DOI: 10.1002/cbic.200900309

hChAT: A Tool for the Chemoenzymatic Generation of Potential Acetyl/Butyrylcholinesterase Inhibitors

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Nervous system disorders, such as Alzheimer's disease (AD)[1] and schizophrenia,[2] are believed to be caused in part by the loss of choline acetyltransferase (ChAT) expression and activity, which is responsible for the formation of the neurotransmitter acetylcholine (ACh). ACh is biosynthesized by ChAT by acetylation of choline, which is in turn biosynthesized from L-serine.[3] ACh is involved in many neurological signaling pathways in the parasympathetic, sympathetic, and voluntary nervous system.[4] Because of its involvement in many aspects of the central nervous system (CNS), acetylcholine production and degradation has become the target of research for many neurological disorders including AD.[5] In more recent studies, a decrease in acetylcholine levels, due to decreases in ChAT activity,[6] has been observed in the early stages of AD.[7–9] An increase in butyrylcholinesterase (BChE) has also been observed in AD.[6] Depending on the location in the brain, an increase and decrease of muscarinic acetylcholine receptors (M1–M5) has been shown.[10] Due to the decrease in acetylcholine, acetylcholinesterase (AChE) and BChE inhibitors have become targets for AD drug development.[11–13] Analogues of acetylcholine with AChE inhibitory activity have been shown to be one of the most direct treatments for the suppression of neurodegenerative diseases, such as AD. Although the benefits of these agents are modest, three compounds, donepezil (Aricept®, Eisai/Pfizer),[14] rivastigmine (Exelon®, Novartis),[15] and galantamine (Reminyl®, Shire/Johnson & Johnson), are available as US FDA approved drugs (Figure 1).[16–18] However, the synthesis workload invested in the preparation of these drugs has limited and delayed progress for the preparation of libraries of potentially more active novel acetylcholine analogues for cholinesterase (ChE) inhibition.

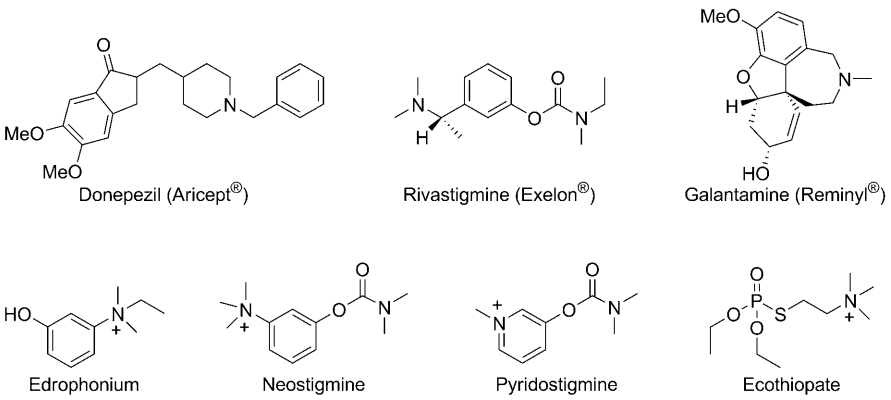

Figure 1. Bioactive acetylcholine analogues.

Other reversible AChE inhibitors, such as edrophonium, neostigmine, pyridostigmine, and ecothiopate, have chemical structures that rely on the features and motifs that compose acetylcholine (Figure 1). They all possess an alkylated ammonium unit similar to that of acetylcholine. Furthermore, all of the compounds have an oxygen atom that is separated by either two carbons from the ammonium group, as is the case in acetylcholine, or three carbons. Neostigmine, pyridostigmine, and ecothiopate all have a carbonyl or a phosphate analogue at an equivalent position to that of the acetyl group of acetylcholine. These successful examples suggest that the following motifs should be considered in the search for and design of novel acetylcholine-based bioactive compounds: 1) the compounds should have an ammonium functional group, and 2) the ammonium group should be placed in a two C–C distance from the acyl group. A variety of acyl groups at the oxygen might improve the activity. As such, the design and synthesis of acetylcholine-based compound libraries has a great potential for the discovery of novel acetylcholine analogues that can act as AChE inhibitors and/or as specific receptor agonists.

Previously, Kim et al.[19] briefly explored the substrate promiscuity of human choline acetyltransferase (hChAT) and showed that the enzyme requires two major aspects of the choline compound. Choline derivatives must maintain the ethanolamine core both in number of atoms and substitution pattern. Investigations of the hChAT cosubstrate (acetyl-CoA) promiscuity have not been reported.

In this study, we developed a chemoenzymatic method that provides facile and rapid access to a large number of novel acetylcholine analogues that would otherwise require several synthesis and purification steps, which would significantly increase the efforts for the preparation of libraries to be used for high-throughput screening. We used hChAT to generate novel acylcholine compounds using a variety of choline and CoA

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900309.

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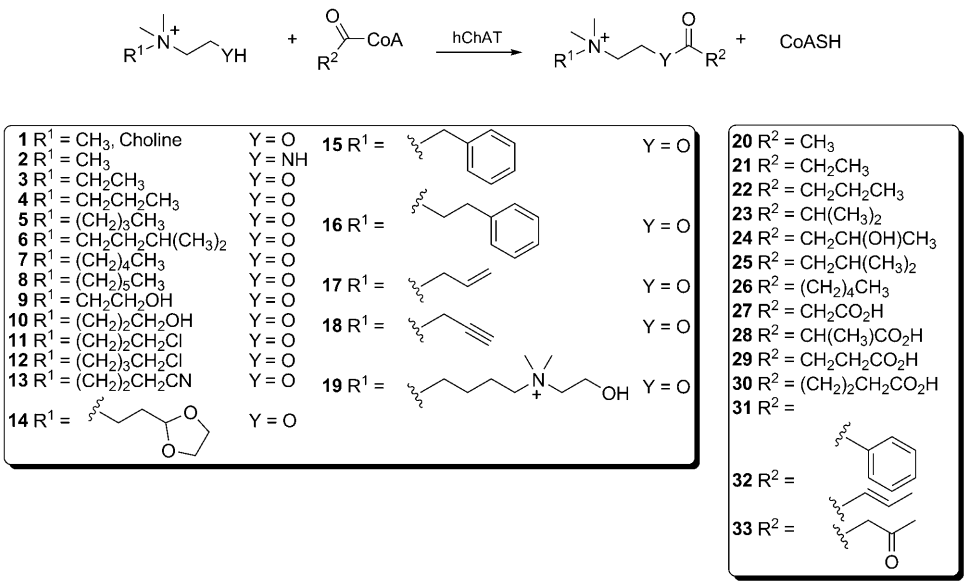
analogues (Scheme 1). Earlier work has set the precedent that acetylated choline analogues can behave as AChE inhibitors, for example, hemicholinium-3 (HC), which when acetylated forms acetylseco-HC.[13] Using our proposed chemoenzymatic strategy with various combinations of CoA derivatives and choline analogues, we generated a library of compounds with potential AChE inhibitory activity and demonstrated the hChAT cosubstrate promiscuity.



Scheme 1. Various combinations of choline and CoA analogues.

Shown in Scheme 1 are the proposed combinations of choline (substrates) and CoA (cosubstrates) analogues. It is reported that HC is a substrate for hChAT.[19] The results of the reactions with the various combinations tested are shown in Table 1. Choline analogues **5**–**8**, **10**–**15**, **17**, and **19** did not react well with acetyl-CoA when using hChAT, and were therefore not pursued further with other CoA analogues. The unreactive aspect of compounds **5**–**8**, **12**, and **19** imposes a limit to the length of the choline derivative. Propylcholine (**4**) reacted well with acetyl-CoA, however, the addition of a methylene as in butylcholine (**5**) removed much of the reactivity. In addition, compounds **10**, **11**, **13**, and **14** demonstrate that substituting the terminal proton of propylcholine with one or more heteroatoms also disrupts the ability of hChAT to acetylate these compounds. Interestingly, benzylcholine (**15**) does not become acetylated and phenylethylcholine (**16**) does. The ethylphenyl group resembles HC, which is known to fit into the active site, and implies that too much rotational freedom prohibits the choline analogues from binding to the hChAT active site. These results suggest that the channel might be smaller than originally thought. We also tested a few compounds with double substitutions. Diethylcholine and dibutylcholine (not shown) demonstrated no activity with hChAT; this led us to conclude that the choline analogues require two methyl groups or a constrained cyclic structure for catalysis to take place.[19]

On the CoA side, we observed that compounds **28**–**30** and **33** did not work with any of the choline analogues tested. The data show that having an extended carbonyl in the CoA derivative will also hinder the binding of the CoA. This is the first report of hChAT cosubstrate promiscuity. Other acetyltransferases have also been shown to be cosubstrate promiscuous, including aminoglycoside acetyltransferases (AACs)[20] and *N*-acetyltransferases from the Gcn-5 family.[21]

From the kinetic data (Table 2), we can see that hChAT has a similar K_m for choline as previously reported.[19] In our hands choline has a K_m of (0.59 ± 0.12) mM with a catalytic turnover (k_{cat}) of (1.04 ± 0.02) s^{-1} and yields an overall efficiency (k_{cat}/K_m) of 1.78 $mM^{-1}s^{-1}$; the previously reported K_m is 0.17 mM.[19] It is important to note that the previously reported enzyme had the N-terminal tag cleaved from the protein and we chose to leave the tag intact. Experiments were also performed to determine the kinetic parameters of acetyl-CoA, which displayed

Table 1. Compounds tested and generated by using hChAT.

Substrate	Cosubstrate									
	20	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**31**	**32**
1	34[a]	35[a]	36[b]	37[c]	38[c]	39[c]	40[b]	41[a]	45[b]	46[a]
2	47[a]	48[a]	49[a]	50[b]	51[b]	52[c]	53[b]	54[b]	55[a]	56[a]
3	57[a]	58[a]	59[b]	60[c]	61[c]	62[c]	63[b]	64[a]	65[b]	66[a]
4	67[a]	68[a]	69[b]	70[c]	71[c]	72[c]	73[c]	74[c]	75[c]	76[b]
9	77[a]	78[a]	79[a]	80[b]	81[b]	82[b]	83[b]	84[a]	85[b]	86[a]
16	87[a]	88[b]	89[b]	90[b]	91[b]	92[c]	93[c]	94[c]	95[c]	96[c]
18	97[a]	98[b]	99[b]	100[b]	101[c]	102[b]	103[b]	104[c]	105[c]	106[c]

[a] Indicates a good increase in the spectrophotometric assay over 30 min. [b] Indicates a moderate increase in the spectrophotometric assay over 30 min. [c] Indicates poor or no increase in the spectrophotometric assay over 30 min.

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Table 2. Kinetic parameters of hChAT.

Choline analogue	CoAs	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$\mu M^{-1} s^{-1}$]
	CoA analogue kinetic parameters[a]			
choline (**1**)	AcCoA	20.5 ± 0.9[b]	2.4 ± 0.4	0.115
	ProCoA	31.7 ± 9.3	0.31 ± 0.001	0.010
	CrotCoA	7.1 ± 2.4	0.104 ± 0.012	0.015
	MalCoA	19.9 ± 1.5	0.122 ± 0.014	0.006
aminocholine (**2**)	AcCoA	19.4 ± 1.1	1.99 ± 0.0118	0.103
	ProCoA	12.7 ± 5.2	0.197 ± 0.065	0.016
	BenzCoA	2.9 ± 0.6	0.177 ± 0.008	0.061
	CrotCoA	8.6 ± 1.7	0.231 ± 0.022	0.027
ethylcholine (**3**)	AcCoA	14.8 ± 3.9	0.219 ± 0.013	0.015
propylcholine (**4**)	AcCoA	3.8 ± 1.0	0.074 ± 0.006	0.020
OH-ethylcholine (**9**)	AcCoA	18.5 ± 6.2	0.760 ± 0.180	0.041
propargylcholine (**18**)	AcCoA	10.1 ± 0.9	0.122 ± 0.013	0.012
	Choline analogue kinetic parameters[a]			
Choline analogue	CoAs	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{-1} s^{-1}$]
choline (**1**)	AcCoA	0.59 ± 0.12[b]	1.04 ± 0.02	1.78
aminocholine (**2**)	AcCoA	0.34 ± 0.03	16.2 ± 0.04	47.6
OH-ethylcholine (**9**)	AcCoA	1.2 ± 0.5	0.723 ± 0.208	0.60

[a] Kinetic parameters were determined at pH 7.5. [b] Errors were calculated from at least three independent trials of kinetic experiments.

a K_m of (20.5 ± 0.9) μM, a k_{cat} of (2.4 ± 0.4) s^{-1}, and a catalytic efficiency of 0.115 $\mu M^{-1} s^{-1}$ (Table 2).

We also determined the kinetic parameters of *n*-propionyl-CoA, crotonyl-CoA, and malonyl-CoA (Table 2). In most cases the K_m of the CoA derivative was equal to or higher than acetyl-CoA, however, in all cases the k_{cat} was much lower (tenfold) and led to a reduced catalytic efficiency. In the case of crotonyl-CoA, the K_m was lower (7.1 ± 2.4 μM) than acetyl-CoA. Perhaps the double bond allows for binding interactions that are missing in the other CoA analogues. Kinetic parameters were also determined for acetyl-CoA by using other choline analogues and no significant change was seen in the binding; this shows that regardless of the modification to the choline, the binding of acetyl-CoA remains unchanged.

We wanted to investigate aminocholine (**2**) because of the potential of the amide analogues of acetylcholine to serve as AChE and/or BChE inhibitors. We found that aminocholine had a K_m of (0.34 ± 0.03) mM, which is identical to choline, a similar k_{cat} of (16.2 ± 0.04) s^{-1}, which gives a catalytic efficiency of 47.6 $mM^{-1} s^{-1}$. The increased catalytic turnover can be explained from the increased nucleophilicity of the nitrogen compared to oxygen. Also interesting about aminocholine is its ability to accept additional cosubstrates not accepted by choline. In addition to *n*-propionyl-CoA, crotonyl-CoA, and malonyl-CoA, aminocholine also accepts benzoyl-CoA and butyryl-CoA. When the kinetic parameters for all derivatives were determined, we observed that all had similar or lower K_m values compared to that of acetyl-CoA (Table 2). The tested cosubstrate analogues also have decreased catalytic turnovers as expected, and yield lower catalytic efficiencies. Confirmation of completion of the chemoenzymatic reactions were performed by TLC (Figure 2, representative example with aminocholine (**2**) and acetyl-CoA).

In summary, we have presented evidence that hChAT exhibits its relaxed specificity with respect to both the substrate and cosubstrate. We have provided experimental kinetic data for the choline derivatives, acetyl-CoA and CoA derivatives. This work delineates the limits of the hChAT enzyme as a tool for the rapid and easy production of libraries of acetylcholine analogues as potential ChE inhibitors. To further increase the diversity and scope of both substrates and cosubstrates that can be used for the rapid chemoenzymatic generation of high-throughput acetylcholine analogue libraries our observations establish the groundwork for expanding the promiscuity of the hChAT enzyme.

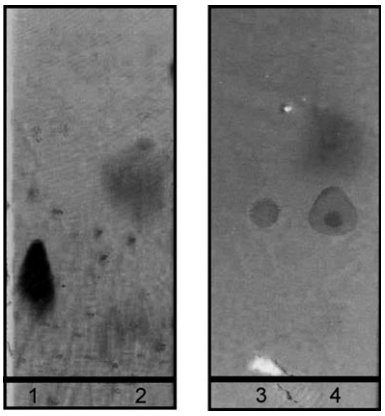

Figure 2. TLCs of aminocholine (**2**; lane 1, $R_f = 0.27$), acetylaminocholine (**47**; lane 2, $R_f = 0.45$), choline (**1**; lane 3, $R_f = 0.36$), and acetylcholine (**34**; lane 4, $R_f = 0.64$ as a mixture with **1**) produced by using hChAT, and visualized with Dragendorff's reagent; eluent system: 6:2:2 MeCN/MeOH/ammonium nitrate (0.5 M).

Experimental Section

Materials: All alkyl halides, ethanolamines, THF, choline, aminocholine, CoA derivatives, and buffer components were obtained from Sigma–Aldrich. Methanol and acetonitrile were obtained from Fisher Scientific. All compounds were used without any further purification. The hChAT was over-expressed in *E. coli* BL21 (DE3) cells (Invitrogen) and the pProExHTa plasmid containing the His-tagged enzyme was kindly provided by Prof. Brian Shilton (University of Western Ontario, ON, Canada). NMR spectra were recorded by using a Varian 400-MR and a 300 MHz Bruker DPX-300. IR measurements were taken by using a Perkin–Elmer SpectrumBX FT-IR system.

Expression and purification of recombinant hChAT: The purified plasmid hChAT-pProExHTa (generously provided by Brian H. Shilton, University of Western Ontario, ON, Canada) was transformed into *E. coli* BL21 (DE3) competent cells for protein expression and purification. Production of the protein was performed as previously reported.[22] Briefly, LB medium (1 L) supplemented with ampicillin (100 $\mu g\,mL^{-1}$) was inoculated with 1 mL of an overnight culture of the transformant containing the hChAT-pProExHTa construct, and incubated with shaking (200 rpm) at 37 °C. The cultures were

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grown to an OD_{600} of ~0.5, induced with isopropryl-β-thiogalactopyranoside (IPTG; 0.4 mL of 1 M stock; final concentration 0.4 mM) and shaken for an additional 16 h at 18 °C. Cells were harvested by centrifugation (6000 rpm, 5 min, 4 °C, Beckman Coulter Aventi JE centrifuge, F10 rotor) and resuspended in buffer A (300 mM NaCl, 50 mM Na_2HPO_4, pH 8.0, adjusted at room temperature, 10% glycerol). Resuspended cells were lysed (one pass at 10000–15000 psi, Avestin EmulsiFlex-C3 high-pressure homogenizer) and the cell debris was removed by centrifugation (16000 rpm, 60 min, 4 °C, Beckman Coulter Aventi JE centrifuge, JA-17 rotor). The supernatant was then incubated with Ni-NTA agarose resin (3 mL; Qiagen) at 4 °C for 2 h with gentle rocking. The resin was loaded onto a column and washed with buffer A (10 mL) containing 5 mM imidazole, followed with buffer A (10 mL) containing 20 mM imidazole. The desired protein was eluted from the column in a stepwise imidazole gradient (5 mL fractions of 20 mM (2×), 40 mM (3×), 250 mM imidazole (3×)). Fractions containing the pure desired protein (as determined by SDS-PAGE; Figure S1) were combined and dialyzed at 4 °C against buffer A (3×1 L) for 1 h each. The dialyzed protein was concentrated by using Amicon Ultra PL-10, flash frozen, and stored at −80 °C. The protein concentration was determined by using a Nanodrop spectrometer (Thermo Scientific). Protein yield was 1.5 mg L^{-1} of culture.

Determination of CoA derivatives and substrate promiscuity: A previously developed spectrophotometric assay was used to monitor the formation of 4-thiopyridone from the reaction of 4,4′-dithiodipyridine with the CoA formed from acylation of choline compounds.[20] The assay exploits the absorbance at 324 nm of 4-thiopyridone, which results from reaction (200 μL) of hChAT (60 nM) with DTDP (2 mM), CoA derivative (200 μM) and choline or choline analogue (2 mM) in HEPES (50 mM, pH 7.5, adjusted at room temperature). The mixture was incubated at 37 °C for 25 to 30 min, and measurements were taken every 30 s in a high-throughput fashion by using a Spectra Max M5 microplate reader. Representative examples of "good", "moderate", and "poor" cosubstrates with various choline analogues are displayed in Figures S2–S4 in the Supporting Information.

Confirmation of reaction completion by TLC: Reactions (100 μL) were carried out at 37 °C in HEPES (50 mM, pH 7.5, adjusted at room temperature) in the presence of acetyl-CoA (11 mM), choline analogue (10 mM), and hChAT (36 μM), overnight. Aliquots (10 μL) of each reaction were loaded onto a TLC plate (EMD, Silica gel F254 250 mm thickness). The eluent system utilized was 6:2:2 MeCN/MeOH/ammonium nitrate (0.5 M), and visualization was achieved by using Dragendorff's reagent (solution A: 0.17 g $BiNO_3$ in 2 mL AcOH, 8 mL H_2O; solution B: 4 g KI in 10 mL AcOH, 20 mL H_2O; A + B were mixed and diluted to 100 mL in H_2O).

Determination of kinetic parameters: Kinetic parameters were determined by using the aforementioned assay. The kinetic parameters for the CoA analogues were determined by holding the choline or choline derivatives constant (2 mM) and varying the CoA concentration (0 to 0.25 mM). Parameters for choline and its analogues were determined by holding the acetyl-CoA concentration constant (1 mM) and varying the choline or choline analogue concentration (0 to 1 mM). Parameters were calculated by using a Lineweaver–Burke regression of the initial rates (representative examples are shown in Figures S5–S8).

Synthesis of choline analogues; general procedure: In general 2-(dimethylamino)ethanol was dissolved in THF or MeOH (10 mL). The alkyl halide was added to this solution. The reaction was stirred at room temperature, overnight. If the reaction was carried out in THF the product was filtered and dried in vacuo. When MeOH was used, the MeOH was evaporated under reduced pressure and the compound recrystallized from MeOH/EtOAc. The product was then filtered and dried. These methods were inspired by the work of Dasgupta et al.[23] See the Supporting Information for precise quantities, yields, and characterization of all compounds.

Acknowledgements

We gratefully acknowledge Dr. Brian H. Shilton (University of Western Ontario, ON, Canada) for the generous gift of the hChAT-containing plasmid. Dr. Mi Hee Lim and Allana Mancino (University of Michigan) are also acknowledged for use of equipment.

Keywords: acylation · Alzheimer's disease · coenzyme A cosubstrates · enzyme promiscuity · inhibitors

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Received: May 18, 2009
Published online on July 27, 2009

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