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Crystallographic evidence for doxorubicin binding to the receptor-binding site in *Clostridium botulinum* neurotoxin B

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The neurotoxins of *Clostridium botulinum* and tetanus bind to gangliosides as a first step of their toxin activity. Identifying suitable receptors that compete with gangliosides could prevent toxin binding to the neuronal cells. A possible ganglioside-binding site of the botulinum neurotoxin B (BoNT/B) has already been proposed and evidence is now presented for a drug binding to botulinum neurotoxin B from structural studies. Doxorubicin, a well known DNA intercalator, binds to the neurotoxin at the receptor-binding site proposed earlier. The structure of the BoNT/B–doxorubicin complex reveals that doxorubicin has interactions with the neurotoxin similar to those of sialyllactose. The aglycone moiety of the doxorubicin stacks with tryptophan 1261 and interacts with histidine 1240 of the binding domain. Here, the possibility is presented of designing a potential antagonist for these neurotoxins from crystallographic analysis of the neurotoxin–doxorubicin complex, which will be an excellent lead compound.

1. Introduction

C. botulinum and tetanus neurotoxins belong to the same class of neurotoxins and share significant sequence homology. They possess similar structural and functional domains and have a similar mechanism of toxicity (Simpson, 1986). However, botulinum neurotoxins act at the neuromuscular junction (NMJ) causing flaccid paralysis, while tetanus toxin acts at the central nervous system (CNS) causing spastic paralysis (Schiavo *et al.*, 2000). These neurotoxins are potential biowarfare agents and are also a significant public health problem. There are no known antidotes available either for tetanus or for botulinum neurotoxins.

C. botulinum toxins follow a four-step mechanism (Montecucco *et al.*, 1994); they bind to the neuronal cells, are internalized into the vesicles and translocated into the cytosol, where they attack specific components of SNARE proteins to cleave them at specific peptide bonds causing inhibition of formation of SNARE complex and thereby blocking neurotransmitter release (Söllner *et al.*, 1993). Clostridium neurotoxins comprise two chains, an N-terminal light chain of 50 kDa (LC) and a C-terminal heavy chain of 100 kDa (HC), held together by a single disulfide bond. The heavy chain is responsible for binding, internalization and translocation, while the light chain is responsible for catalytic activity inside the cytosol. Neurotoxins bind to the neuronal cells *via* gangliosides and negatively charged lipids on the surface of the cell (Menestrina *et al.*, 1994). A double-receptor model, with low-affinity binding to gangliosides and high-affinity binding to a protein receptor, has been proposed and a protein receptor has been identified (Montecucco, 1986). The catalytic domain is a zinc endopeptidase containing an HE*xx*H zinc-binding motif (Schiavo *et al.*, 1994).

Antagonists for these neurotoxins could act in three ways (Adler *et al.*, 1998). They could either be molecules which attach to the binding site, thereby inhibiting binding of neurotoxins to gangliosides, or they may act before internalization to prevent internalization or they could be inhibitors which would stop the catalytic action by blocking the active site or by chelating the active-site zinc.

In an attempt to identify suitable small-molecule ligands that bind to the C-fragment of clostridium tetanus neurotoxin (TeNT), several small molecules were screened by computational chemistry and then tested with modeling, docking and electrospray ionization mass spectroscopy (ESI-MS) (Lightstone *et al.*, 2000). Of the many compounds tested, doxorubicin, a DNA-intercalator molecule, was identified to bind with a binding constant of 9.4 µ*M*. Also, from the declustering potential used in ESI-MS, it was concluded that it binds in a hydrophobic pocket. It has been shown that it competes with gangliosides for binding. Here, we present crystallographic evidence for doxorubicin binding to clostridium neurotoxins. We used BoNT/B in our studies since (i) a high-resolution structure was available, (ii)

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Table 1
Data and refinement statistics.

Unit-cell parameters (Å,°)	a = 76.27, b = 122.93, c = 95.42, β = 112.95
Space group	$P2_1$
Resolution range (Å)	50.0–2.5
Total No. of reflections	197506
No. of unique reflections	52722
R_{sym}	6.7 (31.9)
Completeness	99.1 (95.2)
Average $I/\sigma(I)$	14.3
R factor	0.217
R_{free}	0.276
No. of protein atoms	10617
No. of water molecules	354
No. of heterogen atoms	45
Average B factor (Å^2)	
Protein	29.8
Water molecules	27.5
Heterogens	37.8
RMSD	
Bond lengths (Å)	0.007
Bond angles (°)	1.23

all clostridium neurotoxins share significant sequence homology at the C-terminus and (iii) they all possess similar structure (Lacy *et al.*, 1998; Swaminathan & Eswaramoorthy, 2000*b*; Umland *et al.*, 1997).

2. Materials and methods

BoNT/B crystals were obtained as described previously (Swaminathan & Eswaramoorthy, 2000*a*). PEG 4000 was used as precipitant in MES buffer pH 6.0 to grow the crystals. The protein–doxorubicin complex crystals were prepared by soaking BoNT/B crystals in mother liquor containing doxorubicin. The best soaking condition was obtained when crystals were soaked for 36 h in mother liquor containing 50 m*M* doxorubicin.

Data were collected from a crystal at beamline X12C of National Synchrotron Light Source, Brookhaven National Laboratory with the use of a CCD-based detector (Brandeis B1.2). The data collection and processing were performed using *MARMAD* (Skinner & Sweet, 1998) and *HKL*/*DENZO* (Otwinowski & Minor, 1997). The data-collection statistics are given in Table 1. Crystals of BoNT/B are prone to non-isomorphism even among crystals from the same crystallization well. As the comparison of structure factors of crystals soaked in doxorubicin with those of the native crystal gave an R_{merge} of 0.49, the structure of the complex was determined by the molecular-replacement method using the native structure as a model with *AMoRe* (Navaza & Saludjian, 1997). The model was refined with *CNS* (Brunger *et al.*, 1998) until convergence. The σ-weighted difference Fourier density map was calculated and checked for the possible bound drug. With the available information about the sialyllactose-binding site and the presence of a continuous residual density in the difference density map, the binding site of doxorubicin was identified and the ligand was modeled with the program *O* (Jones *et al.*, 1991). The model was refined after including doxorubicin and 354 water molecules. The final R and R_{free} are 0.22 and 0.28, respectively. The refinement parameters are included in Table 1. The structure was examined with *PROCHECK* (Laskowski *et al.*, 1993) and the coordinates have been submitted to the PDB (PDB code 1i1e). The $2F_o - F_c$ map for doxorubicin is shown in Fig. 1. However, the density is very weak for two terminal atoms which is not unusual as the drug may be disordered at the tail part of the binding.

3. Results and discussion

The botulinum neurotoxin B molecule comprises three structural domains arranged almost linearly, with the translocation domain (HC_N) in the middle flanked by the binding (HC_C) and the catalytic (LC) domains. The catalytic domain and the N-terminal domain of the heavy chain HC_N are held together by a loop (also called the belt region) which is a part of the HC_N domain. The HC_C has minimal interaction with the HC_N domain and is tilted away from the translocation domain. The region between the binding and translocation domains is filled with water molecules, which seems to be a possible interaction site for receptors (Swaminathan & Eswaramoorthy, 2000*b*). The HC_C domain consists of two structural subdomains, a β-sheet/jelly-roll domain and a trefoil domain. The C-terminal half of the HC_C domain contains the binding site for gangliosides.

The ganglioside recognition in TeNT was identified as the carboxy-terminal 34 residues of the C fragment (residues 1282–1315), and the sequence homology among clostridium neurotoxins is very high near the C-terminal region (Shapiro *et al.*, 1997). The photoaffinity labeling occurred predominantly at His1292 of TeNT, which corresponds to Glu1265 of BoNT/B. The gangliosides, especially the 1b series (*e.g.* GT1b or GD1b) showed good affinity for binding to the C-fragment of TeNT. The structures of the binding domains are also very similar in the crystal structures determined so far (Lacy *et al.*, 1998; Swaminathan & Eswaramoorthy, 2000*b*; Umland *et al.*, 1997). In BoNT/A, tryptophan fluorescent quenching is accompanied by ganglioside binding, suggesting that a solvent-exposed tryptophan may be present near the binding site (Kamata *et al.*, 1997). Trp1288 of TeNT or Trp1261 of BoNT/B is present in this C-terminal region and is exposed to the solvent. Crystallographic evidence for a ganglioside-binding site has been shown in the crystal structure of the BoNT/B–sialyllactose complex (Swaminathan & Eswaramoorthy, 2000*b*). The sialic acid of sialyllactose binds in the above-mentioned region and stacks between Trp1261 and His1240 in BoNT/B. However, sialic acid does not make any contact with Glu1265 or residues near Glu1265 in the primary sequence as suggested by biochemical

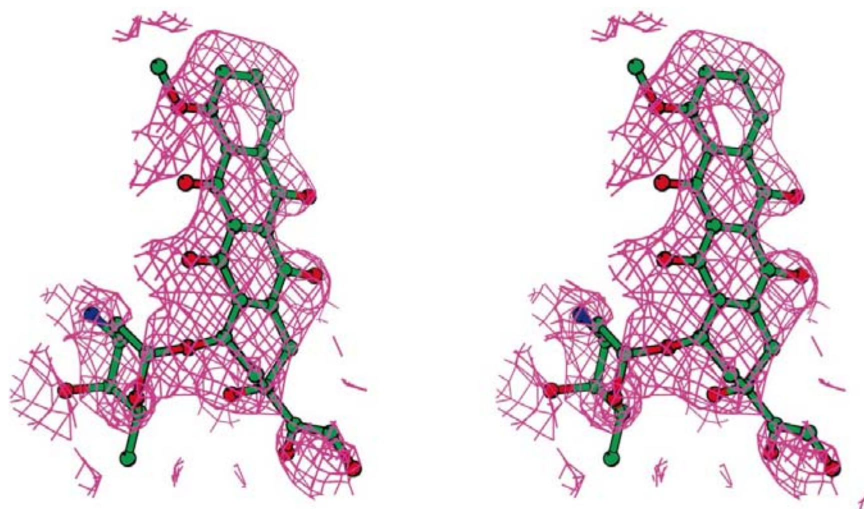


Figure 1
Stereographic view of $2F_o - F_c$ map for doxorubicin. The map is contoured at 1σ. This figure was created with *BOBSCRIPT* and *MOLSCRIPT* (Kraulis, 1991).

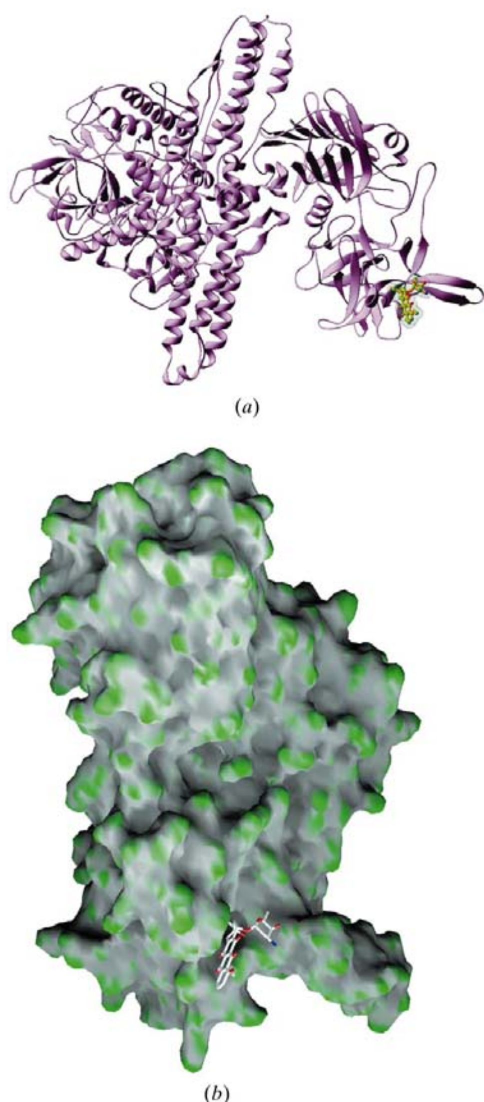


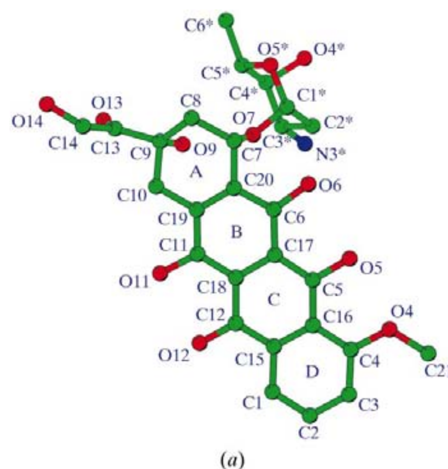
Figure 2
(*a*) *RIBBONS* (Carson, 1991) representation of the intact BoNT/B molecule. The bound doxorubicin molecule is shown as a ball-and-stick model, with the van der Waals surface as a dotted surface. (*b*) *GRASP* (Nicholls *et al.*, 1991) representation of the surface curvature of the C-fragment of BoNT/B with doxorubicin shown as a stick model. The orientation of the molecule is similar to that of (*a*).

studies (Shapiro *et al.*, 1997). A pocket is formed between His1240 and Trp1261 in BoNT/B and this pocket is present in all clostridium neurotoxins for which structures are known. In view of these facts, it may be concluded that this binding site is common to all clostridium neurotoxins.

Doxorubicin (Dox) binds in a cavity formed by residues Glu1188, Glu1189, His1240, Tyr1260 and Trp1261 (Fig. 2). This binding site is the same as that for sialyllactose for BoNT/B and possibly for gangliosides for all clostridium neurotoxins. Doxorubicin interacts with the protein through O13 and O14 of its hydroxy acetyl group, which is buried in the cavity. The numbering scheme of Dox is given in Fig. 3(*a*). His1240 makes a hydrogen bond with Dox and Trp1261 is stacked with the planar aglycone moiety of the Dox, with the D ring facing the solvent region. Most of the O atoms and the N atom in Dox are hydrogen bonded to the protein (Fig. 3*b*). O14 hydrogen bonds with Gly1238 N and Cys1257 O, O13 hydrogen bonds to His1240 N, and O9 and O11 are bonded to His1240 ND1 and Ser1259 OG, respectively. Glu 1188O forms bifurcated hydrogen bonds with O11 and O12 of the doxorubicin. The pyranose ring interacts with a symmetry-related molecule of the protein (Fig. 3*b*). N3*, O4* and O5* are hydrogen bonded with Glu331 OE1, Asp332 O and Ser333 N, respectively. Also, O6 interacts with Glu331 O of the symmetry-related molecule. Table 2 lists the interactions between the neurotoxin and doxorubicin.

As there is no antidote available for botulism at present and the preventive measures are also experimental, toxin–potential drug interaction studies are important. The crystal structure of the BoNT/B–sialyllactose complex identified the potential receptor-binding site as the cleft between Trp1261 and His1240 (Swaminathan & Eswaramoorthy, 2000*b*). This binding pocket with a tryptophan exposed to solvent is commonly found in all the BoNTs and TeNT. The present study showed that Dox binds to the same site of sialic acid binding. The hydroxyl O atoms O14 and O13 interact with the protein at the same site as sialyllactose. His1240 and Trp1261 are on either side of Dox as with the sialyllactose. Even though the binding site was predicted by a previous study (Lightstone *et al.*, 2000), the orientation of the molecule seems to be different from that proposed. In particular, the direction in which the amino group points is different; while it is pointing toward the interface of the two subdomains of the C-fragment in the model proposed, it is pointing away from the interface in the crystal structure. This may provide an additional clue in designing molecules for inhibiting neurotoxin binding to the membranes.

The widely used anticancer drugs anthracycline antibiotics, daunomycin and doxorubicin are known to interact with DNA with the aglycone ring intercalating between the base pairs of DNA (Cirilli *et al.*, 1992). In the case of the BoNT/B–doxorubicin complex, the aglycone moiety is stacked with Trp1261. As the activity of the anthracycline antibiotics varies with even a small modification in its structure, more derivatives of these drugs may have to be studied with BoNT/B to find a potential drug.



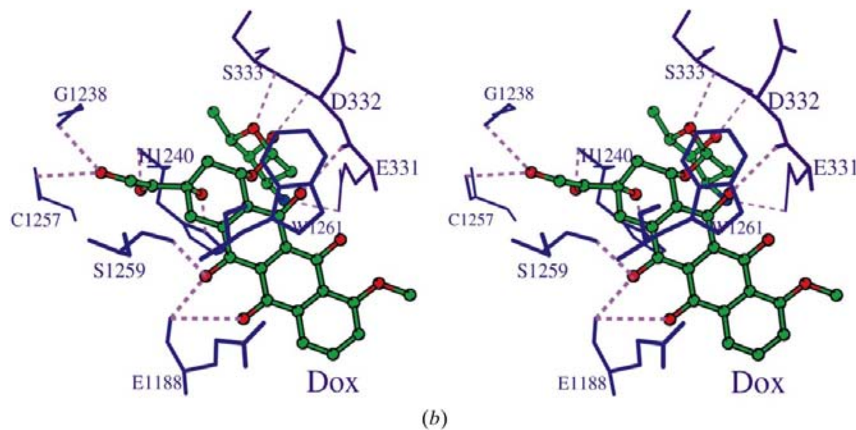


Figure 3
(*a*) The numbering scheme of doxorubicin. (*b*) Stereoview of the interactions of doxorubicin with the protein. Hydrogen-bonding contacts are shown as dashed lines. While amino-acid residues interacting with doxorubicin are shown as a stick model, doxorubicin is shown as a ball-and-stick model.

Table 2
Doxorubicin–protein interactions.

Doxorubicin	Protein	Distance (Å)
O14	Cys1257 O	3.19
O14	Gly1238 N	2.79
O13	His1240 N	3.01
O9	His1240 ND1	2.40
O11	Ser1259 OG	2.79
O11	Glu1188 O	2.67
O12	Glu1188 O	3.28
Symmetry-related molecule		
N3*	Glu331 OE1	3.05
O4*	Asp332 O	2.74
O5*	Ser333 N	2.97
O6	Glu331 O	3.42

4. Conclusions

The present study has defined the interactions between doxorubicin and the neurotoxin. Also, the difference in orientation of doxorubicin from that of the previous studies (Lightstone *et al.*, 2000) underscores the importance of crystallographic study for understanding the interactions of drug molecules with toxins. Even though the affinity of doxorubicin for neurotoxins may not be strong, it certainly presents itself as a strong lead compound since a number of analogues of doxorubicin have already been synthesized and may present better candidates (Cirilli *et al.*, 1992). With the knowledge that doxorubicin competes with gangliosides to bind to the toxin and that the mechanism is similar to the ganglioside binding, it would be a potential lead compound for drug design to treat botulism.

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