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Toward the discovery of dual inhibitors for Botulinum neurotoxin A: Concomitant Targeting of endocytosis and light chain protease activity

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Dyngo-4a™ has been ascribed to be an endocytic inhibitor for BoNT/A neurotoxicity through dynamin inhibition. Herein, we demonstrate this molecule to have a previously unrecognized dual activity against BoNT/A, dynamin-protease inhibition. To establish the importance of this dual activity, detailed kinetic analysis of Dyngo-4a's inhibition of BoNT/A metalloprotease is described as well as cellular and animal toxicity studies. The research presented is the first polypharmacological approach to counteract BoNT/A intoxication.

Clostridium botulinum, an anaerobic bacterium, produces Botulinum neurotoxin (BoNT), which is considered to be the most lethal of known toxins. BoNT is a 150 kDa protein consisting of 100 kDa heavy chain and 50 kDa light chain.[1] The heavy chain is responsible for cellular surface recognition, toxin internalization and translocation of light chain. The light chain (LC), a zinc metalloprotease is the etiological agent responsible for neurotoxicity, namely, proteolysis of SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) proteins.[2] Upon cleavage of SNAREs, vesicles containing acetylcholine are unable to fuse with the presynaptic neuromuscular junction. As a result, release of acetylcholine is blocked, leading to flaccid paralysis (botulism), and potential death.

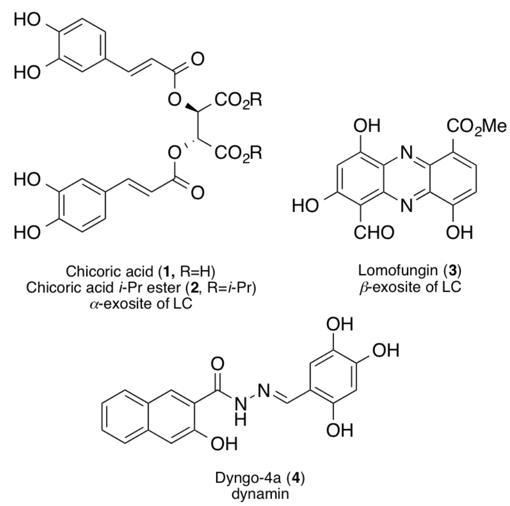

Fig. 1 Structures and targets of exosite inhibitors (**1**-**3**) and endocytic inhibitor (**4**).

Among the eight serotypes for the BoNTs (A-H), A, B, and E cause botulism in humans.[3] BoNT/A and E target SNAP-25 (synaptosome-associated protein-25 kDa), and B targets synaptobrevin. In particular, intoxication from BoNT/A persists up to several months and its potency is greater than any other serotypes (LD_{50}: 1 ng/kg).[4] BoNT/A is sold under the trade name of BOTOX® and has been used for treatment of medical conditions such as migraine and facial wrinkle reduction. Yet, it is also recognized as a potential bioweapon by Center for Disease Control and Prevention (CDC) as one of the six category A agents.[5] Current treatments for BoNT/A intoxication are limited to antitoxin and vaccination, which can readily remove toxin from circulation; however, such treatments become ineffective once cellular internalization of the toxin takes place.[6]

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With increasing threats of terrorism in the last two decades, discovery of therapeutic treatments for BoNT/A intoxication is of utmost importance and urgency, which cannot be overstated.[7] Because BoNT/A's metalloprotease is the agent responsible for its neurotoxicity our laboratory as well as others have been actively investigating the development of LC inhibitors including active site and exosite inhibitors.[8] In terms of active site inhibitors, we have developed hydroxamic acids, taking advantage of its strong coordinating nature to zinc.[9] We have also discovered exosite inhibitors from natural product screens: chicoric acid and lomofungin (**1** & **3**, Fig 1) are prototypical examples.[10] Although, it has been a major challenge to translate potent active site enzyme inhibitor efficacy to cellular and ultimately animal lethality models, we recently demonstrated that a prodrug approach has enabled hydroxamic acids to gain traction such that it facilitated cellular uptake and attenuated LC activity.[11]

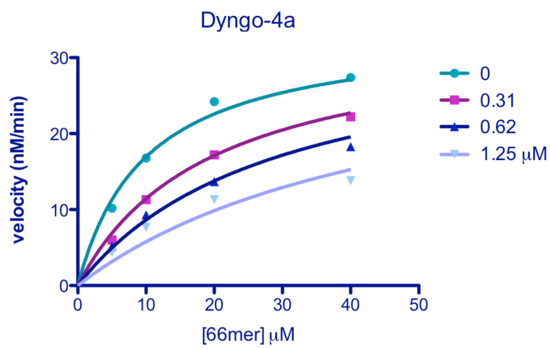


Fig 2. Global fit using a competitive inhibition mechanism.

An alternative rational approach to neutralize BoNT/A is to prevent its internalization from the cellular surface. In this regard, Meunier and co-workers reported how the dynamin inhibitor, Dyngo-4a™, thwarted BoNT/A internalization in hippocampal neurons and delayed onset of botulism in an *in vivo* mouse model (**4**, Fig 1).[12] Dyngo-4a is a pharmacologically refined analogue of Dynasore, which is a known inhibitor for dynamin.[13] Dynamin is a mechanochemical GTPase responsible for the vesicle scission step in endocytosis.[14] From the standpoint of BoNT/A neurotoxicity, Dyngo-4's mechanism of action was attributed to the fact that BoNT/A mainly utilizes a dynamin-dependent endocytic pathway to enter neuronal cells.[12]

In the effort to search for molecules that could inhibit the protease of BoNT/A, we turned our focus to the polyphenolic scaffold embedded within Dyngo-4a. We suspected that this Dyngo-4a's phenolic architecture could serve as an exosite LC inhibitor, based on its structural similarity to chicoric acid. Thus, Dyngo-4a was tested *in vitro* employing both FRET-based SNAPtide and LC/MS-based 66mer assays.[15] As anticipated the compound was inactive in the SNAPtide test, while it showed inhibitory activity in the 66mer assay, indicating that Dyngo-4a's binding was exosite driven. To further define Dyngo-4a's inhibition profile, detailed kinetics of the compound were examined with varied concentrations of 66mer substrate (Fig 2). Dyngo-4a showed a competitive inhibition mechanism with the K_i value of 0.32 ± 0.05 μM.

As a means to understand Dyngo-4a's proteolytic mechanism of inhibition, we investigated Dyngo-4a's ability to access the α and β-exosites of BoNT/A's LC. To begin, a dual inhibition assay between Dyngo-4a and chicoric acid *i*-Pr ester

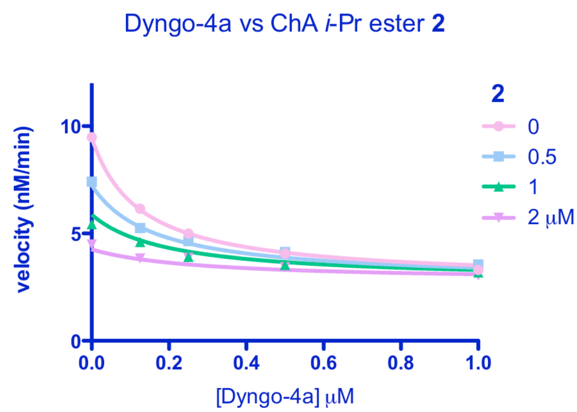


Fig 3. Mutually exclusive fit for Dyngo-4a and chicoric acid *i*-Pr ester.

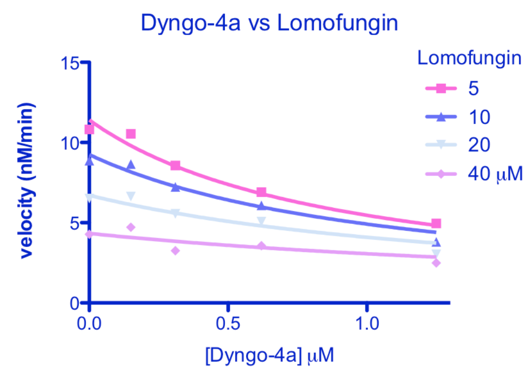
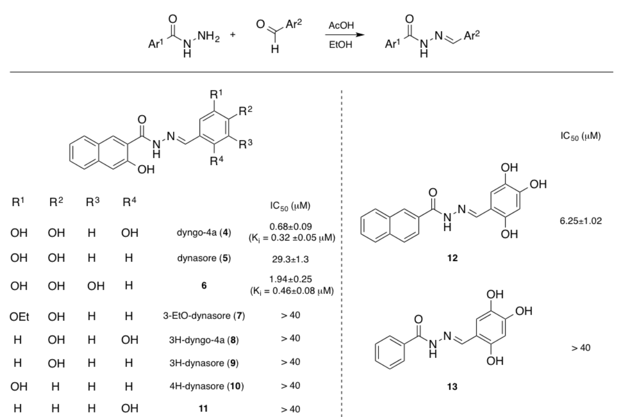


Fig 4. Mutually exclusive fit for Dyngo-4a and lomofungin.



R1	R2	R3	R4		IC50 (μM)
OH	OH	H	OH	dyngo-4a (**4**)	0.68±0.09 (K_i = 0.32 ±0.05 μM)
OH	OH	H	H	dynasore (**5**)	29.3±1.3
OH	OH	OH	H	**6**	1.94±0.25 (K_i = 0.46±0.08 μM)
OEt	OH	H	H	3-EtO-dynasore (**7**)	> 40
H	OH	H	OH	3H-dyngo-4a (**8**)	> 40
H	OH	H	H	3H-dynasore (**9**)	> 40
OH	H	H	H	4H-dynasore (**10**)	> 40
H	H	H	OH	**11**	> 40

Scheme 1. SAR study examining Dyngo-4a as the inhibitor-scaffold and BoNT/A protease. The top panel presents the general synthetic route used to prepare Dyngo-4a analogues. All assays were conducted as previously described.[15]

(**2**, Fig 1), an α-exosite inhibitor, was undertaken.[16] As a frame of reference, **2** presents a competitive inhibition mechanism with the K_i value of 1.8 ± 0.3 μM. Here we observed mutually exclusive binding, indicating that the two molecules cannot bind simultaneously (Fig 3). A parallel assay now engaging lomofungin, a presumed β-exosite inhibitor also presented mutual exclusivity (Fig 4). Moreover, Dyngo-4a demonstrated complete inhibition of the BoNT/A LC at 20 μM, with a competitive inhibition mechanism, whereas chicoric acid only displayed partial inhibition with a non-competitive profile.[10a] In a final attempt to further define BoNT/A LC and Dyngo-4a's interactions a competitive assay between Dyngo-4a and an active site hydroxamate inhibitor, previously validated from crystallographic analysis, was conducted.[9(b),(d)] Here non-mutually exclusive binding was observed, i.e. both can bind simultaneously with an enhancement factor (α) of 1.6 ± 0.8 (Supporting Information). Although, we are currently unable to unambiguously assign the location within BoNT/A LC that Dyngo-4a resides, our data taken in total suggests that Dyngo-4a imparts itself within one or both of the exosite regions of the BoNT/A LC.

Augmenting these kinetic studies we conducted an SAR examination of Dyngo-4a to probe what functional groups were essential for its activity (Scheme 1). A variety of analogues were either purchased or synthesized, the latter through a condensation between a hydrazide and an aldehyde under acidic conditions (Scheme 1), and tested in the 66mer assay. As expected, the three phenolic groups were found to be important for inhibitory activity. Interestingly, **6**, having an altered phenolic substitution pattern exhibited virtually equal potency and competitive inhibition mechanism with the K_i value of 0.46 ± 0.08 μM (Supporting Information). Moreover, Dyngo-4a analogues presenting only one/two hydroxyl groups were inactive. Strikingly, although the hydroxyl functionality displayed within the naphthyl moiety was not crucial as a point of inhibitory action, the naphthyl scaffolding was required for inhibition (**12** & **13**, Scheme 1).

Having established that Dyngo-4a is a promising BoNT/A protease inhibitor, we evaluated the compound in a cellular assay using human induced pluripotent stem cells (hiPSC)-derived neurons.[17] This platform was chosen due to the high sensitivity to BoNT, a steep dose-response curve, and species-relevant assay with a more pure and defined population of neurons than other cell-based assays. To distinguish between its endocytic and protease inhibitory activities, the assay was conducted in two different modes. In one assay, Dyngo-4a was added 1 h before the toxin exposure to examine its endocytic inhibition; and in the other, the inhibitor was added 45 min after the toxin exposure to assess its protease inhibition. Dyngo-4a showed SNAP-25 protection, albeit at a relatively high concentration of 200 μM when it was added before toxin exposure. Unfortunately, similar inhibition was not observed when added after toxin exposure. Post exposure cellular rescue using protease inhibitors has been rare.[8,11] We also hypothesize that a lack of inhibition might also be due to our inability to examine higher concentrations of Dyngo-4a. This is especially pertinent with the sensitive nature of hiPSC neurons to foreign substances, which has been noted, and indeed Dyngo-4a showed cellular toxicity above 250 μM.

With kinetic, SAR, and cellular studies unfolded, we summoned a mouse lethality model, hoping to further probe the importance of non-endocytic efficacy engendered within Dyngo-4a. Thus, Dyngo-4a was injected 2.5-3h post intoxication wherein the mice begin to labor (pinched abdomens and labored breathing), and survival time was monitored and summarized in Fig 5. Remarkably, we observed multiple rodents survived the challenge. Although, other mechanisms might come into play that are unknown at this time, clearly Dyngo-4a has an effect on survival that would not be governed in this time frame by dynamin inhibition of BoNT/A internalization.

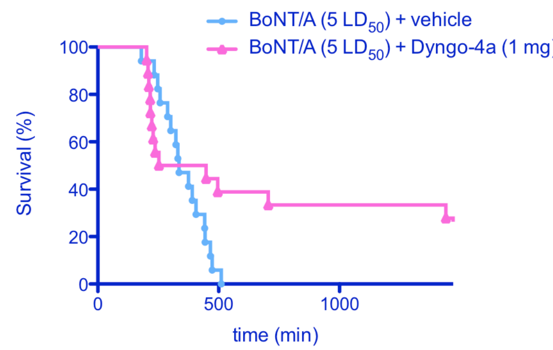


Fig 5. Mouse lethality study of Dyngo-4a protection.

Conclusions

In summary, Dyngo-4a, a heretofore inhibitor of BoNT/A neurotoxicity previously thought to function solely via the blocking of heavy chain internalization through the inhibition of dynamin, has now been found to also be a regulator of the BoNT/A protease. This unrecognized BoNT/A LC inhibition by Dyngo-4a, engenders this molecule to now be the first reported pharmacological antagonist that can blunt multiple processes associated with BoNT/A neurotoxicity. Kinetic examination of Dyngo-4a and the BoNT/A LC while complex and challenging posits Dyngo-4a's mode of inhibition through the protease's exosite. Although, the results from cell-based assay using hiPSC neurons suggest predominant endocytic inhibition, survival of mice was observed following a lethal injection of BoNT/A, implicating protease inhibition by Dyngo-4a.

While unintended off-target activities can lead to toxicity and jeopardize drug discovery efforts, in recent years the number of drugs targeting multiple targets has been increased, and polypharmacological strategies have risen to address challenging goals, as represented by the success of multikinase inhibitors.[18] Making a case for Dyngo-4a is tantalizing in that a single pharmacophore, a polyphenol, targets two completely different cellular proteins, one endogenous and the other exogenous. We believe that a rational dual inhibition design strategy targeting endocytosis and the BoNT/A LC presents enormous potential to combat one of the most potent toxins known to man. We are hopeful that this newly found dual inhibitor activity stimulates the discovery of optimized new generation endocytic-protease inhibitors of not only BoNT/A but also the other BoNT serotypes.

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