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Evaluating Fmoc-amino acids as selective inhibitors of butyrylcholinesterase

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Abstract

Cholinesterases are involved in neuronal signal transduction, and perturbation of function has been implicated in diseases, such as Alzheimer's and Huntington's disease. For the two major classes of cholinesterases, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), previous studies reported BChE activity is elevated in patients with Alzheimer's disease, while AChE levels remain the same or decrease. Thus, the development of potent and specific inhibitors of BChE have received much attention as a potential therapeutic in the alleviation of neurodegenerative diseases. In this study, we evaluated amino acid analogs as selective inhibitors of BChE. Amino acid analogs bearing a 9-fluorenylmethyloxycarbonyl (Fmoc) group were tested, as the Fmoc group has structural resemblance to previously described inhibitors. We identified leucine, lysine, and tryptophan analogs bearing the Fmoc group as selective inhibitors of BChE. The Fmoc group contributed to inhibition, as analogs bearing a carboxybenzyl group showed ~tenfold higher values for the inhibition constant (K_i value). Inclusion of a *t*-butoxycarbonyl on the side chain of Fmoc tryptophan led to an eightfold lower K_i value compared to Fmoc tryptophan alone suggesting that modifications of the amino acid side chains may be designed to create inhibitors with higher affinity. Our results identify Fmoc-amino acids as a scaffold upon which to design BChE-specific inhibitors and provide the foundation for further experimental and computational studies to dissect the interactions that contribute to inhibitor binding.

Keywords

Butyrylcholinesterase; Cholinesterase; Fmoc-amino acid; Tryptophan; Inhibition

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Compliance with ethical standards

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Introduction

Cholinesterases and the inhibition of cholinesterases have been the subject of numerous studies over the past several decades [e.g., reviewed in (Pagano et al. 2015; Hogan 2014; Pinho et al. 2013; Huang et al. 2013; Anand and Singh 2013; Musial et al. 2007)]. The enzymes are divided into two major subfamilies: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE hydrolyzes acetylcholine resulting in the termination of neurotransmission and the enzyme also been suggested to be involved in activities, such as cell differentiation and development (Nizri and Brenner 2013; Halliday and Greenfield 2012; Abreu-Villaca et al. 2011; Jiang and Zhang 2008; Dori and Soreq 2006; Meshorer et al. 2002; Grisaru et al. 1999). While the biological role of BChE is less well understood, there has been significant interest in the development of molecules that selectively inhibit BChE relative to AChE, as BChE activity was found to increase in Alzheimer's patients, while AChE activity decreases or remains the same (Mushtaq et al. 2014).

X-ray structures show that the overall AChE and BChE structures are similar, but several structural differences in the active sites and active-site gorges have been identified (Nicolet et al. 2003; Soreq et al. 1992; Dvir et al. 2010; Sussman et al. 1991). While the active site of each enzyme has a serine nucleophile as part of a serine, histidine, glutamate catalytic triad, and two backbone amides from glycine residues are situated to form hydrogen bonds with the substrate carbonyl group in an 'oxyanion hole', the size of the active-site cavities is different for the two enzymes. In BChE, aliphatic residues, such as leucine and isoleucine, are found, where two phenylalanine residues are found at the corresponding positions in AChE, and the active-site gorge is larger in BChE relative to AChE. These structural differences are suggested to allow the accommodation of larger substrates in the BChE active site relative to AChE (Macdonald et al. 2012; Radic et al. 1993; Vellom et al. 1993; Saxena et al. 1997). Indeed, over six decades ago, Augustinsson reported that substrates larger than propionylcholine were not efficiently hydrolyzed by AChE, while BChE efficiently catalyzes the hydrolysis of this substrate and the larger butyrylcholine commonly used in biochemical studies (Augustinsson 1948). In addition to the differences in the size of the active-site cavities, the larger gorge leading to the BChE active site has been investigated in studies of inhibitor development [e.g., (Saxena et al. 1997)]. In particular, significant attention has focused on a site at the outer end of the gorge known as the 'peripheral binding site', as this site has been suggested to be important for substrate binding, and studies in acetylcholinesterase have focused on developing inhibitors that interact with this distal binding site (Mallender et al. 2000; Szegletes et al. 1999).

Over the past decades, numerous compounds have been synthesized and tested as cholinesterase inhibitors, including natural products and variants synthesized based on natural products [e.g., (Brus et al. 2014; Huang et al. 2013; Pinho et al. 2013; Anand and Singh 2013; Carolan et al. 2010; Kamal et al. 2008)]. These classes of inhibitors include compounds that have been used as therapeutics, such as donepezil, galantamine, rivastigmine, and tacrine (Fig. 1) (Anand and Singh 2013). Although the structures of cholinesterase inhibitors are widely varied, as shown by the few examples in Fig. 1, many reoccurring features, such as aromatic groups, are found in different inhibitor classes. A model for the importance of aromatic groups in cholinesterase inhibitors is that these groups

may lead to favorable binding interactions with aromatic groups within the cholinesterase active site. Based on this observation and previous studies, we postulated that molecules bearing the aromatic 9-fluorenylmethyloxycarbonyl (Fmoc) group might provide a series of compounds that inhibit cholinesterase (Fig. 2). Indeed, a previous study by Zhao et al. evaluated a series of β -carboline and quinolone alkaloids derivatives that bear structural resemblance to the Fmoc-moiety and they found several compounds inhibited BChE and/or AChE (Zhao et al. 2013). We chose to investigate Fmoc-amino acids, as amino acid analogs provide a scaffold upon which to readily vary functional groups and molecular size. The Fmoc group is used extensively in solid-phase peptide synthesis, and numerous Fmoc-protected amino acids and amino acid analogs are commercially available or can be readily synthesized (Fields and Noble 1990; Behrendt et al. 2016). Herein, we report that Fmoc-amino acids inhibit BChE and that the inhibitors are selective for BChE relative to AChE. The compounds show competitive inhibition, but the site or sites of interaction for the analogs, such as the active site, peripheral binding site, or another site, remains to be determined. Nevertheless, the results provide a foundation upon which to evaluate amino acid analogs as BChE-selective inhibitors. Furthermore, the results provide a basis for future computational modeling studies to evaluate the mode or modes of interaction and to make predications to guide the design of more potent inhibitors.

Materials and methods

Materials

All reagents were of the highest purity commercially available (97 %). All buffers were prepared with reagent grade materials or better. Bovine serum albumin (BSA) was from Thermo Fisher Scientific. Butyrylcholinesterase from equine serum and acetylcholinesterase from *Electrophorus electricus* were from Sigma-Aldrich. *S*-butyrylthiocholine and *S*-acetylthiocholine were from Alfa Aesar. Fmoc- and Boc-amino acids were from AAPPTec (Louisville, KY), and the amino acids were from Fisher Scientific and Alfa Aesar. Thin-layer chromatography and NMR spectroscopy were used to analyze amino acid analog purity. Absorbance measurements were made using a PerkinElmer Lambda 25 spectrophotometer fitted with a PCB-1500 water Peltier system (PerkinElmer) for temperature control. Cuvettes were from Starna Cells (Atascadero, CA). Water for all solutions was purified using a Barnstead Nanopure Diamond water system.

Relative enzyme activity measurements

Activity measurements were performed based on the method of Ellman (Ellman et al. 1961). Lyophilized butyrylcholinesterase from equine serum or lyophilized acetylcholinesterase from *E. electricus* was dissolved in cold 10 mM sodium phosphate, pH 7.5. Reactions were conducted at 25 °C in 100 mM sodium phosphate, pH 7.5, 1 mM MgCl₂, 0.2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 1.2 μ M BSA, and 100 μ M butyrylthiocholine or 100 μ M acetylthiocholine.

To evaluate the effect of amino acids and analogs on cholinesterase activity, stock solutions containing the amino acid or analog were prepared in methanol. An aliquot of the stock solution of the amino acid or analog was added to the reaction mixture (without enzyme) to

give a final concentration of 200 μM with 2 % (vol/vol) methanol as a cosolvent. The cosolvent was included to increase Fmoc-amino acid solubility. For the control reaction without an amino acid analog, methanol was added to a final concentration of 2 % (vol/vol). Reactions were initiated by adding enzyme (final concentration 50 nM BChE or 20 nM AChE), and initial rates were determined by monitoring continuously at 412 nm. A molar absorptivity of $14,150 \text{ M}^{-1} \text{ cm}^{-1}$ was used to calculate product formation (Riddles et al. 1983). Relative activity was determined by dividing the initial rate for reaction in the presence of each amino acid or analog by the reaction with 2 % methanol and without amino acid or analog. At least three determinations using independently prepared solutions of the amino acids or analogs were measured and averaged. The enzyme and substrate concentrations were varied, typically fourfold, to test if the relative activity was affected by the enzyme and/or substrate concentrations. The relative activities determined at the different enzymes and substrate concentrations tested were experimentally indistinguishable.

Inhibition constant (K_I) and half maximal inhibitory concentration (IC_{50}) value determinations

Reactions were conducted at 25 °C in 100 mM sodium phosphate, pH 7.5, 1 mM MgCl_2 , 0.2 mM DTNB, 1.2 μg BSA, 100 μM butyrylthiocholine, and 40 nM butyrylcholinesterase. Solutions of the Fmoc-amino acid analog at varying concentrations were prepared in methanol and added to the reaction mixture to give a final methanol concentration of 2 % (vol/vol). The reactions were initiated by the addition of enzyme, and the initial rates were determined by monitoring continuously at 412 nm as described above. The K_I values were determined by fitting the initial rates as a function of inhibitor concentration (typically 12–14 concentrations) as previously described (Segel 1974). For the calculation of the IC_{50} dose–response curve, the Fmoc-amino acid concentrations were varied over a minimum of six orders of magnitude. IC_{50} values were determined by fitting the initial rates as a function of inhibitor concentration using the log(inhibitor) versus the response variable slope equation. At least three determinations using independently prepared inhibitor solutions were averaged to determine K_I and IC_{50} values. All calculations were completed using KaleidaGraph (Synergy Software).

van der Waals volume calculations

Calculations of van der Waals volumes of Fmoc-amino acids were performed using the approach described by Zhao et al. (2003). The number of atoms, bonds, and rings for each compound were determined, and the volume was calculated using the Atomic and Bond Contributions of the van der Waals volume (VABC) method (Zhao et al. 2003). To compare to the previous studies that used the program VOIDOO (version 3.1) to calculate the van der Waals volume (Kleywegt and Jones 1994), the VABC method was used to calculate the van der Waals volume for ethopropazine and compared to the literature value (Saxena et al. 1997).

Results and discussion

We first tested if Fmoc analogs of 19 of the 20 common amino acids inhibit BChE. The Fmoc analog of cysteine was not tested, because the cysteine sulfhydryl group interferes

with the assay used to measure enzyme activity. We used a concentration of 200 μM Fmoc-amino acid in 2 % methanol (vol/vol) to evaluate the effect of each Fmoc-amino acid on BChE activity. Methanol was included in the reaction as a cosolvent to increase the aqueous solubility. We chose a concentration of 200 μM Fmoc-amino acid for the initial survey, as all the analogs were soluble under these conditions. The effect of each Fmoc-amino acid on BChE activity was determined by comparing enzyme activity in the presence of 200 μM Fmoc-amino acid to BChE activity in the presence of 2 % methanol to control for the methanol used as the cosolvent. As shown in Fig. 3a, two Fmoc-amino acids, Fmoc-Leu and Fmoc-Lys, showed the greatest decrease in activity. Several other Fmoc-amino acids decreased BChE activity, including Fmoc-Ile, Fmoc-Phe, Fmoc-Trp, and Fmoc-Tyr. The results show that analogs bearing different side-chain functional groups were found to exhibit the greatest inhibition, including analogs bearing a hydrophobic group (leucine), aromatic group (tryptophan), and cationic group (lysine). Differences in the effect on BChE activity were observed within the series of analogs with hydrophobic side chains. Specifically, Fmoc-Leu showed the greatest reduction of activity, but the constitutional isomer Fmoc-Ile showed an approximately two-fold smaller decrease in activity. A similar smaller effect was observed for Fmoc-Val. Nevertheless, the larger aliphatic side chains appear to facilitate inhibition compared to smaller side chains, as BChE activity in the presence of Fmoc-Ala or Fmoc-Gly was not experimentally distinguishable from activity in the absence of an Fmoc-amino acid. The presence of an aromatic group appeared to contribute to inhibitor binding, as Fmoc-Phe, Fmoc-Tyr, and Fmoc-Trp all showed a reduction in enzyme activity with Fmoc-Trp showing the greatest effect. Other Fmoc-amino acids did not have a significant effect on BChE activity, including analogs bearing carboxylate and amide groups, such as Fmoc-Asn, Fmoc-Gln, Fmoc-Asp, and analogs bearing a hydroxyl group, such as Fmoc-Thr. In addition, relative activity was reduced but within 10 % of reaction without an amino acid added for several analogs, including Fmoc-Pro, Fmoc-Met, and Fmoc-Ser.

We next individually measured the inhibition constants (K_I values) for the analogs that showed the greatest relative decrease in BChE activity, and the results are summarized in Table 1. (IC_{50} values are reported in Supplementary Table 3). The K_I values of 115 and 150 μM for Fmoc-Leu and Fmoc-Lys, respectively, suggest that a hydrophobic or cationic group is important for inhibition. The K_I value for Fmoc-Ile was 194 μM suggesting an approximately two-fold difference in the inhibition properties for the two constitutional isomers, Fmoc-Leu and Fmoc-Ile. As a natural substrate for the enzyme, acetylcholine, bears a cationic ammonium group, analogs bearing a positively charged group, such as the cationic Lys side chain, may be favorable for binding and indeed Fmoc-Lys was one of the more potent inhibitors with a K_I value of 150 μM . Although Fmoc-Arg is expected to bear a cationic group under the reaction conditions, in the presence of 200 μM Fmoc-Arg, the relative activity was indistinguishable from the reaction without Fmoc-amino acid present (Fig. 3). Minimally, two models may account for the different behaviors shown by Fmoc-Lys and Fmoc-Arg. One model is that the larger guanidinium group of the arginine side chain is not able to access the active site, because it is not accommodated in the active-site gorge compared to the smaller ammonium group of Lys. However, the calculation of van der Waals volumes described below and shown in Supplementary Table 4 suggests that all Fmoc-amino

acid analogs tested can be accommodated in the BChE active-site gorge. As the arrangement of hydrogen-bonding groups for the Arg side chain differs compared to Lys, another model is that the differences in these hydrogen-bonding arrangements may prevent Arg from making favorable interactions analogous to Lys, and/or the arrangement could lead to the Arg side chain making unfavorable interactions. Future computational modeling studies will be needed to dissect the physical basis for the difference in inhibition for Fmoc-Lys and Fmoc-Arg.

Do the Fmoc-amino acids selectively inhibit BChE?

To evaluate if the Fmoc-amino acids selectively inhibit BChE relative to AChE, we next tested if the analogs affect AChE activity. An evaluation analogous to that described above for BChE was performed using AChE. In this experiment, we evaluated the effect of the presence of 200 μM Fmoc-amino acid on AChE activity relative to a reaction without an amino acid analog. All reactions contained 2 % methanol (vol/vol) as cosolvent. The results in Fig. 3b show that AChE activity in the presence of 200 μM Fmoc-amino acid was indistinguishable from the activity in the absence of the amino acid analog for all compounds tested. These results indicate that the K_i values for inhibitor binding to AChE are $>200 \mu\text{M}$ and suggest that the Fmoc-amino acids that decreased BChE activity are selective for the inhibition of BChE.

As noted above, the active site and active-site gorge of BChE are larger than those in AChE. The simplest model for selective inhibition of BChE is that the Fmoc-amino acids are too large to be accommodated by the smaller active-site gorge in AChE. Saxena et al. previously used X-ray data to calculate the volume of the active-site gorges for AChE from *Torpedo californica* and a model to calculate the volume of the active-site gorge in human BChE (Saxena et al. 1997). They reported that the volume of the active-site gorge for AChE is $302.31 \text{ \AA}^3$, while the BChE active-site gorge is $501.91 \text{ \AA}^3$, suggesting that the BChE active-site gorge is $\sim 200 \text{ \AA}^3$ larger than that for AChE. In the same study, a small molecule, ethopropazine, was shown to have a 9000-fold difference in the K_i value between BChE and AChE. Calculation of the van der Waals volume for ethopropazine showed a volume of $317.6 \text{ \AA}^3$, and it was suggested that the smaller AChE active-site gorge was unable to accommodate the bulky inhibitor leading to selective binding (Saxena et al. 1997).

We carried out a similar analysis using the available data for the active-site gorges of AChE and BChE from Saxena et al. (Saxena et al. 1997). Although our experiments were performed using AChE from *E. electricus*, a comparison of X-ray structures show that the two structures are nearly superimposable [an overlay of the structures of AChE from *T. californica* (PDB ID 1EA5) and *E. electricus* (PDB ID 1EEA) gave an overall RMSD for of $0.328 \text{ \AA}$]. We first calculated the van der Waals volumes for the Fmoc-amino acid analogs using the approach described by Zhao et al. (2003). To compare to the literature, we used the approach of Zhao et al. to calculate the van der Waals volume for ethopropazine and determined a van der Waals volume of $305.6 \text{ \AA}^3$ similar to $317.6 \text{ \AA}^3$ reported by Saxena et al. (1997). The results listed in Table 2 show that the van der Waals volumes for all the Fmoc-amino acids that inhibited BChE are larger than the calculated molecular volume for the AChE active-site gorge, but smaller than the calculated molecular volume for the BChE

active-site gorge (van der Waals volumes for all Fmoc analogs tested are given in Supplementary Table 4). Together, the results suggest that the Fmoc-amino acids that selectively inhibit BChE are too bulky to be accommodated in the smaller gorge but can be accommodated by BChE.

Is the Fmoc group important for BChE inhibition?

As described in the “Introduction”, Fmoc-amino acids were tested as BChE inhibitors as the aromatic Fmoc group bears structural features found in other cholinesterase inhibitors. An additional structural feature that may contribute to inhibition of BChE is the carbamate group, as carbamates have been used as cholinesterase inhibitors (Verma et al. 2015; Hartsel et al. 2012; Wong et al. 2013; Bar-On et al. 2002). Nevertheless, several Fmoc-amino acids did not show an inhibitory effect under the conditions tested (e.g., Fmoc-Ala). An alternate model for the observed inhibition is the leucine, lysine, and tryptophan side chains that lead to enzyme inhibition independent of the Fmoc group. To test this possibility, we examined if amino acids without the Fmoc group inhibit BChE.

The ability of amino acids to inhibit BChE was tested by comparing BChE activity in the presence of 200 μ M amino acid relative to the activity without amino acid as described above. The results summarized in Supplementary Table 5 show that relative to the control activity was unchanged in the presence of all amino acids tested (a similar result shown in Supplementary Table 6 was observed for AChE). These results suggest that the side chains of Lys, Leu, and Trp alone do not lead to BChE inhibition and support the model that the Fmoc group is important for inhibition. An alternate model, however, for the observed differences of the amino acid and Fmoc-amino acid is the cationic amine group in the amino acid that is unfavorable for binding and inhibiting the enzyme. To evaluate further the importance of the Fmoc and carbamate group in inhibition, we investigated the ability of carboxybenzyl (Cbz)-protected amino acids to inhibit BChE.

Amino acids bearing the Cbz group maintain a carbamate group analogous to the Fmoc-amino acids, but a phenyl group replaces the fluorenyl moiety found in the corresponding Fmoc-amino acids (Fig. 2). The ability of each Cbz-containing amino acid to inhibit BChE was evaluated as described for the Fmoc analogs, and the results are shown in Fig. 4. While Cbz-protected Ile, Val, Phe, and Tyr showed 5 % reduction in activity compared to reaction in the absence of an amino acid analog, activity was reduced by 22, 8, and 13 % in the presence of Cbz analogs of Leu, Trp, and Lys, respectively. We next determined the K_i values for the three analogs that showed a >5 % decrease in activity, and the results are summarized in Table 3. The results show that the Cbz analogs of Leu, Trp, and Lys do inhibit BChE, but the inhibition constants are ~tenfold higher than the corresponding Fmoc analogs. A model to account for this observation is that interactions of the active site or peripheral binding site (or another binding site) with the aromatic moiety contribute to favorable binding, and the larger fluorenyl group relative to the phenyl group leads to more favorable interactions with the Fmoc analogs. Together, these results suggest that the importance of the larger fluorenyl group and additional experimental approaches to vary the size of the aromatic moiety in combination with computational modeling will be aid in identifying the binding site or sites and to dissect the interactions important for binding.

As an additional experiment to survey amino acid analogs, we tested if analogs bearing the *tert*-butyloxycarbonyl (Boc) protecting group inhibit BChE. The Boc-protected analogs were selected, as the Boc protecting group is commonly used in solid-phase peptide synthesis allowing ready access to the compounds. The results summarized in Supplementary Table 8 show that the Boc-protected amino acids had little effect on BChE activity. A specific feature for differences in the Boc versus Fmoc-amino acids cannot be discerned from these results alone, as multiple features are varied, such as aromaticity and size, but the bulky aliphatic *t*-butyl moiety of the Boc group may introduce unfavorable steric effects. A future direction will be to explore if analogs bearing smaller aliphatic groups can act as inhibitors.

Evaluating the effect of modifying side-chain substituents on BChE inhibition

An attractive feature of evaluating amino acids analogs as cholinesterase inhibitors is that the analogs offer the ability to explore readily the effect of incorporating a variety of hydrophobic and aromatic substituents on the amino acid side chains. Previously reported analogs often bear hydrophobic and/or aromatic groups and these groups may interact with the hydrophobic and aromatic groups in the BChE active site or at another binding site (Andersson et al. 2013; Berg et al. 2011; Kaboudin et al. 2009; Law et al. 2007). To test if including hydrophobic or aromatic groups on the amino acid side chains of Fmoc-amino acids lead to more potent inhibitors, we evaluated the inhibition properties for a series of commercially available compounds bearing side-chain protecting groups commonly used in peptide synthesis—*t*-butyl, Boc, and Cbz.

Figure 5 shows the results of evaluating the Fmoc-amino acid analogs relative to analogs without the modified side chains. The greatest decrease in relative activity was observed for Fmoc-Trp(Boc)-OH compared to Fmoc-Trp. Measurement of the K_I value shows that the incorporation of a Boc group on the indole nitrogen reduces the K_I value approximately eightfold. The Boc group on the Trp indole nitrogen introduces a carbamate group and steric bulk relative to the indole hydrogen. It is possible that the hydrogen on the indole nitrogen makes unfavorable interactions and removal of this hydrogen leads to a better inhibitor. An alternate model is favorable hydrogen bonding, and/or van der Waals interactions are introduced by the carbamate and/or *t*-butyl group of the Boc modification.

While the introduction of a Boc group on the Trp indole nitrogen led to a better inhibitor, the K_I values determined for Fmoc-Tyr and Fmoc-Lys bearing side-chain protecting groups, *t*-butyl and carboxybenzyl, respectively, were similar to the corresponding compounds without side-chain protecting groups. Although the carboxybenzyl group introduces a carbamate group, and carbamate groups are parts of numerous cholinesterase inhibitors, the positive charge of the lysine chain may contribute to binding interactions in the BChE active site analogous to the cationic group of the choline substrate and loss of the cationic-side chain in the Fmoc-Lys(Boc)-OH may lead to higher K_I value. Together, these results identify that modifications of the amino acid side chain can lead to a better inhibitor than the Fmoc-amino acid alone, and indicate the complexity associated with identifying the types of interactions, e.g., hydrogen bonding, aromatic, van der Waals interactions, that may lead to developing more potent inhibitors.

Conclusions

We identified several Fmoc-amino acids that inhibit BChE revealing the potential of amino acid analogs as a scaffold to develop potent and selective BChE inhibitors. The aromatic fluorenyl group appears to be important for inhibitor binding, as the Fmoc analogs were better inhibitors than the corresponding Cbz analogs, but interactions in addition to the Fmoc group may contribute to inhibitor binding, as Fmoc-Gly and Fmoc-Ala showed little effect on BChE activity. The results with Fmoc-Leu and Fmoc-Trp identify these compounds as good candidates for further optimization, and the Fmoc-Trp analog bearing a Boc group on the side chain was promising to develop even better inhibitors. The cationic-side chain of Fmoc-Lys may introduce a cationic group in the choline-binding site of the enzyme active site and may provide a scaffold to incorporate features of the substrate in the peptide inhibitors. In addition, peptides containing an Fmoc group may provide an attractive scaffold to design inhibitors as the functional groups, and arrangement of functional groups within the inhibitor can be readily varied.

While our result show that Fmoc-amino acids act as BChE-selective inhibitors, the site or sites of inhibitor binding are not revealed by this study. As described above, in addition to the active site, much attention has been focused on the importance of a site distal to the active site, known as the ‘peripheral binding site’, and inhibitors designed to interact with the active site and peripheral binding site in cholinesterases have been reported (Berg et al. 2011; Nicolet et al. 2003; Xie et al. 2013; Zhao et al. 2011). We are currently performing computational modeling studies to evaluate potential binding sites and to make predictions that can be tested experimentally using a larger library of compounds to dissect further the interactions important for binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Tacrine **Donepezil** **Rivastigmine** **Galantamine**

Fig. 1.
Examples of cholinesterase inhibitors

Fmoc-amino acid

Cbz-amino acid

Boc-amino acid

Fig. 2.
Fmoc-, Cbz-, and Boc-amino acids. The 'R' group was varied in this study

Fig. 3.

Effects of Fmoc-amino acids on **a** BChE and **b** AChE activities. The Fmoc-amino acids are identified using single-letter amino acid notation. Relative activity is the activity in the presence of 200 μ M Fmoc-amino acid relative to the activity in the absence of the analog. Values are from Supplementary Tables 1 and 2

Fig. 4.

Evaluating the effect of varying the amino group modifications on inhibition of BChE.

Amino acids lacking a group on the amine (*hyphen*), Cbz (Z), and Fmoc (F) are compared.

Relative activity is the activity in the presence of the amino acid or analog compared to the activity in the absence of an analog. Values are from Supplementary Tables 1, 5, and 7

Fig. 5. Evaluating the effect of introducing side-chain modifications on inhibition of BChE. **a** Structures of the Fmoc-amino acids evaluated. **b** K_i values determined for inhibition of BChE. *Minus symbol* indicates that the Fmoc-amino acid side chain is unmodified, and *plus symbol* indicates the Fmoc-amino acid side chains bear the modifications shown in **a**. Values are from Supplementary Tables 1 and 9

Table 1

Inhibition constants for Fmoc-amino acids

Fmoc-amino acid	K_I (μM)
Fmoc-Leu-O ⁻	115 ± 11
Fmoc-Lys-O ⁻	150 ± 10
Fmoc-Ile-O ⁻	194 ± 13
Fmoc-Val-O ⁻	313 ± 14
Fmoc-Phe-O ⁻	330 ± 15
Fmoc-Tyr-O ⁻	307 ± 13
Fmoc-Trp-O ⁻	193 ± 21

Table 2

Calculated volumes of the AChE and BChE active-site gorges from Saxena et al. and calculated van der Waals volumes of Fmoc-amino acids

Enzyme	Volume of active-site gorge ($\text{\AA}^3$) ^a
<i>Torpedo</i> AChE	302.31
Human BChE	501.91
Fmoc-amino acid	van der Waals volume ($\text{\AA}^3$)
Fmoc-Leu-O ⁻	336.65
Fmoc-Lys-O ⁻	348.97
Fmoc-Ile-O ⁻	336.65
Fmoc-Val-O ⁻	319.35
Fmoc-Phe-O ⁻	357.37
Fmoc-Tyr-O ⁻	366.16
Fmoc-Trp-O ⁻	377.07

^aData from Saxena et al

Table 3Inhibition constants (K_I values) for the inhibition of BChE by Cbz-amino acids

Fmoc-amino acid	K_I (μM)
Cbz-Leu-O ⁻	1150 ± 130
Cbz-Lys-O ⁻	1535 ± 502
Cbz-Trp-O ⁻	4795 ± 630