The Mechanism of Inhibition of Botulinum Neurotoxin Type A by Two Quinolinol Compounds

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ABSTRACT

Quinolinol-based compounds are a promising starting point for discovery of effective inhibitors of the clostridial neurotoxin, botulinum neurotoxin type A light chain (BoNT/A LC). Insights into the mechanism of inhibition by quinolinol compounds facilitate interpretation of docking data and inhibitor optimization. In this study, a fluorogenic substrate of BoNT/A, SNAPtide, was used to study the mechanism by which two new quinolinol compounds, MSU58 and MSU84, with IC₅₀ values of 3.3 μM and 5.8 μM, respectively, inhibit BoNT/A LC. Kinetic studies and model discrimination analysis showed both compounds to be competitive inhibitors of BoNT/A LC with inhibition constants (Kᵢ) 3.2 μM and 6.2 μM for MSU58 and MSU84, respectively. The kinetic rate constant for substrate and inhibitor binding and release were also determined. These data indicate that the inhibitors bind in the BoNT/A LC active site and that inhibitor binding is mutually exclusive with the binding of the substrate. This is the first study to report the competitive inhibition of BoNT/A LC by quinolinol compounds. These data help define the inhibitor binding pocket and, along with structure activity relationship studies, provide immediate direction for further compound synthesis.
MONTCLAIR STATE UNIVERSITY

Mechanism of inhibition of botulinum neurotoxin type A by two quinolinol compounds

by

Yacoba Vroom Teschemaker Minnow

A Master’s Thesis Submitted to the Faculty of

The College of Science and Mathematics

Montclair State University

In Partial Fulfillment of the Requirements

For the degree of

Master of Science in Pharmaceutical Biochemistry

January 2017
THE MECHANISM OF INHIBITION OF BOTULINUM NEUROTOXIN TYPE A BY TWO QUINOLINOL COMPOUNDS

A THESIS

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by
Yacoba Vroom Teschemaker Minnow
Montclair State University
Montclair, NJ
2017
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CHAPTER 1: INTRODUCTION

Botulinum neurotoxins (BoNTs) cause botulism, a deadly condition characterized by flaccid paralysis. They have been categorized as Category A biowarfare agents by the Centers of Disease Control and Prevention \[^{1-2}\]. While worldwide there are only about 1000 cases reported yearly \[^{3-4}\], due to BoNT’s potency and potential ease of production for bioterrorism activity, inhibitors are needed for the clinic \[^{4-9}\]. Current therapy for exposure to the toxin relies on inhibitory antibodies, which have several limitations including a limited supply of the antitoxin, unknown long term effects, and a short application window (approximately 24 hours post exposure) \[^{4-5,10}\]. Challenges in BoNT inhibitor discovery include the large peptide substrate-enzyme interface, which makes it more difficult to define a site where an inhibitor may bind, and conformational flexibility of BoNT \[^{4,11}\]. Quinolinol compounds have been explored as BoNT inhibitors and optimization efforts can be aided by a deeper understanding of the mechanism by which compounds inhibit BoNT activity.

BoNTs are produced by the anaerobic bacterium *Clostridium botulinum* and are endoproteases that cause flaccid neuromuscular paralysis by blocking acetylcholine release at the neuromuscular junction. BoNTs are secreted as holotoxins, consisting of a ~100 kD heavy chain (HC) and a ~50 kD light chain (LC), linked by a disulfide bond \[^{12-14}\] (Figure 1). The LC is a zinc-dependent metalloprotease while the HC facilitates the targeting and internalization of LC into cells \[^{14}\]. There are seven antigenically distinct serotypes of BoNTs labelled A-G; serotype A is the most potent and most prevalent toxin in humans \[^{10,15}\]. When BoNT enters the bloodstream, the holotoxin is transported to the neurotransmitter junction and enters the presynaptic neuron. The LC is liberated.
intracellularly and cleaves soluble N-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) peptides and prevents acetylcholine release into the neuromuscular junction, resulting in neuromuscular paralysis (botulism) \[^{10}\]. Intoxication with botulinum toxins may occur naturally through ingestion of contaminated food (foodborne botulism), through wounds contaminated with clostridia, or by deliberate introduction through injection or inhalation \[^{7}\].

**FIGURE 1:** Crystal structure of BoNT/A showing the 100 kD HC (green), 50 kD LC (blue, cyan), the receptor binding domain (yellow, red orange) and the catalytic zinc (dark grey). The crystal structure was obtained from the Protein Data Bank (PDB # 3BTA) and modified with Chimera (UCSF, San Francisco, CA).

The natural substrate of BoNT/A is a large 25 kDa peptide (SNAP-25, amino acid residues 1-206) \[^{15-16}\]. Studies have shown that although the presence of the entire length of the natural substrate enhances substrate recognition and specificity, only the amino acid residues 197-202 are in direct contact with the catalytic site of BoNT/A LC. Catalytic cleavage occurs at the scissile bond between residues 197 and 198 \[^{2,16}\]. Crystal structures of truncated substrate analogs (aa residues 197-202) bound to BoNT/A LC.
(PDB ID # 3DDA, 3DDB) revealed multiple potential sub-binding sites (subsites) in the active site \[^2\]. The crystal structure # 3DDA shows that the carbonyl oxygen and N-terminal NH\(_2\) of Gln197 of the substrate chelate the catalytic zinc in the BoNT/A LC active site. The 8-hydroxyquinoline (quinolinol moiety) was suggested to form a close interaction with the zinc cation to exclude any water present at the active site \[^9,17\], possibly mimicking the interaction between Gln197 of the substrate with the catalytic zinc. The salt bridge formed between the guanidinium of Arg198 sidechain and Asp370 sidechain (BoNT/A LC residue) also appears to be crucial for catalytic activity \[^2\].

Burnett and coworkers suggested that there are two binding subsites (1 and 2) in the catalytic region of BoNT/A LC that are important for the proper binding and orientation of small molecule inhibitors. The hydrophobic pocket of subsite 1 is occupied by the aromatic sidechains Phe162, Phe177 and Phe193 and the methyl side chain of Thr219. Hydrophobic interactions with a methyl substituent of a chemical compound are typical interactions found in this site \[^9\]. Subsite 2 is a deep pocket and consists of Met164, Thr175, Arg230, Pro238 and His226, which is required for catalysis. Several polar residues, Glu55, Gln161, Glu163, Lys175 and Arg176, are also present in subsite 2 and may participate in ionic interactions or water mediated hydrogen bonding with an ionizable amine in an inhibitor \[^9\]. Burnett and coworkers proposed that potent inhibitors of BoNT/A LC must interact with residues present in both binding subsites 1 and 2 in the substrate binding region \[^9\].

Efforts in small molecule and peptidic BoNT type A (BoNT/A) inhibitor discovery have been ongoing since the late 1990's \[^8-9,13,18\]. Silhar and coworkers reported that a hydroxamate compound used in combination with the non-competitive
natural product inhibitor chicoric acid resulted in synergistic inhibition of BoNT/A LC but hydroxamate compounds raise toxicity concerns due to promiscuous zinc chelation \cite{19, 20}. Recently, quinolinol compounds have received attention as inhibitors of BoNT/A LC \cite{4, 17, 21} in part because of structural dissimilarity to other known metalloprotease inhibitors. Moreover, quinolinol compounds have low molecular weights, offer several points for structure activity investigation and can be readily synthesized by a multicomponent condensation \cite{17}. Caglic and coworkers screened 188 quinolinol compounds for inhibitory activity against BoNT/A LC and found that 80\% of the most active compounds had IC$_{50}$ values below 10 \textmu M with few in the sub-micromolar range \cite{17}. Structure activity relationship studies revealed that the quinolinol moiety alone was not sufficient to elicit any inhibitory activity against BoNT/A LC. Their analysis and modeling led to the hypothesis that aryl groups at R$_2$ and R$_3$ of the template (Figure 2) promote proper conformation, permit binding into the active site and that substitutions at positions R$_2$ and R$_3$ (Figure 2) with bulky heterocyclic groups improved inhibitory activity \cite{17}. However, no detailed enzymatic or kinetic studies were carried out to investigate the mechanism by which quinolinols inhibit BoNT/A.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{fig2.png}
\caption{Structures of the quinolinol scaffold (left) and compounds 4-chloro- \textit{N}-(4-fluorophenyl) methyl] pyridin-3-amine (MSU58) and 4-chloro-(3-fluorophenyl) methyl benzenesulfonamide (MSU84) (right).}
\end{figure}
There are three types of inhibition (competitive, noncompetitive, and uncompetitive) that are used to describe how an inhibitor binds to the target enzyme. Characterization of the mechanism of inhibition of BoNT/A LC by quinolinol compounds can provide insights on how these compounds interact with the target. For example, knowing whether inhibitor and substrate binding are mutually exclusive events and whether an inhibitor binds in the substrate binding site or another distinct site would facilitate structure activity relationship studies, the design of more potent inhibitors, and the interpretation of inhibitory data. This understanding is particularly needed for BoNT/A LC, a challenging target with an unusually large substrate-enzyme interface where a small molecule may need to possess multiple functionalities to block the binding of substrate. Molecular docking studies performed by Roxas-Duncan and coworkers with five quinolinol analogs predicted that they interact with the zinc cation in the hydrophobic pocket of the LC, blocking the active site [4]. Conversely, subsequent enzyme binding studies by Lai and coworkers reported noncompetitive inhibition of BoNT/A LC by quinolinol compound 7-(phenyl(8-quinolinylamino) methyl)-8-quinolinol (QAQ) [21]. Noncompetitive inhibitors bind to a site distinctly different from the substrate binding site and bind equally well to free enzyme and to enzyme-substrate complex. Optimization efforts of noncompetitive inhibitors must focus on the site where the inhibitor binds, distinct from the substrate binding site, and consider the structural changes that result from substrate binding.

In this work, we determine the inhibition mechanisms of two novel quinolinol inhibitors MSU58 and MSU84 (Figure 2) against BoNT/A LC metalloprotease activity using a commercially available fluorescent resonance energy transfer (FRET) substrate,
SNAPtide. While the docking studies with several quinolinol inhibitors suggest that these compounds block the active site zinc, experimental kinetic studies on QAQ support noncompetitive inhibition. To increase the understanding of the mechanism of action of quinolinol derivatives against BoNT/A LC, we apply the FIQ correction and determine the mechanism of inhibition by new compounds, MSU58 and MSU84.

CHAPTER 2: METHODS AND MATERIALS

Materials
A recombinant BoNT/A LC (product #610) (residues 1-429 of the full length BoNT/A), its fluorogenic substrate SNAPtide (FITC/DABCYL) (product #521) and the unquenched peptide (product #528) were obtained from List Biological Laboratories (Campbell, CA). The inhibitors, MSU58 and MSU84 were synthesized in the Montclair State University medicinal chemistry laboratory. All other buffers and reagents were obtained from Fisher Scientific (Hampton, NH).

Preparation of buffers, substrate (SNAPtide), enzyme (BoNT/A LC) and unquenched product

Enzyme dilution buffer (50 mM K-HEPES with 0.05 % Tween 20, pH 7.4) and assay buffer (40 mM K-HEPES 0.01 % Tween 20, pH 7.4), were made from the free acid form of HEPES (H-HEPES) and potassium-HEPES (K-HEPES). Each buffer solution was filter sterilized using a Corning 0.22 μM filter and refrigerated. A 2.5 mM stock solution of SNAPtide was prepared by reconstituting 200 nmoles of the commercial product in 80% DMSO and a 2 μM stock solution of BoNT/A LC, by reconstituting 10
μg in enzyme dilution buffer, per manufacturer’s instructions. The unquenched peptide (50 nmoles) was reconstituted in DMSO to make a 500 μM stock solution. All the solutions were stored in aliquots at -20 °C and all assays were conducted in 40 mM K-HEPES 0.01 % Tween 20 pH 7.4 at room temperature unless otherwise stated.

**Determination of kinetic parameters**

The kinetic parameters and standard curves were measured concurrently in Costar half area black 96 well plates (Costar #3694, Corning Inc). The fluorescent signal was monitored in a Biotek (Synergy H1, # 15061913) plate reader set (490 nm excitation, 523 nm emission; gain (sensitivity) of 50) for 90 minutes at 1 minute intervals with shaking for 30 seconds between each read [22]. Unquenched peptide was diluted in assay buffer to final concentrations ranging from 0.3 μM to 3 μM to obtain a standard curve. The slope of the graph of fluorescent signal versus concentration of the unquenched peptide was used to convert the fluorescent signals of the assay wells to initial velocities (V₀) in μM/min.

The V₀ measurements were conducted in triplicate at different substrate concentrations, with maximum substrate concentration of 50 μM, in a 50 μL well volume containing 4.2 nM BoNT/A LC. Diluted substrate solutions were prepared in 16 % DMSO in assay buffer and each substrate solution contributed 1.6 % DMSO to the well. The final concentration of DMSO in the well was 3.2 % (1.6 % from SNAPtide and 1.6 % from inhibitor). Control experiments demonstrated that BoNT/A LC could tolerate increasing DMSO concentrations up to 6 % DMSO in assay buffer with no significant reduction in reaction rate.
Determination of inhibition constants ($K_i$) and dissociation constant ($K_D$)

The $K_i$ of MSU58 and MSU84 was determined using concentrations of 0, 3, 6 and 9 μM of each inhibitor. A 10 mM stock of inhibitor was initially diluted in DMSO and then subsequently in assay buffer to make a 90 μM inhibitor solution. Serial dilutions were made in 16 % DMSO in assay buffer and each inhibitor dilution contributed a final DMSO concentration of 1.6 % in a 50 μL final assay volume containing 4.2 nM BoNT/A LC and multiple SNAPtide concentrations ranging from 2.9 μM to 50 μM.

The inhibitor, assay buffer and BoNT/A LC were preincubated for 30 minutes on a plate shaker at room temperature after which the SNAPtide was added and placed in the Synergy H1 plate reader for a kinetic read. Controls for the assay include, BoNT/A LC and SNAPtide in the absence of inhibitor, SNAPtide alone, assay buffer alone and the unquenched peptide at varying concentrations. The reciprocals of the $V_o$ were plotted against the inhibitor concentration to form Dixon plots, from which the $K_i$ values were estimated. The $K_i$ values were also determined from the IC$_{50}$ values, using the Cheng-Prusoff equation [23].

To determine the $K_D$, the fluorescence of BoNT/A LC (0.2 μM) in 250 μL of 40 mM K-HEPES pH 7.4 in the presence of varying concentrations of MSU58 and MSU84 (0.5 μM to 23 μM) was monitored at 280 nm excitation and 323 nm emission in the fluorimeter. The fluorescent signals were corrected for inner filter effect using correction factors obtained from the fluorescence of 0.2 μM of tryptophan in 250 μL 40 mM K-HEPES pH 7.4 in the presence of 0.5 μM to 23 μM MSU58 and MSU84. The $K_D$ was determined by fitting the data in KaleidaGraph (Synergy Software, Reading, PA) to the
equation: \(-\frac{m_2}{1 + (m_1/m_0)} + m_3\); where \(m_1\) = Estimated \(K_D\); \(m_2\) = Minimum signal value; \(m_3\) = Maximum signal value

**FIQ Correction**

In order to correct for the quenching effect of cleaved DABCYL molecules on the total fluorescence of the product, FIQ correction factors were determined using the methods described in \([24, 25]\). Briefly, SNAPtide dilutions were made similar to that used in the kinetic assays (2.9 \(\mu\)M to 50 \(\mu\)M) and the unquenched peptide prepared to a final concentration of 0.5 \(\mu\)M unquenched peptide in a final volume of 50 \(\mu\)L. Fluorescent endpoint readings of each SNAPtide concentration in assay buffer (50 \(\mu\)M to 0 \(\mu\)M in 50 \(\mu\)L assay volume, 2:3 fold dilution) and 0.5 \(\mu\)M of unquenched peptide in the presence of each SNAPtide concentration were measured after 15 minutes incubation at 25 °C as described in the previous section. The endpoint reading of 0.5 \(\mu\)M unquenched product was measured total signal of the unquenched product in the absence of SNAPtide. The experiment was performed in triplicate.

To calculate the correction factors, the signal of 0.5 \(\mu\)M unquenched peptide in the presence of SNAPtide was subtracted from the signal of each representative SNAPtide concentration to obtain the apparent signal of the 0.5 \(\mu\)M unquenched peptide in the presence of each SNAPtide concentration. The signal of the unquenched peptide in the presence of each SNAPtide concentration was divided by the total signal from the 0.5 \(\mu\)M unquenched peptide, to give a ratio of how much signal reaches the detector in the presence of each SNAPtide concentration. This gave the FIQ correction factor and the
values can be found in (Table 2). The initial velocities were corrected by dividing with the FIQ calculated at each substrate concentration \[^{[24]}\].

*Turbidimetric Solubility Assay*

To investigate the time dependent solubility of MSU58 and MSU84, a 1:2 fold serial dilution of inhibitors was made to obtain concentrations in the range of 10 \(\mu\)M to 40 \(\mu\)M in a final volume of 100 \(\mu\)L in a clear 96 well polypropylene plate. Inhibitor dilutions were made with final a DMSO concentration of 3.2 % to mimic assay conditions with control wells containing 3.2 % DMSO in assay buffer. The plate was shaken for 5 minutes on a plate shaker after which a time course absorbance reading is measured at 600 nm at 25 °C.

The solubility of MSU58 and MSU84 in the assay buffer was investigated using the procedure described in \[^{[26]}\]. Concentrations used were in the range 0.16 \(\mu\)M to 100 \(\mu\)M in a final volume of 100 \(\mu\)L in a clear 96 well polypropylene plate by adding 1 \(\mu\)L inhibitor to 99 \(\mu\)L assay buffer. Final DMSO concentration was 1 %.

*Model discrimination analysis using DynaFit4 software*

DynaFit4 software (BioKin Ltd, Watertown, MA) was used to determine the most plausible inhibition mechanism. A DynaFit script specifying the fit parameters and concentration of the reactants was prepared for each likely inhibition model (Competitive, noncompetitive and uncompetitive). For each model, the catalytic activity \(k_{cat}\) was fixed to the value determined from the kinetic experiments. First order rate constants \(k_{on}\) and \(k_{off}\) and the second order rate constants in each model were allowed to
vary as adjustable parameters. The experimental data was then exported to DynaFit and analyzed through a non-linear least squares regression.

CHAPTER 3: RESULTS AND DISCUSSION

This study was aimed at identifying the mechanism by which the two quinolinol compounds (MSU58 and MSU84) inhibit BoNT/A LC and determining the number and nature of inhibitor binding sites present on the enzyme. The results will benefit structure activity relationship (SAR) studies and drug optimization efforts towards the development more potent BoNT/A LC inhibitors.

*Steady-state kinetics of BoNT/A LC with SNAPtide support competitive inhibition.*

The kinetic parameters ($K_M$, maximum velocity ($V_{max}$) and $k_{cat}$) of BoNT/A LC were determined using the substrate analog SNAPtide, a FRET peptide with an FITC fluorescent donor on the N-terminus and a DABCYL quencher at the C-terminus. BoNT/A LC catalyzes the cleavage of SNAPtide, releasing the FITC from the DABCYL; the resulting increase in fluorescence emission was measured to monitor product formation over time. The fluorescence emission intensities were converted to $V_0$ values using standard curves determined in the same experiment. The $V_0$ values were determined at multiple SNAPtide concentrations and data was fitted to the Michaelis-Menten equation using GraphPad Prism 6 software (Figure 3).
FIGURE 3: Effect of inhibitors MSU58 and MSU84 on BoNT/A LC kinetics. BoNT/A LC activity was measured in the presence of (●) 0 μM, (■) 3 μM, (▲) 6 μM and (▼) 9 μM MSU58 (a-c) and MSU84 (d-f) at 490/523nm in a BioteK Synergy H1 plate reader. Figures of three independent experiments with each inhibitor, conducted in 40 mM K-HEPES containing 0.01% Tween 20 at room temperature are illustrated. The $V_0$ values were corrected for FIQ (Results in
data file: MSU58 data > botox assay 05.23.16.581; botox assay 05.23.16.582; botox assay 06.14.16.58.1 and MSU84 data > botox assay 04.27.16.84ii; botox assay 04.11.16.84; botox assay 08.09.16.84)

A $k_{cat}$ for BoNT/A LC on SNAPtide was determined to be 0.21 s$^{-1}$ measured at room temperature in 40 mM K-HEPES pH 7.4 (Table 1); this value is in qualitative agreement with the $k_{cat}$ of 0.28 s$^{-1}$ reported previously by Feltrup and Singh at 37 °C in 20 mM HEPES, 0.1 % Tween 20 pH 7.6 [24]. The $K_M$ we determined (46.4 ± 0.8 μM) was approximately twice that determined by Feltrup and Singh (~ 22 μM); the different ranges of substrate concentrations used in the assays (1 - 50 μM here and 1 - 10 μM, by Feltrup and coworkers, respectively) in addition to the different buffer and Tween 20 concentrations may be responsible for the difference in $K_M$ values. The $K_M$ value of 46.4 ± 0.8 μM determined here agrees with the value of 42.3 ± 2.4 reported by Lai and coworkers in an excess of zinc acetate [21]. In the presence of 0, 3, 6 and 9 μM inhibitor (Table 1), the $V_o$ versus the SNAPtide concentration curves shifted to the right for both inhibitors, indicating an increase in $K_M$ with increasing inhibitor concentration (Figure 3). Presence of the inhibitor did not significantly affect $V_{max}$. Lineweaker-Burk plots (Figure 4) further show an increasing $K_M$ and unchanged $V_{max}$ with increasing inhibitor concentrations. A decrease in the $k_{cat}/K_M$ in the presence of increasing inhibitor concentration was observed for both inhibitors (Table 1). These findings are consistent with competitive inhibition.
**FIGURE 4:** Representative Lineweaver-Burk Plots of the reciprocal of the initial velocity (1/V₀) min/nM versus the reciprocal of the SNAPtide concentration (1/ [SNAPtide] (µM⁻¹)) in the presence of varying concentrations of MSU88 (A) and MSU84 (B) (♦) = 0 µM, (■) = 3 µM, (▲) = 6 µM and (x) = 9 µM. Data was obtained at room temperature in 40 mM K-HEPES pH 7.4 containing 0.01% Tween 20. Results are representative of three independent experiments (Data file: MSU58 data > botox assay 05.23.16.581 and MSU84 data > botox assay 04.27.16.84i). See Appendix A3 for full description.

**TABLE 1** Kinetic parameters of the BoNT/A LC catalyzed reaction in the presence of MSU58 and MSU84

<table>
<thead>
<tr>
<th>[I] (µM)</th>
<th>K_M (µM)</th>
<th>V_max (nM/min)</th>
<th>k_cat (s⁻¹)</th>
<th>k_cat/K_M (mM⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSU58</td>
<td>MSU84</td>
<td>MSU58</td>
<td>MSU84</td>
</tr>
<tr>
<td>0</td>
<td>46 ± 3</td>
<td>45 ± 11</td>
<td>46 ± 1</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>3</td>
<td>47 ± 3</td>
<td>55 ± 9</td>
<td>44 ± 4</td>
<td>57 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>99 ± 13</td>
<td>106 ± 18</td>
<td>54 ± 5</td>
<td>73 ± 2</td>
</tr>
<tr>
<td>9</td>
<td>104 ± 8</td>
<td>94 ± 24</td>
<td>48 ± 2</td>
<td>57 ± 7</td>
</tr>
</tbody>
</table>

Increasing K_M and a constant V_max were observed for both inhibitors and are consistent with competitive inhibition. k_cat values were calculated from the equation V_max/[E_T], where [E_T] was the enzyme concentration in the assay (4.2 nM). Values are ± S.E for an average of three independent experiments.
**Modified FIQ correction for higher SNAPtide concentrations.**

It was necessary to correct the data for FIQ, which is the tendency of the quencher on the intact or cleaved SNAPtide to absorb some of the light fluoresced by the FITC in another molecule of a cleaved SNAPtide \(^{24-25}\). This correction had not been reported when the previous and only kinetic studies on inhibition mechanism of a quinolinol compound on BoNT/A LC were conducted on QAQ. Feltrup and Singh outlined a correction using the slope of the FIQ correction factors versus SNAPtide concentration \(^{24}\). We used higher substrate concentrations than were used by Feltrup et al. At [SNAPtide] > 10 or 15 μM, the FIQ correction factors versus the SNAPtide concentration relationship was not linear (Figure 5) and we were not able to use the slope to correct our data. Consequently, we calculated the correction factors (Table 2) as outlined by Liu et al.\(^{25}\). The uncorrected \(K_M\) (17.8 ± 1.6 μM) was 2.6-fold lower than the FIQ-corrected \(K_M\) (46.4 ± 0.8 μM) (Figure 6). The correction also resulted in a 2.3 times higher \(k_{cat}\) value of 0.21 ± 0.02 s\(^{-1}\) compared to 0.09 ± 0.01 s\(^{-1}\) (uncorrected \(k_{cat}\)). Feltrup and Singh reported a 1.96-fold and 2.12-fold increase in the \(K_M\) and \(k_{cat}\) respectively, as a result of the FIQ correction, in agreement with the results here.\(^{24}\) The FIQ correction is necessary when using SNAPtide as the substrate to study BoNT/A LC kinetics but the slope cannot be applied when concentrations of substrate in excess of 20 μM are used. A previously described FIQ correction was employed and found to significantly affect value of kinetic parameters \(^{24-25}\).
FIGURE 5: FIQ correction factors versus SNAPtide concentration are shown. The FIQ correction factors were calculated as described by Feltrup and Singh. The relationship is linear up to 10 - 15 μM but not linear at concentrations > 20 μM. The experiment was performed at room temperature using 40 mM K-HEPES at pH 7.4. (Data file: FIQ factors > FIQ factors 08.09.16).

TABLE 2: FIQ correction factors

<table>
<thead>
<tr>
<th>[SNAPtide] (μM)</th>
<th>FIQ correction factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.0</td>
<td>0.56 ± 0.009</td>
</tr>
<tr>
<td>33.3</td>
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<td>22.2</td>
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<td>9.9</td>
<td>0.82 ± 0.008</td>
</tr>
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<td>6.6</td>
<td>0.94 ± 0.044</td>
</tr>
<tr>
<td>4.4</td>
<td>0.92 ± 0.017</td>
</tr>
<tr>
<td>2.9</td>
<td>0.93 ± 0.033</td>
</tr>
<tr>
<td>0.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Fluorescent Internal Quenching correction factors determined for correcting the initial velocities at the substrate concentrations used in the assay. Calculated factors are an average of three experiments conducted at room temperature in 40 mM K-HEPES containing 0.01% Tween 20 at pH 7.4. (Data file: > FIQ factors > FIQ factors 08.09.16).
**FIGURE 6:** The change in $K_M$ (Figure A) and $k_{cat}$ (Figure B) before and after FIQ correction in the presence of different concentrations of MSU58. FIQ correction factors were determined at conditions similar to the assay conditions. Values are the averages of three independent experiments with S.E. (Data file: >MSU58 data > botox assay 06.14.16.58.1)

**$K_I$ and $K_D$ of MSU58 and MSU84 with BoNT/A LC**

The IC$_{50}$ values for MSU58 and MSU84 were determined to be $3.3 \pm 0.3 \, \mu M$ and $5.8 \pm 2 \, \mu M$ ($\pm$S.E, N = 14, 4) respectively, using $[S]$ of 0.625 $\mu M$ (Figure A1 in Appendix). A decrease in percent inhibition at concentrations $>10 \, \mu M$ was observed for MSU84 and was found to be due to a time-dependent precipitation of MSU84 at concentrations $>10 \, \mu M$ (Figure A2(b) in Appendix). The better inhibition by MSU58 compared to MSU84 may be due to the 3-pyridyl substituent in MSU58. Structure activity relationship studies by Caglic and coworkers reported that a 3-pyridyl substituent at R$_2$ resulted in a 60 % more potent inhibitor against BoNT/A LC. Docking studies revealed a hydrogen bond between the 3-pyridyl substituent and the amino group of Arg363 $^{[4,17]}$. Interaction with Arg363 is also important for stabilizing the binding of the substrate for catalysis $^{[2]}$. Caglic and coworkers also suggested that a methyl substituent at the R$_4$ position of the
quinolinol moiety may account for 85% increased inhibitory activity; introduction of a methyl group at R₄ of MSU58 and MSU84 could be explored in future studies [¹⁷].

The Kᵢ of each inhibitor was obtained by converting the IC₅₀ to Kᵢ using the Cheng-Prusoff equation (See Table 3 caption) and also by employing Dixon plots [²³]. The Kᵢ values calculated for MSU58 and MSU84 were 3.3 ± 1.1 μM and 5.7 ± 4.6 μM, respectively (Table 3). As expected, Kᵢ was similar to the IC₅₀ because the [SNAPtide] used in the screening of the inhibitors was << Kₘ [²⁷, ²⁸]. In the Dixon plots, the reciprocals of the V₀ from the kinetic analysis were plotted against the inhibitor concentrations (Figure 7). Data at each substrate concentration was fitted individually to a linear equation and the negative X-axis value of the point of intersection of the lines in the upper left quadrant gave an estimate of the Kᵢ. For each Dixon plot, the negative X-axis value of the intersection point of all pairs of individual lines were determined and the average of these values was used to obtain the Kᵢ of 3.2 ± 0.5 μM for MSU58 and 6.2 ± 0.9 μM for MSU84. These values correspond to those determined using the Cheng-Prusoff equation (Table 3) and indicate that both compounds inhibit BoNT/A LC protease activity in the single digit micromolar range, with MSU58 having a slightly higher inhibitory effect compared to MSU84. It can also be seen in Figure 7 that the lines intersect in the second quadrant above the negative X-axis, supporting competitive enzyme inhibition model for each inhibitor [²⁹]. A Kᵅ of 1.9 ± 0.5 μM and 7.5 ± 1.1 μM was determined for MSU58 and MSU84 respectively. These values are similar to the Kᵢ of 3.2 ± 0.5 μM for MSU58 and 6.2 ± 0.9 μM for MSU84 and agree with competitive inhibition. Representative Kᵅ curves for MSU58 and MSU84 are illustrated in Figure 8.
FIGURE 7: Dixon Plots of MSU58 (a-c) and MSU84 (d-f) with $K_i$ values. Figures represent three independent experiments with each inhibitor, performed at room temperature in 40 mM K-HEPES pH 7.4, containing 0.01 % Tween 20. $1/V_0$min/nM was plotted against the concentration of the inhibitor for varying the SNAPtide (2.9 μM – 50.0 μM). (Data file: MSU58 data: botox assay 05.23.16.581; botox assay 05.23.16.582; botox assay 06.14.16.58.1 and MSU84 data: botox assay 04.27.16.84ii; botox assay 04.11.16.84; botox assay 08.09.16.84)
TABLE 3: The IC$_{50}$ values and $K_i$ of MSU58 and MSU84

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$ (µM)</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dixon plot</td>
<td>Cheng-Prusoff equation</td>
</tr>
<tr>
<td>MSU58</td>
<td>3.3 ± 0.3 (14)</td>
<td>3.2 ± 0.5 (3)</td>
</tr>
<tr>
<td>MSU84</td>
<td>5.8 ± 2.3 (4)</td>
<td>6.2 ± 0.9 (3)</td>
</tr>
</tbody>
</table>

The $K_i$ was determined from Dixon plots and related to the $K_i$ value determined from the Cheng-Prusoff equation using predetermined IC$_{50}$ values of each compound. Cheng-Prusoff equation is given by; $IC_{50} = K_i (1+ [S]/K_M)$ [23]. Values are ± S.E (N).
FIGURE 8: Representative $K_D$ curves for MSU58 (A) and MSU84 (B). The fluorescent signal was plotted against the concentration of each inhibitor and fitted to the equation: $\frac{-m_2}{1+(m_1/m_0)} + m_3$ in KaleidaGraph. The experiment was conducted in 40 mM K-HEPES pH 7.4 at room temperature. Results are representative of three independent experiments (Data file: > Kd expts > BoNT A LC 58 trial 2 12.02.16 and >BoNT A LC 84 trial 3 12.02.16).

**Model Discrimination Analysis**

To determine whether the noncompetitive and uncompetitive inhibition mechanisms could be reasonably ruled out for MSU58 and MSU84, model discrimination analysis was performed. The model discrimination function in the DynaFit software uses statistical methods to determine the most probable inhibition model among a set of models \[^{30}\]. The Akaike information criterion (AIC) was used; the model with the lowest AIC number is defined as the best model \[^{30}\]. DynaFit was used to calculate the difference between the AIC numbers of each model and the best model ($\Delta$AIC) and to determine the Akaike weights $w^{(AIC)}$ \[^{30-31}\]. The most plausible model has the highest...
\(w^{(AIC)}\) with 1.0 being the maximum possible \(w^{(AIC)}\) value \([31]\). Results of model discrimination analysis in Table 4 shows \(w^{(AIC)}\) for both MSU58 and MSU84 to be 1.00 for the competitive model of inhibition and 0.00 for either noncompetitive or uncompetitive inhibition, indicating that the competitive model of inhibition is the most likely mechanism of inhibition of BoNT/A LC by either MSU58 and MSU84 and that the other models may be ruled out. This is consistent with results from Table 1, the Lineweaver-Burk plots (Figure 4) and the Dixon plots in Figure 7.

**TABLE 4:** Results of model discrimination analysis using DynaFit

(I)

<table>
<thead>
<tr>
<th>Model</th>
<th>np</th>
<th>(\Delta AIC)</th>
<th>MSU58</th>
<th>MSU84</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Competitive</td>
<td>36</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Uncompetitive</td>
<td>35</td>
<td>1217.7</td>
<td>1002.4</td>
<td>1094</td>
</tr>
<tr>
<td>Noncompetitive</td>
<td>36</td>
<td>108.8</td>
<td>23.2</td>
<td>2550.8</td>
</tr>
</tbody>
</table>

Table 4 is split into (I) and (II). The \(\Delta AIC\) of the best model is equal to 0.00; \(w^{(AIC)}\) for the competitive model = 1.00 supports the finding that both MSU58 and MSU84 are competitive inhibitors of BoNT/A LC.
Kinetic rate constants

DynaFit was used to perform a non-linear regression analysis to determine the kinetic rate constants associated with the BoNT/A LC catalyzed reaction by fitting the experimental data to a least squares fit of the mechanism shown in Scheme 1 \[^{[30]}\]. Table 5 displays the average best fit association and dissociation rate constants ($k_{on}$ and $k_{off}$) values and inhibitor association and dissociation rate constants ($k_{a.i}$ and $k_{d.i}$) of three independent experiments with compounds MSU58 and MSU84. The constants $k_{a.i}$ and $k_{d.i}$ are on the same timescale as the catalytic activity and these compounds do not exhibit time-dependent inhibition. The $k_{cat}$ value was fixed to the experimentally determined value 0.21 s\(^{-1}\). The $k_{on}$ and $k_{off}$ (Table 5) are related to the $K_M$ and $k_{cat}$ ($K_M = \frac{k_{off} + k_{cat}^2}{k_{on}}$); the calculated $K_M$ is 43.3 $\pm$ 8.2 $\mu$M; this value is close to the value of 46.4 $\pm$ 0.8 $\mu$M determined from fitting the data to the Michaelis-Menten equation. Table 5 also shows average values of the $k_{a.i}$ and $k_{d.i}$, which are related to $K_I$ ($K_I = \frac{k_{d.i}}{k_{a.i}}$). These values were calculated to be 6.2 $\pm$ 1.3 $\mu$M for MSU58 and 5.0 $\pm$ 1.4 $\mu$M for MSU84.

\[ E + S \xrightarrow{k_{on}} E.S \xrightarrow{k_{cat}} E + P \]

\[ + \]

\[ \downarrow \]

\[ k_{a.i} \quad k_{d.i} \]

\[ E.I \]

\textbf{SCHEME 1:} The competitive mechanistic model by which MSU58 and MSU84 inhibit BoNT/A LC.
TABLE 5: DynaFit determined rate constants and kinetic constants

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$k_{on}$ ($\mu$M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_M$ (μM)</th>
<th>$k_{d,i}$ (μM$^{-1}$ s$^{-1}$)</th>
<th>$k_{d,i}$ (s$^{-1}$)</th>
<th>$K_I$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSU58</td>
<td>0.04 ± 0.003</td>
<td>2.0 ± 0.1</td>
<td>55 ± 7</td>
<td>0.13 ± 0.01</td>
<td>0.8 ± 0.1</td>
<td>6.2 ± 1.3</td>
</tr>
<tr>
<td>MSU84</td>
<td>0.06 ± 0.01</td>
<td>1.7 ± 0.4</td>
<td>32 ± 12</td>
<td>0.14 ± 0.02</td>
<td>0.7 ± 0.1</td>
<td>5.0 ± 1.4</td>
</tr>
</tbody>
</table>

DynaFit determined rate constants and determined kinetic constants; values are averages of three independent data ± S.E. The experimental data were fit to the model in Scheme 1 and the $k_{cat}$ was fixed to 0.21 s$^{-1}$. The $K_M$ and $K_I$ values in the table were calculated from the on and off rate constants and agree with the experimentally found values.

Time-dependent Precipitation of MSU84

A phenomenon that was observed during the determination of IC$_{50}$ values for MSU58 and MSU84, was the markedly decrease in percent inhibition at higher concentration of the compound following an expected increase in percent inhibition with increasing concentration of MSU84 (Figure 9). This effect was not observed for MSU58. To investigate this phenomenon, a turbidimetric assay was performed as described under the methods and materials section. The time dependence of solubility was monitored at $A_{600}$ nm (absorbance) over 4 hours under assay conditions. The results, displayed in Figure 9(b), revealed that MSU84 begins to precipitate out of solution with time at higher concentrations (20 μM and 40 μM). At these concentrations, the compound molecules begin to cluster and aggregate, increasing the turbidity of the solution and may be the underlying reason for the observed decrease in percent inhibition at those concentrations of MSU84. To ensure that precipitation did not impact inhibition mechanism studies, the concentrations of MSU84 used in the kinetic assay were kept below 20 μM. The solubility assay also revealed that MSU84 was not soluble beyond 20 μM whereas no solubility issues were observed for MSU58 up to 100 μM (Figure 10).
FIGURE 9: Time-dependent precipitation of MSU84. Turbidity reaction monitored for (a) MSU58 and (b) MSU84. □ = 10 µM and ◼ = 40 µM. The absorbance was measured at 600 nm for 4 hours with a 3.2 % final DMSO concentration. Figures represent absorbance values at the highest concentration (40 µM) and the concentration closest to that used in the kinetic assay (10 µM). Error bars are the Standard errors of the average of two experiments. (Data file: > Solubility > Timecourse Turbidity assay 6.28.16)

FIGURE 10: Solubility of MSU58 and MSU84. The solubility of MSU58 (♦) and MSU84(◼) in the assay buffer (40 mM K-HEPES pH 7.4) at room temperature. The figure is the average of three independent experiments with standard errors. (Data file: > Solubility > Solubility 11.30.16 plate 3)
CONCLUSION

Many reports suggest that quinolinol based compounds block the BoNT/A LC active site via metal chelation by the 8-hydroxy quinolinol moiety but not previously supported by kinetic experiments. Conversely, noncompetitive inhibition by quinolinol compound QAQ was reported based on kinetic studies [21]. Others have suggested that quinolinol compounds do not inhibit BoNT/A LC by excluding the interaction of the zinc with water, which is necessary for substrate cleavage [4]. MSU58 and MSU84 were found to be competitive inhibitors of BoNT/A LC. We found that it was necessary to do the FIQ correction when determining the kinetic parameters using SNAPtide and that the non-linearity of the relationship between the correction factors and SNAPtide concentrations beyond 20 μM SNAPtide requires that the FIQ factors be determined as suggested by Liu et al., and as we have done here when using higher SNAPtide concentrations that are required for full Michaelis-Menten analysis. It was determined that the phenomenon where MSU84 gives a lower percent inhibition at the highest concentration in the screening assay was due to the slow precipitation of this compound at concentrations of 20 and 40 μM. This study is the first to report experimental data demonstrating competitive inhibition of BoNT/A LC by a quinolinol compound. In future SAR studies and drug optimization efforts, the addition of a methyl group to the R₄ position of the quinolinol moiety in MSU58 and MSU84 may yield more potent BoNT/A LC inhibitors.
REFERENCES


23. Cheng, Y.; Prusoff, W. H., Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 1973, 22 (23), 3099-108.


APPENDIX

A1: Determination of IC$_{50}$

Inhibitor stock solutions were made in DMSO at 10 mM concentration and diluted to make a 1 mM solution in DMSO for the assay. Serial dilutions of the inhibitor (1:2 fold) were made in DMSO in clear polypropylene plates and transferred to the half area black well plates to obtain concentrations in the range of 1.6 μM to 20 μM in a final well volume of 50 μL. Each assay well also contained 4.2 nM BoNT/A LC and 0.625 μM SNAPtide and a final DMSO concentration in the assay of 2%. Experiments were conducted a minimum of four independent experiments in the BioTek Synergy 2 for 90 minutes; set at 485 nm excitation and 528 nm emission with 5 minutes shaking between reads. The percent inhibition versus compound concentration graphs were used to determine the IC$_{50}$ values in Microsoft Excel using the Solver function and the four-parameter logistic equation $[^{32}]$ (Figure A1).

![Figure A1](image)

**FIGURE A1:** Representative dose-response curves for determining the IC$_{50}$ of compounds MSU58 and MSU84. The curves were fit to a four parameter logistic equation using the solver function in Microsoft Excel $[^{32}]$. The curve fitting for MSU84 was done not including the 20 μM data point. The experiments were performed in 40 mM K-HEPES pH 7.4 at room temperature.
**A2: Lineweaver-Burk analysis supports competitive inhibition**

Double reciprocal plots (Lineweaver-Burk plots) were created in Microsoft Excel (Figure 4). The points of intersection on the positive y-axis and negative x-axis represent the reciprocals of the $V_{max}$ ($1/V_{max}$) and $K_M$ ($1/ K_M$), respectively. The point of intersection on the y-axis did not change in the presence of increasing inhibitor, indicating that the $V_{max}$ of the reaction was independent of inhibitor concentration for both MSU58 and MSU84. On the other hand, the points of intersection on the negative x-axis change with inhibitor concentration, corresponding to an increasing $K_M$ with an increase in the concentration of inhibitor. This is characteristic of the competitive mechanism of inhibition and agrees with the trend observed in Table 1, suggesting that both MSU58 and MSU84 compete with SNAPtide for the active site of BoNT/A LC. This agrees with the earlier docking studies that suggested that quinolinol compounds interact with the zinc cation in the hydrophobic pocket of the active site of BoNT/A LC $^{[4,17]}$.

**FIGURE A2:** Representative DynaFit traces for the competitive inhibition model for MSU58 and MSU84. Figures are a graph of the fluorescent signal versus the time (s) for data sets obtained from three independent experiments.
A3: Compound synthesis

**MSU58.** A mixture of 4-fluorobenzaldehyde (0.11 ml, 1 mmol) and 3-aminopyridine (94 mg, 1 mmol) was stirred in a 50 ml RBF for 30 min before adding 5-chloro-8-quinolinol (144 mg, 0.8 mmol). The mixture was heated to 120 °C whereupon the reaction became homogenous after approximately 1 hour; stirring continued for another 6 hours at 120 °C. After TLC indicated that the quinolinol was consumed, the flask was cooled. The contents were dissolved in minimum amount of methylene chloride and loaded on a silica gel column. Chromatography was performed using 30% ethyl acetate in hexanes. The desired product was obtained in 56% yield and greater than 97 % purity.

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.81-8.83 (dd, 1H), 8.46-8.50 (dd, 1H), 8.17-8.18 (d, 1H), 7.94-7.96 (dd, 1H), 7.63 (s, 1H), 7.53-7.58 (m, 1H), 7.43-7.48 (m, 2H), 7.01-7.10 (m, 3H), 6.90-6.94 (m, 1H), 6.07-6.09 (d, 1H).

**MSU84.** A mixture of 3-fluorobenzaldehyde (0.11 ml, 1 mmol) and benzene sulfonamide (157 mg, 1 mmol) was stirred in a 50 ml RBF for 30 min and was added 5-chloro-8-quinolinol (144 mg, 0.8 mmol). The mixture was heated to 120 °C whereupon the reaction became homogenous after approximately 1 hour; stirring continued for another 6 hours at 120 °C. After TLC indicated that the quinolinol was consumed, the flask was cooled. The contents were dissolved in minimum amount of methylene chloride and loaded on a silica gel column. Chromatography was performed using 30% ethyl acetate in hexanes. The product was obtained in 53% yield and greater than 96 % purity.

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.76-8.78 (dd, 1H), 8.40-8.44 (dd, 1H), 7.62-7.65 (m, 2H), 7.53-7.57 (dd, 1H), 7.25 (s, 1H), 7.24 (s, 1H), 7.06-7.14 (m, 3H), 6.89-7.00 (m, 2H),
6.02-6.05 (d, 1H), 5.78-5.81 (d, 1H). Compound MSU 58 was synthesized to greater than 97% purity.