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L-Hypaphorine and D-hypaphorine: Specific antiacetylcholinesterase activity in rat brain tissue

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ABSTRACT

Acetylcholinesterase (AChEis) inhibitors are used to treat neurodegenerative diseases like Alzheimer's disease (AD). L-Hypaphorine (L-HYP) is a natural indole alkaloid that has been shown to have effects on the central nervous system (CNS). The goal of this research was to synthesize L-HYP and D-HYP and test their anticholinesterasic properties in rat brain regions. L-HYP suppressed acetylcholinesterase (AChE) activity only in the cerebellum, whereas D-HYP inhibited AChE activity in all CNS regions studied. No cytotoxic effect on normal human cells (HaCaT) was observed in the case of L-HYP and D-HYP although an increase in cell proliferation. Molecular modeling studies revealed that D-HYP and L-HYP have significant differences in their binding mode positions and interact stereospecifically with AChE's amino acid residues.

Alzheimer's disease (AD) causes a lack of neurotransmitters, which are responsible for transmitting nerve stimulation from one neuron to another. Acetylcholine (ACh) (Fig. 1) is the primary neurotransmitter deficient in this disease.[1] ACh is important in the modulation of many functions throughout the central nervous system (CNS), including excitatory effects, attention, and cognition. The choline acetyltransferase (ChAT) is produced with choline (Ch) and acetyl coenzyme A as substrates. Acetylcholinesterase (AChE) terminates ACh neurotransmission by rapidly hydrolyzing ACh, yielding Ch and acetic acid.[2] Although there is no cure for AD, the use of Acetylcholinesterase inhibitors (AChEis) represents a significant advancement in the treatment of this pathology.[3] AChE remains one of the main well validated molecular targets for the treatment of neuromuscular disease and myasthenia gravis.[4] The AChEis prevent the degradation of ACh by neurotransmitters and consequently increases neurotransmission in cholinergic structures and other cholinergic systems.[1]

Several AChEis are based on natural products. The alkaloids, for example, are found in many medicinal plants and used as alternative medicines in many countries.[1,5] Several AChEis, such as galantamine, rivastigmine, and donepezil, have been approved by the Food and Drug Administration (FDA). Memantine, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, is another example. It is used in clinical procedures to improve memory in some people with AD.[6] The presence of L-hypaphorine (L-HYP) was found during phytochemical studies of *Erythrina mulungu* (*Erythrina verna*) (Fig. 1). Different biological studies show that *Erythrina* alkaloids act on the peripheral cholinergic system.[7] L-HYP is also present in large quantities in other species of *Erythrina* genus,[5,8,9] and its extracts have shown sedative and anticonvulsant effects.[7,10] L-HYP is present in human nutrition through the ingestion of lentils, peanuts, peanut butter, and chickpeas, and can be found in breast milk at a concentration up to 1.24 μM.[11] The alkaloid has many reported biological activities, such as anti-inflammatory,[12] antihyperglycemic and,[13] holds potential for treating obesity and insulin resistance.[14] Moreover, L-HYP is linked to the bioenergy cascade of neurotransmitters

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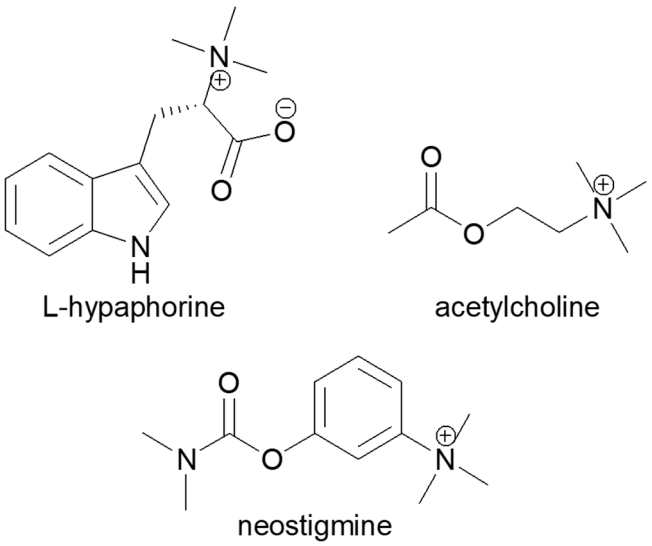


Fig. 1. Structure of L-hypaphorine (L-HPY), acetylcholine (ACh) and neostigmine (NEO).

in the brain, according to the results of sleep induction in rats.[8]

Bel-Kassaoui and co-workers (2008) investigated the neurotoxic action of L-HYP in goats.[15] This study was based on the possibility that L-HYP could be responsible for the neurotoxic action of *Astragalus lusitanicus*. The results showed no intoxication effect in the animals.[15] However, there are no detailed studies of the effect of L-HYP on enzymes of the cholinergic system, such as AChE. L-HYP and ACh have interesting bioisosteric relationships. L-HYP is also similar to that of neostigmine (NEO), a potent AChE inhibitor with a quaternary ammonium group (Fig. 1).[16]

As a result, because these structures have different profiles of AChE activity, this study proposed looking into the effect of L-HYP on AChE activity in the cerebral cortex, cerebellum, striatum, and hippocampus.[17] In addition, this work also proposed the synthesis and evaluation of the effect of D-HYP on AChE activities in view of the importance of the analysis of enantiomers for evaluating biological effects.[18,19]

L-HPY and D-HPY were prepared from L-tryptophan (L-Trp) and D-tryptophan (D-Trp), respectively, following the procedure depicted on Scheme 1. The synthesis consisted of the *N*-methylation reaction of L-Trp or D-Trp with CH_3I in the presence of base at room temperature (rt) in order to form a quaternary ammonium salt. The hypaphorines hydrochlorides (HYP·HCl) were obtained by adding a 2 M HCl solution under constant agitation and cooled to 0 °C. All compounds were purified by recrystallization with ethanol in high yields. The structures were confirmed by ^1H and ^{13}C NMR, and ESI-HRMS analyses (Appendix A. Supplementary data). The enantiomeric purities were documented based on optical rotation data (Appendix A. Supplementary data) and liquid enantioselective chromatography (Chirex® (*S*)-LEU/(*S*)-NEA column).

Even at basic ($NaHCO_3$) and acid (2 M HCl) pHs, the HYPs were obtained enantiomerically pure (Fig. 2), and no racemization reaction occurred (Fig. 2, Figs. S15-17 Supplementary Data). The HYP products were subjected to liquid enantioselective chromatography analyses, as well as mixtures, to confirm the chromatography efficiency in separating the enantiomers (Fig. 2). Because each product had only one chromatographic peak, it was easy to prove that they are enantiomerically pure and that racemization did not occur throughout the reactions.

L-HYP and D-HYP were evaluated in the form of hydrochloride salts (L-HYP·HCl and D-HYP·HCl), which are more soluble in water, to determine their AChE inhibitory activity. In each structure, the inhibition of AChE activity was investigated separately (i.e., brain, cerebral cortex, cerebellum, striatum and hippocampus). The AChE occurs throughout the CNS and is found in these structures in different

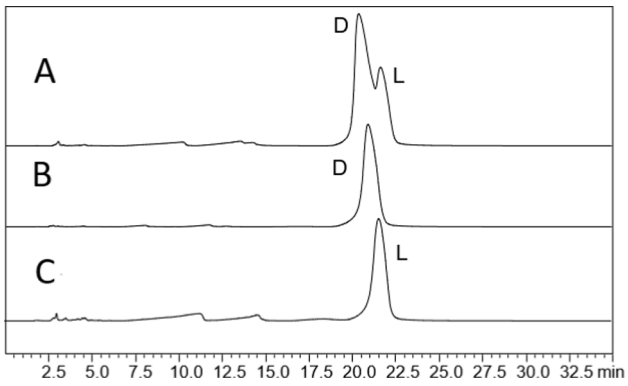


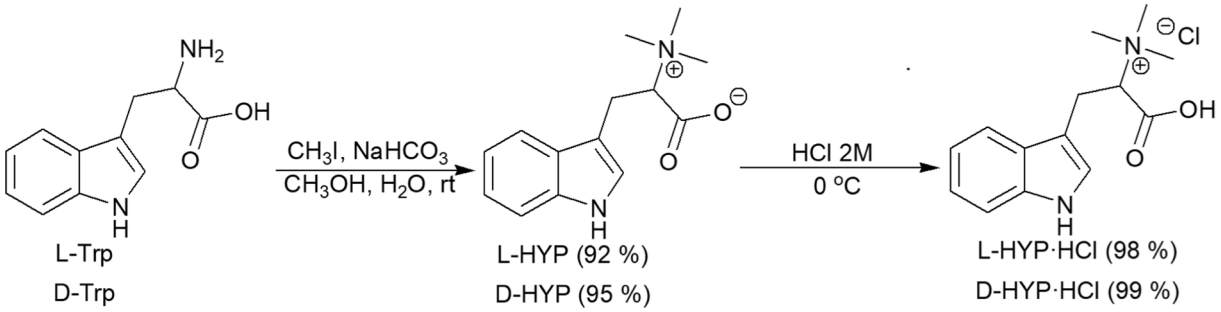
Fig. 2. Chromatograms at the wavelength 290 nm from D-HYP·HCl and L-HYP·HCl on the Chirex® (*S*)-LEU/(*S*)-NEA column. (A) Mixture of enantiomers D-HYP·HCl and L-HYP·HCl (2:1 w/w); (B) D-HYP·HCl; (C) L-HYP·HCl.

Table 1
IC_{50} of compounds on inhibition of AChE activity in different brain structures.

Compounds	IC_{50}[a] (μM ± S.E.M)			
	Cortex	Cerebellum	Striatum	Hippocampus
L-HYP·HCl	>1000	18.63 ± 0.14^A	>1000	>1000
D-HYP·HCl	0.24 ± 0.28^A	19.12 ± 0.03^A	34.60 ± 0.34^A	0.11 ± 0.32^A
NEOBr[b]	0.65 ± 0.14^B	37.18 ± 0.07^B	3.17 ± 0.04^B	0.06 ± 0.07^B

[a] Concentration of the compound (L-HYP·HCl, or D-HYP·HCl, or NEOBr) to inhibit 50% of AChE activity.

[b] Neostigmine bromide. The results are presented as $IC_{50} \pm$ standard error of the mean (S.E.M). Different capital letters in the column indicate a significant difference between the compounds (*t*-student, $p < 0.001$ or in the one-way ANOVA test of repetitive measurements with post-test of Tukey, $p < 0.05$). The concentration of substrate was saturating (1 mM) and the content of protein (mean ± SD) in cerebral cortex, cerebellum, striatum and hippocampus was 23.6 ± 1.72 μg, 14.8 ± 1.72 μg, 18.4 ± 2.8 μg, and 10.4 ± 1.92 μg, respectively.



Scheme 1. Synthesis of L-HYP (isomer *S*), D-HYP (isomer *R*) and their hydrochlorides (HYP·HCl). Reaction yields are in parentheses.

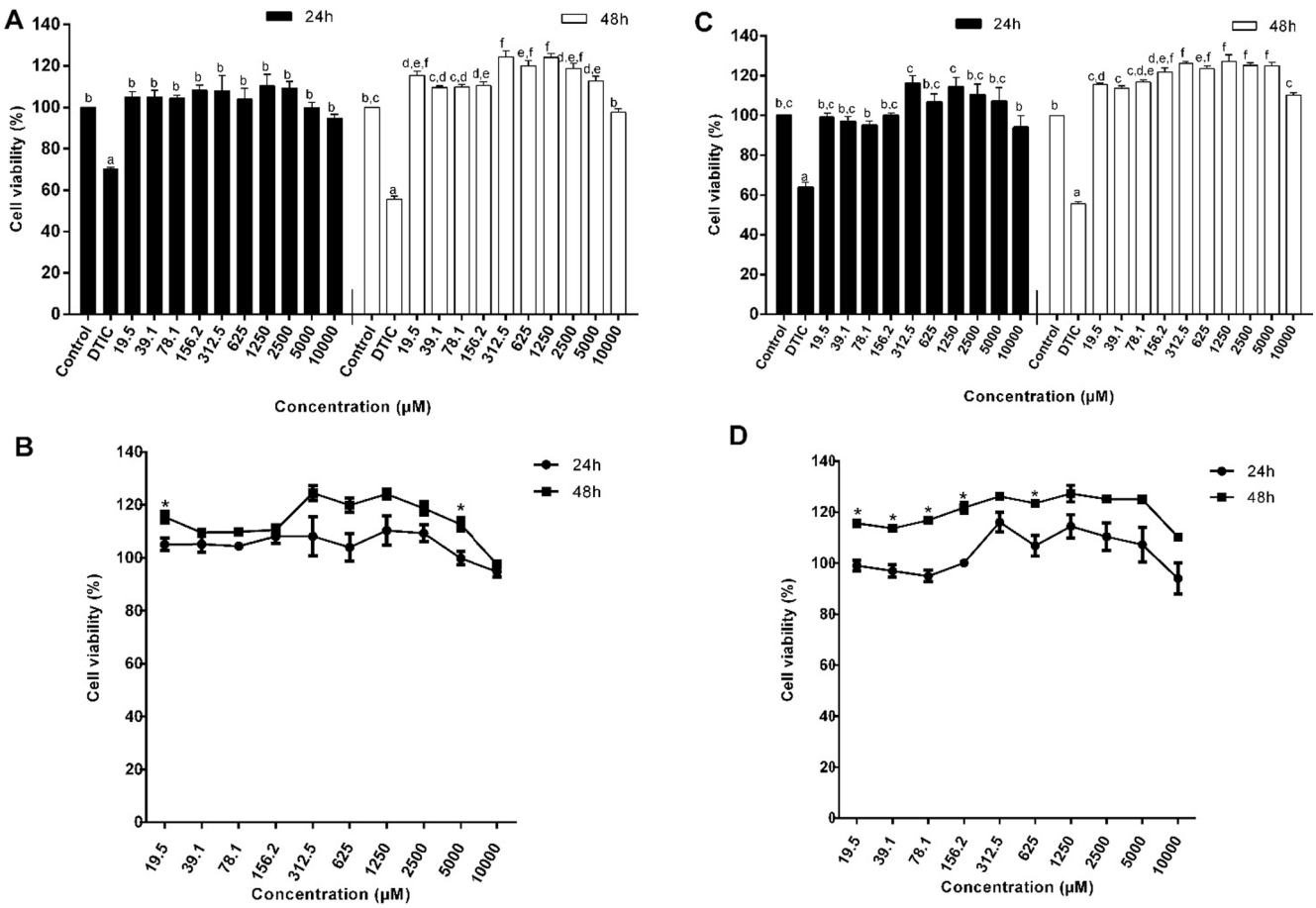


Fig. 3. Cell viability of normal human cells (HaCaT) treated with hypaphorine (HYP). (A) Effect of different concentrations L-HYP·HCl on HaCaT cells. (B) Comparison of the effects of L-HYP·HCl on cell viability in 24 h and 48 h. (C) Effect of different concentrations of D-HYP·HCl on HaCaT cells. (D) Comparison of the effects of D-HYP·HCl on cell viability in 24 h and 48 h. DTIC: dacarbazine. Different letters denote a significant difference between the treatments (ANOVA/Tukey, $p < 0.05$).

amounts.[20] The analysis of AChEis in different brain structures is crucial as it may demonstrate selective effects of inhibition.[17,21] Therefore, it is important for the understanding of the role of drugs in the treatment of neurodegenerative diseases that affect specific brain structures such as AD,[1] cerebellar degenerative disorders,[22] Huntington's disease,[23] and Parkinson's disease.[24] The concentration that inhibits 50% of AChE activity hydrolysis was determined by selecting brain areas in which the investigated compounds had an inhibitory effect on AChE activity. The neostigmine bromide (NEOBr) IC_{50} has also been determined in the four brain structures to compare the inhibitory potential of D-HYP·HCl and L-HYP·HCl (Table 1).

L-HYP·HCl promoted AChE inhibition had a selective effect with an IC_{50} of 18.63 ± 0.14 μM only in cerebellum. This effect outperformed the inhibitor NEOBr, which had an IC_{50} of 37.18 ± 0.07 μM. The cerebellum has the lowest cholinergic activity of all the brain areas; yet, the cholinergic system in this structure is vital for vestibulo-ocular reflexes, as well as motor coordination and cardiovascular regulation.[25]

Cerebellar cholinergic disorders are among the neuropsychiatric disorders that can cause cholinergic abnormalities, such as decreased ACh levels, leading to pathological alterations.[26] Autism spectrum disorder (ASD) is one such example.[25,27] Although there is still only a small understanding of the pathophysiology of ASD and cholinergic system, lower levels of L-HYP in ASD and associated allergic diseases have been observed.[28] The relationship between ACh and L-HYP levels in the bodies of ASD patients has not yet been reported. The result achieved in our investigations however showed that there is a particular AChE inhibition activity in the brain that indicates the interaction of L-HYP to ACh.

D-HYP·HCl inhibited AChE activity in all brain regions tested (Table 1). In the cortex and cerebellum, this enantiomer was more active than NEOBr, with IC_{50} values of 0.24 ± 0.28 and 19.12 ± 0.03 μM, respectively. The IC_{50} values of the enantiomers in the cerebellum did not differ significantly, indicating that they have comparable activity. However, unlike L-HYP·HCl, the inhibitory activity of D-HYP·HCl was not restricted to a specific brain structure. On hippocampus AChE, D-HYP·HCl showed the lowest IC_{50} (0.11 ± 0.32 M) when compared to NEOBr. The D-enantiomer has a higher activity in the cortex and hippocampus. The cholinergic system in these brain regions is the most affected during the early stages of Alzheimer's disease, causing memory loss, speech problems, and social behavior issues as the disease progresses.[29] Although D-HYP·HCl had lower inhibitory activity in the striatum when compared to other brain structures, its effect, even at higher concentrations, may contribute to cholinergic signaling, as the striatum contains high levels of ACh and plays an important role in motor control and learning.[30]

L-HYP, which is found in extracts of *Vaccaria segetalis* and *E. mulungu*, has recently been shown to have anti-inflammatory properties.[31,32] It is worth noting that in AD, an inflammatory process plays a significant role in the disease's onset and progression.[33] Moreover, evidence suggests that centrally acting AChEis used in the treatment of AD can modulate peripheral immune responses in order to activate the cholinergic anti-inflammatory pathway.[34] HYPs could act as an AChEi, increasing ACh levels and promoting ACh rebalance. As a result, ACh could build up in the synaptic cleft. Several studies on the effects of AChEis found that they slow gray matter atrophy in the hippocampus, cortex, and basal forebrain.[35]

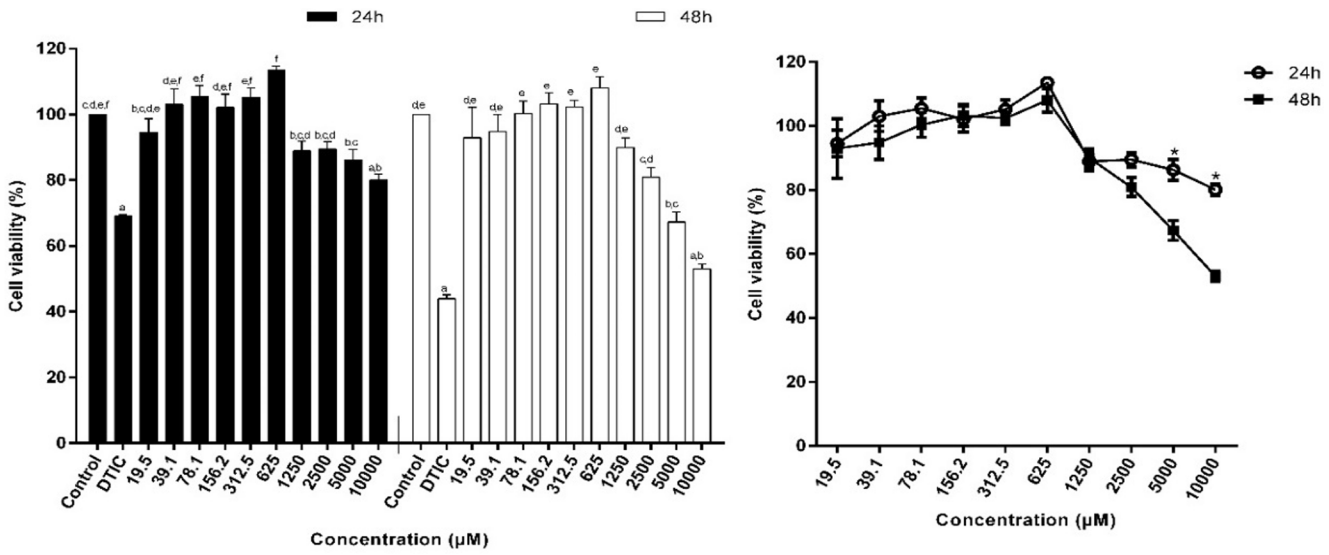


Fig. 4. Cell viability of normal human cells (HaCaT) treated with NEOBr. (A) Effect of different concentrations of NEOBr on HaCaT cells. (B) Comparison of the effects of NEOBr on cell viability in 24 h and 48 h. DTIC: dacarbazine. Different letters denote a significant difference between the treatments (ANOVA/Tukey, $p < 0.05$).

Previously, studies with racemic mixtures and pure enantiomers were conducted to evaluate the inhibition of AChE activity, and the results revealed that enantiomers may have different effects.[36] Studies show that AChE activities can vary in different brain structures due to various AChE isoforms. A plausible biological mechanism by which the same AChEi could act selectively on different brain structures remains unanswered.[17] The biological mechanism by which the same AChEi can act selectively on various brain areas is currently unknown.[17] The striatum is the most AChE brain structure due to the dense intrinsic cholinergic neurons[20] that can be linked to several factors such as: age[37] stresses,[38] malnourishment,[39] hypothyroidism,[40] and ethanol intake.[41]

Consequently, ideal AChE inhibitors should be highly specific for the various brain structures, have minimal effects on the peripheral cholinergic system, and cause no toxicity in other organs. The use of specific AChEis could lead to the development of more effective cognitive stimulants.[42,43] The survey of biological activity of pure enantiomers is important since studies show that the use of pure drugs has the advantage that the total dose administered is reduced, the dose–response ratio is simplified and the toxicity due to the inactive isomer is lessened.[44] This is also critical in drug development, in order to enhance clinical benefit while minimizing pharmacological adverse effects.[19]

Cell viability assays in the presence of L-HYP·HCl, D-HYP·HCl (Fig. 3) and, NEOBr (Fig. 4) were performed in normal human cells (HaCaT).[45] Dacarbazine (DTIC) was used as a positive control and was cytotoxic to 29.74 and 44.46% of the cells treated for 24 and 48 h, respectively. L-HYP·HCl and D-HYP·HCl showed no cytotoxic effect on HaCaT in the two periods analyzed (24 and 48 h) and did not alter the cell viability rate after 24 h of experiment ($p < 0.05$). The enantiomers promoted an increase in cell number after 48 h of the experiment (Fig. 3A and C). The comparison between the two periods demonstrated an increase ($p < 0.05$) in cell proliferation for the 19.5 and 5000 μM concentrations of L-HYP·HCl after 48 h (Fig. 3B) and, 19.5–156.2 and 625 μM concentrations of D-HYP·HCl after 48 h (Fig. 3D). The increased cell proliferation in the experiments observed with L-HYP·HCl and D-HYP·HCl may be associated with the concentration of ACh on HaCaT cells. HaCaT are keratinocytes and have a functional nonneuronal cholinergic system, including AChE, ChAT and, cholinergic receptors.[46] The result led us to assume L-HYP·HCl and D-HYP·HCl inhibit HaCaT's AChE activity and increase ACh concentration, which is related to increased cell viability, proliferation and, migration by the presence of this neurotransmitter in keratinocytes.[45,47] This explains in part the multiplication of cells observed in our experiments.

The results of the cell viability experiment for NEOBr (Fig. 4) showed that the compound exhibited cytotoxicity at concentrations of 10,000 μM (24 h), 5000 and 10,000 μM (48 h) (Fig. 4A). In the comparison between the two time periods, an increase ($p < 0.05$) in cytotoxicity was observed for the doses of 5000 and 10,000 μM (Fig. 4B).

When we compared the cytotoxic effects of L-HYP·HCl, D-HYP·HCl, and NEOBr, we discovered that the enantiomers were not cytotoxic and performed better than NEOBr. It is worth noting that NEO is one of the most commonly used AChE inhibitor drugs in perioperative medicine, especially after the administration of neuromuscular blockers.[48] More detailed studies of the increase in cell proliferation in response to AChE inhibition in keratinocytes are needed to confirm the suggested hypothesis comprising inhibitors such as NEOBr. The cytotoxicity, as well as genotoxic and apoptotic effects of NEOBr exhibited on HaCaT were also observed in human embryonic renal cells (HEK-293).[49] These toxic effects of NEO may account for the increased cell proliferation not observed on HaCaT upon treatment with NEOBr.

The differences in potency and selectivity demonstrated by L-HYP and D-HYP among the tested brain structures is an attractive result. However, these results are not rare in literature. Although chiral bioactive isomers, they have markedly different pharmacologic, toxicologic, pharmacokinetic and, metabolic behavior. One enantiomer may produce the aimed therapeutic effect and, the other may be inactive, exhibit lower potency and, even toxic effects.[50] The different biological properties of each enantiomer may be due to distinct binding modes at the active site of the target. Therefore, molecular modeling is an excellent tool for better understanding our biological outcomes.

The position of the ligand at the active site of AChE is crucial for its inhibitory capacity. The AChE has two main binding sites: one is a hydrophobic pocket with Ser, His and, Glu as the more significant amino acid residues and, the other, composed of Tyr, Trp and, Asp, is known as a peripheral anionic site of AChE.[51,52] The literature vastly reports these two pockets and, an array of binding modes are observed as a consequence of the structural diversity of the studied inhibitors. The differences between the structural features make difficult the description of a structure–activity relationship for the compounds. However, some amino acids are considered a key to the AChE inhibition as His440, Glu327, Ser200, Trp84, Trp279 and, Tyr121. Most of the inhibitors can interact with more than one site.[4] Due to the quantity of AChEis, the

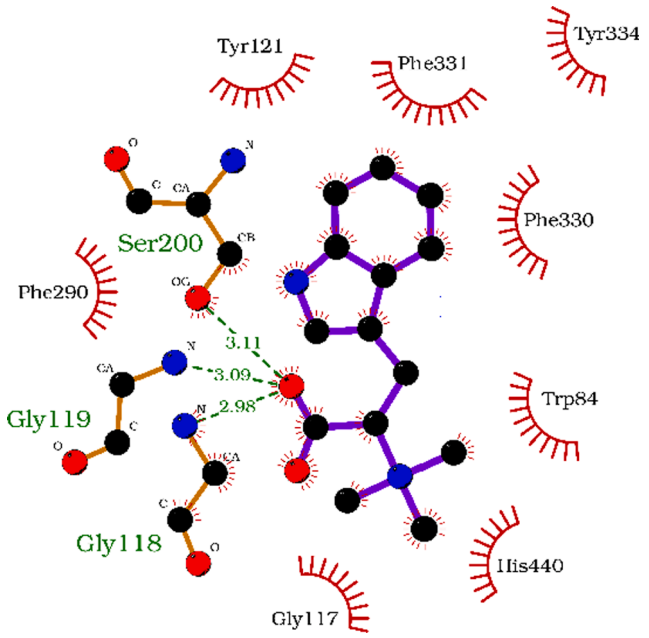


Fig. 5. Intermolecular interactions between D-HYP and AChE (PDB ID: 1QTI). The 2D diagram was provided by the program LigPlot+. Hydrogen bonds are shown as green dotted lines.

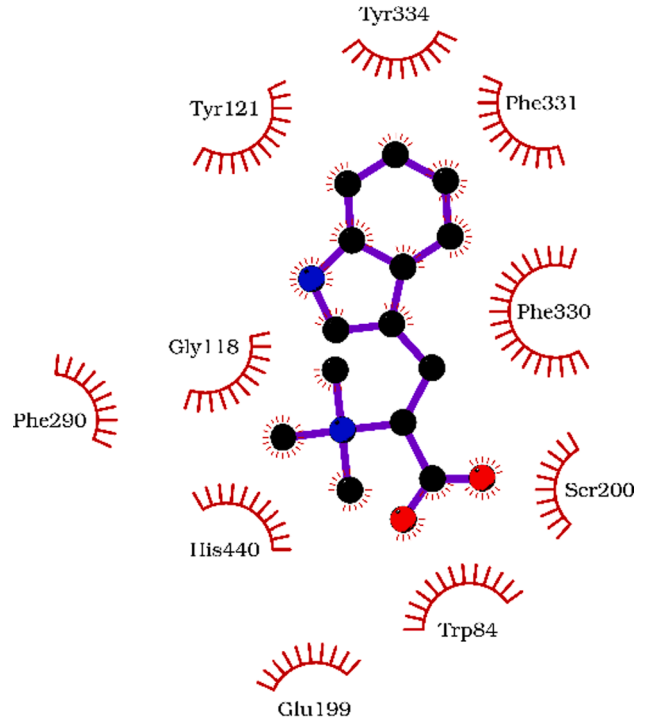


Fig. 6. Intermolecular interactions between L-HYP and AChE (PDB ID: 1QTI). The 2D diagram was provided by the program LigPlot+.

crystal of galantamine in complex with AChE (PDB ID: 1QTI)[53] was used in our molecular modeling studies since this compound is an alkaloid. The analyses considered the high structural similarity between the quaternary ammonium group D-HYP and L-HYP and ACh as well.

After the molecular docking simulations and geometry optimization, L-HYP and D-HYP presented remarkable differences in their binding mode positions interacting in a stereospecific way with the amino acid residues of AChE. The analysis of D-HYP at the active site of AChE

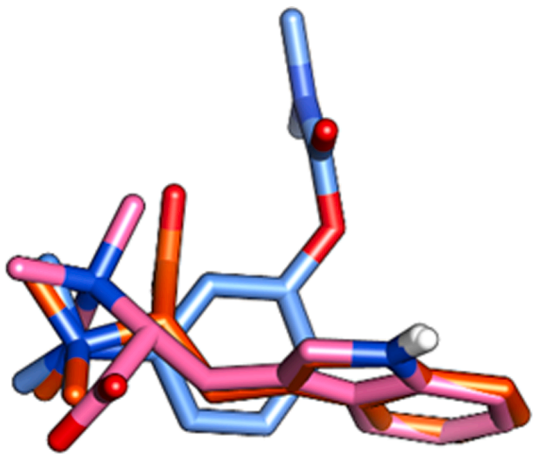


Fig. 7. Overlay of NEO (blue), L-HYP (pink) and D-HYP (orange).

showed many hydrophobic interactions with the amino acid Phe331, Phe330, Trp84, His440 and, Gly117 and hydrogen bonds with Ser200, Gly119 and, Gly118 (Fig. 5). Both are considered strong intermolecular interactions and, the sum of the results in a very stable complex between D-HYP and AChE. Such stability at the active site may explain the high activity demonstrated by D-HYP in the AChE inhibitory assay.

In contrast, the output of molecular simulations of L-HYP (Fig. 6) showed that the enantiomer binds to the active site through hydrophobic interactions and does not make hydrogen bonds as D-HYP (Fig. 5). The main difference between the binding mode position of D-HYP and L-HYP is the location of the carboxylic acid group. The absence of hydrogen bonds may be responsible for the lower inhibition capacity of L-HYP. The selective action of this enantiomer on the cerebellum is unclear yet. The study of the intermolecular interaction alone between the ligand and AChE seems insufficient since even ACh can have action in different brain regions depending on the physiological situation.[41]

It is also important to emphasize that NEO, used in the biological tests, has a different binding mode position when compared with D-HYP and L-HYP (Fig. 7). However, the standard compound occupies the same pocket at the active site of AChE. A direct comparison between the biological activities of L-HYP, D-HYP and, NEO is feasible. It is possible to verify a better overlap of the quaternary ammonium group of NEO and D-HYP, corroborating the results of AChE inhibition observed for these compounds concerning D-HYP.

L-HYP and D-HYP act differently to AChE inhibition in brain structures. L-HYP inhibited AChE only in the cerebellum, while D-HYP was also active in the cortex, striatum and, hippocampus. *In silico* results show that the location of the carboxylic acid group affects the binding mode of D-HYP and L-HYP. The compounds were not cytotoxic to HaCaT cells and, their role in increasing cell viability may be associated with the availability of ACh. The function of HYPs in the nonneuronal cholinergic system depends on additional research. L-HYP has many biological properties and, its action seems to have an importance for cognitive functionality, specialty in the cerebellum. In addition, D-HYP is a potent AChEi and can be considered a prototype for the development of drugs for neurodegenerative diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data (synthetic procedures and analytical/spectral data for compounds synthesized, procedures for *in silico* studies, AChE inhibitory and cytotoxicity assays) to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128206.

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