SUPPORTING INFORMATION

General: The Johns Hopkins Clinical Compound Library (JHCCL) purchased from Dr. David Sullivan (JHU Bloomberg School of Public Health) as 10 mM stocks in DMSO or water in a 96-well plate format. Lomofungin was purchased from Santa Cruz Biotechnology, Inc. Stock solutions of lomofungin were prepared in DMSO. Note, DMSO stock solutions of lomofungin are stable and may be stored in the dark at – 20 °C, as determined by NMR spectroscopy.

Evaluation of LC/A inhibitors with a FRET-based assay[1]: LC/A $(1\text{-}425)^2$ activity was measured in black 96-well microtiter plates (CoStar; Corning, Inc.) by use of a Molecular Devices SpectraMax GeminiEM plate reader. Assays contained 40 mM HEPES (pH 7.4), 45 nM enzyme, and varying concentrations of inhibitor in a final volume of 100 μL with a total DMSO concentration of 0.5-2%. Assay mixtures were preincubated for 5 min at 23 °C and were initiated by the addition of 5 μM SNAPtide® (List Biological Laboratories, Inc.). Fluorimeter parameters consisted of an λ_{ex} = 490 nm (slit width = 2 nm), λ_{em} = 532 nm (slit width = 2 nm), and a cutoff filter at 495 nm. Initial rates were measured from the linear region of each assay, typically from data collected over a range of 100 to 300 s.

Evaluation of LC/A inhibitors with an LC/MS assay[3]: BoNT/A LC/A $(1\text{-}425)^2$ at 75 pM enzyme was assayed at 23°C, pH 7.4, in 40 mM HEPES in 200 μl volumes with a total DMSO concentration of 2%. After incubation for 30 min, 25 μl aliquots were withdrawn and quenched by the addition of 3 μl of 15% aqueous TFA, 13C – labeled product standard was added to a 1 μM final concentration. Sample analysis was done by use of an Agilent 1100 LC/MS system. A 20 μl sample was injected onto a Zorbax 300SB-C8 column (4.6x50 mm, 5 μm, Agilent Technologies) subjected to a gradient (A to B where A = 0.1 % formic acid in water and B = 0.1 % formic acid in acetonitrile) of 2.5 % B from 0 to 2.5 min, 2.5 % B to 97.5 % B from 2.5 to 10 min, and 97.5 % B from 10 to 13 min at a constant flow rate of 0.5 ml/min. A column-solvent equilibration time of 4 min was conducted prior to the next sample analysis. Mass spectral acquisition included a solvent front delay of 2.5 min. Operational parameters were: positive

single ion monitoring of m/z 460.9 and 462.9 corresponding to the M+2 peak of the reaction product and labeled internal standard respectively, nitrogen as a nebulizing and drying gas (20 psi, 3 l/min), HV capillary voltage at 4 kV and the drying gas temperature to 300 °C. Run analysis and quantitation was by use of Chemstation software (Agilent). Enzyme velocities were determined from a linear fit of product formation versus incubation time. The inhibition constant, K_i was determined by a non-linear least squares global fit of the equation 1 to the initial rates of product formation for a matrix of 66-mer substrate and inhibitor concentrations bracketing $K_{m(app)}$ and $K_{i(app)}$. Combination studies were conducted with varying concentrations of lomofungin and D-chicoric acid or 2,4-dichlorocinnamic hydroxamate, and with the 66-mer substrate held constant at approximately K_m.

$$v = \frac{V_{MAX}\mathrm{S}}{(\mathrm{S}+\mathrm{K_M})\left(1+\frac{\mathrm{I}}{\mathrm{K_I}}\right)} \qquad \text{(Eq. 1)}$$

Neuronal cell-based BoNT inhibition: Primary cultures of cerebellar granule cells were prepared from 7 day-old Sprague-Dawley rats[4] essentially by the method of Farkas et al[5]. Briefly, after aseptically removing cerebella from the skulls, tissue was freed from the meninges and incubated in 0.05% trypsin solution for 12 min at 37 °C. After a brief centrifugation, cells were triturated in neuronal culture medium (DMEM/F12 containing 10% FBS, 500IU/ml penicillin, 500 µg/ml streptomycin and 1:50 dilution of B27 supplement (Gibco)) and filtered through a sterile cell strainer mesh with 40 µm pore size (BD Falcon).[6-8] Cell number was determined by trypan blue exclusion, and cells were seeded at a density of $7x10^4$ cells per well onto 24 well poly-D-lysine coated plate in neuronal culture medium. The cultures were maintained at 37 °C in a humidified atmosphere of 7% CO_2. After 24 hr of culturing, cytosine arabinoside (Sigma) was added to a final concentration of 20 µM to prevent astrocytic proliferation. The neurons were cultured for 8-10 days before use. For the assay, the final volume of each well was adjusted to 0.5ml with neuronal culture medium, varying concentrations of lomofungin (1-10 µl of a DMSO stock) or DMSO alone were added, followed by 0.2 nM BoNT/A holotoxin (3 µl

of a PBS stock) or PBS alone and the cells were incubated for 4 hr at 37 °C in an atmosphere of 5% CO_2. The cells were harvested into a preweighed Eppendorf screw-cap vial, pelleted by centrifugation, and the supernatant was removed. Four microliters of M-PER (Pierce) and 2 µl of SDS-PAGE sample buffer were added for each mg of cell pellet, and samples were boiled for 10 min. Proteins were separated on a 15% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane for standard western blotting. SNAP-25 was detected with rabbit anti-SNAP-25 antibody (1:5000; Sigma) followed by HRP-conjugated goat anti-rabbit IgG (1:5000; GE Healthcare) using a ECL kit (GE Healthcare), Figure S1. Band densities for SNAP-25 and its cleaved product were normalized and relative intensities determined using scanning densitometry (Kodak IS2000 imager).

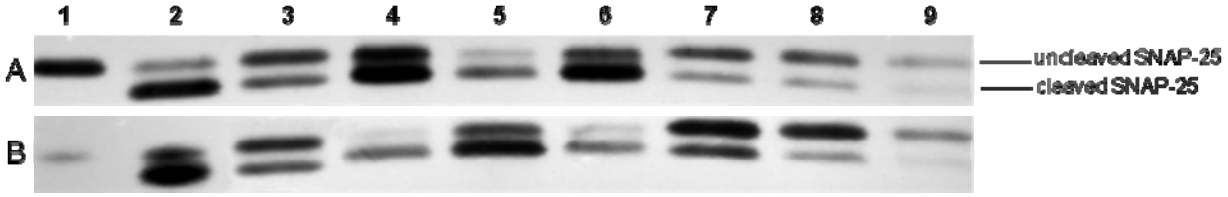

Figure S1. Western blot analysis demonstrating the efficacy of lomonfungin inhibition of BoNT/A in primary rat cerebellar neurons. A and B represent results from duplicate experiments. Lane 1, control cells with no BoNT/A; lane 2, 0.2 nM BoNT/A; lane 3 to 9, 0.2 nM BoNT/A with control compound (lane 3), 30 µM lomofungin (lane 4), 60 µM lomofungin (lane 5), 90 µM lomofungin (lane 6), 150 µM lomofungin (lane 7), 180 µM lomofungin (lane 8), 240 µM lomofungin (lane 9).

Derivation of rate equations for the lomofungin and D-chicoric acid inhibitor combination:

Abbreviations:

D-Chicoric Acid (C), Free Enzyme (E), Total Enzyme present (E_T), Enzyme-Chicoric Acid complex (EC), Enzyme-Lomofungin complex (EL), Enzyme- D-Chicoric Acid-Lomofungin complex (ECL),

Derivation of an equation describing nonmutually exclusive inhibition with partial response.

1) $E_T = E + EC + EL + ECL$ kinetically relevant species

2) $E_T = E + E\frac{C}{IC_{50}C} + E\frac{L}{IC_{50}L} + E\alpha\frac{C}{IC_{50}C}\frac{L}{IC_{50}L}$ species expressed as their rapid equilibrium forms*

3) $E_T = \left(1 + \frac{C}{IC_{50}C} + \frac{L}{IC_{50}L} + \alpha\frac{C}{IC_{50}C}\frac{L}{IC_{50}L}\right)E$ factoring out free enzyme

4) $v \propto \left(E + \delta EC\right)$ kinetically competant forms ($\delta < 1$)

5) $\frac{v}{Vo} = \frac{E}{E_T}\left(1+\delta\frac{C}{IC_{50}C}\right)$ relative velocity is equal to the ratio of free over total enzyme

6) $\frac{v}{Vo} = \frac{1+\delta\frac{C}{IC_{50}C}}{1 + \frac{C}{IC_{50}C} + \frac{L}{IC_{50}L} + \alpha\frac{C}{IC_{50}C}\frac{L}{IC_{50}L}}$ substitution of equation 3 into equation 5

Derivation of an equation describing mutually exclusive inhibition with partial response.

1) $E_T = E + EC + EL$ kinetically relevant species

2) $E_T = E + E\frac{C}{IC_{50}C} + E\frac{L}{IC_{50}L}$ species expressed as their rapid equilibrium forms*

3) $E_T = \left(1 + \frac{C}{IC_{50}C} + \frac{L}{IC_{50}L}\right)E$ Factoring out free enzyme concentration

4) $v \propto \left(E + \delta EC\right)$ kinetically competant forms ($\delta < 1$)

5) $\frac{v}{Vo} = \frac{E}{E_T}\left(1+\delta\frac{C}{IC_{50}C}\right)$ relative velocity is equal to ratio of free over total enzyme

6) $\frac{v}{Vo} = \frac{1+\delta\frac{C}{IC_{50}C}}{1+\frac{C}{IC_{50}C}+\frac{L}{IC_{50}L}}$ substitution of equation 3 into equation 5

* see reference[9]

Analysis of the combination Study of lomofungin with D-chicoric acid: Most inhibitors encountered are capable of producing complete inhibition i.e., at saturating inhibitor concentration the observed enzyme velocity is zero. Taking the inverse of velocity and plotting this as a function of inhibitor concentration results in a straight line. This phenomenon is of great utility when examining inhibitors in combination. As mentioned in the text of this manuscript, diagnostic patterns emerge that allow the analyst to readily distinguish between mutually exclusive and nonmutually exclusive binding. Rarely though occasionally, an inhibitor is discovered that at saturation produces only partial inhibition. Such an observation has mechanistic implications. This observation means that inhibitor binding does not preclude substrate binding but the presence of bound inhibitor reduces the enzyme's catalytic efficiency by impacting k_{cat} or Km or both. Unfortunately, a plot of inverse rate versus a partial inhibitor's concentration will result in a curved line and complicate the interpretation of an inhibitor combination study.

In this supplementary material, we have included the fits of two binding models to the lomofungin – D-chicoric acid inhibitor combination study. In Figure S2 panel A a fit to a nonmutually exclusive binding model is presented. As shown, the data are well characterized by the fit. Initially though, we examined the fit to the mutually exclusive binding model. This is a simpler interaction model in that the interaction parameter (α) has the value of zero. The interaction parameter reflects the shift in potency of one inhibitor upon binding of the other. When α assumes the value of zero, the implication is that upon the first inhibitor's binding the second inhibitor is prevented from binding. As seen in Figure S2 panel B, the best fit to a mutually exclusive binding model omits the curvature observed in the empirical data as well as, the fractional velocity takes on a physically unrealistic negative value implying there is no reasonable solution by this model.

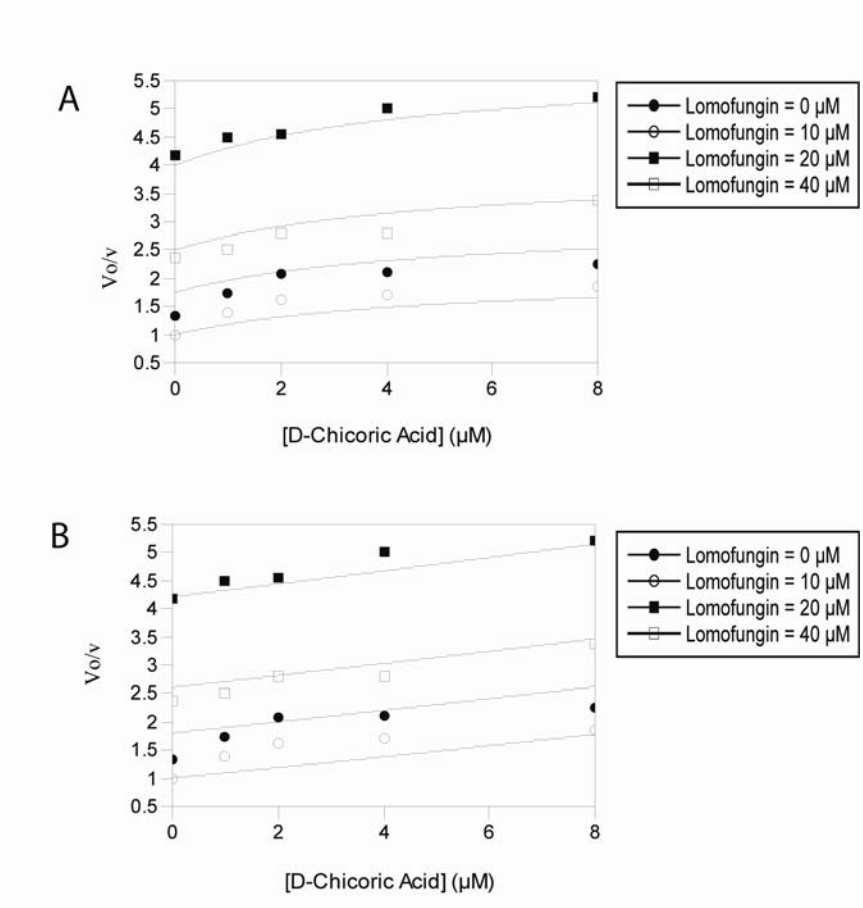

Figure S2. Combination studies with lomofungin and D-chicoric acid. (A) Data fit to a nonmutually exclusive binding model. (B) Data fit to a mutually exclusive binding model.

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