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Picolinic Acids as β -Exosite Inhibitors of Botulinum Neurotoxin A Light Chain

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Abstract

In developing small-molecule inhibitors of botulinum neurotoxin serotype A light chain (BoNT/A LC), substituted picolinic acids were identified. Extensive investigation into the SAR of the picolinic acid scaffold revealed 5-(1-butyl-4-chloro-1H-indol-2-yl)picolinic acid (CBIP), which possessed low micromolar activity against BoNT/A. Kinetic and docking studies demonstrated binding of CBIP to the β -exosite: a largely unexplored site on the LC that holds therapeutic relevance for botulism treatment.

Graphical Abstract

A series of novel substituted picolinic acids demonstrated low micromolar inhibition of botulinum neurotoxin A light chain at the β -exosite.

Botulinum toxin (BoNT) is an exotoxin produced by the bacterium *Clostridium botulinum* and is considered one of the most deadly known substances; it is estimated that a 1 ng/kg dose of the toxin can kill an adult human.¹ Although eight different serotypes of the toxin (A–H) have been characterized, serotype A causes the most severe symptoms and is the most common cause of potentially fatal foodborne botulism.² Improper canning techniques are to blame for the most recent botulism cases in the United States.³ Because of its remarkable toxicity to humans, BoNT poses a bioterrorism threat and attempts at weaponizing the toxin have been made by former Iraqi and Soviet Union government programs.⁴ In response, the CDC has mandated surveillance and control measures for mitigating botulism outbreaks, which includes a supply of antitoxin.⁵ Equine BoNT antitoxin is the only FDA-approved therapy for botulism but is minimally effective more than 24 h following the onset of symptoms.⁶ Ironically, BoNT serotype A (BoNT/A), is used

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in small, controlled quantities as the commercial product BOTOX[®] for cosmetic purposes and to treat a variety of conditions involving muscle spasms.^{7, 8} BoNT/A intoxication occurs upon endocytosis into neurons via the 100 kDa heavy chain,⁹ followed by cleavage of the 25 kDa SNAP-25 SNARE protein via the 50 kDa zinc-metalloprotease light chain (LC). As a consequence, acetylcholine signaling is disrupted causing paralysis.^{10, 11} Since the action of the LC directly interferes with neurotransmission to muscles, the LC has remained the primary target of medicinal chemistry campaigns: the most common strategy being chelation of the active-site zinc with hydroxamates, quinolinols or peptidomimetics to inhibit LC-mediated proteolysis of SNAP-25.^{12–17} Despite promising preclinical data, none of these compounds have progressed to clinical trials, begging the need for alternative LC inhibition strategies at other sites on the enzyme; preliminary reports of α - and β -exosite inhibition have shown promise.^{18–21}

Previously we have identified lomofungin, a natural product, as a β -exosite inhibitor of BoNT/A.²⁰ We believe lomofungin holds ample therapeutic potential, but it contains reactive functionalities such as an aldehyde, ester and multiple phenols which limit *in vivo* stability (Fig. 1). In searching for more stable scaffolds containing the same pharmacophores, we identified picolinic acid (PA) as a potential alternative core molecule that was amenable to library preparation. As an indication of its safety and *in vivo* compatibility, PA is found endogenously in mammals.²² Until now, PA has not been explored as an inhibitor scaffold in any context, however, picolinamide derivatives have been reported.²³

In order to assess activity of inhibitors, a robust LCMS-based assay was used to quantify the 9-mer cleavage peptide produced by BoNT/A LC proteolysis of a 66-mer peptide substrate, homologous to the entire BoNT/A LC binding region of the SNAP-25 protein.²⁴ Preliminary virtual screening and docking studies suggested that 5-substituted PA analogues could form favorable interactions with the BoNT/A LC β -exosite (Fig. S1); therefore, a series of these compounds was synthesized and tested in the 66-mer cleavage assay to probe the SAR. Efficiency and scope were key facets in designing the synthetic methodology used for generated the library. In considering these criteria, we chose a 2-indolyl fragment to append to the 5-position of the PA core. Virtually any indolyl fragment could be attached to a 5-bromopicolinate via a Suzuki-Miyaura reaction; special conditions developed by the Hartwig group enabled cross coupling of reactive 2-indolyl pinacol boronate esters with a variety of aryl halides.²⁵ We exploited this methodology to create a series of over 40 different 5-(1H-indol-2-yl)picolinates (Scheme 1 and SI). In addition to introducing library diversity through substituents on the indole ring (R^2), subsequent alkylation of the indole nitrogen opened the possibility of adding substituents via S_N2 of the corresponding alkyl halide (R_1 , Scheme 1). A *tert*-butyl ester was used to protect the picolinic acid for the Suzuki and S_N2 couplings, and was quantitatively removed with TFA in the final step.

In testing a sublibrary containing various R_1 groups, we found that most lipophilic functionalities improved inhibitor potency over the weakly active, unsubstituted parent compound **1a-OH** (Fig. 2, Table S1). Although not the most potent inhibitors, *N*-alkyl substituted compounds (Et **2a**, Pr **3a**, Bu **4a**) were chosen for further investigation due to their higher ligand efficiency and ease of synthesis. In selecting butyl as the R_1 group, we synthesized a second sublibrary to investigate the SAR of R_2 indole ring substituents (Fig 2,

Table S2). A two-fold increase in inhibition was observed when introducing chloro, methyl or trifluoromethyl groups at the 3-and 4-positions (**4b**, **c**, **h**, **m**), however, bulkier functionalities like 3-CN (**4g**) and 4-OMe (**4n**) caused a drastic reduction in inhibitor potency (Fig. 2, Table S2).

In identifying some promising R₁ and R₂ substituents independently, we further probed the SAR by creating a third sublibrary with different R₁ and R₂ combinations (Table S3). Generally, PAs with longer R₁ chain length and R₂=3-Me and 4-Cl aryl groups (**4a**, **b**) possessed the greatest potency. These compounds showed similar activity against the truncated and full length enzyme, but some possessed >3-fold inhibition versus the full length enzyme. This result suggests that the extra 23 amino acids found in the full length enzyme interact minimally with the PA inhibitors.

Following the comprehensive investigation of indolylpicolinate SAR, compound **4c**, dubbed 'CBIP' was chosen as our lead compound because of its low micromolar IC₅₀ and predicted increased metabolic stability over a similarly potent compound **4b**; a 4-Cl substituent may decrease possible 5-hydroxylation by tryptophan hydroxylase.²⁶ Final modifications were made to the CBIP structure to ensure that no additional inhibitory activity could be gained (Fig. 2). Promisingly, removal of some of the key functionalities in the CBIP molecule such as the 5-indolyl fragment, the *N*-butyl fragment (**1a-OH**), the pyridine nitrogen (**18**) and the acid carbonyl (**19**) led to drastic reduction in enzyme inhibition. Repositioning of the indolyl (**22**) and carboxyl group (**17**) also caused decreased inhibitor potency. The SAR investigation revealed that the CBIP structure was optimally designed for BoNT/A LC inhibition. Furthermore, a new direction for inhibitor discovery was elucidated which included replacement of the indolyl group with a dibenzofuranyl group (**21**, Fig. 2). Since conversion of the acid to an ester (**20**) led to reduced inhibition, **21** still relies on the picolinic acid warhead for inhibitor-activity, suggesting that it is still binding to the enzyme in the same manner as CBIP.

A kinetic evaluation of CBIP inhibition was conducted in the 66-mer assay which included direct comparison to lomofungin. Dose response curves of both compounds revealed an 8-fold increase in potency of CBIP over lomofungin (Fig. 3).

Previously, kinetic studies have revealed that lomofungin shows non-mutually exclusive inhibition in combination with α -exosite inhibitor chicoric acid *i*-Pr ester (ChAE) and a hydroxamate active-site inhibitor, indicating that it binds at the BoNT/A β -exosite.²⁰ We evaluated the inhibition kinetics of CBIP in the presence of both lomofungin and the ChAE as tool compounds to elucidate possible exosite binding.^{21, 27} A global fit of both data sets gave values for the enhancement factor (α) which were <1 for the CBIP-lomofungin combination and >1 for the CBIP-ChAE combination (Fig. 4). These results suggest that CBIP binds mutually exclusively with lomofungin but non-mutually exclusively with chicoric acid; therefore, CBIP likely binds at the β -exosite of BoNT/A LC,^{21, 27, 28} while direct interaction at the Zn-containing active site was not observed.[‡] Interestingly, low

[‡]Active site binding was ruled out due to inactivity of the compounds in a the SNAPtide assay: a FRET-based assay (List Biological Labs) with a 13 amino acid substrate that interacts only with the BoNT/A LC active site.

concentrations of lomofungin reduced inhibition of CBIP, suggesting that lomofungin actually binds more tightly to the enzyme than CBIP despite being a poorer inhibitor (Fig. S2).

Docking experiments were performed to elucidate the possible binding mode of CBIP within the β -exosite. Briefly, lowest energy conformers of CBIP and lomofungin were generated using OMEGA^{29–31} and were docked with FRED^{32–34} into the β -exosite which has been structurally characterized.³⁵ The predicted poses of both compounds are shown in Fig. 5. In corroboration with a previous computational study on lomofungin-BoNT/LC binding,³⁶ the key interaction of both molecules appears to be a hydrogen bond (H-bond) with the phenol OH of Tyr250. Furthermore, the conformation of the Tyr250 loop has been shown to be profoundly influenced by substrate binding to the LC;³⁵ therefore, a potentially stronger interaction of CBIP relative to lomofungin with Tyr250 may disrupt SNAP-25 binding, resulting in the superior inhibitory activity of CBIP. A second possible interaction was observed with Asn418 as an H-bond donor. Hydrophobic interactions were present between Leu207 and the CBIP indole ring, while the butyl chain appeared to interact in a shallow groove formed by the backbone of Phe369 and Asn368 (see rotated CBIP image in Fig. S3). On the other hand, lomofungin could not reach this groove (extending into the page), explaining its lack of inhibition compared to CBIP, while the extra H-bond with Leu207 accounts for the tighter binding of lomofungin relative to CBIP (Fig. 5, Fig. S2). Overall, the predicted CBIP pose is consistent with the observed SAR; a variety of 5-, 6-, and *N*-indole substituents were tolerated due to a lack of steric constraint by the enzyme at these positions. The 3- and 4-positions, however, can sterically clash with Arg425 to produce greater substituent selectivity (Fig. 5). Furthermore, alteration of the PA warhead drastically decreased enzyme inhibition by disrupting the critical PA H-bond with the enzyme.

In sum, we were successful in designing a more drug-like and potent exosite inhibitor (CBIP) as a substitute to the natural product, lomofungin. A comprehensive evaluation of picolinic acid SAR identified CBIP as a lead compound. CBIP showed low μ M inhibition against BoNT/A LC while binding to the same site as lomofungin, putatively through a key interaction with Tyr250. The more favourable physiochemical properties of CBIP could lend to its potential efficacy and stability in future *in vivo* studies for ameliorating BoNT/A toxicity. The presence of the picolinic carboxylic acid group opens the possibility for a prodrug strategy with hydrolysable esters. Additionally, SAR studies have suggested that future PA-based inhibitor design could be achieved by swapping the 5-indolyl group with another heteroaromatic group, or by adding an H-bond donating group at the PA ring 4-position to enhance inhibitor-enzyme interaction via Leu207 (Fig. 5).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Structures of lomofungin and picolinic acids

Fig. 2.
Structure-Activity Relationship (SAR) mapping of 5-substituted picolinic acids against BoNT/A LC

Fig. 3.
Potency comparison between CBIP and lomofungin against BoNT/A LC

Fig. 4.
Inhibition kinetics of CBIP in combination with α -exosite inhibitor chicoric acid *i*-Pr ester (ChAE) and β -exosite inhibitor lomofungin

Fig. 5.
Predicted poses of lomofungin (light blue) and CBIP (tan) docked into the β -exosite of BoNT/A LC

Scheme 1.

Synthesis of substituted 5-(1H-indol-2-yl)picolinates

Reagents and conditions: a) B_2Pin_2 , $[Ir(cod)OMe]_2$, dtbpy, THF, 80 °C, 18 h; b) *tert*-butyl 5-bromopicolinate, $P(o-Tol)_3$, $Pd(dba)_2$, Na_2CO_3 , 1:9 H_2O/THF , rt, 18 h; c) R_1-X , Cs_2CO_3 , DMF, 80 °C, 1 h (X=I) or 18 h (X=Br); d) 1:1 TFA/DCM, rt, 2 h.