Identification of Clinically Viable Quinolinol Inhibitors of Botulinum Neurotoxin A Light Chain

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ABSTRACT: Botulinum neurotoxins (BoNT) are the most potent toxins known and a significant bioterrorist threat. Few small molecule compounds have been identified that are active in cell-based or animal models, potentially due to toxin enzyme plasticity. Here we screened commercially available quinolinols, as well as synthesized hydroxyquinolines. Seventy-two compounds had IC_{50} values below 10 μM, with the best compound exhibiting submicromolar inhibition (IC_{50} = 0.8 μM). Structure–activity relationship trends showed that the enzyme tolerates various substitutions at R_1 but has a clear preference for bulky aryl amide groups at R_2, while methylation at R_3 increased inhibitor potency. Evaluation of the most potent compounds in an ADME panel showed that these compounds possess poor solubility at pH 6.8, but display excellent solubility at low pH, suggesting that oral dosing may be possible. Our data show the potential of quinolinol compounds as BoNT therapeutics due to their good in vitro potencies and favorable ADME properties.

INTRODUCTION

Clostridium botulinum neurotoxin serotype A (BoNT/A), the most potent toxin known to man (LD_{50} ≈ 0.5 ng/kg), disrupts neurotransmission by cleaving proteins involved in the exocytosis of the neurotransmitter acetylcholine from motor neurons at the neuromuscular junction. Inhibition of acetylcholine release, in turn, results in flaccid paralysis and may lead to host death due to heart or respiratory failure. Because of its extraordinary toxicity and ease of production in large quantities, BoNT/A is considered a potential bioterrorism weapon.2

The mechanism of action of BoNT includes binding to motor neuron surface receptors, cellular uptake via endocytosis, and escape of the N-terminal catalytic domain from endosomes into the cytosol. Once in the cytosol, the BoNT/A light chain (BoNT/A LC), a zinc-dependent metalloproteinase, cleaves its cognate protein substrate, SNAP-25 (Synaptosomal Associated Protein of 25 kDa). SNAP-25 is a member of the SNARE (Soluble NSF Attachment Protein Receptor) complex of proteins involved in acetylcholine exocytosis. Cleavage of SNAP-25 disrupts acetylcholine neurotransmission from motor neurons to their target muscle cells, ultimately resulting in flaccid paralysis.3

Because of the rapid internalization and intracellular site of action of the toxin, treatments that inhibit holotoxin binding and/or internalization, including the only existing treatment of BoNT intoxication with passive immunization with equine or human neutralizing antibodies, have a limited window of application and become inefficient once the toxin is internalized into the target neuronal cell. However, compounds that target intracellular BoNT/A LC activity have a much larger window of application due to the extremely long half-life of the BoNT/A LC. Therefore, we and others have focused primarily on targeting BoNT/A LC enzymatic activity and have reported a number of small molecule inhibitors of BoNT/A LC that are highly efficient in vitro, but when used ex vivo or in vivo, most of these compounds fail because of their poor pharmacokinetic properties.4,5 Consequently, there is a great demand to identify and develop new inhibitors of BoNT/A LC with improved in vivo properties that would not only show good in vitro inhibition of the isolated toxin but could also reverse symptoms of botulism in an intoxicated host.

Most reported inhibitors of BoNT/A LC share a common motif, that is, a Zn^{2+} chelator that prevents water-catalyzed proteolysis of the peptide bond of the protein substrate SNAP-25. One such chelator is the hydroxamate moiety used in BoNT/A LC inhibitors developed by our laboratory and others.4-6 Although hydroxamate-based inhibitors were shown to exhibit excellent in vitro potency against the recombinant enzyme with K_{i} in the nanomolar range (K_{i} of one of the most potent hydroxamate compounds to date, (2E)-3-(3-chloro-6-fluoro-benzo[b]thiophen-2-yl)-N-hydroxy-(E)-acylamide, is 77 nM),6 these compounds generally suffer from poor bioavailability, short metabolic half-lives that seldom exceed one hour,4 and high cytotoxicity.5 In addition, we have recently reexamined a series of hydroxamate inhibitors and found an
inconsistency in structure–activity relationship (SAR) trends across similar hydroxamate compounds, suggesting a relative plasticity of the BoNT/A LC active site and cautioning against making generalizations across compound series. Quinolinol compounds have been reported as an alternative class of BoNT/A LC inhibitors and were shown to have good ex vivo properties in cell-based, as well as tissue-based, assays. However, SAR for this series of compounds and further pharmacokinetic data have not been reported. In light of the light chain active site plasticity, we examined the SAR of BoNT/A LC quinolinol inhibitors and assessed the pharmacologic parameters of the best inhibitors in ADME studies.

RESULTS AND DISCUSSION

Traditional approaches for the discovery and development of BoNT/A LC inhibitors have been based on screening efforts to obtain novel chemotypes that target the catalytic activity of the LC metalloprotease. Although such approaches generally result in compounds with improved in vitro potency compared to existing inhibitors, these compounds have displayed poor efficacy when advanced into cellular models, presumably due to various limitations including low aqueous solubility and poor cell permeability. In addition, these compounds were shown to suffer from disappointing pharmacokinetic properties (e.g., half-life < 1 h) when tested in animal models (T.J.D. and K.L.H., unpublished data). Furthermore, our recent analysis of the SAR present in the cinnamyl hydroxamate inhibitor series has demonstrated that crystal structure-guided optimization of BoNT/A LC inhibitors can overlook compounds with greater potency than the parent lead. In this case, optimized molecules experimentally found to have increased potencies were predicted to be poor inhibitors that were unable to fit into the active site.

We hypothesized that enzyme flexibility of the BoNT/A LC might have resulted in a similar phenomenon in other inhibitor series; to test this, we analyzed the quinolinol series of inhibitors. The quinolinol moiety is a known chelating motif and therefore represents a viable moiety for a metalloprotease inhibitor. Quinolinol inhibitors (Figure 1) have been modeled in order to optimize activity while still retaining drug-like properties; (c) the compounds would essentially be devoid of potentially reactive or known problematic moieties including aldehydes, ketones, charged compounds, and carboxylic and sulfonic acids.

The inhibitory activity of the quinolinol compounds was tested in the SNAPtide assay, an established assay for identification of BoNT/A LC inhibitors. The assay measures changes in fluorescence produced by cleavage of a fluorogenic substrate that consists of a synthetic peptide derived from the amino acid sequence of SNAP-25 flanking the natural BoNT/A LC cleavage site as well as a fluorophore/quencher pair. This assay is a useful primary screen for BoNT/A inhibitor screening campaigns because of its simplicity, automation potential, and ability to screen numerous compounds simultaneously. However, the assay cannot be utilized to determine more rigorous kinetic constants, such as the $K_i$. Because the $K_i$ of the SNAPtide substrate is well above 1 mM, any assay with this substrate is performed under substrate-limiting conditions.

One hundred eighty-eight commercially available quinolinol derivatives were initially screened at a final concentration of 10 μM. Interestingly, more than half of the compounds tested (54% hit rate) exhibited greater than 50% inhibition of BoNT/A LC activity toward the SNAPtide substrate, implying the enzyme can tolerate various substitutions of the main quinolinol scaffold and suggesting significant plasticity of the active site cleft. The $Z'$-factor, a measure of statistical predictability of results obtained by a high-throughput method, of our primary screen was 0.92, where a $Z'$-factor between 0.5 and 1.0 signifies a screening assay with excellent quality.

Subsequently, a secondary follow-up screen was performed on a subset of 90 compounds, including 25 compounds identified in the primary screen as less potent (i.e., <50% inhibition) or inactive. Using this assay, IC₅₀ values were determined for all 90 active compounds (Table 1 and Supplemental Table 1). Of this set, 80% of the compounds showed IC₅₀ values <10 μM, with the best compounds exhibiting submicromolar IC₅₀. Seven compounds that exhibited between 25% and 50% inhibition in the primary screen at 10 μM, were shown to have IC₅₀ values <10 μM, resulting in a false-negative discovery rate of the primary screen of 7.8%. The IC₅₀ of compound 4, the most potent compound in the library, was 0.8 ± 0.1 μM.

Despite the promiscuity of the enzyme for various substitutions on the main quinolinol backbone (Figure 1), some structure–activity relationships could be deduced from the inhibitory data (Table 1). Compounds with a five- or six-membered heterocycle at both R₁ and R₂ showed good inhibition, and compounds that did not have a 6-membered heterocycle at R₂ had severely decreased inhibitory activity (1–3, 15, 22, 27–29). The enzyme tolerated virtually any substitution on the R₁ heterocycle, including electron donating (4–12) and electron withdrawing (15–37) groups on the R₁ phenyl ring; moreover, substitutions of the R₁ phenyl ring with heterocyclic pyridyl (40–53) were tolerated. In addition, a heterocyclic pyridine-3-yl ring at the R₂ position (41, 43, 45, 47, 49, 51, 53) was favored over the pyridine-2-yl substitution (40, 42, 44, 46, 48, 50, 52) with the pyridine-3-yl compounds being up to 60% more potent (Table 1), suggesting that 3-N atom of the pyridine ring forms favorable interactions with the side chains of LC active site residues. Presumably, this interaction is with Arg363, which has been reported to interact with the R₂ moiety of quinolinol inhibitors based on molecular modeling parameters of the best inhibitors in ADME studies.

### Figure 1. General structure of 8-hydroxy-quinoline compounds that are potent BoNT/A LC inhibitors. Substitutions investigated in the study are numbered R₁ through R₄ in shaded circles.

| Compound | IC₅₀ (μM) | Hit Rate |
|----------|----------|----------|
| 4        | 0.8 ± 0.1 | 100%     |

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Table 1. Inhibitory Activities of 8-Hydroxy-quinolines toward BoNT/A LC

| Compound | Chembridge or ChemDiv ID | R₁ | R₂ | R₃ | R₄ | IC₅₀ (µM) |
|----------|--------------------------|----|----|----|----|-----------|
| 1        | 5924147                  |    | H  | H  |    | >100      |
| 2        | 5925700                  |    | H  | H  |    | >100      |
| 3        | 5928516                  |    | H  | H  |    | >100      |
| 4        | 5838194                  |    | H  | H  |    | 0.8 ± 0.1 |
| 5        | 6033702                  |    | H  | H  |    | 3.6 ± 0.3 |
| 6        | 6637624                  |    | CH₃| H  |    | 1.6 ± 0.1 |
| 7        | 6377743                  |    | CH₃| H  |    | 10.7 ± 1.1|
| 8        | 7630436                  |    | CH₃| H  |    | 0.9 ± 0.1 |
| 9        | 6377946                  |    | H  | H  |    | 13.4 ± 0.3|
| 10       | 7330066                  |    | CH₃| H  |    | 2.1 ± 0.3 |
| 11       | 6381500                  |    | H  | H  |    | 4.6 ± 0.3 |
| 12       | 7633631                  |    | CH₃| H  |    | 1.1 ± 0.2 |
| 13       | 6381368                  |    | CH₃| H  |    | 1.4 ± 0.1 |
| 14       | 6636291                  |    | CH₃| H  |    | 3.5 ± 0.6 |
| 15       | 5845249                  |    | H  | H  |    | >100      |
| 16       | 6380954                  |    | H  | H  |    | 3.3 ± 0.3 |
| 17       | 7633962                  |    | CH₃| H  |    | 2.0 ± 0.2 |
| 18       | 7629244                  |    | H  | H  |    | 1.5 ± 0.2 |
| 19       | 7319416                  |    | CH₃| H  |    | 1.2 ± 0.1 |
| 20       | 6376791                  |    | H  | H  |    | 6.2 ± 0.5 |
| 21       | 6635878                  |    | CH₃| H  |    | 3.0 ± 0.2 |
| 22       | 5924214                  |    | H  | H  |    | >100      |
| 23       | 6379137                  |    | H  | H  |    | 7.1 ± 0.4 |
| 24       | 6378600                  |    | CH₃| H  |    | 2.8 ± 0.2 |
| 25       | 6372490                  |    | H  | H  |    | 2.5 ± 0.2 |
| 26       | 6637173                  |    | CH₃| H  |    | 2.1 ± 0.2 |
| 27       | 5848587                  |    | H  | H  |    | >100      |
| 28       | 5926670                  |    | H  | H  |    | >100      |

| Compound | Chembridge or ChemDiv ID | R₁ | R₂ | R₃ | R₄ | IC₅₀ (µM) |
|----------|--------------------------|----|----|----|----|-----------|
| 29       | 5926377                  |    | H  | H  |    | >100      |
| 30       | 5928396                  |    | H  | H  |    | 7.1 ± 1.2 |
| 31       | 6380808                  |    | H  | H  |    | 3.5 ± 0.4 |
| 32       | 7633178                  |    | CH₃| H  |    | 2.5 ± 0.3 |
| 33       | 6378687                  |    | CH₃| H  |    | 4.5 ± 0.7 |
| 34       | 6634547                  |    | H  | H  |    | 4.3 ± 0.4 |
| 35       | 7630693                  |    | CH₃| H  |    | 1.0 ± 0.1 |
| 36       | 6646208                  |    | H  | H  |    | 5.6 ± 0.4 |
| 37       | 6633429                  |    | CH₃| H  |    | 1.9 ± 0.3 |
| 38       | 7021092                  |    | CH₃| H  |    | 2.0 ± 0.2 |
| 39       | 7719526                  |    | CH₃| H  |    | >100      |
| 40       | 8397-0126                |    | H  | H  |    | 5.6 ± 0.8 |
| 41       | 8397-0127                |    | H  | H  |    | 1.9 ± 0.3 |
| 42       | 8397-0165                |    | H  | H  |    | 5.1 ± 0.6 |
| 43       | 8397-0166                |    | H  | H  |    | 2.9 ± 0.4 |
| 44       | 8397-0270                |    | H  | H  |    | 7.8 ± 1.3 |
| 45       | 8397-0271                |    | H  | H  |    | 4.2 ± 1.0 |
| 46       | 8397-0298                |    | H  | H  |    | 5.6 ± 1.3 |
| 47       | 8397-0299                |    | H  | H  |    | 3.0 ± 0.3 |
| 48       | 8397-0558                |    | H  | H  |    | 5.1 ± 1.0 |
| 49       | 8397-0559                |    | H  | H  |    | 4.4 ± 0.3 |
| 50       | 8397-0572                |    | H  | H  |    | 5.2 ± 0.7 |
| 51       | 8397-0573                |    | H  | H  |    | 2.3 ± 0.4 |
| 52       | 8397-0664                |    | H  | H  |    | 16.7 ± 1.9|
| 53       | 8397-0665                |    | H  | H  |    | 6.7 ± 1.1|

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The potency of inhibitors, where the presence of a methyl group in inhibition of BoNT/A LC, we conducted additional sub-
the quinolinol molecule were absolutely required for the LC active site cleft.

Group forms favorable interactions with the side chains of the compound 38 BoNT/A LC. An interesting might interact with an aromatic residue or the zinc cation of thiophen-2-yl substitution at the R1 position, but only in potent inhibition of BoNT/A LC. In contrast, the 6-methyl compound became virtually inactive. However, bulky aryl amide substitutions (compounds 14, 16–21, 23–26, 30–53) rendered the inhibitors active, suggesting that the R3 group might interact with an aromatic residue or the zinc cation of BoNT/A LC. An interesting finding came from analyzing compounds 38 and 39. Both compounds bear a 5-methyl-thiophen-2-yl substitution at the R3 position, but only compound 38, with unsubstituted R3 2-pyridyl ring, resulted in potent inhibition of BoNT/A LC. In contrast, the 6-methyl group on the R2 ring (39) rendered the compound completely inactive, suggesting the methyl group produces a significant steric clash with the walls of the BoNT/A LC binding pocket. However, this trend is not globally true; comparison of compounds 13 and 14 shows that methylation of the R2 2-pyridyl ring does not result in dramatically reduced inhibition.
The presence or absence of a methyl group at the R3 position (compound pairs 5–6, 9–10, 11–12, 16–17, 18–19, 20–21, 23–24, 25–26, 31–32, 34–35, 36–37) had a clear effect on the potency of inhibitors, where the presence of a methyl group decreased the IC50 values of corresponding inhibitors with otherwise the same structure by as much as 85% (13.4 vs 2.1 μM for compounds 9 and 10). This suggests that the R3 methyl group forms favorable interactions with the side chains of the LC active site cleft.

In an attempt to more completely understand which parts of the quinolinol molecule were absolutely required for the inhibition of BoNT/A LC, we conducted additional substructure analysis of portions of the parent scaffold and synthesized the required compounds (Table 2). 8-Hydroxy-quinoline (54) served as the simplest compound bearing only the quinoline backbone with the chelating motif, but without any additional structural determinants that could facilitate binding of the inhibitor into the active site cleft of the protease and thus increase its potency. As expected, 8-hydroxy-quinoline showed poor inhibition of SNAPtide cleavage at inhibitor concentrations ≤30 μM (Table 2), suggesting the quinoline backbone by itself is not sufficient for efficient inhibition. In addition, when the prototype quinolinol compound (57) that bears an aryl group on each of the R1 and R2 positions was tested, it exhibited relatively good potency of 2.2 μM, implying that both the R1 and R3 substitutions are required for productive docking of the inhibitor into the active site and, consequently, potent inhibition (Table 2). When the R2 group was removed altogether (56), the molecule lost most of its inhibitory activity (IC50 = 27 μM), suggesting that the R1 moiety is needed for either proper molecular conformation or favorable interaction of the inhibitor with BoNT/A LC. Unfortunately, multiple attempts to synthesize an analogous molecule in which R2 was missing failed, yielding only starting material.

An important finding came from a compound where the 8-hydroxy group of the compound 57 was protected with an isopropyl group (Supplemental Figure 1), rendering the compound completely inactive (IC50 >100 μM, data not shown), confirming the necessity of the 8-hydroxy group and suggesting its role in chelation of the catalytic zinc.

Further analysis of the structures of quinolinol inhibitors uncovered a distinct similarity to molecules that have been advanced into clinical testing for indications unrelated to botulism. The compound 8-hydroxy-quinoline (54), although not a very potent BoNT/A LC inhibitor, and the 8-hydroxy-quinoline backbone, are structurally related to two clinically approved drugs, clioquinol (58; Figure 2) and chloroxine (60; Figure 2), both of which possess good pharmacokinetic properties. Clioquinol, 5-chloro-7-iodo-8-hydroxy-quinoline, was produced as a topical antiseptic and marketed as an oral intestinal amebicide as early as the 1930s. It has good in vivo properties, including oral bioavailability, single-dose concentrations are dose-related, and the drug half-life in humans is 11–14 h. In addition, clioquinol treatment has been successfully used to improve the diseased state of Alzheimer patients in a pilot phase 2 clinical trial. When clioquinol (58) was tested for in vitro inhibitory activity toward BoNT/A LC, it exhibited moderate inhibition with an IC50 value of 20.3 μM (Table 2), notably improved inhibition compared to the activity of 8-hydroxy-quinoline (54; IC50 > 30 μM). Similarly, chloroxine, 5,7-dichloro-8-hydroxy-quinoline (60), a clinically approved antibacterial drug used in shampoos for dandruff and seborrheic dermatitis, showed moderate inhibition of BoNT/A LC was removed altogether (56), the molecule lost most of its inhibitory activity (IC50 = 27 μM), suggesting that the R1 moiety is needed for either proper molecular conformation or favorable interaction of the inhibitor with BoNT/A LC. Unfortunately, multiple attempts to synthesize an analogous molecule in which R2 was missing failed, yielding only starting material.

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LC with even more potent IC$_{50}$ of 11.5 μM relative to clioquinol (Table 2).

We next questioned if the SAR trends observed in our screening set would hold true with clioquinol and chloroxine. Interestingly, simple 2-methyl substitutions of clioquinol and chloroxine significantly improved inhibitory activities of both compounds (Table 2). 2-Methyl-clioquinol (59) showed a 31% improvement in potency (IC$_{50}$ = 14.1 μM) when compared to the unmethylated clioquinol (58), whereas 2-methyl-chloroxine (i.e., 2-methyl-5,7-dichloro-8-hydroxy-quinoline; 61) showed a moderate improvement of 12% (IC$_{50}$ = 10.1 μM) when compared to the unmethylated chloroxine (60), confirming the observed trend with previously examined quinolinol compound pairs (Tables 1 and 2). While these are not as potent as many of the hydroxamate leads, it should be pointed out that the improved IC$_{50}$ values of the methylated analogues of clioquinol and chloroxine (59 and 61) are comparable to the observed EC$_{50}$ values of quinolinol compounds tested in a cell-based BoNT/A neurotoxicity assay. Therefore, although these methylated analogues have lower activity, they still may be potent enough to inhibit intracellular toxin function in cell-based models. Moreover, benzimidazole acrylonitrile-based$^{18}$ and d-cysteine-based$^{18}$ inhibitor concentrations required to exhibit 50% protection in cellular assays have been shown to be around 30 μM, concentrations similar to the IC$_{50}$ values of clioquinol and chloroxine (58 and 60). Collectively, these data suggest that the two existing drugs with extensive pharmacokinetic and pharmacodynamic data and detailed toxicity profiles could be repurposed for further cellular and in vivo testing as well as serve as an attractive template for developing more potent BoNT/A LC inhibitors with superior in vivo properties to those of the existing BoNT/A LC inhibitors.

Table 3. In Vitro ADME Properties of Selected Quinolinol BoNT/A LC Inhibitors

| compd | metabolic stability, human | metabolic stability, rat | plasma stability, human | plasma stability, rat | aqueous solubility |
|-------|---------------------------|--------------------------|--------------------------|----------------------|-----------------|
|       | metabolic rate (μM/min·mg) | half-life (min) | half-life (hr) | remaining at 3 h | metabolic rate (μM/min·mg) | half-life (min) | half-life (hr) | remaining at 3 h | pH 6.8 (nM) | pH 1.0 (μM) |
| 34    | 0.215                     | 25.5                     | 24.4                     | 90.9                 | 0.130                     | 42.2                     | 51.1                     | 100          | 74.1            | 1049            |
| 35    | 0.047                     | 116                     | 42.0                     | 92.2                 | 0.271                     | 20.3                     | 109                     | 98.5         | 65.9            | 997             |
| 36    | 0.242                     | 22.7                     | 6.1                      | 53.4                 | 0.487                     | 11.3                     | 5.0                      | 42.2         | 5.3             | 102             |
| 37    | 0.324                     | 17.0                     | 10.7                     | 66.2                 | 0.627                     | 8.8                      | 5.0                      | 86.6         | 33.8            | 76.7             |
| 56    | 0.203                     | 27.1                     | 14.2                     | 96.1                 | 0.149                     | 36.8                     | 12.9                     | 76.3         | 111             | 1144            |
| 57    | 0.186                     | 29.6                     | 11.4                     | 78.7                 | 0.637                     | 8.6                      | 13.5                     | 67.3         | 78.0            | 1174            |
| 58    | 0.086                     | 63.9                     | 42.4                     | 100                  | 0.071                     | 76.9                     | 136                      | 83.9         | <1.0            | 38.1             |
| 60    | 0.112                     | 48.9                     | 18.6                     | 100                  | 0.100                     | 55.0                     | 67.7                     | 100          | 532             | 326              |
| 61    | 0.190                     | 28.8                     | 24.5                     | 92.2                 | 0.142                     | 38.6                     | 115                      | 96.9         | 394             | 1098            |

Nine compounds, including clioquinol and chloroxine, were selected based on their initial in vitro potency, absence of known reactive groups, and availability of compound pairs with or without the R$_3$ methyl group, and advanced into a set of in vitro ADME assays including aqueous solubility, metabolic stability in human and rat liver microsomes, and human and rat plasma stability (Table 3). These assays were selected as they directly affect formulation and in vivo exposure, respectively. Moreover, compounds were assayed in the context of human and mouse liver microsomal and plasma metabolic stability, due to potential species differences in liver and plasma metabolism, as well as to inform future toxicology and clinical studies designs.

Aqueous solubility of the nine quinolinol inhibitors was determined at two different pH values to evaluate the feasibility of oral administration in possible in vivo assays and preclinical studies (Table 3). All compounds exhibited extremely low aqueous solubility at pH 6.8, ranging between <1 nM (58) and 0.5 μM (60), concentrations that are well below their IC$_{50}$ values, suggesting none of the compounds would be soluble in the environment of the small intestine. However, when the pH was reduced to 1.0, compound solubility markedly increased, ranging between 38 μM (58) to almost 1.2 mM (57), suggesting that quinolinol compounds may be amenable to oral dosing paradigms. This finding is in agreement with oral dosing as the preferred route of administration of clioquinol, where serum levels of clioquinol in patients with Alzheimer’s disease ranged between 13 to 25 μM at total oral daily dosages of 250 to 750 mg that were well below the dose associated with neurological toxicity (20–30 mg/kg/day).$^{14,15}$

In general, all of the compounds, with the exception of compound 36, were highly stable in human and rat plasma, with stability t$_{1/2}$ in plasma averaging 23.5 ± 4.5 h for human and 63.8 ± 18.2 h for rat plasma, respectively. All of the compounds tested, save compounds 35 and 58, showed high metabolic rates both in human as well as in rat liver microsomes. Collectively, the half-lives of the compounds in either species did not exceed 60 min, with the exception of compounds 35 (human t$_{1/2}$ = 116 min) and 58 (human t$_{1/2}$ = 64 min, rat t$_{1/2}$ = 77 min), which is comparable to the published half-lives of hydroxamate-based BoNT/A LC inhibitors.$^4$

The results of this study indicate that 8-hydroxy-quinoline compounds represent an attractive scaffold for design of potent BoNT/A LC inhibitors. A screen of 188 quinolinol compounds from commercially available small molecule repositories against recombinant BoNT/A LC showed that a broad range of substitutions is well tolerated by the enzyme, further validating...
the hypothesis that BoNT/A LC flexibility can significantly impact small molecule inhibitor design. Indeed, flexibility of BoNT/A LC substrate binding site has previously been suggested by X-ray crystallography and implied in our data through observed inconsistency in SAR trend across hydroxamate compound series. However, the inhibitory profile of two clinically approved molecules with established pharmacokinetics, cloquinol and chloroxine, demonstrated that these compounds could be repurposed for BoNT/A testing and may serve as a scaffold for future design and optimization. The most potent compound from our screen (4) displayed a submicromolar IC$_{50}$ which is the most potent inhibition of BoNT/A LC by a quinolinol lead. Despite the poor solubility of the entire series of compounds at pH 6.8, improved solubility at pH 1.0 argues for testing in oral dosing models to enhance in vivo exposure. Presently, pharmacoki- netic analysis of these compounds is currently underway and will be reported in due course.

**EXPERIMENTAL PROCEDURES**

**Materials.** 8-Hydroxy-quinoline (54; cat. no. 252565, 99% purity), 2-methyl-8-hydroxy-quinoline (55; cat. no. H57602, 98% purity), 5,7-dichloro-2-methyl-8-hydroxy-quinoline (61; cat. no. 130532, 98% purity), and 5-chloro-7-iodo-2-methyl-8-hydroxy-quinoline (59; cat. no. PH004692) were purchased from Sigma-Aldrich, 5-chloro-7-iodo-8-hydroxy-quinoline (58; cat. no. A11137, 98% purity) was purchased from Alfa Aesar, 5,7-dichloro-8-hydroxy-quinoline (60; cat. no. 11343, 99% purity) was obtained from Acros Organics. All ChemBridge compounds were certified to have met the high quality standard of ≥90% NMR identification and had a purity level of at least 90%. Purity of ChemDiv compounds was confirmed by the supplier by LC/MS and NMR and had a purity of >95%. All synthesized compounds assayed for enzyme activity were purified by semipreparative reversed phase HPLC and found to be greater than 95% pure based on analysis by 300 MHz. $^1$H NMR and analytical scale reversed phase HPLC with diode array detection.

**General Procedures.** LC/MS data were determined with a Waters Alliance 2695 HPLC/MS (Waters Symmetry C18, 4.6 × 75 mm, 3.5 μm) with a 2996 diode array detector from 210–400 nm; the solvent system is 5%–95% MeCN in water (with 0.1% TFA) over nine minutes using a linear gradient, and retention times are in minutes. Mass spectrometry was performed on a Waters ZQ using electrospray in positive mode. Preparative reversed phase HPLC was performed on a Waters Sunfire column (19 × 50 mm, C18, 5 μm) with a 10 min mobile phase gradient of 10% acetonitrile/water to 90% acetonitrile/water with 0.1% TFA as buffer using 214 and 254 nm as detection wavelengths. Injection and fraction collection were performed with a Gilson 215 liquid handling apparatus using Trilution LC software.

7-((Pyridin-2-yl)amino)methyl)quinolin-8-ol (57).

To a solution of 2-aminopyridine (33 mg, 0.23 mmol) and 8-hydroxyquino-
line-7-amine (24 mg, 0.23 mmol, 23 μL) and 2-
aminopyridine (22 mg, 0.23 mmol) in 95% ethanol (1 mL) was added a solution of benzaldehyde (24 mg, 0.23 mmol, 23 μL) and 59 (57).

**Aqueous Solubility.** Aqueous solubility was determined by the method of Zhou et al. using a miniaturized shake flask approach at pH 1.0 and 6.8 and an analyte concentration of 1.0 mM. Aqueous solutions of analyte were incubated at room temperature in the chamber of a Whatman (Piscataway, NJ) Mini-UniPrep syringeless filter for 24 h while shaking gently (600 rpm). Subsequent to incubation, filter plungers were pushed down to the bottom of the syringeless filter chamber assemblies, allowing filtrate to enter the plunger compartment. Following an additional 30 min incubation at room temperature, filtrates were diluted with 50:50 acetonitrile/water + 0.1% formic acid and analyzed by LC–MS/MS. Analyte concentrations were determined by the interpolation of peak area ratio from a calibration curve formed by matrix spiked with authentic reference material, over a calibration range of 0.05–12.5 μM (pH 6.8) or 0.5–2500 μM (pH 1.0).

**LC–MS/MS Analysis.** LC–MS/MS analysis was conducted using an Agilent (Santa Clara, CA) 6460 triple quadrupole mass
spectrometer coupled with an Agilent liquid chromatography (LC) system. The LC system consists of a binary pump, degasser, column heater, and autosampler. Chromatographic separation was performed on a Waters XBridge C18 (3.5 μm, 3.0 × 50 mm) or an Agilent Zorbax SB-Aq (4.6 × 50 mm) analytical column using a ballistic gradient of mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 1.0 mL/min. Mobile phase was heated to a temperature of 45 °C.

**ASSOCIATED CONTENT**

1. **Supporting Information**

Inhibitory activities of additional 8-hydroxy-quinolines; structures of compound 57 and its inactive analogue. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

BoNT, botulinum neurotoxin; LC, light chain metalloprotease; SAR, structure–activity relationship; ADME, absorption, distribution, metabolism, excretion

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