Small Molecules Showing Significant Protection of Mice against Botulinum Neurotoxin Serotype A

ABSTRACT

Botulinum neurotoxin serotype A (BoNTA) causes a life-threatening neuroparalytic disease known as botulism that could afflict large, unprotected populations if the toxin were employed in an act of bioterrorism. Current post-exposure therapy is limited to symptomatic treatment or passive immunization that is effective for treating infant botulism at a cost of US $45,300 per treatment regimen. Antibodies can neutralize the extracellular but not the intracellular BoNTA. Moreover, antibody production, storage, and administration in a mass casualty scenario.
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Small Molecules Showing Significant Protection of Mice against Botulinum Neurotoxin Serotype A

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Abstract

Botulinum neurotoxin serotype A (BoNTA) causes a life-threatening neuromotoric disease known as botulism that could affect large, unprotected populations if the toxin were employed in an act of bioterrorism. Current post-exposure therapy is limited to symptomatic treatment or passive immunization that is effective for treating infant botulism at a cost of US $45,300 per treatment regimen. Antibodies can neutralize the extracellular but not the intracellular BoNTA. Moreover, antibody production, storage, and administration in a mass casualty scenario pose logistical challenges. Alternatively, small-molecule inhibitors of BoNTA endopeptidase (BoNTAe) are sought to antagonize the extracellular or intracellular toxin. While several such molecules reportedly demonstrated efficacy in protecting cells against BoNTA, there is scant information to show that small molecules can significantly protect mammals against BoNTA. Herein we report the development of effective small-molecules BoNTAe inhibitors with promising in vivo pharmacokinetics. One such molecule has an in vivo half-life of 6.5 hours and is devoid of obvious sign of toxicity. Pre-treatment with this molecule at 2 mg/kg protected 100% and 70% of treated mice against BoNTA at 5 times of its median-lethal dose during the periods of 2 and 4 half-lives of the inhibitor, respectively. In contrast, 40% and 0% of untreated mice survived during the respective periods. Similar levels of protection were also observed with two other small molecules. These results demonstrate that small molecules can significantly protect mice against BoNTA and support the pursuit of small-molecule antagonists as a cost-effective alternative or as an adjunct to passive immunity for treating botulism.


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Competing Interests: Y-PP, JD, SW, JGP, JJS, and CBM are inventors of a filed provisional patent application titled “Small-Molecule Botulinum Toxin Inhibitors” that covers the inhibitors described in this paper, but this does not alter the adherence to all the PLoS ONE policies on sharing data and materials.

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†‡ Current post-exposure therapy is limited to symptomatic treatment or passive immunization that is effective for treating infant botulism at a cost of US $45,300 per treatment regimen
Antibodies can neutralize the extracellular but not the intracellular BoNTA. Moreover, antibody production, storage, and administration in a mass casualty scenario pose logistical challenges. To antagonize the extracellular or intracellular BoNTA, small molecules [13–20] have been developed to inhibit BoNTA endopeptidase (BoNTAc) – the catalytic domain of BoNTA that specifically cleaves a critical component of the neurosecretory apparatus required for acetylcholine release [21]. While several such molecules have demonstrated efficacy in protecting cells against BoNTA [13,15,20], there is scant information to show that small molecules can significantly protect mammals against BoNTA, although an in vivo study of small-molecule BoNTAc inhibitors has been reported [22].

Herein, we report the development of effective small-molecule BoNTAc inhibitors with in vivo half-life of 4–6 hours. These inhibitors showed 100% and 70% of protection of mice against BoNTA at 5 times of its median-lethal dose during the periods of 2 and 4 half-lives of the inhibitors at an inhibitor concentration of 2 mg/kg, respectively. We also discuss the prospect of small-molecule inhibitors as a cost-effective alternative or as an adjunct to passive immunity for treating botulism.

**Results**

**Design and Synthesis**

We previously reported a serotype-specific, small-molecule BoNTAc inhibitor, H3H (structure shown in Figure 1), which has a $K_{i}^{app}$ value of 3.8±0.77 μM and was resulted from our lead identification and optimization as summarized in Figure 1 [14,23]. One drawback of H3H is insolvility in water. In optimizing H3H for water solubility and higher potency in inhibiting BoNTAc, we encountered problems in derivatizing H3H caused by chemical instability under acidic conditions (pH<2.0) that was presumably due to the proton at position 3 of the indole ring. These problems hampered the structural modifications of H3H guided by insights from computer simulations or the crystal structures of inhibitor-bound BoNTAc complexes.

Recognizing the synthesis step as the rate-determining step of the optimization, we set out to first establish a facile synthetic scheme that can lead to a group of inhibitor analogues and then use computer simulations of the inhibitor-bound BoNTAc complexes to prioritize the syntheses of the analogues. This was different from what we did earlier, namely, first finding alternative analogues on the basis of computer simulations and then determining whether the alternatives were synthetically accessible.

Accordingly, we developed a simple synthetic scheme shown Figure 2 that begins with a known intermediate used for the synthesis of H3H [14]. The new scheme, which readily leads to a handful of new analogues of H3H by varying substituents $R_1$, $R_2$, and $R_3$, enabled us to address the problems of water solubility and chemical instability of H3H by introducing hydrophilic groups and replacing the position-3 proton of the indole ring with a fluoride atom [24], respectively. Preliminary multiple molecular dynamics simulations (10 1-ns-long simulations) suggested that two of such analogues, F3A and F4H (structures shown in Figure 1), might be able to interact favourably with the active site of BoNTAc. The simulation results were later supported by the extended multiple molecular dynamics simulations (10 10-ns-long simulations) described below.

Therefore, we made F4H and F3A with relative ease according to the scheme shown in Figure 2. Gratifyingly, we found that both F4H and F3A are water soluble at concentrations up to 5.0 mM and stable under acidic conditions.

**Computer Simulation**

Subsequent extended multiple molecular dynamics simulations (10 10-ns-long simulations) of BoNTAc in complex with F4H or F3A suggested that both inhibitors have (1) the hydroxamate coordinating the zinc ion embedded in the active site, (2) the hydroxamate forming a hydrogen bond to Glu224, (3) the cation-pi interaction of the thiophene-substituted phenyl group with Arg363, (4) the pi-pi interactions of the thiophene-substituted phenyl group with Phe194 and Tyr366, (5) the interaction of the ketone oxygen atom with Asp370 that is bridged by at least one
water molecule, and (6) the cation-pi and pi-pi interactions of the indole-substituted phenyl group with Lys66 and Gln162, respectively (Figure 3). The main differences between the two inhibitor complexes are that (1) the thiophene-substituted phenyl group has stronger pi-pi interactions (judged by distance) with Tyr366 and Phe194 in F4H-BoNTAe than in F3A-BoNTAe, (2) Tyr366 forms a hydrogen bond with the carbonyl oxygen atom of the hydroxamate in F4H-BoNTAe but not in F3A-BoNTAe, and (3) the interaction between the ketone oxygen atom and Asp370 is bridged by one or two water molecules in F4H-BoNTAe or F3A-BoNTAe, respectively. The coordinates of the simulation-generated F4H-BoNTAe and F3A-BoNTAe complexes are available in Datasets S1 and S2, respectively.

Biological Evaluation

High performance liquid chromatography (HPLC)-based BoNTAe inhibition assays [25] showed that F4H is as potent as H3H in inhibiting BoNTAe, and F3A is less potent than H3H (Table 1). Furthermore, H3H, F4H, and F3A showed no acute toxicity to mice. We therefore performed in vivo pharmacokinetic studies on all three inhibitors. Interestingly, the exposures of F4H and F3A to mice are nearly the same but slightly less than that of H3H, as measured by the area under the time-concentration curve (AUC), even though the maximum concentration (Cmax) for each inhibitor are different (Table 1). The nearly identical half-lives (t1/2<6 hours) of F4H and F3A are longer than that of H3H.
60% of the extracellular toxin. 

lasting antibodies [27] that are effective to neutralize the shortened if the compound were used in combination with long-
one day for multiple days. This treatment could therefore be plausible that the problem with a long

molecule BoNTAe inhibitors [22]. The work described above has been only one article to date reporting an

nanomolar potencies are difficult to obtain [19], and (3) there

days [7]), (2) small-molecule BoNTAe inhibitors with low

mice treated with any of the three inhibitors survived during the 12-hour period (≈2t_{1/2} for F4H) after the BoNTA challenge. During this period, the inhibitors are expected to work optimally according to the time course of the inhibitor concentration in mouse plasma. In contrast, 60% of the untreated mice died during the 12-hour period. Consistently, all untreated mice died 24 hours (≈4t_{1/2} for F4H) after the challenge, whereas 70% and 60% of the F4H-treated mice survived 24 hours and 48 hours (≈8t_{1/2} for F4H) after the challenge, respectively (Figure 4). Furthermore, 10% of the mice treated with any of the three inhibitors survived without symptoms of botulism until they were euthanized on day 5 (Figure 4).

Discussion

Small-molecule BoNTAe inhibitors have been pursued actively by different research groups [13–20], but concern remains with regard to the feasibility of the small-molecule therapy for botulism, primarily because (1) BoNTA has a long in vivo half-life (t_{1/2} >31 days [7]), (2) small-molecule BoNTAe inhibitors with low nanomolar potencies are difficult to obtain [19], and (3) there has been only one article to date reporting an in vivo study of small-molecule BoNTAe inhibitors [22]. The work described above offers the following insights into the prospect of the small-molecule botulism therapy, although additional studies are needed to determine if the observed protection of mice against BoNTA by the pre-treatment of F4H, H3H, or F3A involves inhibition and clearance of extracellular toxin depots, uptake by intoxicated neurons, or both routes.

F4H, H3H, and F3A have in vivo half-lives of 4–6 hours, and all mice treated with any of the three inhibitors survived during the 12-hour period (≈2t_{1/2} for F4H) after the BoNTA challenge. It is therefore plausible that the problem with a long in vivo half-life of BoNTA can be mitigated by treating with an F4H-like compound one dose per day for multiple days. This treatment could be shortened if the compound were used in combination with long-lasting antibodies [27] that are effective to neutralize the extracellular toxin.

F4H showed 82±6% inhibition of BoNTAe at the inhibitor concentration of 20 μM. However, with one 2-mg/kg intraperitoneal injection, F4H showed 100, 70, and 60% protection of mice against BoNTA during the 12-, 24-, and 48-hour periods after the toxin challenge, respectively. This suggests that small-molecule BoNTAe inhibitors with low nanomolar potencies might not be necessary; inhibitors with low micromolar or high nanomolar potencies may suffice.

All three different inhibitors protected 100% of treated mice during the 12-hour period (≈2t_{1/2} for F4H) and 10% of the mice during the standard 5-day observation period, with a single intraperitoneal injection of the inhibitor against a supralethal BoNTA challenge. Furthermore, 90% of the F3A-treated mice, 40% of the H3H-treated mice, and 40% of the F4H-treated mice died 48 hours after the toxin challenge, respectively (Figure 4). The in vivo potencies appeared to be consistent with the in vitro potencies of the three inhibitors (Table 1). These results support the hypothesis that protection of mice against BoNTA can be achieved by treatment with a small-molecule BoNTAe inhibitor and are incentive to improve BoNTAe inhibitor structures and dosing regimen to optimize in vivo efficacies.

In summary, the present work demonstrates that small-molecule inhibitors can significantly protect mice against BoNTA and encourages the pursuit of small-molecule BoNTAe inhibitors for alternative or complementary treatment of botulism.

Materials and Methods

The animal experiments were performed with an approved protocol by the Institutional Animal Care and Use Committee at the Walter Reed Army Institute of Research (IACUC number: B02-06) that is in compliance with the Animal Welfare Act and other United States federal statutes and regulations involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition.

Reagents

Hexanes (Hex), ethyl acetate (EtOAe), and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Pittsburgh, PA). BSA, HEPES buffer, and zinc chloride were purchased from Sigma-Aldrich (St. Louis, MO). Dithiothreitol was obtained from BioRad (Hercules, CA). All commercially available reagents were used as received. Recombinant BoNTAe was provided by Dr. Leonard Smith of the United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD.

Chemical Synthesis

General Description. The 1H NMR (400 MHz) and 13C NMR (100 MHz) spectra were recorded on a Mercury 400 spectrometer from Varian (Palo Alto, CA). Chemical shifts are

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% BoNTAe inhibition</th>
<th>C_{max} (ng/mL)</th>
<th>C_{24} (ng/mL)</th>
<th>AUC_{max} (hr-ng/mL)</th>
<th>T_{1/2} (hr)</th>
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<tbody>
<tr>
<td>H3H</td>
<td>78±4</td>
<td>497.4</td>
<td>3.0</td>
<td>1547.3</td>
<td>4.35</td>
</tr>
<tr>
<td>F4H</td>
<td>82±6</td>
<td>738.4</td>
<td>&lt;0.5</td>
<td>1386.4</td>
<td>6.50</td>
</tr>
<tr>
<td>F3A</td>
<td>47±1</td>
<td>256.0</td>
<td>7.0</td>
<td>1385.9</td>
<td>6.25</td>
</tr>
</tbody>
</table>

1 The inhibition assays were conducted at an inhibitor concentration of 20 μM. doi:10.1371/journal.pone.0010129.t001
ESI. IR spectra were obtained on a ThermoNicolet Avatar 370 FT-IR (Waltham, MA) using KBr pellets. Medium pressure liquid chromatography (MPLC) was performed with Biotage SP-1 (Milford, MA) using silica gel (EM Science, 230–400 mesh). HPLC was carried out on a 5-μm C18 column (analytical: 4.60×250 mm, HyperClone; semi-preparative: 21.2×250 mm, Gemini) from Phenomenex (Torrance, CA) eluting with linear gradient of 80% of solution A (1000 mL of H2O and 1 mL of TFA) to 100% of solution B (100 mL of H2O, 900 mL of MeCN and 1 mL of TFA) over 20 minutes at a flow rate of 1.0 mL/min (analytical) or over a specified amount of time at a flow rate of 10 mL/min (semi-preparative) with UV detection at 254 nm on a Beckman Coulter System Gold HPLC system (166P detector and 125P solvent module) from Beckman Coulter (Brea, CA). KCN is highly toxic and must be handled with extreme care by trained personnel.

Methyl 2-(2-bromo-5-(1-(4-(1,3-dioxoisindolin-2-yl)butyl)-3-fluorophenyl)thiophen-3-yl)acetate (2). To a solution of methyl 2-bromo-5-(1-(4-(1,3-dioxoisindolin-2-yl)butyl)-2-phenyl-1H-indole-6-carbonyl)thiophen-3-yl)acetate [1 in Figure 2] [14] (156 mg, 0.24 mmol) in 3 mL CHCl3 was added 1-fluoropyridinium triflate (78 mg, 0.28 mmol), and then the mixture was stirred at room temperature for 6 days. The resulting mixture was diluted with 40 mL Et2O, washed with brine (2×10 mL), dried over MgSO4, filtered, and then concentrated in vacuo. MPLC purification (Hex:EtOAc/9:1) gave 2 (66 mg, 41%) as a yellow solid foam. 1H NMR (CDCl3) δ 7.94 (s, 1H), 7.78–7.75 (m, 2H), 7.71–7.63 (m, 4 H), 7.57 (s, 1H), 7.50–7.39 (m, 5H), 4.25 (t, J = 7.2 Hz, 2H), 3.71 (s, 3H), 3.69 (s, 2H), 3.49 (t, J = 6.6 Hz, 2H), 1.67–1.60 (m, 2H), and 1.50–1.43 (m, 2H) (see Figure S1 for proton NMR spectrum of 2); 13C NMR (CDCl3) δ 187.42, 170.49, 168.57, 143.91, 141.87 (βCF = 244.0 Hz), 136.06, 135.98, 135.02, 134.26, 132.30 (γCF = 7.0 Hz), 132.08, 131.54, 129.95, 129.17, 128.44 (βCF = 3.0 Hz), 127.60 (βCF = 21.0 Hz), 123.48, 122.40 (βCF = 3.0 Hz), 112.66, 52.61 (q, J = 10.7 Hz), 43.64, 37.25, 35.07, 27.32, and 25.76; IR cm⁻¹ 2921.2, 1707.6, and 1393.0; LRMS-EI m/z 672 and 674 (12% each, [M]+), 160 (100%, [CH3NPh]+); HRMS-ESI calculated for C26H25BrFN4O3Na+[M+Na]+ 674.0622, found 674.0619.

Methyl 2-[(1-[(4-(1,3-dioxoisindolin-2-yl)butyl)-3-fluoro-2-phenyl-1H-indole-6-carbonyl)-2-(4-hydroxyphenyl)thiophen-3-yl]azetidin-1-yl] (3a). A mixture of 2 (42 mg, 0.062 mmol), Pd[PPh3]4 (8 mg, 0.007 mmol), CsF (28 mg, 0.18 mmol), 4-hydroxyphenylboronic acid (13 mg, 0.094 mmol), and H2O (200 μL) in 1,2-dimethoxyethane (8 mL) was degassed with N2 for 10 minutes and then refluxed for 6 hours. The resulting suspension was poured into H2O (10 mL) and then extracted with 70 mL Et2O. The organic layer was washed with brine (2×10 mL), dried over MgSO4, and then concentrated in vacuo. MPLC purification (Hex:EtOAc/5:1) of the residue gave 3a as a yellow solid foam (34 mg, 79%). 1H NMR (CDCl3) δ 7.78–7.66 (m, 7H), 7.50–7.38 (m, 7H), 6.94 (d, J = 8.4 Hz, 2H), 6.27 (s, 1H), 4.26 (t, J = 7.0 Hz, 2H), 3.69 (s, 5H), 3.48 (t, J = 6.8 Hz, 2H), 1.6–1.59 (m, 2H), and 1.50–1.43 (m, 2H) (see Figure S2 for proton NMR spectrum of 3a); 13C NMR (CDCl3) δ 188.36, 171.87, 168.57, 157.05, 149.86, 141.93 (βCF = 244.6 Hz), 141.28, 137.87, 137.80, 134.24, 132.38, 132.35, 132.10, 131.05, 130.98 (βCF = 3.0 Hz), 129.98, 129.16, 128.36 (βCF = 3.0 Hz), 127.28 (βCF = 15.3 Hz), 125.25, 123.49, 121.42, 119.98 (βCF = 15.3 Hz), 116.93, 116.17, 112.64, 52.53 (q, J = 9.9 Hz), 43.61, 37.27, 34.49, 27.35, and 25.77; IR cm⁻¹ 3391.2, 2929.4, 2851.8, 1711.7, and 1442.0; LRMS-ESI m/z 687 (100%, [M]+), 439 (65%); HRMS-ESI calculated for C30H26BrF2N2O2Na+[M+Na]+ 709.1779, found 709.1767.

Figure 4. The survival curves of mice treated with placebo or a BoNTAe inhibitor. F3A: top, H3H: middle, and F4H: bottom. doi:10.1371/journal.pone.0010129.g004

reported in ppm using either tetramethylsilane or the solvent peak as an internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, brs = broad singlet, d = doublet, t = triplet, brt = broad triplet, q = quartet, m = multiplet), coupling constant, and integration. Low-resolution mass spectra were recorded using either Hewlet Packard 5973 Mass Spectrometer with SIS Direct Insertion Probe (Palo Alto, CA) or Waters QZ/EMD 1000 Mass Spectrometer (Milford, MA). High-resolution mass spectra were obtained on a Bruker BioTOF II
2-(5-[(1-(4-Aminobutyl)-3-fluoro-2-phenyl-1H-indole-6-carbonyl)-2-(4-hydroxyphenyl)thiophen-3-yl]-N-hydroxycetamide (F4H). To a stirred solution of 3x (34 mg, 0.049 mmol) in THF/MeOH (3 mL/5 mL), 1 mL of 30% aqueous NH$_2$OH was added, followed by a catalytic amount (two crystals) of KCN. The resulting mixture was stirred for 23 hours at room temperature, and then filtered through a short Celite column. HPLC purification of the filtrate gave F4H-TFA as a yellow amorphous solid (20 mg, 60%). The semi-preparative and analytical HPLC retention times of F4H-TFA are 14.00 and 14.57 minutes, respectively (see Figure S3 for chromatograms of F4H-TFA before and after the HPLC purification).

1H NMR (CD$_3$OD) $\delta$ 8.11 (s, 1H), 7.78 (s, 1H), 7.73 (d, $J$ = 8.4 Hz, 1H), 7.66 (dd, $J$ = 1.2, 8.4 Hz, 1H), 7.59–7.58 (m, 4H), 7.54–7.50 (m, 1H), 7.39 (d, $J$ = 8.6 Hz, 2H), 6.92 (d, $J$ = 8.6 Hz, 2H), 4.36 (s, $J$ = 7.2 Hz, 2H), 3.51 (s, 2H), 2.79 (t, $J$ = 7.2 Hz, 2H), 1.76–1.69 (m, 2H), and 1.50–1.43 (m, 2H) (see Figure S4 for proton NMR spectrum of F4H; $^{13}$C NMR (CD$_3$OD) $\delta$ 188.30, 169.26, 160.90 (q, CF$_3$CO$_2$H), 154.38, 150.74, 150.11, 141.75 ($\delta_{C3}$ = 243.1 Hz), 140.72, 137.85, 132.29, 132.15 ($\delta_{C3}$ = 5.3 Hz), 130.73, 130.53, 129.83, 129.02, 128.45 ($\delta_{C3}$ = 3.8 Hz), 127.30 ($\delta_{C3}$ = 20.5 Hz), 123.36, 120.58, 116.94 ($\delta_{C3}$ = 16.0 Hz), 116.65, 115.71, 113.10, 43.29, 31.38, 32.29, 27.11, and 24.70; IR cm$^{-1}$ 3438.5, 2927.7, 1677.0, 1608.9, 1551.6, 1428.1, 1250.1, 1209.2, 1138.2; LRMS-EI m/z 557.2468 [M$^+$]H$^+$, 557.1857, found 557.2040.

Methyl 2-[2-(3-aminophenyl)-5-[1-(1,3-dioxoisodolin-2-yl)butyl]-3-fluoro-2-phenyl-1H-indole-6-carbonyl]thiophen-3-yl]acetate (3y). A mixture of 2 (20 mg, 0.03 mmol), Pd(PPh$_3$)$_4$ (7 mg, 0.006 mmol), Cs$_2$CO$_3$ (13 mg, 0.09 mmol), 3-aminophenylboronic acid (6 mg, 0.04 mmol), and H$_2$O (60 mL) in 1,2-dimethoxyethane (4 mL) was degassed with N$_2$ for 10 minutes and then refluxed until all the starting ester had been consumed (3 hours). The resulting black suspension was poured into H$_2$O (10 mL) and then extracted with 40 mL EtOAc. The organic layer was washed with brine (2 × 20 mL), dried over MgSO$_4$, and then concentrated in vacuo. MPLC purification (Hex:EtOAc:Ac$/$O = 1:1:1) of the residue gave 3y as a yellow solid foam (15 mg, 74%).

1H NMR (CDCl$_3$) $\delta$ 7.99 (s, 1H), 7.78–7.76 (m, 2H), 7.71–7.67 (m, 5H), 7.51–7.37 (m, 5H), 7.24 (s, $J$ = 7.6 Hz, 1H), 6.88 (d, $J$ = 7.2 Hz, 1H), 6.83 (s, 1H), 6.73 (d, $J$ = 7.2 Hz, 1H), 4.26 (t, $J$ = 7.0 Hz, 2H), 3.72 (s, 2H), 3.69 (s, 3H), 3.48 (t, $J$ = 6.8 Hz, 2H), 1.66–1.58 (m, 2H), and 1.50–1.45 (m, 2H) (see Figure S5 for proton NMR spectrum of 3y); $^{13}$C NMR (CDCl$_3$) $\delta$ 188.12, 171.73, 168.51, 149.72, 147.07, 141.92 ($\delta_{C3}$ = 245.0 Hz), 141.74, 137.49, 134.19, 134.09, 132.46, 132.36 ($\delta_{C3}$ = 6.0 Hz), 132.13, 130.93, 129.96, 129.15, 129.05, 128.58 ($\delta_{C3}$ = 3.0 Hz), 127.21 ($\delta_{C3}$ = 10.7 Hz), 123.46, 121.39, 119.94 ($\delta_{C3}$ = 6.1 Hz), 116.91, 116.89, 115.80 ($\delta_{C3}$ = 15.3 Hz), 112.59, 52.41, 43.61, 37.25, 34.50, 27.36, and 25.77; IR cm$^{-1}$ 3456.5, 3366.6, 2945.7, 1711.7, 1601.4 and 1393.0; LRMS-EI m/z 557.2017, found 557.2040.

F3A was obtained according to the RESP procedure [29] with ab initio calculations at the HF/6-31G*//HF/6-31G* level using the

**in Vitro Evaluation**

**Pharmacokinetics Study.** The in vivo pharmacokinetic parameters were determined by dosing 6 Balb/c mice intraperitoneally with a test inhibitor at 2 mg/kg at which concentration no obvious sign of toxicity was observed. Blood was collected by cardiac puncture at 0.5, 1, 2, 4, 8, and 24 hours and the plasma was separated and kept frozen at −80°C until processing. Each experiment was repeated three times. The plasma was thawed and extracted with two volumes of ice-cold acetonitrile to precipitate plasma proteins and release the inhibitor. The organic phase was analyzed by liquid chromatography mass spectrometry and the concentration of the inhibitor was determined based on a standard curve run in parallel. The stability of the inhibitor in acetonitrile was determined prior to analyzing pharmacokinetic samples. Pharmacokinetic values were determined with WinNonLin software from Pharcite based on the plasma concentration curve.

**Protection Study.** The protection studies were carried out by using a standardized mouse model of botulism [26]. Briefly, groups of Balb/c mice were given a single 2-mg/kg intraperitoneal injection of H3H, F4H, F3A or dimethyl sulfoxide as a control and, after 30 minutes, each mouse was challenged intraperitoneally with BoNTA at 5 times of its median-lethal dose. Dimethyl sulfoxide was used as a carrier vehicle because H3H is water insoluble. All mice were examined twice daily for survival, behaviour, motor activity, breath, and extracocular symptoms of botulism. The numbers of mice in the treated and control groups were 10 and 5, respectively. Survival curves were constructed based on the number of survivors and statistically analyzed using GraphPad Prism 5.0 (Graphpad Software, Inc.).

**Computer Simulations**

**Model Preparation.** The atomic charges of F4H and F3A were calculated according to the RESP procedure [29] with the HF/6-31G*/HF/6-31G* level using the
Gaussian 98 program [30]. The starting structure of inhibitor-BoNTAe was generated by (1) manually docking the inhibitor into the BoNTAe active site and (2) replacing the active-site zinc ion with the tetrahedral zinc ion using the cationic dummy atom approach [23,31-33]. In the manual docking, the hydroxamate group was placed near the tetrahedral zinc ion, the thiophene-substituted phenyl group was placed near Arg363, and the ammonium group was placed near Glu64. The BoNTAe structure used for the docking was taken from the crystal structure of an inhibitor-bound BoNTAe (Protein Data Bank Code: 3BOO [34]) whose conformations of missing residues 62-67 were taken from the crystal structure of a BoNTAe mutant in complex with SNAP-25 (Protein Data Bank Code: 1XTG [35]). For BoNTAe, His323 and His227 were treated as HIN (histidinate) [32,36,37]; His39, His230, and His269 were treated as HID; all other His residues were treated as HIP; Glu261 and Glu351 were treated as GLH [32,36,37]. A total of 111 crystallographically determined water molecules (named HOH) located inside the enzyme were included for simulations. The topology and coordinate files of the water-containing inhibitor-BoNTAe complex were generated by the PREP, LINK, EDIT, and PARM modules of the AMBER 5.0 program [38]. The complex was refined by energy minimization using a dielectric constant of 1.0 and 100 cycles of steepest-descent minimization followed by 100 cycles of conjugate-gradient minimization. The refined complex was solvated with 13,617 and 13,540 TIP3P water molecules (named WAT) [39] for F4H and F3A, leading to a system of 48,096 and 47,866 atoms, respectively. The WAT molecules were obtained from solvating the complex using a pre-equilibrated box of 216,000 TIP3P molecules, whose hydrogen atom charge was set to 0.4170, where any water molecule was removed if it had an oxygen atom closer than 2.2 Å to any solute atom or a hydrogen atom closer than 2.0 Å to any solute atom, or if it was located further than 9.0 Å along the x-, y-, or z-axis from any solute atom.

**Multiple Molecular Dynamics Simulations.** The solvated complex system was energy-minimized for 100 cycles of steepest-descent minimization followed by 100 cycles of conjugate-gradient minimization to remove close van der Waals contacts in the system, then heated from 0 to 300 K at a rate of 10 K/ps under constant temperature and volume, and finally simulated independently with a unique seed number for initial velocities at 300 K under constant temperature and pressure using the PMEMD module of the AMBER 8.0 program [40] with the AMBER force field (ff99SB) [41,42]. All simulations used (1) a dielectric constant of 1.0, (2) the Berendsen coupling algorithm [43], (3) a periodic boundary condition at a constant temperature of 300 K and a constant pressure of 1 atm with isotropic molecule-based scaling, (4) the Particle Mesh Ewald method to calculate long-range electrostatic interactions [44], (5) a time step of 1.0 fs, (6) the SHAKE-bond-length constraints applied to all the bonds involving the H atom, (7) saving the image closest to the middle of the “primary box” to the restart and trajectory files, (8) formatted restart file, and (9) default values of all other inputs of the PMEMD module. Ten different molecular dynamics simulations (each lasted 10 ns) were carried out for the BoNTAe in complex with F4H or F3A on a cluster of Apple Mac Pros with 80 Intel Xeon cores (3.0 GHz).

**Simulation Analysis.** For each of the 10 simulations of F4H-BoNTAe or F3A-BoNTAe, 100 instantaneous conformations were saved at 10-ps intervals during the last 1-ns period. A total of 1,000 instantaneous conformations of F4H-BoNTAe or F3A-BoNTAe from the 10 simulations were subjected to a cluster analysis using the average-linkage algorithm (epsilon = 2.0 Å and RMS on all atoms of F4H or F3A) [45]. This analysis identified 7 and 4 clusters for the F4H and F3A conformations, respectively. The numbers of the F4H conformations in Clusters 1–7 are 200, 100, 123, 27, 150, 30, and 70, respectively; the numbers of the F3A conformations in Clusters 1–4 are 600, 299, 1, and 100, respectively. The representative conformations of F4H-BoNTAe and F3A-BoNTAe from their most populated clusters overlay reasonably well (see Figure 3) and are considered as plausible complex structures in water. The coordinates of the representative conformations are available from Datasets S1 and S2. The coordinates of other conformations are available upon request.

**Supporting Information**

**Figure S1** Proton NMR spectrum of 2. Found at: doi:10.1371/journal.pone.0010129.s001 (0.79 MB PDF)

**Figure S2** Proton NMR spectrum of 3x. Found at: doi:10.1371/journal.pone.0010129.s002 (0.56 MB PDF)

**Figure S3** Chromatograms of F4H-TFA before and after the HPLC purification. Found at: doi:10.1371/journal.pone.0010129.s003 (0.17 MB PDF)

**Figure S4** Proton NMR spectrum of F4H. Found at: doi:10.1371/journal.pone.0010129.s004 (0.54 MB PDF)

**Figure S5** Proton NMR spectrum of 3y. Found at: doi:10.1371/journal.pone.0010129.s005 (0.50 MB PDF)

**Figure S6** Chromatograms of F3A-2TFA before and after the HPLC purification. Found at: doi:10.1371/journal.pone.0010129.s006 (0.17 MB PDF)

**Figure S7** Proton NMR spectrum of F3A. Found at: doi:10.1371/journal.pone.0010129.s007 (0.43 MB PDF)

**Dataset S1** Coordinates of simulation-generated model of F4H-BoNTAe. Found at: doi:10.1371/journal.pone.0010129.s008 (0.46 MB TXT)

**Dataset S2** Coordinates of simulation-generated model of F3A-BoNTAe. Found at: doi:10.1371/journal.pone.0010129.s009 (0.47 MB TXT)

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**Author Contributions**

Conceived and designed the experiments: Y-PP JD JJS CBM. Design of F4H and F3A: Y-PP SW JGP. Syntheses of F4H, F3A, and H3H: SW. Performed the experiments: Y-PP JD SW JGP MPN JJS. Analyzed the data: Y-PP JD SW JGP MPN JJS CBM. Wrote the paper: Y-PP JD CBM. Contributed with revisions: Y-PP JD SW JGP MPN JJS CBM.
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