EVALUATION OF QUINOLINOID ANTIMALARIALS DRUGS AS BUTYRYLCHOLINESTERASE INHIBITORS

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Abstract

Expression of butyrylcholinesterase (BChE), a serine hydrolase related to the deposit of amyloid-β plaques in the brain, progressively increases in patients with Alzheimer's Disease (AD). Recent studies on BuChE activity in AD patients has shown a positive correlation between BuChE inhibition and improved memory and cognitive function. Synthetic quinolinoids have been shown to inhibit BuChE. We hypothesized that known, Food and Drug Administration (FDA) or European Union (EU) approved antimalarial quinolinoids might also inhibit BuChE and could thus, potentially, be applied in "off-label" treatment of AD. Here 8 antimalarial quinolinoids were evaluated for effectiveness as inhibitors of BChE. Enzyme activity was monitored using Ellman's assay and visible spectroscopy with two substrates, butyrylthiocholine (BuSCh) and acetylthiocholine (AcSCh). The majority of quinolinoids tested inhibited BChE, but not as well as tacrine, a strong BChE inhibitor. The IC_{50} values for the 8 quinolinoid inhibitors tested here ranged from 0.56 μM to 53 μM. The most effective antimalarial quinolinoid was quinidine with both BuSCh and AcSCh substrates.

Introduction

Malaria is a disease caused by a parasite and commonly spread to humans through infected mosquito bites. Each year, millions of people are infected and hundreds of thousands die (1). The oldest documented treatment for malaria was the use of a cinchona bark tea, containing the alkaloid quinine, in the 17th century (2). Quinine, as well as quinidine, cinchonine and cinchonidine are all present in cinchona bark extracts. What these compounds share in common is the heterocycle quinoline ring, thus all are "quinolinoids". Western medicine transitioned from quinine to chloroquine in the 1940s, then to non-quinolinoids like sulfadoxine and artemisinin from the 1970s to 1990s (3). Since then, numerous other quinolinoids including quinacrine, primaquine, amodiaquine and mefloquine have been approved for malaria treatment in the US and the EU (4).

Acetylcholinesterase (AChE) is critical to the proper functioning of human cholinergic synapses present in the central and peripheral nervous systems. This enzyme is located in the synapse and catalyzes the hydrolysis of acetylcholine into acetic acid and choline. By breaking down acetylcholine, AChE inhibits neurotransmission in cholinergic neurons. Prior studies suggest that quinolinoids can function as inhibitors of AChE (5, 6). Butyrylcholinesterase (BChE) is another known human enzyme that catalyzes the hydrolysis of acetylcholine as well as butyrylcholine and non-choline esters, but its primary *in vivo* function is presently unknown (7). BChE has also been shown to be associated with Alzheimer's Disease, as well as with the hormone ghrelin, cocaine-resistance, and chemical warfare agent toxicity (7-10). Thus, BChE activity has been shown to exert a direct effect on a wide variety of pathologies.

In addition to carboxylic acid esters, BChE catalyzes the hydrolysis of thioesters including acylthiocholines; both AcSCh and BuSCh are substrates for BChE. Acylthiocholines can be effective colorimetric substrates since the free sulfhydryl group of the hydrolysis product can then react with colorless 5,5′-dithiobis-2-nitrobenzoate (DTNB) to generate thionitrobenzoate (TNB), which is a yellow chromophore in alkaline solution (11). Thus, the inhibition of BChE using quinolinoids can be measured spectrophotometrically based on the rate at which TNB is produced. Tacrine is a known quinolinoid AChE and BChE inhibitor previously used for the treatment of Alzheimer's (12). In this work, eight different FDA- or EU-approved antimalarial quinolinoids were tested as inhibitors of BChE (scheme 1). These results suggest that one of these compounds, cinchonine, might be used as a lead compound for development of future drugs targeting Alzheimer's disease, or perhaps other pathologies like ghrelin regulation that are correlated with BChE activity.

Experimental Methods

Human BChE (B4186), BuSCh iodide (B3253), AcSCh iodide (01480), antimalarials (quinine, quinidine, cinchonine, chlo-
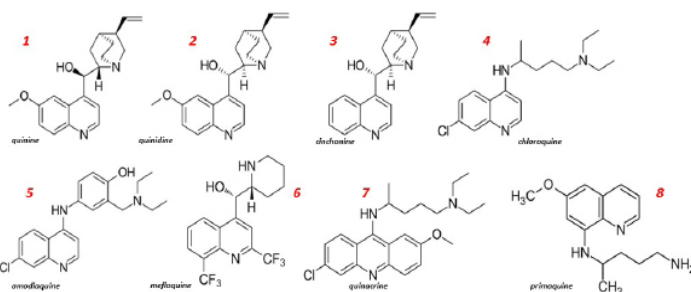
Scheme 1. Structures of antimalarials.

roquine, amodiaquine, mefloquine, quinacrine and primaquine) and DTNB (D3180) were purchased from Sigma-Aldrich (St Louis, MO).

Butyrylcholinesterase activity was monitored at 25 °C in 0.1 M phosphate containing 10 mM $MgCl_2$ adjusted to pH 7.5 with a BChE concentration of 20.8 mg/mL (~350 nM). Enzyme activity was measured with concentrations of BuSCh and AcSCh substrates from 10 mM to 100 mM with 240 mM DTNB in the assay and monitored by visible spectroscopy at 408 nm using an Agilent BioTek Epoch Microplate Spectrophotometer with Gen 2 software. Each solution was measured in triplicate in 96-well plates and enzyme activity was monitored for at least 2 min in 5 s intervals.

Antimalarial stock solutions were made in 10 mM HCl containing 10% DMSO. BChE inhibition with each of the quinolinoids was observed after a 15 min incubation at ambient conditions. For initial examination, all quinolinoid concentrations were 300 μM, BChE concentration was 20.8 μg/mL, substrate concentration was 60 μM, and DTNB concentration was 50 μM. A positive control was prepared with 20.8 μg/mL BChE, 50 μM DTNB, 60 μM substrate, and a volume of 10% DMSO buffer equivalent to the amount of quinolinoid solution added to the analyte solution; the concentration of DMSO never exceeded 2.0 % in the final enzyme assay, and this has been shown to have less than 8% inhibition of the BChE (11). A negative control was also prepared with 20.8 μg/mL BChE, 50 μM DTNB, 60 μM substrate, and 30 μM tacrine. Tacrine was diluted in 10% DMSO buffer in a volume equivalent to the amount of quinolinoid solution added to the analyte solution. Each solution was measured in triplicate for 2 min at

5 s intervals at 408 nm.

Inhibitor activity was reassessed by titrating BChE with quinolinoids ranging from 10 nM to 1.0 mM. Each assay solution contained 20.8 μg/mL BChE, 50 μM of DTNB, and 60 μM of AcSCh or BuSCh. Each solution was measured in triplicate for 2 min at 5 s intervals at 408 nm.

Protein docking was assessed via SwissDock (13). Swiss-Dock is based upon EADock DSS which samples the entire protein surface for binding grooves and estimates CHARMM energies within grids that can include water and ions in the docking simulation. Two crystal structures from the Protein Data Bank (PDB), PDB IDs 1P0I and 2XMC, were used to obtain the Gibb's Free Energy of binding for each antimalarial inhibitor (14, 15). The 2XMC entry is a mutant esterase with greater activity against organophosphates than native BChE.

Results

TNB Spectrum and Standard Curve
The UV-Vis absorbance of TNB was evaluated over a range of wavelengths (300 - 700 nm) (Figure 1A). The wavelength for
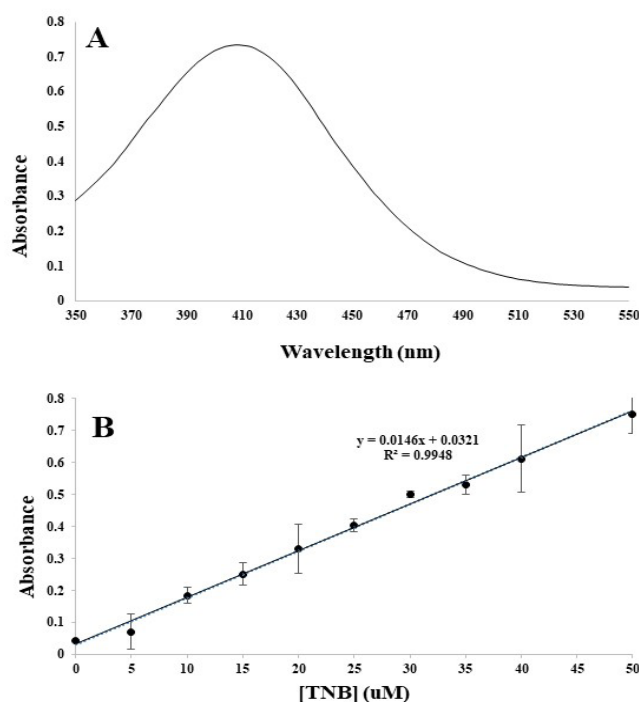

Figure 1. Spectroscopic analysis of TNB. **A.** Absorbance spectrum of TNB (50 μM) solution in phosphate buffer at pH 7.5. The wavelength for maximum TNB absorbance was determined to be 408 nm. B. Standard curve of TNB absorbance at 408 nm versus TNB concentration (0-50 μM). All data were obtained with an Agilent BioTek Epoch Microplate Spectrophotometer. A linear relationship with high correlation was observed between absorbance and concentration of TNB.
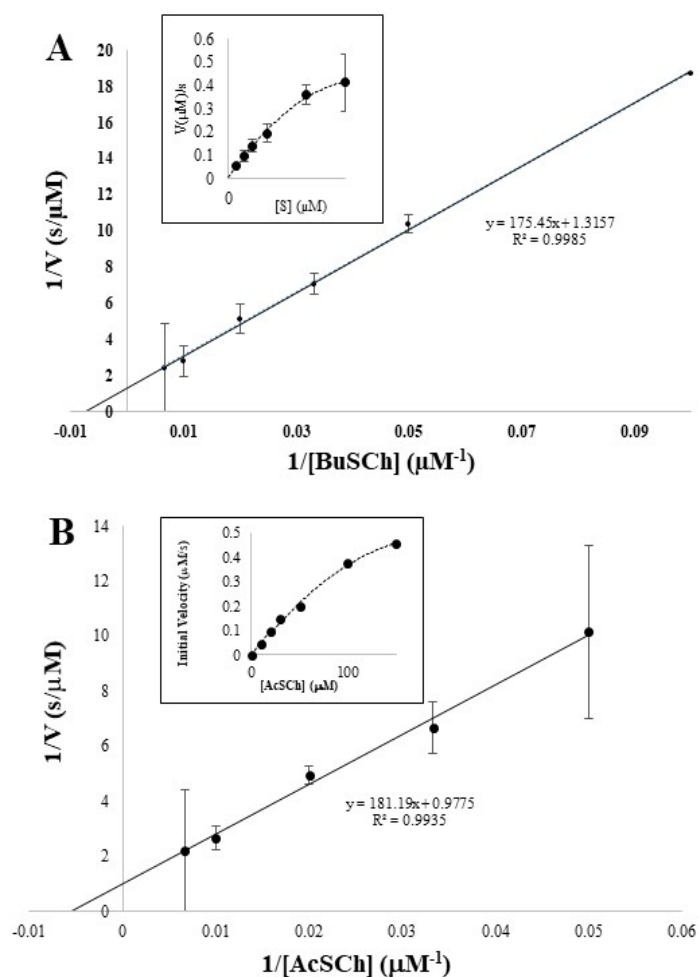

Figure 2. Lineweaver-Burk plots of BChE activity with Michaelis-Menten plots inset. **A.** Lineweaver-Burke plot of BChE using BuSCh as the substrate. **B.** Michaelis-Menten plot of Lineweaver-Burke plot of BChE using AcSCh as the substrate. BChE activity was determined from TNB spectrophotometrically at 408 nm. Kinetic parameters of K_m, V_{max} and k_{cat} were obtained from this enzyme activity data.

maximum absorbance of TNB within this range was determined to occur at 408 nm and the molar extinction coefficient was determined to be 13,800 $M^{-1}cm^{-1}$ in the phosphate buffer. Standards of increasing TNB substrate concentration (0-50 μM) were analyzed with UV-vis at 408 nm (Figure 1B), demonstrating a linear relationship between absorbance and concentration through at least 1.0 absorbance units.

Michaelis-Menten Initial Velocity Kinetic Analysis

Initial reaction rates of the uninhibited reaction between human BChE and either BuSCh or AcSCh of varied substrate concentration (0-150 μM) were conducted (Figure 2). In figure 2A, the substrate was BuSCh, and in figure 2B AcSCh. The enzyme obeys Michaelis-Menten kinetics and data were replotted in Lineweaver-Burke format. A strong linear relationship between the inverses of substrate concentration and reaction rate with high correlation was obtained (0.9983). Enzyme activity was characterized by K_m (76.0 μM for BuSCh, 199 μM for AcSCh), V_{max} (1.29 μM/s for BuSch, 1.0794 μM/s for AcSCh) resulting in k_{cat} values of 61.9 s^{-1} for BuSCh and 51.8 s^{-1} for AcSCh.

Screen of BChE Against a Panel of Quinolinoid Antimalarial Drugs & Tacrine

Inhibition of BChE was compared between a control in the absence of quinolinoid, and 300 μM concentrations of the various quinolinoids or 3 μM tacrine (Figure 3). Inhibition for each analyzed compound demonstrated significant reduction in enzyme activity (> 80%). Quinidine indicated the highest level of inhibition among the quinolinoids of human BChE activity (0.243% with BuSCh, 0.041% with AcSCh) at the 300 μM concentration. However none of the antimalarials were as effective as 3 μM tacrine (0.01% with BuSCh, 0.001% with AcSCh). The weakest inhibitors were mefloquine and primaquine.

IC_{50} Values of Antimalarial Drugs

Initial BChE reaction rates in the presence of varied inhibitor concentrations (10 nM to 1 mM) were evaluated, generating IC_{50} curves for quantitative comparison of inhibitor potency (Figure 4). Quinidine demonstrated the greatest potency by inhibiting 50% of the BChE in the presence of BuSCh substrate with a concentration of only 562 nM. Quinidine also demonstrated the potent inhibition of BChE in the presence of AcSCh with 50% reduction in enzyme
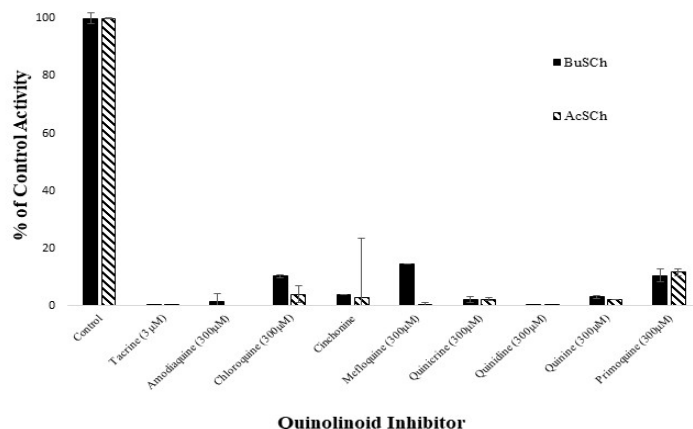
Figure 4. IC_{50} curves of quinolinoid inhibitors of BChE. **A.** Inhibitors using BuSCh as the substrate. **B.** Inhibitors using AcSCh as the substrate. IC_{50} curves were obtained using the BChE DTNB enzyme assay. All data were collected in triplicate, but for clarity, data points and error bars for only the most and least potent inhibitors are shown. With both substrates, the most potent inhibitor was quinidine and the least potent was mefloquine.

Figure 3. Quinolinoid inhibition of BChE. All antimalarials were tested at 300 mM and tacrine was tested at 3 mM using both BuSCh and AcSCH as substrates. At this relatively high concentration, all compounds showed significant inhibition, but quinidine (2) and amodiaquine (5) were most effective.
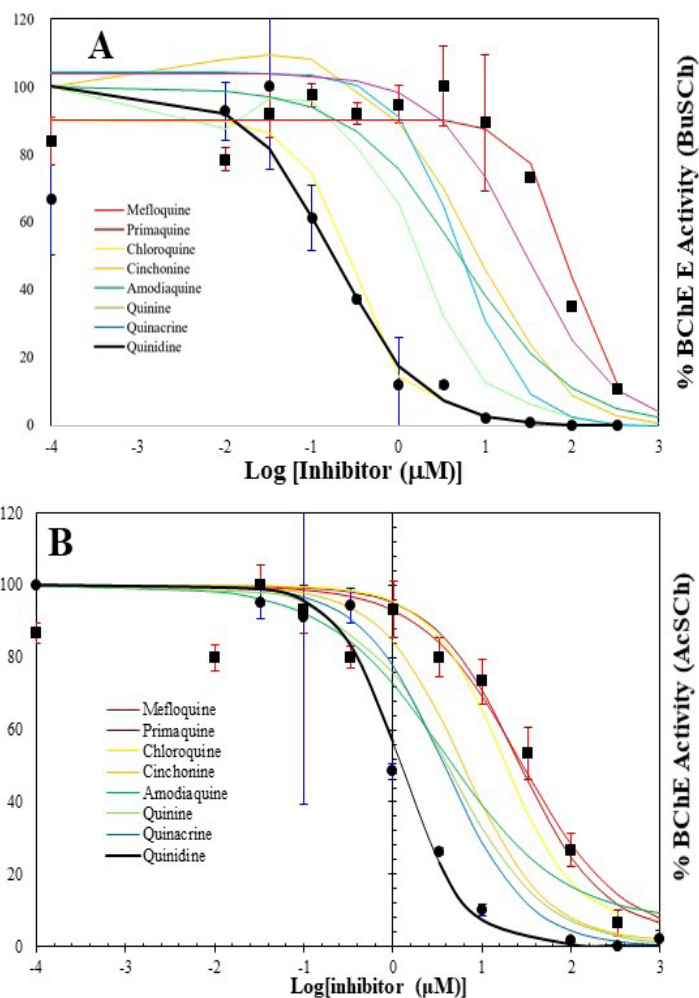
Figure 5. Simulation of binding between human BChE (2XMC) and chloroquine. This figure was provided by SwissDock protein modeling software and visualized using ChimeraX. The majority of inhibitors bind between the alpha helices at the top, which is known to be the catalytic site.

activity accomplished at 0.770 μM. Mefloquine and primaquine were the weakest inhibitors with IC$_{50}$ values >300 mM.

To determine if quinolinoids bound to the same location on BChE, an *in silico* analysis of binding sites was performed using SwissDock. The lowest energy binding site for nearly all of the antimalarial quinolinoid inhibitors is in a groove at the catalytic site on both 1POI and 2XMC between groups of alpha helices (Figure 5). Both acetylcholine and butyrylcholine, as well as tacrine, a known competitive inhibitor (16), bind near to this same site on BChE. Only amodiaquine demonstrated a lowest energy binding significantly far away from the catalytic site.

In silico *Docking of Antimalarials to Human BChE*

The lowest energy binding site found through SwissDock was compared to IC$_{50}$ values obtained through the BChE enzyme assay (Figure 6). All of these quinolinoids had similar Gibbs Free Energy changes upon binding ligands of about -32 kJ/mol. No relationship was found between ΔG of binding and IC$_{50}$ values for either substrate as indicated by the low correlation coefficients for both substrates (0.237 for AcSCh, 0.0018 for BuSCh).

Discussion

The experimentally determined wavelength of maximum absorbance for TNB (408 nm) was in agreement with literature (17). Kinetic analysis was conducted for at least 2 min in all cases. While there was some non-linearity after 1 min, especially at lower substrate concentrations, only the first 45-60 s of data was used to determine enzyme velocities from slopes in A$_{408}$ versus time plots. The Michaels parameter values of K$_m$, V$_{max}$ and k$_{cat}$ were similar to previously published values; the small discrepancies were most likely due to slight differences in buffer and assay reagent concentrations (18). The k$_{cat}$/K$_m$ value for both substrates was about 10^7 s^{-1}, which shows that the enzyme does not perform with catalytic perfection. According to these findings, AcSCh and BuSCh were both viable substrates for the enzyme, and therefore could likely be used as substitutes for AcCh and BuCh.

As expected, tacrine was effective at inhibiting the enzymatic activity of BChE in reference to both substrates at a low concentration (3 mM). The antimalarial quinolinoids displayed inhibitive

properties of varying degrees, but at a much higher concentration (300 μM). Nearly all showed greater than 80% inhibition of BChE activity. In general, inhibition was greater when AcSCh was used as the substrate. This would be expected from the K$_m$ values, since BChE shows a higher affinity for BuSCh than AcSCh. For both substrates, the most promising antimalarials at the given concentration were quinidine and quinacrine.

In general, an IC$_{50}$ concentration of 100 nM, or less, is approaching an acceptable level for drug efficacy. By this standard, only quinidine was a reasonable lead compound for BChE inhibition. Still, with an IC$_{50}$ of 562 nM, it was more potent than 146 natural products from coumarins, flavonoids and stilbenes analogs screened in previous work (19). In comparison, the IC$_{50}$ of tacrine for BChE with BuSCh and AcSCh was previously shown to be 254 and 55 nM, respectively (20), but tacrine also suffers from toxic side effects (21).

The SwissDock molecular docking analysis determined that nearly all of the antimalarials docked at the catalytic site on BChE, with similar albeit slightly varying affinities. However, it was shown in figure 6 that there was no significant amount of correlation between free energy of binding and the IC$_{50}$ value of the antimalarial. This finding demonstrates the short coming of *in silico* binding affinity studies. It is relevant that the two weakest inhibitors from experimentation, primaquine and mefloquine, both have substitutions that can interfere with the quinoline N atom binding, and also had among the lowest binding affinities by SwissDock.

Conclusion

Other synthetic 4-aminoquinolines, including chloroquine, have been examined previously as BChE inhibitors (22). However except for chloroquine, none of those compounds has been approved for use by the FDA or the EU as drugs. Our results suggest that quinidine may be a lead compound for future studies to generate more selective BChE inhibitors. However, like tacrine, the known risk of quinidine toxicity likely renders it unacceptable as is for "off-label" treatment for disorders that result from excessive BChE activity (23).

Figure 6. Comparison of *in vitro* and *in silico* data. A plot of Gibbs Free Energy of binding of quinolinoids versus IC$_{50}$ for enzyme inhibition for each of the flavonoids shows no correlation for either substrate.

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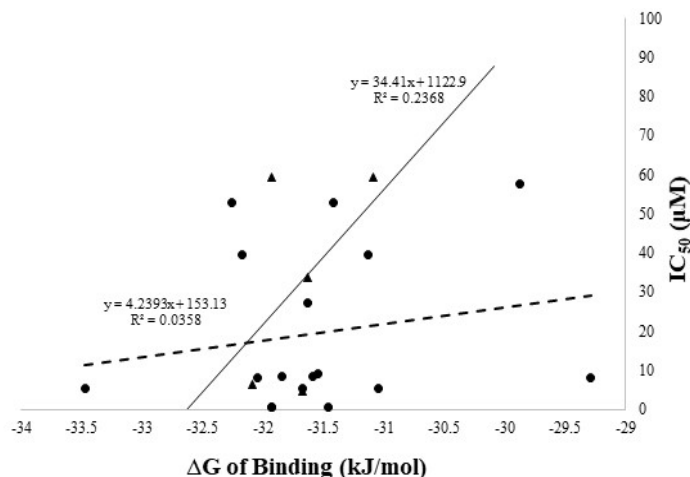7. Ha, Zhe Y., Shintu Mathew, and Keng Yoon Yeong. *Current*

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