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Systematic Discovery of Molecular Probes Targeting Multiple Non-orthosteric and Spatially Distinct Sites in the Botulinum Neurotoxin Subtype A (BoNT/A)

Saede Dadgar¹ and Wely B. Floriano¹

¹Department of Chemistry at Lakehead University and Thunder Bay Regional Research Institute, Thunder Bay ON P7B 5E1, Canada

Abstract

The development of molecular probes targeting proteins has traditionally relied on labeling compounds already known to bind to the protein of interest. These known ligands bind to orthosteric or allosteric sites in their target protein as a way to control their activity. Binding pockets other than known orthosteric or allosteric sites may exist that are large enough to accommodate a ligand without significantly disrupting protein activity. Such sites may provide opportunities to discriminate between subtypes or other closely related proteins, since they are under less evolutionary pressure to be conserved. The Protein Scanning with Virtual Ligand Screening (PSVLS) approach was previously used to identify a novel inhibitor and a fluorescent probe against the catalytic site of the botulinum neurotoxin subtype A (BoNT/A). PSVLS screens compound databases against multiple sites within a target protein, and the results for all the sites probed against BoNT/A, not only the catalytic site, are available online. Here, we analyze the PSVLS data for multiple sites in order to identify molecular probes with affinity for binding pockets other than the catalytic site of BoNT/A. BoNT/A is a large protein with a light (LC) and a heavy (HC) chain that can be assayed separately. We used scintillation proximity assay (SPA) to test experimentally 5 probe candidates predicted computationally to have affinity for different non-orthosteric binding regions within the HC and LC, and one compound predicted not to have affinity for either domain. The binding profiles obtained experimentally confirmed the targeting of multiple and spatially distinct pockets within BoNT/A. Moreover, inhibition assay results indicate that some of these probes do not significantly interfere with the catalytic activity of BoNT/A.

Keywords

molecular probes; non-orthosteric binding; botulinum toxin; molecular docking; small molecule; biomarkers; allosteric regulation; holistic binding

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To whom correspondence should be addressed: Wely B. Floriano, Department of Chemistry, Lakehead University, 950 Oliver Rd, Thunder Bay, ON P7B 5E1, Canada, Phone: (807) 766-7215; wely.floriano@lakeheadu.ca.

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1. INTRODUCTION

A primary (orthosteric) ligand binds to the orthosteric site of its cognate protein to induce or suppress response. Traditionally drug discovery have targeted orthosteric sites in proteins as a way to control their activity. However, this approach fails to produce ligands with enough subtype specificity because of the high degree of conservation generally associated to functionally active sites. Since subtype specificity is important for many pharmacologically relevant targets such as G protein coupled receptors, alternative approaches have been sought. Recently, structure-based drug discovery efforts have shifted towards the targeting of allosteric sites as a way to bypass the high degree of conservation of functional sites among protein subtypes and closely related homologs [1–11]. Allosteric sites are spatially distinct from the orthosteric site. Binding of a ligand to an allosteric site induces conformational changes that affect the binding of the orthosteric ligand and/or the response of the target protein. Allosteric sites are still functional sites in the sense that they modulate the primary function of the protein. However they are under less evolutionary pressure to be conserved than orthosteric sites and, hence, their appeal as target sites for subtype specificity. Conceptually, other binding pockets within a protein may exist that are large enough to accommodate a ligand without producing a significant change in the activity associated to an orthosteric site. Such sites may provide unique characteristics that allow the discrimination of a protein from other closely related proteins. This type of selectivity is especially important in the context of target-specific molecular probes (TS-MPs), which are recognition agents that detect a biomolecule of interest. TS-MPs are usually obtained by labeling a known ligand with a radioisotope or a fluorophore. Because they are based on known ligands, TS-MPs often interfere with the function of the protein they are targeting, which prevents their use to monitor the biological effects of novel ligands. Moreover, they are often plagued by the same specificity problems their originating ligand may have. In the context of TS-MPs, the possibility of finding specific probes for non-interfering/non-orthosteric sites is very attractive and may represent a shift in the current paradigm of probe discovery. A key question, however, remains to be answered: would ligands targeting non-orthosteric and non-allosteric sites have enough specific affinity for the target protein to enable their use as molecular probes? In order to gain insights into this question, we targeted non-orthosteric sites in the botulinum neurotoxin subtype A structure for the identification of molecular probe candidates.

Botulinum neurotoxin (BoNT), one of the most known lethal toxins and a causative agent of botulism disease, is produced by the bacterium *Clostridium botulinum* [12]. Botulism is a serious disease which affects the peripheral nerve system by cleavage of a group of proteins (SNARE). These proteins are involved in membrane fusion at synaptic terminal and their cleavage consequently inhibits acetylcholine secretion resulting in respiratory muscles paralysis and ultimately death [13]. There are eight types of BoNT classified by the letters A - H and BoNT/A is known as the most potent one [14]. BoNT is a polypeptide molecule with a molecular weight of 150 kDa. Activated BoNT is cleaved in two smaller chains, a heavy (HC) and a light chain (LC) with molecular weights of 100 and 50 kDa, respectively. The active site of BoNT with zinc-endopeptidase activity is located in the LC (catalytic domain), whereas the HC contains the binding domain of BoNT [14–15]. BoNT/A and B

have been approved for use as therapeutic drugs [16]. In recent years, several studies have attempted to identify new inhibitors of BoNT due to its various applications in different areas such as medical, pharmaceutical, cosmetic and the potential threat of using BoNT as a biological weapon [16–18]. However, most of these studies focused on identification of novel recognition agents and inhibitors for the active site of BoNT/A. Our group has also targeted the active site of BoNT/A [19], successfully identifying a novel inhibitor and a fluorescent recognition agent. In that work, a library consisting of 1,624 compounds including commercially available radiolabeled ligands was computationally screened against BoNT/A using the Protein Scanning with Virtual Ligands Screening (PSVLS) method. The holistic binding scoring method was applied to the PSVLS results in order to identify novel ligands specifically targeting the catalytic site of BoNT/A.

Here we report the analysis of the PSVLS results in Dadgar et al., 2013 [19] for all 33 binding regions explored by PSVLS, with the intent of identifying non-orthosteric ligands for BoNT/A. Non-orthosteric ligands may serve as subtype-specific molecular probes that take opportunity of unique binding pockets in BoNT/A compared to other subtypes or other homologous proteins (potential confounders in a detection assay). They also provide new opportunities for controlling the function of BoNT/A without direct catalytic inhibition. We selected four ligands based on their PSVLS binding profiles. A previously identified inhibitor [19], paclitaxel, was used as positive control. D-fructose, a non-binder [19], was used as negative control. Selected ligands were tested against BoNT/A HC and BoNT/A LC separately using scintillation proximity assay (SPA). Our results demonstrate that PSVLS/holistic binding scoring approach is an accurate and reliable method to identify molecular probe candidates targeting orthosteric and non-orthosteric sites in BoNT/A.

2. METHODS AND PROCEDURES

2.1 Protein Scanning with Virtual Ligand Screening (PSVLS)

PSVLS was used to screen a virtual library of 1,624 commercially-available radiolabeled ligands against BoNT/A in order to identify new inhibitors and molecular probes targeting the catalytic site of BoNT/A [19], a metalloprotease. The results of that screening for all 33 binding sites of BoNT/A were further analyzed in the present work with the intent of identifying non-orthosteric ligands for BoNT/A. PSVLS is a computational protocol for virtual screening that targets multiple sites within the 3-dimensional (3D) structure of the target protein. PSVLS takes the 3D structures of a target protein and of a library of chemical compounds, scans the protein for potential binding sites, and screens the virtual ligand library for chemical compounds with high affinity for any of the potential binding sites [20–21]. The core computational methodology used in PSVLS is HierVLS [20], a multi-step hierarchical protocol for virtual ligand screening of large (> 500,000) chemical compounds libraries [20]. HierVLS uses a hierarchical approach: a large number of bound protein-ligand 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 (for increased accuracy). The last step is the calculation of the binding energy including solvation for the best surviving bound configuration of each ligand. Binding energies are calculated using a force field-based

scoring function. For PSVLS, we apply HierVLS not only to the active site of the target protein, but also to other available pockets, which can be used for detection of the protein or as alternative sites for controlling biological activity. These alternative binding sites are identified as enclosed empty volumes large enough to accommodate small molecules. PSVLS provides force field-based binding affinities and bound structures for each ligand in the screening library, in each one of the potential binding sites in the target protein. The centers of the 33 binding sites identified in BoNT/A (pdb code 2NYY) are shown in Figure 1 as solid spheres, with the HC and LC domains of BoNT/A labeled.

2.2 Selection of Probe candidates for experimental testing

Principal component analysis (PCA) was used to identify ligands with overall affinity for BoNT/A and, thus, likely to bind experimentally to BoNT/A. This approach takes into account the affinity of the probe candidate for the target protein as a whole and it is not site-specific. PCA is used to reduce the binding energies across all regions into a single predictive score for each chemical compound screened. The first principal component (PC1), which has the largest variance, represents the ligands' overall affinity for the protein. A non-binder/binder threshold is set as one standard deviation below the mean value (zero for the transformed data) of the entire screening library. The underlying assumption is that the screening of a random library will mostly produce scores that represent non-specific interactions between the ligands and the protein, with only a small number of compounds displaying "true" binding interactions and thus passing the threshold.

The ligands in the subset with a PC1 score passing the non-binder/binder threshold are further analyzed in terms of their individual holistic scores [19] at each binding region. This secondary analysis allows for the selection of ligands that preferentially bind to specific sites within the protein. For this second subset of ligands, practical aspects such as commercial availability with a tritium label, price, safety, and easy handling are considered in order to select the final set of ligands for experimental testing.

2.3 Materials for Experiments

BoNT/A LC and HC were purchased from List Biological Laboratories inc. Radioligands were obtained from American Radiolabeled Chemicals inc., except [^3H] aminopterin which was purchased from Moravsek Biochemicals. The specific activity of [^3H] aminopterin (ethanol:water 4:6), [^3H] desmosine (water), [^3H] paclitaxel (ethylacetate), [^3H] solanesol (hexane), [^3H] solanesyl pyrophosphate (IPA:NH₄OH:H₂O) and [^3H] fructose-D (ethanol) were 38.4 Ci/mmol, 5 Ci/mmol, 60 Ci/mmol, 20 Ci/mmol, 20 Ci/mmol and 5 Ci/mmol, respectively. The assay buffer contained 20 mM HEPES buffer at pH 7.4, 0.1% Tween 20, 0.3 mM ZnCl₂, and was used to prepare the dilutions of radioligands. ChromaLink™ Biotin One-Shot Antibody labeling kit was purchased from Solulink and used to biotinylate BoNT/A LC and BoNT/A HC. The hydrolysis buffer contained 20 mM HEPES buffer at pH 7.4 and 1% Tween 20, and was used to make dilutions of BoNT/A HC and BoNT/A LC. Streptavidin coated PolyVinyltoluene (PVT) scintillation proximity assay (SPA) beads were supplied by PerkinElmer, inc.

2.4 Biotinylation of BoNT/A HC and BoNT/A LC

BoNT/A LC (30 µg) and BoNT/A HC (50 µg) were biotinylated using the ChromaLink™ Biotin One-Shot Antibody Labeling Kit according to Solulink's instructions. Two parameters were necessary to determine the concentration of the proteins: the mass extinction coefficient (E1%) for BoNT/A HC and LC, and their absorbance at 280 nm. The E1% of BoNT/A HC was reported to be 17.1 by Weatherly, G. T. et al [22]. E1% value of BoNT/A LC was roughly estimated to be 10. The biotin molar substitution ratio of biotinylated BoNT/A LC and biotinylated BoNT/A HC was calculated to be 2 and 4.3, respectively, using the MSR calculator of Solulink. The total biotin-labeled BoNT/A HC recovered after biotinylation was 28.3 µg and the total labeled BoNT/A LC was 43.9 µg. The biotinylation kit has a chromophore with maximum absorbance at 354 nm, which overlaps the emission spectrum of the PVT SPA (360–490 nm). To correct for this effect, the same concentrations of chromophore present in samples were added to controls in the SPA experiments.

2.5 Scintillation proximity assays

Three formats of adding reagents were tested in preliminary experiments in order to optimize the SPA for BoNT/A: coupling SPA beads and BoNT/A before adding radioligands, adding all three reagents at the same time, and incubating BoNT/A with radioligand before the addition of SPA beads. The most suitable format was found to be delayed addition of SPA beads which also reduces non-specific binding. Preliminary experiments were conducted to optimize the ratio of protein and SPA beads, which is one of the critical factors in SPA to obtain a maximum signal to noise ratio. We used [³H] paclitaxel to optimize the amount of beads necessary to assay BoNT/A LC, whereas [³H] aminopterin was used to optimize bead amount for BoNT/A HC. The concentration of SPA beads was varied between 0.5 – 2.5 mg/ml while the concentrations of BoNT/A HC and [³H] aminopterin were fixed at 17 nM and 1 µM, respectively. The final volume of each sample was 200 µl and the samples were counted at the speed of 1 well/min. The concentrations of [³H] paclitaxel and BoNT/A LC were constant in each sample at 6 nM and 10 nM, respectively. The final concentration of 2 mg/ml of SPA beads was found to be optimal for both BoNT/A LC and BoNT/A HC.

Radioligand and protein (BoNT/A LC or HC) were incubated for 30 minutes at room temperature to pre-equilibrate before the addition of SPA beads to decrease non-specific binding. The SPA beads were reconstituted in assay buffer with a concentration of 50 mg/ml and stored at 4 °C. SPA beads working solutions at 5 mg/ml were prepared on the day of each experiment. The concentration of SPA beads and biotinylated BoNT/A HC (or BoNT/A LC) were fixed in all samples at 2 mg/ml and 17 nM (or 10 nM), respectively. All the experiments were done in 96-well, white and flat bottom Wallac microplates, and each sample was run in duplicate at room temperature. Scintillation was counted using a MicroBeta² plate counter. To obtain comparable data from different runs, a correction for radioactive half-life was applied automatically by the MicroBeta² plate counter. [³H] paclitaxel was used as a positive control at 50 nM concentration and [³H] fructose was used as a negative control at the concentration of 3 µM. These concentrations were chosen based on previous experiments [19]. Non-specific binding of radioligands to the beads was

determined in the absence of BoNT/A. Specific binding was calculated by subtracting non-specific binding to the beads from total binding. [^3H] aminopterin, [^3H] desmosine, [^3H] solanesol, and [^3H] solanesyl pyrophosphate were initially tested at the concentrations of 1 μM , 2 μM , 300 nM, and 600 nM, against BoNT/A HC (or LC). Additionally, [^3H] aminopterin, and [^3H] desmosine were assayed at two lower concentrations (250 nM and 50 nM) for binding to BoNT/A HC in a separate assay. The effective concentration (EC_{50}) of [^3H] paclitaxel against BoNT/A LC was previously determined using SPA[19]. EC_{50} of [^3H] aminopterin and of [^3H] desmosine were determined against BoNT/A HC with varying concentrations of 50nM-1 μM and 10nM-4 μM , respectively. The samples were counted for 18 hours, but the specific binding remained constant after 10 hours. The data was analyzed using Prism 6 (GraphPad, Inc), and EC_{50} was determined by a non-linear regression model. The upper limit of the concentration curves was limited by the maximum concentrations that could be achieved in the assays, given specific activities and the concentrations of the commercial compounds tested.

3. RESULTS

3.1 Selection of Probe Candidates for Experimental Testing

PCA was applied to the PSVLS results in Dadgar and co-authors (2013) to reduce the force field scores of each ligand bound to each of the 33 binding regions identified in the structure of BoNT/A (Figure 1) into one score, corresponding to the first principle component (PC1) which carries the most variance in the data. Ligands with a PC1 score passing the non-binder/binder threshold (Figure 2) are expected to bind experimentally to BoNT/A, and were further analyzed in terms of their individual holistic scores at each binding region (Figure 3). The objective of the PCA was to identify ligands with strong overall affinity for BoNT/A to increase the chances of finding true positive compounds. The objective of the per-region analysis was to identify ligands preferentially binding to spatially distinct regions of BoNT/A belonging to each of the two domains, HC and LC. These domains are structurally stable and functional as separate units, and can be assayed independently of each other. The PSVLS-predicted binding profiles for selected ligands can thus be tested experimentally.

In addition to the previously tested [19] paclitaxel (positive control) and fructose (negative control), we selected aminopterin, desmosine, and solanesyl pyrophosphate (SolanesylPP). All of these compounds passed the non-binder/binder threshold. The region-specific binding energies for these compounds (Figure 3) indicate preferential binding to the HC (aminopterin and desmosine), LC (paclitaxel), or both BoNT/A chains (solanesylPP). Aminopterin is a known anticancer drug and has been used to treat leukemia [23]. Desmosine is a rare amino acid produced by elastin breakdown. The release of desmosine is accelerated in patients with inflammatory conditions such as chronic obstructive pulmonary disease (COPD), and pulmonary tuberculosis (TB) [24–29]. SolanesylPP is predicted to be the strongest binder among the ligands selected. As expected, paclitaxel passed the PC1 non-binder/binder threshold, and its region-specific binding energies (Figure 3) indicate preferential binding to BoNT/A LC, where the catalytic site is located. This is in agreement with previously reported inhibition assays which confirmed paclitaxel as an inhibitor of BoNT/A [19]. Also as expected, fructose did not pass the non-binder/binder threshold.

Solanesol, a derivative of solanesyl pyrophosphate, was selected for testing to contrast against its parent compound, even though solanesol's PC1 score is higher than the threshold (albeit lower than the average score for the library). Solanesol is extracted from tobacco leaves and has been used as a primary material to synthesize vitamin K and coenzyme Q10[30]. The ligands selected for experimental testing are listed in Table 1.

3.2 Experimental Binding to BoNT/A HC and LC using SPA

Preliminary SPA was performed to investigate the binding of selected ligands against BoNT/A LC and BoNT/A HC. Aminopterin was tested at 1000, 250 and 50 nM, and desmosine was tested at 2000, 250 and 50 nM against BoNT/A HC. Since no binding to BoNT/A LC was observed for aminopterin at 500 nM and for desmosine at 2000 nM, lower concentrations of these compounds were not tested. Solanesol was tested at 1000, 300, 50 and 10 nM against BoNT/A HC, and at 300nM against BoNT/A LC. SolanesylPP was tested at (600, 300, 50 and 10 nM against BoNT/A HC, and at 600nM against BoNT/A LC. A summary of these results is provided in Figure 4. The lowest positive concentration tested is provided for positive ligands, whereas the highest concentration tested is shown for negative ligands. [³H] aminopterin and [³H] desmosine both show high affinity for BoNT/A HC, but very low binding to BoNT/A LC. [³H] SolanesylPP (right axis in Figure 4) exhibits binding not only to BoNT/A HC but also to BoNT/A LC. In contrast, [³H] solanesol did not bind to BoNT/A HC or LC at the concentrations tested. [³H] paclitaxel, which was shown to be a BoNT/A inhibitor, [19] only shows binding to BoNT/A LC at the concentrations tested. In addition, [³H] D-fructose, which was selected as a negative control, does not bind to either BoNT/A HC or BoNT/A LC at the concentrations tested.

Positive ligands were further tested for concentration-dependence, except solanesylPP. Pyrophosphates are good complexing agents and it is very possible that the positive results observed in the binding assays for solanesylPP are due to non-selective interactions to Ca²⁺ in BoNT/A LC, as suggested by the ligand-protein structure generated by PSVLS (an interactions diagram is provided as supplemental material). The [³H] paclitaxel binding curve against BoNT/A LC is shown for comparison to the other curves in Figure 5(B). From this curve, the EC₅₀ of paclitaxel against BoNT/A LC is estimated at 17 nM. The binding curve for [³H] aminopterin against BoNT/A HC is shown in Figure 5(A) and indicates that the specific binding is dependent on the concentration of [³H] aminopterin, with an EC₅₀ of 703 ± 98 nM. Binding of [³H] desmosine also show concentration-dependency (Figure 5(C)), with an EC₅₀ of 1.6 ± 0.3 μM against BoNT/A HC.

3.3 Agreement between Predicted and Experimental Binding Profiles

The PSVLS-predicted binding profiles along with the SPA experimental results are summarized in Table 2. Aminopterin is predicted to bind preferentially to BoNT/A HC, which is confirmed experimentally by SPA (EC₅₀ (BoNT/A HC) = 0.7 μM). Aminopterin did not score well in any region in the LC, consistent with SPA results that show no binding to BoNT/A LC at 1μM and supported by reported inhibition assays at 10 μM [19]. Desmosine shows good holistic scores to both HC and LC. Binding to HC is confirmed experimentally by SPA, with an EC₅₀ (BoNT/A HC) of 1.6 μM. Desmosine was not found to bind to BoNT/A LC in the SPA assay at 2 μM or lower. However, binding of desmosine

to BoNT/A LC is suggested by the weak inhibition (25%) observed at 10 μM [19]. This should be confirmed by additional experiments at higher concentrations of desmosine.

According to holistic scores, paclitaxel has a strong preference for BoNT/A LC, which is confirmed by SPA (EC_{50} of 17 nM), and by previously published results [19] of a FRET inhibition assay (IC_{50} of 5.2 μM). The apparent discrepancy between the EC_{50} and the IC_{50} values comes from the nature of the assays. The IC_{50} reported in Dadgar et al. [19] refers to the concentration of paclitaxel that inhibits the turnover of 8 μM of fluorescently labeled substrate by 50%. This is a value that depends on the concentration of the substrate. Higher concentrations of the substrate will increase the IC_{50} of the inhibitor well above its equilibrium constant [31]. In contrast, the EC_{50} reported reflects the concentration of radiolabeled paclitaxel that produces 50% of the maximum signal, which is obtained with excess ligand and corresponds to maximum receptor occupancy. This value is dependent on the concentration of the receptor (BoNT/A in this assay was kept at 7 nM).

The holistic scores for solanesylPP suggest that this compound binds to both BoNT/A HC and LC, in agreement with SPA results at 0.6 μM . However, its binding to both domains may be the result of non-specific effects such as chelation of metal ions coordinated within the protein. Fructose scores very poorly in all binding regions and has consistently produced a negative response in all our experiments. Solanesol did not pass the PC1 non-binder/non-binder threshold but its best region-specific holistic score in region 6, located in the LC, is above the region-specific threshold. No binding was observed for solanesol at 0.3 μM to either BoNT/A HC or LC. We were unable to test solanesol at higher concentrations or in an inhibition assay against BoNT/A LC due to insufficient materials to confirm its status.

The observed binding of aminopterin or desmosine to BoNT/A HC could potentially prevent the binding of BoNT/A to cell surface receptors. This is an undesirable outcome for molecular probes, since they should ideally be non-interfering. The location of the recognition site for cell surface receptors in BoNT/A HC can be inferred from the experimentally determined structure of BoNT/A in complex with ganglioside co-receptor GT1b (pdb code 2VU9). In Figure 6, we show the PSVLS-predicted structures for paclitaxel, aminopterin and desmosine superposed to BoNT/A in complex with GT1b. GT1b binds to a shallow groove in the ligand binding domain (LBD) of the heavy chain of BoNT/A. Paclitaxel is predicted to bind near the catalytic site (CS) of BoNT/A in the light chain. The PSVLS results for aminopterin indicate 3 regions in BoNT/A HC with similar binding affinities (R7, R8, and R27), either of which could be the *de facto* binding site for aminopterin. The PSVLS results for desmosine place its binding to BoNT/A HC in region 10 which is nearby region 27, shown in Figure 6 with aminopterin bound to it. None of the predicted binding sites overlap with bound GT1b. Thus, the mapping of the best PSVLS conformations for aminopterin and desmosine onto the structure of the BoNT/A in complex GT1b suggests that these two compounds are unlikely to interfere with the binding of the toxin to cell surface receptors. In contrast, the predicted structure of paclitaxel bound to BoNT/A LC suggests interference with catalytic activity, given its proximity to the zinc ion in the catalytic site, consistently with previously reported inhibition assays.

Overall, there is a very clear agreement between predicted and experimentally determined binding profiles for the selected ligands. This agreement indicates that PSVLS is an efficient methodology for the identification of ligands targeting non-orthosteric sites, as well as orthosteric sites.

BoNT/A is a large protein, with 33 identified binding pockets, and has a catalytic site notoriously known to be somewhat promiscuous due to its plasticity [32]. These characteristics make BoNT/A a good proof-of-concept target for a non-orthosteric ligands discovery platform. However these characteristics are also responsible for the atypically high discovery rate attained in this project (4 positive compounds out of 1,624 virtually screened). For this reason, further validation that includes more typical targets must be performed for the discovery platform presented here.

4. DISCUSSION AND CONCLUSIONS

Target-specific molecular probes are organic compounds that bind to a particular protein (the target) and report its presence in the form of a detectable signal, such as fluorescence or scintillation caused by radioactivity. Molecular probes can be utilized in medical imaging to locate regions where the target protein is present or it is more/less abundant than normal, indicating a disease state. They can also be utilized in laboratory assays to identify and quantify a protein that is associated to a disease or condition. The current paradigm of molecular probes discovery is to modify existing medicinal drugs known to bind to the protein of interest. However, this approach has a number of disadvantages: not all medicinal drugs are suitable for labeling; labeling with an additional chemical group may negatively impact binding affinity to the target; the molecular probe may have biological activity against the target protein, precluding its use for monitoring the effects of novel drugs against the target; not all proteins for which a molecular probe is desirable have known ligands.

We applied PSVLS, a virtual screening approach, to identify molecular probes targeting non-orthosteric sites within the whole (HC and LC) structure of BoNT/A[19]. We tested experimentally 6 ligands with predicted binding profiles that included orthosteric and non-orthosteric binding site preferences, as well as ligands not expected to bind favorably to BoNT/A. Because BoNT/A is a large protein with two distinct domains that can be separated and tested independently of each other, we were able to confirm experimentally the PSVLS-predicted binding profiles. Our results demonstrate that the PSVLS approach has the ability to identify probe candidates for multiple, spatially distinct binding regions within the structure of the target protein. This enables the use of PSVLS for identification of probes that take advantage of unique non-conserved sites, thus increasing selectivity. Non-orthosteric binding sites are not under the same evolutionary pressure to be conserved as do orthosteric sites and, thus, present better opportunities for the discovery and/or design of highly selective molecular probes. Moreover, some of the regions target by PSVLS may not interfere upon ligand binding with the main functional site of the target protein. This enables the use of the PSVLS probes for monitoring treatment response through medical imaging without the risk of affecting the actual treatment. Since PSVLS does not require prior knowledge of compounds that bind to the protein target of interest, we anticipate that this method will be able to identify molecular imaging probes for newly identified protein

biomarkers from genomics/proteomics studies. The only condition for applying PSVLS to newly identified biomarkers is the availability of an experimental structure, or the possibility of modeling the biomarker's structure using well-established methods for protein structure modeling. The early identification of molecular probes will accelerate the validation of novel biomarkers by enabling scientists to develop target-specific assays to conduct their studies.

Similar to most docking protocols, PSVLS does not fully account for structural flexibility of the protein, which is an important feature of BoNT toxins [33–35]. In PSVLS, the protein structure is only allowed to relax and conform to the bound ligand in the last step of the protocol, which consists of an all-atoms energy-minimization. Although our results clearly support the use of this protocol for probe discovery, extending the calculations to consider structural plasticity, especially of the BoNT/A LC domain, could provide valuable insights into the expected *ex/in vivo* behavior of these probes. Additional calculations could include molecular dynamics of the probe-protein complexes obtained from PSVLS, or repeating PSVLS for multiple conformers of the target protein. Both approaches are, however, beyond the scope of the present study.

In conclusion, the two main outcomes from this study are: (a) a set of novel molecular probes against BoNT/A with non-overlapping binding sites. These probes are potentially suitable for the development of detection assays using solid support, radiolabeled probes, or other technologies. (b) A computer-assisted protocol for identification of non-orthosteric molecular probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Lane R, Chubukov P, Liu W, Canals M, Cherezov V, Abagyan R, et al. Structure-Based Ligand Discovery Targeting Orthosteric and Allosteric Pockets of Dopamine Receptors. *Mol Pharmacol*. 2013
2. Christopoulos A. Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat Rev Drug Discov*. 2002; 1:198–210. [PubMed: 12120504]
3. Bond CJ, Jurica MS, Mesecar A, Stoddard BL. Determinants of allosteric activation of yeast pyruvate kinase and identification of novel effectors using computational screening. *Biochemistry*. 2000; 39:15333–43. [PubMed: 11112519]
4. McKay DB, Chang C, Gonzalez-Cestari TF, McKay SB, El-Hajj RA, Bryant DL, et al. Analogs of methyllycaconitine as novel noncompetitive inhibitors of nicotinic receptors: pharmacological characterization, computational modeling, and pharmacophore development. *Mol Pharmacol*. 2007; 71:1288–97. [PubMed: 17308033]

5. Ramirez UD, Myachina F, Stith L, Jaffe EK. Docking to large allosteric binding sites on protein surfaces. *Adv Exp Med Biol.* 2010; 680:481–8. [PubMed: 20865533]
6. Arencibia JM, Pastor-Flores D, Bauer AF, Schulze JO, Biondi RM. AGC protein kinases: from structural mechanism of regulation to allosteric drug development for the treatment of human diseases. *Biochim Biophys Acta.* 2013; 1834:1302–21. [PubMed: 23524293]
7. Liu F, Li F, Ma A, Dobrovetsky E, Dong A, Gao C, et al. Exploiting an allosteric binding site of PRMT3 yields potent and selective inhibitors. *J Med Chem.* 2013; 56:2110–24. [PubMed: 23445220]
8. Tomita N, Hayashi Y, Suzuki S, Oomori Y, Aramaki Y, Matsushita Y, et al. Structure-based discovery of cellular-active allosteric inhibitors of FAK. *Bioorg Med Chem Lett.* 2013; 23:1779–85. [PubMed: 23414845]
9. Mahmoud MM, Ali HI, Ahn KH, Damaraju A, Samala S, Pulipati VK, et al. Structure-Activity Relationship Study of Indole-2-carboxamides Identifies a Potent Allosteric Modulator for the Cannabinoid Receptor 1 (CB1). *J Med Chem.* 2013
10. Shiryayev SA, Cheltsov AV, Gawlik K, Ratnikov BI, Strongin AY. Virtual ligand screening of the National Cancer Institute (NCI) compound library leads to the allosteric inhibitory scaffolds of the West Nile Virus NS3 proteinase. *Assay Drug Dev Technol.* 2011; 9:69–78. [PubMed: 21050032]
11. Laine E, Martinez L, Ladant D, Malliavin T, Blondel A. Molecular motions as a drug target: mechanistic simulations of anthrax toxin edema factor function led to the discovery of novel allosteric inhibitors. *Toxins (Basel).* 2012; 4:580–604. [PubMed: 23012649]
12. Lebeda FJ, Cer RZ, Mudunuri U, Stephens R, Singh BR, Adler M. The Zinc-Dependent Protease Activity of the Botulinum Neurotoxins. *Toxins.* 2010; 2:978–97. [PubMed: 22069621]
13. Fasshauer D, Sutton RB, Brunger AT, Jahn R. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *P Natl Acad Sci USA.* 1998; 95:15781–6.
14. Sharma SK, Ferreira JL, Eblen BS, Whiting RC. Detection of type A, B, E, and F Clostridium botulinum neurotoxins in foods by using an amplified enzyme-linked immunosorbent assay with digoxigenin-labeled antibodies. *Appl Environ Microb.* 2006; 72:1231–8.
15. Ahmed SA, Olson MA, Ludivico ML, Gilsdorf J, Smith LA. Identification of residues surrounding the active site of type A botulinum neurotoxin important for substrate recognition and catalytic activity. *The protein journal.* 2008; 27:151–62. [PubMed: 18213512]
16. Dhaked RK, Singh MK, Singh P, Gupta P. Botulinum toxin: Bioweapon & magic drug. *Indian J Med Res.* 2010; 132:489–503. [PubMed: 21149997]
17. Eubanks LM, Dickerson TJ, Janda KD. Technological advancements for the detection of and protection against biological and chemical warfare agents. *Chem Soc Rev.* 2007; 36:458–70. [PubMed: 17325785]
18. Ward AB, Molenaers G, Colosimo C, Berardelli A. Clinical value of botulinum toxin in neurological indications. *Eur J Neurol.* 2006; 13:20–6. [PubMed: 17112346]
19. Dadgar S, Ramjan Z, Floriano WB. Paclitaxel Is an Inhibitor and Its Boron Dipyrromethene Derivative Is a Fluorescent Recognition Agent for Botulinum Neurotoxin Subtype A. *J Med Chem.* 2013; 56:2791–803. [PubMed: 23484537]
20. Floriano WB, Vaidehi N, Zamanakos G, Goddard WA 3rd. HierVLS hierarchical docking protocol for virtual ligand screening of large-molecule databases. *J Med Chem.* 2004; 47:56–71. [PubMed: 14695820]
21. Avila, A. Eshu: A Set of Analysis Tools For The Orunmila Ligand-Protein Docking And Scoring System. Pomona: California State Polytechnic University Pomona; 2009.
22. Weatherly GT, Bouvier A, Lydiard DD, Chapline J, Henderson I, Schrimsher JL, et al. Initial purification of recombinant botulinum neurotoxin fragments for pharmaceutical production using hydrophobic charge induction chromatography. *Journal of chromatography A.* 2002; 952:99–110. [PubMed: 12064550]
23. Smith A, Hum M, Winick NJ, Kamen BA. A case for the use of aminopterin in treatment of patients with leukemia based on metabolic studies of blasts in vitro. *Clin Cancer Res.* 1996; 2:69–73. [PubMed: 9816092]

24. Devenport NA, Reynolds JC, Parkash V, Cook J, Weston DJ, Creaser CS. Determination of free desmosine and isodesmosine as urinary biomarkers of lung disorder using ultra performance liquid chromatography-ion mobility-mass spectrometry. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences*. 2011; 879:3797–801.
25. Lamerz J, Friedlein A, Soder N, Cutler P, Dobeli H. Determination of free desmosine in human plasma and its application in two experimental medicine studies. *Analytical biochemistry*. 2013; 436:127–36. [PubMed: 23399390]
26. MacLay JD, McAllister DA, Rabinovich R, Haq I, Maxwell S, Hartland S, et al. Systemic elastin degradation in chronic obstructive pulmonary disease. *Thorax*. 2012; 67:606–12. [PubMed: 22374923]
27. Satta J, Laurila A, Paakko P, Haukipuro K, Sormunen R, Parkkila S, et al. Chronic inflammation and elastin degradation in abdominal aortic aneurysm disease: an immunohistochemical and electron microscopic study. *European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery*. 1998; 15:313–9.
28. Seddon J, Kasproicz V, Walker NF, Yuen HM, Sunpath H, Tezera L, et al. Procollagen III N-terminal Propeptide and Desmosine are Released by Matrix Destruction in Pulmonary Tuberculosis. *The Journal of infectious diseases*. 2013; 208:1571–9. [PubMed: 23922364]
29. Turino GM, Ma S, Lin YY, Cantor JO, Luisetti M. Matrix elastin: a promising biomarker for chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine*. 2011; 184:637–41. [PubMed: 21757624]
30. Taylor MA, Fraser PD. Solanesol: added value from Solanaceous waste. *Phytochemistry*. 2011; 72:1323–7. [PubMed: 21459392]
31. Neubig RR, Spedding M, Kenakin T, Christopoulos A. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol Rev*. 2003; 55:597–606. [PubMed: 14657418]
32. Smith GR, Caglic D, Capek P, Zhang Y, Godbole S, Reitz AB, et al. Reexamining hydroxamate inhibitors of botulinum neurotoxin serotype A: extending towards the beta-exosite. *Bioorg Med Chem Lett*. 2012; 22:3754–7. [PubMed: 22542019]
33. Kumar R, Kukreja RV, Cai S, Singh BR. Differential role of molten globule and protein folding in distinguishing unique features of botulinum neurotoxin. *Biochim Biophys Acta*. 2014; 1844:1145–52. [PubMed: 24568862]
34. Kukreja RV, Singh BR. Comparative role of neurotoxin-associated proteins in the structural stability and endopeptidase activity of botulinum neurotoxin complex types A and E. *Biochemistry*. 2007; 46:14316–24. [PubMed: 18004882]
35. Cai S, Kukreja R, Shoesmith S, Chang TW, Singh BR. Botulinum neurotoxin light chain refolds at endosomal pH for its translocation. *The protein journal*. 2006; 25:455–62. [PubMed: 17115255]

Figure 2.

PC1 scores for a library of 1,624 compounds computationally screened against BoNT/A whole structure (HC and LC). Dashed lines mark one standard deviation above/below the mean score for the library. Ligands selected for experimental testing are highlighted.

Figure 3. Holistic Scores (HS) per region for selected ligands passing the PC1 non-binder/binder threshold.

Figure 4. Specific binding of each radioligand to BoNT/A HC (solid) and BoNT/A LC (checkered) using SPA. Solanesyl.PP is plotted on the right y-axis. Samples are compared to the negative control (fructose).

Figure 5.

SPA binding curves (A) for paclitaxel against BoNT/A LC (7 nM), and for (B) aminopterin and (C) desmosine against BoNT/A HC (17 nM). The EC_{50} of [3H] paclitaxel was determined to be 17 nM. The EC_{50} for [3H] aminopterin was determined to be 703 nM. The EC_{50} for [3H] desmosine was estimated at 1.6 μM . Non-linear regression was used to estimate EC_{50} .

Figure 6.

PSVLS-predicted structures superposed to BoNT/A in complex with ganglioside co-receptor GT1b (pdb code 2VU9). Paclitaxel (brown carbons), desmosine (orange carbons), aminopterin (pink carbons) and GT1b (green carbons) are shown in space-filled representation. The translocation domain (TD) and the ligand binding domain (LBD) are part of BoNT/A HC, whereas the catalytic site (CT) is located within BoNT/A LC. Based on the structural superposition, the regions targeted by paclitaxel, aminopterin and desmosine show no overlap with the binding region for GT1b. Paclitaxel's positioning near the catalytic zinc ion (pink sphere labeled Zn^{++}) is consistent with experimentally confirmed inhibition of catalytic activity.

Table 1

Common name, structure, and molecular weight of the selected ligands from PSVLS.

Ligand	Chemical structure	Molecular weight (g/mol)
Aminopterin		440.41
Desmosine		526.60

Ligand	Chemical structure	Molecular weight (g/mol)
Paclitaxel		853.90
Solanesol		631.06
Solanesyl pyrophosphate		791.02
D-(-)fructose		180.16

Table 2

Predicted versus experimental binding profiles for selected ligands from PSVLS. Lowest positive concentration refers to the lowest concentration tested that gave a positive response. Highest concentration tested refers to the highest concentration tested that gave a negative response. Inhibition results for aminopterin, desmosine, paclitaxel and fructose are from Dadgar et al.[19] and were obtained using FRET for 10 μ M of each ligand against BoNT/A LC (10 nM). (+) indicates positive response, whereas (–) indicates negative response in the SPA binding assays.

Compound Name	Computational Binding Preference (score) ¹	BoNT/A LC	BoNT/A HC	Lowest Positive Concentration	Highest Negative Concentration	EC ₅₀	% Inhibition at 10 μ M[19]
Aminopterin	R27 (–4.7) HC R8 (–4.8) HC R7 (–4.7) HC	–	+	1000 nM ⁴	500nM ³	703 nM	11
Desmosine	R6 (–5.5) LC R23 (–4.9) LC R10 (–4.8) HC R1 (–4.8) LC	–	+	2000 nM ⁴	3000 nM ³	1.6 μ M	25
Solanesol	R6 (–6.0) LC	–	–	NA	300 nM	NA	NA
Solanesyl pyrophosphate	R27 (–13.8) HC R3 (–13.4) HC R7 (–13.4) HC R2 (–12.9) LC R10 (–12.9) HC	+	+	600 nM ²	NA	ND	NA
Paclitaxel	R14 (–11.0) LC	+	–	50 nM	50 nM	17 nM	95
Fructose	R17 (–1.9) HC	–	–	NA	3000 nM	NA	0

¹ Score corresponds to holistic scores [19] at the best region(s) among the 33 regions scanned. Multiple regions are listed if the difference in binding energies was 1 unit or less. Scores not passing the per-region non-binder/binder threshold are shown in grey italic.

² The lowest positive concentration of SolanesylPP was 600 nM for both BoNT/A HC and LC.

³ Ligands were tested at higher concentration against BoNT/A LC, producing a negative response; no lower concentrations were tested afterwards.

⁴ Positive ligands at higher concentration were tested against BoNT/A HC at two lower concentrations (250 nM and 50 nM), producing negative responses.

NA = not applicable; ND = not determined; LC = light chain, including the catalytic site among others; HC = heavy chain.