JOURNAL OF
EMERGING INVESTIGATORS

Article

Modular mimics of neuroactive alkaloids - design, synthesis, and cholinesterase inhibitory activity of rivastigmine analogs

Erika Yu[1,2], Shloka Raghavan[1,2], Shreya Anand[1,3], Harrison Xu[1,4], Tvisha Nepani[1,5], Niharika Nambiar[1,4], Julia Vu[1,6,] Alivia Zhang[1,7], Suhani Babu[1,4], Elena Brierley-Green[1,8], Anushka Peer[1,9], Alice Finkelstein[1,10], Adrienne Ferguson[1,11], Udbhav Avadhani[1,12], Sanhita Nittala[1,13], Edward Njoo[1]

[1] Department of Chemistry, Biochemistry, & Physics, Aspiring Scholars Directed Research Program, Fremont, CA
[2] Amador Valley High School, Pleasanton, CA
[3] Los Altos High School, Los Altos, CA
[4] Dougherty Valley High School, San Ramon, CA
[5] Milpitas High School, Milpitas, CA
[6] The Quarry Lane School, Dublin, CA
[7] American High School, Fremont, CA
[8] Palo Alto High School, Palo Alto, CA
[9] James Logan High School, Union City, CA
[10] Carlmont High School, Redwood City, CA
[11] Prospect High School, San Jose, CA
[12] Leigh High School, San Jose, CA
[13] California High School, San Ramon, CA

SUMMARY

The treatment of neurological diseases has evolved to include neuroactive alkaloids isolated from naturally occurring phytochemical sources. While some of these compounds have gone on to clinical use themselves, others have inspired the development of synthetic analogs, which might possess greater potency or better pharmacological features than the natural product itself. One such naturally occurring alkaloid, physostigmine, which is found in the Calabar bean plant *Physostigma venenosum*, has been demonstrated to be a potent cholinesterase inhibitor. However, some of physostigmine's characteristics limit its therapeutic potential, prompting the development of rivastigmine, a similarly structured synthetic compound. The research in our group focused on the synthetic optimization of rivastigmine and its analogs, utilizing computer modeling and biological assays to determine the most favorable analog for inhibition of acetylcholinesterase (AChE), the enzyme that breaks down the neurotransmitter acetylcholine (ACh) to terminate neuronal transmission and signaling between synapses. Patients with Alzheimer's Disease have lower levels of ACh, which has been associated with symptoms like memory impairment and confusion. The inhibition of AChE allows for ACh accumulation and continued signaling in parasympathetic nervous system. Through our studies, we determined that rivastigmine and its analogs were less effective at inhibiting AChE than physostigmine, and their biological activity is governed by sterics.

INTRODUCTION

For decades, neuroactive alkaloids isolated from naturally occurring phytochemical sources have been crucial in the identification and optimization of small molecules with potency in treating neurological disorders (1,2). Some natural products have gone on to inspire the development of synthetic analogs, which might possess greater potency or better pharmacological features than the natural product itself. One such naturally occurring alkaloid, physostigmine, which is found in the calabar bean plant *Physostigma venenosum*, has been demonstrated to be a potent cholinesterase inhibitor (3). Specifically, physostigmine possesses an N-methylcarbamate moiety, which reversibly acylates the active site serine in acetylcholinesterases, rendering such enzymes inactive in breakdown capabilities of acetylcholine, a neurotransmitter (4). This results in acetylcholine accumulations near cholinergic synapses in the central nervous system, allowing for continued chemical messaging in the parasympathetic nervous system (5).

Despite its potency as an enzyme inhibitor, physostigmine's low bioavailability, short half life of 16 minutes due to autohydrolysis of the N-methylcarbamate, and acute toxicity has limited its therapeutic potential (6 - 9). These challenges have prompted the development of rivastigmine, a synthetic amino carbamate small molecule that is inspired by and structurally analogous to physostigmine (**Figure 1**). In 2000, rivastigmine was approved by the United States Food and Drug Administration (FDA) to treat neurodegenerative diseases such as dementia, Alzheimer's disease, and Parkinson's disease (10 - 12). Acetylcholinesterase (AChE), the enzyme that breaks down acetylcholine (ACh), has two binding sites for ACh: an anionic site and an esteratic site.

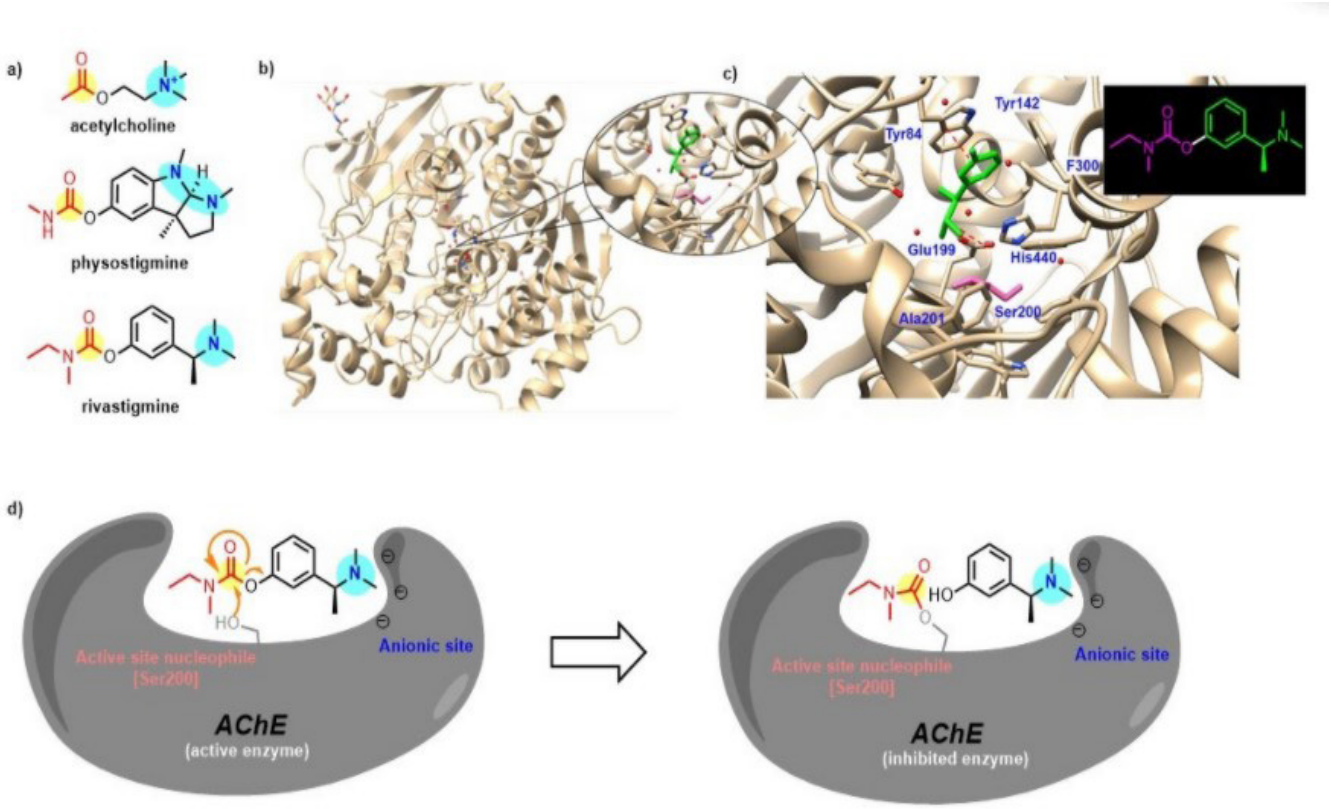

Figure 1. Rivastigmine is an FDA approved small molecule neuroactive therapeutic competitive inhibitor of acetylcholinesterase and butyrylcholinesterase. (a) Acetylcholine, the endogenous substrate of acetylcholinesterase (AChE), relies on a cationic site (highlighted in blue) and a hydrolyzable carbonyl (highlighted in yellow) for molecular recognition and binding to the active site of AChE. Physostigmine, a neuroactive natural product that competitively inhibits AChE, and rivastigmine, its synthetic mimic, share this pharmacophore template with tertiary amine functionalities (blue), which are protonated and hence cationic at physiological pH, and a hydrolyzable carbamate (yellow). (b) X-ray crystal structure of rivastigmine bound to AChE color coded (PDB:1GQR). (c) The magnified image of rivastigmine bound to AChE shows the residues that mediate the binding: Tyr84, Tyr142, F300, Glu199, His440, Ala201, and Ser200. The pink and green coloration seen in the image correspond to the carbonyl side (highlighted in pink) and the amine side (highlighted in green) of rivastigmine. (d) Binding of rivastigmine and similar substrates is mediated by its electrostatic interactions with the anionic binding site in the enzyme. Reaction with serine 200 causes covalent acylation of the active site nucleophile, which results in the inhibition of enzyme activity.

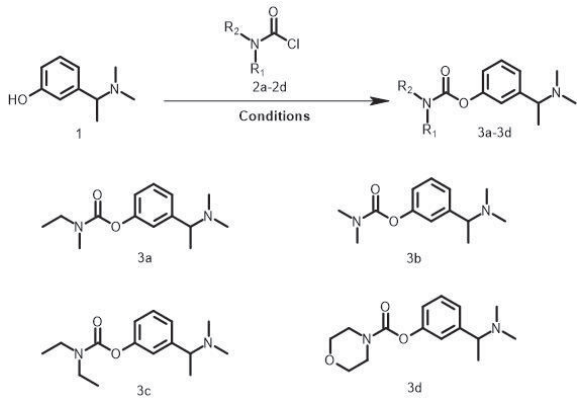

Figure 2. Summary of synthetic efforts. Rivastigmine (3a) and analogs (3b, 3c, 3d) were synthesized through one step acylation of 3-(1-(dimethylamino)ethyl)phenol (1) with the corresponding n, n-dialkyl carbamoyl chloride (2a-2d) while avoiding the use of pyrophoric hydride metals.

Rivastigmine acts as a competitive inhibitor as it binds to the enzyme's active site in a similar manner to ACh, preventing the hydrolysis of ACh by binding at the esteratic part of the catalytic site (14, 15). AChE then cleaves the rivastigmine molecule, releasing the resulting phenol (15).

Here we report the synthetic optimization and cholinesterase inhibitory activity of rivastigmine and four of its analogs to physostigmine and rivastigmine. Moreover, we employed computer modeling to give structural insight into the biochemical behavior of these compounds. We hypothesized the N,N-dimethyl carbamate analog to demonstrate the greatest inhibitory activity among the analogs. From our biological assays, we found the N,N-dimethyl carbamate analog did indeed inhibit acetylcholinesterase the most compared to rivastigmine and the other analogs, leading us to conclude that the differences in biological activity is due to sterics. We suggest a focus on synthesizing rivastigmine analogs with less steric bulk to improve inhibitory effects.

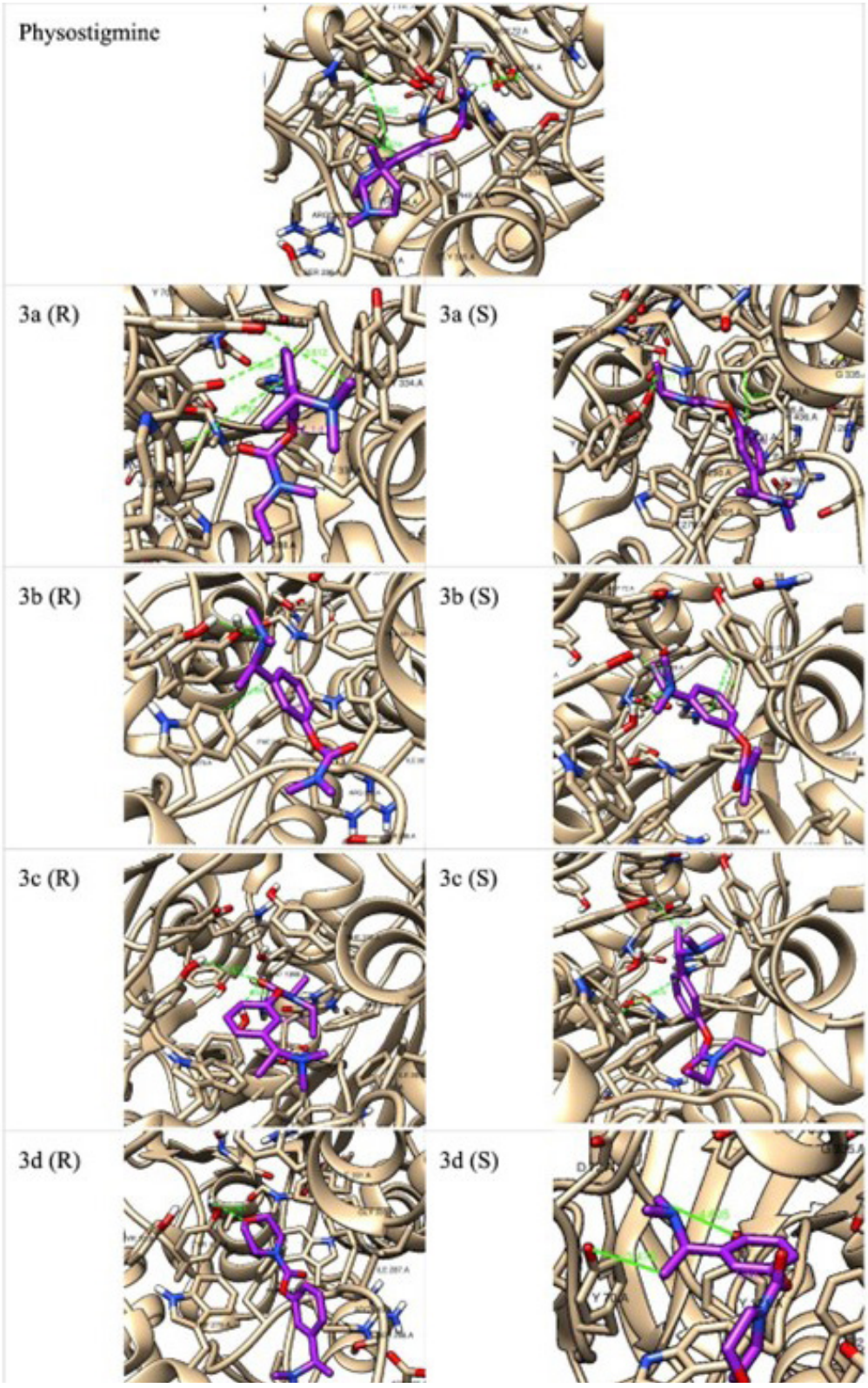

Figure 3. Molecular binding observed through docking experiments. Computational studies allow us to predict the behavior of our carbamate compounds when testing their efficacy with in vivo and ex vivo assays. Each panel on the left represents a synthesized analog with its s-enantiomer form on the right. The compound is colored purple, the protein AChE is colored tan, and all relevant interactions are colored green.

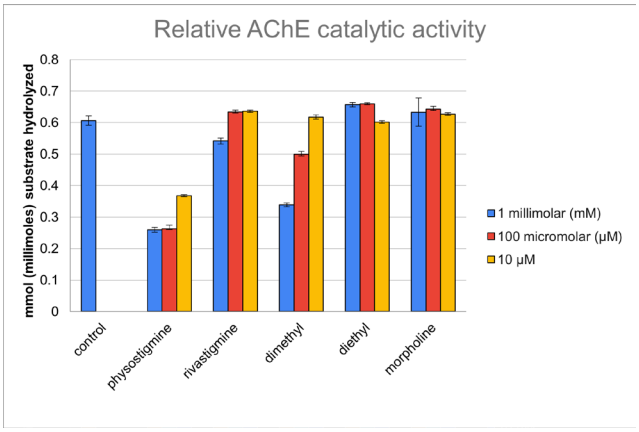

Figure 4. Comparison of acetylcholinesterase inhibitory activity between physostigmine, rivastigmine, and compounds 3b, 3c, and 3d. Bar graph showing millimoles (mmol) of substrate hydrolyzed (n=3). The control consisted of the 15% DMSO solution. Physostigmine and each of the analogs were diluted to 1 millimolar (mM), 10 micromolar (μM), and 100 μM in a 15% DMSO solution.

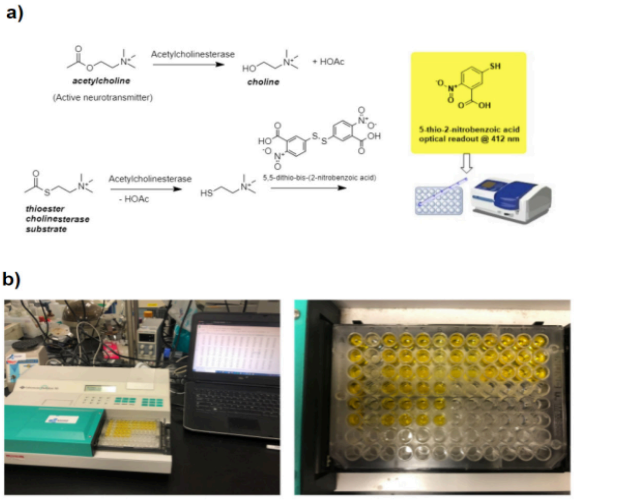

Figure 5. Methodology used for in vitro studies of acetylcholinesterase inhibitory ability of compounds 3a, 3b, 3c, 3d, and physostigmine. (a) AChE catalyzes the hydrolysis of acetylcholine into choline and acetic acid. Similarly, AChE catalyzes the hydrolysis of acetylthiocholine into an intermediate that results in the breakdown of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) into 5-thio-2-nitrobenzoic acid, which was used to colorimetrically determine the activity of AChE. (b) On the left, a plate reader is pictured, which was used to obtain quantitative data regarding absorbance (n = 3). On the right, a well plate is pictured, containing compounds after DTNB has been added, which results in varying intensities of pigmentation.

RESULTS

Chemical Synthesis

Previous reports of the synthesis of rivastigmine involved the acylation of 3-(1-(dimethylamino)ethyl)phenol and N-Ethyl-N-methylcarbamoyl chloride in the excess of sodium hydride (16). While it has shown to give the desired carbamate in high yields, sodium hydride is pyrophoric and difficult to handle due to its sensitivity to air and moisture (16). Therefore, we sought to identify alternative conditions to synthesize rivastigmine and analogs thereof that might avoid the use of sodium hydride.

Our efforts began with the acetylation of 3-(1-(dimethylamino)ethyl)phenol and N-Ethyl-N-methyl carbamoyl chloride, accomplished with pyridine and 4-dimethylaminopyridine (DMAP) in dichloromethane (DCM). This resulted in 57.3% yield. We also attempted synthesizing rivastigmine without using a base, but this effort was unsuccessful as no conversion was observed by thin layer chromatography (TLC).

Afterwards, we worked on the optimization and synthesis of three analogs of rivastigmine (referred to as compounds 3b, 3c, and 3d). Compound 3b was successfully synthesized with 3-(1-(dimethylamino)ethyl)phenol and dimethylcarbamoyl chloride, using DMAP in catalytic amounts, and DCM as a solvent (Figure 2). This resulted in 26.4% yield. No product was yielded using toluene as a solvent. However, Compound 3c was most successfully synthesized with 3-(1-(dimethylamino) ethyl)phenol and diethylcarbamoyl chloride, using DMAP in catalytic amounts, and toluene as a solvent (**Figure 2**). This resulted in 40.6% yield. By using pyridine as a base and DCM as a solvent, there was only 17.3% yield. Compound 3d was successfully synthesized with 3-(1-(dimethylamino)ethyl) phenol and 4-morpholinecarbonyl chloride, using no base, DMAP, and DCM, which yielded 58.3% (**Figure 2**).

Computation Results

Once we synthesized our analogs, we utilized computer modeling to obtain insight into their biochemical behavior. Molecules that performed well, those that bound more spontaneously to acetylcholinesterase, had various interactions with the enzyme's binding pocket. Physostigmine demonstrated both aromatic and hydrogen bonding interactions with the best performing conformer, including aromatic face-to-edge pi-stacking with Phe 290 at a distance of 3.574 Å and parallel-displaced pi-stacking at a distance of 3.7 Å to Trp 279 (Figure 3). Hydrogen bonding occurred between physostigmine and Tyr 70 (4.358 Å) (**Figure 3**).

Binding interactions for the r-enantiomers of the analogs were also both aromatic and hydrogen bonding, as well as some cation/anion-pi interactions (Figure 3). Compound 3c had hydrogen bonding with Tyr 70 (4.1 Å) and Tyr 121 (3.5 Å) (**Figure 3**). Compound 3b also had hydrogen bonding with Tyr 70 (3.1 Å), as well as parallel-displaced face-to-face aromatic stacking with Trp 279 (Figure 3). Compound 3a also had hydrogen bonding with Tyr 70 (3.6 Å) and Tyr 121 (3.6 Å), and face to face pi-stacking with Trp 279 (5.7 Å) (**Figure 3**). Compound 3d only demonstrated hydrogen bonding with Tyr 121 (3.1 Å) (**Figure 3**). Therefore, commonalities between the r-enantiomers of the molecules were aromatic interaction with Trp 279, as well as various Tyr hydrogen bonding interactions.

Among the s-enantiomers of our compounds, the r-enantiomers displayed many of the same types of interactions (**Figure 3**). Compound 3c demonstrated hydrogen bonding with Tyr 70 (3.6 Å) and face-to-face pi-stacking with Trp 279 (**Figure 3**). Compound 3b and compound 3d were similar in their interactions, with hydrogen bonding with Tyr 70 (3.1 Å), but they differed in that 3b had hydrogen bonding with Tyr 121 (4.0 Å), and they both had had parallel displacement pi-stacking interactions with Tyr 334 and Trp 279, respectively (**Figure 3**). Compound 3a was similar to its other enantiomer with hydrogen bonding interactions with Tyr 70 (2.8 Å), as well as face to face pi-stacking with Trp 279 (3.8 Å) (**Figure 3**).

Colorimetric Assay

We sought to test our computational predictions using *in vivo* colorimetric assays to test acetylcholinesterase inhibitory activity using difference concentrations (1mM, 100μM, and 10μM) of physostigmine, rivastigmine, and compounds 3b-3d. We found that physostigmine outperformed rivastigmine and all of our analogs, with strong inhibition observed at all concentrations tested (**Figure 4**). However, among the analogs, compound 3b exhibited the greatest inhibitory activity. Compound 3a, in comparison, only exhibited a slight inhibition. In addition, compound 3c and 3d had effectively zero inhibitory activity even after two hours of incubation.

DISCUSSION

In this study, we synthesized and evaluated a library of racemic rivastigmine analogs for acetylcholinesterase inhibitory activity. Namely, we explored the role of alkyl substitution at the carbamate fragments. Through our experiments, we determined that physostigmine outperformed rivastigmine and all of our analogs. Our analog with the smallest alkyl substituents (compound 3b, with an N,N-dimethyl carbamate) performed better than those with bulkier alkyl substituents. This was consistent with our initial hypothesis, although all of our compounds performed unexpectedly poorly in comparison to physostigmine. Within the 15-minute compound exposure window, the inhibitory activity of rivastigmine at 1 millimolar (mM) was minimal, and almost zero inhibition of AchE was observed at 100 micromolar (μM) and 10 μM of rivastigmine. Similarly, our diethyl analog (compound 3c) and morpholine analog (compound 3d) did not result in any meaningful enzymatic inhibition even at 1 mM. This suggests that the efficacy of these compounds in inhibiting acetylcholinesterase is governed by a steric factor introduced by the alkyl chains attached to the nitrogen of the carbamate. Physostigmine, which only has a n-monomethyl, was the most potent of all the compounds. This might be either attributed to greater binding affinity, less steric encumbrance at the reaction center, or a combination of these factors.

Additionally, for the compounds that exhibited inhibitory activity, a dose dependency was observed, wherein higher concentration of compounds results in greater inhibition of acetylcholinesterase. This is consistent with expectation, particularly if the enzyme reaction is governed by Michaelis–Menten kinetics.

The results presented herein established the role of carbamate sterics in the inhibitory activity of physostigmine, rivastigmine and its analogs, providing insight into the future development and discovery of more potent AChE inhibitors for treatment of neurodegenerative disease.

Since physostigmine was the most effective inhibitor, with a n-monomethyl carbamate, the synthesis of a desethyl analog of rivastigmine is likely to demonstrate greater inhibitory activity than rivastigmine itself against ACHe. Moreover, while rivastigmine is a dual cholinesterase inhibitor with no selectivity between acetylcholinesterase and butyrylcholinesterase, it is possible that our analogs might exhibit more selectivity of one over the other based on sterics. Finally, the S-enantiomer of rivastigmine is more biologically active than the R-enantiomer (17). Our current synthesized analogs were made from a racemic mixture. Whether this role of stereochemistry is also operative in our analogs remains to be seen. The synthesis of new analogs inspired by the work here, S-enantiomer analogs as well as cholinesterase selectivity experiments, are currently underway in our laboratory and shall be reported in the due course of time.

MATERIALS AND METHODS

Computer Modeling

Each of the target analogs were screened computationally with AChE in both configurations due to the chirality of rivastigmine. The geometries of the R- and S- enantiomers of each analog compounds were constructed on Avogadro, an open-source molecular modeling software (17). Following this, they were preoptimized by molecular mechanics at 10,000 steps with Universal Force Field (UFF). These structures were thermodynamically minimized via density functional theory (DFT) at the B3LYP / def2-SVP level of theory. DFT calculations were performed on ORCA, an ab initio software suite for quantum chemistry calculations (19). The optimized structures were docked to the active site of acetylcholinesterase to predict binding affinities of the analogs. Molecular docking was performed on AutoDock Vina, and were visualized on UCSF Chimera (19-23). All computer modeling was performed on a Dell PowerEdge 710 server with a 24 core Intel Xeon X5660 processor at 2.80 GHz and 32GB RAM.

Synthesis

Following computational screening, each of the analogs was synthesized using the mechanism outlined (Figure 2). The respective carbamoyl chlorides were used to synthesize compounds 3a, 3b, 3c, and 3d. Two different conditions were attempted for each analog: the presence and absence of base, and the usage of DCM or toluene as solvent. DMAP was used as a catalyst for all reactions.

In vitro Assay

Compounds 3a, 3b, 3c, 3d, and physostigmine were diluted to 1 mM, 10 µM, and 100 µM in 15% DMSO and 85% deionized water. Fifty µL of each compound was added to a Thermo Fisher 96 well plate through a multi-channeled pipette. Fifty µL of the acetylcholinesterase enzyme was diluted in 12 mL of 0.1 M Tris Buffer and pipetted into each well (Figure 5). This process was done twice, with the first well plate being incubated for 120 minutes and the second for 75 minutes.

Fifty µM of 0.1 M Tris buffer (pH 7) was added to 0.1 M of AK Scientific S-acetylthiocholine iodide (95% purity) to make the substrate solution. After substrate solution was added, the well plate was incubated for 12.5 minutes.

Subsequently, 25 µL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) solution was added into each well. The well-plate was placed in a Labsystems MultiSkan MS plate reader in order to measure absorbance (Figure 5). Each trial was done in triplicates. Following the first set of scans, 25 µL of DTNB solution was added again to the well plate and was placed in the plate reader to be scanned another 3 times. All data points were reported as triplicates.

ACKNOWLEDGMENTS

We would like to thank Aspiring Scholars Directed Research Program (ASDRP) for supporting this project.

Received: March 28, 2022
Accepted: May 30, 2022
Published: August 22, 2022

REFERENCES

1. Matsuura, Hélio Nitta, and Fett-Neto, Arthur Germano. “Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action.” Plant Toxins, 7 Aug. 2015, pp. 1–15., doi:10.1007/978-94-007-6728-7_2-1.
2. Hussain, Ghulam *et al.* “Role of Plant Derived Alkaloids and Their Mechanism in Neurodegenerative Disorders.” International Journal of Biological Sciences vol. 14,3 341-357. 9 Mar. 2018, doi:10.7150/ijbs.23247
3. Andrade, Olyn A. “Physostigmine.” NCBI, U.S. National Library of Medicine, 25 July 2021.
4. Triggle, David. “The Pharmacology of Physostigmine.” CNS Drug Reviews, Wiley-Blackwell, 14 July 2021.
5. Nordberg, A, and A L Svensson. “Cholinesterase inhibitors in the treatment of Alzheimer's disease: a comparison of tolerability and pharmacology.” Drug Safety vol. 19,6 (1998): 465-80. doi:10.2165/00002018-199819060-00004
6. Somani, S M, and S N Dube. “Physostigmine--an overview as pretreatment drug for organophosphate intoxication.” International Journal of Clinical Pharmacology, Therapy, and Toxicology vol. 27,8 (1989): 367-87.
7. Coelho Filho, João M, and Jacqueline Birks. “Physostigmine for Dementia Due to Alzheimer's Disease.” Cochrane Database of Systematic Reviews, 2001, doi:10.1002/14651858.cd001499.
8. Birks, Jacqueline S, *et al.* “Rivastigmine for Alzheimer's Disease.” Cochrane Database of Systematic Reviews, 2015, doi:10.1002/14651858.cd001191.pub4.
9. Henderson, Emily J *et al,* “The ReSPonD trial--rivastigmine to stabilise gait in Parkinson's disease a phase II, randomised, double blind, placebo controlled trial to evaluate the effect of rivastigmine on gait in patients with Parkinson's disease who have fallen.” BMC neurology vol. 13 188. 3 Dec. 2013, doi:10.1186/1471-2377-13-188
10. Jann, M W. “Rivastigmine, a new-generation cholinesterase inhibitor for the treatment of Alzheimer's disease.” Pharmacotherapy vol. 20,1 (2000): 1-12. doi:10.1592/phco.20.1.1.34664
11. Colović, Mirjana B *et al,* “Acetylcholinesterase inhibitors: pharmacology and toxicology.” Current neuropharmacology vol. 11,3 (2013): 315-35. doi:10.2174/1570159X11311030006
12. Onor, Maria Luisa *et al.* “Rivastigmine in the treatment of Alzheimer's disease: an update.” Clinical interventions in aging vol. 2,1 (2007): 17-32. doi:10.2147/ciia.2007.2.1.17
13. Venkatasubban, Kunisi S., *et al.* “Decarbamoylation of Acetylcholinesterases Is Markedly Slowed as Carbamoyl Groups Increase in Size.” ScienceDirect, Oct. 2018, https://doi.org/10.1016/j.abb.2018.08.006.
14. Venkatasubban, K. S., Johnson, J. L., Thomas, J. L., Fauq, A., Cusack, B., & Rosenberry, T. L. (2018). Decarbamoylation of acetylcholinesterases is markedly slowed as carbamoyl groups increase in size. Archives of Biochemistry and Biophysics, 655, 67–74. https://doi.org/10.1016/j.abb.2018.08.006
15. Colović, Mirjana B *et al.* “Acetylcholinesterase inhibitors: pharmacology and toxicology.” Current neuropharmacology vol. 11,3 (2013): 315-35. doi:10.2174/1570159X11311030006
16. Sodium Hydride; MSDS No. 223441 [Online]; Sigma-Aldrich: St. Louis, MO, Jan 13, 2020.
17. Cordato, D.J., *et al.* “Stereochemistry in Clinical Medicine: A Neurological Perspective.” Journal of Clinical Neuroscience, vol. 10, no. 6, 8 Oct. 2003, pp. 649–654., doi:10.1016/j.jocn.2002.10.001.
18. Marcus D Hanwell, Donald E Curtis, David C Lonie, Tim Vandermeersch, Eva Zurek and Geoffrey R Hutchison; “Avogadro: An advanced semantic chemical editor, visualization, and analysis platform” Journal of Cheminformatics 2012, 4:17.
19. Neese, Frank, *et al.* “The ORCA Program System.” WIREs Computational Molecular Science, vol. 2, no. 1, 2011, pp. 73–78. doi:10.1002/wcms.81.
20. Trott, Oleg, and Arthur J Olson. “AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading.” Journal of computational chemistry, vol. 31, no. 2, 2010, pp. 455-61. doi:10.1002/jcc.21334