70] for CRM197 and Gly to Asp at position 128 for CRM176. Mutation in CRM197 almost completely reduces its toxicity [14], thus, it became the most widely used and well studied non-toxic derivative of DT. However, a lot of another non-toxic CRMs were described in further works (Table 1) which contain substitutions in their C-domains and can be potentially used for the creation of another non-toxic single-point mutant by means of site-directed mutagenesis.

Another set of 11 CRMs was obtained by nitrosoguanidine mutagenesis of β-corynephage [73], among which CRM107 was shown to selectively kill cerebellar Purkinje neurons [74]. Besides, CRMs 102 and 103 were characterized, as they were used in the development of immunotoxins [75]. The sequences of the rest of the mentioned above proteins are unknown as the particular features of these mutants did not attract the attention of researchers.

Some other CRMs produced in *C. diphtheria* possess the unique and potentially valuable properties. For instance, CRM26 is even smaller than CRM30 and represents SbA with a little bit more truncated T-domain [76, 77]. CRM1001 which possess the transition of Cys471 to Tyr in R-domain was also produced in *C. diphtheria* [78, 79]. CRM1001 was shown to bind the proHB-EGF of the target cells as well as DT but is deficient in cell entry resulting in a reduced toxic effect [78].

Recombinant DT derivatives produced in the foreign host cells. Only DT derivatives from the *C. diphtheriae* cells were listed above. However, production of proteins in their natural producers can be rather inconvenient. Recombinant analogs produced in heterologous systems are much more easy to obtain in the laboratory. Therefore, a variety of recombinant forms of DT were created.

Native tox^+ gene of DT and some of its truncated forms were expressed in *E. coli* [80–82]. Perhaps, the creation of strains of *E. coli* with the native DT gene can be rather dangerous for humans and the environment. Moreover, it is noteworthy that due to the probability of a reverse mutation, production of the single-point full-length DT mutants can potentially provide the same threat.

The gene of CRM228 was inserted in *pKTHI637* vector and cloned in *Bacillus subtilis* cells for secretion in bacterial culturing media [83]. Two truncated forms of CRM228 which contain no R-domain were also described in [83], from which one form contained the C-terminal cysteine residue for conjugation of chemical linkage of targeting molecules. Thus, it was demonstrated that this expression system with *B. subtilis* host cells is completely suitable for the production of the full-length and truncated toxoids and possibly, the entire DT molecules. According to the opinion of the author of this review, production of DT derivatives in the culturing media is the most reasonable biotechnological solution, because folding of the proteins, in this case, can occur in the most correct way. However, there is a report that production of proteolytically split CRM197 by *B. subtilis* may occur [84].

Some studies are devoted to the production of recombinant CRM197 in the T7 RNA polymerase-based expression system and *E. coli* as a host cell [85]. In this case, recombinant CRM197 is accumulated in the cytoplasm and most frequently — in the inclusion bodies [86, 87]. In some cases, it was possible to obtain

Table 1. **The most important DT derivatives which were produced in *C. diphtheria* cells**

DT derivative	Structure alterations	Function alterations	References
CRM45	Deletion of the C-terminal portion Gln387 — Ser560	Loss of the receptor-binding activity, however weak cytotoxicity is preserved	[61, 70]
CRM30	Deletion of the unknown C-terminal portion, possibly Ala280 — Ser560	Loss of the receptor-binding activity, however weak cytotoxicity is preserved	[61]
CRM26	Deletion of the unknown C-terminal portion larger than in CRM30	Loss of the receptor-binding activity, however weak cytotoxicity is preserved	[76,77]
CRM228	Substitutions Gly79 to Asp, Glu162 to Lys, Ser197 to Gly, Lys200 to Ser, Asn389 to Phe, Gly431 to Ser, Asn507 to Asp and Lys528 to Ser in C- and R-domains	Loss of the SbA catalytic activity and receptor-binding activity	[61, 72]
CRM197	Substitution of Gly52 to Gln in the C-domain	Loss of SbA catalytic activity	[61, 70]
CRM176	Substitution of Gly128 to Asp in the C-domain	Partially reduced catalytic activity of native SbA	[61]
CRM107	Substitutions Leu390 to Phe and Ser525 to Phe in R-domain	Deficient binding to DT receptor, however selectively kills the Purkinje neurons, about 10 times less toxic to Vero and Jurkat cells than CRMs 102 and 103	[73 75]
CRM103	Substitution of Ser508 to Phe in R-domain	Retained full enzymatic activity but had defective receptor binding, weak toxicity	[75]
CRM102	Substitutions Pro308 to Ser and Ser508 to Phe in T- and R-domains	Retained full enzymatic activity but had defective receptor binding, weak toxicity	[75]
CRM1001	Substitution of Cys471 to Tyr in the R-domain resulter in the absence of a disulphide bond between Cys461 and Cys 461	Preserving the ability to bind DT receptor, but deficient in the internalization step of intoxication	[78, 79]

CRM197 protein in the soluble fraction of *E. coli* cell lysate [88, 89].

Recombinant SbB and SbA of DT — another well-studied DT derivatives. There are several studies in which for some reasons production of SbA [90, 91] or SbB [90, 92, 93] was established in *E. coli*.

R-domain is the part of DT molecule of the smallest M_r which preserves the ability to bind the DT receptor. An attempt was made to obtain a mutated R-domain, the binding of which to the DT receptor would have an enhanced affinity [94]. Besides, R-domain was cloned in *E. coli* for the purposes of enhancement of bioavailability of curcumin to cells [95]. Curcumin, a perspective for cancer treatment secondary metabolite of plant cells is poorly soluble in water, however, it's solubility can be effectively increased when it is adsorbed to protein and also R-domain. Cloning of R-domain was also described in [96] for characterization of its interaction with the DT receptor.

Of the particular interest are fluorescent derivatives of DT fused to some fluorescent proteins (EGFP, mCherry, etc.), which were described in works [60, 97]. Such labeled fragments of the toxin molecule can be successfully used to study binding of living cell receptors, the expression levels of DT receptor, as well as its internalization by endocytosis in cells [43, 44, 98].

The information about the most important DT derivatives produced in foreign host cells is summarized in Table 2.

Application of DT derivatives for studying the biological functions of native toxin. The most of DT recombinant derivatives with specific mutations and functional tags have been developed specifically to study the biological properties of the native toxin and the interaction of the eukaryotic cells with its

Table 2. **Fragments and analogs of DT molecule which were produced by cells of a foreign producents**

DT derivative	Host cells	Specific features	References
CRM197	*E. coli*	Non-toxic DT analog	[86–89]
	B. subtilis	Extracellular secretion, non-toxic	[84]
CRM228	*B. subtilis*	Extracellular secretion, non-toxic	[83]
Truncated forms of CRM228 with no R-domain (with and without C-terminal Cys residue)	*B. subtilis*	Extracellular secretion of the C- and T-domains combination for development of targeted toxins, non-toxic	[83]
SbA	*E. coli*	Preserves catalytic activity, non-toxic (as it unable to translocate across lipid bilayer by itself)	[90, 91]
SbB	*E. coli*	Preserves receptor-binding and pore-forming activities, non-toxic	[90, 92, 93]
T-domain	*E. coli*	Preserves pore-forming activity, non-toxic	[111, 116–121]
R-domain	*E. coli*	Preserves receptor-binding activity, non-toxic	[94–96]

molecules. For today all possible derivatives that have a certain defective function of the native toxin have been identified. For instance, a variety of mutations were introduced by site-directed mutagenesis into the recombinant derivatives of DT in order to study the biological functions of various amino acid residues. Among them, there should be noted mutations in the active site of C-domain [99–105] and T-domain [75, 106–115].

However, until now, the question regarding the mechanism of translocation of the subunit A DT to the cytosol through the lipid membrane remains unresolved. It is supposed that translocation of the polypeptide chain of the SbA moves through a protein-conducting channel, which is formed by a T-domain of DT. Recombinant T-domain and its pore-forming activity in lipid bilayers have been extensively studied in black lipid membranes [111, 116–121]. In classical works on DT conductivity, it was suggested that at least two T-domains participate in the formation of a single pore [20, 21]. However, recently appeared a message that just a single T-domain is completely sufficient for the formation of a typical DT channel in black lipid membranes [122].

Nonetheless, the most unclear thing about the SbA transport is not how the translocation channel is arranged itself, but what is the force that pulls the polypeptide chain through this channel. There are some findings that lethal and edema factors of anthrax toxin could be translocated by a proton–protein symport through the channel which is formed by protective antigen, the third component of this toxin [123]. It is natural to assume that the polypeptide chain of subunit A can also be transported by a similar mechanism. Similar ideas were already presented in [20, 124] and [125]. There are some findings that certain factors from the host cell can directly participate in the transport of SbA and possibly facilitate this process [126].

Specific cell ablation with DT and its catalytic domain. As it was already mentioned above, mice are resistant to the cytotoxic action of DT. Toxin-resistant animals survive when they are administered DT doses that lead to the death of cells in their organism that contain on their surface a receptor that is normally expressed only in sensitive species. This fact allowed the development a technique for specific ablation of cells in the body of transgenic mice using native DT — the toxin receptor-mediated cell knockout (TRECK) [127].

The first step of TRECK is generation of transgenic mice expressing human DT receptor under the control of a cell type-specific promoter. DT is injected into the transgenic mice at the desired time points to ablate those cells in which the promoter is active. One disadvantage of this method was that due to the high immunogenicity of DT, repeated injections which are necessary for complete cell ablation were ineffective. To solve this complication, the authors created a murine line with the immune tolerance against DT [127]. The receptor of DT deficient in epidermal growth factor-like biological activity but which preserves its ability of binding DT [128] was also created for this purpose to avoid

potential problems with DT receptor acting as a growth factor in mice.

Specific cell ablation in multicellular organisms serves mainly to study the functions of certain cell populations which express a specific marker that is non-expressed in other cell types in the body of laboratory animals. TRACK was used for generation of a murine model of type 1 diabetes [129], a similar conditional cell ablation was used by another collective of authors for depletion of dendritic cells [130, 131]. A large amount of work was done by this approach to study the *in vivo* functions of murine myeloid cells [132].

Expression of active SbA directly in the cytoplasm — is another strategy for specific cell ablation [133–137] which does not require application of native DT. The gene of the SbA in cells of the transgenic organisms can be inserted under the controllable promoter, so that gene expression can be induced by a certain factor [134, 137] (conditional cell ablation), or the promoter can be activated by itself during ontogenesis only in certain specific types of cells [133, 136, 138] (non-conditional, promotor-dependent ablation). The last approach is frequently used not only in animals but also in plant organisms to study the expression of certain genes in different plant cells [138].

Derivatives of DT as vaccine components

Formalin-treated DT is a component in combined pertussis-diphtheria-tetanus vaccines (DTaP and Tdap) [139]. DTaP is a vaccine that helps children younger than age 7 develop immunity. Tdap is a booster immunization given at age 11 that offers continued protection from those diseases for adolescents and adults.

The mechanism of formaldehyde detoxification is based on the reactivity of the carbonyl group regarding the primary amine groups on the protein (i.e. side chain of lysine and an N-terminal amino group of the polypeptide chain). During a reaction, a metilol intermediate is formed, which condenses with water to form a Schiff base. Then the Schiff base interacts mainly with a 5-position of the tyrosine ring to form stable covalent methylene bridges. In detoxification protocols for vaccine production, the resulting Schiff-base is stabilized by glycine or lysine [140]. Manufacturing of the anatoxin for vaccination, which for the first glance has a very simple principle, is a highly standardized multi-week and multi-stage process that is carefully regulated. Resulted anatoxin is tested in numerous assays to ensure that the toxicity has been completely neutralized. For more than 100 years, since the production of anatoxins for vaccination was incepted, the standard protocol for DT, tetanus and pertussis toxins inactivation did not change much [140]. Recombinant genetically inactivated DT, tetanus and pertussis toxins were proposed for development of the next-generation of DTaP and Tdap vaccines [141, 142].

Conjugate vaccines are created by covalently attaching a poor antigen to a strong antigen thereby eliciting a stronger immunological response to the poor antigen. The strong antigen to which the target poor antigens are conjugated is called the carrier [143]. Diphtheria anatoxin, tetanus toxoid, and CRM197 are also used as carriers in several widely used, routine childhood and adult conjugate vaccines against encapsulated bacteria such as *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitidis* [144, 145].

Derivatives of DT as anticancer agents

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 [146]. The non-toxic to DT-sensitive species CRM197 turned out to be promising 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 [147–149] and increases the survival of patients with progressive cancer [4, 150, 151]. There is a lot of evidence that CRM197 is effective in suppressing the cancer of breast [149, 152, 153], oral cavity [154], stomach [155], immune cells [156] and ovaries [147, 148].

CRM197 was introduced into a medical practice for the treatment of human cancer as the main component of BK-UM medication [2–4]. Recombinant CRM197 was produced in *E. coli* which greatly facilitates obtaining of this protein for the manufacturing of diphtheria toxoid-based HB-EGF-targeted medications [86, 88, 89].

The effect of CRM197 on tumors is implemented by the interaction of this protein with soluble HB-EGF. It was demonstrated that proHB-EGF is often overexpressed in transformed cells and that HB-EGF promotes the development of a malignant phenotype. The gene of HB-EGF is considered to be strongly responsible for chemotherapy resistance [157] and oncogenic transformation [59]. Cell treatment with CRM197 leads to reduced malignant potential since when CRM197 is bound to HB-EGF is unable to interact with its cell receptor EGFR [155,157,158]. Nowadays,

it is generally accepted that the mechanism of the CRM197 antitumor action is blocking of the soluble HB-EGF.

However, as a medicine for intraperitoneal administration, CRM197 possess an essential disadvantage, as, like the native DT, it is also highly immunogenic to humans. The anticancer potential of less immunogenic than CRM197 toxin derivatives was not studied properly.

DT-based targeted toxin therapy

Monoclonal antibodies specific for tumor cell surface antigens or their Fv-fragments have been linked to toxins or toxin subunits to generate a new class of therapeutic drugs called immunotoxins. Most often antibodies and their Fv to clusters of differentiation proteins 3 (mostly to CD3ε) [159, 160], 19 [161, 162], and 22 [163, 164] are used as immunotoxin targets. The most known immunotoxin which is based on DT is Resimmune [165]. More information on DT-based immunotoxins could be found in works [161, 162, 166, 167]. Another noteworthy immunotoxin, Moxetumomab pasudotox, was developed based on *Pseudomonas aeruginosa* exotoxin A (PE) [164].

Not only antibodies can be used for targeted toxin therapy — different ligands of the overexpressed receptors in cancer cells, like growth factors, hormones, cytokines and some other specific molecules can be employed as well.

Sometimes, a complete DT molecule was combined with a targeting molecule [168,169]. Since only C- and T-domains are necessary for translocation of SbA, a variety of truncated fragments with no R-domain were obtained. At this point it should be noted that despite R-domain is absent in some DT derivatives, such fragments can still exhibit toxicity in certain cell cultures [170].

For substitution of R-domain instead of antibodies most commonly were used such factors as vascular endothelial growth factor [171, 172], α-melanocyte-stimulating hormone [173], interleukin-2 [174], interleukin-3 [175–177] and interleukine-13 [178], granulocyte-macrophage colony-stimulating factor [179–182], urokinase [183] and even transferrin [184]. In introduction to the medical practice, only interleukin-2 fused to the first 388 amino acids of DT (Denileukin diftitox or Ontak) was successful [185–188]. Interleukin-3 fused to the same DT fragment also demonstrated good results in clinical trials [189, 190], however, it was not introduced in cancer therapy.

DT derivatives, used to construct the targeted toxins are also should possess a high immunogenicity, as they contain the sufficient part of DT molecule.

Immunogenicity of DT derivatives. It remains unclear why DT possesses such strong immunogenic properties compared to other proteins. There is no detailed comparison of the immunogenicity of individual functional domains of the DT molecule, but attempts of such studies have been already done [191]. There are some not systematic data on immunogenicity of different fragments of DT [91, 192, 193] or on the immunodominant areas of DT surface [194], however, it is unknown exactly, which of functional domains is the most immunogenic.

Investigation of the immunogenicity of individual fragments of the DT is valuable for medicine since CRM197 and DT fragments without R-domain for targeted toxins are repeatedly administered in cancer therapy.

When immunogenic DT-based means administered repeatedly, they are fast eliminated from the bloodstream. Directed modification of DT [161] and PE [195–198] is carried out in order to reduce such immunogenicity.

Therefore, the search for DT derivatives that retain the most pronounced anti-tumor effects and possess the least immunogenicity is very perspective. Besides, it is also important to compare the immunogenicity between the variety of derivatives of other toxins (ricin, PE, etc.) used for targeted toxin therapy in order to find those that are the least immunogenic.

In a biological study, DT derivatives are used to investigate the function of respective components of the entire toxin molecule. The least understood question concerning DT functions is the translocation of SbA through the lipid bilayers. The phenomenon of the resistance of some mammalian species to DT has found a peculiar application for a specific ablation of certain cell types in multicellular organisms.

DT is excellent for use in vaccines, both anti-diphtheria and as a carrier protein for antigens of other pathogenic microorganisms. However, the use of DT in medicine is much broader.

Catalytically active SbA of DT complexed to the T-domain is used for the construction of recombinant means for targeted intoxication of cancer cells, like immunotoxins. The peculiarity of the anticancer effect of DT compared to other toxins of different origin is that its non-toxic derivatives, like CRM197, also exert the antitumor effect. Anticancer

properties of the non-toxic DT derivatives are explained by the involvement of DT receptor, which is inactivated by binding to a DT R-domain, in cancerogenesis and versatile range of other cell physiological functions. Therefore, in the anticancer therapy, it is necessary to use simultaneously both distinct functions of DT: toxic for directional cell elimination by targeted toxins and blocking of the soluble HB-EGF for reducing para- and autocrine activation of EGFR in malignant cells.

Thus, DT is suitable for developing on its basis the newest biomedical products and biotechnological application for specific cell elimination, because it has one of the highest toxicity among other toxins and it is easy to obtain its active recombinant forms. However, the main obstacle in application of DT derivatives for the purposes other than immune prophylaxis, like cancer therapy and specific cell ablation — is high immunogenicity. Thereover, the search for the least immunogenic recombinant derivatives of DT is of a high importance for biomedicine.

REFERENCES

1. *Alouf J.E., Freer J.H. The Comprehensive Sourcebook of Bacterial Protein Toxins, Second Edition*. 2nd ed. Academic Press, 1999.
2. *Fukagawa S., Yotsumoto F., Odawara T., Manabe S., Ishikawa T., Yasunaga S., Miyamoto S.* Antitumour Effects of Intravenous Administration of BK-UM, a Novel Inhibitor of HB-EGF, in Ovarian Cancer Therapy. *Anticancer Res.* 2017, 37 (7), 3891–3896. doi:10.21873/anticanres.11770.
3. *Miyamoto S., Yotsumoto F., Ueda T., Fukami T., Sanui A., Miyata K., Nam S.O., Fukagawa S., Katsuta T., Maehara M., Kondo H., Miyahara D., Shirota K., Yoshizato T., Kuroki M., Nishikawa H., Saku K., Tsuboi Y., Ishitsuka K.* BK-UM in patients with recurrent ovarian cancer or peritoneal cancer: a first-in-human phase-I study. *BMC Cancer*. 2017, 17 (1), 89. doi:10.1186/s12885-017-3071-5.
4. *Nam S.O., Yotsumoto F., Miyata K., Suzaki Y., Yagi H., Odawara T., Manabe S., Ishikawa T., Kuroki M., Mekada E., Miyamoto S.* Preclinical Study of BK-UM, a Novel Inhibitor of HB-EGF, for Ovarian Cancer Therapy. *Anticancer Res*. 2014, 34 (8), 4615–4620.
5. *Selim S.A., Mohamed F.H., Hessain A.M., Moussa I.M.* Immunological characterization of diphtheria toxin recovered from Corynebacterium pseudotuberculosis. *Saud. J. Biol. Sci*. 2016, 23 (2), 282–287. doi:10.1016/j.sjbs.2015.11.004.
6. *Greenfield L., Bjorn M.J., Horn G., Fong D., Buck G.A., Collier R.J., Kaplan D.A.* Nucleotide sequence of the structural gene for diphtheria toxin carried by corynebacteriophage beta. *Proc. Natl. Acad. Sci. U.S.A.* 1983, 80 (22), 6853–6857.
7. *Freeman V.J.* Studies on the virulence of bacteriophage infected strains of Corynebacterium diphtheriae. *J. Bacteriol*. 1951, 61 (6), 675–688.
8. *Freeman V.J., Morse I.U.* Further observations on the change to virulence of bacteriophage-infected avirulent strains of Corynebacterium diphtheriae. *J. Bacteriol*. 1952, 63 (3), 407–414.
9. *Guedon E., Helmann J.D.* Origins of metal ion selectivity in the DtxR/MntR family of metalloregulators. *Mol. Microbiol.* 2003, 48 (2), 495–506.
10. *Spiering M.M., Ringe D., Murphy J.R., Marletta M.A.* Metal stoichiometry and functional studies of the diphtheria toxin repressor. *Proc. Natl. Acad. Sci. U.S.A.* 2003, 100 (7), 3808–3813. doi:10.1073/pnas.0737977100.
11. *Smith W.P., Tai P.C., Murphy J.R., Davis B.D.* Precursor in cotranslational secretion of diphtheria toxin. *J. Bacteriol.* 1980, 141 (1), 184–189.
12. *Schneewind O., Missiakas D.M.* Protein secretion and surface display in Gram-positive bacteria. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 2012, 367 (1592), 1123–1139. doi:10.1098/rstb.2011.0210.
13. *Collier R.J.* Diphtheria toxin: mode of action and structure. *Bacteriol. Rev*. 1975, 39 (1), 54–85.
14. *Uchida T., Pappenheimer A.M., Greany R.* Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem.* 1973, 248 (11), 3838–3844.
15. *Bell C.E., Eisenberg D.* Crystal structure of nucleotide-free diphtheria toxin. *Biochemistry*. 1997, 36 (3), 481–488. doi:10.1021/bi962214s.
16. *Eisenberg D., Bell C.E., Bennett M.J., Collier R.J., Schlunegger M.P., Steere B.A., Weiss M.S.* A Structure-Based Model of Diphtheria Toxin Action. In: *Protein Toxin Structure*. Springer, Berlin, Heidelberg; 1996:25–47.
17. *Choe S., Bennett M.J., Fujii G., Curmi P.M., Kantardjieff K.A., Collier R.J., Eisenberg D.* The crystal structure of diphtheria toxin. *Nature*. 1992, 357 (6375), 216–222. doi:10.1038/357216a0.

18. *Simpson J.C., Smith D.C., Roberts L.M., Lord J.M.* Expression of mutant dynamin protects cells against diphtheria toxin but not against ricin. *Exp. Cell Res.* 1998, 239 (2), 293–300. doi:10.1006/excr.1997.3921.
19. *Olsnes S., Sandvig K.* How protein toxins enter and kill cells. In: *Immunotoxins (Frankel A.E. ed.). Martinus Nijhoff Publishing, Boston.* 1987, 39–74.
20. *Kagan B.L., Finkelstein A., Colombini M.* Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes. *Proc. Natl. Acad. Sci. U.S.A.* 1981, 78 (8), 4950–4954.
21. *Donovan J.J., Simon M.I., Draper R.K., Montal M.* Diphtheria toxin forms transmembrane channels in planar lipid bilayers. *Proc. Natl. Acad. Sci. U.S.A.* 1981, 78 (1), 172–176.
22. *Naglich J.G., Metherall J.E., Russell D.W., Eidels L.* Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell*. 1992, 69 (6), 1051–1061.
23. *Abraham J.A., Damm D., Bajardi A., Miller J., Klagsbrun M., Ezekowitz R.A.* Heparin-binding EGF-like growth factor: characterization of rat and mouse cDNA clones, protein domain conservation across species, and transcript expression in tissues. *Biochem. Biophys. Res. Commun.* 1993, 190 (1), 125–133. doi:10.1006/bbrc.1993.1020.
24. *Louie G.V., Yang W., Bowman M.E., Choe S.* Crystal structure of the complex of diphtheria toxin with an extracellular fragment of its receptor. *Mol. Cell*. 1997, 1 (1), 67–78.
25. *Higashiyama S., Lau K., Besner G.E., Abraham J.A., Klagsbrun M.* Structure of heparin-binding EGF-like growth factor. Multiple forms, primary structure, and glycosylation of the mature protein. *J. Biol. Chem*. 1992, 267 (9), 6205–6212.
26. *Mitamura T., Higashiyama S., Taniguchi N., Klagsbrun M., Mekada E.* Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J. Biol. Chem*. 1995, 270 (3), 1015–1019.
27. *Shen W.H., Choe S., Eisenberg D., Collier R.J.* Participation of lysine 516 and phenylalanine 530 of diphtheria toxin in receptor recognition. *J. Biol. Chem.* 1994, 269 (46), 29077–29084.
28. *Nakamura K., Iwamoto R., Mekada E.* Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin alpha 3 beta 1 at cell-cell contact sites. *J. Cell Biol.* 1995, 129 (6), 1691–1705.
29. *Iwamoto R., Mekada E.* Heparin-binding EGF-like growth factor: a juxtacrine growth factor. *Cytokine Growth Factor Rev.* 2000, 11 (4), 335–344.
30. *Hasuwa H., Shishido Y., Yamazaki A., Kobayashi T., Yu X., Mekada E.* CD9 amino acids critical for upregulation of diphtheria toxin binding. *Biochem. Biophys. Res. Commun*. 2001, 289 (4), 782–790. doi:10.1006/bbrc.2001.6053.
31. *Iwamoto R., Higashiyama S., Mitamura T., Taniguchi N., Klagsbrun M., Mekada E.* Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. *EMBO J*. 1994, 13 (10), 2322–2330.
32. *Cha J.H., Brooke J.S., Ivey K.N., Eidels L.* Cell surface monkey CD9 antigen is a coreceptor that increases diphtheria toxin sensitivity and diphtheria toxin receptor affinity. *J. Biol. Chem*. 2000, 275 (10), 6901–6907.
33. *Cobbett L.* The Resistance of Rats to Diphtheria Toxin. *Br. Med. J*. 1899, 1 (1998), 902–903.
34. *Pappenheimer A.M.* Diphtheria Toxin. *Ann. Rev. Biochem*. 1977, 46 (1), 69–94. doi:10.1146/annurev.bi.46.070177.000441.
35. *Moehring T.J., Moehring J.M.* Interaction of diphtheria toxin and its active subunit, fragment A, with toxin-sensitive and toxin-resistant cells. *Infect. Immun.* 1976, 13 (5), 1426–1432.
36. *Cha J.H., Brooke J.S., Eidels L.* Toxin binding site of the diphtheria toxin receptor: loss and gain of diphtheria toxin binding of monkey and mouse heparin-binding, epidermal growth factor-like growth factor precursors by reciprocal site-directed mutagenesis. *Mol. Microbiol.* 1998, 29 (5), 1275–1284.
37. *Morris R.E., Saelinger C.B.* Diphtheria toxin does not enter resistant cells by receptor-mediated endocytosis. *Infect. Immun.* 1983, 42 (2), 812–817.
38. *Mitamura T., Umata T., Nakano F., Shishido Y., Toyoda T., Itai A., Kimura H., Mekada E.* Structure-function analysis of the diphtheria toxin receptor toxin binding site by site-directed mutagenesis. *J. Biol. Chem.* 1997, 272 (43), 27084–27090.
39. *Naglich J.G., Metherall J.E., Russell D.W., Eidels L.* Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell*. 1992, 69 (6), 1051–1061.
40. *El Hage T., Decottignies P., Authier F.* Endosomal proteolysis of diphtheria toxin without toxin translocation into the

cytosol of rat liver in vivo. *FEBS J*. 2008, 275 (8), 1708–1722. doi:10.1111/j.1742-4658.2008.06326.x.
41. *Heagy W.E., Neville D.M.J*. Kinetics of protein synthesis inactivation by diphtheria toxin in toxin-resistant L cells. Evidence for a low efficiency receptor-mediated transport system. *J. Biol. Chem*. 1981, 256 (24), 12788–12792.
42. *Didsbury J.R., Moehring J.M., Moehring T.J*. Binding and uptake of diphtheria toxin by toxin-resistant Chinese hamster ovary and mouse cells. *Mol. Cell. Biol*. 1983, 3 (7), 1283–1294.
43. *Labyntsev A.J., Kolybo D.V., Yurchenko E.S., Kaberniuk A.A., Korotkevych N.V., Komisarenko S.V*. Effect of the T-domain on intracellular transport of diphtheria toxin. *Ukr. Biochem. J*. 2014, 86 (3), 77–87.
44. *Labyntsev A.J., Korotkevych N.V., Kolybo D.V., Komisarenko S.V*. Effect of diphtheria toxin T-domain on endosomal pH. *Ukr. Biochem. J*. 2015, 87 (4), 13–23.
45. *Higashiyama S., Abraham J.A., Miller J., Fiddes J.C., Klagsbrun M*. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science*. 1991, 251 (4996), 936–939.
46. *Iwamoto R., Mekada E*. ErbB and HB-EGF signaling in heart development and function. *Cell Struct. Funct*. 2006, 31 (1), 1–14.
47. *Raab G., Klagsbrun M*. Heparin-binding EGF-like growth factor. *Biochim. Biophys. Acta*. 1997, 1333 (3), F179-199.
48. *Higashiyama S., Abraham J.A., Klagsbrun M*. Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. *J. Cell Biol*. 1993, 122 (4), 933–940.
49. *Mine N., Iwamoto R., Mekada E*. HB-EGF promotes epithelial cell migration in eyelid development. *Development*. 2005, 132 (19), 4317–4326. doi:10.1242/dev.02030.
50. *Marikovsky M., Vogt P., Eriksson E., Rubin J.S., Taylor W.G., Joachim S., Klagsbrun M*. Wound fluid-derived heparin-binding EGF-like growth factor (HB-EGF) is synergistic with insulin-like growth factor-I for Balb/MK keratinocyte proliferation. *J. Invest. Dermatol*. 1996, 106 (4), 616–621.
51. *Shirakata Y., Kimura R., Nanba D., Iwamoto R., Tokumaru S., Morimoto C., Yokota K., Nakamura M., Sayama K., Mekada E., Higashiyama S., Hashimoto K*. Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing. *J. Cell. Sci*. 2005, 118 (Pt 11), 2363–2370. doi:10.1242/jcs.02346.
52. *Tokumaru S., Higashiyama S., Endo T., Nakagawa T., Miyagawa J.I., Yamamori K., Hanakawa Y., Ohmoto H., Yoshino K., Shirakata Y., Matsuzawa Y., Hashimoto K., Taniguchi N*. Ectodomain shedding of epidermal growth factor receptor ligands is required for keratinocyte migration in cutaneous wound healing. *J. Cell Biol*. 2000, 151 (2), 209–220.
53. *Kimura R., Iwamoto R., Mekada E*. Soluble form of heparin-binding EGF-like growth factor contributes to retinoic acid-induced epidermal hyperplasia. *Cell Struct. Funct*. 2005, 30 (2), 35–42.
54. *Asakura M., Kitakaze M., Takashima S., Liao Y., Ishikura F., Yoshinaka T., Ohmoto H., Node K., Yoshino K., Ishiguro H., Asanuma H., Sanada S., Matsumura Y., Takeda H., Beppu S., Tada M., Hori M., Higashiyama S*. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat. Med*. 2002, 8 (1), 35–40. doi:10.1038/nm0102-35.
55. *Miyagawa J., Higashiyama S., Kawata S., Inui Y., Tamura S., Yamamoto K., Nishida M., Nakamura T., Yamashita S., Matsuzawa Y*. Localization of heparin-binding EGF-like growth factor in the smooth muscle cells and macrophages of human atherosclerotic plaques. *J. Clin. Invest*. 1995, 95 (1), 404–411. doi:10.1172/JCI117669.
56. *Takemura T., Hino S., Kuwajima H., Yanagida H., Okada M., Nagata M., Sasaki S., Barasch J., Harris R.C., Yoshioka K*. Induction of collecting duct morphogenesis in vitro by heparin-binding epidermal growth factor-like growth factor. *J. Am. Soc. Nephrol*. 2001, 12 (5), 964–972.
57. *Das S.K., Wang X.N., Paria B.C., Damm D., Abraham J.A., Klagsbrun M., Andrews G.K., Dey S.K*. Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. *Development*. 1994, 120 (5), 1071–1083.
58. *Powell P.P., Klagsbrun M., Abraham J.A., Jones R.C*. Eosinophils expressing heparin-binding EGF-like growth factor mRNA localize around lung microvessels in pulmonary hypertension. *Am. J. Pathol*. 1993, 143 (3), 784–793.
59. *Fu S. l., Bottoli I., Goller M., Vogt P.K*. Heparin-binding epidermal growth factor-like growth factor, a v-Jun target gene, induces oncogenic transformation. *Proc. Natl. Acad. Sci. U.S.A*. 1999, 96 (10), 5716–5721.
60. *Kaberniuk A.A., Labyntsev A.I., Kolybo D.V., Oliĭnyk O.S., Redchuk T.A., Korotkevych N.V., Horchev V.F., Karakhim S.O., Komisaren-*

ko S.V. [Fluorescent derivatives of diphtheria toxin subunit B and their interaction with Vero cells]. *Ukr. Biokhim. Zh. (1999)*. 2009, 81 (1), 67–77. (In Ukrainian).
61. *Uchida T., Pappenheimer A.M., Harper A.A.* Diphtheria toxin and related proteins. 3. Reconstitution of hybrid "diphtheria toxin" from nontoxic mutant proteins. *J. Biol. Chem.* 1973, 248 (11), 3851–3854.
62. *Kageyama T., Ohishi M., Miyamoto S., Mizushima H., Iwamoto R., Mekada E.* Diphtheria toxin mutant CRM197 possesses weak EF2-ADP-ribosyl activity that potentiates its anti-tumorigenic activity. *J. Biochem.* 2007, 142 (1), 95–104. doi:10.1093/jb/mvm116.
63. *Qiao J., Ghani K., Caruso M.* Diphtheria toxin mutant CRM197 is an inhibitor of protein synthesis that induces cellular toxicity. *Toxicon*. 2008, 51 (3), 473–477. doi:10.1016/j.toxicon.2007.09.010.
64. *Kimura Y., Saito M., Kimata Y., Kohno K.* Transgenic mice expressing a fully nontoxic diphtheria toxin mutant, not CRM197 mutant, acquire immune tolerance against diphtheria toxin. *J. Biochem.* 2007, 142 (1), 105–112. doi:10.1093/jb/mvm115.
65. *Mekada E., Uchida T.* Binding properties of diphtheria toxin to cells are altered by mutation in the fragment A domain. *J. Biol. Chem*. 1985, 260 (22), 12148–12153.
66. *Hu V.W., Holmes R.K.* Single mutation in the A domain of diphtheria toxin results in a protein with altered membrane insertion behavior. *Biochim. Biophys. Acta*. 1987, 902 (1), 24–30.
67. *Malito E., Bursulaya B., Chen C., Surdo P.L., Picchianti M., Balducci E., Biancucci M., Brock A., Berti F., Bottomley M.J., Nissum M., Costantino P., Rappuoli R., Spraggon G.* Structural basis for lack of toxicity of the diphtheria toxin mutant CRM197. *Proc. Natl. Acad. Sci. USA*. 2012. doi:10.1073/pnas.1201964109.
68. *Salmas R.E., Mestanoglu M., Unlu A., Yurtsever M., Durdagi S.* Mutated form (G52E) of inactive diphtheria toxin CRM197: molecular simulations clearly display effect of the mutation to NAD binding. *J. Biomol. Struct. Dyn.* 2016, 34 (11), 2462–2468. doi:10.1080/07391102.2015.1119060.
69. *Papini E., Colonna R., Schiavo G., Cusinato F., Tomasi M., Rappuoli R., Montecucco C.* Diphtheria toxin and its mutant crm 197 differ in their interaction with lipids. *FEBS Lett.* 1987, 215 (1), 73–78.
70. *Giannini G., Rappuoli R., Ratti G.* The amino-acid sequence of two non-toxic mutants of diphtheria toxin: CRM45 and CRM197. *Nucl. Acids Res.* 1984, 12 (10), 4063–4069.
71. *Bigio M., Rossi R., Nucci D., Antoni G., Rappuoli R., Ratti G.* Conformational changes in diphtheria toxoids. Analysis with monoclonal antibodies. *FEBS Lett.* 1987, 218 (2), 271–276.
72. *Kaczorek M., Delpeyroux F., Chenciner N., Streeck R.E., Murphy J.R., Boquet P., Tiollais P.* Nucleotide sequence and expression of the diphtheria tox228 gene in Escherichia coli. *Science*. 1983, 221 (4613), 855–858.
73. *Laird W., Groman N.* Isolation and characterization of tox mutants of corynebacteriophage beta. *J. Virol.* 1976, 19 (1), 220–227.
74. *Riedel C.J., Muraszko K.M., Youle R.J.* Diphtheria toxin mutant selectively kills cerebellar Purkinje neurons. *Proc. Natl. Acad. Sci. U.S.A.* 1990, 87 (13), 5051–5055.
75. *Greenfield L., Johnson V.G., Youle R.J.* Mutations in diphtheria toxin separate binding from entry and amplify immunotoxin selectivity. *Science*. 1987, 238 (4826), 536–539.
76. *Bacha P., Murphy J.R.* Isolation and characterization of extragenic suppressor strains of Corynebacterium diphtheriae. *J. Bacteriol*. 1978, 136 (3), 1135–1142.
77. *Bacha P., Reichlin S.* Systemic toxicity of diphtheria toxin-related fragments (CRM26, CRM45), a hormone-toxin hybrid protein (TRH-CRM45), and ricin A. *Proc. Soc. Exp. Biol. Med.* 1986, 181 (1), 131–138.
78. *Dell'Arciprete L., Colombatti M., Rappuoli R., Tridente G.* A C terminus cysteine of diphtheria toxin B chain involved in immunotoxin cell penetration and cytotoxicity. *J. Immunol.* 1988, 140 (7), 2466–2471.
79. *Papini E., Schiavo G., Tomasi M., Colombatti M., Rappuoli R., Montecucco C.* Lipid interaction of diphtheria toxin and mutants with altered fragment B. 2. Hydrophobic photolabelling and cell intoxication. *Eur. J. Biochem.* 1987, 169 (3), 637–644.
80. *Shafiee F., Rabbani M., Behdani M., Jahanian-Najafabadi A.* Expression and purification of truncated diphtheria toxin, DT386, in Escherichia coli: An attempt for production of a new vaccine against diphtheria. *Res. Pharm. Sci*. 2016, 11 (5), 428–434. doi:10.4103/1735-5362.192496.
81. *Barbieri J.T., Collier R.J.* Expression of a mutant, full-length form of diphtheria toxin in Escherichia coli. *Infect. Immun*. 1987, 55 (7), 1647–1651.
82. *Shmelev V.A., Perovskaia O.N., Kopylov P.K., Nosova L.I., Popov S.G.* [Synthesis, secretion, and proteolytic degradation of diphtheria toxin in Escherichia coli]. *Mol. Gen. Mikrobiol. Virusol.* 1991, (10), 3–8.
83. *Hemilä H., Glode L.M., Palva I.* Production of diphtheria toxin CRM228 in B. subtilis. *FEMS Microbiol. Lett.* 1989, 53 (1–2), 193–198.

84. *Zhou J., Petracca R.* Secretory expression of recombinant diphtheria toxin mutants in B. Subtilis. *J. Tongji Med. Univ.* 1999, 19 (4), 253–256.
85. *Studier F.W.* Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.* 1991, 219 (1), 37–44.
86. *Stefan A., Conti M., Rubboli D., Ravagli L., Presta E., Hochkoeppler A.* Overexpression and purification of the recombinant diphtheria toxin variant CRM197 in Escherichia coli. *J. Biotechnol.* 2011, 156 (4), 245–252. doi:10.1016/j.jbiotec.2011.08.024.
87. *Manoilov K.Y., Gorbatiuk O.B., Usenko M.O., Shatursky O.Y., Borisova T.O., Kolybo D.V.* [The characterization of purifed recombinant protein CRM197 as a tool to study diphtheria toxin]. *Dopov. Nats. akad. nauk Ukr.* 2016, (9), 124–133. doi:10.15407/dopovidi2017.02.088. (In Ukranian).
88. *Mahamad P., Boonchird C., Panbangred W.* High level accumulation of soluble diphtheria toxin mutant (CRM197) with co-expression of chaperones in recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 2016, 100 (14), 6319–6330. doi:10.1007/s00253-016-7453-4.
89. *Dukhovlinov I.V., Fedorova E.A., Bogomolova E.G., Dobrovolskaya O.A., Chernyaeva E.N., Al-Shekhadat R.I., Simbirtsev A.S.* Production of recombinant protein CRM197 in Escherichia coli. *Russian Journal of Infection and Immunity*. 2015, 5 (1), 37. doi:10.15789/2220-7619-2015-1-37-44. (In Russian).
90. *Kaberniuk A. A., Oliinyk O. S., Redchuk T. A., Romaniuk S. I., Kolybo D. V., Komisarenko S. V.* Cloning of recombinant subunits of Corynebacterium diphtheriae diphtheria toxin and their expression in Escherichia coli. *Dopov. Nats. akad. nauk Ukr.* 2008, 3, 160–166. (In Ukrainian).
91. *Lee C.W., Lee S.F., Halperin S.A.* Expression and Immunogenicity of a Recombinant Diphtheria Toxin Fragment A in Streptococcus gordonii. *Appl. Environ. Microbiol.* 2004, 70 (8), 4569–4574. doi:10.1128/AEM.70.8.4569-4574.2004.
92. *Nascimento D.V., Lemes E.M.B., Queiroz J.L.S., Silva Jr. J.G., Nascimento H.J., Silva E.D., Hirata Jr. R., Dias A. a. S.O., Santos C.S., Pereira G.M.B., Mattos-Guaraldi A.L., Armoa G.R.G.* Expression and purification of the immunogenically active fragment B of the Park Williams 8 Corynebacterium diphtheriae strain toxin. *Braz. J. Med. Biol. Res.* 2010, 43 (5), 460–466. doi:10.1590/S0100-879X2010007500032.
93. *Johnson N., Pickett M.A., Watt P.J., Clarke I.N., Heckels J.E.* Construction of an epitope vector utilising the diphtheria toxin B-subunit. *FEMS Microbiol. Lett.* 1997, 146 (1), 91–96.
94. *Suzuki K., Mizushima H., Abe H., Iwamoto R., Nakamura H., Mekada E.* Identification of diphtheria toxin R domain mutants with enhanced inhibitory activity against HB-EGF. *J. Biochem.* 2015, 157 (5), 331–343. doi:10.1093/jb/mvu079.
95. *Kumar A., Das G., Bose B.* Recombinant receptor-binding domain of diphtheria toxin increases the potency of curcumin by enhancing cellular uptake. *Mol. Pharm.* 2014, 11 (1), 208–217. doi:10.1021/mp400378x.
96. *Esbensen Q.Y., Falnes P.O., Olsnes S., Madshus I.H.* Subcloning and characterization of the binding domain of fragment B of diphtheria toxin. *Biochem. J.* 1993, 294 (Pt 3)663–666.
97. *Labyntsev A.J., Korotkevych N.V., Manoilov K.J., Kaberniuk A.A., Kolybo D.V., Komisarenko S.V.* Recombinant fluorescent models for studying the diphtheria toxin. *Russ. J. Bioorg. Chem.* 2014, 40 (4), 401–409. doi:10.1134/S1068162014040086.
98. *Labyntsev A.I., Korotkevich N.V., Kaberniuk A.A., Romaniuk S.I., Kolybo D.V., Komisarenko S.V.* [Interaction of diphtheria toxin B subunit with sensitive and insensitive mammalian cells]. *Ukr. Biokhim. Zh. (1999).* 2010, 82 (6), 65–75. (In Ukranian).
99. *Blanke S.R., Huang K., Collier R.J.* Active-site mutations of diphtheria toxin: role of tyrosine-65 in NAD binding and ADP-ribosylation. *Biochemistry*. 1994, 33 (51), 15494–15500.
100. *Wilson B.A., Blanke S.R., Reich K.A., Collier R.J.* Active-site mutations of diphtheria toxin. Tryptophan 50 is a major determinant of NAD affinity. *J. Biol. Chem.* 1994, 269 (37), 23296–23301.
101. *Blanke S.R., Huang K., Wilson B.A., Papini E., Covacci A., Collier R.J.* Active-site mutations of the diphtheria toxin catalytic domain: role of histidine-21 in nicotinamide adenine dinucleotide binding and ADP-ribosylation of elongation factor 2. *Biochemistry*. 1994, 33 (17), 5155–5161.
102. *Tweten R.K., Barbieri J.T., Collier R.J.* Diphtheria toxin. Effect of substituting aspartic acid for glutamic acid 148 on ADP-ribosyltransferase activity. *J. Biol. Chem.* 1985, 260 (19), 10392–10394.
103. *Johnson V.G., Nicholls P.J.* Histidine 21 does not play a major role in diphtheria toxin catalysis. *J. Biol. Chem.* 1994, 269 (6), 4349–4354.
104. *Papini E., Schiavo G., Rappuoli R., Montecucco C.* Histidine-21 is involved in diphtheria toxin NAD+ binding. *Toxicon.* 1990, 28 (6), 631–635.

105. *Fu H., Blanke S.R., Mattheakis L.C., Collier R.J.* Selection of diphtheria toxin active-site mutants in yeast. Rediscovery of glutamic acid-148 as a key residue. *Adv. Exp. Med. Biol.* 1997, 41945–52.
106. *Zhao G., London E.* Behavior of diphtheria toxin T domain containing substitutions that block normal membrane insertion at Pro345 and Leu307: control of deep membrane insertion and coupling between deep insertion of hydrophobic subdomains. *Biochemistry*. 2005, 44 (11), 4488–4498. doi:10.1021/bi047705o.
107. *Kaul P., Silverman J., Shen W.H., Blanke S.R., Huynh P.D., Finkelstein A., Collier R.J.* Roles of Glu 349 and Asp 352 in membrane insertion and translocation by diphtheria toxin. *Protein Sci.* 1996, 5 (4), 687–692. doi:10.1002/pro.5560050413.
108. *Johnson V.G., Youle R.J.* A point mutation of proline 308 in diphtheria toxin B chain inhibits membrane translocation of toxin conjugates. *J. Biol. Chem.* 1989, 264 (30), 17739–17744.
109. *O'Keefe D., Collier R.J.* Cloned diphtheria toxin within the periplasm of Escherichia coli causes lethal membrane damage at low pH. *Proc. Natl. Acad. Sci. U.S.A.* 1989, 86 (1), 343–346.
110. *O'Keefe D.O., Cabiaux V., Choe S., Eisenberg D., Collier R.J.* pH-dependent insertion of proteins into membranes: B-chain mutation of diphtheria toxin that inhibits membrane translocation, Glu-349----Lys. *Proc. Natl. Acad. Sci. U.S.A.* 1992, 89 (13), 6202–6206.
111. *Rodnin M.V., Kyrychenko A., Kienker P., Sharma O., Posokhov Y.O., Collier R.J., Finkelstein A., Ladokhin A.S.* Conformational Switching of the Diphtheria Toxin T Domain. *J. Mol. Biol.* 2010. doi:10.1016/j.jmb.2010.07.024.
112. *Antignani A., Youle R.J.* Endosome fusion induced by diphtheria toxin translocation domain. *Proc. Natl. Acad. Sci. U.S.A.* 2008, 105 (23), 8020–8025. doi:10.1073/pnas.0711707105.
113. *Cabiaux V., Mindell J., Collier R.J.* Membrane translocation and channel-forming activities of diphtheria toxin are blocked by replacing isoleucine 364 with lysine. *Infect. Immun.* 1993, 61 (5), 2200–2202.
114. *Ren J., Sharpe J.C., Collier R.J., London E.* Membrane Translocation of Charged Residues at the Tips of Hydrophobic Helices in the T Domain of Diphtheria Toxin†. *Biochemistry*. 1999, 38 (3), 976–984. doi:10.1021/bi981576s.
115. *Senzel L., Gordon M., Blaustein R.O., Oh K.J., Collier R.J., Finkelstein A.* Topography of Diphtheria Toxin's T Domain in the Open Channel State. *J. Gen. Physiol.* 2000, 115 (4), 421–434.
116. *Kienker P.K., Wu Z., Finkelstein A.* Topography of the TH5 Segment in the Diphtheria Toxin T-Domain Channel. *J. Membr. Biol.* 2016, 249 (1–2), 181–196. doi:10.1007/s00232-015-9859-9.
117. *Kienker P.K., Wu Z., Finkelstein A.* Mapping the membrane topography of the TH6-TH7 segment of the diphtheria toxin T-domain channel. *J. Gen. Physiol.* 2015, 145 (2), 107–125. doi:10.1085/jgp.201411326.
118. *Vargas-Uribe M., Rodnin M.V., Kienker P., Finkelstein A., Ladokhin A.S.* Crucial Role of H322 in Folding of the Diphtheria Toxin T-Domain into the Open-Channel State. *Biochemistry*. 2013, 52 (20), 3457–3463. doi:10.1021/bi400249f.
119. *Rodnin M.V., Kyrychenko A., Kienker P., Sharma O., Vargas-Uribe M., Collier R.J., Finkelstein A., Ladokhin A.S.* Replacement of C-terminal histidines uncouples membrane insertion and translocation in diphtheria toxin T-domain. *Biophys. J.* 2011, 101 (10), L41-43. doi:10.1016/j.bpj.2011.10.018.
120. *Wu Z., Jakes K.S., Samelson-Jones B.S., Lai B., Zhao G., London E., Finkelstein A.* Protein translocation by bacterial toxin channels: a comparison of diphtheria toxin and colicin Ia. *Biophys. J.* 2006, 91 (9), 3249–3256. doi:10.1529/biophysj.106.085753.
121. *Finkelstein A., Oh K.J., Senzel L., Gordon M., Blaustein R.O., Collier R.J.* The diphtheria toxin channel-forming T-domain translocates its own NH2-terminal region and the catalytic domain across planar phospholipid bilayers. *Int. J. Med. Microbiol.* 2000, 290 (4–5), 435–440. doi:10.1016/S1438-4221(00)80059-4.
122. *Gordon M., Finkelstein A.* The number of subunits comprising the channel formed by the T domain of diphtheria toxin. *J. Gen. Physiol.* 2001, 118 (5), 471–480.
123. *Finkelstein A.* Proton-coupled protein transport through the anthrax toxin channel. *Philos Trans. R Soc. Lond. B Biol. Sci.* 2009, 364 (1514), 209–215. doi:10.1098/rstb.2008.0126.
124. *Deleers M., Beugnier N., Falmagne P., Cabiaux V., Ruysschaert J.M.* Localization in diphtheria toxin fragment B of a region that induces pore formation in planar lipid bilayers at low pH. *FEBS Lett.* 1983, 160 (1–2), 82–86.
125. *Shiver J.W., Donovan J.J.* Interactions of diphtheria toxin with lipid vesicles: determinants of ion channel formation. *Biochim. Biophys. Acta*. 1987, 903 (1), 48–55.

126. *Murphy J.R.* Mechanism of diphtheria toxin catalytic domain delivery to the eukaryotic cell cytosol and the cellular factors that directly participate in the process. *Toxins (Basel)*. 2011, 3 (3), 294–308. doi:10.3390/toxins3030294.
127. *Saito M., Iwawaki T., Taya C., Yonekawa H., Noda M., Inui Y., Mekada E., Kimata Y., Tsuru A., Kohno K.* Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat. Biotechnol.* 2001, 19 (8), 746–750. doi:10.1038/90795.
128. *Furukawa N., Saito M., Hakoshima T., Kohno K.* A diphtheria toxin receptor deficient in epidermal growth factor-like biological activity. *J. Biochem.* 2006, 140 (6), 831–841. doi:10.1093/jb/mvj216.
129. *Matsuoka K., Saito M., Shibata K., Sekine M., Shitara H., Taya C., Zhang X., Takahashi T.A., Kohno K., Kikkawa Y., Yonekawa H.* Generation of mouse models for type 1 diabetes by selective depletion of pancreatic beta cells using toxin receptor-mediated cell knockout. *Biochem. Biophys. Res. Commun.* 2013, 436 (3), 400–405. doi:10.1016/j.bbrc.2013.05.114.
130. *Jung S., Unutmaz D., Wong P., Sano G.-I., De los Santos K., Sparwasser T., Wu S., Vuthoori S., Ko K., Zavala F., Pamer E.G., Littman D.R., Lang R.A.* In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity*. 2002, 17 (2), 211–220.
131. *Bennett C.L., van Rijn E., Jung S., Inaba K., Steinman R.M., Kapsenberg M.L., Clausen B.E.* Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *J. Cell Biol.* 2005, 169 (4), 569–576. doi:10.1083/jcb.200501071.
132. *Ruedl C., Jung S.* DTR-mediated conditional cell ablation-Progress and challenges. *Eur. J. Immunol.* 2018, 48 (7), 1114–1119. doi:10.1002/eji.201847527.
133. *Aguila H.L., Hershberger R.J., Weissman I.L.* Transgenic mice carrying the diphtheria toxin A chain gene under the control of the granzyme A promoter: expected depletion of cytotoxic cells and unexpected depletion of CD8 T cells. *Proc. Natl. Acad. Sci. U S A.* 1995, 92 (22), 10192–10196.
134. *Brockschnieder D., Lappe-Siefke C., Goebbels S., Boesl M.R., Nave K.-A., Riethmacher D.* Cell depletion due to diphtheria toxin fragment A after Cre-mediated recombination. *Mol. Cell. Biol.* 2004, 24 (17), 7636–7642. doi:10.1128/MCB.24.17.7636-7642.2004.
135. *Nakamura S., Terashima M., Kikuchi N., Kimura M., Maehara T., Saito A., Sato M.* A new mouse model for renal lesions produced by intravenous injection of diphtheria toxin A-chain expression plasmid. *BMC Nephrol.* 2004, 54. doi:10.1186/1471-2369-5-4.
136. *Lang R.A., Bishop J.M.* Macrophages are required for cell death and tissue remodeling in the developing mouse eye. *Cell*. 1993, 74 (3), 453–462.
137. *Brockschnieder D., Pechmann Y., Sonnenberg-Riethmacher E., Riethmacher D.* An improved mouse line for Cre-induced cell ablation due to diphtheria toxin A, expressed from the Rosa26 locus. *Genesis*. 2006, 44 (7), 322–327. doi:10.1002/dvg.20218.
138. *Frank A.C., Johnson M.A.* Expressing the Diphtheria Toxin A Subunit from the HAP2(GCS1) Promoter Blocks Sperm Maturation and Produces Single Sperm-Like Cells Capable of Fertilization. *Plant Physiol.* 2009, 151 (3), 1390–1400. doi:10.1104/pp.109.144204.
139. *Talbird S.E., Graham J., Mauskopf J., Masseria C., Krishnarajah G.* Impact of tetanus, diphtheria, and acellular pertussis (Tdap) vaccine use in wound management on health care costs and pertussis cases. *J. Manag. Care Spec. Pharm.* 2015, 21 (1), 88–99, 99a-c. doi:10.18553/jmcp.2015.21.1.88.
140. *Rappuoli R., Bagnoli F. Vaccine Design: Innovative Approaches and Novel Strategies. Horizon Scientific Press*, 2011.
141. *Robbins J.B., Schneerson R., Keith J.M., Miller M.A., Kubler-Kielb J., Trollfors B.* Pertussis vaccine: a critique. *Pediatr. Infect. Dis. J.* 2009, 28 (3), 237–241. doi:10.1097/INF.0b013e31818a8958.
142. *Mooi F.R.* Bordetella pertussis and vaccination: the persistence of a genetically monomorphic pathogen. *Infect. Genet. Evol.* 2010, 10 (1), 36–49. doi:10.1016/j.meegid.2009.10.007.
143. *Pichichero M.E.* Protein carriers of conjugate vaccines. *Hum. Vaccin. Immunother.* 2013, 9 (12), 2505–2523. doi:10.4161/hv.26109.
144. *Shinefield H.R.* Overview of the development and current use of CRM(197) conjugate vaccines for pediatric use. *Vaccine*. 2010, 28 (27), 4335–4339. doi:10.1016/j.vaccine.2010.04.072.
145. *Bröker M., Costantino P., DeTora L., McIntosh E.D., Rappuoli R.* Biochemical and biological characteristics of cross-reacting material 197 CRM197, a non-toxic mutant of diphtheria toxin: use as a conjugation protein in vaccines and other potential clinical applications. *Biologicals*. 2011, 39 (4), 195–204. doi:10.1016/j.biologicals.2011.05.004.
146. *Buzzi S., Maistrello I.* Inhibition of growth of Erlich tumors in Swiss mice by

diphtheria toxin. *Cancer Res.* 1973, 33 (10), 2349–2353.
147. *Tang X.-H., Deng S., Li M., Lu M.-S.* Cross-reacting material 197 reverses the resistance to paclitaxel in paclitaxel-resistant human ovarian cancer. *Tumour Biol.* 2016, 37 (4), 5521–5528. doi:10.1007/s13277-015-4412-0.
148. *Yagi H., Yotsumoto F., Sonoda K., Kuroki M., Mekada E., Miyamoto S.* Synergistic anti-tumor effect of paclitaxel with CRM197, an inhibitor of HB-EGF, in ovarian cancer. *Int. J. Cancer.* 2009, 124 (6), 1429–1439. doi:10.1002/ijc.24031.
149. *Nam S.O., Yotsumoto F., Miyata K., Fukagawa S., Odawara T., Manabe S., Ishikawa T., Kuroki M., Yasunaga S., Miyamoto S.* Anti-tumor Effect of Intravenous Administration of CRM197 for Triple-negative Breast Cancer Therapy. *Anticancer Res.* 2016, 36 (7), 3651–3657.
150. *Buzzi S., Rubboli D., Buzzi G., Buzzi A.M., Morisi C., Pironi F.* CRM197 (nontoxic diphtheria toxin): effects on advanced cancer patients. *Cancer Immunol. Immunother.* 2004, 53 (11), 1041–1048. doi:10.1007/s00262-004-0546-4.
151. *Tsujioka H., Fukami T., Yotsumoto F., Ueda T., Hikita S., Takahashi Y., Kondo H., Kuroki M., Miyamoto S.* A possible clinical adaptation of CRM197 in combination with conventional chemotherapeutic agents for ovarian cancer. *Anticancer Res.* 2011, 31 (7), 2461–2465.
152. *Lian C., Ruan L., Shang D., Wu Y., Lu Y., L P., Yang Y., Wei Y., Dong X., Ren D., Chen K., Liu H., Tu Z.* Heparin-Binding Epidermal Growth Factor-Like Growth Factor as a Potent Target for Breast Cancer Therapy. *Cancer Biother. Radiopharm.* 2016, 31 (3), 85–90. doi:10.1089/cbr.2015.1956.
153. *Yotsumoto F., Oki E., Tokunaga E., Maehara Y., Kuroki M., Miyamoto S.* HB-EGF orchestrates the complex signals involved in triple-negative and trastuzumab-resistant breast cancer. *Int. J. Cancer.* 2010, 127 (11), 2707–2717. doi:10.1002/ijc.25472.
154. *Dateoka S., Ohnishi Y., Kakudo K.* Effects of CRM197, a specific inhibitor of HB-EGF, in oral cancer. *Med. Mol. Morphol.* 2012, 45 (2), 91–97. doi:10.1007/s00795-011-0543-6.
155. *Sanui A., Yotsumoto F., Tsujioka H., Fukami T., Horiuchi S., Shirota K., Yoshizato T., Kawarabayashi T., Kuroki M., Miyamoto S.* HB-EGF inhibition in combination with various anticancer agents enhances its antitumor effects in gastric cancer. *Anticancer Res.* 2010, 30 (8), 3143–3149.
156. *Kunami N., Yotsumoto F., Ishitsuka K., Fukami T., Odawara T., Manabe S., Ishikawa T., Tamura K., Kuroki M., Miyamoto S.* Antitumor effects of CRM197, a specific inhibitor of HB-EGF, in T-cell acute lymphoblastic leukemia. *Anticancer Res.* 2011, 31 (7), 2483–2488.
157. *Wang F., Liu R., Lee S.W., Sloss C.M., Couget J., Cusack J.C.* Heparin-binding EGF-like growth factor is an early response gene to chemotherapy and contributes to chemotherapy resistance. *Oncogene.* 2007, 26 (14), 2006–2016. doi:10.1038/sj.onc.1209999.
158. *Zhou Z.N., Sharma V.P., Beaty B.T., Roh-Johnson M., Peterson E.A., Van Rooijen N., Kenny P.A., Wiley H.S., Condeelis J.S., Segall J.E.* Autocrine HBEGF expression promotes breast cancer intravasation, metastasis and macrophage-independent invasion in vivo. *Oncogene.* 2014, 33 (29), 3784–3793. doi:10.1038/onc.2013.363.
159. *Frankel A.E., Woo J.H., Ahn C., Foss F.M., Duvic M., Neville P.H., Neville D.M.* Resimmune, an anti-CD3ε recombinant immunotoxin, induces durable remissions in patients with cutaneous T-cell lymphoma. *Haematologica.* 2015, 100 (6), 794–800. doi:10.3324/haematol.2015.123711.
160. *Wolska-Washer A., Robak P., Smolewski P., Robak T.* Emerging antibody-drug conjugates for treating lymphoid malignancies. *Expert Opin. Emerg. Drugs.* 2017, 22 (3), 259–273. doi:10.1080/14728214.2017.1366447.
161. *Schmohl J.U., Todhunter D., Taras E., Bachanova V., Vallera D.A.* Development of a Deimmunized Bispecific Immunotoxin dDT2219 against B-Cell Malignancies. *Toxins (Basel).* 2018, 10 (1),. doi:10.3390/toxins10010032.
162. *Zheng Q., Wang Z., Zhang H., Huang Q., Madsen J.C., Sachs D.H., Huang C.A., Wang Z.* Diphtheria toxin-based anti-human CD19 immunotoxin for targeting human CD19+ tumors. *Mol. Oncol.* 2017, 11 (5), 584–594. doi:10.1002/1878-0261.12056.
163. *Kreitman R.J., Tallman M.S., Robak T., Coutre S., Wilson W.H., Stetler-Stevenson M., FitzGerald D.J., Santiago L., Gao G., Lanasa M.C., Pastan I.* Minimal residual hairy cell leukemia eradication with moxetumomab pasudotox: phase 1 results and long-term follow-up. *Blood.* 2018, 131 (21), 2331–2334. doi:10.1182/blood-2017-09-803072.
164. *Wayne A.S., Shah N.N., Bhojwani D., Silverman L.B., Whitlock J.A., Stetler-Stevenson M., Sun W., Liang M., Yang J., Kreitman R.J., Lanasa M.C., Pastan I.* Phase 1 study of the anti-CD22 immunotoxin moxetumomab pasudotox for childhood acute lymphoblastic leukemia. *Blood.* 2017, 130 (14), 1620–1627. doi:10.1182/blood-2017-02-749101.

165. *Woo J.H., Lee Y.-J., Neville D.M., Frankel A.E.* Pharmacology of anti-CD3 diphtheria immunotoxin in CD3 positive T-cell lymphoma trials. *Meth. Mol. Biol.* 2010, 651157–175. doi:10.1007/978-1-60761-786-0_10.
166. *Thompson J., Hu H., Scharff J., Neville D.M.* An anti-CD3 single-chain immunotoxin with a truncated diphtheria toxin avoids inhibition by pre-existing antibodies in human blood. *J. Biol. Chem.* 1995, 270 (47), 28037–28041.
167. *Wang Z., Wei M., Zhang H., Chen H., Germana S., Huang C.A., Madsen J.C., Sachs D.H., Wang Z.* Diphtheria-toxin based anti-human CCR4 immunotoxin for targeting human CCR4(+) cells in vivo. *Mol Oncol.* 2015, 9 (7), 1458–1470. doi:10.1016/j.molonc.2015.04.004.
168. *Testa U., Riccioni R., Biffoni M., Diverio D., Lo-Coco F., Fo R., Peschle C., Frankel A.E.* Diphtheria toxin fused to variant human interleukin-3 induces cytotoxicity of blasts from patients with acute myeloid leukemia according to the level of interleukin-3 receptor expression. *Blood.* 2005, 106 (7), 2527–2529. doi:10.1182/blood-2005-02-0540.
169. *Wen Z.L., Tao X., Lakkis F., Kiyokawa T., Murphy J.R.* Diphtheria toxin-related alpha-melanocyte-stimulating hormone fusion toxin. Internal in-frame deletion from Thr387 to His485 results in the formation of a highly potent fusion toxin which is resistant to proteolytic degradation. *J. Biol. Chem.* 1991, 266 (19), 12289–12293.
170. *Zhang Y., Schulte W., Pink D., Phipps K., Zijlstra A., Lewis J.D., Waisman D.M.* Sensitivity of cancer cells to truncated diphtheria toxin. *PLoS ONE.* 2010, 5 (5), e10498. doi:10.1371/journal.pone.0010498.
171. *Ramakrishnan S., Olson T.A., Bautch V.L., Mohanraj D.* Vascular endothelial growth factor-toxin conjugate specifically inhibits KDR/flk-1-positive endothelial cell proliferation in vitro and angiogenesis in vivo. *Cancer Res.* 1996, 56 (6), 1324–1330.
172. *Wild R., Yokoyama Y., Dings R.P.M., Ramakrishnan S.* VEGF-DT385 toxin conjugate inhibits mammary adenocarcinoma development in a transgenic mouse model of spontaneous tumorigenesis. *Breast Cancer Res. Treat.* 2004, 85 (2), 161–171. doi:10.1023/B:BREA.0000025407.02896.ec.
173. *Murphy J.R., Bishai W., Borowski M., Miyanohara A., Boyd J., Nagle S.* Genetic construction, expression, and melanoma-selective cytotoxicity of a diphtheria toxin-related alpha-melanocyte-stimulating hormone fusion protein. *Proc. Natl. Acad. Sci. U.S.A.* 1986, 83 (21), 8258–8262.
174. *Williams D.P., Wen Z., Watson R.S., Boyd J., Strom T.B., Murphy J.R.* Cellular processing of the interleukin-2 fusion toxin DAB486-IL-2 and efficient delivery of diphtheria fragment A to the cytosol of target cells requires Arg194. *J. Biol. Chem.* 1990, 265 (33), 20673–20677.
175. *Chan C.H., Blazar B.R., Greenfield L., Kreitman R.J., Vallera D.A.* Reactivity of murine cytokine fusion toxin, diphtheria toxin390-murine interleukin-3 (DT390-mIL-3), with bone marrow progenitor cells. *Blood.* 1996, 88 (4), 1445–1456.
176. *Black J.H., McCubrey J.A., Willingham M.C., Ramage J., Hogge D.E., Frankel A.E.* Diphtheria toxin-interleukin-3 fusion protein (DT(388)IL3) prolongs disease-free survival of leukemic immunocompromised mice. *Leukemia.* 2003, 17 (1), 155–159. doi:10.1038/sj.leu.2402744.
177. *Cohen K.A., Liu T.F., Cline J.M., Wagner J.D., Hall P.D., Frankel A.E.* Toxicology and pharmacokinetics of DT388IL3, a fusion toxin consisting of a truncated diphtheria toxin (DT388) linked to human interleukin 3 (IL3), in cynomolgus monkeys. *Leuk. Lymphoma.* 2004, 45 (8), 1647–1656. doi:10.1080/10428190410001663572.
178. *Li Y.M., Vallera D.A., Hall W.A.* Diphtheria toxin-based targeted toxin therapy for brain tumors. *J. Neurooncol.* 2013, 114 (2), 155–164. doi:10.1007/s11060-013-1157-8.
179. *Chadwick D.E., Williams D.P., Niho Y., Murphy J.R., Minden M.D.* Cytotoxicity of a recombinant diphtheria toxin-granulocyte colony-stimulating factor fusion protein on human leukemic blast cells. *Leuk. Lymphoma.* 1993, 11 (3–4), 249–262. doi:10.3109/10428199309087002.
180. *Hotchkiss C.E., Hall P.D., Cline J.M., Willingham M.C., Kreitman R.J., Gardin J., Latimer A., Ramage J., Feely T., DeLatte S., Tagge E.P., Frankel A.E.* Toxicology and pharmacokinetics of DTGM, a fusion toxin consisting of a truncated diphtheria toxin (DT388) linked to human granulocyte-macrophage colony-stimulating factor, in cynomolgus monkeys. *Toxicol. Appl. Pharmacol.* 1999, 158 (2), 152–160. doi:10.1006/taap.1999.8691.
181. *Hall P.D., Willingham M.C., Kreitman R.J., Frankel A.E.* DT388-GM-CSF, a novel fusion toxin consisting of a truncated diphtheria toxin fused to human granulocyte-macrophage colony-stimulating factor, prolongs host survival in a SCID mouse model of acute myeloid leukemia. *Leukemia.* 1999, 13 (4), 629–633.

182. *Feuring-Buske M., Frankel A., Gerhard B., Hogge D.* Variable cytotoxicity of diphtheria toxin 388-granulocyte-macrophage colony-stimulating factor fusion protein for acute myelogenous leukemia stem cells. *Exp. Hematol.* 2000, 28 (12), 1390–1400.
183. *Ramage J.G., Vallera D.A., Black J.H., Aplan P.D., Kees U.R., Frankel A.E.* The diphtheria toxin/urokinase fusion protein (DTAT) is selectively toxic to CD87 expressing leukemic cells. *Leuk. Res.* 2003, 27 (1), 79–84.
184. *Hagihara N., Walbridge S., Olson A.W., Oldfield E.H., Youle R.J.* Vascular protection by chloroquine during brain tumor therapy with Tf-CRM107. *Cancer Res.* 2000, 60 (2), 230–234.
185. *Kreitman R.J.* Immunotoxins for targeted cancer therapy. *AAPS J.* 2006, 8 (3), E532-551. doi:10.1208/aapsj080363.
186. *Wayne A.S., FitzGerald D.J., Kreitman R.J., Pastan I.* Immunotoxins for leukemia. *Blood.* 2014, 123 (16), 2470–2477. doi:10.1182/blood-2014-01-492256.
187. *Turturro F.* Denileukin diftitox: a biotherapeutic paradigm shift in the treatment of lymphoid-derived disorders. *Expert Rev Anticancer Ther.* 2007, 7 (1), 11–17. doi:10.1586/14737140.7.1.11.
188. *Duvic M., Talpur R.* Optimizing denileukin diftitox (Ontak) therapy. *Future Oncol.* 2008, 4 (4), 457–469. doi:10.2217/14796694.4.4.457.
189. *Urieto J.O., Liu T., Black J.H., Cohen K.A., Hall P.D., Willingham M.C., Pennell L.K., Hogge D.E., Kreitman R.J., Frankel A.E.* Expression and purification of the recombinant diphtheria fusion toxin DT388IL3 for phase I clinical trials. *Protein Expr. Purif.* 2004, 33 (1), 123–133. doi:10.1016/j.pep.2003.09.003.
190. *Hogge D.E., Yalcintepe L., Wong S.-H., Gerhard B., Frankel A.E.* Variant diphtheria toxin-interleukin-3 fusion proteins with increased receptor affinity have enhanced cytotoxicity against acute myeloid leukemia progenitors. *Clin. Cancer Res.* 2006, 12 (4), 1284–1291. doi:10.1158/1078-0432.CCR-05-2070.
191. *Orr N., Galen J.E., Levine M.M.* Expression and Immunogenicity of a Mutant Diphtheria Toxin Molecule, CRM197, and Its Fragments in Salmonella typhi Vaccine Strain CVD 908-htrA. *Infect Immun.* 1999, 67 (8), 4290–4294.
192. *Kaberniuk A.A., Oliinyk O.S., Kolybo D.V., Komisarenko S.V.* Toxin-neutralizing properties of antibodies to diphtheria toxin recombinant subunits A and B and a new method of their estimation. *Ukr. Biokhim. Zh.* (1999). 2009, 81 (3), 92–101. (In Ukrainian).
193. *Pavlov P.V., Leonova A.G.* Effect of the products of protein splitting in the culture medium on toxin formation. II. Antigen and immunogenic properties of diphtheria toxins (toxoids) obtained on the medium, digested by 2 enzymes, during culture of the Weisensee strain. *Zh. Mikrobiol. Epidemiol. Immunobiol.* 1961, 32, 95–99. (In Russian).
194. *Romaniuk S.I., Kolibo D.B., Komisarenko S.V.* Perspectives of application of recombinant diphtheria toxin derivatives. *Bioorg. Khim.* 2012, 38 (6), 639–652.
195. *Liu W., Onda M., Lee B., Kreitman R.J., Hassan R., Xiang L., Pastan I.* Recombinant immunotoxin engineered for low immunogenicity and antigenicity by identifying and silencing human B-cell epitopes. *Proc. Natl. Acad. Sci. U.S.A.* 2012, 109 (29), 11782–11787. doi:10.1073/pnas.1209292109.
196. *Mazor R., Onda M., Pastan I.* Immunogenicity of therapeutic recombinant immunotoxins. *Immunol. Rev.* 2016, 270 (1), 152–164. doi:10.1111/imr.12390.
197. *Onda M., Beers R., Xiang L., Nagata S., Wang Q.-C., Pastan I.* An immunotoxin with greatly reduced immunogenicity by identification and removal of B cell epitopes. *Proc. Natl. Acad. Sci. U.S.A.* 2008, 105 (32), 11311–11316. doi:10.1073/pnas.0804851105.
198. *Flavell D.J.* Countering immunotoxin immunogenicity. *Br. J. Cancer.* 2016, 114 (11), 1177–1179. doi:10.1038/bjc.2016.84.

БІОЛОГІЧНІ ВЛАСТИВОСТІ ТА МЕДИЧНЕ ЗАСТОСУВАННЯ ПОХІДНИХ ДИФТЕРІЙНОГО ТОКСИНУ

К. Ю. Манойлов

Інститут біохімії ім. О. В. Палладіна
НАН України, Київ

E-mail: manoilov.inbox@gmail.com

Метою огляду був аналіз даних літератури, пов'язаних із практичним застосуванням різноманітних похідних дифтерійного токсину. Дослідження взаємодії дифтерійного токсину з чутливими і резистентними клітинами ссавців проводили вже протягом відносно тривалого часу, однак й дотепер існують деякі невирішені проблеми, що стосуються молекулярних механізмів його функціонування. Нативний дифтерійний токсин і частини його молекули, які зберігають токсичність, використовують як інструменти у новітніх біотехнологічних методах специфічного знищення підтипів клітин у багатоклітинних організмах. Нові рекомбінантні похідні дифтерійного токсину періодично отримують у лабораторіях у всьому світі. У біологічних дослідженнях аналоги дифтерійного токсину є зручними засобами для вивчення функцій природного токсину. Нетоксичний аналог дифтерійного токсину, протеїн CRM197, вже введено в клінічну практику як компонент вакцин і протипухлинний агент. Терапія спрямованими токсинами на основі дифтерійного токсину є потенційно перспективною для лікування раку, тому вивчення його похідних має велике значення для біотехнології та медицини.

Ключові слова: клітинна абляція, CRM197, дифтерійний токсин, імуногенність, терапія спрямованими токсинами, токсоїд.

БИОЛОГИЧЕСКИЕ СВОЙСТВА И МЕДИЦИНСКОЕ ПРИМЕНЕНИЕ ПРОИЗВОДНЫХ ДИФТЕРИЙНОГО ТОКСИНА

К. Ю. Манойлов

Институт биохимии им. А. В. Палладина
НАН Украины, Киев

E-mail: manoilov.inbox@gmail.com

Целью обзора был анализ данных литературы, связанных с практическим применением различных производных дифтерийного токсина. Исследования взаимодействия дифтерийного токсина с чувствительными и резистентными клетками млекопитающих проводили в течение относительно длительного времени, однако до сих пор существуют некоторые нерешенные проблемы, касающиеся молекулярных механизмов его функционирования. Нативный дифтерийный токсин и части его молекулы, которые сохраняют токсичность, используют в качестве инструментов в новейших биотехнологических методах специфического уничтожения подтипов клеток в многоклеточных организмах. Новые рекомбинантные производные дифтерийного токсина периодически получают в лабораториях по всему миру. В биологических исследованиях аналоги дифтерийного токсина представляют собой удобные средства для изучения функций природного токсина. Нетоксичный аналог дифтерийного токсина, протеин CRM197, уже введен в клиническую практику как компонент вакцин и противоопухолевый агент. Терапия направленными токсинами на основе дифтерийного токсина является потенциально перспективной для лечения рака, поэтому изучение его производных имеет большое значение для биотехнолоии и медицины.

Ключевые слова: клеточная абляция, CRM197, дифтерийный токсин, иммуногенность, терапия направленными токсинами, токсоид.