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Identification of Novel Small Molecules That Bind to Two Different Sites on the Surface of Tetanus Toxin C Fragment

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A combination of computational methods, electrospray ionization mass spectroscopy (ESI-MS), and NMR spectroscopy has been used to identify novel small molecules that bind to two adjacent sites on the surface of the C fragment of tetanus toxin (TetC). One of these sites, Site-1, binds gangliosides present on the surface of motor neurons, while Site-2 is a highly conserved deep cleft in the structures of the tetanus (TeNT) and botulinum (BoNT) neurotoxins. ESI-MS was used to experimentally determine which of the top 11 computationally predicted Site-2 candidates bind to TetC. Each of the six molecules that tested positive was further screened, individually and as mixtures, for binding to TetC in aqueous solutions by NMR. A trNOESY competition assay was developed that used doxorubicin as a marker for Site-1 to provide insight into whether the predicted Site-2 ligands bound to a different site. Of the six predicted Site-2 ligands tested, only four were observed to bind. Naphthofluorescein-di- β -galactopyranoside was insoluble under conditions compatible with TetC. Sarcosine-Arg-Gly-Asp-Ser-Pro did not appear to bind, but its binding affinity may have been outside the range detectable by the trNOESY experiment. Of the remaining four, three [3-(*N*-maleimidopropionyl)-biocytin, lavendustin A, and Try-Glu-Try] bind in the same site, presumably the predicted Site-2. The fourth ligand, Ser-Gln-Asn-Tyr-Pro-Ile-Val, binds in a third site that differs from Site-1 or predicted Site-2. The results provide a rational, cost- and time-effective strategy for the selection of an optimal set of Site-1 binders and predicted Site-2 binders for use in synthesizing novel bidentate antidotes or detection reagents for clostridial neurotoxins, such as TeNT and BoNT.

Introduction

Tetanus toxin (TeNT)¹ and the botulinum toxins (BoNTs) are structurally and functionally related members of the family of clostridial neurotoxins. The recent interest in these neurotoxins arises from the increased frequency of BoNT's use in medicine, occasional dairy cattle and wildfowl deaths that have resulted from toxin ingestion, and the potential threat that this protein might be used by terrorist groups or other nations as a biological

weapon (1, 2). Both toxins selectively concentrate at the synapse of axons in vertebrate motor neurons and are the most potent toxins known to man (3). The entry of these toxins into neuronal cells requires the initial binding of the toxin to gangliosides on the cell surface. Thus, effective inhibitors that block neuronal cell binding can be developed for use as antidotes or serve as molecular recognition materials for affinity-based chemical sensors that detect and identify these highly toxic proteins.

The *Clostridium tetani* and *Clostridium botulinum* bacteria synthesize TeNT and BoNTs, respectively, as single 150 kDa polypeptides that are subsequently clipped into two chains held together by a single disulfide bond. The toxins enter the neuron through processes involving specific recognition, endocytosis and intracellular transport (3). TeNT targets inhibitory neurons within the central nervous system and spinal cord, causing a spastic paralysis, while the BoNTs target peripheral sensory neurons resulting in flaccid paralysis (4). The heavy chain binds specifically to presynaptic neuronal cells, presumably to gangliosides located on the surface of the cell (3). After the entire toxin is internalized into the cells via vesicles, the light chain is translocated into the cytosol and inhibits neurotransmitter release by

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¹ Abbreviations: TeNT, tetanus neurotoxin; TetC, tetanus toxin C fragment consisting of residues 875–1315; BoNT, botulinum neurotoxin; ACD, Available Chemicals Directory; ESI-MS, electrospray ionization mass spectroscopy; trNOESY, transferred nuclear Overhauser effect spectroscopy; trNOE, transferred nuclear Overhauser effect; Sar-RGDSP, Sarcosine-Arg-Gly-Asp-Ser-Pro; SQNYPIV, Ser-Gln-Asn-Tyr-Pro-Ile-Val; MP-biocytin, 3-(*N*-maleimidopropionyl)biocytin; NF-GalPyr, naphthofluorescein-di- β -galactopyranoside; DMSO, dimethyl sulfoxide; τ_c , rotational correlation time; pdb, protein data-bank.

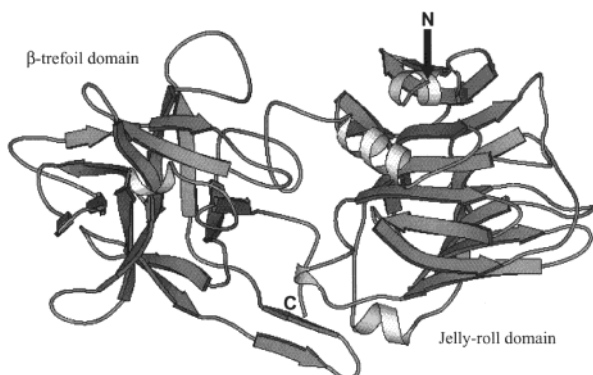


Figure 1. Overall fold of TetC in the high-resolution crystal structure (15) used in the DOCKING procedures. The figure was generated using the program Molscript (57).

targeting and cleaving one of three proteins, VAMP, SNAP-25, or syntaxin (5–7).

TeNT has been shown to specifically bind gangliosides of the G1b series, GD1b or GT1b (8–11). The receptor binding subunit of TeNT is a 51 kDa polypeptide comprising the C-terminal 452 amino acids of the heavy chain (H₂), more commonly referred to as the C fragment (12, 13). In particular, the last 34 residues of the C fragment participate in ganglioside recognition, with residue His1293 identified as being critical for binding (13, 14). Two crystal structures of the tetanus toxin C fragment (TetC, residues 875–1315) have been determined and reported in the protein databank, pdb access codes 1AF9 and 1A8D (15, 16), (<http://www.rcsb.org/pdb/>). These structures show that TetC consists of two subdomains, a lentil lectin-like N-terminal jellyroll domain and a C-terminal β -trefoil domain (16) (Figure 1). Structures of TetC in complex with the individual ganglioside components (4) *N*-acetylgalactosamine (pdb access code: 1D0H), galactose (pdb access code: 1DIW), lactose (pdb access code: 1DLL), and sialic acid (pdb access code: 1DFQ) and with a ganglioside GT1b derivative (17) (pdb access code: 1FV2) are also available. In these complexes, *N*-acetylgalactosamine, galactose, lactose, and sialic acid were observed to bind to four different sites on the surface of TetC which are widely spaced along one edge of the C-terminal β -trefoil subdomain (4) (Figure 2A). Two separate binding sites were identified for *N*-acetylgalactosamine. One of these sites is also the site where sialic acid binds. In comparison, a derivative of the ganglioside GT1b containing glucose, galactose, *N*-acetylgalactosamine, and sialic acid subunits has been shown to bind to a single site—the same site in which lactose binds (4, 17) (Figure 2A,B).

Previously, computational docking studies and mass spectrometry experiments suggested that the antitumor drug doxorubicin might bind in the same site as lactose and the ganglioside GT1b derivative, which we have designated as Site-1 (Figure 2C) (18). Site-1 is a common surface feature found in the structures of both TetC and BoNT, and recent crystal structures of BoNT/B ligand complexes show that doxorubicin (19) and 3'-sialyllactose (20) bind in this site. In addition, preliminary results obtained from the crystal structure of TetC in complex with doxorubicin indicate that this ligand also binds to Site-1 on TetC (S. Swaminathan, personal communication).

In the current study, computational methods were used to identify a second binding site, designated as Site-2,

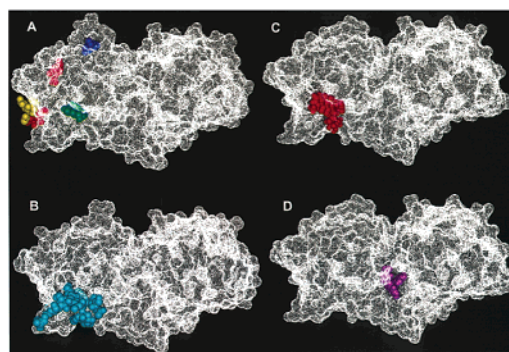


Figure 2. TetC/ligand complexes with the views showing the protein in the same orientation as in Figure 1. The surfaces were generated using the Connolly option in INSIGHTII (Accelrys, San Diego, CA). (A) Crystal structures of TetC in complex with sialic acid, *N*-acetylgalactosamine, galactose, and lactose (4). (B) Crystal structure of the TetC/ganglioside GT1b analogue complex with the ganglioside shown binding to Site-1 (17). (C) Predicted structure of the TetC/doxorubicin complex with doxorubicin shown binding in Site-1. (D) Predicted structure of the TetC/lavendustin complex with lavendustin shown binding in Site-2.

on the surface of TetC adjacent to Site-1 that is present on all clostridial neurotoxins. If ligands could also be identified that bound to this second “pocket”, combinations of ligands that bind Site-1 and Site-2 could be used to develop bidendate reagents for use as molecular sensors that bind to and detect the entire family of clostridial neurotoxins with increased affinity and specificity (21). Although the individual ligands that comprise a linked bidendate compound may only bind weakly to the protein, as expected because of their small size, the free energy of binding of the linked compound is, in principle, the sum of the free energies of each fragment plus a term due to linking (22). Thus, linked compounds with $<\mu\text{M}$ dissociation constants should be obtained by linking two fragments that each dissociate in the $>\mu\text{M}$ range (21). Linking two compounds together so that each bind individually to pockets that have been identified as unique to clostridial neurotoxins would also be expected to increase the specificity of the ligand for these proteins. This is particularly important when a ligand is being developed that can distinguish TeNT or BoNT from other proteins that may have similar structural domains but are essential to cell function.

The computational and experimental strategy used to determine the best sets of molecules to use in designing a bidendate ligand that binds to the targeting domains of the clostridial neurotoxins can greatly reduce the cost and time of the synthetic chemistry effort required. The approach can be scaled up to examine a larger number of ligands or be applied to prepare novel bidendate ligands for other target receptors.

Experimental Procedures

Recombinant Protein, Ligands, and Other Reagents.

Caution: The following chemicals are hazardous and should be handled carefully. The antitumor drug doxorubicin is an inhibitor of reverse transcriptase and RNA polymerase and is an immunosuppressive agent, and it intercalates in DNA (23–25). Lavendustin A is a protein tyrosine kinase inhibitor (26).

A recombinant form of TetC (Roche Molecular Biochemicals, Indianapolis, IN) was used in the ligand binding studies. 3'-Sialyllactose, doxorubicin hydrochloride, sarcosine-Arg-Gly-Asp-Ser-Pro (Sar-RGDSP), 3-(*N*-maleimidopropionyl)biocytin (MP-

biocytin), lavendustin A, and naphthofluorescein-di- β -galactopyranoside (NF-GalPyr) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The following compounds were purchased from Calbiochem-Novabiochem Corp (La Jolla, CA): Ser-Gln-Asn-Tyr-Pro-Ile-Val (SQNYPIV) and sialic acid. Deuterated dimethyl sulfoxide (DMSO) and D₂O were purchased from Isotech Inc. (Miamisburg, OH). Structures of the compounds were drawn in ChemWindow DB (BioRad/Sadtler, Philadelphia, PA).

Computer Modeling Methods. Coordinates obtained from the crystal structure of TetC (pdb code 1A8D) were used for the modeling and docking studies. Four surface binding sites, including Site-1 and Site-2, were identified by calculating the solvent-accessible surface and using the SPHGEN routine from DOCK 4.01 (27), which packs clusters of spheres into structural pockets. A sphere-atom matching scheme in the DOCK 4.01 program (28–30) was then used to computationally screen the Available Chemical Directory (ACD, version 97.2), which contained more than 200 000 commercially available compounds, and predicts which molecules will likely bind to Site-2. The procedure used to computationally dock each compound has been previously described in detail by Lightstone et al. (18). Briefly, different orientations of each ligand within a site were scored for best intermolecular van der Waals and electrostatic force field potentials using AMBER (31) and for contact scoring (32), which is based on a simplified Lennard-Jones function. The top 1% (~1000 compounds) from the force field and contact scoring lists were examined in more detail, one at a time, using computer graphics and visual inspection. 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 binding site.

Electrospray Ionization Mass Spectroscopy (ESI-MS). ESI-MS was used to experimentally determine which of the computationally predicted ligands bind to TetC. Aliquots of the TetC used for binding studies were hydrolyzed in HCl, and the amount of protein present was determined by quantitative amino acid analysis (Structural Protein Laboratory, University of California, Davis, CA). Both TetC and ligands were dissolved in 3 mM ammonium acetate (pH 7.6) buffer/12% v/v methanol.

Twenty microliter samples in which the protein concentration (3–13 μ M) was kept constant and the TetC:ligand ratio was varied from 10:1 to 1:10 in 5 steps were analyzed by ESI-MS on a Mariner orthogonal acceleration time-of-flight instrument (PE Biosystems, Framingham, MA) within 20 min of mixing. The sample infusion rate was 1 μ L/min through 60 and 25 μ m i.d. capillaries. The spectra were acquired at room temperature and summed over 25 scans. The ion intensities were normalized to the protein peak [bound protein/(unbound protein)]. The multiply charged spectra were deconvoluted with the Biospec Data Explorer software (PE Biosystems). Instrument settings, such as gas flow rates, the number of scans, and declustering potentials, were optimized and kept constant for each set of experiments involving a specific complex. A range of declustering voltages was tested for each complex, since mild settings were necessary to probe noncovalent interactions.

Transferred Nuclear Overhauser Effect Spectroscopy (trNOESY) Experiments. All spectra were measured on a Varian INOVA 600 MHz spectrometer at 2, 10, 20, 30, and 37 °C. The phase-sensitive 2D-NOESY experiments of ligands or mixtures of ligands in the absence of protein were carried out at 900 ms mixing times, while mixtures of ligands in the presence of TetC were carried out at 200 and 300 ms mixing times. Long mixing times are necessary for detection of NOEs for small molecules (<1–2 kDa MW) because the product of ω_0 ($2\pi \times$ spectrometer frequency) and τ_c (rotational correlation time) is less than 1 ($\omega_0\tau_c < 1$); in contrast, shorter mixing times are required for large molecules or ligands binding to large molecules because $\omega_0\tau_c \gg 1$ (33). A total of 300 increments were collected in t_1 , each with 48 or 64 scans and 1024 complex data points collected in t_2 . NMR data were processed using VNMR software (Varian Inc., Palo Alto, CA), and the two-dimensional

Table 1. Ligands Tested Positive by ESI-MS for Noncovalent Complex Formation with TetC

predicted Site-1	predicted Site-2
doxorubicin ^{a,b}	Tyr-Glu-Trp
3'-sialyllactose ^b	lavendustin A
D-(+)-cellobiose ^a	Sar-Arg-Gly-Asp-Ser-Pro
neohesperidin diHCl ^a	naphthofluorescein
	di-(β -D-galactopyranoside)
Gly-Arg-Gly-Asp-Ser ^a	3-(N-maleimidopropionyl)biocytin
hemorphin-5 ^a	Ser-Gln-Asn-Tyr-Pro-Ile-Val
etoposide phosphate	

^a Reported in (18). ^b Binds Site-1 in BoNT/B (19).

frequency domain matrixes were analyzed using FELIX (version 97, Accelrys, San Diego, CA).

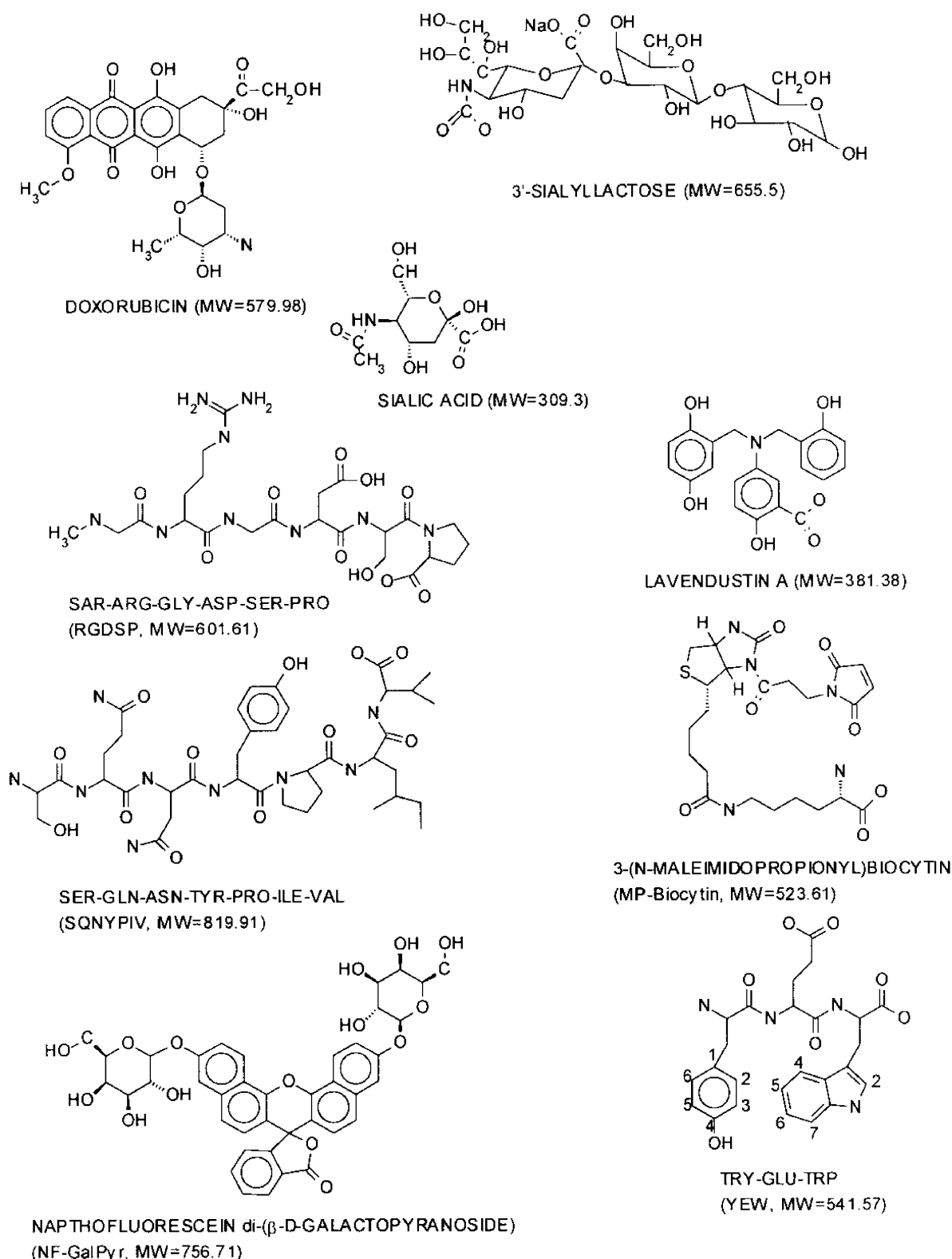
Dry (lyophilized) protein samples were dissolved in 100% D₂O for NMR measurements. Those ligands (MP-biocytin, lavendustin A, and NF-galactopyranoside) that were only moderately soluble in water were first dissolved in a small volume (100 μ L) of deuterated DMSO before addition of an aliquot (between 13 and 38 μ L) to 1 mL of TetC in D₂O. The addition of this small amount of DMSO would not be expected to affect the stability of the protein, as previously demonstrated by other NMR studies (21). Complexes were prepared by one of two methods. In one set of experiments, 3 mg of TetC was dissolved in 1.0 mL of D₂O (57.9 μ M) and centrifuged for 5 min in an Eppendorf microcentrifuge to remove insoluble material prior to adding the ligands. Mixtures of ligands were also prepared by adding the ligands to ~500–800 μ L of D₂O prior to the addition of 1–3 mg of TetC (38–72 μ M). The concentrations of the ligands used in the preparation of the mixtures were ~0.2–1 mM. Different molar ratios of TetC to doxorubicin were tested, ranging from 1:5 to 1:50, to determine the best ratios for use in our experiments. The final molar ratios of TetC:ligand for all subsequent experiments were kept between 1:16 and 1:22 since these ratios provided a good sensitivity for detection of trNOEs for a large range of structurally unrelated ligands.

Results and Discussion

Computational Docking and Mass Spectrometry.

Previously, we reported the results of our computational docking runs and ESI-MS screening of ligands binding to Site-1 (18). Site-1 is a common surface feature in the structures of the targeting domains of both TeNT and the BoNTs, and it has been determined by others to be the site where a ganglioside GT1b analogue (17), 3'-sialyllactose (20), and lactose (4) bind. The six predicted Site-1 binders that were confirmed to bind to TetC by mass spectroscopy are listed in Table 1.

In the current study, the location of the Site-2 binding pocket and the identities of the ligands predicted to bind to this pocket are reported. Site-2 is a deep surface pocket that is located between the C-terminal β -trefoil and N-terminal jellyroll subdomains and has been identified as the most highly conserved pocket in the structures of both TetC and BoNTs (Figure 2D) (34). It is proximal to Site-1, making the ligands that bind to this site attractive candidates for linking to Site-1 ligands. To identify a set of ligands that bind to Site-2, computational docking methods were used to screen the entire Available Chemicals Directory (ACD) and predict a set of small molecules that might bind to Site-2. A variety of structurally diverse ligands were chosen to represent the spectrum of possible candidates. The interactions involved in each complex were ranked by energy and contact scores and the top 1000 compounds were visually examined qualitatively to assess the interactions they form with Site-2. Due to their limited availability or prohibitive cost, the 11 compounds

Chart 1. Structures and Molecular Weights of Ligands Used in This Study

out of the top 34 with best fit to the Site-2 pocket were checked experimentally for binding activity by electrospray ionization mass spectroscopy (ESI-MS). Ligand binding, as defined in the ESI-MS experiments, was confirmed when a new mass peak appeared at the expected mass/charge (m/z) ratio for the ligand/TetC complex. An example of the results obtained for one of these ligands, a representative spectrum for the TetC/SQNYPIV complex, is shown in the Supporting Informa-

tion (Figure S1). Of the 11 ligands tested, 6 were positive for binding activity (55%) (Chart 1 and Table 1). One of these ligands, lavendustin A, is shown docked into Site-2 in the predicted structure of the TetC/lavendustin complex (Figure 2D).

Transferred NOESY (trNOESY) Experiments. While ESI-MS has been used with increasing frequency to detect protein/ligand complexes (35, 36), it was necessary for several applications, in which the TetC would

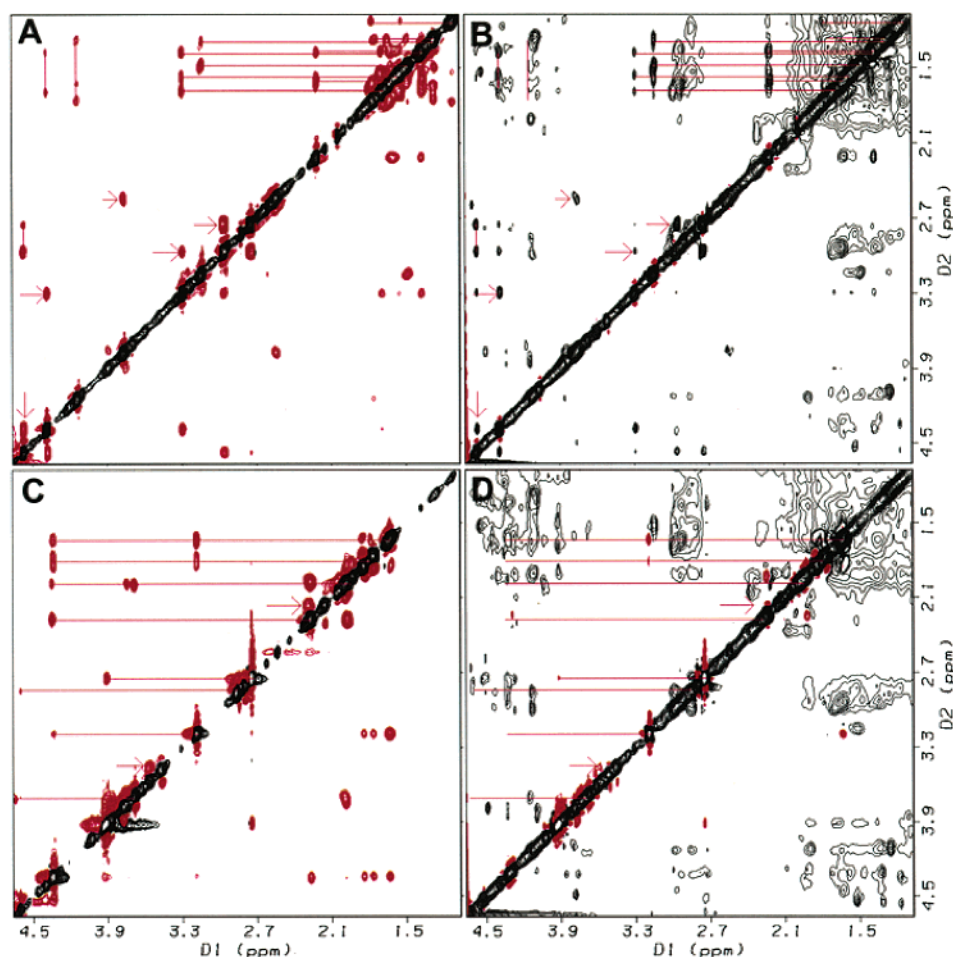


Figure 3. trNOESY experiment. (A) 900 ms NOESY spectrum of MP-biocytin shows weak negative cross-peaks. (B) Addition of TetC results in the cross-peaks in the 300 ms trNOESY to flip their sign to positive, indicating that MP-biocytin binds to TetC. (C) 900 ms NOESY spectrum of Sar-RGDSP shows weak negative cross-peaks. (D) Addition of TetC results in these cross-peaks in the 300 ms trNOESY remaining very weak and negative, indicating that Sar-RGDSP does not bind. The black NOE cross-peaks belong to TetC, as determined by comparison with several spectra of TetC and different ligands. The lines and arrows in the spectra indicate the positions of the cross-peaks belonging to the ligand in the absence of TetC. The 900 ms spectra in panels A and C are plotted at 2 times lower level than the 300 ms trNOESY spectra in panels B and D for presentation purposes. All spectra were acquired at 30 °C. Concentrations and TetC:ligand ratios are given in Table 2.

be present in an aqueous environment, to confirm whether binding activity could also be detected in solution. In addition, the synthesis of novel bidendate ligands requires that the individual compounds are compatible with one another and with TetC under similar solvent conditions. Thus, mixtures containing Site-1 and Site-2 ligands that exhibited binding activity by ESI-MS were further screened for binding activity in the solution state using the transferred nuclear Overhauser effect spectroscopy (trNOESY) experiment (37–39).

TrNOESY experiments (40–42) are routinely used to detect ligand binding to a target protein under conditions of fast exchange (ligands that bind with μM to mM dissociation constants). The intensity of each intraligand NOE cross-peak is governed by the population-weighted cross-relaxation rate. A strong positive NOE cross-peak is observed for binders (black peaks, Figure 3), as opposed to weakly negative (Figure 3) or zero NOE cross-peaks for the same mixture of compounds in the absence of TetC. Thus, the sign flip of the NOE cross-peak between the free versus bound states acts as a simple binary filter to distinguish binders from nonbinders (43, 44). However, positive NOE cross-peaks for small molecules in the absence of TetC were sometimes observed when the molecule contains protons attached to large ring systems,

such as the protons attached to the four-ring system of doxorubicin (boxed peaks, Figure S2A). These protons exhibit less internal motion than those located in more flexible long carbon chains. However, although the sign of the trNOE cross-peaks corresponding to these aromatic resonances remained the same regardless of whether TetC was present or not, their intensities were always much stronger for the bound ligand (Figure S2B). Thus, both the sign and intensity of the cross-peaks were taken into consideration when distinguishing ligands that bind from those that do not.

Initial experiments were carried out with doxorubicin and TetC to determine optimal solvent conditions, protein:ligand molar ratios, and temperatures necessary for routine and high-throughput data collection. Doxorubicin was used as our primary test case for several reasons. It contains both aromatic and aliphatic protons that are well-resolved in the NMR spectra, and thus any changes in trNOEs, either in intensity or in chemical shift, can be readily monitored under different experimental conditions. More importantly, the crystal structure of the doxorubicin/BoNT complex has confirmed that doxorubicin binds in Site-1 (19).

The six predicted Site-2 ligands (Table 1) identified by MS were tested for binding to TetC in aqueous solutions

Table 2. Summary of NMR trNOE Experiments

figure	ligand	[Lig] (μ M)	[TetC] (μ M)	[TetC]:[Lig]	binding
Figure 3					
A,B	MP-biocytin	1232.2	71.8	1:17	yes
C,D	Sar-RGDSP	1187.3	74.2	1:16	no
Figure 4					
A	doxorubicin	1081.4	54.3	1:20	yes
B	Sar-RGDSP	1116.3	50.6	1:22	no
C	SQNYPIV	1050.6	47.4	1:22	yes
D	MP-biocytin	1015.3	46.2	1:22	no
E	lavendustin A	993.3	45.0	1:22	yes
F	NF-GalPyr	926.6	43.3	1:21	maybe ^a
Figures 5 and S3					
	lavendustin A	1158.3	67.8	1:17	yes
	Tyr-Glu-Trp	1171.2	67.8	1:17	yes
Figure 6					
ABDE	sialic acid	386.7	22.3	1:17	no
BC,E	doxorubicin	365.6	21.5	1:17	yes,no ^b
EF	3'-sialyllactose	351.9	20.7	1:17	yes

^a Precipitation was observed, resulting in a decrease in the intensities of all cross-peaks. ^b Doxorubicin binding was observed in 6B and 6C, but not in 6E.

using NMR, either individually or as mixtures containing different combinations of the compounds and in the presence or absence of the Site-1 binder doxorubicin (Table 2). In addition, sialic acid and two known Site-1 binders, doxorubicin and 3'-sialyllactose, were tested for binding to TetC. The chemical structures and molecular weights for these compounds are given in Chart 1.

The trNOESY Competition Assay. The ultimate goal of our computational and screening experiments was to facilitate the selection of the best pairs of ligands to link together in order to synthesize a bidendate molecule that binds with high specificity to clostridial neurotoxins. Previously, Fesik's laboratory (45, 46) had developed and demonstrated a powerful NMR-based screen, SAR (structure-activity relationships) by NMR, to identify small molecules that bind to proximal sites on a protein. However, the limitations of the SAR-by-NMR method are that it requires high concentrations of soluble, purified ¹⁵N-labeled protein. Moreover, the method is limited to studies of proteins having molecular masses <40 kDa. For those target proteins that do not meet these criteria, other NMR screening methods for binding activity, such as saturation-transfer difference spectroscopy (47–49) and diffusion-based experiments (50–52), have been developed. Although these methods do not require large amounts of pure, labeled protein and are not limited by the size of the protein, they do not provide any information about the location of the binding site. Thus, we developed a trNOESY competition assay that uses a ligand known to bind to one site to identify those compounds that bind to a different site. The experiment determines if binding is disrupted by a competing ligand binding to the same site, as evidenced by an absence of trNOEs from one ligand and the presence of trNOEs for the other ligand. Using this approach, ligands could be grouped into sets, those binding to Site-1 and those binding to another site, presumably the predicted Site-2. Unlike the SAR-by-NMR method, however, these assays cannot identify the exact site of ligand binding.

Several limitations of trNOESY competition assays need to be considered: (1) The possibility that two or more ligands can bind the same site simultaneously cannot be completely ruled out based only on their lack

of competition. Recently, Ma et al. (53) have proposed that a protein can preexist in ensembles of sub-states, as a result of its conformational flexibility, and present a range of different binding site shapes to the incoming ligands, such that one site may recognize and bind multiple diverse ligands. (2) An assumption has been made that the ligands are binding specifically to one site only, which may not always be the case. For example, *N*-acetylgalactosamine binds to two different sites in the crystal structure of the TetC complex (Figure 2A) (4). Similarly, if a predicted Site-2 ligand is binding with specificity to more than one site, a stronger competitor can displace it from one site, but not from the other sites. In such a case, both ligands would bind simultaneously to TetC. (3) Nonspecific association of a ligand with TetC is also possible, especially at very high concentrations of ligand.

Nevertheless, for the purpose of identifying potential pairs of ligands that might bind to distinct sites, the trNOESY competition assays can prove to be extremely useful. The experiments are carried out in aqueous solutions using small quantities of unlabeled protein, and the influence of one ligand binding in one site on the binding of a second ligand binding in another site can be qualitatively evaluated. The solubility and compatibility of the ligands with protein dissolved in the same aqueous solutions can also be assessed. In cases where ligands both exhibit binding activity simultaneously, the assay can aid in identifying a suitable ligand pair that can be linked to produce a bidendate molecule with each component having specificity for two different sites.

Influence of Doxorubicin Binding in Site-1 on Predicted Site-2 Binders. The strategy used to identify TetC ligand pairs is based on the following assumption. Since doxorubicin is known to bind in Site-1, if doxorubicin and a predicted Site-2 ligand are observed to bind simultaneously to TetC and the addition of doxorubicin to the TetC/Site-2 ligand complex does not disrupt ligand binding, then the ligand must bind to a site other than Site-1. Using this approach, ligands that bind to sites other than Site-1 were identified by adding doxorubicin to TetC (Figure 4A), and then a series of five predicted Site-2 binders (Table 1) were added sequentially to the TetC/doxorubicin complex (Figure 4B–F). In subsequent experiments, the influence of doxorubicin binding in Site-1 on the binding of ligands to a second site was evaluated.

In the first set of trNOESY experiments, doxorubicin was observed to remain bound to Site-1 throughout the additions of all five of the predicted Site-2 ligands (Figure 4). The compounds SQNYPIV and lavendustin A both bind to TetC, as evidenced by the presence of strong positive trNOEs belonging to these ligands, individually and when doxorubicin, Sar-RGDSP, and MP-biocytin are present (Figure 4A,C,E). Sar-RGDSP and MP-biocytin did not bind to TetC when in a mixture. Sar-RGDSP also tested negative for binding activity when added to TetC alone (Figure 3C,D), while MP-biocytin tested positive when added as the only ligand (Figure 3A,B). Thus, MP-biocytin binding appears to be disrupted by the presence of one of the other ligands.

Lavendustin A appears to be partially displaced by addition of NF-GalPyr (Figure 4F), suggesting that these two compounds may bind in the same site. However, it is difficult to tell from these experiments if NF-GalPyr actually binds to TetC. Although new positive cross-peaks

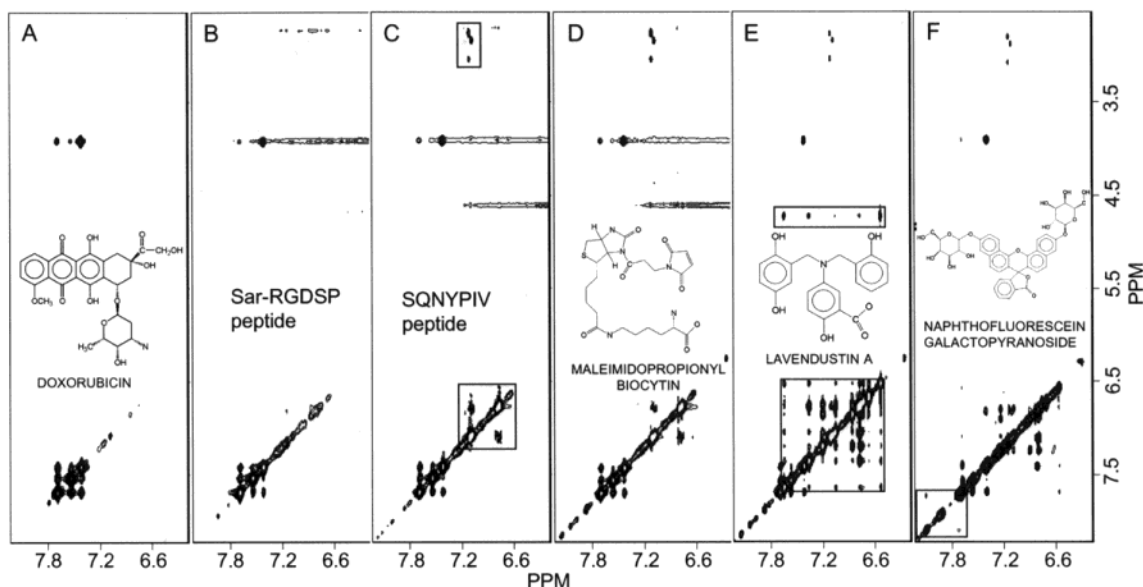


Figure 4. Effect of doxorubicin binding in Site-1 on predicted Site-2 ligands binding to TetC: Expanded regions of the 2D trNOESY spectra of TetC/ligand complexes at 200 ms mixing time and 20 °C showing binding of doxorubicin (panel A, Site-1 binder) and a series of predicted Site-2 binders added sequentially. The identities of the compounds are shown in each panel, and the concentrations and TetC:ligand molar ratios are given in Table 2. The presence of new strong and positive cross-peaks (boxed) indicates binding. SQNYPIV (panel C) and lavendustin A bind (panel E), but both appear to be partially displaced by addition of NF-GalPyr (panel F).

appear in the spectrum upon addition of NF-GalPyr (boxed peak, Figure 4F), these cross-peaks may correspond to aromatic protons belonging to a large ring system and are positive in the unbound state. In addition, when NF-GalPyr was added to the mixture, a precipitate formed, and this precipitation may have reduced the concentrations of TetC and the other ligands, resulting in the observation of weaker NOE cross-peaks in the spectrum. While NF-GalPyr was observed to be insoluble in aqueous solutions, the ligand remained in solution in 35% DMSO. Experiments could not be performed at this concentration of DMSO, however, because it precipitated TetC. Additional binding studies were not performed with NF-GalPyr due to the incompatibility of the protein and ligand under similar solvent conditions.

In summary, the results from this set of experiments indicate that both lavendustin A and SQNYPIV are good candidates for linking with doxorubicin to create a bidentate TetC ligand, while Sar-RGDSP, MP-biocytin, and NF-GalPyr are not.

Influence of Adding Mixtures of Site-2 Ligands on Binding Activity. Mixtures of two to three compounds were next tested for binding to TetC to verify the results obtained from the previous set of experiments in which the predicted Site-2 ligands were added sequentially after doxorubicin was added. These studies provide important information about how different compounds behave in the presence of each other and with TetC under the same solvent conditions. The simultaneous binding of two (or more) compounds would suggest that these ligand pairs should be good candidates to link together to form a bidentate ligand.

In mixtures containing the Site-1 binder doxorubicin and the predicted Site-2 binders lavendustin A and MP-biocytin, strong positive trNOEs were observed for doxorubicin and lavendustin A in the presence of TetC, but not for MP-biocytin (Figure S2A,S2B). This result suggests that although MP-biocytin binds to TetC when not in a mixture (Figure 3B), it does not bind when another ligand with higher affinity for the same binding site is

present. Additional experiments show that MP-biocytin binds TetC in the presence of doxorubicin (Figure S2C, S2D). Therefore, MP-biocytin and lavendustin A must be competing for the same site, with lavendustin A having the higher affinity. This was confirmed by testing a mixture of MP-biocytin and lavendustin A with TetC and only observing lavendustin A binding activity (Figures S2B). For the sake of discussion, we will assume that both lavendustin A and MP-biocytin are binding in the predicted Site-2, although the trNOESY experiments do not identify the exact location of the site.

SQNYPIV binds in the presence of the Site-1 binder doxorubicin (Figure 4C) and the predicted Site-2 binders MP-biocytin (Figure S2F) and lavendustin A (Figure 4E), suggesting that SQNYPIV binds in a third independent site. This result, however, does not rule out the possibility that SQNYPIV is binding nonspecifically or with low affinity to Site-1 and Site-2. Yet the results indicate that this peptide would still be a good candidate for pairing with either a Site-1 or a Site-2 ligand.

When a mixture of Tyr-Glu-Trp and lavendustin A was tested for binding to TetC (Figures 5 and S3), both compounds were observed to bind, but numerous intermolecular NOEs between lavendustin A and Tyr-Glu-Trp were detected. In particular, the aromatic protons of lavendustin A show NOEs to the aliphatic protons of all three amino acids in Tyr-Glu-Trp (Figure 5) and to the aromatic ring of tryptophan (Figure S3), while the aliphatic protons of lavendustin A show NOEs predominantly to tyrosine aliphatic and aromatic protons (Figure 5). These results indicate that lavendustin A and Tyr-Glu-Trp are most likely binding to the same site, presumably the predicted Site-2, with approximately the same binding affinity, and that the intermolecular NOEs between them are protein mediated indirectly through fast exchange mechanisms (54). We cannot, however, completely exclude the second possibility—that is, lavendustin A and Tyr-Glu-Trp are simultaneously binding to different sites within 5 Å of one another. To address this question further, quantitative analyses of the trNOE

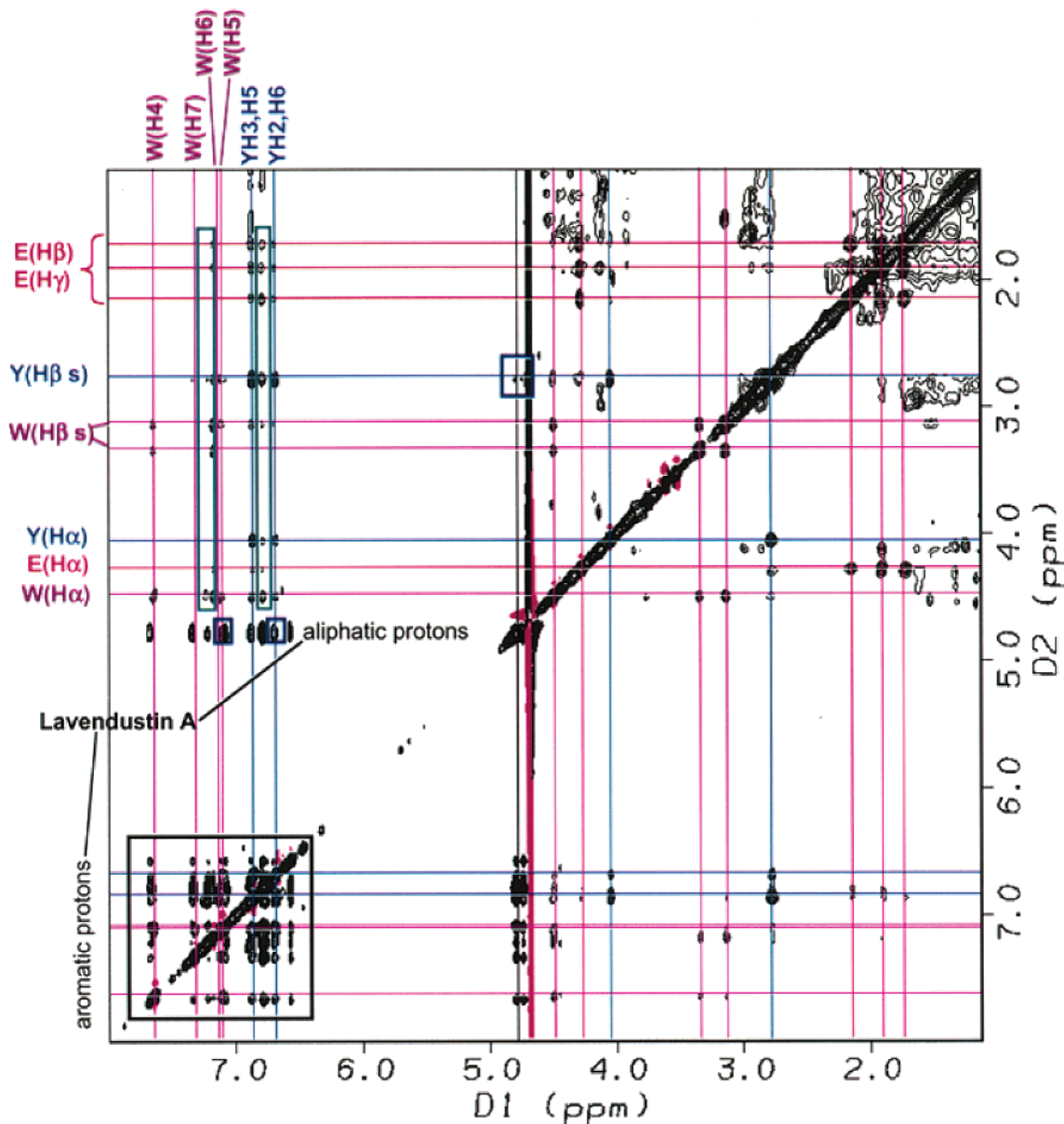


Figure 5. Interligand NOEs: 300 ms mixing time trNOESY spectrum at 30 °C of a mixture of lavendustin A and Try-Glu-Trp (YEW) in the presence of TetC. The numbering scheme of the residues in the Try-Glu-Trp peptide is given in Chart 1. Interligand NOE's between lavendustin A aromatic protons and Try-Glu-Trp aliphatic protons are boxed. Interligand NOEs between the aliphatic protons of lavendustin A and Tyr(H β 's), Tyr(H2,H6), and Trp(H6) are boxed. Small chemical shift changes are observed for both ligands when in the absence or presence of TetC. An expanded plot of the aromatic region (black box) is shown in the Supplementary Information (Figure S3).

data using a complete relaxation matrix approach (53, 54) will be carried out in future studies.

Of the remaining predicted six Site-2 ligands tested, Sar-RGDSP binding to TetC was not detected by the trNOESY experiments. Sar-RGDSP did not bind when alone (Figure 3C,D) or when in a mixture with doxorubicin (Figure 4B) or Trp-Glu-Trp (Figure S2H), suggesting that the binding affinity of this ligand to TetC (if it does occur in solution) is outside the range that can be detected by this method. Since four suitable ligands that do bind to a site other than Site-1 have been identified (lavendustin A, Tyr-Glu-Trp, MP-biocytyl, and SQNYPIV), no further binding studies were carried out with Sar-RGDSP and TetC.

Temperature Effects and Competition between Site-1 Ligands. These results show that the trNOESY competition assay can be useful in classifying predicted

Site-2 ligands with respect to binding site, but care must be employed in interpreting the data if binding is not observed. An important limitation of the trNOESY competition assay is that binding can only be detected for those compounds that have mM to μ M dissociation constants. For example, sialic acid does not appear to bind to TetC, either alone or when mixed with doxorubicin, as evidenced by the presence by the weak negative cross-peaks for this ligand in the 300 ms trNOESY spectra (Figure 6A). However, a recent crystal structure of a TetC/sialic acid complex by Emsley et al. (4) shows that sialic acid binds to a site that is adjacent to Site-1 (Figure 2A). One possible explanation for the discrepancies between the NMR and X-ray results is that sialic acid binds TetC with $<10^3$ M or $>10^6$ M affinity, which is outside the range of binding that can be detected by trNOESY experiments. Changing the temperature can

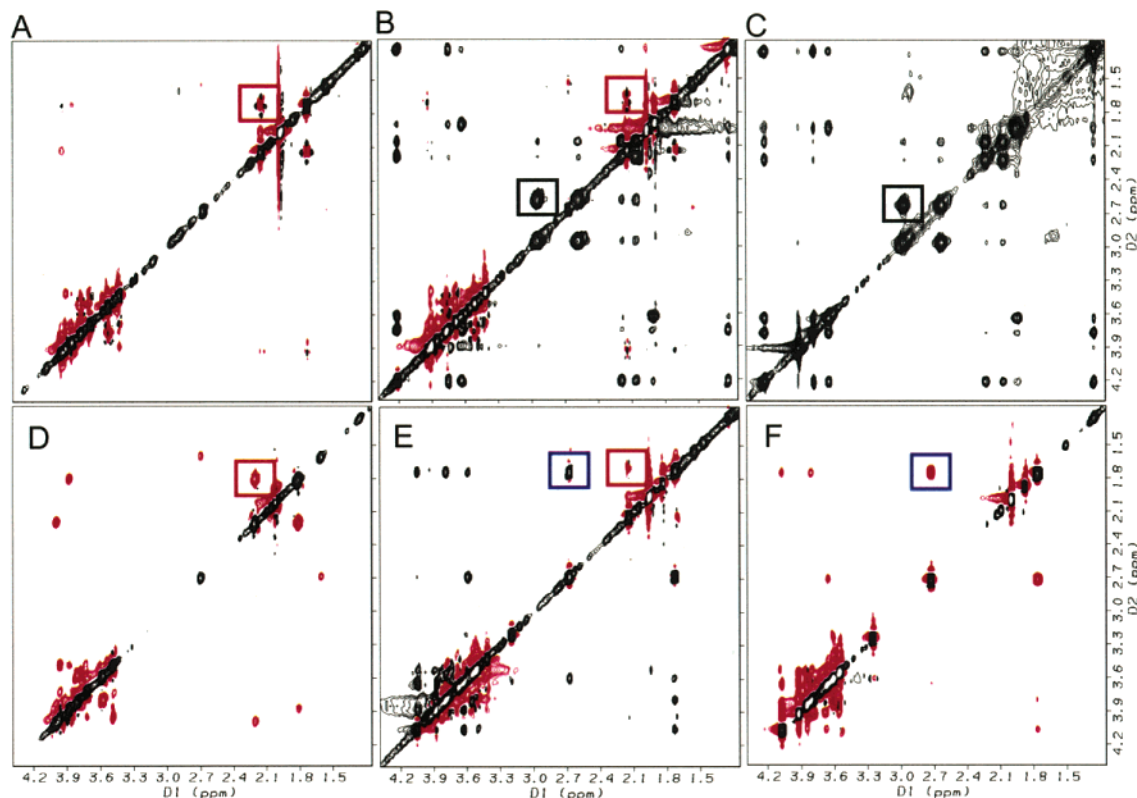


Figure 6. Temperature effects and Site-1 competition assay. (A) Sialic acid does not appear to bind to TetC at 2, 10, 20, 30, or 37 °C. The representative 300 ms trNOESY at 10 °C is shown. (B) Addition of doxorubicin to a mixture of TetC and sialic acid results in doxorubicin binding (black cross-peaks) while sialic acid does not bind as shown in the 300 ms trNOESY spectrum at 2 °C. (C) A 300 ms trNOESY at 20 °C of doxorubicin and TetC is shown for comparison. The 900 ms spectra at 30 °C of sialic acid and 3'-sialyllactose are shown in (D) and (F), respectively, for comparison. (E) Addition of 3'-sialyllactose to the mixture of sialic acid and doxorubicin in (B) shows that 3'-sialyllactose displaces doxorubicin from binding TetC at 2, 10, 20, or 30 °C. A representative 300 ms trNOESY spectrum at 2 °C is shown. One representative cross-peak corresponding to each ligand has been boxed: sialic acid; doxorubicin; 3'-sialyllactose.

alter the rate of exchange between ligand and TetC to thereby increase the possibility for detection of trNOEs in some cases. However, sialic acid did not bind to TetC at 2, 10, 20, 30, or 37 °C, as evidenced by the presence of negative cross-peaks in the spectra at all of these temperatures. When doxorubicin was added to the mixture of sialic acid and TetC, binding was observed for doxorubicin, confirming that the integrity of the protein was intact (Figure 6B). Since 3'-sialyllactose is known to bind Site-1 in BoNT, we next added this ligand to the mixture of TetC, sialic acid, and doxorubicin. In this case, 3'-sialyllactose was observed to displace doxorubicin from Site-1 (Figure 6E). This result suggests that 3'-sialyllactose has a higher binding affinity than doxorubicin for Site-1.

Conclusion. The ultimate goal of this work was to use a combination of computational, ESI-MS, and NMR methods to obtain an optimal set of small molecules for use in rational design of a bidentate ligand that binds with high specificity and affinity to the *Clostridium* neurotoxins. In the present work, a new site (Site-2), which is highly conserved in the structures of these toxins, was identified by computational mapping of the TetC surface. The ACD was computationally screened for small molecules to dock into Site-2, and a small subset of structurally diverse molecules with best fit were screened experimentally for binding activity by ESI-MS. The 6 out of 11 ligands that tested positive for binding by MS were further screened individually and in mixtures for binding in solution by NMR. If the trNOESY

experiment indicated the ligand bound, competitive assays were used to provide insight into whether two or more compounds bind to the same site or to different sites. However, the possibility of noncompeting ligands binding to the same site could not be excluded, and the exact binding site on TetC could not be determined. Nevertheless, in the cases where competition could be observed, the relative binding affinities of two or more ligands binding to the same site could be estimated. The NMR binding studies were also carried out under biologically relevant conditions and provided information on whether ligands and target protein are compatible in similar solvents.

The results suggest that the best ligands from each site to use for preparing bidentate detection agents for the *Clostridium* neurotoxins are doxorubicin or 3'-sialyllactose (Site-1), lavendustin A, Trp-Glu-Try, and MP-biocytin (predicted Site-2), and SQNYPIV, (third independent site). Doxorubicin, 3'-sialyllactose, and lavendustin A have a distinct advantage over the peptides SQNYPIV and Trp-Glu-Try because they are not subject to degradation by peptidases, and over MP-biocytin since they remain bound when the other soluble compounds are present. In addition, doxorubicin may prove to be more useful in certain applications than 3'-sialyllactose because it tends to bind TetC over a wider range of temperatures, solvent conditions, and concentrations (data not shown). These properties are especially useful in the development of robust chemical sensors that will be exposed to environmental samples or body fluids. The

results obtained by funneling a large database of potential compounds down to a few of the most likely combinations to link together should significantly reduce the effort and cost of the synthetic chemistry involved.

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Supporting Information Available: ESI-MS spectra of TetC and TetC/SQNYPIV complex, NOESY spectra of various mixtures of ligands in the absence and presence of TetC, and the expanded region of the aromatic region of NOESY spectra of the Tyr-Glu-Trp, lavendustin A, and TetC mixture showing intermolecular NOEs (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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