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Paclitaxel is an Inhibitor and its Boron Dipyrromethene Derivative is a Fluorescent Recognition Agent for *Botulinum* Neurotoxin Subtype A

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Abstract

We have successfully identified one new inhibitor and one new fluorescent recognition agent for the *botulinum* neurotoxin subtype A (BoNT/A) using the virtual screening protocol Protein Scanning with Virtual Ligand Screening (PSVLS). Hit selection used an in-house developed Holistic Binding scoring method. Selected hits were tested experimentally for inhibitory activity using Fluorescence Resonance Energy Transfer (FRET) assays against the light chain (catalytic domain) of BoNT/A. Ligand binding was determined against the light and heavy chain BoNT/A complex either through radiolabeled ligand binding assays (non-fluorescent ligands) or fluorescence intensity assays (fluorescent ligands). These experimental assays have confirmed one compound (paclitaxel) to inhibit BoNT/A's proteolytic activity experimentally with an IC₅₀ of 5.2 μM. A fluorescent derivative was also confirmed to bind to the toxin, and therefore is a suitable candidate for the rational design of new detection agents and for the development of fluorescence-based multi-probe detection assays.

Keywords

botulinum neurotoxin subtype A; botulism; *botulinum* toxin inhibitors; detection agents; fluorescent probes; virtual screening; holistic binding; probe discovery

1. INTRODUCTION

Botulinum neurotoxin (BoNT) is considered the most poisonous toxin known (<http://www.cdc.gov>). It is extremely potent and lethal, and it is also easy to produce, transport, and use for weaponry. ^{1–2} Inhalation or ingestion of BoNT causes a neurological syndrome (botulism) that can lead to death by paralysis of the breathing muscles within 24 hours. ³

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Supporting information. The following data are provided as supplementary material in a single pdf file: 2D ligand-protein interaction diagrams for (a) paclitaxel, (b) pac-BDP, (c) pac-FL, and (d) pac-OG bound to BoNT/A, force field and Holistic Scores for each ligand in the screening library docked to each of 33 binding sites in the target protein, and force field and Holistic Scores for the best conformer per ligand found to be in the catalytic site of the target protein. This material is available free of charge at <http://pubs.acs.org>.

The toxin prevents the release of the transmitter acetylcholine (ACh) in synapses and neuromuscular junctions, thus impeding communication between neurons and muscle cells, which results in muscle paralysis.² The toxin prevents release of the transmitter ACh by cleavage of specific soluble N-ethylmaleimide-sensitive attachment protein receptor (SNARE) through the toxin's light chain (a zinc-dependent endoprotease). All seven BoNT subtypes, labeled A to G, prevent ACh release.^{2, 4–8} However, the specific SNARE protein and cleavage site targeted vary among the seven serotypes. Serotypes A, C and E cleave synaptosome-associated protein of 25 kDa (SNAP-25), each at a unique peptide bond. Serotype C also cleaves syntaxin. Serotypes B, D, F, and G target synaptobrevin (also known as vesicle-associated membrane protein). All serotypes require substrates with a minimum of 40 amino acids for efficient cleavage.⁹ The subtype A toxin is responsible for the highest mortality rate in botulism.¹⁰ *Botulinum* neurotoxins are synthesized as inactive single chain proteins, cleaved, and released as two-chain (a 100 kDa heavy chain and a 50 kDa light chain) complexes. The heavy (HC) and the light chain (LC) are bound together by one disulfide bond (this complex is referred to throughout the text as whole BoNT/A).

The mechanism of action and potency of BoNT makes it an effective therapeutic agent. Successful treatment of over 50 conditions involving hyperactivity of nerves communicating to various muscles or glands have been reported in the literature in recent years.^{11–17} These conditions range from incontinence, spasticity, and focal dystonia (sustained contraction of muscles), to pain. BoNT is particularly useful for managing symptoms of Parkinson's Disease (PD), such as tremors, dystonias, sialorrhea (drooling), and constipation.^{13–14, 18–21} However, the most notorious use of BoNT is cosmetic to temporarily reduce the appearance of wrinkles.²² Under brand names such as Botox® (Allergan, Inc.) and Dysport® (Medicus Pharmaceutical Co.) BoNT injections have become the most performed non-surgical cosmetic procedure in the world.^{22–25} Although effective, the clinical use of BoNT is not without risk. Recent findings have shown that locally injected BoNT may affect unintended sites, either directly or indirectly,^{26–32} and may induce long-term undesirable effects.^{33–34}

BoNT's cosmetic, medical and homeland security importance³⁵ has fueled an intense search for small molecule inhibitors of its protease activity. Several studies^{35–43} have reported novel inhibitors for BoNT/A which are promising drug candidates, all of them with activities in the micromolar range. Four small molecule inhibitors based on the 4-amino-7-chloroquinoline scaffold of anti-malarial drugs³⁶ were discovered using structure-based pharmacophore elucidation and optimization. These inhibitors were found to have IC₅₀s from 3.2 to 17 μM against the *botulinum* neurotoxin subtype A light chain (BoNT/A LC). Computer-assisted design of BoNT/A-specific hydroxamate inhibitors led to a compound that discriminates between BoNT/A LC and BoNT/B LC.⁴² Pharmacophore search was also used to identify BoNT/A LC inhibitors from the National Cancer Institute Open Repository (NCIOR).³⁷ This search led to the identification of compound NSC 240898 (K_d = 4.6 μM), deemed to be a promising lead for the development of novel therapeutics. A similar search was used to identify another inhibitor from the NCIOR possessing a previously unidentified scaffold, diazachrysenone.⁴¹ This scaffold was used to refine existing pharmacophore models, leading to the identification of a non-zinc coordinating inhibitor with K_i = 600 nM.^{38, 41} Peptidomimetics successfully resulted in a potent inhibitor (K_i = 41 nM) of BoNT/A LC.⁴³ High-throughput screening was successful⁴⁰ in identifying two lead compounds with *in vivo* activity that represent previously unrecognized chemical scaffolds for the development of therapeutic agents to treat BoNT exposure. Out of 12 compounds found to inhibit BoNT/A LC experimentally with IC₅₀s in the 1–95 μM range, only 6 caused 35% or more decrease in SNAP-25 cleavage in cell-based assays. Surprisingly, only 2 of the 12 confirmed inhibitors showed *in vivo* activity.⁴⁰ High-throughput screening combined with Structure-activity Relationship (SAR) studies were used to develop inhibitors containing the hydroxamate moiety on different scaffolds in an attempt to minimize the promiscuity for

other proteases inherent to this metal chelator.³⁹ Irreversible inhibitors of BoNT/A LC have also been reported.⁴⁴

Despite all the efforts, the discovery and development of new inhibitory agents for therapeutic use has had limited success translating from *in vitro* to *in vivo* activity.^{40, 45–46} The inhibitory activity of most of these small molecule inhibitors is based on the coordination of the zinc cation (Zn^{+2}) in the active site of the toxin,^{37, 40, 42, 46} which is a common feature of zinc-dependent metalloproteases. These compounds tend to lack sufficient affinity because their binding is mostly due to zinc coordination and not anchored to specific atomic interactions to amino acids within the pocket of the protein. The identification of inhibitors with a different mode of binding is, thus, desirable.

In this paper, we report the successful identification of a new inhibitor and a new fluorescent recognition agent for BoNT/A using a computer-assisted discovery methodology. These compounds were identified by an in-house developed methodology, Protein Scanning with Virtual Ligand Screening (PSVLS), based on the *HierVLS* protocol for virtual screening,^{47–48} and a new in-house developed scoring function called Holistic Binding (Floriano et al., in preparation). The fluorescent derivative was shown experimentally to bind to BoNT/A complex, the form in which the toxin is distributed throughout the body, and is useful for the development of novel detection assays for BoNT/A. The inhibitor reported here represents a previously unidentified class of inhibitors and may lead to development of high-specificity BoNT/A inhibitors for therapeutic use. The results presented in this study indicate that Holistic Binding is an efficient approach for the identification of recognition agents for BoNT.

2. METHODS AND PROCEDURES

2.1. Overall Strategy

The PSVLS methodology was used to computationally screen a library of 1,624 chemical compounds. This virtual library was designed to include compounds readily available commercially with a radioactive label (3H or ^{14}C) to facilitate experimental confirmation of lead candidates. Binding scores for each chemical compound (ligand) in the screening library were calculated using the Holistic Binding scoring function, which requires the molecular docking of each ligand into all available binding pockets within the target protein. Ligands were ranked by Holistic Binding scores and 5 were selected for experimental confirmation of binding. These included 1 compound predicted to be a strong inhibitor, 2 compounds predicted to have borderline affinity for the catalytic site of the toxin, 1 compound known to delay onset of botulism and predicted to have borderline affinity by Holistic Binding, and 1 compound predicted to be inactive (a “non-binder”). Selected hits were tested for binding to the target protein and for inhibition of catalytic activity. Ligand binding was determined using radiolabeled ligand binding assays, while inhibition of catalytic activity was determined using Fluorescence Resonance Energy Transfer (FRET) assays. Inhibition assays were performed for the recombinant light chain of BoNT/A, which is catalytically active, while binding assays used non-activated BoNT/A heavy and light chain complex. Confirmation of binding to whole BoNT/A was important because the catalytic site is expected to be partially occluded in the whole BoNT/A,^{49–51} which may affect efficacy of the inhibitor *in vivo*. Three commercially available fluorescent derivatives of one confirmed hit (paclitaxel) were docked computationally to whole BoNT/A, and tested experimentally for binding to whole BoNT/A using fluorescence intensity. The objective was to investigate whether any of these derivatives might be useful as a recognition agent in a detection assay.

In addition to in-house developed software for PSVLS and Holistic Binding score calculations, the programs Yasara (www.yasara.org) and MOE (www.chemcomp.com) were used for generation and manipulation of 3D structures, and for structural analysis of docked complexes. All chemical compounds were purchased from Sigma Aldrich (www.sigmaaldrich.com) and Cedarlane Labs (www.cedarlanelabs.com). Toxin was obtained from List Biological Laboratories (www.listlabs.com) and from Metabionics (www.metabionics.com). PSVLS calculations were performed on High Performance Computer Clusters running the Linux operating system.

2.2. Computational Screening

Selection and preparation of the ligand database to be computationally screened—In order to facilitate the experimental validation of the PSVLS/Holistic Binding scoring methodology, we constructed a database of chemical compounds that are readily available for purchasing with a ^{14}C or a ^3H radioactive label. Compounds for virtual screening were selected from the Available Chemicals Directory (ACD) (Symyx, Inc.) through DiscoveryGate (www.discoverygate.com). We searched for compounds containing “ H^3 ” or “ C^{14} ”. Chemical structures were downloaded in sdf format (2D) and converted to three-dimensional (3D) structures using MOE (www.chemcomp.com). Ligand structures were stripped of counter-ions. Gasteiger atomic charges were assigned to each ligand in the database. The ligands were subject to geometry optimization followed by energy minimization using the Dreiding force field.⁵² The final optimized 3D structures were saved as multiple-ligands in mol2 format and used for virtual screening.

Preparation of the target protein for structure-based virtual screening—The experimentally-determined 3D structure of the target protein, *botulinum* neurotoxin subtype A, was downloaded from the RCSB Protein Data Bank (www.rcsb.com). We used the structure identified by the PDB code 2NYY (2.6 Å resolution) which corresponds to whole BoNT/A (heavy and light chains). The structure was checked for missing atoms and/or segments, and overall structural quality using the software packages Yasara (www.yasara.org) and/or MOE (www.chemcomp.com). Ligands, co-factors, and counter ions present in the pdb file were removed, and missing atoms/segments were added to the structure. Missing segments Ala490-Glu492 and Thr623-Lys626 were copied from PDB structure 3BTA (BoNT/A at 3.2 Å resolution). The complete structure was assigned CHARMM22⁵³ atomic charges and energy-minimized for 2,000 steps of conjugate-gradient minimization using the Dreiding force field.⁵²

Protein Scanning with Virtual Ligand Screening (PSVLS)—In this in-house developed approach, the 3D structure of a target protein is scanned computationally for potential binding sites using the program *Pass*.⁵⁴ This program is one of many methods available for identification of binding pockets in proteins.^{55–64} *Pass* is freely distributed, efficient, and its outputs are compatible with the downstream applications in PSVLS, making it a convenient choice for our approach. *Pass* uses a geometric approach to identify voids in protein structures fast and accurately. The size, shape and burial extend of these voids are used to assess their likelihood to represent binding pockets. A virtual library of chemical compounds is screened against each available binding pocket identified by *Pass* within the target protein using molecular docking. Virtual screening is performed using the *HierVLS* protocol,⁴⁷ with parameters adjusted to produce more accurate results at the expense of computational time. *HierVLS* is a computational protocol for fast screening of large chemical compound libraries.⁴⁷ *HierVLS* uses a hierarchical approach: a large number of bound configurations are created in the least computationally expensive step for each ligand. Subsequent steps reduce the number of bound configurations per ligand while increasing the computational time required to process them, thus increasing accuracy. The

last step includes the calculation of the binding energy with solvation for the best surviving bound configuration of each ligand. PSVLS generates 3D structures and calculates force field-based binding energies for each ligand bound to each binding region of the target protein. For each binding region, i , force field-based binding energies (FF_BE) are calculated as the difference between the sum of potential (PE) and solvation (SE) energies of the bound state minus the unbound state:

$$FF_BE_i = (PE + SE)_{BoundComplex} - (PE + SE)_{UnboundProtein} - (PE + SE)_{UnboundLigand} \quad (EQ\ 1)$$

Ligands with favorable (more negative) calculated binding affinities to the target binding site are potentially active compounds.

The 1,624 ligands in the virtual library were computationally screened against each one of the 33 binding regions identified in the BoNT/A structure (pdb code 2NYY) using PSVLS. This virtual screening generated $1,624 \times 33 = 53,592$ bound structures and respective calculated binding energies for analysis. PSVLS calculations were performed through the *Orunmila* (formerly known as *Cassandra*)⁴⁸ graphical user interface on a Linux-based High Performance Computer Cluster. The average time for each ligand docked into all 33 binding regions of the BoNT/A structure was 35 hours on one core of a Intel Core 2 Quad core processor, and each run generated >200 GB of data.

Selection of PSVLS Hits—The force-field based scores calculated in PSVLS for each ligand bound to a targeted site within the protein reflects the affinity of the ligand for that particular site. Once in close proximity of each other, however, on-target but off-site binding events may take place. These events are reflected in the binding constants measured experimentally but are not directly considered, to our knowledge, by any of the current methods for calculating binding affinities. In our Holistic Binding scoring approach, the binding scores for each ligand are calculated as a function of the force field-based binding energies calculated by PSVLS at each available binding pocket. The force field-based binding energies are scaled by a propensity factor (second term in equation (2)) that reflects the ratio between the force field-based binding energy for region i and the sum of the force field-based binding energies for all n regions:

$$HBS_i = FF_BE_i \times \frac{FF_BE_i}{\sum_{j=1}^n FF_BE_j} \quad (EQ\ 1)$$

The result of this approach is a set of ligands ranked by binding affinity for a particular binding site relative to all other potential sites within the target protein.

The results of PSVLS/Holistic Binding scoring were analyzed in order to identify potential inhibitors targeting the catalytic region of BoNT/A. Ligands with less than 70% of their molecular surface buried into the binding region within the protein were eliminated from the list, regardless of binding energy. Overly charged ligands and ligands with phosphate groups were excluded from consideration to avoid bias introduced by the limitations of the force field in describing correctly the inter-atomic interactions for these ligands. Remaining ligands were ranked according to their calculated Holistic Binding scores. Ligands with better (more negative) Holistic Binding scores are more likely to bind experimentally to the target protein.

2.3. Experimental Confirmation of Selected Hits

Inhibition Assay—Catalytic activity of BoNT/A was determined using fluorescence resonance energy transfer (FRET) in an assay that requires no separation of free from bound ligands. SNAPtide® (FITC/DABCYL) (List Laboratories, Inc), a synthetic quenched fluorescent peptide containing the native cleavage site for *botulinum* toxin type A, was used in competition assays against the ligands selected for testing. Cleavage of the substrate by BoNT/A releases the fluorophore (FITC) and full fluorescence is restored. The increase in fluorescence intensity is directly proportional to the amount of cleavage that has occurred and thus allows for accurate measurement of enzymatic activity.

Light chain BoNT/A recombinant protein (BoNT/A LC) was purchased from List Laboratories, Inc., and reconstituted in a buffer of 20 mM HEPES and 0.1% TWEEN-20 at pH 8.2. Enzymatic activity was determined prior to ligand binding experiments for 10 nM of BoNT/A LC in a FRET assay using SNAPtide® unquenched calibration peptide (List Laboratories, Inc.). The correlation coefficient for a linear fitting of concentration versus Relative Fluorescence Units (RFU) was 0.996 for a concentration range from 0.0125 μ M to 0.8 μ M of substrate.

SNAPtide® substrate was reconstituted following the manufacturer's instructions. A final concentration of 8 μ M SNAPtide® per well was used for the competition assays. Ligands were purchased from Sigma-Aldrich, Inc., and from Cedarlane Laboratories. These were: paclitaxel [CAS 33069-62-4] from *Taxus brevifolia* (95% purity) and semi-synthetic (97% purity); D-(-)-fructose [CAS 57-48-7] (99% purity); desmosine [CAS 10019-68-8] (99.5%±0.2% purity); aminopterin [CAS 54-62-6] (96% purity); monensin [CAS 22373-78-0] (90–95% purity). Ligands were prepared as 1 mM stock solutions and diluted appropriately. DMSO, deionized water, 2.0M aqueous NaOH, or ethanol was used as solvent, according to the solubility profiles of each ligand. Whenever DMSO was used as solvent, its final concentration in the assay was kept at less than 2% of the volume, to prevent inhibition of cleavage by the solvent. Ligands were tested in triplicate for a fixed 10 nM concentration of toxin in a volume of 250 μ l per well. For the competition assays, substrate was added to the mixture of toxin and ligand. Assays were performed in 96-well black polystyrene microplates (OptiPlate, PerkinElmer, Shelton, CT, USA, <http://las.perkinelmer.com/>). FRET assays were run at 37°C after 1 hour of incubation. Excitation wavelength was set to 490 nm and emission was set to 523 nm, with a cutoff filter at 495 nm. Fluorescence intensity was recorded 1 hour after mixing using a Synergy 4 microplate reader. The percent of inhibition caused by each ligand at each concentration tested was calculated relative to the fluorescence intensity measured for uninhibited cleavage of SNAPtide®, which was determined in triplicate for the same concentrations of substrate and toxin used in the competition assays.

Dose-response curve—A dose-response curve was determined for paclitaxel using the FRET assay described above. A final concentration of 8 μ M SNAPtide® and 10 nM of BoNT/A LC in a total volume of 200 μ l per well was used for the competition assays. Assays were conducted in triplicate at 37°C. Paclitaxel and toxin were incubated for 30 minutes prior to the addition of SNAPtide®. The DMSO effect on the assay was taken into account using controls with concentrations varied from 0.0008% to 40%. EDTA (ethylenediaminetetraacetic acid) was used as an inhibitor control at varying concentrations (10 mM–10 μ M). EDTA is known to have an inhibitory effect on BoNT/A LC enzymatic activity.^{65–66}

Immobilization of BoNT/A to magnetic beads for separation—For assays requiring separation of free from bound ligands, biotinylated BoNT/A (heavy and light

chains) was immobilized onto streptavidin-coated magnetic purification beads (BcMag-streptavidin from Bio-Clone, Inc., www.bioclon.com). Biotinylated whole BoNT/A was purchased (Metabio, Inc.) as 1 µg/ml solution in standard buffer. The beads were washed three times with deionized water prior to toxin immobilization, and the supernatants were discharged. Magnetic beads (2 µl) and whole BoNT/A solutions corresponding to 0.2 µg of toxin were incubated for 30 minutes at room temperature at pH 7.4. Loaded (toxin-attached) beads were washed with buffer (25 mM HEPES, 0.3 mM ZnCl₂, and 0.2% TWEEN-20) to remove the excess of protein. Protein concentration was determined for the supernatants of the immobilization and washing steps by absorbance at 280 nm using 200 µl aliquots. The amount of streptavidin released from the beads during washing steps was estimated for aliquots without toxin. Loaded beads were re-suspended in buffer prior to use. For separation, microcentrifuge tubes containing suspended beads were placed in a magnetic separator (Bio-Clone, Inc.) until the supernatant became clear and could be aspirated (2–3 minutes). For all assays, non-specific binding to the beads was determined by mixing ligands with beads, without toxin, under the same conditions of the assays being performed. Total binding was determined based on reading of the beads and supernatant fractions.

Ligand Binding Fluorescence Intensity (FI) Assays—Fluorescent paclitaxel derivatives were tested for binding against whole BoNT/A using fluorescence intensity assays. The toxin was attached to magnetic beads to facilitate separation. The samples containing ligand and toxin were read relative to reference samples of the fluorescent ligands in buffer. Buffer was used as blank. Non-fluorescent paclitaxel was used as negative control. Ligands were added to toxin-loaded suspended beads and incubated for 1 hour prior to reading. Beads were separated from supernatant using a magnetic separator. Fluorescence intensity was determined for both fractions. Bead fractions were re-suspended in buffer for reading. Fluorescent derivatives of paclitaxel were purchased from Invitrogen, Inc.: Oregon Green 488 paclitaxel [CAS 301844-13-3] (pac-OG); BODIPY® FL paclitaxel [CAS N/A] (pac-FL); BODIPY® 564/570 paclitaxel [CAS N/A] (pac-BDP). Stock solutions for the ligands were prepared according to manufacturer's recommendations. Ligands were tested in triplicate at 10 µM, for a fixed 10 nM concentration of toxin. For these assays, the whole BoNT/A toxin (List Laboratories, Inc.) was used. Whole BoNT/A was reconstituted in a buffer of 20 mM HEPES, 0.3 mM ZnCl₂, and 0.2% TWEEN-20 at pH 8.2. BoNT/A was not activated with DTT for this assay. The MW of whole BoNT/A is 150,000 Daltons. Ligands and toxin were incubated in the same buffer used to reconstitute the toxin for 1 hour at 37°C prior to reading. The concentration of toxin was determined by absorbance at 280 nm. Excitation and emission wavelengths were set according to the fluorophores to be tested (excitation/emission): BODIPY® FL (505nm/515nm), BODIPY® 564/570 (565nm/571nm), and Oregon Green 488 (496nm/524nm). The BODIPY® fluorophores are boron dipyrromethene derivatives. BODIPY® FL is 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacene-3-propionic acid, whereas BODIPY® 564/570 is 4,4-difluoro-5-styryl-4-bora-3a,4a-diazas-indacene-3-propionic acid. Oregon Green® 488 (2',7'-difluorofluorescein) is a fluorescein analog.

Radiolabeled Ligand Binding (RLB) Assays—We used radiolabeled ligands to probe binding of selected ligands to whole (heavy and light chains) BoNT/A. Ligands found to inhibit catalytic activity of BoNT/A LC do not necessarily bind to whole BoNT/A because the catalytic site is believed to be inaccessible in whole BoNT/A,⁶⁷ which is the form found in the bloodstream.⁶⁸ Since binding to whole BoNT/A may contribute positively to *in vivo* activity of the inhibitors, we tested confirmed inhibitors for binding to the heavy and also to the light chain BoNT/A complex.

Radiolabeled ligands were purchased from Sigma (St. Louis, MS): D-fructose-UL-¹⁴C at 1.0 mCi/ml, with specific activity of 300 mCi/mmol, and purity 95%, as determined using

HPLC by the manufacturer; and Paclitaxel-(2-benzoyl-ring-UL- ^{14}C) at 0.25 mCi/ml, specific activity of 50.7 mCi/mmol, and purity of 99.9% determined using HPLC by the manufacturer. The assay buffer was 25 mM HEPES with 0.2% TWEEN-20 at pH 8.2. Radiolabeled ligands were incubated with whole BoNT/A attached to magnetic beads for 1 hour at room temperature. D-(-)-fructose was tested at 10 μM (1.2 uCi) and paclitaxel was tested at 25 μM (0.5 uCi). The final assay volume was 400 μl per vial. The supernatant fraction was separated from the beads fraction using a magnetic separator. Aliquots of 3 ml of EconoSafe scintillation cocktail (Sigma-Aldrich, Inc.) were added to each vial. Counting was performed using both fractions in duplicate samples, on a Beckman LS6500 scintillation counter. Each vial was counted for 10 minutes. This counting time was determined sufficient to reduce the standard deviation to less than 1% of the mean counts per minute. Total and free radiolabeled ligand concentrations were estimated from counting beads and supernatant fractions. Non-specific binding to the beads was determined as described earlier.

Scintillation Proximity Assay (SPA)—We used SPA to confirm the dose-dependent binding of paclitaxel to BoNT/A LC. Streptavidin-coated Polyvinyltoluene SPA beads were purchased from PerkinElmer, Inc., and reconstituted in 20 mM HEPES buffer at pH 7.4, 0.1% Tween 20, and 0.3 mM ZnCl_2 . Paclitaxel [2-benzoyl ring-3H] at 1 mCi/ml, with specific activity of 60 mCi/mmol, and purity of 99% determined using HPLC by the manufacturer was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). BoNT/A LC (30 μg) was biotinylated using ChromaLink™ Biotin One-Shot Antibody Labeling Kit (Solulink, Inc). At each ligand concentration, Paclitaxel [2-benzoyl ring-3H] and 7 nM biotinylated BoNT/A LC were incubated for 15 minutes at room temperature before addition of SPA beads at 2 mg/ml per well. The assay was run in triplicate at the final volume of 200 μl per well, with readings at 1 hour intervals. Scintillation was counted on a MicroBeta2 plate counter (PerkinElmer, Inc) and corrected for non-specific binding/non-proximity effects.

3. RESULTS AND DISCUSSION

3.1. PSVLS/Holistic Binding scores

Results from PSVLS consist of 3D structures for each ligand bound into each binding cavity of the target protein, and their respective force field-based binding energies. A total of 53,592 protein ligand complexes were generated for the 1,624 screened ligands bound to 33 binding cavities in the BoNT/A structure. The 3D structure for BoNT/A used in the PSVLS calculations and the location of the centers for the 33 binding cavities identified in this structure are shown in Figure 1. Some of these binding regions correspond to known binding sites for substrates (Catalytic Site), and for polysialogangliosides and protein receptors which bind to the Receptor Binding Domain. The other sites are uncharacterized and may or may not function as allosteric sites. They are, however, cavities large enough to accommodate the binding of a small organic molecule. Holistic Binding scores were calculated for each binding site of BoNT/A by combining the force field-based energies, corrected for solvation, according to equation EQ 2. At each binding site targeted for docking, these scores represent the binding affinity of a ligand for that particular site relative to all the other sites. In this paper, we report only the analysis of the Holistic Binding scores for the catalytic site of BoNT/A, even though ranked lists of ligands were also obtained for other sites. The Holistic Binding scores for each one of the 1,624 ligands screened bound to the catalytic site of BoNT/A are shown in Figure 2. The mean Holistic Score for the entire library in the catalytic site is -1.9 kcal/mol. The mean scores for the other 32 regions ranged from -0.9 kcal/mol to -2.8 kcal/mol.

Most ligands in a random library are not expected to have specific affinity for the target protein. Using the binding energy definition in equation (EQ 1), these “non-binders” should

produce scores equal to or above zero (unbound state being more energetically favorable than bound state). However, non-specific intermolecular interactions with the protein may produce below zero scores for these “non-binders”. In terms of score distribution, this means that the vast majority of ligands in a library will score within certain values while very few ligands, if any, with specificity for the protein target will produce much better scores (more negative, in the case of our scoring function). The threshold score that discriminates binders and non-binders can be estimated from the frequency distribution diagram shown in Figure 3. Most ligands (68%) scored near -2 kcal/mol. The vast majority (87.5%) scored -3.0 kcal/mol and should be considered non-binders. If we optimistically consider that 2% or less of the library may be binders, then ligands with scores equal to or lower than -5 kcal/mol should be considered for experimental testing. This value corresponds to the mean of binding energies (shown as solid line in Figure 2) plus two times the standard deviation for the data set (shown as dashed line in Figure 2). This binder (at or below) versus non-binder (above) threshold is region-specific, and must be determined for each binding region of interest. It is also case-specific and may vary with target protein, screening library, scoring function, and/or virtual screening method.

3.2. Relative Contribution of Other Regions to the Holistic Scores

The contribution from FF binding energies in other regions to the Holistic Scores at the catalytic site can be summarized as follows: except for paclitaxel, all of the other ligands selected had 3 to 8 kcal/mol difference between the FF energy at the catalytic site and at the next best non-catalytic site. This translates into less than 1 kcal/mol difference in Holistic Scores. For paclitaxel, the catalytic region has the highest FF binding energy (-90.9 kcal/mol), followed by three non-catalytic regions with FF binding energies around 80 kcal/mol, which are considerably worse than the FF binding energy in the catalytic site. All three non-catalytic regions are at the interface between the catalytic and translocation domains. The differences in FF energies relative to the catalytic site yield Holistic Scores differences from 2.5 to 2.3 kcal/mol. Aminopterin and fructose have better FF binding energies in other regions of the target protein than in the catalytic site, and this trend is preserved in the Holistic Scores.

3.3. Selection of PSVLS Hits for Experimental Testing

Five ligands were selected for experimental testing (Figure 4). Selection was based on their Holistic Binding scores and ranking at the catalytic site. We chose to focus on the catalytic site because of the existence of a well-established experimental assay to validate the computational predictions about inhibitory activity. After experimental confirmation of the initial 5 ligands, 3 fluorescent derivatives of the best confirmed ligand (paclitaxel) were also docked, scored and tested experimentally for binding. All ligands were re-ranked after inclusion of these fluorescent derivatives to the ranking list. The ligands selected for experimental testing along with the rationale for their selection is presented below. These data are summarized in Table 1.

Paclitaxel has the 6th best score in the catalytic site, and the top three (3) scores are its derivatives. The 4th and 5th best ranking ligands were known complexing agents for removal of heavy metal ions and a surfactant. They were expected to have low selectivity for BoNT/A for reasons discussed earlier and were, therefore, not selected for experimental testing. The catalytic region has the highest binding affinity to paclitaxel (-11 kcal/mol), followed by three non-catalytic regions (27, 5 and 23) with affinities between -8.5 kcal/mol and -8.7 kcal/mol, which are considerably worse than the binding affinity in the catalytic site. These regions contributed the most for the adjustment of the FF binding energy into the catalytic site holistic score. The score for paclitaxel in the catalytic region is well below the

non-binder/binder threshold for the catalytic region (-5 kcal/mol) and, for this reason, we expect paclitaxel to bind strongly and inhibit experimentally BoNT/A LC.

Desmosine binds to the catalytic site of BoNT/A with a score (-5.5 kcal/mol) just above our non-binder/binder threshold. This compound should display some inhibitory activity experimentally.

Aminopterin binds to the catalytic site of BoNT/A with a score (-4.2 kcal/mol) above (worse than) the non-binder/binder threshold, but significantly lower (more favorable) than the median value for the library (-1.9 kcal/mol). Hence, it is expected to exhibit low inhibitory activity, if any.

Monensin, a sodium and proton selective ionophore antibiotic, is known to delay the onset of botulism. Two mechanisms have been suggested for Monensin's ability to delay muscular paralysis: control of endosomal acidification which does not involve direct binding to the toxin,⁶⁹ and inhibition of toxin-induced channel formation.⁷⁰ In our calculations, monensin was found to bind to the catalytic site of BoNT/A with a score of -4.1 kcal/mol. This value is above (worse than) the binder/non-binder threshold of -5 kcal/mol, but it is much better than the median (non-binder) value of -1.9 kcal/mol, similarly to aminopterin. We expect this compound to have low to no inhibitory activity.

Fructose has a score of -1.8 kcal/mol at the catalytic site, which places this compound in the "non-binder" category. Hence, this compound is expected to be inactive experimentally as inhibitor of BoNT/A catalytic activity. The best score for fructose across all binding regions is -1.9 kcal/mol (region 17). Therefore, fructose is a good choice for negative control in the binding experiments targeting both whole BoNT/A as well as BoNT/A LC. The experimental confirmation of a non-binder is a good test of the prediction power of the overall methodology.

3.4. Inhibition of BoNT/A Catalytic Activity by Selected Hits

The results of the FRET assay against the light chain (catalytic domain) of BoNT/A are shown in Table 2. These preliminary experimental assays indicated that paclitaxel inhibits the botulism toxin's proteolytic activity experimentally by 95% at $10\text{ }\mu\text{M}$ (semi-synthetic paclitaxel). Additional assays have found that paclitaxel isolated from natural sources inhibit BoNT/A LC by 85% at $10\text{ }\mu\text{M}$. A dose-response curve, shown in Figure 5(a), was constructed for paclitaxel using the FRET assay. A four-parametric nonlinear regression model (Prism5, GraphPad Inc.) determined the IC_{50} for paclitaxel to be $5.2\text{ }\mu\text{M} \pm 1.1\text{ }\mu\text{M}$ over two independent experiments. The dose-response curve for EDTA (known to inhibit catalytic activity) is also shown in Figure 5(a) for comparison. Paclitaxel is solubilized using DMSO as co-solvent, which was found to be well tolerated in the SNAPtide assay for up to 25% DMSO.⁷¹ Our results indicate that the fluorescence intensity produced by fixed concentrations of toxin and SNAPtide is stable up to 4.8% DMSO. At higher DMSO concentrations, the effect of the solvent needs to be taken into account. The concentration-dependency of paclitaxel binding to BoNT/A LC was confirmed by a dose-response curve, shown in Figure 5(b), constructed using the SPA assay described earlier.

Paclitaxel is a marketed drug used for cancer treatment. It targets tubulin, interferes with the dynamic instability of microtubules and, thus, prevents cell division.⁷² The use of paclitaxel as a therapeutic agent for botulism is likely to be undesirable because of its insolubility and its toxic effects. However, paclitaxel may provide a good starting point for the rational design of novel inhibitors for therapeutic use. Other terpene (farnesyl pyrophosphate -5.7 kcal/mol), terpene precursor (geranylgeranyl pyrophosphate -5.2 kcal/mol), and taxane (docetaxel -5.8 kcal/mol) were found to have favorable Holistic Binding scores in our

calculations. Although they have not yet been confirmed experimentally, these compounds may provide additional assistance for rational design of potent BoNT/A inhibitor. The finding that paclitaxel inhibits BoNT/A may also have implications for the development of new anti-cancer treatments. Zinc deficiency was found to reduce paclitaxel efficacy in LNCaP prostate cancer cells.⁷³ This fact combined with our results for BoNT/A inhibition suggests that paclitaxel inhibit zinc-dependent proteases present in early-stage cancerous cells, in addition to its well-documented role as a disruptor of microtubule function. LNCaP cells are a model for early-stage prostate cancer and this additional role for paclitaxel may help in the identification of early-stage biomarkers for prostate cancer. The zinc-dependent effect was not observed in PC3 cell lines, which are a model for late-stage prostate cancer.⁷³

As predicted by the analysis of Holistic Binding scores, fructose shows no inhibitory activity against BoNT/A. The ligands predicted to be borderline for binding showed low inhibitory activity. Monensin displayed 38% inhibition at 10 μ M, which is better than anticipated and suggests that direct inhibition may be a secondary mechanism to the ones already proposed for this ligand's ability to delay onset of botulism.^{69–70} Desmosine and aminopterin showed low inhibitory activity (25% and 11% at 10 μ M), consistent with their Holistic Scores. Both are at the non-binder/binder threshold. Aminopterin showed intrinsic fluorescence at 100 μ M with emission peak at 463 nm, and may interfere with fluorescence readings at high concentrations.

The force field binding energies (FF_BE) and ranking for each one of the compounds discussed above is also provided in Table 1 for comparison. The non-binder/binder threshold for the catalytic region of BoNT/A using FF_BE values is -106 kcal/mol. Under the FF_BE ranking, only aminopterin would have been considered a “binder”, and the identification of paclitaxel would have required the experimental testing of 5.6% of the screening library. These results emphasize the better performance of the holistic binding scheme compared to traditional force field-based scoring.

3.5. Paclitaxel Fluorescent Derivatives

In addition to the search for novel potent inhibitors, fast and reliable detection of BoNT/A is also of concern. The standard test for detection of BoNT is still the mouse lethality assay.⁷⁴ This assay is laborious, expensive, time-consuming, and ethically controversial. Other technologies are available^{74–80} but none is ideal for fast, low cost and portable devices that accurately detect the presence of the toxin in a variety of field, clinical and food samples. Existing detection technologies are based on the catalytic activity of BoNT/A. However, the BoNT/A catalytic site is believed to be occluded when the light and heavy chain are connected.⁶⁷ Since the toxin only releases its light chain under reducing conditions, the efficacy of these assays depends on the breakage of the disulfide bond connecting the light and heavy chains. Therefore, the discovery of small molecules suitable as recognition agents for BoNT/A is desirable.

Upon experimental confirmation of paclitaxel as an inhibitor of BoNT/A, we investigated fluorescent derivatives of paclitaxel that could be used as recognition agents for detection of BoNT/A. Three commercially available derivatives were tested experimentally for binding to whole BoNT/A. They were tested against whole BoNT/A because the catalytic site of BoNT/A is believed to be partially occluded in whole BoNT/A, and a good recognition agent must be able to detect the whole toxin. This is the form of the toxin before the catalytic domain is translocated with the help of the translocation domain into the cytosol, where neurotransmission is inhibited as a result of SNARE protein cleavage by the catalytic domain.⁶⁷ The paclitaxel fluorescent derivatives were also submitted to PSVLS/Holistic Binding scoring. The intention of these calculations was to verify whether the Holistic

Binding approach could have identified the experimentally positive compounds as “good binders”. All three derivatives scored well (Table 3), but only one of them was found to effectively bind to whole BoNT/A (Table 3). Analysis of the ligand-protein interactions for the best PSVLS complex of each paclitaxel fluorescent derivative reveals that hydrogen bonds to Gln162 are a common feature. The paclitaxel moiety is buried in the binding site in the structures of pac-BDP and pac-FL but more exposed to solvent in the pac-OG structure. The fluorophore (BDP, FL or OG moiety) is exposed in the structure of pac-BDP, somewhat exposed in pac-FL and buried within the binding cavity in pac-OG. This fluorescence intensity assay results against whole BoNT/A follow the same trend. Hydrogen bonds to Gln162, Lys166, Glu64 and Arg536 anchors pac-OG to the site and are likely responsible for a more favorable calculated binding affinity compared to pac-BDP and pac-FL. The ligand-protein interaction diagrams are available as supporting information.

These fluorescent paclitaxel derivatives are used for imaging microtubule formation and for labeling microtubules in solution. Their direct use as BoNT/A recognition agent is limited, as their known target (tubulin) could cause a false positive reading in the assays. However, the experimentally confirmed conjugate (pac-BDP) can be used as a lead for the design of highly-specific fluorescent probes for BoNT/A. This compound can also be useful in multi-probe fluorescence-based detection assays, where elimination of false positives can be accomplished by means of combinatorial response.

3.6. Binding of Paclitaxel to Whole BoNT/A

Inhibitors unable to bind to BoNT/A before the catalytic domain reaches the cytosol may be less effective than inhibitors that target whole BoNT/A. In order to probe whether paclitaxel binds to whole BoNT/A in addition to the exposed BoNT/A LC, radiolabeled ligand binding experiments were carried out for ^{14}C -paclitaxel and ^{14}C -fructose against non-activated BoNT/A complex. Fructose was used as negative control as it is not expected to bind to any form of BoNT/A. The toxin was attached to magnetic beads for separation. Absorption at 280 nm was used to confirm that the beads were loaded with toxin prior to the assay. Paclitaxel was tested at 50 μM and 25 μM , while fructose was tested at 50 μM and 10 μM . The amount of toxin was kept constant. Specific binding of paclitaxel to the whole toxin was consistent at both concentrations (7%). Specific binding of fructose to BoNT/A was 5% at 10 μM and 3% at 50 μM . Binding to non-loaded beads (non-specific binding) was 29% (25 μM) and 23% (50 μM) for paclitaxel, and 3% for fructose at both concentrations. The low solubility of paclitaxel in water is likely responsible for this relatively high non-specific binding. These results (Table 3) indicate that paclitaxel does not bind to whole BoNT/A, even though it inhibits the catalytic activity of the toxin as confirmed by the FRET assay. However, further tests are necessary to eliminate the possibility that the immobilization of the toxin prevented the binding of paclitaxel by making its binding site less accessible. In addition, full biochemical characterization of this previously unidentified inhibitor is necessary to assess its potential as therapeutic agent for botulism.

4. CONCLUSION

We used the Holistic Binding computer-assisted approach to successfully identify a previously unknown inhibitor of BoNT/A catalytic activity, paclitaxel. We showed experimentally that paclitaxel binds (SPA assay) and inhibits (FRET assay) BoNT/A light chain but does not bind to whole BoNT/A (radiolabeled ligand binding assay), while its fluorescent derivative pac-BDP binds to the heavy and light chain BoNT/A complex (fluorescence intensity assay).

The predictions from Holistic Binding are consistent with experimental observations, supporting the use of this technique for discovery of novel inhibitors and recognition agents

for BoNT/A. The concept of non-binder/binder thresholds determined by the mean and standard deviation values for the virtual library was shown to be an effective way to select hits for experimental testing in the case of BoNT/A. Further evaluation of false positive and false negative rates is needed to adequately determine its predictive power. Nonetheless, we expect that non-binder/binder thresholds will be useful in the context of other virtual screening approaches as well.

The success rate of a VLS run largely depends on the size and diversity of the chemical library,⁸¹ and on the fortuitous presence of true binders (positives). In general, more is better and the screening of large libraries has a better chance to yield true binders. Our screening of a small library may seem to have been more successful than one could have expected. However, based on reported success rates for first-tier High Throughput Screening (0.2% to 1.91%),^{65, 82–83} a library of the size we virtually screened might have 3–31 positive compounds. The ability to enrich the top of a ranked list with whatever true binders are present in the library is the main challenge of VLS. The *HierVLS* method used at the core of PSVLS was shown to yield good enrichment factors.⁴⁷ Our present results, albeit not conclusive, indicate that the Holistic Binding approach is efficient at concentrating true binders at the top of the ranked list.

The Holistic Binding approach is a time- and cost-efficient alternative to other discovery approaches, such as HTS. For example, a recent HTS⁶⁵ of 16,544 compounds found 34 BoNT/A inhibitors in the first-tier screening (0.2% hit rate), which were reduced to 9 inhibitors upon a confirmatory ELISA assay (0.05% hit rate). The best inhibitor in this group showed 95% inhibition of the toxin in a FRET assay with 50nM BoNT/A and 5 μ M SNAPtide® substrate. In contrast, our virtual screening of a library ten times smaller readily identified an inhibitor with comparable performance in a similar FRET assay. Although the fortuitous presence of active ligands in the libraries being (experimentally or computationally) screened is certainly a determinant of success, our methodology was shown to be effective at identifying active ligands at a more accessible cost. VLS generally requires expensive High Performance Computer Clusters (HPCCs). However, the recent availability of service-based cloud computing environments such as Amazon Elastic Compute Cloud (EC2) (aws.amazon.com/ec2/) is expected to make virtual screening more affordable.

The need for novel fluorescent recognition agents for BoNT/A is reinforced by the recent development of a multi-wavelength spatial LED illumination based detector that utilizes SNAPtide® as probe.⁸⁴ The detector technology for a fluorescent platform is already in place, but its applicability is curtailed by the limitations associated to the available probes. Although reliable, SNAPtide® only works if the catalytic domain of the toxin is activated and fully available for binding. The development of small-molecule probes capable of working in a wide range of conditions is, thus, desirable and will have immediate application. Within this goal, pac-BDP is currently being used by our group as a lead compound for the structure-based design of new chemical entities with improved affinity and selectivity for BoNT/A. The existence of an experimentally determined structure for paclitaxel-tubulin (pdb codes 2HXF, 2HXX and 1IA0) along with our predicted ligand-BoNT/A structure enables the rational design of analogs with reduced affinity for tubulin and increased affinity for BoNT/A. The same strategy is being applied for the design of novel BoNT/A inhibitors based on paclitaxel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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8. ABBREVIATIONS USED

BoNT	<i>Botulinum</i> neurotoxin
BoNT/A	<i>Botulinum</i> Neurotoxin Subtype A
CAS	Chemical Abstracts Service
EF	Enrichment Factor
FF_BE	force field-based binding energies
HBS	Holistic Binding Score
HC	heavy chain
HierVLS	Hierarchical Virtual Ligand Screening
LED	light-emitting diode
LC	light chain
NCIOR	National Cancer Institute Open Repository
Pa-OG	paclitaxel, 2',7'-difluorofluorescein conjugate
Pa-BDP	paclitaxel, 4,4-difluoro-5-styryl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid conjugate
Pa-FL	paclitaxel, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid conjugate
PE	potential energy
PSVLS	Protein Scanning with Virtual Ligand Screening
RLB	Radiolabeled Ligand Binding
SE	solvation energy
SNARE	specific soluble N-ethylmaleimide-sensitive attachment protein receptor
SNAP-25	synaptosome-associated protein of 25 kDa
VLS	Virtual Ligand Screening

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Figure 1.

Structure of *botulinum* neurotoxin subtype A (BoNT/A) (heavy and light chains) and location of the centers for the 33 binding cavities targeted by *PSVLS*. The structure for BoNT/A corresponds to pdb code 2NYY. Color scheme: Catalytic Domain (Light chain; residues 2–410) is cyan; Translocation Domain (residues 411–871) is gray; Receptor Binding Domain motif 1 (residues 872–1087) is blue; Receptor Binding Domain motif 2 (residues 1088–1293) is green. Zinc in the Catalytic Site is represented as pink spheres. Yellow spheres represent the centers of binding regions identified and targeted for molecular docking by Protein Scanning with Virtual Ligand Screening (PSVLS). Some of these binding regions correspond to known binding sites for substrates (Catalytic Site), and for polysialogangliosides and protein receptors which bind to the Receptor Binding Domain. Figure 1 was generated using the molecular modeling software Yasara (www.yasara.org).

Figure 2.

Holistic Binding scores at the catalytic site for BoNT/A complexes from *PSVLS*. More negative numbers correlate to better binding. Solid line labeled “mean” represents mean value of Holistic Score for the ligand library. Dashed line represents the mean plus two standard deviations (non-binder/binder threshold). Ligands scoring below this threshold may bind experimentally to the target protein.

Figure 3.

Frequency distribution of Holistic Binding scores at the catalytic site. Bars represent the percent of PSVLS complexes that fall into a score bin centered at the plotted value. The Holistic Binding score mean value for this compound library was -1.9 kcal/mol. 87.5% of ligands have scores above -1.0 kcal/mol. 9.6% of the ligands have scores of -4.0 ± 1 kcal/mol. The top 2.8% have scores of -5.0 kcal/mol or better. Biologically active compounds are more likely to be found among the high-ranking (most negative) candidates.

Figure 4.

Chemical structures of the lead compounds discovered by Protein Scanning with Virtual Ligand Screening (PSVLS) combined with Holistic Binding scoring. These compounds were tested experimentally for inhibition and/or binding to BoNT/A. (a) paclitaxel; (b) aminopterin; (c) desmosine; (d) fructose; (e) monensin.

Figure 5.

(a) Inhibition curve for paclitaxel and EDTA (positive control) against the FRET substrate SNAPtide® (8 μM). The IC_{50} for paclitaxel against the light chain (catalytic domain) of BoNT/A (10 nM) was estimated to be 5.2 μM using a four-parametric nonlinear regression model (Prism5, GraphPad Inc.). (b) Specific binding curve for Paclitaxel [2-benzoyl ring-3H] against the light chain (catalytic domain) of BoNT/A (7 nM) obtained in a SPA assay. Curve-fitting was performed with a four-parametric nonlinear regression model (Prism5, GraphPad Inc.). These results confirm the concentration-dependency of paclitaxel binding to BoNT/A LC.

Table 1

Holistic Binding (HB) scores and predicted activity for inhibitor candidates selected for experimental testing. Scores were obtained from virtual screening of the *botulinum* neurotoxin subtype A (BoNT/A), heavy and light chains, against 1,624 chemical compounds. A discussion of the rationale used for hit selection is presented in the text.

Ligand	HB Score (kcal/mol)	HB Ranking (catalytic site) ¹	HB Predicted Activity Catalytic Activity	HB Predicted Activity Binding to BoNT/A LC with Inhibition of	FF Score (kcal/mol)	FF Ranking (catalytic site) ²
Paclitaxel	-11.0	6	Yes (strong)		-90.9	91
D-(-)-fructose	-1.8	735	No		-29.1	1285
Desmosine	-5.5	28	May be (low to none)		-115.6	38
Aminopterine	-4.2	63	May be (low to none)		-118.6	36
Monensin	-4.1	70	May be (low to none)		-79.8	163

¹Ranking based on Holistic Binding scores for the catalytic site of BoNT/A. More negative scores are better. The threshold of binding energy used to consider a compound to be a "binder" in the catalytic site is -5 kcal/mol. Ligands with scores more negative than this threshold have better chance to bind experimentally to the target. Ranking reflects the inclusion of fluorescent derivatives of paclitaxel to the ranking list after paclitaxel was selected for testing and confirmed experimentally to bind to BoNT/A.

²Ranking based on raw Force Field (FF) scores for the catalytic site of BoNT/A, provided for comparison. The threshold of binding energy to consider a compound to be a "binder" in the catalytic site for FF-based scores is -106 kcal/mol. Among the compounds in this table, only aminopterine would be predicted as a "binder" by the FF-based scores. Using this scoring scheme, 5.6% of the ranked library would have to be tested experimentally to identify paclitaxel as a BoNT/A inhibitor.

Table 2

Inhibition of BoNT/A light chain catalytic activity by selected hits.

Ligand	% Inhibition at 10 μ M ^I
Paclitaxel	95
D-(-)-fructose	0
Desmosine	25
Aminopterin	11
Monensin	38

^I Percent inhibition was calculated in FRET assays using 8 μ M of SNAPtide. The concentration of BoNT/A LC recombinant protein was 10 nM. Assays were performed at 37°C, pH 8.2, in triplicate. Toxin was added to solutions of buffer, substrate and ligands. Samples were incubated for 1 hour prior to readings.

Table 3

Binding of fluorescent recognition agent candidates against the *botulinum* neurotoxin subtype A non-activated heavy and light chain complex (whole BoNT/A). Binding to the catalytic domain (light chain) of BoNT/A and inhibition of catalytic activity does not assure binding to whole BoNT/A, which is necessary for reliable detection.

Ligand	Holistic Binding Score at the Catalytic Site (Ranking ¹) (kcal/mol)	Experimental Binding Against Non-activated Whole BoNT/A (% Bound Ligand)	
		Fluorescence Intensity Assay ²	Radiolabeled Ligand Binding Assay ³
D-(-)-fructose	-1.8 (735)		3
Paclitaxel (Pa)	-11.0 (6)		7
Pac-OG	-22.0 (1)	4	
Pac-BDP	-16.0 (2)	46	
Pac-FL	-12.4 (3)	22	

¹Ranking based on Holistic Binding scores for the catalytic and for the best non-catalytic binding site of BoNT/A. More negative scores are better. Derivatives were added to the ranking list after paclitaxel was selected for testing and confirmed experimentally to bind to BoNT/A. All ligands were re-ranked after inclusion of paclitaxel fluorescent derivatives to the ranking list.

²Radiolabeled Ligand Binding. Paclitaxel and fructose were tested in duplicate at 50 μ M (paclitaxel[14C] and fructose[14C]), 25 μ M (paclitaxel[14C]) and 10 μ M (fructose[14C]) for binding to non-activated 0.1 μ g/ml whole BoNT/A, at room temperature and two pHs (5.5 and 7.4), with consistent results. Biotinylated toxin was attached to streptavidin-coated magnetic beads for separation of free and bound ligands.

³Fluorescence Intensity (FI). Fluorescent ligands were tested at 10 μ M against non-activated 0.1 μ g/ml whole BoNT/A. The toxin was attached to magnetic beads for separation. FI was measured in duplicate relative to the reference sample of each fluorescent ligand, after 1 hour incubation at room temperature and pH 7.4. Excitation/Emission wavelengths: Pac-FL (505nm/515nm); Pac-BDP (565nm/571nm); Pac-OG (496nm/524nm).