Published in final edited form as:

Neurotox Res. 2015 May ; 27(4): 384–398. doi:10.1007/s12640-015-9526-z.

Src Family Kinase Inhibitors Antagonize the Toxicity of Multiple Serotypes of Botulinum Neurotoxin in Human Embryonic Stem Cell-Derived Motor Neurons

Erkan Kiris^{1,2,3}, James C. Burnett^{4,5}, Jonathan E. Nuss², Laura M. Wanner², Brian D. Peyser⁵, Hao T. Du³, Glenn Y. Gomba², Krishna P. Kota², Rekha G. Panchal², Rick Gussio⁵, Christopher D. Kane^{2,6,7}, Lino Tessarollo³, and Sina Bavari²

Erkan Kiris: erkan.kiris@nih.gov; Sina Bavari: sina.bavari.civ@mail.mil

¹Geneva Foundation, Tacoma, WA, USA

²Department of Molecular and Translational Sciences, US Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD 21702, USA

³Neural Development Section, Mouse Cancer Genetics Program, Center for Cancer Research, NCI, Frederick, MD 21702, USA

⁴Leidos Biomedical Research, Inc., Computational Drug Development Group (CDDG), NCI, Frederick, MD 21702, USA

⁵CDDG, Developmental Therapeutics Program, NCI, Frederick, MD 21702, USA

⁶Henry M. Jackson Foundation, Bethesda, MD, USA

⁷DoD Biotechnology High Performance Computing Software Applications Institute (BHSAI), Telemedicine and Advanced Technology Research Center (TATRC), US Army Medical Research and Materiel Command (USAMRMC), Frederick, MD 2170, USA

Abstract

Botulinum neurotoxins (BoNTs), the causative agents of botulism, are potent inhibitors of neurotransmitter release from motor neurons. There are currently no drugs to treat BoNT intoxication after the onset of the disease symptoms. In this study, we explored how modulation of key host pathways affects the process of BoNT intoxication in human motor neurons, focusing on Src family kinase (SFK) signaling. Motor neurons derived from human embryonic stem (hES) cells were treated with a panel of SFK inhibitors and intoxicated with BoNT serotypes A, B, or E (which are responsible for >95 % of human botulism cases). Subsequently, it was found that bosutinib, dasatinib, KX2-391, PP1, PP2, Src inhibitor-1, and SU6656 significantly antagonized all three of the serotypes. Furthermore, the data indicated that the treatment of hES-derived motor neurons with multiple SFK inhibitors increased the antagonistic effect synergistically. Mechanistically, the small molecules appear to inhibit BoNTs by targeting host pathways necessary for intoxication and not by directly inhibiting the toxins' proteolytic activity.

Importantly, the identified inhibitors are all well-studied with some in clinical trials while others are FDA-approved drugs. Overall, this study emphasizes the importance of targeting host neuronal pathways, rather than the toxin's enzymatic components, to antagonize multiple BoNT serotypes in motor neurons.

Keywords

Motor neurons; Human embryonic stem cells; Src inhibitors; Botulinum neurotoxins; Cell-based assay; Drug discovery

Introduction

Botulinum neurotoxins (BoNTs) are among the most toxic of known biological substances. There are seven biochemically distinct BoNT serotypes (A–G) that are capable of temporarily disabling motor neurons, leading to impaired muscle function and potentially fatal respiratory arrest (Dolly et al. 2009). Currently, there are no therapies to counter BoNT intoxication once the toxin has internalized into the neuron. This presents a critical, unmet medical need, as exposure to BoNTs can occur via multiple routes (Rossetto et al. 2014). BoNTs are naturally occurring and can sometimes contaminate food or liquids (Wein and Liu 2005). It is also known that BoNTs have been weaponized, resulting in their designation as category A biothreat agents (Arnon et al. 2001). Finally, BoNTs are increasingly being used to treat a range of medical conditions (Kostrzewa and Segura-Aguilar 2007; Chen 2012), thereby increasing the potential for accidental, but therapeutically untreatable, overdosing.

Once a patient's diaphragm muscles are paralyzed following BoNT intoxication, mechanical ventilation is the only life-saving option during the weeks to months that it takes for the toxins to desist. It is estimated that the medical cost of such care can be as high as \$350,000 per patient for every 2 weeks of treatment (Wein and Liu 2005). Several antibodies targeting BoNTs have been approved by the FDA to treat botulism (Larsen 2009) but must be administered soon after exposure to prevent paralysis. Other drug development efforts, which are still in the experimental stages, have mostly focused on directly inhibiting the proteolytic activity of the BoNT light chain (LC) (Capek et al. 2011; Kiris et al. 2014a; Opsenica et al. 2013; Videnovic et al. 2014; Li et al. 2011a). Using in vitro assays, a range of chemically distinct LC inhibitors have been identified (Li et al. 2011b; Patel et al. 2014) but have generally fared poorly in animal models. Importantly, at least four distinct BoNT serotypes (A, B, E, and F) can cause botulism in humans, but current LC drug development efforts disproportionately focus on serotype A. However, BoNT/B and /E account for as many cases of human botulism as the BoNT/A (Sobel et al. 2004). As a result, there is a significant interest in developing novel therapeutics that can antagonize multiple BoNT serotypes and/or promote motor neuron-muscle connection post-intoxication.

We propose that an alternative, viable therapeutic approach is the targeting of host pathways in motor neurons that may be essential for BoNT intoxication. However, such an approach requires a physiologically relevant, experimentally tractable cell-based system to screen and characterize potential therapeutics. Given that BoNTs naturally target neurons, such models

should use these cells. To this end, previous cell-based studies have mostly used either primary embryonic murine spinal neurons or human neuroblastoma cell lines (Pellett 2013). Primary neurons are attractive cellular models for BoNT studies but are difficult to obtain in sufficient numbers for drug screening. Conversely, neuroblastoma cell lines are easily cultured but are biologically distinct from motor neurons and significantly less sensitive to BoNTs. Recently, we and others demonstrated that neurons derived from mouse embryonic stem (ES) cells are a highly sensitive and virtually unlimited cell source for BoNT experimentation (Kiris et al. 2014b; Pellett 2013). However, for studies aimed at modulating host factors, with a focus on translation to the clinic, it is preferable to use human motor neurons. More recently, Whitemarsh et al demonstrated the utility of a human-induced pluripotent stem cell-derived neuronal system, which is mainly composed of glutamatergic and GABAergic neurons, for BoNT studies (Whitemarsh et al. 2012). In this study, we established a human ES-derived motor neuron(hES-MNs)-based system for BoNT drug screening and experimentation.

The overall sequence of events during BoNT intoxication and the proteolytic targets are known: BoNT/A and E cleave SNAP-25; BoNT/B, D, F, and G cleave VAMP-2; and BoNT/C cleaves both SNAP-25 and syntaxin (Montecucco and Molgo 2005; Sun et al. 2012; Breidenbach and Brunger 2004; Brunger et al. 2008; Sikorra et al. 2008). However, our mechanistic understanding of the host signaling pathways involved in BoNT intoxication and/or recovery remains minimal. Some studies have suggested that BoNT activity depends on its phosphorylation by Src (Ibanez et al. 2004; Blanes-Mira et al. 2001; Encinar et al. 1998; Ferrer-Montiel et al. 1996; Toth et al. 2012). Src family kinases (SFKs) play critical roles in many cellular functions in the nervous system, including the development and maintenance of neurons, synaptic plasticity, axon guidance, and neurotransmission (Ohnishi et al. 2011). In motor neurons, SFKs are highly expressed and modulate NMDA receptor ion channels, nicotinic acetylcholine receptors, axonal outgrowth, and neurotransmitter release (Kao et al. 2009; Wiesner and Fuhrer 2006). Small molecule SFK inhibitors represent an expanding class of compounds that are well characterized and include an increasing number of FDA-approved drugs (Aleshin and Finn 2010; Sen and Johnson 2011).

Here, we examined how SFK inhibitors affect BoNT intoxication using hES-MNs. Specifically, commercially available, pharmaceutically active SFK inhibitors that interfere with BoNT activity in hES-MNs were identified. Furthermore, we found that (1) several SFK inhibitors can antagonize multiple BoNT serotypes in a dose-dependent manner, and (2) combinations of the small molecules provide greater protection against BoNT-mediated cleavage of SNARE proteins. These findings suggest that compounds targeting signaling pathways in neurons, rather than toxin itself, could potentially be used to inhibit multiple BoNT serotypes.

Materials and Methods

Directed Differentiation of Human ES Cells into Motor Neurons

H9 human ES cells were purchased from WiCell Research Institute, and cultured and differentiated as described previously (Li et al. 2008; Hu and Zhang 2009; Lee et al. 2007),

but with modifications. Briefly, ES cells were cultured on mitomycin-inactivated primary mouse embryonic fibroblast (MEF) cells in H9-ES medium, which consists of Advanced DMEM/F12 supplemented with 20 % knockout serum replacer, β -mercaptoethanol (final 0.1 mM), 0.5 % Glutamax, 1 % non-essential amino acids, and 4 ng/ml of basic fibroblast growth factor (bFGF). Unless otherwise stated, all reagents were obtained from Invitrogen. For embryoid body (EB) formation, the ES cell colonies were first removed from MEF monolayer by incubating with dispase (1 U/ml). The colonies were then resuspended in H9 medium without bFGF and seeded onto low-adherence dishes for the first 4 days to form EBs. On day 4, culture medium was switched to the H9-neural induction medium including 1:1 Advanced DMEM/F12 plus Neurobasal medium supplemented with 1 % Glutamax, 1 % non-essential amino acids, 1 % N2 supplement, 0.2 mM ascorbic acid, and 2 μ g/ml heparin and EBs were maintained in the suspension culture. Following 6 days of neural induction (differentiation day 10), EBs were treated with retinoic acid (RA) (0.1 μ M, Sigma-Aldrich) for 5 days. Between days 15 and 28, in addition to 0.1 μ M RA, sonic hedgehog (Shh) (100 ng/ml, R&D Systems) protein, or its agonist (Hh-Ag1.5) (0.1 μ M) (Cellagentech) was also supplemented to the medium to induce motor neuron specification. On day 28, EBs were transferred to dishes including fresh H9-neural induction media, supplemented with 2 % B27 supplement, cAMP (1 μ M, Sigma-Aldrich), IGF-1 (Peprotech), GDNF (100 ng/ml, R&D Systems), BDNF, CNTF, and NT3 (each at 10 ng/ml, from Chemicon). On day 30, EBs were dissociated using trypsin and plated onto matrigel (BD Biosciences) coated dishes to allow neurite elongation prior to experimentation. On Day 32, the motor neuron medium was supplemented with 2 μ M cytosine β -D-arabinofuranoside (Ara-C) (Sigma), and cells were cultured for 24 h to inhibit unwanted proliferating cells. On Day 33, Ara-C was completely removed, and cells were cultured in fresh motor neuron medium for 2 days (Day 35).

Immunoblotting Analyses

Immunoblotting was performed using standard protocols to characterize hES-MNs. Briefly, H9 ES cells and differentiated cells were harvested at different stages, and the total protein concentration was determined using a BCA assay kit (Thermo Scientific). Equal amounts of protein were separated on SDS-PAGE, transferred to PVDF membranes, and probed overnight with primary antibodies against Oct4 (Sigma), Tau (Tau5, for total tau) (Thermo Scientific) and β -III tubulin (Tuj1) (Covance), Choline Acetyltransferase (ChAT) (Millipore), p75 (Millipore), and GAPDH (Millipore) (as shown in Fig. 1a), as well as for SNAP-25 (Covance), SV2A (Millipore), Syntaxin (Sigma), and VAMP-2 (Millipore or R&D Systems) (as shown in Fig. 2a). Similarly, Western blotting was performed to assess the effects of SFK inhibitors treatments on downstream phosphorylation events (Fig. 7b). hES-MNs (Day 35) were treated with the compounds (30 μ M) for 30 min and then intoxicated with 500 pM BoNT/A for 4 h. Samples were then processed and subjected to immunoblotting, using antibodies against phospho-paxillin (Tyr118), phospho-p130^{CAS} (Tyr410), phospho-FAK (Tyr576/577), phospho-FAK (Tyr397), phospho-p38MAPK (Thr180/Tyr182), phospho-Akt (Ser473), phospho-GSK3 β (Ser9), and phospho-STAT3 (Tyr705) (all from Cell Signaling Technology). The neuron-specific β -III tubulin (Covance) was used as loading control. The immunoblotting results were visualized with a Syngene gel documentation and analysis system.

Immunocytochemistry

hES-MN cultures (day 35) were fixed in 4 % paraformaldehyde-PBS for 10 min and permeabilized with 0.1 % Triton X-100. After being blocked with 10 % serum, the cells were then incubated overnight with the following primary antibodies: Tau5 (Thermo Scientific), LIM3 (Millipore), ChAT (Millipore), NeUN (Millipore), Hb9 [Developmental Studies Hybridoma Bank (DSHB)], Isl1 (DSHB), and β -III tubulin (Covance) (as shown in Fig. 1b), as well as antibodies for SNAP-25 (BD Biosciences), SV2A (Millipore), Syntaxin (Sigma), VAMP-2 (R&D Systems), and MAP2 (Millipore) (as shown in Fig. 2b) in PBS with 10 % serum according to the manufacturers' suggested working concentrations. On the following day, appropriate secondary antibodies, conjugated with Alexa488 and Alexa594, were incubated with the cells for 2 h at room temperature. Image acquisition was performed using Zeiss or Opera (PerkinElmer) confocal microscopes.

BoNT Intoxication and Immunoblotting Analyses to Quantify BoNT-Mediated Proteolysis

hES-MN cultures (day 35) were intoxicated with increasing concentrations of either BoNT/A or BoNT/B or trypsin-activated BoNT/E (MetaBiologics) and incubated at 37 °C for 4 h (Fig. 3). Following intoxication, samples were processed, and SNAP-25 (Covance) and VAMP-2 (R&D Systems) protein cleavages were quantified using standard immunoblotting procedures—as described previously (Huang et al. 2011; Pellett et al. 2007). Quantification of changes in total VAMP-2 protein levels was calculated by normalizing the total VAMP-2 band intensity values to corresponding GAPDH levels relative to non-toxin treated control conditions run on each gel. For inhibitor studies, *Triticum vulgaris* Lectin (TVL) and bafilomycin were used at titrated concentrations and added to the cultures 30 min prior to intoxication. Both reagents were obtained from Sigma. An antibody that neutralizes BoNT/A (4A2-4) (produced at the US Army Medical Research Institute of Infectious Diseases) and the control antibody (Anti staphylococcal enterotoxin B) (Toxin Technology) were simultaneously applied with 1 nM BoNT/A to the cultures.

SFK Inhibitors

Bosutinib (SKI-606), Dasatinib (BMS-354825), KX2-391, and Saracatinib (AZD0530) were obtained from Selleck Chemicals. PP1, PP2, Src Inhibitor-1, and SU6656 were from Sigma.

Statistical Analyses

Student's *t* test was used to calculate statistical significance (*P*) (GraphPad Prism version 6.01). *P* < 0.05 was considered statistically significant, and values are reported as mean $\pm$ SEM.

Quantitative Analysis of Combined Drug Effects Using a Combination Index Method

hES-MNs were treated with vehicle or SFK inhibitors, either single or combined at different concentrations and different ratios as described in Fig. 6b. Bosutinib was not included in combination studies as this compound resulted in increased SNAP-25 cleavage at lower concentrations (Fig. 5). After a 30 min incubation period, the neurons were intoxicated with 500 pM BoNT/E and incubated for an additional 4 h. Samples were then subjected to immunoblotting to determine the extent of SNAP-25 protection under each condition. Using

the SNAP-25 protection data obtained from combined compound treatments, along with the protection data obtained from single compound treatments, Fraction-affected (Fa) values were calculated. Combination index (CI) values were quantitatively determined using the Fa values for 9 dose combinations for each drug pair, using CompuSyn software (ComboSyn, Inc.). The resulting values were plotted using GraphPad software. Based on the Chou–Talalay equation, $C < 1$, $C = 1$, and $C > 1$ indicate synergism, additivity, and antagonism, respectively (Chou 2010). Linear correlation co-efficiency (r) values were higher than 0.9 in all CI calculations. Additionally, synergy measurements were performed using Bliss independence as the null model, reporting the parameter β that minimizes the following metric (Cokol et al. 2011):

$$\sum (fu_{1x,2y} - \beta \times fu_{1x} \times fu_{2y})^2,$$

where fu_{1x} is the fraction unaffected in the presence of compound 1 at concentration x , fu_{2y} is the fraction unaffected in the presence of compound 2 at concentration y , and $fu_{1x,2y}$ is the fraction unaffected in the presence of drugs 1 and 2 at concentrations x and y , respectively. Fraction affected (fa) is calculated as

$$\frac{E - C}{1 - C},$$

where E is the experimental fraction of intact SNAP-25, and C is the control (toxin-only) fraction of intact SNAP-25. Fraction unaffected (fu) is $1 - fa$.

Measuring BoNT/A LC Inhibition Using In Vitro HPLC-Based Assay

BoNT/A LC proteolytic activity was determined using a high-performance liquid chromatography (HPLC)-based assay, as described previously (Nuss et al. 2010). Briefly, this assay utilizes an N-terminal acetylated, C-terminal aminated synthetic peptide that corresponds to the minimum substrate requirements of the BoNT/A LC. Proteolytic assay mixtures consisted of buffer (50 mM HEPES (pH 7.4) + 0.2 mg/ml BSA), recombinant BoNT/A LC, peptide substrate, and SFK inhibitor. A known LC inhibitor, MV150, was used as the positive control. Assay reactions were incubated at 37 °C for 10 min and then quenched with trifluoroacetic acid. Samples were then analyzed by reverse-phase HPLC. The cleavage products were separated using a Shimadzu Prominence ultra fast liquid chromatography XR system with reverse-phase (C18:50 × 2.1 mm, 1.9 μm) chromatography. Percent LC inhibition was calculated by comparing SFK inhibitor samples to DMSO controls. Mean values were obtained from at least three independent assays.

Results

Motor Neurons Derived from Human ES Cells Express the BoNT/A Receptor and Intracellular Targets

Human ES cells were differentiated into motor neurons and analyzed by Western blots at different stages of the differentiation process to quantify changes in the protein levels characteristic of stem cells, neuronal cells, and motor neurons (Fig. 1a). The pluripotency

marker Oct4, which is highly expressed in ES cells, was down-regulated during differentiation. In contrast, the neural markers Tau (Tau5, for total tau) and β -III tubulin, along with ChAT, an enzyme that is required for the synthesis of the neurotransmitter acetylcholine, and p75, a low-affinity neurotrophin receptor expressed in motor neurons, were up-regulated in response to motor neuron inducing factors.

To characterize the differentiation process at the single cell level, immunocytochemistry was performed to examine the expression of markers for neural cells and more specifically motor neurons (Fig. 1b). Immunostaining for the neural markers Tau, β -III tubulin, and NeUN indicated that the majority of the differentiated cells were neurons. Motor neurons were identified by the markers ISL1, HB9, and LIM3. All of the cells that were positive for motor neuron markers were also positive for neural markers (Fig. 1b). Confocal microscopy further indicated that ChAT was expressed by HB9+ cells, suggesting that these cells were producing acetylcholine. Using confocal imaging, quantification of cells expressing motor neuron markers at day 35 indicated that ~50 % of the cells in culture were motor neurons (data not shown), which is similar to results from previous studies (Li et al. 2008; Hu and Zhang 2009). Overall, these data indicated that the majority of the differentiated cells at day 35 were neurons, and that a sizeable subset consisted of motor neurons.

Next, we investigated the expression levels of the intracellular BoNT targets during directed differentiation. Western blot analysis exhibited that the levels of BoNT target proteins (SNAP-25, VAMP-2, and Syntaxin) increased during differentiation (Fig. 2a). Confocal microscopy further confirmed the presence of these proteins in the differentiated neurons (Fig. 2b). It has been shown that SV2 (isoforms A–C) receptors are critical for the entry of multiple BoNT serotypes into neurons (Dong et al. 2006; Mahrhold et al. 2006; Peng et al. 2011; Fu et al. 2009; Dong et al. 2008; Strotmeier et al. 2014). Here, our results exhibited that BoNT receptor SV2A was expressed in the differentiated neurons (Fig. 2a, b). Although we have not determined the expression of other SV2 serotypes, it is highly likely that SV2B and C are also expressed in ES-derived motor neurons as it has been shown that all three isoforms of SV2 receptors are endogenously expressed in primary motor neurons (Dong et al. 2006). Taken together, the endogenous expression of all BoNT intracellular targets suggested that hES-MN cultures should be sensitive to intoxication by all BoNT serotypes.

Human ES-Derived Motor Neurons are Highly Sensitive to BoNT/A, BoNT/B, and BoNT/E in a Dose-Dependent Manner

The sensitivity of hES-MNs to increasing concentrations of BoNT/A (Fig. 3a), /B (3b), and /E (3c) was evaluated using Western blot analysis. A dose-dependent increase in SNAP-25 cleavage was observed with increasing concentrations of BoNT/A (Fig. 3a) and /E (Fig. 3c). Notably, the activity of BoNT/A and/E in hES-MNs was similar to that of both primary spinal cord neurons and mouse ES-derived motor neurons (Keller et al. 2004; Kiris et al. 2011). BoNT/B cleaves VAMP-2, a 116 amino acid protein, between residues Gln⁷⁶–Phe⁷⁷, leading to a decrease in the amount of full-length VAMP-2 (Huang et al. 2011; Pellett et al. 2007). Changes in normalized total VAMP-2 protein levels relative to non-toxin-treated control conditions was therefore used to determine BoNT/B activity, and a dose-dependent decrease in VAMP-2 levels upon exposure to increasing levels of BoNT/B

was observed. These data demonstrate that hES-MNs are highly sensitive to intoxication by different BoNT serotypes.

Human ES-Derived Motor Neurons Can be Used to Evaluate Inhibitor-Mediated SNAP-25 Protection During BoNT/A Intoxication

To determine if hES-MNs could provide a cell system that effectively identifies inhibitors of BoNT/A-mediated SNAP-25 cleavage, we tested three known BoNT/A inhibitors: TVL (Coffield and Yan 2009), anti-BoNT/A antibody 4A2-4 (Pless et al. 2001), and bafilomycin (Kiris et al. 2011). TVL non-specifically prevents BoNT/A binding to its receptors, thereby inhibiting toxin entry into the neuronal cytosol, whereas 4A2-4 prevents uptake by directly binding to BoNT/A. Pre-treatment with both entry inhibitors resulted in dose-dependent decreases in SNAP-25 proteolysis (Fig. 3d). Bafilomycin is an ATPase inhibitor that prevents endosome acidification, thereby preventing BoNT delivery into the neuronal cytoplasm. Again, we found that pre-treatment of hES-MNs with increasing concentrations of bafilomycin prevented SNAP-25 cleavage in a concentration-dependent manner (Fig. 3d). These data indicate that hES-MNs can be used to evaluate the efficacy of inhibitors of BoNT/A-mediated SNAP-25 proteolysis.

SFK Inhibitors Attenuate BoNT-Mediated Substrate Cleavage

Having established that hES-MNs are susceptible to BoNT-mediated intoxication, we next sought to determine whether modulation of SFK pathways affect BoNT activity in motor neurons. Eight compounds known to inhibit SFK activity (Zhang and Yu 2012; Aleschin and Finn 2010; Sen and Johnson 2011) were first tested for dose-dependent effects on SNAP-25 cleavage during BoNT/A exposure. The commercial designations and structures of the compounds are shown in Fig. 4. Bafilomycin was used as a positive control. Doses ranging from 1 to 30 μ M of compound were applied to cells 30 min prior to intoxication. Cells were intoxicated for 4 h and toxin-mediated protein cleavage was assessed by Western blotting. Seven of the SFK inhibitors (Bosutinib, Dasatinib, KX2-391, PP1, PP2, Src inhibitor-1, and SU6656) provided statistically significant decreases in BoNT/A-mediated SNAP-25 cleavage (Student's *t* test) (Fig. 5a). The same seven compounds also significantly protected VAMP-2 and SNAP-25 during BoNT/B or BoNT/E exposure, respectively (Fig. 5b, c). Interestingly, Saracatinib, which does not interfere with BoNT/A-mediated SNAP-25 cleavage (Fig. 5a), provided VAMP-2 and SNAP-25 protection during BoNT/B and /E exposure. Overall, the results indicated that seven SFK inhibitors provide dose-dependent protection against all three BoNT serotypes (Fig. 5a–c). SFK inhibitors were examined for toxicity in ES-derived motor neurons using LDH assay (Garwood et al. 2011). Cells were plated in a 384-well format (4×10^4 cells/well) and treated with SFK Inhibitors (30 μ M) for 4 h without BoNT exposure. The compounds did not exhibit noteworthy toxic propensities (data not shown). Additionally, confocal microscopy was conducted to examine morphological indicators of general neuron health after treatment with the toxin alone (500 pM BoNT/A) or the toxin in combination with the compound. The results indicated that the compounds were well tolerated by neurons. Images showing the neuronal morphology of typical neuron cultures that were treated with compounds are shown in Fig. 5d. The cells were stained with β -III tubulin antibody (green), and Hoechst dye (blue) was used to stain the nuclei. In general, the data indicate that compounds known to act on the SFK host

signaling pathway can interfere with the ability of different BoNT serotypes to cleave their respective substrates.

Treatment with Combinations of SFK Inhibitors Increases Substrate Protection

There is heightened interest in drug combinations as it is an attractive strategy promising dose and toxicity reduction in vivo. For proof-of-concept, we hypothesized that it might be possible to increase SNAP-25 protection during BoNT intoxication by administering KX2-391 in combination with other SFK inhibitors. To test this hypothesis, BoNT/E intoxication was utilized because the quantification of BoNT/E-mediated SNAP-25 cleavage is relatively easier when compared to BoNT/A- and /B-mediated SNARE cleavage (Fig. 3). We chose to combine KX2-391 with other SFK inhibitors from this investigation because this compound's mechanism of action differs from that of other small molecules tested in the study (Fig. 6a). More specifically, KX2-391 is a non-ATP-competitive inhibitor that binds to the substrate binding site while the other SFK inhibitors bind to the ATP binding pocket except Src inhibitor-1, which may interact with both sites (Schenone et al. 2011; Sen and Johnson 2011). Additionally, KX2-391 is an orally bioavailable inhibitor that is currently in Phase II clinical trials.

Dose response matrix experiments were used to determine possible compound combination effects (Fig. 6b). Specifically, the compounds were tested at increasing concentrations (1, 10 and 20 μ M) either alone or in all possible combinations. SNAP-25 protection values for both single and combined drug treatments were evaluated using CompuSyn software to calculate combination index (CI) values for all 9 combinations for each pair of drugs, based on Chou-Talalay method (Chou 2010). CI >1, CI = 1, and CI <1 indicate antagonism, additivity, and synergy, respectively. At lower dose combinations (i.e., the combinations including KX2-391 at 1 μ M concentrations), and when fixed drug ratios such as 1:1, 10:10, and 20:20 were considered, CI values were lower than or equal to 1 for almost all of the compound combinations, suggesting synergistic and additive effects. However, it should be noted that some combination analyses resulted in CIs >1. Additionally, Bliss independence model was employed to further define potential synergistic effects. In these measurements, β was less than one for all combinations, indicating overall Bliss synergy. The greatest effect was with SU6656 (β = 0.944), followed by Saracatinib (β = 0.967), Src inhibitor-1 (0.967), Dasatinib (0.977), PP1 (0.997), and PP2 (0.998). The fraction affected as predicted by the Bliss model was less than observed at nine out of nine (9/9) data points for SU6656, 7/9 for Saracatinib, 7/9 for Src inhibitor-1, 5/9 for Dasatinib, 4/9 for PP1, and 3/9 for PP2. Overall, our data suggest that the combination of two SFK inhibitors provides increased SNAP-25 protection during BoNT/E challenge compared to the treatment with the individual compounds alone.

SFK Inhibitors Prevent BoNT Intoxication Through the Modification of Host Cell Pathways

To determine if the SFK inhibitors were acting directly on BoNT/A-mediated cleavage of SNAP-25, a well-characterized HPLC-based in vitro assay for measuring BoNT/A LC proteolytic activity was used to evaluate the compounds (Nuss et al. 2010) (Fig. 7a). A competitive BoNT/A LC inhibitor (MV150) was used as a positive control and demonstrated an IC₅₀ value of 5.2 μ M (data not shown). Conversely, none of the SFK inhibitors demonstrated LC inhibitory activity as they provided an IC₅₀ of greater than 30

μM. Hence, it can be concluded that the SFK inhibitors that were evaluated do not exert their protective effects via direct inhibition of the proteolytic activity of BoNT/A but rather act on host cellular mechanisms.

SFKs modulate other kinases including focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) and Jak-signal transducers, and activators of transcription (STAT) pathways (Sen and Johnson 2011). To determine the effects of the SFK inhibitors on SFK signaling pathways, we examined the phosphorylation patterns of downstream effectors (Ceppi et al. 2009; Congleton et al. 2012). These data indicated that the SFK inhibitors do indeed affect SFK-mediated signaling pathways in motor neurons that are crucial for many cellular mechanisms (Fig. 7a). In particular, the inhibitors lead to notable phosphorylation changes in known downstream effectors Paxillin, p130^{CAS}, FAK, p38MAPK, Akt, GSK3β, and STAT3. No significant changes in the expression level of the neuronal marker β-III tubulin were observed despite the observed changes in phosphorylation. Therefore, the data indicate that SFK inhibitors are affecting various SFK signaling events that can modulate BoNT activity in the neuronal cytosol.

Discussion

The overall objective of this study was to evaluate the potential of SFK inhibitors to antagonize the intoxication effects of multiple BoNT serotypes in motor neurons. To do so, we first developed a simple, reliable, and physiologically relevant hES-MN system for evaluating BoNT neuronal intoxication. It is well established that BoNT-mediated SNARE protein cleavage is sufficient to inhibit neurotransmission (Apland et al. 1999; Bajohrs et al. 2004), indicating that the cleavage products of these target proteins provide critical markers to assess the effects of potential BoNT antagonists. Hence, neuron-based assays can provide a simple format for identifying small molecules that protect SNARE proteins during BoNT exposure (Hakami et al. 2010; Kota et al. 2014). To this end, we and others have shown that mouse ES-derived neurons and more specifically motor neurons can be used for BoNT research (Kiris et al. 2014b; Pellett 2013; McNutt et al. 2011). However, it is important to recognize that there are species-related differences between mouse and human cells, and that these differences in target expression and small molecule potency can be critical to the translational aspects of efficacy and toxicity during drug development. Indeed, the comparison of mouse and human ES-derived neuron-based drug screening assays in other systems have revealed significant translational differences, with human cells yielding more relevant results (McNeish et al. 2010). Therefore, the hES-MN system greatly facilitates BoNT drug screening and experimentation, as this platform offers the ability to identify and validate lead compounds using a system that is physiologically pertinent to human botulism.

In this study, we hypothesized that host motor neuron pathways might be important for facilitating BoNT intoxication, and therefore, that the inhibitors of such pathways could potentially be used to antagonize multiple BoNT serotypes. To test this hypothesis, we conducted a targeted screen using hES-MNs to identify small molecules that protect SNARE proteins during BoNT intoxication by specifically focusing on SFK pathways. SFKs are highly expressed in the nervous system, and a large body of literature has identified SFKs as key proteins for normal motor neuron function (Ohnishi et al. 2011; Kao et al. 2009;

Wiesner and Fuhrer 2006). Here, we show that commercially available, well-studied SFK inhibitors can be used to antagonize multiple BoNT serotypes.

As opposed to the ‘host-targeted BoNT inhibition’ paradigm evaluated in this study, the majority of drug development efforts to counter botulism have focused on directly inhibiting the enzymatic activities of individual serotype metalloprotease components in vitro (Capek et al. 2011; Li et al. 2011b; Kiris et al. 2014a). In general, targeting LC proteolytic activity is a rational approach. However, as evidenced by the lack of clinical candidates, these enzymes are challenging targets for small molecule drug development. Moreover, in vitro BoNT LC assays rely on an inherent assumption that the structures of the toxins in a cell-free environment are similar to their bioactive conformations in the neuronal cytosol. However, this assumption can be problematic as protein structures are determined not only by their primary amino acid sequences and post-translational modifications but also by the cellular environment itself (Bompiani and Dickerson 2014). For example, protein–protein interactions and protein chaperones can all effect protein conformations. In this regard, it is known that three-dimensional structures of SNARE proteins change when bound to other protein subunits (Sudhof 2013). Thus, it may be likely that in vitro assays provide poor approximations of the physiologically relevant conformational states of both LCs and their respective substrates. It is also important to recognize that serotypes other than BoNT/A can cause human botulism, yet drug development efforts have primarily focused on this single serotype. It would therefore be beneficial to develop therapeutic modalities that are concomitantly effective against multiple BoNT serotypes. However, considering the biochemical differences between the BoNT serotypes (Lebeda et al. 2010), the prospect of developing a single small molecule inhibitor that is capable of targeting multiple serotype LCs is unlikely. Despite extensive research on BoNT drug development, there have been only a few compounds that have shown multi-serotype BoNT inhibition, such as toosendanin and bafilomycin (used in this manuscript as a positive control, shown in Fig. 5a–c), both of which are not LC inhibitors (Li et al. 2011b; Fischer et al. 2009; Fischer 2013). In this study, we sought to identify compounds that can act on host motor neuron pathways to mitigate BoNT intoxication. Our results show that compounds targeting host signaling pathways can effectively impair the activities of multiple BoNT serotypes. In particular, the results indicate that host cellular factors are important for the virulence associated with BoNT intoxication and might provide novel avenues for mitigating botulism.

The SFK inhibitors used in this study are well characterized as their chemical and biological properties are well studied (Sen and Johnson 2011; Aleshin and Finn 2010). For example, KX2-391, which was one of the most potent SNAREs protecting compound identified in this study, is already known to be an orally bioavailable Src inhibitor that is in clinical trials (Sen and Johnson 2011). Additionally, another orally bioavailable small molecule, Saracatinib, is also in clinical trials against various conditions including Alzheimer’s disease, while Bosutinib and Dasatinib are FDA-approved anti-cancer therapeutics (Aleshin and Finn 2010; Zhang and Yu 2012). Of the three, Dasatinib, an inhibitor permeable to the blood brain barrier, was of interest as it exhibits motor neuron protection in both in vitro and in vivo models of Amyotrophic Lateral Sclerosis (Katsumata et al. 2012). Importantly, it has been suggested that cancer drugs targeting SFK pathways can be utilized to treat

neurodegenerative diseases (Hebron et al. 2013; Liu et al. 2008; Katsumata et al. 2012). Currently, it is not known whether the identified SFK inhibitors may have therapeutic potential against botulism. Extensive future studies are needed to evaluate such potential. Importantly, drug development efforts can potentially utilize the identified inhibitors or their derivatives as reference compounds to develop more potent multi-serotype BoNT antagonists. Additionally, these inhibitors can be also used as molecular probes to elucidate signaling pathways involved in BoNT intoxication and recovery.

Significantly, the results from the study showed that combining KX2-391 with other SFK inhibitors can increase SNAP-25 protection during BoNT/E intoxication versus single compound treatment (Fig. 6). This finding suggests that inhibitors acting on multiple sites of the same target may contribute additional efficacy during development of a BoNT combination therapy. In general, drug combinations are a promising strategy to overcome the toxicity that results from off-target effects associated with administering high doses of single compounds (Lehar et al. 2009). Importantly, combinational therapy has been explored in the BoNT field (Deshpande et al. 1997; Silhar et al. 2010). With respect to the results from this study, it can be concluded that hES-MNs can provide a sensitive platform to evaluate the combinatorial effects of BoNT inhibitors. Such physiologically relevant cell-based assays can facilitate the discovery of therapeutic ‘cocktails’ to treat BoNT intoxication and can be adapted to explore synergistic and/or additive relationships between a variety of drug candidates that act through different mechanisms. For example, combination regimens including SFK inhibitors, antibodies, and/or LC inhibitors might provide effective strategies to treat BoNT poisoning.

Currently, the precise mechanism(s) underlying SFK inhibitor protection of SNARE proteins during BoNT/A, B, and E intoxication has not been fully elucidated. At this stage in the discovery of host-based therapeutics to counter these toxins, at least three potential mechanisms can be proposed (summarized in Fig. 7c). One hypothesis is that the examined SFK inhibitors may be preventing the Src-mediated phosphorylation of BoNT LCs. Previous studies have reported that BoNT LCs are phosphorylated by Src kinase, and such phosphorylation events might be critical for toxins’ activities and stability (Ibanez et al. 2004; Blanes-Mira et al. 2001; Encinar et al. 1998; Ferrer-Montiel et al. 1996), but there are contradictions regarding the phosphorylation sites and their functional effects (Toth et al. 2012). Additionally, it is not known if the LCs are phosphorylated in the physiological target of BoNTs, i.e., motor neurons. Another possibility is that the antagonism of BoNT-mediated SNARE protein cleavage observed during this study could be due to ‘off-target’ effects. However, the dose-dependent protection against all three BoNT serotypes that was observed (Fig. 5), and the examples of synergistic activity presented herein (Fig. 6) do not synchronize well with this possibility. Finally, a broader but more feasible hypothesis is that SFK signaling pathways are critical for BoNT activity in neurons (Fig. 7c). Specifically, the observation that seven of the eight compounds antagonize the cleavage of SNARE proteins by all three of the BoNT serotypes examined suggests that the toxins’ activities are affected by similar intracellular pathways (Fig. 7c). Data showing that the compounds do not directly inhibit the enzymatic activity of the LC (Fig. 7a) are consistent with this hypothesis. Here, we also show that the inhibitors modulate SFK-mediated signaling events in hES-MNs (Fig. 7b). Therefore, it is plausible to suggest that SFK-mediated signaling pathways are

important determinants of BoNT activity, and that using small molecules to modulate one or more SFK pathways may interfere with a critical step during or after the neuronal intoxication process.

SFKs play crucial roles in a range of cellular functions in motor neurons, including synaptic transmission (Messa et al. 2010), the suppression of which is the ultimate result of BoNT intoxication. Importantly, previous studies suggested that phosphorylation of SNARE proteins is a critical mechanism regulating SNARE localization, conformation, and physical interaction with other SNARE proteins, and thereby SNARE complex formation and neuroexocytosis (Snyder et al. 2006; Shu et al. 2008). Therefore, with respect to understanding SFK inhibitor-mediated protection of BoNT SNARE cleavage, it is possible that the inhibitors might affect SNARE phosphorylation events that can change the localization of the SNARE proteins and/or lead to conformational changes, resulting in the inability of BoNT to cleave the substrate. Indeed, it has been reported that BoNT/A LC localizes to neuronal membrane where it directly binds and cleaves SNAP-25, suggesting that intraneuronal localization of LCs and SNARE proteins might be crucial for BoNT activity (Chen and Barbieri 2011). Currently, it is not known whether other signaling pathway(s) might have a role in BoNT intoxication and/or recovery. It has been suggested that protein kinase C (PKC) activation results in increased Src activity in neurons (Salter and Kalia 2004; Lu et al. 1999), raising the possibility that Src and PKC participate in a mutual pathway, with PKC being located upstream of Src. Although, it is unknown whether PKC activity is critical for the mechanism of action of BoNTs, or the recovery of intoxicated neurons, this signaling appears to be crucial for SNARE and SNARE interacting proteins (Snyder et al. 2006; Lau et al. 2010), and consequently the SNARE complex formation (Yang et al. 2007) and the normal exocytosis of neurotransmitters (Shu et al. 2008) (Fig. 7c). Future studies focused on elucidating the precise mechanism(s) through which SFK inhibitors protect SNARE proteins during BoNT motor neuron intoxication are the critical next steps that will be taken to further develop host-based therapeutics that can be used to antagonize the activities of multiple BoNT serotypes.

In conclusion, our study has shown that (1) hES-MNs can be used effectively to screen for BoNT countermeasures, (2) well-characterized SFK inhibitors antagonize BoNT/A, /B, and /E in a dose-dependent manner, and (3) combinatorial small compound regimens form the basis for an approach whereby human ES-derived motor neuronbased cell testing with mechanistically different BoNT inhibitors can be useful to screen compound combinations to mitigate the effects of botulism. Finally, our study exhibits that small molecules targeting host motor neuron pathways can be useful for antagonizing BoNT intoxication across multiple serotypes. To best of our knowledge, this is the first study reporting that inhibitors targeting SFK signaling pathways can antagonize multiple BoNT serotypes in hES-derived motor neurons.

Acknowledgments

We are indebted to Drs. Esta Sterneck and Balamurugan Kuppusamy for their insightful discussion concerning SFK inhibitors. Also, we thank Rajarshi Guha for R functions to optimize β for synergy calculations, and Veronica Soloveva for helpful discussion. This research was supported by grants from the Defense Threat Reduction Agency and National Institutes of Health (4R33AI101387 - 03). For JCB, this project has been funded in whole or in part

with federal funds from the National Cancer Institute (NCI), National Institutes of Health (NIH), under contract no. HHSN261200800001E. LT has been supported by the Intramural Research Program of the NCI, Center for Cancer Research, NIH.

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Fig. 1.

The generation and characterization of human ES-derived motor neurons. **a** Western blot analyses were performed to detect the expression of markers characteristic for ES cells, Oct4; neuronal cells, Tau and β -III Tubulin; and post-mitotic motor neuron related, ChAT, and p75, during the differentiation process. **b** Immunofluorescence analysis of neuronal and motor neuron-related markers in hES-MNs. Cells (Day 35) were immunostained with antibodies against neuronal marker Tau, NeUN, and β -III Tubulin; motor neuron-related markers LIM3 and ChAT; and post-mitotic motor neuron markers HB9 and ISL1. Cell nuclei were counterstained with DAPI. The *scale bar* is 50 μ m (Color figure online)

Fig. 2.

Intracellular targets of all BoNT serotypes and the SV2A receptor are present in human ES-derived motor neurons. **a** Immunoblotting was performed to determine the expression of BoNT intracellular targets SNAP-25, VAMP-2, and syntaxin, as well as SV2A receptor, during differentiation. **b** Cells such as those in Fig. 1b were immunostained for SNAP-25, VAMP-2, Syntaxin, and the SV2A receptor. *Blue* indicates DAPI-stained nuclei. The *scale bar* is 50 μ m (Color figure online)

Fig. 3.

Human ES-derived motor neurons are highly sensitive to BoNT/A, /B, and /E in a dose-dependent manner. hES-MNs were treated with various concentrations of **a** BoNT/A (0–1000 pM), **b** BoNT/B (0–20,000 pM), and **c** BoNT/E (0–1000 pM). Blots are representative of at least three independent experiments. *Error bars* represent standard errors of the means (SEM). **d** Immunoblotting was performed to determine inhibitor-mediated SNAP-25 protection during BoNT/A intoxication to evaluate the suitability of the system for BoNT inhibitor screening. hES-MNs were treated with the indicated inhibitors and then intoxicated

for 4 h. Positive controls included BoNT/A neutralizing antibodies, *Triticum Vulgaris* Lectin (TVL), and bafilomycin

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Fig. 4.
The chemical structures of SFK inhibitors evaluated in the study

Fig. 5.

SFK inhibitors antagonize multiple BoNT serotypes in a dose-dependent manner. **a** Eight SFK inhibitors were tested for SNARE protein protection during **a** BoNT/A, **b** BoNT/B, and **c** BoNT/E intoxication. hES-MNs were incubated with increasing concentrations of the compounds (1–30 μ M) for 30 min and then treated with BoNTs for 4 h. Immunoblotting was used to measure the extent of SNAP-25 and VAMP-2 protein cleavage. The values are given as mean $\pm$ SEM from at least three independent experiments. *Double asterisk* and *single asterisk* value significant at 99 and 95 % confidence levels, respectively, compared to DMSO + Toxin control conditions. **d** Images of typical cultures treated with the compounds and/or 500 pM BoNT/A. Cells were treated with the compounds for 30 min and then intoxicated with 500 pM BoNT/A for 4 h. Cells were then fixed and immunostained for neuron-specific β -III Tubulin (green) and Hoechst dye (nuclei; blue) (Color figure online)

Fig. 6.

Effects of KX2-391 and six other SFK inhibitors alone and in combination on SNAP-25 protection during BoNT/E intoxication. **a** KX2-391 binds to the substrate binding site while the other SFK inhibitors bind to the ATP binding pocket. (+) Src inhibitor-1 may bind to both sites. **b, c** hES-MNs were treated with KX2-391 either alone or in combination with the other SFK inhibitors at the indicated concentrations for 30 min and intoxicated with 500 pM BoNT/E for 4 h. Samples were then subjected to Western blot analyses to determine SNAP-25 protection. The effects of drug combinations were evaluated using the Chou–Talalay combination index (CI) method. CI values lower than 1 indicate synergism, CIs equal to 1 indicate an additive effect, and CIs higher than 1 indicate antagonism

Fig. 7. SFK inhibitors act on host cellular mechanisms but not directly on BoNT enzymatic activity. **a** A well-established HPLC-based assay was used to determine SFK inhibitor effects on BoNT/A LC activity in vitro. **b** Representative immunoblots exhibiting the effects of SFK inhibitor treatments on downstream phosphorylation events. Neuron-specific β -III tubulin was used as a loading control. **c** Model for the mechanism of action of SFK inhibitors during BoNT challenge in hES-MNs. See discussion for details