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4-Amino-7-chloroquinolines: probing ligand efficiency provides botulinum neurotoxin serotype A light chain inhibitors with significant antiprotozoal activity

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Abstract

Structurally simplified analogs of dual antimalarial and botulinum neurotoxin serotype A light chain (BoNT/A LC) inhibitor bis-aminoquinoline (**1**) were prepared. New compounds were designed to improve ligand efficiency while maintaining or exceeding the inhibitory potency of **1**. Three of the new compounds are more active than **1** against both indications. Metabolically, the new inhibitors are relatively stable and non-toxic. Twelve, **14**, and **15** are more potent BoNT/A LC inhibitors than **1**. Additionally, **15** has excellent *in vitro* antimalarial efficacy, with IC₉₀ values ranging from 4.45-12.11 nM against five *Plasmodium falciparum* (*P.f.*) strains: W2, D6, C235, C2A, C2B. The results indicate that the same level of inhibitory efficacy provided by **1** can be retained/exceeded with less structural complexity. Twelve, **14**, and **15** provide new platforms for the development of more potent dual BoNT/A LC and *P.f.* inhibitors adhering to generally accepted chemical properties associated with the druggability of synthetic molecules.

Keywords

Botulinum neurotoxin; malaria; aminoquinoline inhibitors; dual activity; chemotherapy; cytotoxicity

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ASSOCIATED CONTENT

Supporting information

Two procedures for the determination of the purity of tested compounds are given. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

The compounds presented herein inhibit both the botulinum neurotoxin serotype A light chain (BoNT/A LC) metalloprotease and protozoa *Plasmodium falciparum* (*P.f.*). Accordingly, the Introduction first describes the two targets, and subsequently provides an overview of 4-amino-7-chloroquinoline-based compounds that act as dual BoNT/A LC inhibitors and as antimalarial agents.

Botulinum neurotoxins

Botulinum neurotoxins (BoNTs) are the most potent of bacterial toxins.¹ Seven BoNT serotypes (A-G) are known, and all are initially secreted as polypeptide chains with molecular weights of approximately 150 kDa.² Proteolytic processing results in the active form of the toxins: a disulfide-linked dimer composed of a 100 kDa heavy chain (HC) and 50 kDa light chain (LC).³ The HC binds to pre-synaptic membrane receptors and transports the LC into the neuronal cytosol. The LC is a Zn²⁺ metalloprotease, and depending on the BoNT serotype, cleaves one of three proteins: either synaptosomal-associated protein of 25 kDa (SNAP-25), vesicle-associated membrane protein (VAMP (also referred to as synaptobrevin)), or syntaxin.⁴ These three proteins compose the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex. The BoNT/A and /E LCs cleave SNAP-25,^{4a} the BoNT/C LC cleaves both SNAP-25 and syntaxin, and the BoNT/B, /D, /F, and /G LCs cleave VAMP (also referred to as synaptobrevin).^{4b-f} The BoNT/LC-mediated cleavage of any one of the three SNARE proteins inhibits the release of acetylcholine into neuromuscular junctions, resulting in the flaccid paralysis associated botulism.⁵

Due to ease of production, dissemination, and lethality,⁶ BoNTs are classified as Category A biothreat agents by the US Centers for Disease Control and Prevention (CDC).⁷ Yet the only treatment available for BoNT poisoning at this time is the administration of antitoxin vaccines.^{6,8} However, the vaccines afford prophylactic protection only. Hence, the only life-saving option available (post-neuronal intoxication) is expensive and resource limited long-term mechanical ventilation.⁹ Consequently, there has been/is a significant interest in the development of small molecule inhibitors that can effectively antagonize BoNT/LC proteolytic activity, with the vast majority of research focusing on the discovery and development of inhibitors of the BoNT/A LC (the longest acting of the BoNT/LC serotypes in the neuronal cytosol).^{6,10} As a part of the scientific effort to develop BoNT/A LC countermeasures, 4-amino-7-chloroquinoline (4,7-ACQ)-based compounds have, to date, been identified as some of the most potent, non-Zn²⁺ chelating inhibitors of this LC serotype (Figure 1).^{11,12,13,28}

Malaria

Despite decades of research, malaria remains a devastating tropical disease resulting in over one million deaths each year.¹⁴ Moreover, the prevention and treatment of malaria is becoming increasingly more complicated by the spread of multidrug resistant *P. f.* strains to readily available drugs, and in particular chloroquine (CQ),¹⁵ which has been the standard antimalarial chemotherapeutic for many years¹⁶.

During the complex lifecycle of *P. f.* there are several stages during which therapeutic intervention could be effective.¹⁷ However, since the clinical symptoms of malaria occur during the parasite's asexual intra-erythrocytic phases, the majority of antimalarial drugs target this stage of the lifecycle. Specifically, during the blood stage, protozoa infest red blood cells and digest hemoglobin (Hb) within an acidic food vacuole (FV). Subsequently, two types of proteolytic byproducts are released: 1) small peptides that are transported to the

cytoplasm and 2) the toxic heme moiety ferriprotoporphyrin-IX (Fp(III)-IX). The parasite possesses defenses that protect it against the toxicity of Fp(III)-IX.¹⁸ One involves the sequestering of the toxic heme byproduct into insoluble hemozoin. Two additional mechanisms of detoxification¹⁹ include hydrogen peroxide mediated decomposition of the heme in the FV,^{20,21} and a cytosolic, glutathione-mediated mechanism of heme degradation.²² All 4,7-ACQ-based antimalarial agents, such as CQ, act as inhibitors of hemozoin formation by complexing with the Hb heme. Additionally, it has been suggested that 4,7-ACQ-based drugs may also act as inhibitors of oxidative²³ and glutathione-mediated²⁴ heme degradation. However, it is important to note that the heme detoxification pathway is not directly involved in CQ resistance. Rather, it has been reported that CQ resistance is associated with mutations in drug transporters (PfCRT, Pgh1, and PfMRP) that affect drug accumulation in the parasite—either by reducing drug uptake, or increasing drug efflux, or both.^{17,25} Interestingly, a recent study has indicated that, while reduced CQ accumulation appears to be central to CQ resistance, variable CQ plasma level concentrations in patients is the underlying factor resulting in the evolution of the emergence of CQ resistant *P. f.* strains.²⁶ But regardless of evolutionary cause, research to develop novel 4,7-ACQ-based compounds that bypass CQ resistance is resulting in the synthesis of potentially promising new antimalarial agents, including bisquinolines, side-chain modified 4,7-ACQ-based derivatives, and hybrid 4,7-ACQ compounds.^{18,27}

Dual target inhibition

During the screening of numerous libraries of small molecules to identify lead BoNT/A LC inhibitors,¹¹ we discovered that *N,N*-bis(7-chloroquinolin-4-yl)heteroalkanediamines can provide up to 60% inhibition of the enzyme at 20 μ M concentration (the most potent of the examined compounds, **1**, is shown in Figure 1). This led to the evaluation of antimalarial drugs (Amodiaquine, CQ, Quinacrine, Quinine, and Quinidine) in the BoNT/A LC assay, as an FDA approved drug can be fast-tracked as a treatment for a new indication; however, all were found to be poor inhibitors of the BoNT/A LC.¹¹ Subsequently, we continued to evaluate a variety of 4,7-ACQ-based antimalarial agents to identify novel BoNT/A LC inhibitors (Figure 1).^{12,28} Over time, these discoveries have led to a paradigm whereby 4,7-ACQ-based antimalarial agents are chemically modified with the intent of also generating novel BoNT/A LC inhibitors, and vice versa.¹² Herein we describe a new set of compounds that are derivatives of bis-4,7-ACQ-based antimalarial **1**²⁹ (Figure 1), which inhibits that BoNT/A LC by 60% at 20 μ M concentration.^{11,12,30} However, unlike **1** and other bis-4,7-ACQ-based compounds,^{11,29} the derivatives described in this study were designed to be more 'ligand efficient,' consisting of a single 4,7-ACQ-component tethered via a short nitrogen containing methylene bridge to a single aromatic ring (either a benzene, mono-substituted benzenes, pyridine, or mono-substituted pyridines). Several of the new compounds inhibit both the BoNT/A LC and CQ-resistant *P. f.* strains with potencies that are equivalent to, or greater than, the potency of **1**.

RESULTS & DISCUSSION

Rationale

Repurposing biologically active compounds (with known indications) to identify additional mechanisms of action through which they may be used to inhibit/treat other targets/pathogens is increasingly being pursued as a cost-effective approach to for treating both orphan diseases (*e.g.*, botulism) and diseases that mainly affect third world populations (*e.g.*, malaria).³¹ For example, miltefosine, which was developed to treat breast cancer, was subsequently discovered, via repurposing studies, to be an effective therapeutic for visceral leishmaniasis (a third world disease that is transmitted by sand flies).³² Another example of repurposing is the discovery that Adefovir dipivoxil, a drug used to treat chronic hepatitis B,

is a potent and competitive inhibitor of anthrax edema factor, which contributes to anthrax pathogenesis.³³ Importantly, these examples indicate that a unifying mechanism to rationalize the activities of biologically active compounds that are against more than one target is not paramount for pursuing potentially beneficial research discoveries.

As indicated in the Introduction (*vide supra*), we previously discovered that antimalarial agents such as **1** – **3** (Figure 1) can also inhibit the BoNT/A LC *in vitro*.^{11,12,28,29} Since, we have continued to evaluate antimalarial agents synthesized in our laboratories for BoNT/A LC inhibition, and vice versa. This approach is a beneficial strategy in that it allows for the efficient exploration of new compound designs, for both indications – alone or in tandem – that would have not been considered in the absence of the dual inhibition testing paradigm. For example, had antimalarial **3** (Figure 1) not been tested for BoNT/A LC inhibitory activity, we would not have discovered that compounds possessing a cholate substructure can inhibit this enzyme. Moreover, even though it has been found that 4,7-ACQ-based compounds inhibit the BoNT/A LC and malaria by different mechanisms of action (*i.e.*, inhibition of enzymatic activity (in the case of the presented BoNT/A LC inhibitors) and inhibition of hemozoin formation (in the case of the presented antimalarial agents), this does not preclude simultaneous optimization for both indications. In this particular study, the optimization effort focused on ‘ligand efficiency’ and how it can be applied to simultaneously develop new molecular scaffolds that inhibit both targets (*vide infra*).

Design strategy

Previous studies indicating that a 4,7-ACQ component tethered by a flexible methylene chain to different substructures, including a second 4,7-ACQ component,¹³ cholic acid derivative,^{12,28} or adamantane¹² (Figure 1), can act as both antimalarial agents and BoNT/A LC inhibitors, led us to consider the preparation of structurally simplified (lower molecular weight (MW) and flexibility), more ligand efficient derivatives to ascertain if we could maintain the same (or greater) BoNT/A LC inhibitory potency as **1** (Figure 1, Table 1), while concomitantly also retaining effective antimalarial efficacy. Specifically, as shown in Figure 2, we started by evaluating **1**, and then **2**, in the context of our gas-phase pharmacophore for BoNT/A LC inhibition.¹¹ As shown in Figure 2, **1** and **2** occupy pharmacophore zones 2 and 3. We deliberately did not explore increasing the size of the compounds to encompass pharmacophore zone 1 because we have found that compounds designed to occupy all three pharmacophore zones, although resulting in more potent BoNT/A LC inhibitors *in vitro*,¹³ gravitate toward high MW and excessive flexibility¹³ – both of which are factors that potentially drive synthetic compounds out of the sphere of druggability. When comparing **1** and **2** in pharmacophore zone 2, it is clear that possessing an N1-(7-chloroquinolin-4-yl)propane-1,3-diamine in this zone results in increased BoNT/A LC inhibition (versus an N1-(7-chloroquinolin-4-yl)ethane-1,2-diamine component (*i.e.*, **1** versus **2**, respectively (Figure 2)), with the inclusion of an aliphatic ionizable nitrogen being tantamount for inhibitory activity. Both of these observations have been corroborated in a previous study.¹¹ Moreover, we have also found that that when tested as fragments, N1-(7-chloroquinolin-4-yl)ethane-1,2-diamine (**4**) and N1-(7-chloroquinolin-4-yl)propane-1,3-diamine (**5**),²⁸ as well as CQ,¹¹ are poor BoNT/A LC inhibitors. Therefore, it was known that a single 4,7-ACQ component tethered to a methylene chain containing an ionizable amine is not structurally sufficient to provide meaningful BoNT/A LC inhibition at 20 μ M. Hence, as shown in Figure 2, a second ring system is required as a pharmacophore zone 3 component, with an aromatic sub-structure being favored over an aliphatic ring system. Of central importance to our design efforts, inhibition data for **2** shows that tethering a zone 3 component to the terminal aliphatic nitrogen of the zone 2 component with a single methylene bridge provides increased antimalarial activity, versus **1**, while still providing BoNT/A LC inhibition (Figure 2). This was key to our design strategy, as it indicated that

we could potentially improve ligand efficiency, while retaining inhibitory potency against both targets, by reducing the number of rotatable bonds found in the linker of **1** (Figure 2). Additionally, based on the fact that adamantane can serve as the zone 3 ring component, it was envisioned that replacing either it, or the 4,7-ACQ component of **1**, with a single aromatic ring might also maintain inhibitory efficacy against both targets, while simultaneously improving ligand efficiency (Figure 2). Based on the above observations, a strategy whereby either benzene, mono-substituted benzenes, pyridine, or mono-substituted pyridines –synthetically tethered to a 4,7-ACQ component (via shorter methylene bridges containing a aliphatic amine) - was initiated. The purpose of using benzene and pyridine as the second structural aromatic component was to ascertain the necessity of the nitrogen heteroatom of the second 4,7-ACQ of **1** as it might apply to the new designs; the purpose for evaluating mono-substituted benzene and pyridines species was to probe molecular diversity with respect to the effects of ring substituents on inhibitory potencies. Finally, in the broader sense of ligand efficiency as it pertains to the accessibility of compound preparation, we also considered two additional conditions: 1) the starting materials should be readily available from commercial sources and 2) final product generation should involve only 2 – 3 synthetic steps.

Chemistry

With the exception of **20** – **23**, all compounds were synthesized by coupling commercially available aryl aldehydes with aminoquinolines via reductive amination (Scheme 1). Simple method modifications were required to generate **20** – **23**. The synthesis of **20** and **21** involved: 1) oxidizing (4-chloropyridin-3-yl)methanol³⁴ into an aldehyde intermediate and 2) coupling with the 4,7-ACQ component via reductive amination (Scheme 2). Methoxy substituted derivatives **22** and **23** were obtained in high yield following substitution of the chlorine atom at the aromatic C(4) position of **20** and **21**, respectively. In both Schemes 1 and 2, the yield of the isolated products is provided.

Biological activity

For BoNT/A LC inhibition, the results for the new compounds were compared with previously reported bis-4,7-ACQ inhibitor **1** (Figure 1 and Table 1) as the control (since it is structurally most applicable with respect to BoNT/A LC inhibition and improving ligand efficiency).¹¹ For antimalarial studies, well established standards artemisinin (ART), CQ, and mefloquine (MFQ) were used for comparative purposes. Furthermore, a select sampling of the compounds was examined for toxicity in HepG2 cells and for *in vitro* metabolism in mouse and human liver microsomes. Finally, IC₉₀ values against five *P. f.* strains were determined for two of the most potent compounds.

Inhibition of the BoNT/A LC

All synthesized compounds were examined for % BoNT/A LC inhibition (Table 1) at 20 μ M concentration with a previously described HPLC-based assay that uses an N-terminal acetylated, C-terminal aminated synthetic peptide identical in sequence to residues 187 – 203 of SNAP-25 as the substrate.¹¹ Overall, compounds containing benzene or mono-substituted benzene were generally more potent BoNT/A LC inhibitors than those possessing a pyridine ring. These results appear to indicate that the quinoline heteroatom nitrogen of **1** is not tantamount for providing inhibitor potency. Moreover, for the benzene derivatives, substitution on the aromatic C(4) position with a fluorine (**12**) or a bromine (**14** and **15**) resulted in more potent enzyme inhibition than control for comparison **1** (Table 1).¹¹ By contrast, a tert-butyl or methoxy substitution on the aromatic C(4) (**8** – **11**, respectively) reduced inhibitory efficacy versus **1**.

In general, as indicated above, the pyridine derivatives (Table 1) are less potent than the benzene derivatives, and in particular, **6** – **9** and **12** – **15**. It is hypothesized that the reduced activities of the pyridine derivatives results from unfavorable contact(s) between the polar nitrogens of the compounds' pyridine rings and enzyme residue(s) in the optimal binding site(s) of the tethered aromatic components. For example, the nitrogens in the derivatives' pyridine rings may be engaging in either unfavorable hydrophobic-polar or base-base interactions with surrounding enzyme residues. Moreover, the inhibition data indicates that the new compounds' pyridine components are not occupying the same binding site as that of the nitrogen containing ring of the second 4,7-ACQ component of **1**; otherwise it would be expected that incorporating a pyridine group would result better potency. However, this is not surprising when taking into account that the longer, more flexible linker separating the two 4,7-ACQ components of **1**, versus the shorter, more restrictive tethers between the 4,7-ACQ and aromatic components of the compounds presented herein, permits **1** to sample binding site locations that are not accessible to the new derivatives. In this regard, it is notable that within the pyridine derivatives there is a trend in which compounds with a methylene tether with $n = 2$ (i.e., **17**, **19**, **21**, and **23** (Table 1)), which results in three methylene groups spanning between the 4-amino group of the 4,7-ACQ component and the linker cationic amine, are all more active than corresponding derivatives with $n = 1$ (i.e., **16**, **18**, **20** and **22** (Table 1)). This is consistent with previous data showing that three methylene chains flanking the central amine in the tether of **1** provides increased inhibitory potency versus compounds possessing shorter methylene spacers.¹¹ Furthermore, substitution on the pyridine rings did not result in inhibitor potencies comparable to that of **1** (Table 1).

Taken together, the structure activity data shown in Table 1 indicates that an additional substructure, such as benzene ring, tethered to a 4,7-ACQ component (via an amino containing methylene linker) is critical for the inhibitory potency of this class of non-Zn²⁺ chelating BoNT/A LC inhibitor. Importantly, using a reductionist synthetic strategy, the data shown in Table 1 demonstrates that BoNT/A LC inhibitory efficiency can be maintained with less 'structure,' as **12**, **14**, and **15** are more potent inhibitors in the *in vitro* assay (or biologically equivalent in general terms) compared with control **1**, as well as previously reported **2** (Figure 1) (i.e., the level of inhibition obtained with the new inhibitors is done so with improved structural efficiency).

Antimalarial activity

Antimalarial results from the *in vitro* screening of the compounds against three *P. f.* strains: D6 (CQ susceptible (CQS) strain), W2 (CQ resistant (CQR) strain) and TM91C235 (multi-drug resistant (MDR) strain), are shown in Table 1. The compounds provided a range of antimalarial activities (Table 1).³⁵ Several of are very potent against the CQS D6 strain, with compounds **6**, **10**, **11**, **14**, **15**, **17**, and **23** (Table 1) being more potent than ART, MFQ, and CQ (IC₅₀ < 9 nM). In addition, all of the derivatives are more active against the CQR W2 strain than CQ, with one compound (**15**) being as potent as both ART and MFQ (Table 1). Furthermore, all of the compounds are also more active than CQ against the MDR TM91C235 strain, while **6**, **10**, **14**, **15** are 4 – 8-fold more active than MFQ against this strain, and **6**, **14**, and **15**, are more potent than ART. The excellent antimalarial activities of many of the synthesized compounds from this study against the resistant CQR W2 and MDR TM91C235 strains may indicate that these 4,7-ACQ derivatives are not recognized by parasitic proteins involved in either drug uptake, drug efflux, or both.

Three compounds were particularly interesting with respect to their antimalarial activities: benzene derivative **6**, and 4-bromophenyl derivatives **14** and **15**.

Compound **6** is very active against the CQS D6 strain ($IC_{50} = 6.41$ nM), it is 60 and 30 times more potent than CQ against the CQR W2 strain and MDR TM91C235 strains, respectively; it is 9 times more active than MFQ against the MDR TM91C235 strain, and is slightly more active than ART against the same strain. 4-Bromophenyl aminoquinoline derivatives **14** and **15** are as active as MFQ against the CQR W2 strain ($IC_{50} = 5.93$ nM), >3 times more active against the CQ D6 strain than CQ, and 2-fold more active than ART against the MDR TM91C235 strain. Moreover, taking into account that the compounds presented herein are 4,7-ACQ-based, it is interesting to note that **15** is >100 times more active than CQ against the CQR W2 strain, >3 times more potent than CQ against the CQS D6 strain, and 38 times more active against the MDR C235 strain than CQ.

In contrast to the ACQ-phenyl analogs discussed above, pyridine derivatives **16** – **23** are less active against the three examined *P. f.* strains (Table 1). However, it is noteworthy that: 1) **17** is more potent, or equipotent, against the CQS D6 strain compared to CQ, MFQ, and ART, respectively (Table 1) and 2) **23** is more potent against the CQS D6 strain versus CQ, MFQ, and ART (Table 1). Again, as with BoNT/A LC inhibition, the data in Table 1 indicates that less chemical structure, *i.e.*, compared with our previously reported 4,7-ACQ-based inhibitors (Figure 1), can be used to attain significant antimalarial activity. This point further indicates that, via synthetic modification, ligand efficiency can be used to guide the generation of more potent or equipotent structurally simplified, low molecular weight 4,7-ACQ-based antimalarials.

Toxicity and Metabolic Stability

The 18 reported compounds were evaluated for toxicity using human liver carcinoma cell line HepG2, and relatively low toxicities were observed (IC_{50} values ranged between 2,000 – 16,000 nM (Table 1)). Not surprisingly, the results indicated that less potent BoNT/A LC inhibitors and less active antimalarials agents are generally less toxic to HepG2 cells. Therefore, relative toxicities (*i.e.*, toxicities versus activities with respect to the three examined *P. f.* strains) were evaluated. The corresponding data are given in Table 1 as selectivity indices (SI), and many of the compounds have SI values >400, which is generally considered the lowest SI value when considering a compound for *in vivo* screening. It was gratifying to observe that two of the most potent BoNT/A LC and *P. f.* inhibitors, **14** and **15**, have SI values well above 400, and that **6**, which also provides > 50% BoNT/A LC inhibition and was equal/more potent than either ART, CQ, MFQ against the three examined *P. falciparum* strains, possesses an SI ranging from 1,700 – 3,000 (Table 1).

Metabolic stability studies using both mouse and human liver microsomes (MLM and HLM, respectively) were performed on 12 of the compounds to assess their half-lives ($t_{1/2}$), a key pharmacokinetic parameter that is critical for drug development (Table 1). The most stable of the examined compounds ($t_{1/2}$ 60 min in both HLM and MLM) were **7**, **14**, **15**, **19**, and **23** (Table 1). Of these, when considering inhibition of the BoNT/A LC, activity against the three *P. f.* strains, SI, and microsomal stability, the most promising compounds for more in-depth biological evaluation are bromo substituted derivatives **14** and **15** (Table 1). Specifically, **14** and **15**: 1) are both metabolically stable in vitro, with $t_{1/2}$ 60 min in both HLM and MLM, 2) inhibit the BoNT/A LC by 69 % and 68%, respectively, at 20 μ M concentration (both of these inhibitory potencies are slightly higher than that of control for comparison **1** (Table 1), 3) provide excellent antimalarial activity against the three examined *P. f.* strains – *i.e.*, **14** and **15** are biologically equal to, or more active than, the 3 controls: ART, CQ, and MFQ, and 4) possess SI values ranging from 650 – 1600 nM and 800 – 1250 nM, respectively. Additionally, **7**, which inhibits the BoNT/A LC by > 50 % at 20 μ M concentration, and which is more potent against CQS *P. f.* strain D6 than CQ and MFQ, and **23** a moderate inhibitor of the BoNT/A LC (43.23% at 20 μ M), but a more potent inhibitor

of CQS *P. f.* D6 than ART, CQ, and MFQ (Table 1), also possess in vitro $t_{1/2}$ 60 min in both HLM and MLM. However, both have less than optimal HepG2 cellular toxicity profiles. Finally, **6**, which inhibits the BoNT/A LC by > 50% at 20 μ M, and which is more potent than ART, CQ and MFQ against CQS *P. f.* strains D6 and MDR TM91C235, but slightly less potent than ART and MFQ against the CQR W2 strain, is metabolically stable when exposed to HLM ($t_{1/2}$ 60 min), but less stable when exposed to MLM ($t_{1/2}$ = 23 min, Table 1).

Detailed studies to further evaluate the antimalarial efficacies of **6** and **15**

Of the dual BoNT/A LC and *P. f.* inhibitors, **6**, **14**, and **15** were the most potent. However, with regard to analogs **14** and **15**, both are effectively equipotent as BoNT/A LC inhibitors, but **15** is a slightly more effective antimalarial against the three examined *P. f.* strains versus **14**, and as a result possesses a better SI ratio against CQR W2 and MDR TM91C235, and retains a very high SI ratio against D6 CQS (Table 1). Based on these observations, and the desire to examine inhibitors with different bridge lengths and no ring substitution versus a bromo substitution, **6** and **15** were further evaluated against two additional, virulent *P. f.* strains: MDR TM90C2A and MDR TM90C2B. Both are resistant to CQ, MFQ, and pyrimethamine. Note: for these studies, both IC₅₀ and IC₉₀ values for **6**, **15**, CQ, and MFQ, were calculated against the two additional strains (Table 1).

As shown in Table 1, both **6** and **15** are significantly more potent antimalarial agents against *P. f.* strains TMR TM90C2A and TMR TMC90C2B as compared to CQ and MFQ. Moreover, Table 1 shows that the IC₉₀ values of **6** and **15** are significantly lower than those of CQ and MFQ versus CQR W2, CQS D6, and MDR TM91C235. As touched upon above, when compared to their antimalarial activities, the toxicities of **6** and **15** in HepG2 cells is low with respect to their potent antimalarial efficacies – as indicated by the SI values shown in Table 1. Based on these results, both **6** and **15**, are good candidates for future *in vivo* studies.

CONCLUDING REMARKS

We have previously described 4,7-ACQ-based antimalarial agents that are also efficacious BoNT/A LC inhibitors.¹² Structurally, all of the reported compounds possess a 4,7-ACQ component linked by a flexible tether to a second structural component – either a second aminoquinoline, cholic acid derivative, or adamantane moiety (Figure 1). Moreover, for all reported chemotypes, the aforementioned flexible tether possesses an aliphatic secondary amine.²⁸ The goal of this study was to improve upon the ‘ligand efficiency’ of previously reported bis-4,7-ACQ-based inhibitors **1** by synthesizing a series of simplified analogs (which also took the structure-activity data for **2** into account) possessing a 4,7-ACQ component tethered to either a non-substituted, or mono-substituted, benzene or pyridine component – versus a second 4,7-ACQ component (as observed in the structure of **1**) or and adamantane (as observed for **2**). The syntheses were straightforward and achievable in 2 – 3 steps with commercially available starting material, which is desirable when developing potentially therapeutic synthetic agents.

Moreover, and importantly, the inhibitory potencies of the new derivatives were achieved with less structural complexity (*i.e.*, versus **1** and **2**), thereby facilitating the possibility of synthetically incorporating new structural motifs that will improve potencies without exceeding acceptable MWs. For example, future synthetic efforts will focus on di-substituted derivatives of the *N*-(benzyl-*N*3-(7-chloroquinolin-4-yl)propane-1,3-diamine structure geared toward increasing BoNT/A LC inhibition. Moreover, since this molecular scaffold also results in potent antimalarial agents, any new derivatives will also be evaluated

against resistant *P. f.* strains. This will continue to result in an efficient feedback loop in which the inhibitor activities are constantly being improved.

Additionally, the observed antimalarial activities of the compounds reported in this study is likely attributable to the inhibition of hemozoin formation,³⁶ and the generally excellent *in vitro* activities of several of the simplified 4,7-ACQ analogs against the CQR and MDR *P. f.* strains, as compared to ART, CQ, and MQF, could be due to the fact that the new 4,7-ACQ-based derivatives are not as well recognized by protozoa proteins involved in either drug uptake or drug efflux.

Finally, of the compounds described herein, **6** and **15**, overall (i.e., potencies, toxicities, SI values) are, good candidates for future *in vivo* testing against drug resistant *P. f.* strains. This is an important point, as these compounds would not have been developed if both BoNT/A LC inhibition and antimalarial activity had not been considered during the design of these compounds.

EXPERIMENTAL SECTION

Chemistry

Melting points were determined on a Boetius PMHK apparatus and were not corrected. IR spectra were recorded on a Thermo-Scientific Nicolet 6700 FT-IR Diamond Crystal. NMR: ¹H and ¹³C NMR spectra were recorded on a Varian Gemini-200 spectrometer (at 200 and 50 MHz, respectively) and/or a Bruker Ultrashield Advance III spectrometer (at 500 and 125 MHz, respectively) in the indicated solvent using TMS or TMS-Na salt as the internal standard. Chemical shifts are expressed in ppm (δ) values and coupling constants (*J*) in Hz. ESI MS spectra of the synthesized compounds were recorded on an Agilent Technologies 6210 Time-of-Flight LC/MS instrument in positive ion mode using CH₃CN/H₂O = 1/1 with 0.2 % HCOOH as the carrying solvent solution. The samples were dissolved in pure acetonitrile (HPLC grade). The selected values were as follows: capillary voltage = 4 kV; gas temperature = 350 °C; drying gas = 12.1 min⁻¹; nebulizer pressure = 45 psig; fragmentator voltage = 70 V.

General procedure

Benzaldehyde (200 mg, 1.88 mmol) and **4** (627 mg, 2.83 mmol) or **5**¹² (667 mg, 2.83 mmol) were dissolved in 30 mL of a dry CH₂Cl₂ / MeOH mixture (1:2, v/v), anh. AcOH (162 μL, 2.83 mmol) was added, and the mixture was stirred under Ar atmosphere at room temperature. After 2.5 h, NaBH₄ (428 mg, 11.31 mmol) was added, and stirring at r.t. was continued for another 18 h. Solvent was removed under reduced pressure, and the remainder was dissolved in CH₂Cl₂ (100 mL). The organic layer was washed with 20 mL 2M NH₄OH and water, and was extracted with CH₂Cl₂ (2 × 15 mL). The combined organic layers were washed with brine and dried over Na₂SO₄. Finally, the solvent was removed under reduced pressure, and crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 95/5).

N-Benzyl-*N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (**6**)

Benzaldehyde (200 mg, 1.88 mmol) and **4** (627 mg, 2.83 mmol) were coupled to afford **6** (470 mg, 76%) using AcOH (162 μL, 2.83 mmol) and NaBH₄ (428 mg, 11.31 mmol). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 95/5). Yield: 390 mg (66%). Pale yellow amorphous powder. Mp = 150–152 °C. IR (KBr): 3432m, 3242m, 3063w, 3022w, 2923w, 2853w, 1612m, 1581s, 1545m, 1491w, 1452m, 1430w, 1368w, 1329m, 1279w, 1251w, 1205w, 1168w, 1142w, 1109w, 1084w, 1027w, 879w cm⁻¹. λ_{max}(ε) = 327 (12700), 257 (23300) nm. ¹H NMR (200 MHz, CDCl₃): 8.55-8.45 (m, 1H),

7.94 (s, 1H), 7.70-7.60 (m, 1H), 7.40-7.20 (m, 5H), 6.36-6.32 (m, 1H), 5.88 (NH), 3.84 (s, 2H), 3.40-3.20 (m, CH₂NHCH₂CH₂NHAr), 3.10-2.90 (m, CH₂NHCH₂CH₂NHAr), 1.88 (NH). ¹³C NMR (50 MHz, CDCl₃): 151.98, 149.85, 149.01, 139.85, 134.75, 128.53, 128.05, 127.22, 125.16, 121.30, 117.31, 99.10, 53.24, 46.68, 41.91. (+)ESI-HRMS (*m/z*): [M + H]⁺ 312.12725 (error -3.35 ppm).

***N*-Benzyl-*N*-(7-chloroquinolin-4-yl)propane-1,3-diamine (7)**

Benzaldehyde (200 mg, 1.88 mmol) and **5** (667 mg, 2.83 mmol) were coupled to afford **7** (470 mg, 76%) using AcOH (162 μL, 2.83 mmol) and NaBH₄ (428 mg, 11.31 mmol). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 95/5). Pale yellow amorphous powder. Mp = 104–107 °C. IR (KBr): 3279m, 3068w, 3031w, 2950w, 2894w, 2837w, 1679w, 1608m, 1580s, 1540m, 1472w, 1451m, 1396w, 1367m, 1331w, 1285w, 1248w, 1203w, 1139m, 1102w, 1083w, 1027w, 1027w, 976w, 906w, 886w, 855w, 828w, 807w cm⁻¹. λ_{max}(ε) = 333 (9000), 259 (14600) nm. ¹H NMR (200 MHz, CDCl₃): 8.45-8.40 (m, 1H), 7.87 (s, 1H), 7.65 (NH), 7.60-7.40 (m, 1H), 7.40-7.20 (m, 5H), 7.08-7.02 (m, 1H), 6.26-6.21 (m, 1H), 3.79 (s, 2H), 3.40-3.25 (m, CH₂NHCH₂CH₂CH₂NHAr), 3.10-2.90 (m, CH₂NHCH₂CH₂CH₂NHAr), 2.29 (NH), 2.05-1.80 (m, CH₂NHCH₂CH₂CH₂NHAr). ¹³C NMR (50 MHz, CDCl₃): 151.83, 150.43, 148.83, 139.41, 134.44, 128.56, 128.27, 128.02, 127.31, 124.63, 122.26, 117.38, 98.08, 54.19, 49.16, 43.75, 27.09. (+)ESI-HRMS (*m/z*): [M + 2H]²⁺ 163.57536 (error 4.89 ppm), [M + H]⁺ 326.14368 (error - 5.61 ppm).

***N*-(4-*Tert*-butylbenzyl)-*N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (8)**

4-*Tert*-butylbenzaldehyde (200 mg, 1.23 mmol) and **4** (410 mg, 1.85 mmol) were coupled to afford **8** (300 mg, 66 %) using AcOH (159 μL, 2.77 mmol) and NaBH₄ (280 mg, 7.40 mmol). The crude product was purified using dry-flash chromatography (SiO₂: EtOAc/MeOH = 8/2). Yellow waxy compound. Mp = 42–45 °C. IR (ATR): 3255m, 3061m, 2961m, 2867m, 1611m, 1580s, 1535m, 1452m, 1368m, 1331m, 1275w, 1243w, 1205w, 1169w, 1139w, 1114w, 1080w, 878w, 806w cm⁻¹. ¹H NMR (200 MHz, CDCl₃): 8.52-8.47 (m, 1H), 7.95-7.93 (m, 1H), 7.73-7.66 (m, 1H), 7.40-7.22 (m, 5H), 6.37-6.32 (m, 1H), 5.93 (NH), 3.82 (s, 2H), 3.36-3.25 (m, CH₂NHCH₂CH₂NHAr), 3.10-3.00 (m, CH₂NHCH₂CH₂NHAr), 1.95 (NH), 1.30 (s, 9H). ¹³C NMR (50 MHz, CDCl₃): 152.00, 150.25, 149.87, 149.03, 136.80, 134.77, 128.60, 127.80, 125.43, 125.18, 121.30, 117.33, 99.14, 52.86, 46.72, 41.86, 34.41, 31.28. (+)ESI-HRMS: (*m/z*): [M + H]⁺ 368.18919 (error - 1.05 ppm).

***N*-(4-*Tert*-butylbenzyl)-*N*-(7-chloroquinolin-4-yl)propane-1,3-diamine (9)**

4-*Tert*-butylbenzaldehyde (200 mg, 1.23 mmol) and **5** (436 mg, 1.85 mmol) were coupled to afford **9** (430 mg, 91 %) using AcOH (159 μL, 2.77 mmol) and NaBH₄ (280 mg, 7.40 mmol). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 95/5). Pale yellow amorphous powder. Mp = 115–117 °C. IR (ATR): 3231m, 3146w, 3060m, 3019w, 2947w, 2921w, 2849w, 1610w, 1579s, 1544m, 1448m, 1425m, 1361m, 1327m, 1276w, 1247w, 1198w, 1166w, 1140w, 1115w, 1075w, 879w, 839w cm⁻¹. ¹H NMR (200 MHz, CDCl₃): 8.48-8.42 (m, 1H), 8.00–7.86 (m, 2H), 7.52-7.36 (m, 3H), 7.31-7.22 (m, 2H), 7.06-6.98 (m, 1H), 6.29-6.23 (m, 1H), 3.80 (s, 2H), 3.44-3.34 (m, CH₂NHCH₂CH₂CH₂NHAr), 3.05-2.96 (m, CH₂NHCH₂CH₂CH₂NHAr), 2.24 (NH), 2.02-1.86 (m, CH₂NHCH₂CH₂CH₂NHAr), 1.36 (s, 9H). ¹³C NMR (50 MHz, CDCl₃): 151.85, 150.58, 150.39, 148.85, 136.32, 134.57, 128.13, 128.07, 125.58, 124.78, 122.48, 117.46, 98.05, 53.86, 49.52, 44.06, 34.50, 31.32, 27.09. (+)ESI-HRMS: (*m/z*): [M + H]⁺ 382.20516 (error - 1.84 ppm).

***N*-(7-Chloroquinolin-4-yl)-*N'*-(4-methoxybenzyl)ethane-1,2-diamine (10)**

Anisaldehyde (100 mg, 0.73 mmol) and **4** (243 mg, 1.09 mmol) were coupled to afford **10** (211 mg, 85%) using AcOH (62 μ L, 1.09 mmol) and NaBH₄ (166 mg, 4.38 mmol). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 9/1). Oil. IR (ATR): 3299w, 2934w, 2905w, 2834w, 1610m, 1579s, 1533w, 1512s, 1450m, 1368w, 1330w, 1300w, 1279w, 1245s, 1206w, 1175w, 1138w, 1108w, 1080w, 1033m, 877w, 844w, 808m cm⁻¹. ¹H NMR (200 MHz, CDCl₃): 8.50 (d, *J* = 5.6 Hz, 1H), 7.93 (d, *J* = 2.2 Hz, 1H), 7.68 (d, *J* = 9.0 Hz, 1H), 7.35 (dd, *J* = 9.0 Hz, *J* = 2.2 Hz 1H), 7.28-7.20 (m, 2H), 6.92-6.82 (m, 2H), 6.34 (d, *J* = 5.6 Hz, 1H), 5.90 (NH), 3.80-3.70 (m, 5H), 3.37-3.22 (m, CH₂NHCH₂CH₂NHAr), 3.10-2.95 (m, CH₂NHCH₂CH₂NHAr), 1.99 (NH). ¹³C NMR (50 MHz, CDCl₃): 158.86, 151.98, 149.88, 149.01, 134.81, 131.86, 129.31, 128.60, 125.21, 121.30, 117.33, 113.92, 99.14, 55.24, 52.64, 46.56, 41.90. (+)ESI-HRMS (*m/z* (%)): [M + H]⁺ 342.12327 (error - 39.45 ppm).

***N*-(7-Chloroquinolin-4-yl)-*N'*-(4-methoxybenzyl)propane-1,3-diamine (11)**

Anisaldehyde (100 mg, 0.73 mmol) and **5** (258 mg, 1.09 mmol) were coupled to afford **11** (174 mg, 67%) using AcOH (62 μ L, 1.09 mmol) and NaBH₄ (166 mg, 4.38 mmol). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 9/1). Oil. IR (ATR): 3266w, 2931w, 2832w, 1609m, 1577s, 1510s, 1448s, 1367m, 1329m, 1300m, 1243s, 1174m, 1174m, 1079m, 1031s, 875m, 806s cm⁻¹. ¹H NMR (200 MHz, CDCl₃): 8.46 (d, *J* = 5.6 Hz, 1H), 7.89 (d, *J* = 2.2 Hz, 1H), 7.77 (NH), 7.51 (d, *J* = 9.0 Hz, 1H), 7.30-7.20 (m, 2H), 7.12 (dd, *J* = 9.0 Hz, *J* = 2.2 Hz, 1H), 6.95-6.85 (m, 2H), 6.27 (d, *J* = 5.6 Hz, 1H), 3.83 (s, 3H), 3.77 (s, 2H), 3.45-3.32 (m, CH₂NHCH₂CH₂CH₂NHAr), 3.02-2.92 (m, CH₂NHCH₂CH₂CH₂NHAr), 2.04 (NH), 2.10-1.85 (m, CH₂NHCH₂CH₂CH₂NHAr). ¹³C NMR (50 MHz, CDCl₃): 158.93, 151.98, 150.52, 148.99, 134.54, 131.59, 129.62, 128.25, 124.79, 122.34, 117.51, 114.00, 98.17, 55.28, 53.70, 49.22, 43.99, 27.17. (+)ESI-HRMS (*m/z*): [M + H]⁺ 356.13897 (error - 37.76 ppm).

***N*-(7-Chloroquinolin-4-yl)-*N'*-(4-fluorobenzyl)ethane-1,2-diamine (12)**

4-Fluorobenzaldehyde (200 mg, 1.61 mmol) and **4** (536 mg, 2.42 mmol) were coupled to afford **12** (510 mg, 96 %) using AcOH (138.5 μ L, 2.42 mmol) and NaBH₄ (366 mg, 9.67 mmol). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 95/5). White amorphous powder. Mp = 148–149 °C. IR (ATR): 3215m, 3066m, 3035m, 2951m, 2880w, 2830m, 1580s, 1510m, 1451m, 1430m, 1376m, 1332w, 1290m, 1240w, 1218m, 1164w, 1140w, 1100w, 1077w, 962w, 898w, 850m, 832w, 812m cm⁻¹. ¹H NMR (200 MHz, CDCl₃): 8.56-8.48 (m, 1H), 7.97-7.92 (m, 1H), 7.72-7.62 (m, 1H), 7.40-7.23 (m, 3H), 7.08-6.95 (m, 2H), 6.40-6.32 (m, 1H), 5.84 (NH), 3.81 (s, 2H), 3.40-3.24 (m, CH₂NHCH₂CH₂NHAr), 3.10-2.95 (m, CH₂NHCH₂CH₂NHAr) 1.63 (NH). ¹³C NMR (50 MHz, CDCl₃): 164.47, 159.59, 152.09, 149.79, 149.10, 135.59, 134.81, 129.70, 129.53, 128.74, 125.25, 121.15, 117.31, 115.54, 115.13, 99.20, 52.57, 46.72, 41.95. (+)ESI-HRMS (*m/z*): [M + H]⁺ 330.11677 (error - 0.02 ppm).

***N*-(7-Chloroquinolin-4-yl)-*N'*-(4-fluorobenzyl)propane-1,3-diamine (13)**

4-Fluorobenzaldehyde (200 mg, 1.61 mmol) and **5** (599 mg, 2.54 mmol) were coupled to afford **13** (450 mg, 81 %) using AcOH (138.5 μ L, 2.42 mmol) and NaBH₄ (366 mg, 9.67 mmol). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 95/5). Pale yellow amorphous powder. Mp = 107–109 °C. IR (ATR): 3370w, 3195m, 2947m, 2892m, 2848m, 1979w, 1605m, 1580s, 1540m, 1510m, 1450m, 1365m, 1329w, 1284w, 1225m, 1138w, 1086w, 853m, 822m cm⁻¹. ¹H NMR (200 MHz, CDCl₃): 8.46 (d, *J* = 5.1 Hz, 1H), 7.92-7.88 (m, 1H), 7.59-7.44 (m, 2H), 7.36-7.24 (m, 2H), 7.17-6.98 (m, 3 H), 6.28 (d, *J* = 5.1 Hz, 1H), 3.80 (s, 2H), 3.43-3.32 (m, CH₂NHCH₂CH₂CH₂NHAr),

3.00–2.90 (m, CH₂NHCH₂CH₂CH₂NHAr), 2.13 (NH), 2.20–1.86 (m, CH₂NHCH₂CH₂CH₂NHAr). ¹³C NMR (50 MHz, CDCl₃): 164.52, 159.64, 151.90, 150.43, 148.88, 135.24, 134.63, 130.02, 129.86, 128.27, 124.76, 122.04, 117.40, 115.64, 115.22, 98.25, 53.51, 49.09, 44.37, 43.81, 27.26. (+)ESI-HRMS (*m/z*): [M + 2H]²⁺ 172.56987 (error 0.09 ppm), [M + H]⁺ 344.13246 (error - 0.08 ppm).

***N*-(4-Bromobenzyl)-*N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (14)**

4-Bromobenzaldehyde (200 mg, 1.08 mmol) and **4** (359 mg, 1.62 mmol) were coupled to afford **14** (310 mg, 73%) using AcOH (93 μL, 1.62 mmol) and NaBH₄ (245 mg, 6.48 mmol). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 95/5). Pale yellow amorphous powder. Mp = 147–150 °C. IR (KBr): 3212m, 3061w, 3026w, 2933w, 2835w, 1580s, 1544m, 1487m, 1450m, 1428m, 1368m, 1329m, 1281w, 1247w, 1201w, 1139m, 1109w, 1074w, 1029w, 1010w, 881w, 843w, 807m cm⁻¹. ¹H NMR (200 MHz, CDCl₃): 8.52–8.47 (m, 1 H), 7.94 (d, *J* = 2.2 Hz, 1 H), 7.66 (d, *J* = 9.0 Hz, 1 H), 7.47–7.42 (m, 2 H), 7.34 (dd, *J* = 9.0 Hz, *J* = 2.2 Hz, 1 H), 7.22–7.17 (m, 2 H), 6.37–6.32 (m, 1 H), 5.82 (NH), 3.79 (s, 2 H), 3.40–3.20 (m, CH₂NHCH₂CH₂NHAr), 3.10–2.90 (m, CH₂NHCH₂CH₂NHAr), 1.85 (NH). ¹³C NMR (50 MHz, CDCl₃): 151.96, 149.81, 149.01, 138.85, 134.81, 131.58, 129.73, 128.58, 125.25, 121.19, 120.99, 117.27, 99.14, 52.62, 46.76, 41.99. (+)ESI-HRMS (*m/z*): [M + H]⁺ 390.03719 (error - 1.21 ppm).

***N*-(4-Bromobenzyl)-*N*-(7-chloroquinolin-4-yl)propane-1,3-diamine (15)**

4-Bromobenzaldehyde (200 mg, 1.08 mmol) and **5** (382 mg, 1.62 mmol) were coupled to afford **15** (330 mg, 76%) using AcOH (93 μL, 1.62 mmol) and NaBH₄ (245 mg, 6.48 mmol). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH = 95/5). Pale yellow amorphous powder. Mp = 129–132 °C. IR (KBr): 3304w, 3238m, 3060w, 3019w, 2938w, 2815w, 1610m, 1581s, 1539m, 1487m, 1454m, 1425m, 1362m, 1329m, 1281w, 1226m, 1136m, 1073w, 1010w, 885w, 845m, 809m cm⁻¹. ¹H NMR (200 MHz, CDCl₃): 8.47–8.42 (m, 1 H), 7.90–7.88 (m, 1 H), 7.50–7.40 (m, 3 H), 7.33 (NH), 7.20–7.05 (m, 3 H), 6.29–6.24 (m, 1 H), 3.75 (s, 2 H), 3.40–3.30 (m, CH₂NHCH₂CH₂CH₂NHAr), 3.05–2.80 (m, CH₂NHCH₂CH₂CH₂NHAr), 2.07 (NH), 2.05–1.80 (m, CH₂NHCH₂CH₂CH₂NHAr). ¹³C NMR (50 MHz, CDCl₃): 152.01, 150.30, 149.01, 138.52, 134.57, 131.69, 129.98, 128.40, 124.78, 121.90, 121.15, 117.38, 98.30, 53.53, 48.99, 43.70, 27.35. (+)ESI-HRMS (*m/z*): [M+2H]²⁺ 202.53012 (error - 1.48 ppm), [M+H]⁺ 404.05242, 90 (error 0.14 ppm).

***N*-(7-Chloroquinolin-4-yl)-*N*-(pyridin-4-ylmethyl)ethane-1,2-diamine (16)**

4-Pyridinecarboxaldehyde (200 mg, 1.86 mmol) and **4** (621 mg, 2.79 mmol) were coupled to afford **16** (430 mg, 74%) using AcOH (160 μL, 2.79 mmol) and NaBH₄ (422 mg, 11.16 mmol). Pale yellow amorphous powder. Mp = 178–183 °C. IR (ATR): 3424s, 2921m, 2851m, 1608m, 1582s, 1534m, 1452m, 1370m, 1332w, 1278w, 1247w, 1223w, 1166w, 1139m, 1099w, 1078w, 896w, 847m, 805m cm⁻¹. ¹H NMR (200 MHz, CD₃OD): 8.45–8.40 (m, 2H), 8.36–8.31 (m, 1H), 8.10–8.04 (m, 1H), 7.78–7.75 (m, 1H), 7.45–7.35 (m, 3H), 6.56–6.51 (m, 1H), 3.88 (s, 2H), 3.55–3.45 (m, CH₂NHCH₂CH₂NHAr), 3.00–2.90 (m, CH₂NHCH₂CH₂NHAr). ¹³C NMR (50 MHz, CDCl₃): 151.58, 149.92, 149.57, 148.94, 148.66, 134.72, 127.98, 125.05, 122.77, 121.44, 117.18, 98.90, 51.96, 46.92, 42.02. (+)ESI-HRMS (*m/z*): [M + H]⁺ 313.12220 (error 2.38 pm).

***N*-(7-Chloroquinolin-4-yl)-*N*-(pyridin-4-ylmethyl)propane-1,3-diamine (17)**

4-Pyridinecarboxaldehyde (200 mg, 1.86 mmol) and **5** (658 mg, 2.79 mmol) were coupled to afford **17** (330 mg, 54%) using AcOH (160 μL, 2.79 mmol) and NaBH₄ (422 mg, 11.16 mmol). Pale yellow amorphous powder. Mp = 116–122 °C. IR (ATR): 3426w, 3308w,

3210w, 3027m, 2957w, 2848w, 1607m, 1581s, 1535m, 1448m, 1365m, 1329m, 1280m, 1234m, 1164w, 1136m, 1106w, 1080w, 1046w, 1010w, 929w, 897w, 876w, 849m, 797m cm^{-1} . ^1H NMR (200 MHz, CD_3OD): 8.45-8.40 (m, 2H), 8.36-8.31 (m, 1H), 8.02-7.95 (m, 1H), 7.78-7.74 (m, 1H), 7.45-7.30 (m, 3H), 6.54-6.50 (m, 1H), 3.83 (s, 2H), 3.50-3.40 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NHAr}$), 2.80-2.70 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NHAr}$), 2.05-1.85 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NHAr}$). ^{13}C NMR (50 MHz, CDCl_3): 151.85, 150.19, 149.85, 148.85, 148.46, 134.52, 128.16, 124.63, 122.95, 121.77, 117.26, 98.32, 52.75, 48.72, 43.19, 27.44. (+)ESI-HRMS (m/z): $[\text{M} + \text{H}]^+$ 327.13661 (error - 1.50 ppm).

***N*-(7-Chloroquinolin-4-yl)-*N'*-(pyridin-3-ylmethyl)ethane-1,2-diamine (18)**

3-Pyridinecarboxaldehyde (214 mg, 2.00 mmol) and **4** (665 mg, 3.00 mmol) were coupled to give **18** (270 mg, 43 %) using AcOH (172 μL , 3.00 mmol) and NaBH_4 (454 mg, 12.0 mmol). The crude product was purified using dry-flash chromatography (SiO_2 : $\text{CH}_2\text{Cl}_2/\text{MeOH} = 95/5$). Yellow amorphous powder. Mp = 141–144 °C. IR (ATR): 3432m, 3234m, 3064m, 2922m, 1661w, 1582s, 1450m, 1426m, 1366m, 1328m, 1277m, 1208w, 1164w, 1135w, 1078w, 1026w, 950w, 895w, 864w, 829w. ^1H NMR (200 MHz, CDCl_3): 8.63-8.56 (m, 1H), 8.55-8.45 (m, 2H), 7.96-7.91 (m, 1H), 7.73-7.63 (m, 2H), 7.39-7.21 (m, 2H), 6.39-6.31 (m, 1H), 5.95 (NH), 3.87 (s, 2H), 3.41-3.28 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHAr}$), 3.11-2.95 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHAr}$), 2.71 (NH). ^{13}C NMR (50 MHz, CDCl_3): 151.74, 151.21, 149.88, 149.57, 148.72, 135.77, 135.10, 134.94, 128.34, 127.62, 125.36, 123.48, 122.23, 121.22, 117.22, 99.08, 97.96, 50.71, 46.88, 45.70, 41.97, 38.75, 23.03. (+)ESI-HRMS (m/z): $[\text{M} + \text{H}]^+$ 313.12011 (error - 4.30 ppm).

***N*-(7-Chloroquinolin-4-yl)-*N'*-(pyridin-3-ylmethyl)propane-1,3-diamine (19)**

3-Pyridinecarboxaldehyde (214 mg, 2.00 mmol) and **5** (707 mg, 3.00 mmol) were coupled to afford **19** (280 mg, 43 %) using AcOH (172 μL , 3.00 mmol) and NaBH_4 (454 mg, 12.0 mmol). The crude product was purified using dry-flash chromatography (SiO_2 : $\text{CH}_2\text{Cl}_2/\text{MeOH} = 9/1$). Yellow waxy compound. Mp = 69 – 72 °C. IR (ATR): 3259m, 2938m, 2842m, 1712w, 1583s, 1539m, 1475w, 1449m, 1428m, 1366m, 1331w, 1286w, 1248w, 1199w, 1133w, 1104w, 1086w, 1027w, 987w, 947w, 900m, 854m, 824w cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 8.60-8.45 (m 2H), 8.36 (d, $J = 5.6$ Hz, 1H), 7.86-7.83 (m, 1H), 7.78-7.65 (m 2H), 7.40-7.31 (m, 1H), 7.30-7.29 (m, 1H), 6.35 (d, $J = 5.6$ Hz, 1H), 3.86 (s, 2H), 3.45-3.35 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NHAr}$), 2.95-2.84 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NHAr}$), 2.05-1.89 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NHAr}$). ^{13}C NMR (50 MHz, CDCl_3): 150.99, 150.70, 148.94, 148.03, 136.54, 135.04, 126.98, 124.96, 123.76, 121.99, 117.07, 98.14, 50.96, 49.60, 49.18, 48.74, 48.32, 47.80, 42.21, 27.31. (+)ESI-HRMS (m/z): $[\text{M} + \text{H}]^+$ 327.13596 (error - 3.48 ppm).

***N*-[(4-Chloropyridin-3-yl)methyl]-*N'*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (20)**

4-Chloropyridine-3-carboxaldehyde (198 mg, 1.40 mmol) and **4** (465 mg, 2.10 mmol) were coupled to afford **20** (198 mg, 41%) using AcOH (120 μL , 2.10 mmol) and NaBH_4 (318 mg, 8.40 mmol). The crude product was purified using dry-flash chromatography (SiO_2 : EtOAc/MeOH = 7/3). White amorphous powder. Mp = 115 – 118 °C. IR (KBr): 3291w, 2947w, 1610w, 1580s, 1538w, 1452w, 1428w, 1406w, 1370w, 1331w, 1280w, 1245w, 1202w, 1138w, 1080w, 878w, 809w cm^{-1} . $\lambda_{\text{max}}(\epsilon) = 327$ (8400), 255 (16200) nm. ^1H NMR (200 MHz, CDCl_3 and CD_3OD): 8.55 (s, 1H), 8.41 (s, 1H), 8.38 (s, 1H), 7.89-7.86 (m, 1H), 7.86-7.80 (m, 1H), 7.40-7.32 (m, 2H), 6.41-6.36 (m, 1H), 3.97 (s, 2H), 3.76 (NH), 3.50-3.40 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHAr}$), 3.10-3.00 (m, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHAr}$). ^{13}C NMR (50 MHz, CDCl_3 and CD_3OD): 151.16, 150.43, 150.30, 149.12, 148.19, 144.26, 135.12, 133.01, 127.25, 125.25, 124.78, 121.70, 117.11, 98.67, 47.98, 46.54, 41.75. (+)ESI-HRMS (m/z): $[\text{M} + \text{H}]^+$ 347.08091 (error - 4.51 ppm).

***N*-[(4-Chloropyridin-3-yl)methyl]-*N'*-(7-chloroquinolin-4-yl)propane-1,3-diamine (21)**

4-Chloropyridine-3-carboxaldehyde (198 mg, 1.40 mmol) and **5** (495 mg, 2.10 mmol) were coupled to afford **21** (240 mg, 47%) using AcOH (120 μ L, 2.10 mmol) and NaBH₄ (318 mg, 8.40 mmol). The crude product was purified using dry-flash chromatography (SiO₂: EtOAc/MeOH = 7/3). White amorphous powder. Mp = 127 – 129 °C. IR (KBr): 3300m, 2939m, 2865m, 1609m, 1583s, 1541m, 1473m, 1453m, 1434m, 1410w, 1366m, 1331m, 1286m, 1250w, 1200w, 1136m, 1082m, 1019m, 962w, 905w, 879w, 851m, 802m cm⁻¹. $\lambda_{\text{max}}(\epsilon)$ = 330 (10600), 258 (19600) nm. ¹H NMR (200 MHz, CDCl₃ and CD₃OD): 8.56 (s, 1H), 8.43 (d, *J* = 5.2 Hz, 1H), 8.36 (d, *J* = 5.6 Hz, 1H), 7.84 (d, *J* = 1.6 Hz, 1H), 7.67 (d, *J* = 9.0 Hz, 1H), 7.41 (d, *J* = 5.2 Hz, 1H), 7.22 (dd, *J* = 9.0 Hz, *J* = 1.6 Hz, 1H), 6.35 (d, *J* = 5.6 Hz, 1H), 4.06 (NH), 3.96 (s, 2H), 3.45-3.35 (m, CH₂NHCH₂CH₂CH₂NHAr), 2.95-2.85 (m, CH₂NHCH₂CH₂CH₂NHAr), 2.05-1.90 (m, CH₂NHCH₂CH₂CH₂NHAr). ¹³C NMR (50 MHz, CDCl₃ and CD₃OD): 150.99, 150.63, 150.45, 149.05, 147.99, 144.26, 134.90, 133.01, 126.91, 124.81, 124.76, 121.97, 117.04, 98.07, 48.36, 47.69, 42.17, 27.26. (+)ESI-HRMS (*m/z*): [M + H]⁺ 361.09704 (error - 3.01 ppm).

***N*-(7-Chloroquinolin-4-yl)-*N'*-[(4-methoxypyridin-3-yl)methyl]ethane-1,2-diamine (22)**

A solution of **20** (50 mg, 0.14 mmol) and NaOMe (1.25 g, 23.0 mmol) in 5 mL MeOH was heated to 70 °C for 24 h. Upon cooling at room temperature, solvent was removed under reduced pressure, and crude product was dissolved in CH₂Cl₂. The organic layer was washed with NH₄Cl (pH = 9–10), and the water layer was extracted with CH₂Cl₂ (2×20 mL). The combined organic layers were dried over Na₂SO₄. The mixture was filtered off, solvent was removed under reduce pressure, and crude product was purified by dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH (NH₃) = 18/1). Yield: 42 mg (85%). White amorphous powder. Mp = 81–83 °C. IR (KBr): 3326m, 3287m, 3212m, 3030m, 2958m, 2916m, 2847m, 1584s, 1545m, 1496m, 1450m, 1356w, 1332m, 1288m, 1142m, 1106w, 1025m, 899m, 871m, 841m, 814m cm⁻¹. $\lambda_{\text{max}}(\epsilon)$ = 330 (12500), 255 (23400) nm. ¹H NMR (200 MHz, CDCl₃): 8.51 (d, *J* = 5.6 Hz, 1H), 8.44 (d, *J* = 5.6 Hz, 1H), 8.35 (s, 1H), 7.84 (d, *J* = 2.2 Hz, 1H), 7.71 (d, *J* = 9.0 Hz, 1H), 7.34 (dd, *J* = 9.0 Hz, *J* = 2.2 Hz, 1H), 6.77 (d, *J* = 5.6 Hz, 1H), 6.37 (d, *J* = 5.6 Hz, 1H), 5.98 (NH), 3.83 (s, 2H), 3.81 (s, 3H), 3.39-3.30 (m, CH₂NHCH₂CH₂NHAr), 3.05-2.95 (m, CH₂NHCH₂CH₂NHAr), 2.17 (NH). ¹³C NMR (50 MHz, CDCl₃): 163.90, 152.01, 151.01, 150.43, 149.87, 149.05, 134.81, 128.60, 125.19, 123.43, 121.33, 117.33, 106.09, 99.16, 55.28, 46.41, 45.90, 41.62. (+)ESI-HRMS (*m/z*): [M + 2H]²⁺ 172.06954 (error - 0.63 ppm), [M + H]⁺ 343.13184 (error - 0.51 ppm).

***N*-(7-Chloroquinolin-4-yl)-*N'*-[(4-methoxypyridin-3-yl)methyl]propane-1,3-diamine (23)**

Using the procedure described above, **21** (50 mg, 0.14 mmol) was transformed to **18** (41 mg, 84%). The crude product was purified using dry-flash chromatography (SiO₂: CH₂Cl₂/MeOH (NH₃) = 18/1). Oil. IR (KBr): 3266m, 2946w, 2843w, 1581s, 1541w, 1497m, 1453m, 1367m, 1331m, 1286m, 1246w, 1199w, 1170w, 1138w, 1028m, 903w, 877w, 852w, 815m cm⁻¹. $\lambda_{\text{max}}(\epsilon)$ = 330 (14100), 257 (22400) nm. ¹H NMR (200 MHz, CDCl₃): 8.51 (d, *J* = 5.6 Hz, 1H), 8.47 (d, *J* = 5.6 Hz, 1H), 8.38 (s, 1H), 7.89 (d, *J* = 2.2 Hz, 1H), 7.79 (NH), 7.42 (d, *J* = 9.0 Hz, 1H), 7.09 (dd, *J* = 9.0 Hz, *J* = 2.2 Hz, 1H), 6.83 (d, *J* = 6.2 Hz, 1H), 6.29 (d, *J* = 5.6 Hz, 1H), 3.85 (s, 2H), 3.81 (s, 3H), 3.45-3.35 (m, CH₂NHCH₂CH₂CH₂NHAr), 3.00–2.90 (m, CH₂NHCH₂CH₂CH₂NHAr), 2.10 (NH), 2.00–1.85 (m, CH₂NHCH₂CH₂CH₂NHAr). ¹³C NMR (50 MHz, CDCl₃): 163.92, 152.01, 151.21, 150.65, 150.52, 149.01, 134.55, 128.33, 124.79, 123.28, 121.99, 117.47, 106.19, 98.12, 55.28, 49.07, 46.81, 44.06, 26.93. (+)ESI-HRMS (*m/z*): [M + 2H]²⁺ 179.07815 (error - 3.78 ppm), [M + H]⁺ 357.14791 (error - 0.70 ppm).

HPLC analyses for purity

Compounds were analyzed for purity using a Waters 1525 HPLC dual pump system equipped with an Alltech Select degasser system, and a dual λ 2487 UV-VIS detector. All compounds were > 95% pure. For details see Supporting information.

HPLC-based assay to calculate *in vitro* BoNT/A LC inhibition

A previously reported HPLC *in vitro* assay was used to determine % BoNT/A LC inhibition.^{11,12,13,28} The assay used an N-terminal acetylated, C-terminal aminated, synthetic peptide identical in sequence to residues 187–203 of SNAP-25. Compounds with intrinsic fluorescence quenching capability do not interfere with the activity measurements of the assay because substrate hydrolysis is determined by HPLC separation of cleaved and uncleaved peptide substrate, followed by measurement of the peak areas. All HPLC separations were performed using a Shimadzu Prominence Ultra Fast Liquid Chromatography XR system equipped with a Hypersil Gold Javelin C18 guard column (Thermo Fisher Scientific, Waltham, MA, USA) and a Hypersil Gold C18 reverse-phase analytical column (50 $\times$ 2.1 mm, 1.9 μ m) (Thermo Fisher Scientific, Waltham, MA, USA). Peak areas were measured with LC solution automated integration software (Shimadzu Corporation, Kyoto, Japan) and used to calculate enzyme reaction rates. Assay mixtures consisted of 40 mM HEPES-0.05% Tween (pH 7.3), recombinant BoNT/A LC (MetaBiologics, Madison, WI, USA), peptide substrate, 0.5 mg/ml Bovine Serum Albumin, 1 mM Dithiothreitol, 50 mM excess zinc, and 20 μ M inhibitor. The assays were run at 37 $^{\circ}$ C, quenched by the addition of TFA, and analyzed using reverse-phase HPLC.

In vitro antimalarial activity

The Malaria SYBR Green I - Based Fluorescence (MSF) Assay is a microtiter plate drug sensitivity assay that uses the presence of malarial DNA as a measure of parasitic proliferation in the presence of antimalarial drugs or experimental compounds. As the intercalation of SYBR Green I dye and its resulting fluorescence is relative to parasite growth, a test compound that inhibits the growth of the parasite will result in a lower fluorescence. The D6 (CDC/Sierra Leone), TM91C235 (WRAIR, Thailand), and W2 (CDC/Indochina III) laboratory strains of *P. f.* were used for each drug sensitivity assessment. The parasite strains were maintained continuously in long-term cultures as previously described.³⁷ Pre-dosed microtiter drug plates for use in the MSF assay were produced using sterile 384-well black optical bottom tissue culture plates containing quadruplicate 12 two-fold serial dilutions of each test compound or mefloquine hydrochloride (Sigma-Aldrich Co., Catalog #M2319) suspended in dimethyl sulfoxide. The final concentration range tested was 0.5 – 10000 ng/ml for all assays. Predosed plates were stored at 4 $^{\circ}$ C until used, not to exceed five days. No difference was seen in drug sensitivity determinations between stored or fresh drug assay plates (data not shown). A batch control plate using Chloroquine (Sigma-Aldrich Co., Catalog #C6628) at a final concentration of 2000 ng/ml was used to validate each assay run. A Tecan Freedom Evo liquid handling system (Tecan US, Inc., Durham, NC) was used to produce all drug assay plates. Based on modifications of previously described methods^{38,37}, *P. f.* strains in late-ring or early-trophozoite stages were cultured in predosed 384-well microtiter drug assay plates in 38 μ l culture volume per well at a starting parasitemia of 0.3% and a hematocrit of 2%. The cultures were then incubated at 37 $^{\circ}$ C within a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂, for 72 hours. Lysis buffer (38 μ l per well), consisting of 20mM Tris HCl, 5mM EDTA, 1.6% Triton X, 0.016% saponin, and SYBR green I dye at a 20 $\times$ concentration (Invitrogen, Catalog #S-7567) was then added to the assay plates for a final SYBR Green concentration of 10 $\times$. A Tecan Freedom Evo liquid handling system was used to dispense malaria cell culture and lysis buffer. The plates were then incubated in the dark at room temperature for 24 hours and

examined for the relative fluorescence units (RFU) per well using a Tecan Genios Plus (Tecan US, Inc., Durham, NC). Each drug concentration was transformed into Log[X] and plotted against RFU values. The 50% and 90% inhibitory concentrations (IC₅₀s and IC₉₀s, respectively) were then generated with GraphPad Prism (GraphPad Software Inc., San Diego, CA) using a nonlinear regression (sigmoidal dose-response/variable slope) equation.

***In vitro* metabolism**

Metabolic stability assay sample preparation was performed in a 96-well plate format using a TECAN Genesis robotic sample processor following WRAIR SOP SP 01–09. All incubations were carried out in 0.1 M sodium phosphate buffer (pH 7.4) in the presence of an NADPH-regenerating system (NADP⁺ sodium salt, MgCl₂ × 6H₂O, and glucose-6-phosphate). Compound (10 μM), microsomes (0.5 mg/mL total protein), buffer, and a NADPH-regenerating system were warmed to 37 °C, and the reaction was initiated by the addition of glucose-6-phosphate dehydrogenase (G6PD). Samples were quenched at 0, 10, 30, and 60 min using an equal volume of cold acetonitrile. Samples were centrifuged to pellet the proteins, and the supernatant was analyzed by LC-MS/MS using fast LC gradient methods. Depletion of parent compound signal was monitored relative to signal at time 0. Chromatograms were analyzed using the mass spectrometry software Xcalibur QuanBrowser. To calculate compound half-lives, a first-order rate of decay was assumed. A plot of the natural log (ln) of the compound concentration versus time was generated in which the slope of the line was -k. Half-lives were calculated as 0.693/k. Hepatic microsomal intrinsic clearance was calculated using the formula $CL_{int, in vitro} = (rate/min) \times (mL/0.5 g) \times 52.5 \text{ mg/g liver}$ or $CL_{int, in vitro} = k \times (\text{microsomal protein content per gram of liver}) / (\text{microsomal protein concentration in incubation buffer})$.

***In vitro* toxicity: assessment of compound toxicity in a HepG2 (hepatocellular carcinoma) cells**

The HepG2 target cells for this assay were cultured as follows: HepG2 cells were cultured in complete Minimal Essential Medium (MEM (Gibco-Invitrogen, #11090-099)) that was prepared by supplementing MEM with 0.19% sodium bicarbonate (Gibco-BRL Cat #25080-094), 10% heat inactivated FBS (Gibco-Invitrogen #16000-036), 2 mM L-glutamine (Gibco-Invitrogen #25030-081), 0.1 mM MEM non-essential amino acids (Gibco-Invitrogen #11140-050), 0.009 mg/ml insulin (Sigma #I1882), 1.76 mg/ml bovine serum albumin (Sigma #A1470), 20 units/ml penicillin–streptomycin (Gibco-Invitrogen #15140-148), and 0.05 mg/ml gentamycin (Gibco-Invitrogen #15710-064). HepG2 cells cultured in complete MEM were first washed with 1X Hank's Balanced Salt Solution (Invitrogen #14175-095), trypsonized using a 0.25% trypsin/EDTA solution (Invitrogen #25200-106), assessed for viability using trypan blue, and resuspended at 250,000 cells/mL. Using a Tecan EVO Freedom robot, 38.3 μL of cell suspension was added to each well of clear, cell culture-treated 384-well microtiter plates (Nunc Cat#164688) for a final concentration of 9570 liver cells per well, and plated cells were incubated overnight in 5% CO₂ at 37°C. Drug plates were prepared with a Tecan EVO Freedom using sterile 96 well plates containing twelve duplicate 1.6-fold serial dilutions of each test compound suspended in DMSO. 4.25 μL of diluted test compound was then added to the 38.3 μL of media in each well providing a 10 fold final dilution of compound. Compounds were tested from a range of 57 ng/mL to 10,000 ng/mL for all assays. Mefloquine was used as a plate control for all assays with a concentration ranging from 113 ng/mL to 20,000 ng/mL. After a 48 hour incubation period, 8 μL of a 1.5 mg/mL solution of MTT diluted in complete MEM media was added to each well. All plates were subsequently incubated in the dark for 1 hour at room temperature. After incubation, the media and drug in each well was removed by shaking the plate over the sink, the plates were then left to dry in the hood for 15 minutes. Next, 30 μL of isopropanol

acidified by the addition of HCl (at a final concentration of 0.36%) was added to dissolve the formazan dye crystals created by the reduction of MTT.³⁹ The plates were put on a 3-D rotator for 15–30 minutes. Absorbance was determined in all wells using a Tecan iControl 1.6 Infinite plate reader. 50% Inhibitory concentrations (IC₅₀) were then generated for each toxicity dose response test using GraphPad Prism (GraphPad Software Inc., San Diego, CA) using a nonlinear regression (sigmoidal dose-response/variable slope) equation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

ART	artemisinin
BoNT/A LC	botulinum neurotoxin serotype A light chain
4,7-ACQ	4-amino-7-chloroquinoline
CQ	chloroquine
MFQ	mefloquine
MLM, HLM	mouse and human liver microsomes, respectively
Fe(III)PPIX	protoporphyrin IX

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1BoNT/A LC: 60% inhibition at 20 μ M conc. $IC_{50} = 11.3 \pm 4.7 \mu$ MAntimalarial activity: IC_{50} (D6) 86 nM; IC_{50} (W2) 26 nM **2**BoNT/A LC: 51.4% inhibition at 20 μ M conc. $IC_{50} = 18.9 \pm 3.0 \mu$ MAntimalarial activity: IC_{50} (D6) 4.26 nM; IC_{50} (W2) 8.40 nM **3**BoNT/A LC: 90% inhibition at 20 μ M conc. $IC_{50} = 10.0 \pm 0.80 \mu$ MAntimalarial activity: IC_{50} (D6) 16.87 nM; IC_{50} (W2) 11.38 nM**Figure 1.**

Examples of previously reported 4,7-ACQ-based compounds that concomitantly act as BoNT/A LC inhibitors and antimalarial agents. For compound **1** antimalarial activity was taken from reference 29.

Figure 2.

The gas-phase pharmacophore for BoNT/A LC inhibition (see reference 11) in the context dual BoNT/A LC and malaria inhibitors **1** and **2**, and of new inhibitor designs. Top row: the three-zone pharmacophore for BoNT/A LC inhibition. The second and third rows from the top show how previously reported compounds **1** and **2** fit in zones 2 and 3 of the pharmacophore. Bottom row: structure activity data for **1** and **2** was used to develop new compound designs to increase ligand efficiency while concomitantly maintaining/increasing inhibitory potency against both biological targets.

Scheme 1.

- 6: $n=1$, X=C, Y=CH, R¹=R²=H, 66%
 7: $n=2$, X=C, Y=CH, R¹=R²=H, 76%
 8: $n=1$, X=C, Y=CH, R¹=Bu-*t*, R²=H, 66%
 9: $n=2$, X=C, Y=CH, R¹=Bu-*t*, R²=H, 91%
 10: $n=1$, X=C, Y=CH, R¹=OMe, R²=H, 85%
 11: $n=2$, X=C, Y=CH, R¹=OMe, R²=H, 67%
 12: $n=1$, X=C, Y=CH, R¹=F, R²=H, 96%
 13: $n=2$, X=C, Y=CH, R¹=F, R²=H, 81%
 14: $n=1$, X=C, Y=CH, R¹=Br, R²=H, 73%
 15: $n=2$, X=C, Y=CH, R¹=Br, R²=H, 76%
 16: $n=1$, X=N, Y=CH, R²=H, 74%
 17: $n=2$, X=N, Y=CH, R²=H, 54%
 18: $n=1$, X=C, Y=N, R¹=H, R²=H, 43%
 19: $n=2$, X=C, Y=N, R¹=H, R²=H, 43%

i) a) MnO_2 , CH_2Cl_2 , r.t.; b) **4** or **5**, CH_2Cl_2 , MeOH, AcOH, NaBH_4 , r.t., 18 h;
ii) NaOMe, MeOH, 85 °C, 24 h

Scheme 2.

Table 1
Compound structures, BoNT/A LC % inhibition, antimalarial activity, selectivity index, and hepatic toxicity and metabolism.

compound	n	X	Y	R ₁	R ₂	toxin inhibition BoNT/A LC % inhibition ^b	antimalarial activity (IC ₅₀ (all) and IC ₉₀ ^d (select compounds only), nM)				toxicity against Hep G2 cells (IC ₅₀ , nM) ^h	selectivity index (IC ₅₀) ^j	metabolic stability t _{1/2} (min)	
							CQR W2 ^c	CQS D6 ^d	MDR TM91C235 ^e	MDR TM90C2A ^f	MDR TM90C2B ^g		HLM ^j	MLM ^j
6	1	C	CH	H	H	52.00	10.90, 14.11 ^a	6.41, 9.62 ^a	7.38, 9.30 ^a	9.94, 14.11 ^a	9.30 12.51 ^a	1783/3032/2633	60	23
7	2					51.83	52.17	17.19	39.90	-	-	56/169/73	60	60
8	1	C	CH	Bu- <i>t</i>	H	47.00	42.40	24.19	24.73	-	-	245/429/419	-	-
9	2					50.28	48.44	21.73	31.94	-	-	45/100/68	-	-
10	1	C	CH	OMe	H	18.00	14.33	3.22	16.09	-	-	406/1806/362	27	6
11	2					14.00	56.20	6.18	50.58	-	-	86/778/95	60	18
12	1	C	CH	F	H	68.00	45.78	26.38	55.18	-	-	350/608/291	-	-
13	2					50.09	49.44	13.09	29.37	-	-	110/415/185	-	-
14	1	C	CH	Br	H	69.00	9.47	4.10	9.98	-	-	695/1606/660	60	60
15	2					68.00	5.93, 7.41 ^a	3.95, 4.45 ^a	6.18, 7.66 ^a	7.16, 10.13 ^a	7.91, 12.11 ^a	834/1252/800	60	60
16	1	N	CH	-	H	24.00	375.05	60.70	204.11	-	-	409/2528/752	-	-
17	2					42.29	204.06	8.65	76.41	-	-	176/4146/469	9	9
18	1	C	N	H	H	25.00	225.38	53.39	108.70	-	-	426/1796/882	-	-
19	2					39.89	260.08	-	73.43	-	-	90/ /317	60	60
20	1	C	N	H	C1	23.00	140.81	63.75	83.34	-	-	46/102/78	10	37
21	2					35.87	72.97	25.67	30.97	-	-	52/146/121	16	19
22	1	C	N	H	OMe	19.00	19.00	67.09	7.29	-	-	435/4000/588	38	23
23	2					43.25	86.87	4.76	53.24	-	-	57/1040/93	60	60
1 ^k	-	-	-	-	-	60.00	26 ^l	86 ^l	-	-	-	-	-	-
ART	-	-	-	-	-	-	6.70 ^m	9.00 ^m	13.04 ^m	-	-	-	-	-
CQ ⁿ	-	-	-	-	-	7.00	634.30 (11), 942.50 ^d	14.22 (12), 19.23 ^a	226.21 (11), 475.66 ^a	249.84, 510.46 ^a	307.38, 602.30 ^a	-	-	-

compound	n	X	Y	R ₁	R ₂	toxin inhibition BoNT/A LC % inhibition ^b	antimalarial activity (IC ₅₀ (all) and IC ₉₀ ^a (select compounds only), nM)				toxicity against Hep G2 cells (IC ₅₀ , nM) ^h	selectivity index (IC ₅₀) ^j	metabolic stability t _{1/2} (min)	
							CQR W2 ^c	CQS D6 ^d	MDR TM91C235 ^e	MDR TM90C2A ^f	MDR TM90C2B ^g		HLM ^j	MLM ^j
MFQ ^o	-	-	-	-	-	28.00	6.50 (11), 18.09 ^a	19.98 (12), 43.65 ^a	65.55 (11), 132.73 ^a	51.12, 132.73 ^a	27.98, 75.22 ^a	-		-

^a IC₉₀ (mM).

^b % inhibition calculated at 20 mM conc.

^c *P. falciparum* Indochina W2 clone.

^d *P. falciparum* African D6 clone.

^e *P. falciparum* multidrug resistant C235 strain (Thailand).

^f *P. falciparum* multidrug resistant C2A strain.

^g *P. falciparum* multidrug resistant C2B strain.

^h Hep G2: hepatocellular carcinoma.

ⁱ Selectivity index: SI = IC_{tox}/IC_{Pf} activity.

^j HLM and MLM: human and mouse liver microsomes, respectively.

^k BoNT/A LC, control (see Figure 1 for the structure).

^l IC₅₀ values obtained from reference 29.

^m The average of greater than eight replicates.

ⁿ Control drug: CQ as a diphosphate.

^o MFQ as an HCl salt (P,f : number of replicates given in parentheses for CQ and MFQ).