Identification of Novel Small Molecule Ligands That Bind to Tetanus Toxin

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Tetanus toxin belongs to a family of clostridial protein neurotoxins for which there are no known antidotes. Another closely related member of this family, botulinum toxin, is being used with increasing frequency by physicians to treat severe muscle disorders. Botulinum toxin has also been produced in large quantities by terrorists for use as a biological weapon. To identify small molecule ligands that might bind to the targeting domain of tetanus and botulinum toxins and to facilitate the design of inhibitors and new reagents for their detection, molecular docking calculations were used to screen a large database of compounds for their potential to bind to the C fragment of tetanus toxin. Eleven of the predicted ligands were assayed by electrospray ionization mass spectrometry (ESI-MS) for binding to the tetanus toxin C fragment, and five ligands (45%) were found to bind to the protein. One of these compounds, doxorubicin, was observed to have strong hydrophobic interactions with the C fragment. To check the ligands for their ability to compete with ganglioside binding, each was also tested using a GT1b liposome assay. Doxorubicin was the only ligand found to competitively bind the tetanus toxin C fragment with an appreciable binding constant (9.4 μM).

Introduction

The clostridial neurotoxins include tetanus toxin and seven serotypes of botulinum toxin. These proteins selectively target vertebrate motor neurons and are the most potent toxins known to man (*1*). The toxins are synthesized by the bacteria as a single 150 kDa polypeptide that is subsequently clipped into two chains and held together by a single disulfide bond. Concentrating at the synapse of axons, the toxins enter the neuron through a process that involves specific recognition followed by endocytosis and intracellular transport (*1*). The heavy chain is known to bind specifically to presynaptic neuronal cells, and the light chain penetrates the cell to inhibit neurotransmitter release by targeting and cleaving one of three proteins, VAMP, SNAP-25, or syntaxin (*2*, *3*). Although the mechanism by which the toxin enters the cell is not fully understood, gangliosides have a high selective affinity for these neurotoxins, and it is believed that the initial step must involve the binding of the toxin to gangliosides located on the surface of the cell (*1*). Tetanus neurotoxin (TeNT)[1] has been shown to specifically bind gangliosides of the G1b series, GD1b or GT1b (*4*−*7*). The receptor binding subunit of TeNT has been shown to be the 51 kDa carboxy-terminal domain of the heavy chain (H_C), more commonly termed the C fragment of tetanus toxin (*8*). In addition, the last 34 residues of the H_C have been reported to participate in ganglioside recognition (*8*, *9*). In particular, His1293 has been identified as the critical residue for ganglioside binding (*9*).

The recent interest in clostridial neurotoxins comes from both the increased frequency of their use in medicine (*10*) and the potential threat that they might be used by terrorist groups or other nations as biological weapons (*11*, *12*). Because there currently are no antidotes for these toxins, they represent an increasing hazard to human health. The first step in developing an antidote is to discover inhibitors to tetanus and botulinum toxins. These inhibitors may also serve a useful role as molecular recognition materials for affinity-based chemical sensors in detecting and identifying when these toxins have been deployed. The development of effective inhibitors requires a knowledge of the molecular structure of the proteins and how they function. Structures have recently been determined for both the intact botulinum neurotoxin type A (BoNT/A) (*13*) and the targeting domain of TeNT [PDB

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[1] Abbreviations: ESI-MS, electrospray ionization mass spectroscopy; TeNT, tetanus neurotoxin; H_C, heavy chain of tetanus neurotoxin; BoNT/A, botulinium neurotoxin type A; ACD, Available Chemicals Directory; TOF, time-of-flight; MLVs, multilamellar vesicles; DLS, dynamic light scattering; PBS, phosphate-buffered saline.

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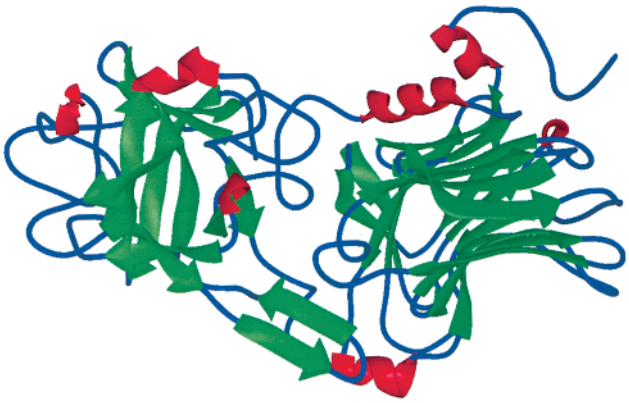

Figure 1. Crystal structure of the C fragment of the tetanus toxin heavy chain, determined to 1.6 Å resolution. α-Helices are shown in red, *β*-sheets shown in green, and loop regions shown in blue.

accession code 1A8D (*14*) and ref *15*]. With such information at hand, computational modeling tools offer the promise of being able to rapidly utilize structural information to discover potential inhibitors that bind specifically to these toxins.

In this study, we have used computational docking tools to identify potential ligand binding sites on the surface of the tetanus toxin C fragment and to screen a small molecule database for ligands that might bind to these sites. A small set of these ligands were then assayed for binding using two experimental techniques, electrospray ionization mass spectroscopy (ESI-MS) and a competitive liposome assay. The results are reported for the first of four ligand binding sites identified on the surface of the C fragment of TeNT.

Materials and Methods

Recombinant Protein, Ligands, and Other Reagents. A recombinant form of the tetanus toxin C fragment was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). The ligands purchased from Sigma Chemical Co. (St. Louis, MO) were *N*-acetylneuraminic acid, d-(+)-cellotetraose, neohesperidin dihydrochloride, oxytetracycline dihydrate, doxorubicin hydrochloride, and bisbenzimide H33258. The following compounds were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA): *N*-acetylneuraminic acid dimer, bisbenzimide H33342, hemorphin-5, and Gly-Arg-Gly-Asp-Ser. Rhodamine 110, (4-chloromethyl)benzoyl amide, L-alanine amide was purchased from Molecular Probes, Inc. (Eugene, OR); L-alanine amide was purchased from Molecular Probes, Inc. (Eugene, OR); thymopoietin II was purchased from Bachem Biosciences, Inc. (King of Prussia, PA), and the laminin binding inhibitor was purchased from Peninsula Laboratories (Belmont, CA). Distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylethanolamine (DMPE), cholesterol (Chol), *N*-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (fluorescein-DPPE), trisialoganglioside (GT1b), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co.

Computer Modeling Methods. Coordinates obtained from the crystal structure of the C fragment of TeNT (PDB accession code 1A8D), which has been determined to 1.57 Å resolution (see Figure 1), were used for the modeling and docking studies. The entire C fragment surface was examined for docking sites to which small molecules could bind by calculating the solvent accessible molecular surface and running the SPHGEN routine from DOCK (*16*), which packs clusters of spheres into the structural pockets. Each cluster of spheres fills the volume of a single pocket and is considered a potential binding site for small molecules. From the resulting 52 clusters of spheres, four sites were identified as potential target sites because of their inclusion or proximity to the last 34 residues of the TeNT C fragment, the proposed residues involved in ganglioside binding (*9*). Following the selection of the site, docking studies were conducted in an effort to identify small molecules that might bind specifically to this site on the protein. The DOCK program (*17*−*19*) screens a database of compounds on the computer, and predicts which molecules will likely bind tightly to the target receptor. The procedure we used is illustrated in Figure 2. Each compound from the database was computationally "docked" into the pocket on this tetanus toxin domain. Different orientations of the ligands within the binding site were examined, but different conformations of the ligands were not examined because only rigid docking was performed.

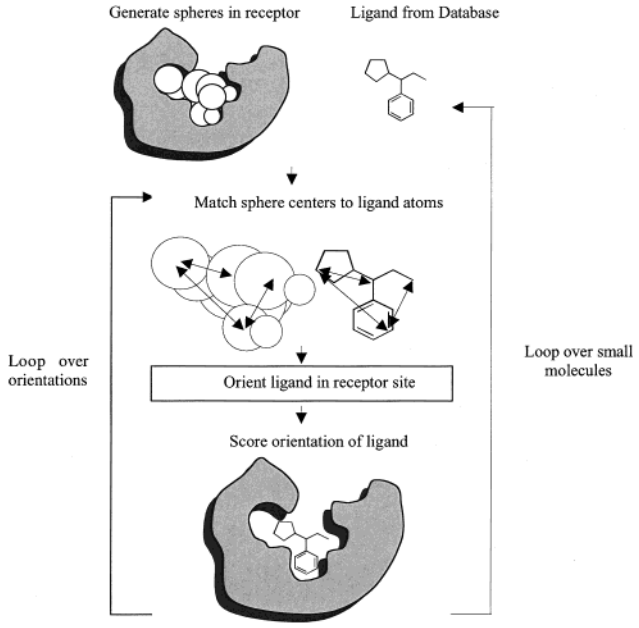

Figure 2. DOCK algorithm. (1) A "negative image" is generated by filling a pocket with spheres. (2) A candidate ligand is retrieved from a database. (3) Internal distances are matched between a subset (usually 3−8) of sphere centers and ligand atoms. (4) The ligand is oriented into the active site. (5) The interaction for that orientation is evaluated by a scoring function; the process is repeated for new orientations. Typically, 10 000 orientations are generated per ligand. The top-scoring orientation is retained. The process is repeated for a new ligand in the database.

The database was prepared from the Available Chemical Database (ACD) version 97.2 (*20*), which contains more than 200 000 commercially available compounds. Partial charges for compounds were generated using Gasteiger Marsili (*21*−*23*) charges in Sybyl (*24*) as described by Meng (*25*). The database was divided by total compound charge, and compounds with an absolute charge of >3 were filtered out. In addition, compounds with <10 or >80 heavy atoms were removed to focus on compounds within the size range for lead drug candidates. A total of 127 475 compounds were included in the final version of the screening database, in five separate lists for each value of total charge.

The DOCK runs were performed on Silicon Graphics workstations with multiple R8000/R10000 processors, and the overall run time ranged from 800 to 1350 CPU hours per site. We used a version of DOCK that has a new sphere-atom matching scheme (*16*). Two scoring schemes were used in the DOCK runs: force-field scoring, which uses the intermolecular van der Waals and electrostatic terms from AMBER (*26*), and contact scoring (*27*), which is based on a simplified Lennard-Jones function. Each ligand orientation was minimized with respect to each scoring scheme that was employed, and separate lists of top scoring ligands were maintained for each scoring scheme. Finally, the top 1% (approximately 1000 compounds) from the force-field list were divided proportionately among the total

charge categories and examined in more detail, one at a time. Compounds from these lists were selected for testing on the basis of visual inspection and evaluation of the compound using computer graphics. A smaller number of compounds from the contact list were also evaluated.

Electrospray Ionization Mass Spectroscopy (ESI-MS). The recombinant tetanus toxin C fragment was dissolved in filtered deionized water (18 MΩ cm^{-1}, Millipore) and centrifuged to remove any insoluble residue. Aliquots of the supernatant were hydrolyzed in HCl, and the protein concentration was determined by quantitative amino acid analysis (Structural Protein Laboratory, University of California, Davis, CA). UV−vis absorption measurements taken on the dissolved protein were used to accurately determine the protein concentration in each experiment. The final C fragment sample was diluted to a final concentration of 3−13 μM in 3 mM aqueous ammonium acetate (pH ~7.6) and 12% methanol. Each ligand was dissolved in the same buffer used to dissolve the protein.

Twenty microliter samples were prepared with various concentration ratios of the ligand to tetanus C fragment. The protein concentration was kept constant in these experiments, while the ligand concentration was varied. Samples were analyzed by ESI-MS on a Mariner orthogonal acceleration time-of-flight (TOF) instrument (PE Biosystems, Framingham, MA) within 20 min of mixing. The sample infusion rate was 1 μL/min, through 60 and 25 μm i.d. capillaries. The ESI mass spectra were acquired at room temperature and summed over 25 scans. The ion intensities in the mass spectrum were normalized to the protein peak [(bound protein)/(unbound protein)]. The multiply charged spectra were deconvoluted with the Biospec Data Explorer software (PE Biosystems). The slope from a plot of (unbound protein)/(bound protein) versus 1/[ligand] was used to obtain a dissociation constant for the ligand (*28*).

Instrument settings, such as gas flow rates, the number of scans, and declustering potentials were optimized and kept constant for each set of experiments corresponding to a specific complex. Mild instrument conditions are necessary to probe noncovalent interactions; therefore, we experimented with a range of declustering voltages. The declustering potential that a complex can withstand without dissociating can provide a qualitative estimate of the strength and nature of the noncovalent interaction.

Liposome Assay. Unilamellar liposomes were prepared with DSPC, Chol, fluorescein-DHPE, and GT1b (41.5:41.5:15:2 molar ratio) by means of extrusion (*29*). Mixtures of various lipids amounting to a total of 10 μmol were prepared in 3 mL of a 9:1 chloroform/methanol mixture in a round-bottomed flask. The lipid solution was dried and then rehydrated in 3 mL of freshly filtered 10 mM phosphate-buffered saline at pH 7.2 and 60 °C to generate extremely large and polydisperse multilamellar vesicles (MLVs). The MLV solution was converted to uniformly sized unilamellar liposomes by extrusion through a 100 nm pore diameter polycarbonate membrane using a pneumatic liposome extruder (Avestin, Inc., Ottawa, ON). Aggregated lipids, residual multilamellar vesicles, and dust particles were removed by centrifugation at 3000 rpm using a benchtop centrifuge.

Hydrodynamic diameters of unilamellar liposomes were estimated by dynamic light scattering (DLS) using a commercial device (Zeta Plus, Brookhaven Instruments Corp., Holtsville, NY). The liposome solution was centrifuged at 3000 rpm for 15 min prior to size measurement. Samples for size measurement were prepared by adding 25 μL of liposome to 2 mL of 10 mM phosphate-buffered saline (PBS) at pH 7.2. The solution was filtered using a 0.2 μm syringe filter and dispensed into a clean plastic cuvette. The scattering data were analyzed using the constrained regularization method of Provencher (*30*), resulting in a size distribution characterized by a mean diameter and variance. The liposome concentration, the number of receptors (GT1b) per liposome, and the number of fluorescein per liposome were determined as described previously (*31*).

To an Immulon 4HBX styrene immunoassay plate (Dynex Technologies, Chantilly, VA) was added 100 μL of 10 μg/mL recombinant tetanus toxin C fragment per well. The microtiter plate was shaken on a plate shaker at 4 °C overnight. The plate was then washed twice for 30 s with PBS using a microplate autowasher (model EL404 from Bio-Tek Instruments). The remaining hydrophobic sites on the polystyrene surface of wells were blocked for 2 h using 1 wt % bovine serum albumin in PBS. After the mixture had been washed with PBS, a mixture containing GT1b liposomes and ligands was added to the wells. The concentration of liposomes in the mixture was kept constant at 1.21×10^{-10} M, and different concentrations of ligands ranging from 10^{-9} to 10^{-3} M were used. The plate was incubated for 2 h at 37 °C and then washed six times with PBS. Methanol (100 μL) containing 0.1% NaOH was added to the wells, and the plate was read in a microplate fluorometer (Cambridge Technology, Inc.).

Results and Discussion

Inhibitors that effectively block the action of clostridial neurotoxins must be targeted to one of two sites on the protein, the ganglioside binding site(s) on the surface of the heavy chain or the active site of the catalytic domain. Molecules that bind to and block the activity of the catalytic domain could be used as antidotes to reduce or eliminate toxicity in exposed individuals. Because the catalytic domain is injected into the motor neurons, these antidotes would need to be delivered into the cell to function efficiently. A more effective method of preventing neurotoxicity would be to block toxin specific recognition of the ganglioside on the surface of the motor neurons by designing a small molecule inhibitor that could bind to the C fragment of the heavy chain of TeNT and interfere with ganglioside binding. By hindering the first step in the delivery of the catalytic domain into the motor neuron, these inhibitors could be administered as prophylactics against the toxins.

Computational Docking. Recently, computational methods such as docking have been used to speed the process of drug discovery and inhibitor design by screening large numbers of molecules and predicting whether they bind into the active sites of target proteins (*32*−*35*). While these efforts have met with moderate success in the design of new drugs that are effective against HIV proteins that are critical for infection and transmission of the disease, it is expected that this approach will prove to be generally useful as a first step in the identification of lead compounds whose binding to the protein can be improved by several orders of magnitude using combinatorial synthesis methods. For the inhibitor to be effective, it needs to specifically recognize the toxin and bind with high affinity. To test the utility of this method for discovering new compounds that compete for ganglioside binding to clostridial neurotoxins, we have used computer docking methods to screen and identify a set of small molecule ligands that bind to the C fragment of TeNT.

The solvent accessible surface of the tetanus toxin C fragment was calculated to identify surface pockets as sites for ligand binding. The volumes of the pockets were calculated by filling them with a combination of spheres with different radii. Fifty-two pockets were identified as potential binding sites for small molecules. Four initial sites were selected as potential target binding sites on the basis of the available experimental data about the residues involved in ganglioside binding. From a fluorescence binding assay, Kamata et al. (*36*) observed that a tryptophan was quenched upon ganglioside binding to

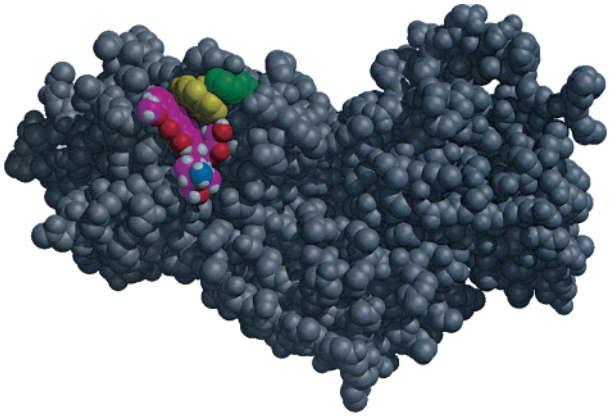

Figure 3. Doxorubicin is shown docked into site 1 of the C fragment of tetanus toxin as predicted by molecular docking calculations. The C fragment is shown in gray. Doxorubicin carbons are shown in magenta, oxygens in red, nitrogens in blue, and the hydrogens in white. Notice that doxorubicin is π stacked with Trp1289 (shown in yellow) and His1293 (shown in green).

BoNt/A. In a recent study of the sequence and structural homology of the clostridial neurotoxins, the sequence of TeNT was shown to be 65% homologous and ~35% identical with the sequences of seven serotypes of botulinum neurotoxin (*37*). Using the information that only a solvent accessible tryptophan could be quenched by ganglioside binding and that binding required the last 34 residues of the carboxy terminal of the C fragment (*8*, *9*), they concluded that the tryptophan being quenched in BoNT/A is Trp1265. Using the same sequence alignment, the equivalent tryptophan in TeNT was found to be Trp1289. In the structure of the TeNT C fragment, Trp1289 is π stacked with His1293, which has also been implicated in ganglioside binding (*9*). On the basis of this experimental evidence, site 1 was the first choice for initiating the docking calculations because it includes Trp1289 and is proximal to His1293, making a bound ligand more likely to interfere with ganglioside binding.

The computational docking program screened the Available Chemicals Directory (ACD) and scored and ranked the molecules by energy and contact. Though the molecules are ranked on the basis of the scores, the scoring function does not predict the binding affinities. Therefore, the top 1% of scored compounds were visually examined. Specific interactions, such as charge and hydrophobic interactions, were qualitatively noted for each compound. A variety of molecules were chosen to represent the spectrum of available compounds even though peptides dominated the top 1% of compounds. Twenty-nine final compounds were selected as potential binders. One of the ligands, doxorubicin, is shown docked into site 1 in Figure 3. Due to their limited availability, only 11 of these ligands were purchased and tested for binding.

Electrospray Ionization Mass Spectroscopy. Some of the first successful analyses of biomolecules by electrospray ionization mass spectrometry were reported by Fenn et al. (*38*), building upon techniques developed in an early work by Dole et al. (*39*). Nonspecific aggregates of proteins, such as salt adducts and those induced at high protein concentrations, were first observed by ESI-MS in the late 1980s. The use of ESI-MS to assess specific noncovalent interactions was suggested later (*40*, *41*). Excellent reviews of the study of noncovalent interactions

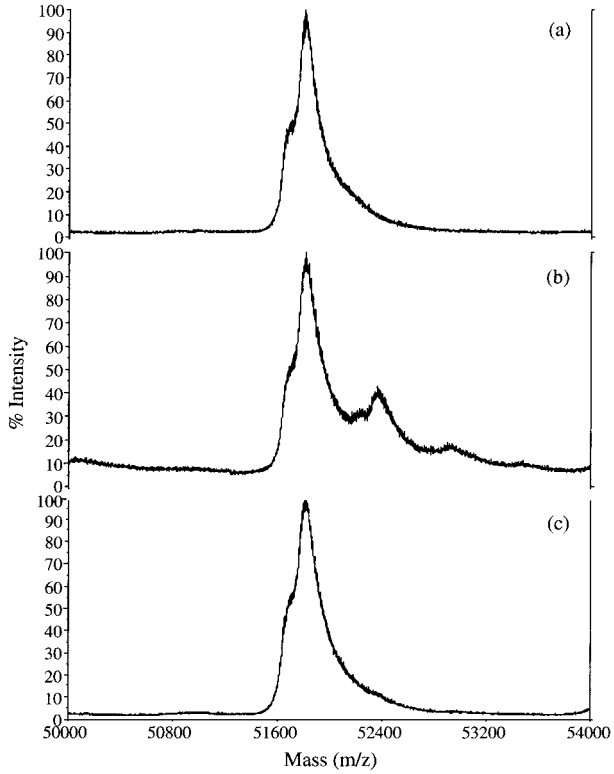

Figure 4. (a) Mass spectrum of the C fragment of tetanus toxin (13 μM, in 3 mM NH_4OAc and 12% CH_3OH at pH 7.6) at 100 V. The molecular mass is 51 800.4 Da. (b and c) The mass spectrum of the complex of doxorubicin and the C fragment of tetanus toxin. (b) At a declustering potential of 100 V, showing ligand binding, the mass of the C fragment is 51 809.6 Da and that of the complex is 52 360.3 Da. Thus, the mass of doxorubicin is 550.7 Da. These masses are within the error of the method since the mass accuracy of 100 ppm is ±5 Da at a mass of 50 000 Da. (c) At 200 V, there is no evidence of ligand binding. The peak that is shown is at 51 797 Da, corresponding to the C fragment of TeNT.

Table 1. Ligands That Were Tested Using ESI-MS for Noncovalent Complex Formation with the Tetanus C Fragment

site 1	
complexes formed	no complexes formed
D-(+)-cellotetraose	rhodamine R110a
doxorubicin HCl	thymopoietin
neohesperidin diHCl	laminin
Gly-Arg-Gly-Asp-Ser	Hoechst 33342
hemorphin-5	Hoechst 33258
	oxytetracycline

by ESI-MS have been published by Loo (*42*) and Smith (*43*). In general, mass spectrometry has been shown to provide specificity, sensitivity, and speed in assaying molecules and complexes (*44*). Electrospray ionization mass spectrometry enables the introduction of liquid samples into a mass spectrometer, and more importantly allows the analysis of highly polar and nonvolatile biological compounds (*45*). These properties of ESI-MS make it an ideal technique for rapid stoichiometry and dissociation constant determinations, especially when solution methods prove difficult (*28*).

Figure 4 shows an ESI spectrum of the C fragment of TeNT and the C fragment−doxorubicin complex. The mass spectrometry results obtained for all the ligands that were tested are summarized in Table 1. Ligand binding, as defined in these experiments, occurs when a new mass peak appears at the expected mass/charge (*m*/

z) ratio for the complex. Only those complexes that are stable to ionization and electrospray are observed. The main concern when using ESI-MS to investigate solution phase characteristics is the induction of complex formation via nonspecific aggregation or gas phase-induced binding. These contributions may arise from either the electrospray process or the solution conditions. To minimize this problem, low ratios of ligand concentration to protein concentration were used. To determine the ligand concentration range, a series of saturation binding curves was generated for each molecule. Complexes were prepared by adding increasing amounts of ligand, while keeping the protein concentration constant, until saturation was achieved. The ratio of bound protein to unbound protein is linearly correlated to the ligand concentration (the inverse was plotted for dissociation constant determination, as stated in Materials and Methods) up to the saturation point, beyond which increasing the concentration of ligand will not produce any more bound protein. Thus, the ligand concentration was kept within the linear region of the saturation binding curves for all binding experiments.

Complexes stabilized by weak binding forces may be labile in the gas phase. To minimize dissociation of the complex, all the samples were electrosprayed at room temperature. The declustering potential was kept between 50 and 200 V to help minimize complex dissociation. All but one of the ligand complexes that were tested withstood the high declustering voltages in the gas phase, indicating that the complexes were primarily stabilized by electrostatic interactions. The only complex that could not withstand the high declustering voltage was the complex with doxorubicin. Doxorubicin could only be observed when the applied nozzle potential was ≤100 V, indicating that the interactions were hydrophobic in nature. The determined binding constant for doxorubicin is 10.6 μM.

Of the 11 tested compounds, five of the ligands were found to bind the heavy chain of TeNT. This success rate of 45% is exceptional, particularly since only 10−40% of the predicted compounds identified in other docking studies were shown to bind to target receptors in the high micromolar range (*46*). These results show that ESI-MS is a reasonably efficient screening method for confirming ligand binding to the targeting domain of tetanus toxin.

Competitive Assays Using Ganglioside-Bearing Liposomes. Bifunctional unilamellar liposomes capable of binding to the targeting domain of tetanus toxin were prepared by incorporating the ganglioside GT1b into the bilayer. To impart signal generation capability, phospholipids (DPPE) conjugated with a fluorescein marker were also incorporated in the bilayer. The average hydrodynamic diameter of the liposomes determined by dynamic light scattering was 129.8 nm. The liposomes contained approximately 4400 GT1b and 33 200 fluorescein molecules per liposome and were very stable; no significant change in size was observed for more than 3 months.

GT1b-carrying liposomes were used in a competitive assay to determine the inhibition characteristics of the ligands that were identified by molecular docking calculations as potential inhibitors for the targeting domain of tetanus toxin. Because these molecules are relatively small, labeling them directly could significantly alter their binding affinities for the tetanus C fragment. This inability to label the ligands eliminates the use of a competitive assay between labeled and unlabeled forms of the compounds to estimate the binding constants. Their small size also precludes a "sandwich" type assay where a secondary labeled receptor is used to bind to the ligand that is bound to the C fragment. We used a competitive assay where liposomes containing GT1b competed with the predicted binding ligands to bind to a limiting amount of the tetanus toxin C fragment. This assay identifies those ligands that inhibit binding of ganglioside to tetanus toxin and determines their binding constants.

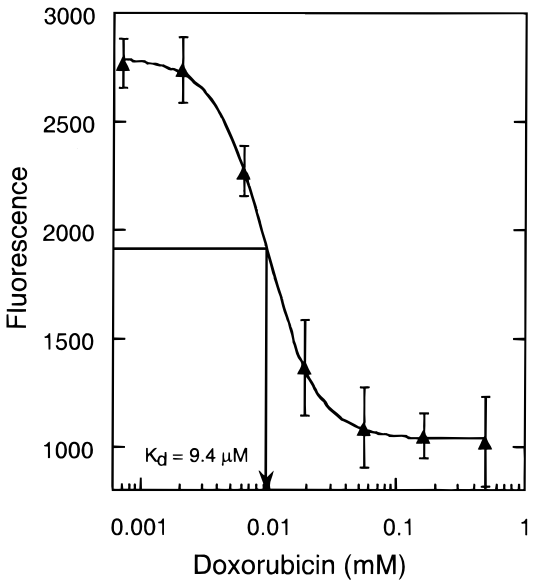

Figure 5. A constant concentration (1.21×10^{-10} M) of GT1b liposomes was allowed to compete with varying concentrations of doxorubicin for the binding of a limited amount of the tetanus toxin C fragment that was adsorbed on a polystyrene plate. A plot of the fluorescence vs the concentration of doxorubicin is shown. The data were fit to a logistic equation, and the dissociation constant (K_d = 9.4 μM) was determined as the concentration of doxorubicin that provides the half-maximal signal.

Figure 5 shows the results of a competitive binding assay where the competing ligands are doxorubicin and liposomes containing GT1b. At low concentrations of doxorubicin, all the liposomes bind to tetanus toxin immobilized on the plate, giving rise to a large signal. As the concentration of doxorubicin increases, doxorubicin begins to compete with liposomes for the binding of available tetanus toxin, thereby reducing the fluorescence signal. For quantitative analysis, the experimental data were fit to a logistic model (*47*), given by

$$F = \beta_2 + \frac{\beta_1 - \beta_2}{1 + (x/\beta_3)^{\beta_4}}$$

where F is the fluorescence signal and x is the concentration of added doxorubicin. β_1 and β_2 are the asymptotic signals as $x \rightarrow 0$ and $x \rightarrow \infty$, respectively. β_3 is the predicted concentration at the response halfway between the two asymptotes, and β_4 is related to the slope. The R^2 value for the fit was 0.9998, indicating a very good correlation between the logistic equation and experimental values. The parameter β_3 can be used as a measure of the affinity for doxorubicin binding to the tetanus toxin C fragment. Using this method, the apparent dissociation constant of the toxin−doxorubicin complex was determined to be 9.4 μM. Out of the five ligands that were

shown to bind to the C fragment by mass spectrometry, only doxorubicin competed for liposome binding.

Summary and Conclusion

These initial efforts to use computational docking to identify new small molecule ligands that bind to clostridial neurotoxins have provided very promising results. Using ESI-MS to verify ligand binding, 45% of the predicted compounds that were tested to bind to the TeNT C fragment were confirmed to actually bind. Further testing of these compounds using a competitive liposome binding assay revealed that one of the molecules, doxorubicin, competed with the ganglioside GT1b for binding to the protein with a dissociation constant of 9.4 μM which is in extremely good agreement with the ESI-MS-determined binding constant of 10.6 μM.

Even though only a small number of predicted ligands were screened and tested in this initial study, the results clearly demonstrate the potential utility of molecular docking calculations as a tool for identifying new ligands that bind to TeNT. Out of 11 compounds that were tested, five were found to bind, and one competed for ganglioside binding. Although the affinity of doxorubicin for TeNT is not sufficiently strong to make it a suitable inhibitor for ganglioside binding, it is expected to be an excellent lead compound since more than 1000 analogues of doxorubicin have already been synthesized for other purposes (*48*). A number of these analogues or new derivatives of doxorubicin may eventually be shown to bind more tightly than the parent compound.

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