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Inhibition *in vivo* of the activity of botulinum neurotoxin A by small molecules selected by virtual screening

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ABSTRACT

To search for small molecular size inhibitors of botulinum neurotoxin A (BoNT/A) endopeptidase activity, we have screened the NCI library containing about 1 million structures against the substrate binding pocket of BoNT/A. Virtual screening (VS) was performed with the software Glide (Grid-based ligand docking energetics) and the findings were confirmed by AutoDock. Ten compounds were found that had favorable energetic and glide criteria and 5 of these compounds were selected for their ability to protect mice *in vivo* against a lethal dose of BoNT/A. Each compound was incubated at different molar excesses with a lethal dose of the toxin and then the mixture injected intravenously into mice. At 4690 M excess, compounds NSC94520 and NSC99639 protected all (100%) the mice from lethal toxicity. Compounds NSC48461 and NSC627733 gave 75% protection. Compound NSC348884 showed the least inhibition of toxicity allowing only a fraction (25%) of the mice to survive challenge with a lethal dose; and in the case of the mice that did not survive there was a considerable delay of mortality. At 2400 M excess compounds NSC94520 remained fully protective while and NSC99639 afforded 75% protection and at 1200 M excess each of these two compounds gave 50% protection. The two compounds gave no protection at 600 or less molar excess. When each compound was administered intravenously at 4690 M excess at different times (from 1 h to 6 h) after the intravenous injection of the active toxin, none of the compounds was able to protect the animals from toxicity. The findings show the value of VS in identifying potential inhibitors of the toxin for further development and improvement.

1. Introduction

Botulinum neurotoxins (BoNTs) produced by the spore-forming anaerobic bacterium *Clostridium botulinum*, are the most toxic substances known. They cause botulism, which is a severe disease characterized by flaccid muscle paralysis due to BoNT-mediated blockage of acetylcholine release at the neuromuscular junctions. So far, seven different serotypes of BoNTs are known to exist (BoNTs A-G). Only serotypes A, B, E and F have been implicated in botulism cases in humans. Serotype A is the most potent amongst the botulinum neurotoxins (Cai and Singh, 2007). The neurotoxins are synthesized as an inactive single polypeptide chain (about 150 kDa) that is activated by post-translational proteolysis to release an active two subunit protein, a heavy chain (H

Abbreviations: BoNT, botulinum neurotoxin; Glide, Grid-based ligand docking energetics; MPA, mouse protection assay; SP, Standard precision; VS, virtual screening; XP, Extra precision.

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~100 kDa) and a light chain (L ~50 kDa) which are linked by an inter-chain disulfide bond (Kumaran et al., 2008a).

Botulinum neurotoxins interact with presynaptic nerve cells by binding first to receptors at the membrane of the nerve ending on the presynaptic neuromuscular junctions. This is followed by internalization via receptor mediated endocytosis. The interchain disulfide bond is then reduced and the L chain, which is a zinc endopeptidase (Schiavo et al., 1992; Fu et al., 1998) is translocated and released into the cytosol. The L chain acts on SNARE proteins (VAMP, syntaxin and SNAP-25), and inhibits neurotransmitter release by destroying the exocytotic docking/fusion machinery. BoNT serotypes A and E cleave SNAP-25 (synaptosomal-associated protein-25 kDa), whereas serotypes B, D, F and G cleave the vesicle-associated membrane protein (VAMP) at specific peptide bonds (for a recent review of BoNT's action see Aoki et al., 2010). BoNT/C has a unique role as it is able to cleave two substrates, SNAP-25 and syntaxin (Williamson et al., 1996).

Although BoNTs are listed as Category A bioterrorism agents by the Center of Disease Control and Prevention, there is to date no suitable post-exposure therapeutic intervention available. Protections have been reported that utilize modified molecule or subunit vaccines (Aoki et al., 2010). In certain cases it may not be desirable to vaccinate a patient who is receiving therapeutic treatment of BoNT, because the protective immune response will render the beneficial treatment ineffective. Also, the vaccine may have a limited effectiveness in neutralizing BoNT's toxicity after the toxin had undergone endocytosis by neuronal cells because the antibodies are not able to enter the nerve cell to neutralize the toxin. Therefore, it is essential to identify new compounds that can act as potent inhibitors of BoNTs and may be potentially useful in blocking the toxic action of the neurotoxin.

The aim of the study was to identify competent inhibitors of BoNT/A using virtual screening (VS) and then testing these compounds in mice *in vivo*. VS is a computational tool used in drug discovery research. It predicts the affinity of compounds from a virtual database to a particular protein target. Analysis of the scores of docked protein ligands helps to identify the structures that would most likely bind to a drug target with a certain affinity.

The substrate binding pocket of BoNT/A was screened against the NCI library containing about 1 million structures [http://www.cancer.gov/]. VS was performed with the software Glide (Grid-based ligand docking energetics) and AutoDock was used for validation.

2. Materials and methods

2.1. Hardware and software

Intel(R)-Pentium(R) 4, CPU 2.60 GHz, RAM: 4.01 GB was used with Linux SUSE 10.0 (operating system), Maestro Version 8.0.308, Glide 4.5. For autodocking we used Autodock Version 3.0.5 and AutodockTools Version 1.4.3 and VMD Version 1.8.5.

2.2. Virtual molecular dynamics

Visual Molecular Dynamics is a molecular visualization program for displaying, animating and analyzing large biomolecular systems using 3-D graphics and built-in scripting [www.ks.uiuc.edu/Research/vmd]. It was used to illustrate the ligand in the BoNT/A binding site.

2.3. ClustalW2

ClustalW2 is a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. It is available at: http://www.ebi.ac.uk/Tools/clustalw2/index.html. In this study ClustalW2 was used to generate an alignment of the catalytic domains of BoNT serotypes A, C, E, F and G. The alignment was used to identify highly conserved regions, especially in the substrate binding site.

2.4. Maestro and Glide

Maestro is a versatile environment for molecular modeling including a multitude of analysis and visualization tools. In this study it was used for VS of small compound libraries in order to identify an inhibitor of the BoNT/A substrate binding site. Maestro comprises the software Glide (Grid-based ligand docking energetics), which performs molecular flexible docking and VS. Glide docks flexible ligands into a rigid receptor structure by rapid sampling of the conformational, orientation, and positional degrees of freedom of the ligand. There are three modes of running Glide which differ in how ligand degrees of freedom are sampled and in the scoring function employed: High-throughput virtual screening (HTVS), standard precision (SP) and extra precision (XP). Each mode is a refinement of the previous mode. The following three basic steps were performed with Glide:

1) The ligand and toxin structure to be used were prepared using LigPrep (for ligand or NCI library) and Protein Preparation Wizard (for BoNT/A). Preparation included adding of hydrogen atoms, desalting, generating tautomers or generating different protonation states (Glide user manual, 2008).

2) The next step comprised the Grid Generation. In this step a distinct area of the BoNT/A structure was defined, in which the docking should be performed. The grid box was manually positioned in a way to comprise the whole ligand binding site of BoNT/A. Glide calculates and stores the affinity potential for specific atom types in each grid point in 3-dimensional grid maps. The generated grid files enable Glide to search for favorable interactions between the ligand and the binding-site region.

3) Afterward the docking step was performed using the generated grid files and the prepared ligands. There are two primary goals of flexible ligand docking: to accurately predict ligand poses and to rank ligands by predicted affinities to the protein.