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BIOLOGICAL PROPERTIES AND MEDICAL APPLICATION OF DIPHTHERIA TOXIN DERIVATIVES

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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.

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БІОЛОГІЧНІ ВЛАСТИВОСТІ ТА МЕДИЧНЕ ЗАСТОСУВАННЯ ПОХІДНИХ ДИФТЕРІЙНОГО ТОКСИНУ

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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

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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.