Indian Journal of Biochemistry & Biophysics
Vol. 62, February 2025, pp. 161-168
DOI: 10.56042/ijbb.v62i2.12118

Inhibition of Botulinum neurotoxin A protease activity by Quercetagetin

Vaidyanathan VV[1]*, Saravanan K[2], Sistla R[2] & Binz T[3]

[1]Vipragen Biosciences Pvt. Ltd., #67B, Hootagalli Industrial Area, Mysore-570 018, Karnataka, India
[2]thinkMolecular Technologies Pvt. Ltd. #03, 1st Cross, Reliaable Tranquil Layout, Bengaluru-560 102, Karnataka, India
[3]Institute of Cellular Biochemistry, Hannover Medical School, Hannover-30625, Germany

Received 27 June 2024; *revised 24 August 2024*

There are concerns on use of Botulinum neurotoxin type A (BoNT/A) as a bioweapon and effective small molecule antidotes to treat toxin poisoning are required. Five phytochemicals with known medicinal properties were evaluated for their ability to bind at the active site of Botulinum neurotoxin A light chain (LC/A). *In silico* docking studies showed that natural flavonoid Quercetagetin interacts with critical amino acid residues in the catalytic pocket and substrate discriminating sub-site regions of the light chain. Using an *in vitro* protease assay with a radiolabeled derivative of the native SNAP-25 substrate and recombinant LC/A, we tested the inhibitory potency of the phytochemicals. Quercetagetin was found to inhibit toxin's protease activity with an IC_{50} of ~ 26 µM. Our study shows that Quercetagetin displays potential to block BoNT/A, and its core benzopyranone scaffold holds promise for developing useful inhibitors to treat botulism.

Keywords: Botulinum toxin type A, *In silico* docking analysis, Inhibition, Protease activity, Quercetagetin

Botulinum neurotoxins (BoNTs) are highly potent toxins produced by anaerobic gram-positive bacilli such as *Clostridium botulinum*[1]. They cause botulism characterized by flaccid paralysis. The clinical syndrome of botulism can occur following ingestion of contaminated food, from colonization of the infant gastrointestinal tract, or from a wound infection. Thus far, several serotypes (designated BoNT/A, B *etc.*, according to the order of their discovery) have been reported, among which toxins A, B, E, and F affect humans. Active BoNTs are composed of a heavy chain (HC/A, H-chain, 100kDa used for cell surface binding) and light chain (LC/A, L-chain, 50 kDa that has a protease activity). The H- and L-chains are held together by a disulphide bond. BoNTs act at neuromuscular junctions where the catalytic L-chain through its Zn-dependent protease activity attacks SNARE proteins (soluble N-ethylmaleimide-sensitive factor-attachment protein-receptor) necessary for membrane fusion during neurotransmitter release. This results in the inhibition of the release of acetylcholine at the peripheral neuromuscular junctions causing classical symptoms such as flaccid paralysis. Specific protein substrates for BoNTs are identified and toxin-target interactions characterized in great detail[2]. Among the BoNTs, BoNT/A is the most potent serotype and it targets SNAP-25 (synaptosome -associated protein of 25kDa), leading to poisoning[3]. Engineered forms of LC/A is being widely used for treatment of muscular and secretory disorders, and cosmetic purposes under different brand names[4].

Due to extreme potency, BoNTs are classified as dangerous bio-warfare agents and could be disseminated via aerosol or by contamination of water or food supplies, causing widespread casualties[5]. Another concern is the serious and long-term adverse events associated with its therapeutic and cosmetic use[6]. Adverse events reported include dysphagia, muscle weakness, and allergic reactions. At present effective antidotes for BoNT/A poisoning are very few and includes anti-toxins antibodies, which acts only on the free circulating toxin, and are ineffective for treating the post-BoNT infected cells. Efforts are on-going either towards developing chemical molecules that block binding of toxin to its cell surface receptors and those that target protease activity of the toxin[7,8]. Design of effective small molecule inhibitors to block BoNT protease activity is challenging for several reasons. Size

*Correspondence:
E-mail: vaidy.vv@vipragen.com
Abbreviations: BoNT/A, Botulinum neurotoxin type A; HC/A, H-chain A; IC_{50}, Inhibitory concentration 50%; LC/A, Light chain A; SNAP-25, synaptosome -associated protein of 25 kDa; SNARE, soluble N-ethylmaleimide-sensitive factor-attachment protein-receptor

and conformational flexibility of the active site, unusually extended toxin-substrate interface area, and discontinuity of substrate recognition motifs (exosites and subsites) are major hurdles. The substrate binding site in LC/A is very large, and ligands with surface areas of >200A^{o} is required for effective interaction. Enzyme-substrate interface is ~4820A^{o}, much larger than typical protein-protein interaction surface area (~1500A^{o}). For reasons cited above, rationally designed peptides and natural phytochemicals with extended structures displaying multiple functional groups are found as effective inhibitors.

Crystal structure of BoNT/A is well described[10-14]. The substrate binding site is in its light chain (LC/A) and this catalytic domain has 3 major components: 1) a Zinc metal ion (Zn^{2+}) located at the active site and is essential for the toxin's protease activity. His223 and His227 of the Zn^{2+} binding motif HEXXH directly coordinate the active site Zinc. Glu224 and Glu262 are also part of the active site. Glu224 coordinates the zinc through a water molecule whereas Glu262 coordinates directly 2) A hydrophobic rim is formed around the active site (subsite S1) by the amino acids Val70, Ile161, Phe163, Phe194 and Phe369. Arg363 and Tyr366 at the vicinity of the active site play an important role in peptide bond cleavage by correct positioning of the substrate 3) The N-terminal loop containing the residues 50-70 is reported to act as a doorway for the LC/A binding site by its opening and closing for any molecule approaching it. This loop prevents the molecule approaching the active site from dissociation by latching on to it. In addition to above, there are other components that influence the catalytic activity. A reactive Cys165 is present approximately 7-8 Å from the zinc ion and is the target for covalent inhibitors which employ a Cys-trapping warhead.

BoNT/A inhibitors identified thus far fall into two main categories; those which chelate Zn ion at the active site and, those which bind to exosite regions of the toxin. A common approach used to identify new scaffolds for drug design is *in silico* screening of natural product libraries using docking studies with BoNT/ LC-A crystal structure[15,16]. From the docking score, compounds are selected for evaluation in *in vitro* toxin assays and promising scaffolds are used for analog design. By this approach, derivatives of hydroxamates, quinolinols and peptidomimetics have been identified and they inhibit the toxin by zinc chelation[17-19]. Compounds such as D-chicoric acid, lomofungin, picolinic acid and nitrophenyl psoralen[20-22] bind to exosite regions in the toxin light chain. This interferes with substrate binding to the toxin and affects its enzyme activity. Although several inhibitors have been reported, many have not progressed further due to low potency, non-specificity and off-target activities[23].

We are interested to identify novel scaffolds from phytochemicals that block/inhibit bacterial toxins as natural products display structural complexity and various pharmacological activities. Natural products and their derivatives identified by virtual screening for binding to target proteins are excellent starting points for new drug discovery. Inhibitors for enzymes such as cyclooxygenase 1, SARS-Cov-2, Leishmaniasis, skin cancer, and bacterial virulence factors have been identified from Indian herbal plants by this approach[24-28]. In this study we have screened 5 well-known phytochemicals and report that Quercetagetin (6-hydroxy Quercetin), a cell-permeable natural flavonol extracted from marigold (*Tagetes erecta* L.) is an inhibitor of BoNT/A light chain protease activity. *In silico* docking experiments show that Quercetagetin makes multiple contacts in the catalytic pocket of the toxin and blocks enzyme activity without zinc chelation. In an *in vitro* assay using recombinant LC/A toxin, we show that Quercetagetin inhibits proteolysis of SNAP-25, the toxin's natural substrate at the neuronal junction. Our results highlight potential and utility of Quercetagetin as a template to design novel small molecule inhibitors for botulinum toxin poisoning.

Materials & Methods

Materials

All phytochemicals used in the study were procured from Sigma (Quercetagetin PHL85766, >90% pure; Quercetin Q4951, >95% pure; Chicoric acid C7243, >95% pure; Curcumin C1386, >65% pure). Stock solutions (10mM) were made in DMSO and stored at -80^{o}C. All other reagents used in the study were of analytical grade.

Methods

Docking procedures for the ligands of Botulinum Neurotoxin A

Selection of crystal structures

All the reported crystal structures of BoNT/A bound with various inhibitors were collected from the protein data bank (PDB) (www.rcsb.org). The conformational landscape of the protease was analyzed using C-α RMSD based clustering methods.

This resulted in eight unique clusters of BoNT/A. From each cluster, a representative crystal structure was selected based on the resolution and completeness of the catalytic domain. By this procedure, the crystal structures of 4KS6, 6XCF, 7KYF, 2ILP, 7KYH, 5V8U, 3QIY, 3QIZ and 3QJ0 were selected to utilize in the docking studies. Among these structures, except the PDB ID 7KYH, remaining protein structures had a resolution less than or equal to 2.3Å. Thus, clustering methods yielded an ensemble of unique conformations of BoNT/A which were used for further modelling of the compounds. All the protein structures contained zinc and the ligands that were modelled carried potential zinc binding motifs.

Docking procedure

ICM Pro software package (Molsoft) was employed to perform molecular docking. Generally, in the protein ligand docking programs, the protein is considered to be rigid. In reality, the segments of the protein structure are known to have movements in response to interactions with ligands and other molecules. Ignoring such movements in the docking of ligands is sometimes pointed out as a key drawback of general docking methods. Ensemble docking which has been implemented with the name 4D docking in the ICM software suite addresses this drawback to some extent. In this procedure, the docking grid consists of several protein molecules with different conformations instead of only one. The ligands are docked on to each of the conformation (member of the ensemble) separately and the best scoring conformation is reported. Thus, the conformational flexibility of the ligand as well as that of the protein is taken into account resulting in the enrichment of docked poses. Usually, the best scoring pose of a ligand is the most complementary to the binding site signifying a potential thermodynamically stable conformation of the protein for that ligand.

The selected eight crystal structures containing various conformations of LC/A were super-imposed and stacked together to account for the flexibility of the binding pocket. The binding site was defined by taking the amino acids within 5 Å surrounding the known inhibitor of LC/A. It comprised of the amino acids – Pro69, Val70, Ile161, Gln162, Phe163, Glu164, Thr220, His 223, Glu224, His227, Glu262, Arg363, Tyr366, Leu367, Asn368, Phe369 and Asp370. The bound inhibitors from the crystal structures were modelled in this receptor and the crystallographic binding modes were reproduced. The docking scores were optimized based on the scores obtained for these known BoNT/A inhibitors. The docking scores of the reported inhibitors were in the range of -27 to -20. All these inhibitors have a zinc binding group in common. The docking scores in ICM-Pro incorporates the following parameters: van der Waals interaction energy, number of torsions in the ligand, the solvation electrostatics energy change upon binding, the hydrogen bond energy, hydrophobic energy in exposing a surface to water, and the desolvation of exposed H-bond donors and acceptors. Since the protease activity of BoNT/A is mediated by zinc, interaction of the inhibitors with zinc was particularly noted in the docking modes.

Generation of recombinant proteins for toxin protease assay

Expression and purification of recombinant proteins

Standard procedures of molecular biology and protein expression were employed to generate C-terminally His_6-tagged LC/A which was purified on Ni-NTA resin, dialyzed against toxin assay buffer (10 mM HEPES, 150 mM K-glutamate, pH 7.2), aliquoted and stored at -70°C[27].

***In vitro* transcription and translation**

Rat SNAP-25A was synthesized *in vitro* from suitable transcription plasmids using the TNT® coupled rabbit reticulocyte lysate system (Promega), in the presence of ^{35}S-methionine (3.7 MBq, 1, 000 Ci/mmol; Hartmann Analytic Germany) in a total volume of 25 µL.

***In vitro* Toxin assay for BoNT/A**

Toxin cleavage of recombinant SNAP-25 protein was done as described earlier[28-29]. The cleavage assay contained 1 µL of the translation mixture of ^{35}S-methionine-labeled rat SNAP-25A with recombinant LC/A. To test inhibitor effects of phytochemicals, LC/A was first incubated with the test item for 10 min at room temperature (21°C). Post incubation, reaction was started by adding radiolabeled substrate. Final reaction was incubated for 60 min at 37°C in a total volume of 10 µL of toxin assay buffer. At the end of incubation, 1 µL of the reaction mixture was withdrawn and mixed with SDS-PAGE sample buffer (120 mMTris-HCl, pH 6.75, 10% (vol/vol) β-mercaptoethanol, 4% (wt/vol) sodium dodecyl sulfate (SDS), 20% (wt/vol) glycerol, 0.014% (wt/vol) bromphenol blue]. Samples were heated for 3 min at 95°C and subjected to SDS–PAGE electrophoresis using 15% gels. Proteins were visualized (radiolabeled) by using a FLA-9000 phosphoimager

(Fujifilm, Japan) and evaluated by the MultiGauge program (Fujifilm). For quantitative analysis of substrate cleavage, assays were done in triplicates (n=3) to generate mean and standard deviation values.

Results

Stabilization of Quercetagetin binding at the active site through H-bonding

We selected five well known natural compounds for screening. Chemical structures of phytochemicals tested is shown in (Fig. 1). Quercetin (Q) and Quercetagetin (QG) are common flavonoids (benzopyranone derivatives). D-Chicoric acid (hydroxyl cinnamic acid) is a caffeic acid derivative. Chlorogenic acid is an ester of caffeic acid and quinic acid (5-caffeoylquinic acid ester). Curcumin is a common curcuminoid used as health supplement, food flavouring and cosmetics. The compounds have structural similarity with several hydroxyl functional groups displayed on ring structure.

Plausible docking modes of the test compounds in the active site of LC/A are shown in (Fig. 2) with corresponding docking scores. Quercetagetin binds in the hydrophobic pocket of the binding site (in the vicinity of Zn metal) with a docking score of -27.0. The two hydroxyl groups at 3' and 4' positions of ring C bind through the H-bond interactions with backbone carbonyl oxygen of Val70 and Gly195, respectively. Rings B and C make π-π-stacking interactions with Phe194. The hydroxyl group at position 5 of ring A is within H-binding distance from Glu224 (the residue involved in the proton transfer to the amide nitrogen in the catalytic mechanism). The OH group at position 6 of ring A makes a H-bond interaction with Phe163 backbone. This OH group is the only structural difference between Quercetagetin and Quercetin. Therefore, this H-bond interaction might play a role in the difference of affinity between them towards BoNT/A. Quercetin binds to the LC/A in a different mode in which the OH group at position 7 of ring A is coordinated to the active site zinc ion. There is an intra-molecular H-bond between carbonyl at 4th position of ring B and the OH group at position 5 of ring A. The OH group at 4' of ring C is involved in an H-bond interaction with Asn362. The docking score of Quercetin binding to the LC/A is -26.6 which is similar to that of Quercetagetin. The docking modes of these two compounds which suggest interactions with zinc and Phe163 provide insights into design of novel BoNT/A inhibitors.

Curcumin binds along with zinc binding site of LC/A with a docking score of 20.4. The carbonyl oxygen atom is coordinated to the zinc ion and one of the phenyl groups is sandwiched between Phe163 and Phe194 through a π-π stacking interaction. It would thus appear that in case of Curcumin and Cholorogenic acid, stabilization of ligand binding via H-bonds is lacking, which may be the reason for their diminished inhibitory potency. Chlorogenic acid has the least affinity for LC/A from the test compounds with a very low docking score of -17.2. One of the OH groups from cyclohexyl is coordinated to zinc ion and has an H-bond interaction with Glu224. The OH group, substituted on the same carbon as of the acid, is having a strong H-bond interaction with Phe163. There are no other stabilizing interactions from the phenyl side of the molecule.

D-chicoric acid has similar binding affinity as Quercetagetin and Quercetin towards LC/A with a docking score of -27.4. One of the carboxylic acids is coordinated to the zinc ion whereas the other is in H-bond with the Phe163 backbone. The two OH groups in one of the phenyl groups are stabilized by

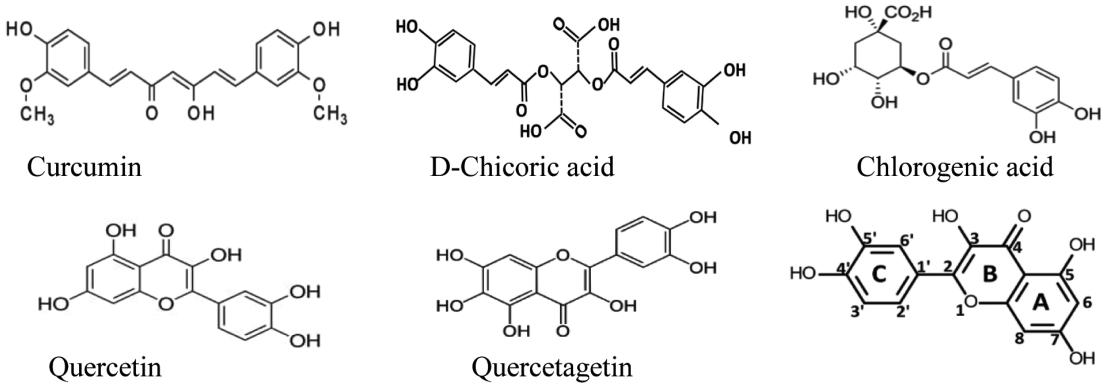

Fig. 1 — Structure of phytochemicals tested for interaction with BoNT/A light chain. Structures were selected for similarity andcomparable position of functional hydroxyl groups. Last structure shows numbering of C atoms and ring specifications in Quercetin

H-bond interactions with Val70 and Gly195 respectively. This phenyl group was also stabilized by a π-π stacking interaction with Phe194. From the other phenyl group, one OH group is in H-bond interaction with Asn368 backbone.

Inhibition of BoNT/A light chain protease activity by Quercetagetin in *in vitro* assays

It was interesting to see if a correlation existed between the docking scores and ligand potency. For this, we evaluated inhibitory effects of phytochemicals on protease activity of LC/A. In this assay, test compounds were pre-incubated with recombinant BoNT/A light chain for 10 min and residual protease activity was measured in an *in vitro* assay using radiolabeled rat SNAP-25A as the substrate. Our *in vitro* assay is based on SDS-PAGE analysis that detects both intact and cleaved SNAP-25 protein. Rat SNAP-25A is a 206 amino acid protein with an observed molecular weight of ~25kDa in SDS-PAGE gels. LC/A cleaves SNAP-25 at Gln197-Arg198, generating a faster moving band of ~ 23kDa which is visualized in 15% SDS-PAGE protein gel using a phosphorimager.

Result of this experiment is shown in (Fig. 3). Except Curcumin, all other compounds were tested at 10 µM. Curcumin was tested at 20 µM concentration. Screening results showed that among the tested phytochemicals, Quercetagetin was most potent and it blocked LC/A activity by ~60% at 10 µM concentration (Fig. 1B, Lane 4). Other phyto-chemicals had low potency and inhibition varied from 2-20%.

In the next set of experiments, Quercetagetin concentration was varied from 3 to 100 µM, to get an

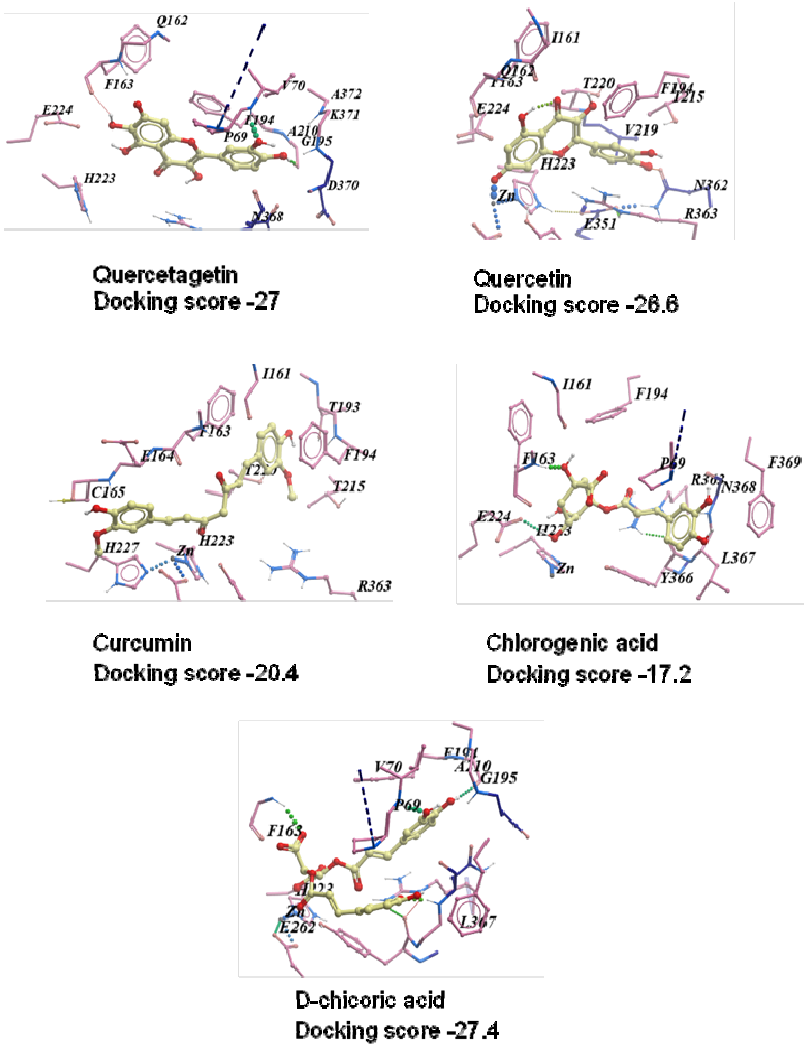

Fig. 2 — The best docking modes of phytochemicals studied in this work as LC/A ligands and their docking scores

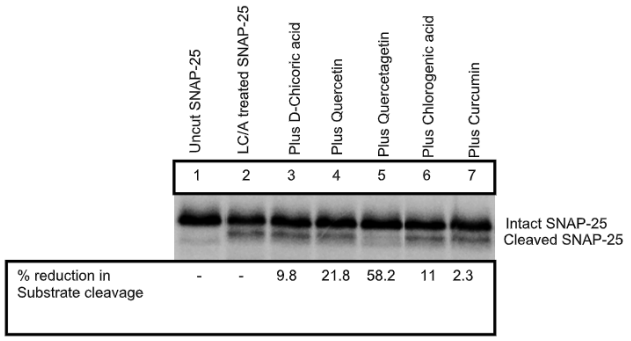

Fig. 3 — Inhibition of BoNT/A light chain activity in *in vitro* assays by phytochemicals. Recombinant LC/A light chain expressed in *E. coli* was pre-incubated with test compounds for 10 min on ice, and residual protease activity was measured using radiolabeled rat SNAP-25 as the substrate. Substrate cleavage was determined by SDS-PAGE and fluorography, and % reduction in substrate cleavage was determined from densitometry scan data. Data shown is representative of 2 separate experiments. Curcumin was tested at 20 µM, while all other compounds were tested at 10 µM concentration

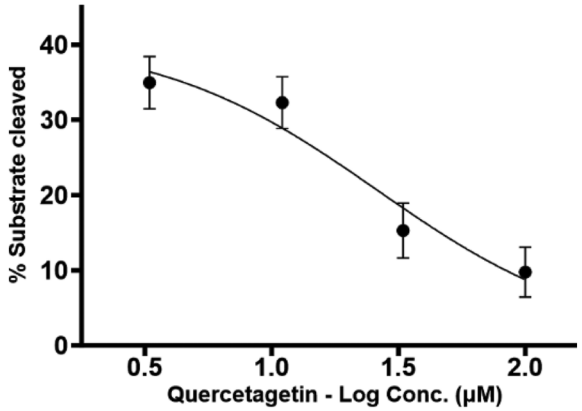

Fig. 4 — Dose dependent inhibition of BoNT/A light chain protease activity by Quercetagetin. Recombinant LC/A light chain expressed in *E. coli* was pre-incubated with various concentrations of Quercetagetin for 10 min on ice, and residual protease activity was measured using radiolabeled rat SNAP-25 as the substrate. Substrate cleavage was determined by SDS-PAGE and fluorography, and % reduction in substrate cleavage was determined from densitometry scan data. Data shown is representative of 2 separate experiments, and values given are mean +/- SD of triplicate estimations

estimate of its IC_{50} value. Result of this experiment is presented in (Fig. 4). The *in vitro* toxin assay is designed in such a way that only about 50% of the substrate (SNAP-25) is cleaved when treated with 1 nM of LC/A for 1 h at 37°C. Without inhibitor treatment, ~53% of substrate was cleaved. After treatment with 3.3 µM of Quercetagetin, only 33% (+/-6) of substrate was cleaved. When the Quercetagetin concentration in the incubation was increased to 11 µM and 33 µM, % substrate cleaved was 32% (+/-6), and 15% (+/-6), respectively. At the highest concentration tested (100 µM Quercetagetin), substrate cleaved reduced to 9% (+/-5.74). This represented almost 90% reduction from control substrate cleavage value. The dose response curve was generated using GraphPad Prism and *in vitro* IC_{50} value was determined as 26 µM.

Discussion

There is a growing feeling that extremely potent toxins such as BoNT/A could be used as a bioweapon and therefore development of small molecule inhibitors is of great urgency. Such inhibitors are also useful to abrogate unintentional overdose scenarios of BoNT/A preparations used in cosmetics and treatment of secretory diseases. Developing inhibitors for BoNT/A toxin commonly involves targeting its enzymatic activity directed towards neuronal protein SNAP-25. Due to large interface between the toxin and its target, it is generally found that effective inhibitors are those with extended structure and several functional groups.

In this study, we have investigated the interaction of five natural products of known medicinal properties with botulinum neuroxin type A light chain (LC/A). We selected phytochemicals that have similar extended structures and positioning of functional groups to aid productive interaction with the target protein (Fig. 1). Docking studies using known crystal structures of LC/A showed that the phytochemicals tested are able to occupy the active site cleft and interact with critical amino acid residues influencing proteolysis. High docking score of ~-27.0 was obtained for Chicoric acid, Quercetin and Quercetagetin, while Curcumin and Chlorogenic acid showed relatively lower scores. Certain differences were seen in the binding of flavonoids Quercetin and its 6-OH derivative Quercetagetin. Binding modes were overall similar except that 6-OH group in Quercetagetin made additional H-bond with Phe163. Phe163 is part of the S1 subsite (5 subsites namely S1-S5 present in the toxin A light chain), involved in stabilizing the substrate binding in the enzyme active site for productive cleavage[14]. Direct interaction with the catalytic Zn^{2+} ion was not seen for Quercetagetin. This is interesting as Benzopyranone ring present in Quercetagetin and Quercetin is a well-known zinc binding structure.

In *in vitro* substrate cleavage assays, Quercetagetin was fairly potent with an IC_{50} of ~26 µM. This value is promising notwithstanding challenges in determining dose response for natural compounds that inherently have low aqueous solubility. Other tested

phytochemicals were weaker inhibitors, although some of them had docking score similar to Quercetagetin. In our docking studies Chicoric acid exhibited the highest docking score of -27.7 but inhibited the enzyme activity weakly. Quercetin which is structurally very similar to Quercetagetin, with a docking score of -26.6 was also a weak inhibitor. Correlating a good dock score with biological efficacy seems to be difficult as docking program only provides estimate of binding affinity[30]. Therefore docking score may not easily translate to inhibitory potency in the absence of information about the mechanism of interaction. Lack of correlation between docking score and biological efficacy is reported for certain classes of enzymes[31]. Active site of LC/A is flexible and able to accommodate a variety of ligands but all such interactions may not be productive. It is possible that additional interaction through 6-OH group of Quercetagetin in the active site region of the toxin, highlighted in this study, stabilizes the enzyme-inhibitor complex efficiently than other naturals tested.

In summary, we show that natural flavonoid Quercetagetin is a promising inhibitor of BoNT/A protease activity. We have shown plausible binding modes for Quercetagetin in the LC/A catalytic site and it's mode of action appears through its interaction with Phe163 leading to unfavourable substrate orientation at the catalytic site. Active-site zinc chelation was not observed in docking studies. This information offers a good starting point for design and evaluation of Quercetagetin analogs with better safety and drug-like properties. Our study also points to the immense potential of natural and ayurvedic medicinal plants as a rich source for novel scaffolds for further exploration as blockers of bacterial toxins.

Acknowledgement

Financial assistance provided to VVV by Alexander von Humboldt Foundation, Germany to undertake part of this study at the Medical University Hannover (MHH) is greatly acknowledged. We thank Dr. Harsha Rajagopal, Vipragen Biosciences, for his help in generating the dose response curve using GraphPad Prism. This paper is dedicated to the memory of Prof. T. Ramasarma.

Conflict of interest

All authors declare no conflict of interest.

References

1 Popoff MR & Bouvet P, Clostridial Toxins. *Future Microbiol*, 4 (2009) 1021.

2 Binz T, Clostridial neurotoxin light chains: devices for SNARE cleavage mediated blockade of neurotransmission. *Curr Top Microbiol Immunol*, 364 (2013) 139.

3 Binz T, Sikorra S. & Mahrhold S, Clostridial Neurotoxins: Mechanism of SNARE Cleavage and outlook on potential substrate specificity reengineering. *Toxins,* 2 (2010) 665.

4 Rasetti-Escargueil C & Palea S, Embracing the versatility of botulinum neurotoxins in conventional and new therapeutic applications. *Toxins,* 16 *(2024)* 261.

5 Cenciarelli O, Riley PW & Baka A, Biosecurity Threat Posed by Botulinum Toxin. *Toxins,* 20 (2019) 681.

6 Eugenia Y. Serious and Long-Term Adverse Events Associated with the Therapeutic and Cosmetic Use of Botulinum Toxin. *Pharmacology*, 95 (2015) 65.

7 Ben David A, Barnea A, Diamant E, Dor E, Schwartz A, Torgeman A & Zichel R, Small molecule receptor binding inhibitors with *in vivo* efficacy against botulinum neurotoxin serotypes A and E. *Int J Mol Sci*, 22 (2021) 8577.

8 Li B, Peet NP, Butler MM, Burnett JC, Moir DT & Bowlin TL, Small molecule inhibitors as countermeasures for botulinum neurotoxin intoxication. *Molecules*, 16 (2010) 202.

9 Jacobson AR, Adler M, Silvaggi NR, Allen KN, Smith GM, Fredenburg RA & Tzipori, S, Small molecule metalloprotease inhibitor with *in vitro*, *ex vivo* and *in vivo* efficacy against botulinum neurotoxin serotype A. *Toxicon*, 137 (2017) 36.

10 Lacy B, Tepp W, Cohen A, Das Gupta B & Stevens R. Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nat Struct Mol Biol*, 5 (1998) 898.

11 Kumaran D, Rawat R, Ahmed SA & Swaminathan S, Substrate binding mode and its implication on drug design for botulinum neurotoxin A. *PLoS Pathog,* 4 (2008) 1.

12 Nicholas RS, Grant EB, Mark SH, Jack PK, Saul T, Kim DJ & Karen NA, Structures of *Clostridium botulinum* neurotoxin serotype A light chain complexed with small-molecule inhibitors highlight active-site flexibility. *Chem Biol*, 14 (2007) 533

13 Silvaggi NR, Wilson D, Tzipori S & Allen KN, Catalytic features of the botulinum neurotoxin A light chain revealed by high resolution structure of an inhibitory peptide complex. *Biochemistry*, 41 (2008) 5736.

14 Chen S, Kim JJP & Barbieri JT. Mechanism of Substrate Recognition by Botulinum Neurotoxin Serotype A. *J Biol Chem*, 282 (2007) 9621.

15 Yalamanchili C, Manda VK, Chittiboyina AG, Guernieri RL, Harrell WA Jr, Webb RP, Smith LA & Khan IA, Utilizing *Ayurvedic* literature for the identification of novel phytochemical inhibitors of botulinum neurotoxin A. *J Ethnopharmacol,* 197 (2017) 211.

16 Patel KB, Cai S, Adler M, Singh BK, Parmar VS & Singh BR, Natural Compounds and Their Analogues as Potent Antidotes against the Most Poisonous Bacterial Toxin. *Appl Environ Microbiol,* 84 (2018) 1.

17 Šilhár P, Silvaggi NR, Pellett S, Čapková K, Johnson EA, Allen KN & Janda KD, Evaluation of adamantine hydroxamates as botulinum neurotoxin inhibitors: synthesis, crystallography, modeling, kinetic and cellular based studies. *Bioorg Med Chem,* 21 (2013) 1344.

18 Minnow YV, Goldberg R, Tummalapalli SR, Rotella DP & Goodey NM, Mechanism of inhibition of botulinum

neurotoxin type A light chain by two quinolinol compounds. *Arch Biochem Biophys,* 618 (2017) 15.

19 Amezcua M, Cruz RS, Ku A, Moran W, Ortega ME, Salzameda NT, Discovery of dipeptides as potent botulinum neurotoxin a light-chain inhibitors. *ACS Med Chem Lett*, 12 (2021)295.

20 Salzameda N, Eubanks L Zakhari J, Tsuchikama K, DeNunzio N, Allen K, Hixon M & Janda KA, Cross-over inhibitor of the botulinum neurotoxin light chain B: A natural product implicating an exosite mechanism of action. *Chem Commun,* 47 (2011) 1713.

21 Li B, Peet NP, Butler MM, Burnett JC, Moir DT & Bowlin TL, Small molecule inhibitors as countermeasures for botulinum neurotoxin intoxication. *Molecules*, 16 (2010) 202.

22 Bremer PT, Xue S & Janda KD, Picolinic acids as β-exosite inhibitors of botulinum neurotoxin A light chain. *Chem Commun,* 52 (2016) 12521.

23 Patel KB, Kononova O, Cai S, Barsegov V, Parmar VS, Kumar R & Singh BR, Botulinum neurotoxin inhibitor binding dynamics and kinetics relevant for drug design. *Biochim Biophys Acta*, 1865 (2021) 129933.

24 Khare C, Jain S, Prajapati H, Dubey N & Ganeshpurkar A, Virtual screening of Ginsenosides for COX-2 inhibition: Insights from *in silico* docking studies. *Indian J Biochem Biophys*, 61 (2024) 581

25 Sharma A. *In silico*-based studies on phytochemicals from native Indian plants as potential inhibitors of SARS-CoV-2. *Indian J Biochem Biophys,* 59 (2022) 653.

26 Jyothi K, Sivaranjani V, Pavithra U, Jayavel S & Muthulakshmi L, Computational studies on new Leishmanial drug targets against Quercetin. *Indian J Biochem Biophys,* 59 (2022) 909.

27 Namasivayam SKR, Gowri Shankar K, Vivek JM, Nizar M & Sudarsan AV, *In silico* and *in vitro* analysis of quorum quenching active phytochemicals from the ethanolic extract of medicinal plants against quorum sensing mediated virulence factors of *Acinetobacter baumannii. Indian J Biochem Biophys,* 56 (2019) 276.

28 Vaidyanathan VV, Yoshino K, Jahnz M, Dörries C., Bade S, Nauenburg S, Niemann H. & Binz, T. Proteolysis of SNAP-25 Isoforms by botulinum neurotoxin types A, C, and E. *J Neurochem,* 72 (1999) 327.

29 Vaidyanathan VV & Binz T, Ability of human SNAP-23 to generate high molecular weight SDS-resistant ternary SNARE complexes is influenced by C-terminal coil content. *Biochem Biophys Rep*, 28 (2021) 101150.

30 Chen YC, Beware of docking. *Trends Pharmacol Sci,* 36 (2015) 78.

31 Spassov DS, Atanasova M & Doytchinova I, Inhibitor Trapping in N-Myristoyltransferases as a Mechanism for Drug Potency. *Int J Mol Sci,* 24 (2023) 11610.