

Corynebacterium diphtheriae: Diphtheria Toxin, the *tox* Operon, and Its Regulation by Fe²⁺ Activation of apo-DtxR

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ABSTRACT Diphtheria is one of the most well studied of all the bacterial infectious diseases. These milestone studies of toxigenic *Corynebacterium diphtheriae* along with its primary virulence determinant, diphtheria toxin, have established the paradigm for the study of other related bacterial protein toxins. This review highlights those studies that have contributed to our current understanding of the structure-function relationships of diphtheria toxin, the molecular mechanism of its entry into the eukaryotic cell cytosol, the regulation of diphtheria *tox* expression by holo-DtxR, and the molecular basis of transition metal ion activation of apo-DtxR itself. These seminal studies have laid the foundation for the protein engineering of diphtheria toxin and the development of highly potent eukaryotic cell-surface receptor-targeted fusion protein toxins for the treatment of human diseases that range from T cell malignancies to steroid-resistant graft-versus-host disease to metastatic melanoma. This deeper scientific understanding of diphtheria toxin and the regulation of its expression have metamorphosed the third-most-potent bacterial toxin known into a life-saving targeted protein therapeutic, thereby at least partially fulfilling Paul Erlich's concept of a magic bullet—"a chemical that binds to and specifically kills microbes or tumor cells."

INTRODUCTION

Over the past 4 decades, perhaps no other infectious disease has been as successfully studied as diphtheria (1, 2). Indeed, the study of diphtheria toxin established the structure-function paradigm for the study of other toxins in the bacterial protein toxin field. Moreover, when coupled with the molecular genetic study of the iron-activated regulatory element, DtxR, that controls

the expression of diphtheria toxin, we now have a detailed understanding of the entire *tox* genetic system, from the regulation of expression to the molecular mechanism of diphtheria toxin action. In this article, we review the development of our current understanding of diphtheria toxin, from its structure-function relationships to its mechanism of entry into the eukaryotic cell cytosol, the molecular mechanism of transition metal ion activation of DtxR and its regulation of *tox* expression, and finally, the protein engineering of diphtheria toxin for the development of highly potent and selective cell-surface receptor-targeted fusion protein toxins for the treatment of human diseases.

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It is well known that to cause clinical disease, the etiologic agent of diphtheria, *Corynebacterium diphtheriae*, must first be infected with one of a closely related family of bacteriophages that carry the structural gene for the toxin. Humans are the only natural host for diphtheria, and disease is readily spread through close contact and aerosolized droplets. While clinical diphtheria remains prevalent in tropical developing countries where immunization with diphtheria toxoid remains sporadic, diphtheria is almost nonexistent in industrialized countries that have continued to maintain a comprehensive immunization program with diphtheria toxoid. Since diphtheria toxin is secreted into the culture medium, the high-yield-producing Park-Williams 8 strain of *C. diphtheriae* has been used almost worldwide to produce diphtheria toxin in sufficient yields to produce diphtheria toxoid (3). Once purified from the spent culture medium, native diphtheria toxin is then treated with formalin at 30 to 38°C for several weeks to produce the nontoxic toxoid. To produce the vaccine, diphtheria toxoid is absorbed to alumina, which acts as an adjuvant, and it is then combined with tetanus toxoid and either heat-killed *Bordetella pertussis* to produce the DPT vaccine or purified pertussis antigens to produce the diphtheria-tetanus-acellular pertussis vaccine.

Along with botulinum, tetanus, and Shiga, diphtheria toxin is one of the most potent bacterial protein toxins known. In humans the 50% lethal dose for diphtheria toxin was found to be approximately 100 ng/kg. This value was determined after a catastrophic accident in Kyoto, Japan, in 1948. Shortly after the end of World War II, a mass immunization program against diphtheria was initiated in Japan. After the production of diphtheria toxin and its distribution into vials for formalin detoxification in the preparation of toxoid, one vial of toxin was either inadvertently not treated or reverted to full toxicity after formalin treatment and was sent into the community as part of the program to immunize children. Of 606 children that were “immunized” from this vial, 68 children, mostly between the ages of 1 and 2, died of diphtheria intoxication (4, 5). Since the concentration of toxin in the vial, the dose that was administered, and the body weight of the children were all known, the human 50% lethal dose for diphtheria toxin could be calculated.

While diphtheria toxoid as a vaccine was available as early as the 1920s, investigations into the development of a simplified medium for toxin production led Alwin M. Pappenheimer, Jr., to make one of the earliest observations that the concentration of iron in the growth medium was critical to obtain maximal yields of the toxin (6). In 1935, Pappenheimer, then a Bradford Fellow at

the Harvard Medical School, started his laboratory at the Massachusetts Antitoxin and Vaccine Laboratory to work on diphtheria toxin production. At that time Pyrex glassware was newly available, and he used Pyrex flasks in his first attempts to produce toxin. While he followed established protocols for medium preparation and incubation temperature and time for maximal yields, Pappenheimer was able to produce only half as much toxin as that reported by his predecessors who used the older soft glassware flasks in the laboratory. The question was obvious: Why did the glassware make a difference in toxin production? Pappenheimer’s experiment to address the question was equally obvious: He broke a flask made of soft glass and ground it into a powder. He then added varying amounts of powdered glass to the Pyrex flasks he was using and determined its effect on toxin production. Remarkably, Pappenheimer found that the addition of as little as 300 µg powdered soft glass resulted in a stimulation of both *C. diphtheriae* growth and toxin yield. Equally importantly, he found that the addition of 5 or 10 mg of powdered glass resulted in the almost complete inhibition of toxin production without a change in the growth of the diphtheria bacillus. The irony (pun intended) of this story is that Pappenheimer wrote his Ph. D. dissertation at Harvard College on the effect of organic bases on the oxidation potential of heme. It is no surprise then that his first experiments were focused on the effect of adding increasing amounts of inorganic iron to the growth medium as a function of diphtheria toxin yield. As shown in his classic 1936 paper (6), the stimulation and subsequent inhibition of toxin production following the addition of either powdered glass or iron salts to the media were superimposable. The ability to produce a maximal yield of diphtheria toxin in the soft glass resulted from the leaching of small levels of iron from the glass into the culture medium. We now know that maximal yields of diphtheria toxin are produced only when iron becomes the growth-rate-limiting substrate. So, as early as 1936, it was realized that with respect to iron, an essential nutrient for the growth of the bacillus, the physiologic state of toxigenic *C. diphtheriae* was a determining factor in the production of diphtheria toxin.

TEMPERATE CORYNEBACTERIOPHAGES CARRY THE STRUCTURAL GENE FOR DIPHTHERIA TOXIN, *tox*, IN LYSOGENIC *C. DIPHTHERIAE*

While it had been known since the earliest observations of *C. diphtheriae* that there were both virulent and nonvirulent strains that could be isolated from healthy

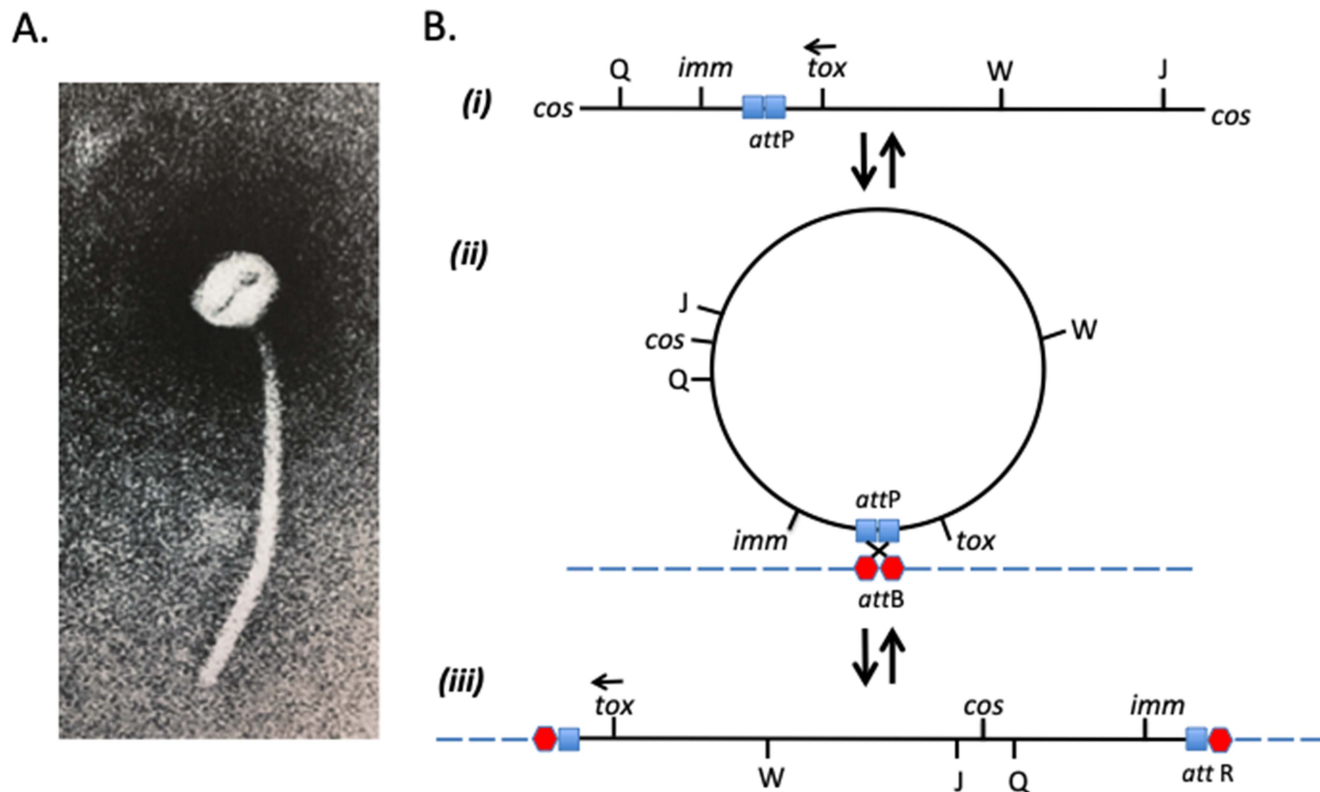
individuals, it was not until the remarkable discovery by Freeman (7) that one could isolate diphtheria toxin-producing strains following the exposure of nontoxic strains to corynebacteriophages. Thus, it became clear that not only the physiologic state of *C. diphtheriae* with respect to the concentration of iron in the growth medium but also its lysogenic state determined the ability of a given isolate to produce diphtheria toxin. As important as Freeman's observation was, the question of whether it was the host bacterium or the lysogenic phage that carried the structural gene for the toxin still remained.

Twenty years after Freeman's initial report, Uchida et al. (8) described the isolation of corynebacteriophage β mutant lysogens of *C. diphtheriae* that produced nontoxic proteins that were serologically related to diphtheria toxin. The discovery of these mutant serologically related cross-reacting materials (CRMs) was extremely important because they not only demonstrated that the structural gene for diphtheria toxin, *tox*, was carried by β -phage, but the isolation of CRMs that

were the result of both nonsense (e.g., CRM45) and missense (e.g., CRM197) mutations established both the genetic orientation of the toxin, N- to C-terminal, and began to give both biochemical and genetic definition to its structural-functional domains.

While corynebacteriophage β has not been nearly as well studied as coliphage λ , it is a temperate phage and, as such, may either enter a vegetative lytic cycle or lysogenize a sensitive host (e.g., the nontoxic, nonlysogenic $C_{7s}(-)^{tox-}$ strain of *C. diphtheriae*) and convert it from a *tox-* to a *tox+* phenotype. Both genetic and molecular genetic evidence suggest that the linear β -genome circularizes by ligation of its cohesive ends (*cos*) and integrates into the host chromosome as a prophage in a manner that corresponds to that for the integration of λ -phage into the *Escherichia coli* genome (9–11). It has been shown that the prophage map is a circular permutation of the vegetative map and that the *imm* marker is positioned at one end of the β -genome and *tox* is at the other end (Fig. 1). The $C_{7s}(-)^{tox-}$ genome carries two separate *attB* sites, *attB1* and *attB2*, and as a result,

FIGURE 1 (A) Electron micrograph of corynebacteriophage β , which has a polyhedral head ~52 nm in diameter and a 270 nm long tail. (B) Genetic map of β -phage in its (i) vegetative phase, (ii) circularized form, and (iii) prophage state. (Modified from reference 9.)



β -phage can integrate and form single lysogens, double lysogens, and in rare instances, triple lysogens (12). In its lysogenic state, most β -phage genes appear to be repressed, and the lysogen is immune to superinfection by homoimmune corynebacteriophages. While triple lysogens are unstable and revert to stable double lysogens, under iron-limiting conditions, the final yield of diphtheria toxin that may be produced is directly related to the number of integrated prophage genomes (12).

CORYNEBACTERIAL DETERMINED DtxR REGULATES EXPRESSION OF THE CORYNEBACTERIOPHAGE *tox* GENE

As noted above, the discovery by Uchida et al. (8) that the structural gene encoding diphtheria toxin was carried by corynebacteriophage β raised the question of whether the iron-sensitive regulation of *tox* expression was mediated by either a corynebacteriophage or corynebacterial determinant. To address that question, Murphy et al. (13) used S-30 extracts of *E. coli* in a coupled transcription-translation system programmed with β -phage DNA to synthesize diphtheria toxin *in vitro*. In this system, diphtheria toxin was expressed in good yield, and remarkably, the *in vitro* synthesis of the toxin was not inhibited by the addition of iron. In contrast, the addition of cell-free extracts of the nontoxigenic, nonlysogenic C7_s(-)^{tox-} strain of *C. diphtheriae* to the *E. coli* system resulted in the inhibition of *tox* expression but not that of other phage gene products. These results clearly suggested that the inhibitory effect of iron was mediated through a bacterial host-determined factor. Furthermore, the subsequent isolation of *C. diphtheriae* mutants which when lysogenized by β -phage constitutively expressed diphtheria toxin in the presence of excess iron (14), as well as the isolation of phage mutants which constitutively expressed diphtheria toxin when lysogenized in wild-type *C. diphtheriae* (15, 16), ultimately led to a hypothetical model for the regulation of *tox* expression (Fig. 2).

DNA sequence analysis of the structural gene for diphtheria toxin revealed a 27-bp interrupted palindromic sequence that overlapped the putative -10 region of the *tox* promoter (17–20). Based on these observations, this upstream region of the gene encoding diphtheria toxin was then designated the *tox* promoter-operator, *toxPO*. In 1989, Fourel et al. (21) used DNase protection assays to show that an element(s) found in crude cell-free extracts of *C. diphtheriae* specifically bound to *toxO*. Mutant strains of *C. diphtheriae* that are *cis*-dominant for toxin production were later found to carry mutations only in the *tox* operator (22–24).

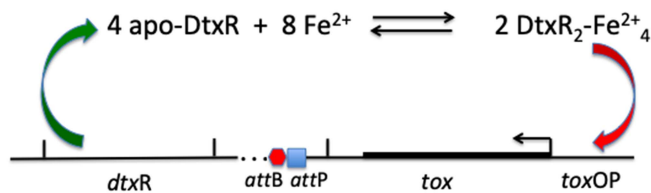


FIGURE 2 Schematic representation of Fe²⁺ activation of apo-DtxR and the binding of two dimers to the *tox* operator, thereby repressing expression of the diphtheria toxin structural gene, *tox*.

To clone the *trans*-acting putative *tox* repressor, Boyd et al. (25) screened genomic libraries of the C7_s(-)^{tox-} strain of *C. diphtheriae* in recombinant *E. coli* that carried a transcriptional fusion between *toxPO* and *lacZ*. This work led to the isolation of the structural gene for the diphtheria *tox* repressor, *dtxR*, which encoded a 226-amino acid protein. DtxR was shown to repress the expression of β -galactosidase from the *toxPO-lacZ* fusion in *E. coli* in an iron-dependent fashion. Schmitt and Holmes (23) subsequently demonstrated the functional activity of DtxR in *C. diphtheriae* by showing that expression of wild-type *dtxR* in the iron-insensitive host-mutant C7hm723($\beta^{\text{tox+}}$) strain resulted in conversion to the wild-type iron-sensitive phenotype.

TRANSITION METAL IONS ACTIVATE DtxR BINDING TO THE *tox* OPERATOR

By using gel mobility shift assays, Tao et al. (26) demonstrated that the activation of apo-DtxR and its subsequent binding to a [³²P]-labeled *toxO* probe required the addition of divalent transition metal ions to both the binding reaction mixture and the gel matrix. Since binding of DtxR to the labeled *toxO* probe was blocked by the addition of excess unlabeled probe, anti-DtxR antibodies, or the chelator 2,2'-dipyridyl, DtxR binding was specific and dependent on metal ion activation. Both Tao and Murphy (27) and Schmitt and Holmes (28) demonstrated that the addition of Co²⁺, Fe²⁺, Ni²⁺, Cd²⁺, or Mn²⁺ activated apo-DtxR and resulted in the protection of the *toxPO* probe in DNase protection assays. Zinc was found to be only a weak activator of apo-DtxR, and Cu²⁺ failed to activate repressor activity.

While apo-DtxR could be activated by a number of divalent transition metal ions, Tao and Murphy (29) demonstrated that the activated DtxR could also bind to a number of closely related DNA target sequences. To determine the consensus operator sequence for DtxR binding, Tao and Murphy used a gel electrophoresis

mobility-shift assay and PCR amplification for *in vitro* affinity selection of randomized DNA sequences from a universe of $>6 \times 10^{10}$ variants. After 10 rounds of *in vitro* selection, each with 30 cycles of PCR amplification, a family of DNA sequences that functioned as DtxR-responsive elements both *in vitro* and *in vivo* was isolated and characterized. The consensus sequence for DtxR binding was found to be as follows: T.AGGTTAGC/GCTAACCT.A. Moreover, since this family of related target sequences was found to bind DtxR with the same apparent affinity as the 27-bp *tox* operator, it was clear that the iron-activated repressor was most likely to function as a global regulatory element in the regulation of iron-sensitive genes in *C. diphtheriae*. Indeed, a number of genes which have upstream DtxR-binding sites, including the operon essential for the expression of siderophores for iron acquisition, have been isolated and characterized (23, 28).

Since the cloning and characterization of DtxR from *C. diphtheriae*, homologous metal ion-activated repressors have been identified in a number of other Gram-positive prokaryotes. IdeR (iron-dependent regulator) which has been found in several species of *Mycobacterium* and is 78% identical and 90% homologous has been isolated and characterized (30). Remarkably, expression of the *C. diphtheriae* hyperactive repressor, DtxR(E175K), in merodiploid strains of both *Mycobacterium tuberculosis* and *Staphylococcus aureus* has been shown to attenuate virulence *in vivo*, presumably by repression of iron-sensitive genes (31–33). These results clearly suggest that there is a high degree of both function and DNA sequence target specificity between DtxR and IdeR. In addition, DtxR homologs have been identified in a number of genera, including the species *Enterococcus faecalis* (34), *Streptococcus mutans* (35), *S. aureus* (36), *Streptomyces pilosus* (37), and *Rhodococcus equi* (38). It is of interest to note that both *M. tuberculosis* and *C. diphtheriae* also express additional DtxR-like proteins which are likely to be activated by different transition metal ions and have different DNA target sequences (39), including the MntR protein in *C. diphtheriae*, which is responsive to Mn^{2+} .

STRUCTURAL BASIS OF TRANSITION METAL ION ACTIVATION OF DtxR REPRESSOR ACTIVITY

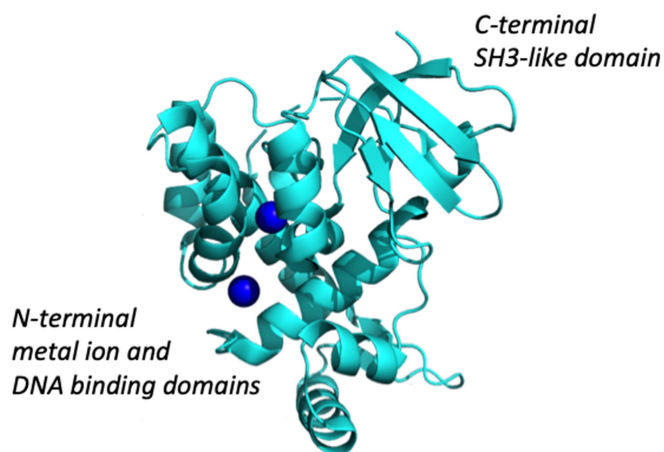
As noted above, DtxR is a 226-amino acid protein. DNA sequence analysis revealed a single cysteine residue, Cys102, and disulfide-linked dimers are inactive. The substitution of Cys102 with all 20 amino acids, except

for Asp, by site-directed mutagenesis results in the complete loss of repressor activity. Further characterization of the wild type and individual mutants demonstrated that Cys102 plays an essential role in the coordination of Fe^{2+} in the activation of apo-DtxR (40). In addition, Wang et al. (24) used bisulfite mutagenesis to inactivate DtxR and found that a high percentage of mutations were found in regions of DtxR that exhibited homology with other repressors. In addition, a number of mutations were also isolated in a predicted α -helical region with the sequence of His98-Cys102-His106 that resembled metal ion-binding motifs in other proteins.

A more complete understanding of the structural basis of metal ion-activated DtxR repressor activity was revealed by the solution of the crystal structures of apo-DtxR and holo-DtxR in complex with *toxPO* (41–44). As shown in Fig. 3, the structure of the C-terminal end of DtxR was found to fold into a SRC homology 3 (SH3)-like domain (45). The SH3 domain was first described as a conserved sequence in the viral adaptor protein v-Crk but has also been found to be present in phospholipases and the cytoplasmic tyrosine kinases, Abl and Src. In general, SH3 domains are found in proteins that either interact with other proteins or mediate the formation of specific protein complexes by binding to proline-rich peptide sequences.

DtxR contains a total of eight α -helices, six of which are contained in the N-terminal two-thirds of the protein. Helices B and C and the loop connecting them form the helix-turn-helix DNA-binding motif. Interestingly, Ni^{2+} -activated DtxR was shown to bind to

FIGURE 3 X-ray crystal structure of Co^{2+} -activated DtxR showing its N-terminal metal ion and DNA-binding domain and the C-terminal SH3-like domain. (Modified from reference 45 [Protein Data Bank ID 1C0W].)



the *toxPO* oligonucleotide as two pairs of dimers (44). In this instance, each DtxR dimer was found to bind to almost opposite faces of the palindromic *toxO* sequence (Fig. 4). As one would anticipate, the solution of the X-ray structures of DtxR and the ternary complex that forms with its binding to *toxO* confirms and extends the earlier observation that its footprint compasses a region of 30 bp immediately upstream of the transcription initiation signal (46).

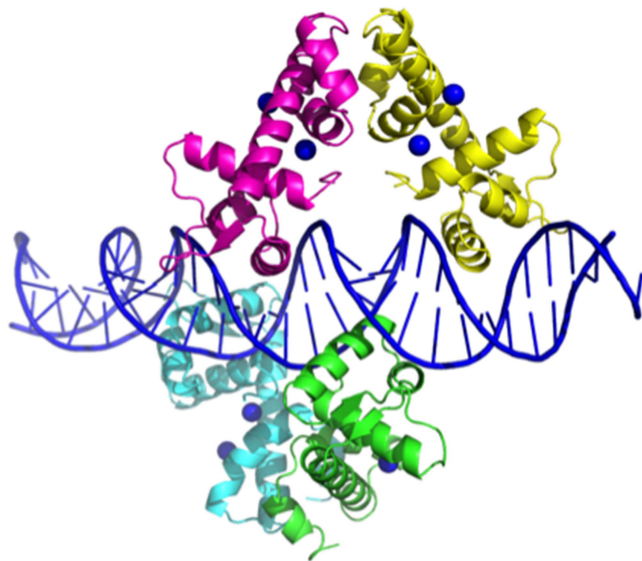
While the overall mechanism of DtxR binding to *toxO* is similar to that of other prokaryotic repressors, there are some unique interactions that should be noted. The C-helix of DtxR is responsible for most interactions with DNA and inserts into the major groove of the DNA double helix. Each helix-turn-helix in the dimer makes a total of nine interactions with backbone phosphate groups. In addition, the guanidinium group of Arg60 binds in the minor groove of DNA and thereby makes a bridge to additional phosphate groups. Since there is a structural rearrangement of activated DtxR upon binding to the *tox* operator, Thr7 in helix A also interacts with a backbone phosphate group, and Ser37 and Pro39 contact methyl groups on thymine bases in the *tox* operator through van der Waals interactions (47).

While saturation and equilibrium dialysis experiments suggested that DtxR contained a single metal ion-

binding site with an apparent dissociation constant of 2×10^{-6} to 9×10^{-7} M (46), X-ray crystallographic analysis of transition metal ion-DtxR complexes clearly revealed two metal ions bound to each monomer (41, 43). Using site-directed mutagenesis, Ding et al. (48) demonstrated that the primary metal ion-binding site of DtxR is composed of Met10, Cys102, Glu105, His106, and a water molecule form an octahedral coordination center. The second metal ion-binding site, or ancillary site, is composed of five residues: His79, His98, Glu83, Glu170, and Gln173 (41, 43, 49). The role played by the ancillary metal ion-binding site was elucidated through the analysis of DtxR(E175K), a hyperactive mutant that remained active *in vivo* even in the presence of the chelator 2,2'-dipyridyl (50). *In vitro* studies with purified apo-DtxR(E175K) demonstrated that this mutant required very low levels of metal ion to transit from an inactive apo- to the active form of the repressor. Love et al. (51) demonstrated that the hyperactive phenotype of DtxR(E175K) was dependent on an intact ancillary metal ion-binding site and that this site facilitated the conversion of the inactive apo-repressor to its active *toxO* binding conformation.

Nuclear magnetic resonance solution structures combined with other biophysical studies have suggested that apo-DtxR exists in a partially unstructured molten globule, which upon coordination with divalent transition metal ions undergoes a structural conversion to a discrete ordered tertiary structure that both dimerizes and is able to bind to the *tox* operator (52). As shown schematically in Fig. 5, in its inactive molten globule apo-form, the SH3-like domain forms an intramolecular complex with a proline-rich peptide segment of the repressor and, in doing so, destabilizes the ancillary metal ion-binding site of the repressor, and this leads to the complete inactivation of the repressor. Upon metal ion-binding to the primary site, the SH3-like domain dissociates from this proline-rich region, thereby allowing a structural conversion and stabilization of the ancillary metal ion-binding site. The resulting conformational changes then allow the binding of the second metal ion to the ancillary site and subsequent dimerization of DtxR and the formation of an active repressor (53).

FIGURE 4 X-ray crystal structure of Ni²⁺-activated DtxR (C102D) bound to the *tox* operator. Due to high thermal values, the C-terminal SH3-like domain is not shown in this Ni²⁺ activated structure. (Modified from reference 44 [Protein Data Bank ID 1DDN].)



STRUCTURE-FUNCTION RELATIONSHIPS OF DIPHTHERIA TOXIN AND THE MOLECULAR MECHANISM OF ITS ACTION

Diphtheria toxin is expressed and secreted into the culture medium as a single polypeptide chain of 62 kDa by *C. diphtheriae*. The toxin is readily purified from the

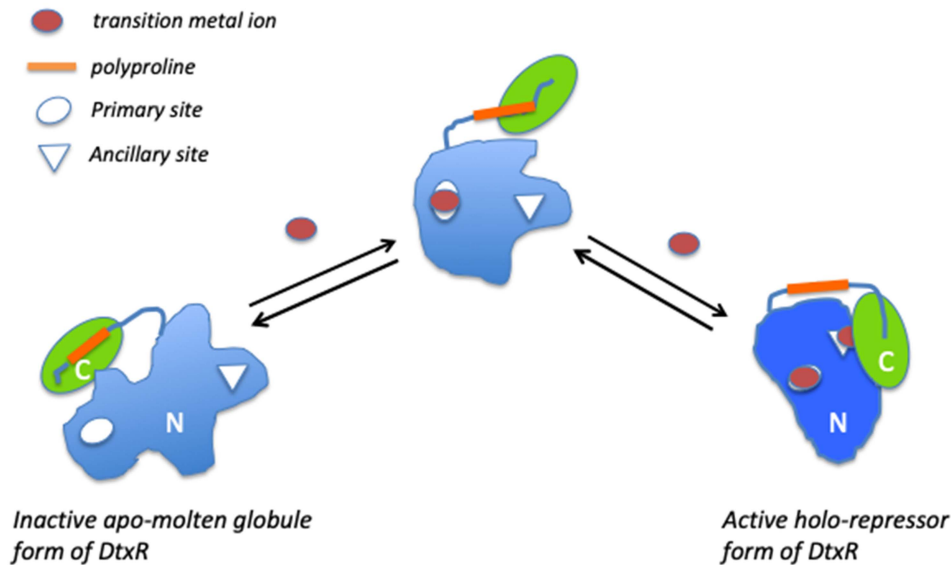
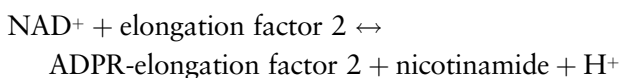


FIGURE 5 DtxR is a two-domain protein that is composed of the N-terminal [blue] and C-terminal SH3-like [green] regions and functionally distinct transition metal ion-binding sites (primary and ancillary). An activating transition metal ion first binds to the primary site. This binding event orients the DNA-binding helices and begins to induce the folding of the disordered N-terminal domain. Subsequent binding of a second metal ion to the ancillary site disassociates and reorients the SH3-like domain away from the poly-proline region of the repressor and completes the folding of the N-terminal domain, resulting in the formation of the dimer interface of the active holo-repressor. (Modified from reference 52.)

spent culture supernatant by ammonium sulfate precipitation followed by ion exchange chromatography on a diethylaminoethyl matrix. While the intact toxin is enzymatically inactive, exposure of purified toxin to trypsin or other serine proteases results in “nicking” of the protein and the subsequent activation of adenosine diphosphate ribosyl (ADPR) transferase. Under reducing and denaturing conditions, nicked diphtheria toxin may be separated into an enzymatically active N-terminal 24-kDa fragment A and its 38-kDa C-terminal fragment B (54, 55). Honjo et al. (56) and Gill et al. (57) demonstrated that nicked diphtheria toxin and fragment A were enzymatically active in catalyzing the following reaction:



Yamaizumi et al. (58), in a series of elegant experiments, loaded erythrocyte ghosts with various concentrations of purified fragment A and a known concentration of fluorescein isothiocyanate-labeled bovine serum albumin. These preloaded red cell ghosts were then fused to diphtheria toxin-resistant mouse L-cells by Sendai virus. Using a fluorescence-activated cell sorter, L-cells that fused with

a single red cell ghost were then isolated and grown for 7 days. Careful analysis of the colony-forming ability of the recipient cells compared to the control cells clearly demonstrated that the delivery of a single molecule of fragment A to the cytosol was sufficient to kill that cell.

Comparison of the nontoxic mutants CRM197 and CRM45 revealed that the C-terminal 38 kDa fragment B of diphtheria toxin carried two functional domains: a hydrophobic domain (59), which under denaturing conditions behaved like an integral membrane protein, and the native receptor-binding domain. As such, it was realized through biochemical and genetic analyses that native diphtheria toxin was a protein with at least three structural-functional domains: (i) catalytic, (ii) trans-membrane or translocation, and (iii) receptor-binding domains. As shown in Fig. 2, this prediction proved to be correct with the determination of the crystal structure of diphtheria toxin (60, 61). While DNA sequence analysis of the *tox* structural gene revealed the presence of a 21-amino acid signal sequence (18–20), the mature form of diphtheria toxin is a 535-amino acid polypeptide. The N-terminal catalytic domain consists of amino acids Gly1 to Cys186 and is composed of eight β -strands that form two β -sheets forming a core structure that is

surrounded by seven short α -helices. The β -sheets that form the central core also form the active site cleft. The catalytic domain is connected to the centrally positioned translocation domain by a 14-amino acid loop that is subtended by a disulfide bond between Cys186 and Cys201. This exposed loop carries an ArgArgValArg protease recognition site for either furin or other trypsin-like proteases. The proteolytic cleavage of this site is essential for the release of the catalytic domain into the eukaryotic cell cytosol (62). The translocation domain encompasses amino acids Cys186 to K385 and is composed of nine α -helices and their connecting loops. The translocation domain plays an essential role in the intoxication process by forming an 18- to 22-Å channel, or pore, through which the catalytic domain is threaded from the lumen of acidified endocytic vesicles into the cytosol. This channel is formed by translocation helices 5 to 7 and 8 to 9, which are highly hydrophobic and form two membrane-soluble “daggers” (61). The loops connecting translocation helices 8 to 9 and 5 to 6 are highly acidic and carry a total of six Asp and Glu residues. Upon internalization of the toxin into an endosomal compartment and the acidification of the vesicle lumen by the vacuolar ATPase, the carboxylic acid groups on these Asp and Glu residues become protonated and lose their respective charges, allowing for membrane insertion and channel formation.

Ratts et al. (3) developed an *in vitro* assay system composed of partially purified endosomes that were preloaded with either diphtheria toxin or the fusion protein toxin DAB389IL-2 in the presence of the vacuolar ATPase inhibitor bafilomycin A1 to probe the requirements necessary for catalytic domain translocation from the endosomal lumen to the external medium. Upon removal of bafilomycin A1 and addition of ATP, the endosomal lumen becomes rapidly acidified; however, catalytic domain translocation to the external medium was shown to require the addition of eukaryotic cytosolic fractions to the assay mixture. Using this assay system, Ratts et al. (3) demonstrated that both coatamer complex 1 (COPI) and thioredoxin reductase were essential for *in vitro* translocation of the catalytic domain. Ratts et al. (63) also described a highly conserved motif, T1, in translocation helix 1 of diphtheria toxin that played an essential role in the delivery of the catalytic domain to the cytosol. In 2010, Trujillo et al. (64) used both site-directed mutagenesis and a COPI complex precipitation assay to demonstrate that the interaction(s) between at least three lysine residues in the T1 motif are essential for both COPI binding and delivery of the catalytic domain to the eukaryotic cell cytosol. Further,

Trujillo et al. (64) demonstrated that substitution of the lysine-rich region in translocation helix 1 with the COPI binding portion of the p23 adaptor cytoplasmic tail results in a mutant form of toxin that displays full wild-type activity against sensitive eukaryotic cells.

The above-described studies demonstrate that diphtheria toxin has evolved to become an extremely efficient nano-machine in which all of its structural domains play an essential role in the intoxication of sensitive eukaryotic cells. The molecular mechanisms leading toward intoxication require at least the following steps: (i) binding of the toxin to its cell surface receptor, the heparin-binding epidermal growth factor precursor, (ii) furin-mediated nicking of the protease-sensitive RVRR site in the 14-amino acid exposed loop between the catalytic and translocation domains, (iii) internalization of toxin into an endosomal compartment that becomes acidified through the action of its vacuolar ATPase, (iv) insertion of translocation helical loops formed by TH5-7 and TH8-9 into a vesicle membrane forming a channel or pore, (v) the insertion of the disulfide-linked C-terminal end of the catalytic domain and N-terminal end of the translocation domain into the channel, (vi) protein::protein interactions between the dibasic KKKXX signatures in translocation helix 1 with COPI, which facilitates the threading and translocation of the catalytic domain through the channel, (vii) reduction of the disulfide bond between the catalytic and translocation domains by thioredoxin reductase, (viii) the release and refolding of the catalytic domain into an enzymatically active ADP-ribosyltransferase, and (ix) the NAD⁺-dependent ADP-ribosylation of elongation factor 2, which results in the cessation of protein synthesis and death of the cell (65).

MOLECULAR EPIDEMIOLOGY OF DIPHThERIA AND DtxR

Outbreaks of clinical diphtheria almost always occur in individuals who have not been immunized with either the DPT or diphtheria-tetanus-acellular pertussis vaccine and who have been exposed to a “carrier” (a person who carries a toxigenic strain of *C. diphtheriae* as part of their normal flora) who has recently traveled to a country where mass immunization against diphtheria is not performed and disease is still prevalent. *C. diphtheriae* is usually transmitted via the aerosol route; close contact with an individual presenting with cutaneous diphtheria may also result in transmission. While colonization of a susceptible individual with a toxigenic strain certainly plays a role in pathogenesis, Pappenheimer and Murphy

(66) demonstrated that transmission of toxigenicity may also occur by *in situ* lysogenic conversion of an autochthonous nontoxigenic strain of *C. diphtheriae* to toxigenicity. It is remarkable that immunization with diphtheria toxoid results in the production of neutralizing antibodies that not only block diphtheria toxin from binding to its cell surface receptor, but also protects against clinical disease (67, 68). It is well known that mass immunization of populations with diphtheria toxoid has led to a dramatic decrease in the incidence of clinical disease (69). For example, in 1958, before mass immunization with diphtheria toxoid in Romania, only 60% of the population was immune to diphtheria toxin, whereas by 1979, the percentage dramatically increased to 97%. At the same time, diphtheria morbidity dropped from ca. 600 per 100,000 in 1958 to only 1 per 100,000 in 1972 (67, 70).

The consequence of either stopping or dramatically reducing a mass immunization program against diphtheria is also known. During the 1990s, the Newly Independent States (NIS) of the former Soviet Union experienced a sweeping epidemic of clinical diphtheria. With the breakup of the Soviet Union, public health immunization programs were dramatically affected and by 1990 only 68% of Russian children had received the appropriate vaccination regimen (71). As a result, by 1995 the incidence of clinical diphtheria in the NIS peaked at over 50,000 cases. From the onset of the diphtheria outbreak in 1990 through 1999, more than 157,000 cases and 5,000 deaths were reported.

Given the increased incidence of clinical diphtheria in the NIS, Kolodkina et al. (72) characterized 129-bp single-strand conformational polymorphism DNA fragments that contained the *toxPO* region from 81 strains of *C. diphtheriae*. From this group, only two strains had mutations. Nucleotide sequence analysis revealed T to C mutations in positions -54 and -184 of the *tox* operon. The -54 mutation was found in the 9-bp interrupted palindromic sequence of *toxPO*, and these two strains had the highest level of toxin production out of the 81 strains characterized. In addition, these investigators also analyzed the nucleotide sequence of the *dtxR* gene. Fifteen strains in this collection were found to carry two missense mutations in DtxR, A147V and L214I in the C-terminal region of the repressor. Interestingly, the *dtxR* alleles in the two strains that expressed the highest levels of diphtheria toxin were not found to carry a nucleotide substitution. This initial study was extended by Kombarova et al. (73), who found seven point mutations in the structural gene for diphtheria toxin. While most of these mutations did not result in an amino

acid substitution, two strains from the Otchakov ribotype carried a G393R mutation in the native receptor-binding domain of the toxin. In contrast, 16 point mutations in *dtxR* were found. Of these, the A147V mutation is characteristic of the epidemic clonal group Sankt-Peterburg/Rossija, which was the dominant strain isolated in the Russian diphtheria epidemic in the 1990s.

It is remarkable that the amino acid sequence of diphtheria toxin encoded by the omega-phage in the Park-Williams strain of *C. diphtheriae*, PW8, which was isolated in 1896 (74), is identical to the amino acid sequence of the toxin produced by all but two of the clinical isolates carrying the G393R mutation that have been characterized from the NIS diphtheria epidemic. While several missense mutations within the *tox* gene have been described, they encode the same amino acid as found in the PW8 allele. It is interesting to note that in the single exception, the G393R substitution falls early on in the toxin's receptor-binding domain and is far removed from the region of that domain which interacts with the toxin's eukaryotic cell surface receptor-binding region. This extraordinary conservation of amino acid sequence strongly suggests the highly evolved nature of the toxin's structure-function relationships. This conservation of amino acid sequence for at least 100 years suggests that the evolution of new *tox* alleles that might encode a toxin sufficiently different to avoid neutralization by antitoxoid antibodies is highly unlikely.

In contrast, this high degree of conservation does not hold for DtxR. In the case of this metal ion-activated repressor, the N-terminal region of *dtxR* was found to carry several missense mutations that did not give rise to an amino acid substitution. In contrast, missense mutations in the C-terminal region of the repressor were often found to encode amino acid substitutions that appeared to subtly affect DtxR activity (51, 75-77).

TURNING THE SWORD OF DIPHTHERIA INTO THE PLOWSHARE OF TARGETED THERAPEUTICS

Shortly after Köhler and Milstein (78) described the methodology to produce monoclonal antibodies, many in the bacterial protein toxin field seized on this technology in an attempt to fulfill Paul Ehrlich's idea of developing "magic bullets," therapeutic compounds that were selectively targeted toward a disease-causing organism or cell (79). In an early series of experiments, Gilliland et al. (50) independently coupled ricin A chain and diphtheria toxin fragment A to monoclonal antibodies directed against a cell surface antigen on colo-

rectal carcinoma cells. While the ricin A chain-coupled immunotoxin was highly potent (i.e., the half maximal inhibitory concentration [IC₅₀] < 10⁻¹² M), the diphtheria toxin fragment A immunotoxin, surprisingly, was found to be at least 1,000-fold less potent (i.e., IC₅₀ ~ 5 × 10⁻⁹ M). These results prompted Bacha et al. (80) to ask whether fragment A alone contained sufficient structural information to deliver the catalytic domain across the endocytic vesicle membrane. Since Boquet et al. (59) had previously demonstrated that under denaturing conditions, the hydrophobic domain in the B fragment of CRM45 displayed properties of an integral membrane protein, Bacha et al. (80) independently coupled fragment A (CRM26) and CRM45 to thyrotropin-releasing hormone (TRH) and assessed their relative cytotoxic potency against GH3 rat pituitary cells. While the fragment A-TRH construct was non-toxic even at concentrations of 10⁻⁷ M, the CRM45-TRH construct was highly active (IC₅₀ ~ 3 × 10⁻⁹ M). These results clearly demonstrated that fragment B translocation domain sequences were required to facilitate the delivery of fragment A, the catalytic domain, into the eukaryotic cell cytosol.

Since additional preparations of CRM45-TRH varied by greater than 10-fold in cytotoxic potency, Murphy et al. (81) turned to recombinant DNA and protein engineering methods to redesign native diphtheria toxin by receptor-binding domain substitution. The hypothesis was that by choosing a surrogate receptor-binding domain that bound to a cell surface receptor that was both limited in distribution and was internalized into an endosomal compartment that became acidified, it might be possible to develop a family of fusion protein toxins that could potentially serve as a new class of targeted toxins for the treatment of human diseases. Moreover, we reasoned that, if successful, these genetically engineered proteins would constitute a unique family of reagents to deplete discrete subsets of cells and to study the molecular process by which the diphtheria toxin catalytic domain was delivered to the cytosol of targeted eukaryotic cells.

To begin to test this hypothesis, Leong et al. (82, 83) demonstrated that cloned fragment A of diphtheria toxin was expressed and secreted by the SecA apparatus in recombinant *E. coli*. Despite the controversial nature of deliberately designing and constructing a recombinant gene that encoded a toxic protein that the world had not seen previously, in 1985, we were finally granted approval to genetically construct, express, and evaluate the biosafety of the first fusion protein toxin under biosafety level 4 (BSL-4) containment. Murphy et al.

(81) described the assembly of a fusion gene that was composed of a portion of diphtheria toxin that encoded first 486 amino acids of diphtheria toxin and a synthetic gene encoding the 13-amino acid polypeptide α -melanocyte-stimulating hormone (α -MSH). While this fusion protein was expressed and secreted into the periplasmic space of recombinant *E. coli*, the yield of full-length fusion protein toxin was extremely low due to proteolytic degradation. Since this degradation appeared to have occurred close to the fusion junction between diphtheria and α -MSH sequences, we reasoned that increasing the molecular size of the substitute receptor-binding domain with a larger polypeptide might at least partially solve the degradation problem by steric hindrance. In 1987, Williams et al. (84) described the genetic construction and properties of a fusion protein toxin in which the 133-amino acid sequence of human interleukin-2 (IL-2) was genetically fused to amino acid 486 of diphtheria toxin. Surprisingly, DAB486IL-2 was expressed, and full-length fusion protein toxin was secreted into the periplasmic space in good yield.

Since the DAB486IL-2 construct used a naturally occurring *SphI* restriction endonuclease site in the *tox* gene as the fusion junction between diphtheria toxin and IL-2 sequences, we then asked how much fragment B sequence was required to deliver the catalytic domain of the fusion protein into the target cell cytosol. Williams et al. (85) constructed a series of in-frame deletion mutants and found that the removal of 97 amino acids from Thr387 to His485 increased both the cytotoxic potency and the apparent dissociation constant of the resulting fusion toxin, DAB389IL-2, for cells that expressed the high-affinity IL-2 receptors (IL-2R). Upon the subsequent solution of the diphtheria toxin X-ray structure (Fig. 6), we realized that quite by chance our internal in-frame deletion of 97 amino acids resulted in an almost perfect receptor-binding domain substitution (Fig. 7). Since that time, more than a dozen diphtheria-based fusion protein toxins have been constructed and characterized using amino acid 388 or 389 as the junction between diphtheria toxin and surrogate receptor-binding domain polypeptides.

In 1999, the U.S. Food and Drug Administration (FDA) approved denileukin diftitox (Ontak; DAB389IL-2) for the treatment of refractory cutaneous T cell lymphoma (CTCL), making it the first-in-class recombinant targeted biologic to achieve approval. Phase III trials conducted with CTCL patients who had failed other therapeutic interventions demonstrated that 30% of the total number of patients had a 50% or greater reduction in their tumor burden for at least 6 weeks following

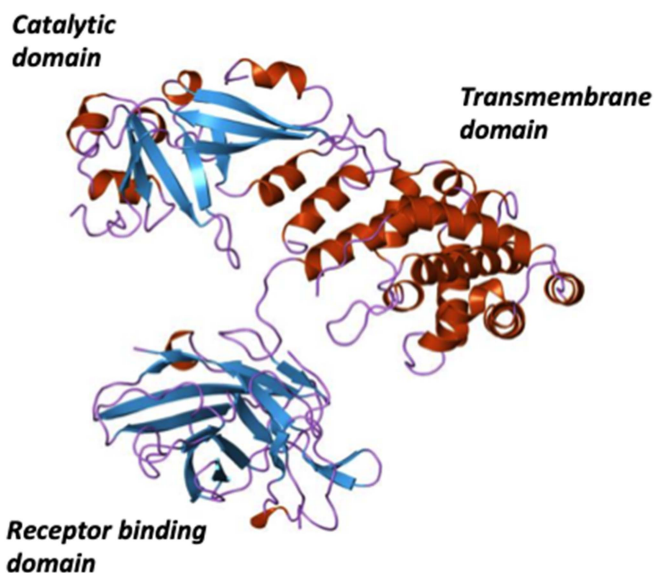


FIGURE 6 X-ray structure of native diphtheria toxin showing its catalytic, translocation, and receptor-binding domains.

treatment (86, 87). In the case of peripheral T cell lymphomas, an aggressive form of non-Hodgkin's lymphoma with a median overall survival of 5.5 months, Ontak has been used both as a monotherapy and in combination with traditional chemotherapy (88). Both Fuentes et al. (89) and Wong et al. (90) reported individual cases where Ontak therapy, when used as a long-term maintenance therapy over a span of 1 to 2 years, resulted in sustained remissions of 9 and 4 years that are ongoing. In addition to its effective use in T cell malignancies, Ontak has also been successfully used in the treatment of steroid-resistant graft-versus-host disease. Ho et al. (91) conducted a phase I trial of Ontak in 30 patients presenting with steroid-resistant graft-versus-host disease and reported that 50% of the patients responded with complete resolution, and an additional 21% of the total number of patients responded with partial resolution of their disease.

While Ontak selectively targets the high and intermediate IL-2R on malignant T cells in both CTCL and peripheral T cell lymphomas, as well as on activated T effector cells in graft-versus-host disease, in recent years it has also shown promise as an immunotherapeutic in the transient depletion of T regulatory cells in solid tumors. Both phase I (92) and phase II (93) studies have shown that Ontak is also effective as an immunotherapeutic as a monotherapy in patients presenting with unresectable stage IV malignant melanoma. In these instances, Ontak was shown to transiently deplete T regulatory cells and, as such, apparently break tolerance

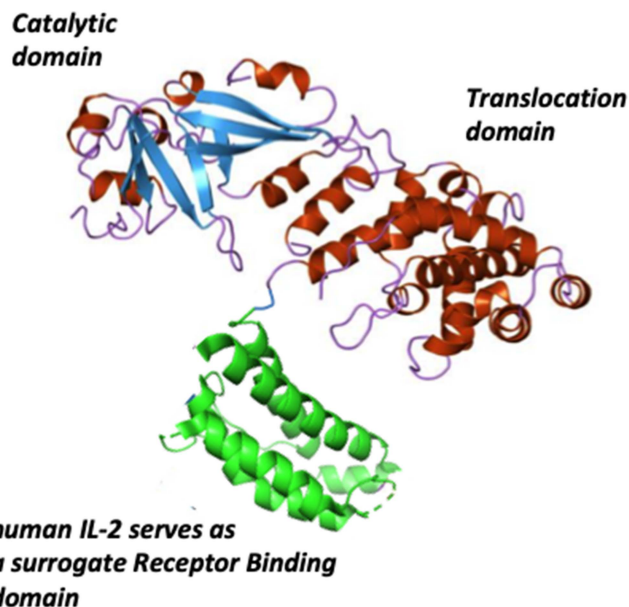


FIGURE 7 Molecular model of denileukin difitox (Ontak, DAB389IL-2). The catalytic and translocation domains are composed of amino acids Gly1 through Thr387 of native diphtheria toxin, to which the 133-amino acid polypeptide of human IL-2 is genetically fused. The additional two amino acids in the construct are the result of the introduction of a unique *SphI* restriction endonuclease site at the fusion junction between diphtheria toxin and human (h)IL-2 sequences.

and allow for a more robust host-mounted T-effector cell antitumor response.

Despite its clinical effectiveness, Ontak was placed on clinical hold in 2011 because of the presence of drug aggregates, contaminating DNA, varying concentrations of Tween20, and batch-to-batch variations in its final formulation. Since Ontak was expressed as inclusion bodies in recombinant *E. coli*, the agent had to be completely denatured and refolded into a biologically active conformation. During the refolding process, it was necessary to add Tween20 to partially prevent the formation of insoluble drug aggregates and allow for refolding of the drug into a biologically active conformation.

We (94) recently solved the production issues associated with refolding of the drug by recloning the structural gene for Ontak, DAB389IL-2, in an *E. coli*-*C. diphtheriae* shuttle vector. Furthermore, we modified the gene by adding back the native *tox* signal sequence so that the fusion protein toxin would be secreted into the culture medium. Finally, we introduced mutations into the downstream half of the palindromic *tox* operator so that expression was constitutive even in medium with high iron concentrations. Thus, this second-generation Ontak, s-DAB389IL-2, is expressed and secreted from

recombinant *C. diphtheriae* in good yield as a fully folded monomeric protein that may be readily isolated and purified from the culture medium. Cheung et al. (94) also demonstrated that s-DAB389IL2 has great potential when used in a sequential combination regimen with anti-PD-1 in the treatment of B16F10 melanoma in the mouse. In these studies, either s-DAB389IL-2 or s-DAB389IL-2(V6A), a vascular leak motif modified mutant, was first used to transiently deplete T regulatory cells, thereby removing one of the immune suppression signals that dampen a T effector cell antitumor response. Anti-PD-1 was then administered to block the PDL-1–PD-1 interaction between the tumor and the immune system, thereby blocking a second suppressive signal to the T effector arm of the immune system. The combined effect of this sequential therapeutic regimen was found to be at least additive and perhaps synergistic in the treatment of this aggressive murine melanoma. These results are encouraging and prestage the use of s-DAB389IL2 (V6A) in clinical medicine in a sequential checkpoint inhibitor blockade for the treatment of solid tumors.

Ontak (denileukin diftitox, DAB389IL-2) and the second-generation form of the biologic, s-DAB389IL-2 (V6A), while not magic and certainly not bullets, have shown remarkable efficacy in the treatment of human malignancies and in murine models of melanoma and colon and renal cell carcinomas, both as a targeted monotherapy against the tumor itself and as an immunotherapeutic to reset immunologic balance by transient depletion of T regulatory cells. In the latter instance, the transient depletion of T regulatory cells appears to break tolerance and thereby allows a robust patient-mounted antitumor response. Thus, through the biochemical and biochemical genetic study of diphtheria toxin, the diphtheria *tox* operon, and the iron-activated repressor DtxR, which regulates expression, and through modern molecular biologic methods, one of nature's most potent bacterial protein toxins has been tamed. Diphtheria toxin, once the primary virulence determinant of a dreaded infectious disease of children, has now been retargeted to selectively eliminate specific disease-causing cells as a protein therapeutic, thereby at least partially fulfilling Paul Erlich's magic bullet concept of a "chemical that binds to and specifically kills microbes or tumor cells" (79).

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