Electronic Supplementary Material (ESI) for Chemical Communications.
This journal is © The Royal Society of Chemistry 2015

Supporting Information

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

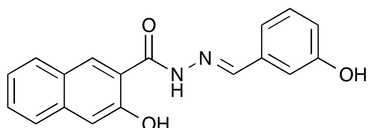
Hajime Seki, Song Xue, Mark S. Hixon, Sabine Pellett, Marek Remeš,
Eric A. Johnson, and Kim D. Janda*

1. Synthesis of Dyngo-4a analogues and characterization data
2. Enzyme assays
3. Cellular assay
4. Mouse lethality assay

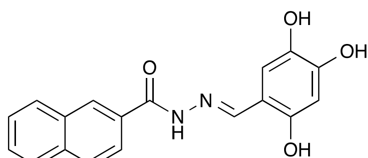
1. Synthesis of dyngo-4a analogues and characterization data

Dyngo-4a (**4**), Dynasore (**5**), 3-EtO-Dynasore (**7**), 3H-Dynasore (**9**) were purchased from Sigma-Aldrich. Compounds **6**, **8**, and **11** were synthesized according to the literatures.[1] All the other compounds (**10**, **12**, and **13**) were synthesized as follows:

Hydrazide (0.30 mmol, 1 equiv), aldehyde (0.30 mmol, 1 equiv), and AcOH (0.1 mL) were mixed in EtOH (3 mL) and refluxed overnight. After cooling down to the ambient temperature, the reaction mixture was poured into ice water. The resulting precipitate was collected by filtration and washed with water and dried as solid.



(*E*)-3-hydroxy-*N'*-(3-hydroxybenzylidene)-2-naphthohydrazide (10)
off-white solid 82.2 mg, 89%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.93 (1 H, s), 11.30 (1 H, s), 9.67 (1 H, s), 8.46 (1 H, s), 8.38 (1 H, s), 7.92 (1 H, d, *J* 8.3), 7.77 (1 H, d, *J* 8.3), 7.52 (1 H, t, *J* 7.2), 7.37 (1 H, t, *J* 7.2), 7.33 (1 H, s), 7.28 (1 H, t, *J* 7.8), 7.25 (1 H, s), 7.15 (1 H, d, *J* 7.6), 6.88 – 6.84 (1 H, m); ^{13}C NMR (151 MHz, DMSO) δ 163.7, 157.7, 154.1, 148.6, 135.8, 135.4, 130.2, 129.9, 128.7, 128.2, 126.8, 125.8, 123.8, 120.3, 119.0, 117.6, 112.8, 110.5; HRMS (ESI-TOF) m/e calcd for $[M+H]^+$ $C_{18}H_{15}N_2O_3$: 307.1077, found 307.1077.



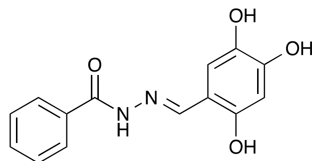
(*E*)-*N'*-(2,4,5-trihydroxybenzylidene)-2-naphthohydrazide (12)[2]
yellow solid 46.8 mg, 48%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.97 (s, 1H), 10.63 (s, 1H), 9.57 (s, 1H), 8.58 (s, 1H), 8.54 (d, *J* = 1.7 Hz, 1H), 8.51 (s, 1H), 8.10 – 8.04 (m, 2H), 8.03 – 7.96 (m, 2H), 7.64 (d, *J* = 1.9 Hz, 2H), 6.92 (s, 1H), 6.35 (s, 1H); ^{13}C NMR (151 MHz, DMSO) δ 162.4, 151.9, 149.3, 148.5, 138.5, 134.3, 132.1, 130.5, 128.9, 128.1, 127.9, 127.9, 127.7, 126.9, 124.2, 114.6, 109.5,

---

[1] **6** & **8**: A. McCluskey, J. A. Daniel, G. Hadzic, N. Chau, E. L. Clayton, A. Mariana, A. Whiting, N. N. Gorgani, J. Lloyd, A. Quan, L. Moshkanbaryans, S. Krishnan, S. Perera, M. Chircop, L. von Kleist, A. B. McGeachie, M. T. Howes, R. G. Parton, M. Campbell, J. A. Sakoff, X. Wang, J. Y. Sun, M. J. Robertson, F. M. Deane, T. H. Nguyen, F. A. Meunier, M. A. Cousin and P. J. Robinson, *Traffic*, 2013, **14**, 1272.
11: L. Caboni, B. Egan, B. Kelly, F. Blanco, D. Fayne, M. J. Meegan, D. G. Lloyd, J. *Chem. Inf. Model*. 2013, **53**, 2116.

[2] Overlap of two carbon peaks at 127.9 ppm was confirmed by ^{1}H^{13}C-HSQC experiment.

103.5; HRMS (ESI-TOF) m/e calcd for $[M+H]^+$ $C_{18}H_{15}N_2O_4$: 323.1026, found 323. 1029.



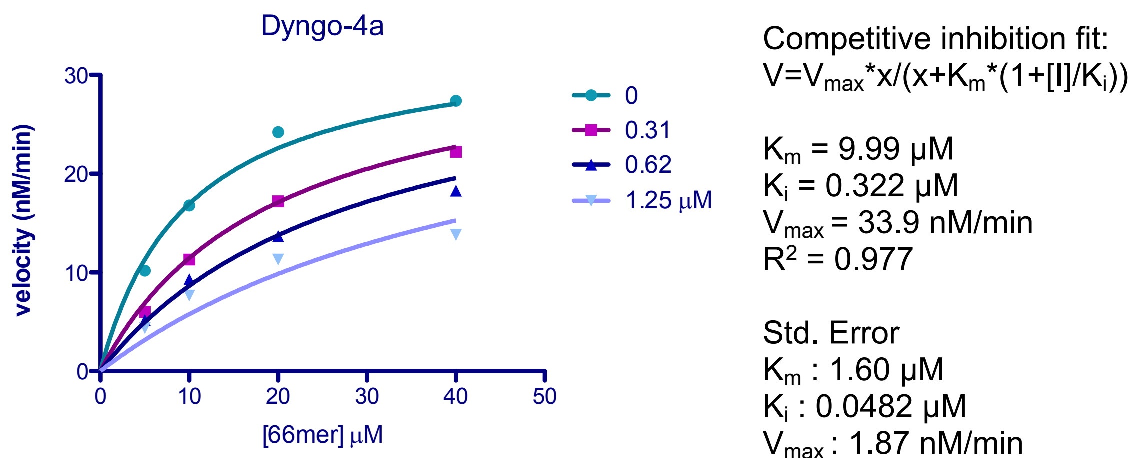
(*E*)-*N'*-(2,4,5-trihydroxybenzylidene)benzohydrazide (13)
brown solid, 44.6 mg, 55%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.79 (s, 1H), 10.62 (s, 1H), 9.55 (s, 1H), 8.56 (s, 1H), 8.44 (s, 1H), 7.95 – 7.83 (m, 2H), 7.58 (s, 1H), 7.52 (dd, J = 8.3, 6.9 Hz, 2H), 6.87 (s, 1H), 6.33 (s, 1H); ^{13}C NMR (151 MHz, DMSO) δ 162.4, 151.9, 149.3, 148.5, 138.5, 133.2, 131.7, 128.5, 127.5, 114.7, 109.4, 103.5; HRMS (ESI-TOF) m/e calcd for $[M+H]^+$ $C_{14}H_{13}N_2O_4$: 273.0875, found 273.0871.

2. Enzyme assays (SNAPtide assay and 66mer assay)

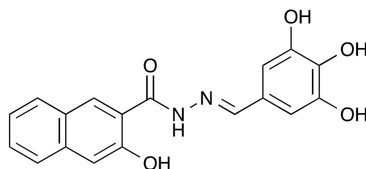
Both assays were conducted as previously described with recombinant Botulinum neurotoxin light chain A (1-425).[3]

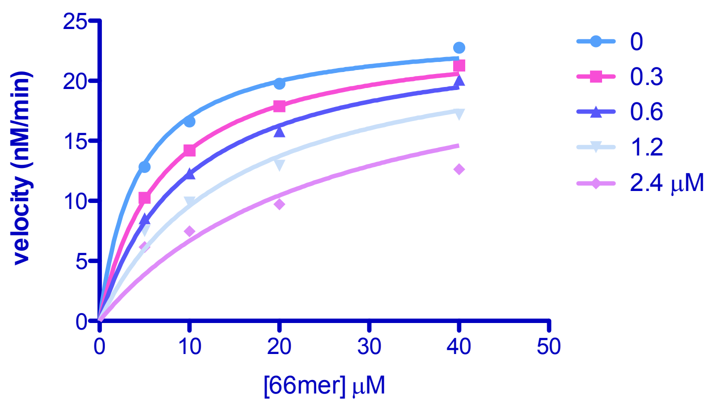
- SNAPtide assay: [BoNT/A] = 37 nM, [SNAPtide] = 5 μM
- 66mer assay: [BoNT/A] = 0.5-1 nM, [66mer] = 5 μM

Prism 5 was used for kinetic analysis.



[3] (a) L. M. Eubanks, M. S. Hixon, W. Jin, S. Hong, C. M. Clancy, W. H. Tepp, M. R. Baldwin, C. J. Malizio, M. C. Goodnough, J. T. Barbieri, E. A. Johnson, D. L. Boger, T. J. Dickerson, K. D. Janda, *Proc. Natl. Acad. Sci.* 2007, **104**, 2602. (b) K. Capkova, M. S. Hixon, L. A. McAllister and K. D. Janda, *Chem. Commun.*, 2008, 3525.

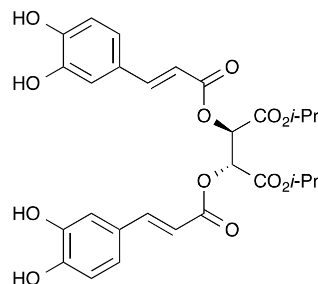


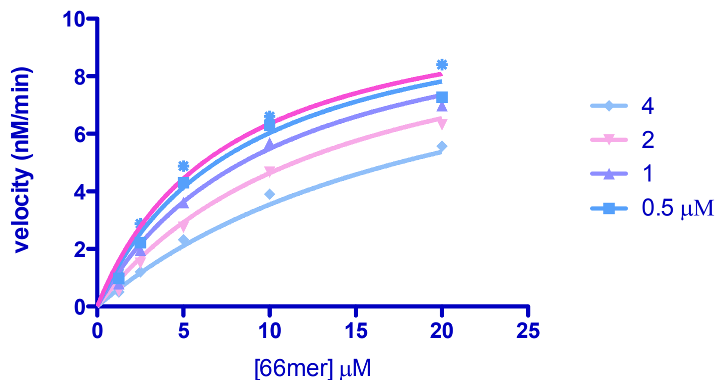


Competitive inhibition fit:
$V=V_{max}*x/(x+K_m*(1+[I]/K_i))$

K_m = 4.25 μM
K_i = 0.462 μM
V_{max} = 24.2 nM/min
R^2 = 0.966

Std. Error
K_m : 0.662 μM
K_i : 0.0789 μM
V_{max} : 0.829 nM/min





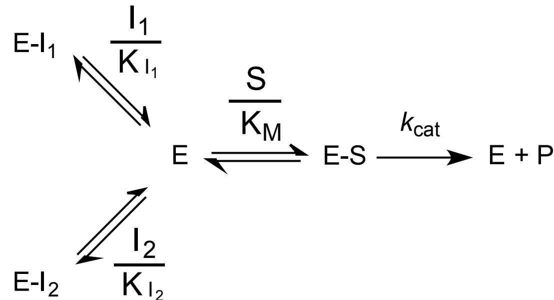
Competitive inhibition fit:
$V=V_{max}*x/(x+K_m*(1+[I]/K_i))$

K_m = 6.60 μM
K_i = 1.78 μM
V_{max} = 11.1 nM/min
R^2 = 0.985

Std. Error
K_m : 0.786 μM
K_i : 0.273μM
V_{max} : 0.500 nM/min

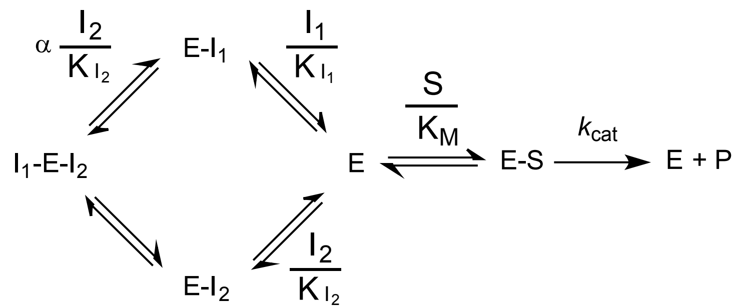
Dual inhibition assay

1) Mechanism of mutually exclusive inhibition:



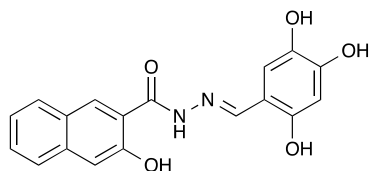
$$\frac{1}{v}=\frac{1}{v_{max}}\left(\frac{I_1}{K_{I_1}}+\frac{I_2}{K_{I_2}}+\frac{S}{K_M}\right)$$

2) Mechanism of non-mutually exclusive inhibition:

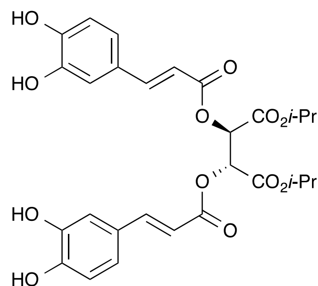


$$\frac{1}{v}=\frac{1}{v_{max}}\left(\frac{I_1}{K_{I_1}}+\frac{I_2}{K_{I_2}}+\alpha\frac{I_1I_2}{K_{I_1}K_{I_2}}+\frac{S}{K_M}\right)$$

α is an enhancement factor, indicating the difference in affinity for I_2 to $E\text{-}I_1$ complex



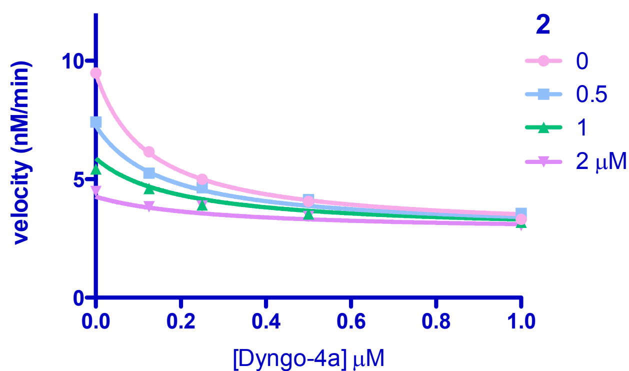
vs



Mutually exclusive fit:

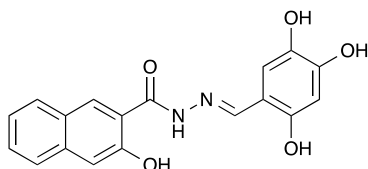
$$V=V_{max}*(1+a*x/K_{i(Dyngo\text{-}4a)})/(1+x/K_{i(Dyngo\text{-}4a)}+[I]/K_{i(ChA\ i\text{-}Pr\ ester)})$$

Dyngo-4a vs ChA *i*-Pr ester **2**

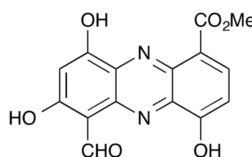


K_i (Dyngo-4a) = 0.124 µM
K_i (ChA *i*-Pr ester)= 1.64 µM
V_{max} = 9.47 nM/min
a = 0.29
R^2 = 0.987

Std. Error
K_i (Dyngo-4a) : 0.0147 µM
K_i (ChA *i*-Pr ester) : 0.110 µM
V_{max} : 0.185 nM/min
a = 0.015

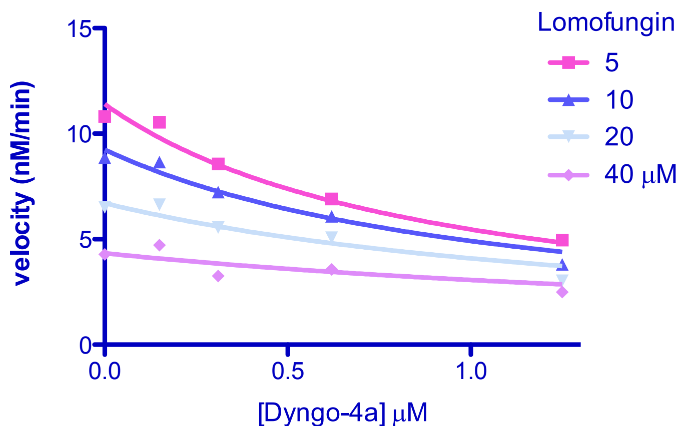


vs



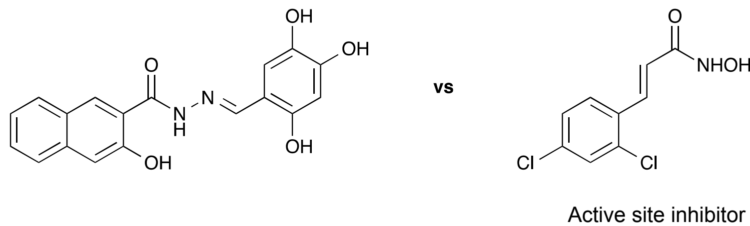
$$\text{Mutually exclusive fit: } V=V_{max}/(1+x/K_{i(Dyngo\text{-}4a)}+[I]/K_{i(lomofungin)})$$

Dyngo-4a vs Lomofungin



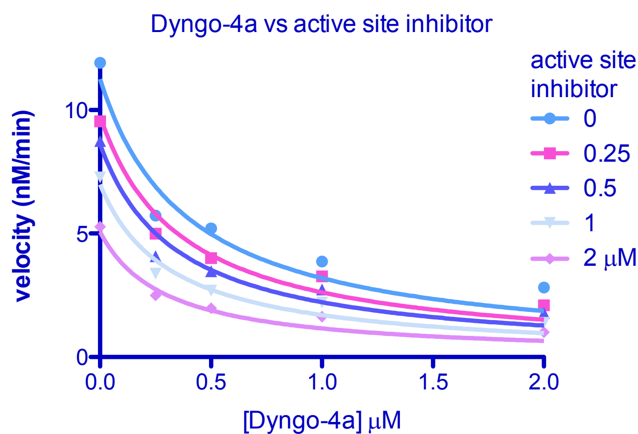
K_i (Dyngo-4a) = 0.71 µM
K_i (Lomofungin)= 16.5 µM
V_{max} = 14.9 nM/min
R^2 = 0.970

Std. Error
K_i (Dyngo-4a) : 0.0777 µM
K_i (Lomofungin) : 1.91 µM
V_{max} : 0.740 nM/min



Non-mutually exclusive fit:[4]

$$V=V_{max}/(1+x/K_{i\,(Dyngo\text{-}4a)}+[I]/K_{i(Active\ site)}+\alpha*x*[I]/(K_{i\,(Dyngo\text{-}4a)}*K_{i(Active\ site)}))$$



K_i (Dyngo-4a) = 0.396 μM
K_i (Active site)= 1.63 μM
V_{max} = 11.3 nM/min
R^2 = 0.959
α: 1.61

Std. Error
V_{max} : 0.488 nM/min
K_i (Dyngo-4a) : 0.0543 μM
K_i (Active site) : 0.302 μM
α: 0.762

---

[4] Statistical F-test justified the inclusion of the enhancement factor (α) and non-mutually exclusive fit as a better model.

3. Cellular assay

Pure Botulinum neurotoxin (BoNT) A1 was prepared from C. botulinum strain Hall A hyper as previously described.[5] The toxin was dissolved in phosphate buffered saline, pH 7.4 and 40 % glycerol, and stored at −20ºC until use. Activity of the BoNT/A1 preparation was determined by the mouse bioassay,[6] and specific toxicity was about 1.25 x 10^8 mouse LD_{50} Units/mg. The inhibitors were dissolved in 100 % DMSO to 50 mM and stored at 4ºC

The hiPSC derived neurons and culture medium were purchased from Cellular Dynamics International (Madison, WI), and cultured in 96-well plates as described for 5 days prior to the assay.[7] For the inhibition assay, 200 LD_{50} Units of BoNT/A1 was added to the cells in 50 µl stimulation medium (modified neurobasal containing 2.2 mM $CaCl_2$ and 56 mM KCl (Invitrogen) and supplemented with B27 and glutamax), and the cells were incubated at 37ºC in a humidified 5 % CO_2 atmosphere for 7.5 min.

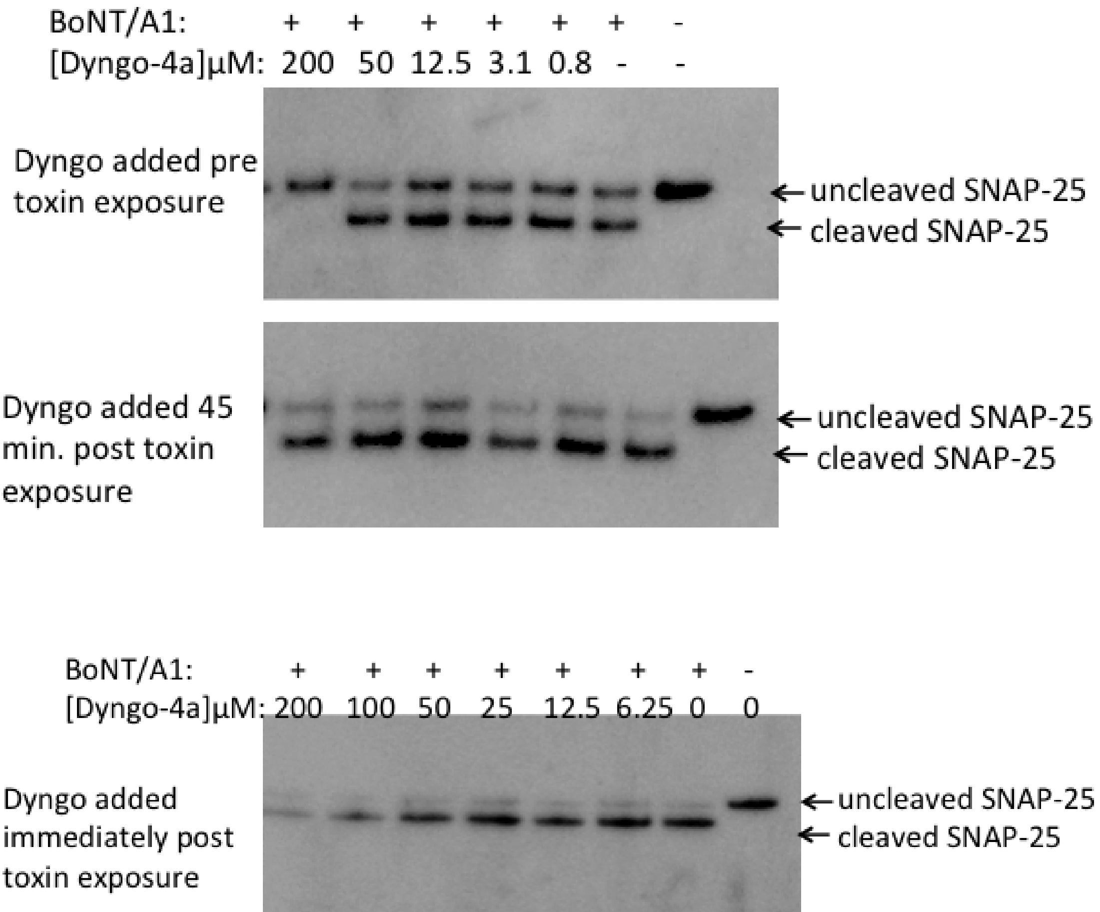
In order to determine whether Dyngo-4a inhibits BoNT/A induced SNAP-25 cleavage via inhibition of endocytosis only or also by inhibition of LC activity, the cells were either pre-incubated with serial dilutions (200, 50, 12.5, 3.125, and 0.8 µM) of Dyngo-4a for 1 h before toxin exposure, or the dilutions were added 45 min post toxin exposure or immediately after toxin exposure (7.5 min required for toxin exposure, and 4 min for wash-out, Dyngo-4a was added at 11.5 min post first addition of toxin). The cells pre-incubated with Dyngo-4a were washed before toxin exposure to remove any extracellular inhibitor. Cells were harvested at 8 h post toxin exposure, and cell lysates analyzed for SNAP-25 cleavage as above. All samples were tested in duplicates.

---

[5] C. J. Malizio, M. C. Goodnough, E. A. Johnson, *Methods Mol. Biol*. 2000, **145**, 27.

[6] (a) C. L. Hatheway Botulism. in: Laboratory Diagnosis of Infectious Diseases. Principles and Practice, Springer-Verlag, New York, 1988, 111. (b) E. J. Schantz, D. A. Kautter, *Anal. Chem.*, 1978, **61**, 96.

[7] R. C. M. Whitemarsh, M. J. Strathman, L. G. Chase, C. Stankewicz, W. H. Tepp, E. A. Johnson, S. Pellet, *Toxicol. Sci.*, 2012, **126**, 426.



4. Mouse lethality assay

The assay was conducted by Dr. Michael C. Goonough, Ph.D. (Metabiologics, Madison, WI).

Dyngo-4a was dissolved in a NMP (*N*-methyl-2-pyrrolidone) and PEG300 (polyethylene glycol 300) with 1:9 ratio, and diluted 10-fold with PBS for the study.[8]

35 female CD-1 mice (~20 g) were challenged with 5 LD_{50} of BoNT/A intraperitoneally. At 2.5 - 3 hours post toxin injection when laboring was observed, Dyngo-4a (1 mg, 0.5 mL) was injected intraperitoneally into 18 mice, and vehicle was given to 17 mice as a control group.

[8] C. B. Harper, S. Martin, T. H. Nguyen, S. J. Daniels, N. A. Lavidis, M. R. Popoff, G. Hadzic, A. Mariana, N. Chau, A. McCluskey, P. J. Robinson and F. A. Meunier, *J Bio.l Chem.*, 2011, **286**, 35966.