doi.org/10.1002/cbdv.202300207 Research Article CHEMISTRY & BIODIVERSITY

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Influence of Chirality of Benzimidazole Amine Hybrids on Inhibition of Human Erythrocytes Carbonic Anhydrase I, II and Acetylcholinesterase

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Novel chiral benzimidazole amine hybrids (**4a**–**4d**) were synthesized from commercially available amine [(*R*)- (+)-phenylethylamine, (−) (*S*)-(-)-phenylethylamine, (−) (*R*)-(-)-cyclohexylethylamine, (*S*)-(+)-cyclohexylethylamine] and 2-(chloromethyl)-N-tosyl-1*H*-benzimidazole. The synthesized compounds (**4a**–**4d**) were characterized by IR, NMR, and LC/MS analysis. The inhibitory effect of **4a**–**4d** on human erythrocytes carbonic anhydrase I (hCA-I), II (hCA-II), and acetylcholinesterase (AChE) activity was investigated. For hCA-I, the IC_{50} values of **4a**–**4d** were found to be 4.895 μM, 1.750 μM, 0.173 μM, and 0.620 μM, respectively, and for hCA-II, the IC_{50} values of **4a**–**4d** were found to be 0.469 μM, 0.380 μM, 0.233 μM, 0.635 μM, respectively. Furthermore, IC_{50} values of **4a**–**4d** on AChE were found as 87.5 nM, 100 nM, 26.92 nM, and 100 nM, respectively. In addition, molecular docking analysis was performed to evaluate the affinity of **4a**–**4d** against hCA-I, hCA-II, and AChE and explain their binding interactions.

Introduction

Hobrecker first synthesized benzimidazole in 1872, since then benzimidazoles and their derivatives became an essential heterocyclic compound found in many natural products.[1–7] Benzimidazole-hybrid has a unique chemical structure demonstrating a broad biological and therapeutic activity.[8–16] In recent years, extensive studies have revealed the importance of hybrid molecules containing benzimidazole structures. These remarkable structures have tremendous pharmacological activity, including anti-inflammatory, antiviral, anthelmintic, antihistaminic, antitubercular, antiulcer and antimicrobial, antiprotozoal, antileishmanial, antiglycation and antioxidant, antimycobacterial, anti-HIV, antitumor, and antiproliferative properties.[16–28] Recently, compounds containing benzimidazole structures have been identified as an anti-hypertensive agent, novel Zika inhibitors, inhibitors targeting HCV NS5B polymerase, antileukemic agents, potent activators of AMP-activated protein kinase anti-hepatitis C, and anticonvulsants.[29–35] Richards et al. demonstrated that the main benzimidazole structure has good efficacy in treating allergies and asthma.[36] In addition to these studies, various studies have reported that benzimidazole derivatives have anticancer effects.[37]

Carbonic anhydrase (CA) (EC 4.2.1.1) regulates the acidity of the chemical environment in the body and prevents body functions from being damaged.[38] Due to these vital physiological properties, extensive studies have been carried out on carbonic anhydrase enzymes. Anti-acetylcholine esterases (anti-AChE) are used as anti-Alzheimer's drugs to treat moderate Alzheimer's disease (AD) because of their enhanced cognitive connectivity and cholinergic neurotransmission in clinical applications. Many CA and AChE inhibitors have been identified and used in clinical practice. However, due to the high side effects of such inhibitors, there is a need for new and effective inhibitors that will reduce the use of unnecessary doses with low side effects.[39–40]

It is well-known that the chiral nature of these compounds is very effective in the biological and pharmacological properties. As a result, chiral compounds have a considerable importance in biological processes. The previous studies have shown that using chiral compounds as a drug reduces the effects of undesirable toxic and ecological and unnecessary excess drug use.[41–43] Many non-chiral have been reported for their inhibition activity against AChE, CA-I, and CA-II, while the information on the influence of chirality on such compounds is limited. This article reveals the application of chiral benzimidazole amine hybrids in inhibition activity for the first time.

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.202300207

Results and Discussion

Chemistry

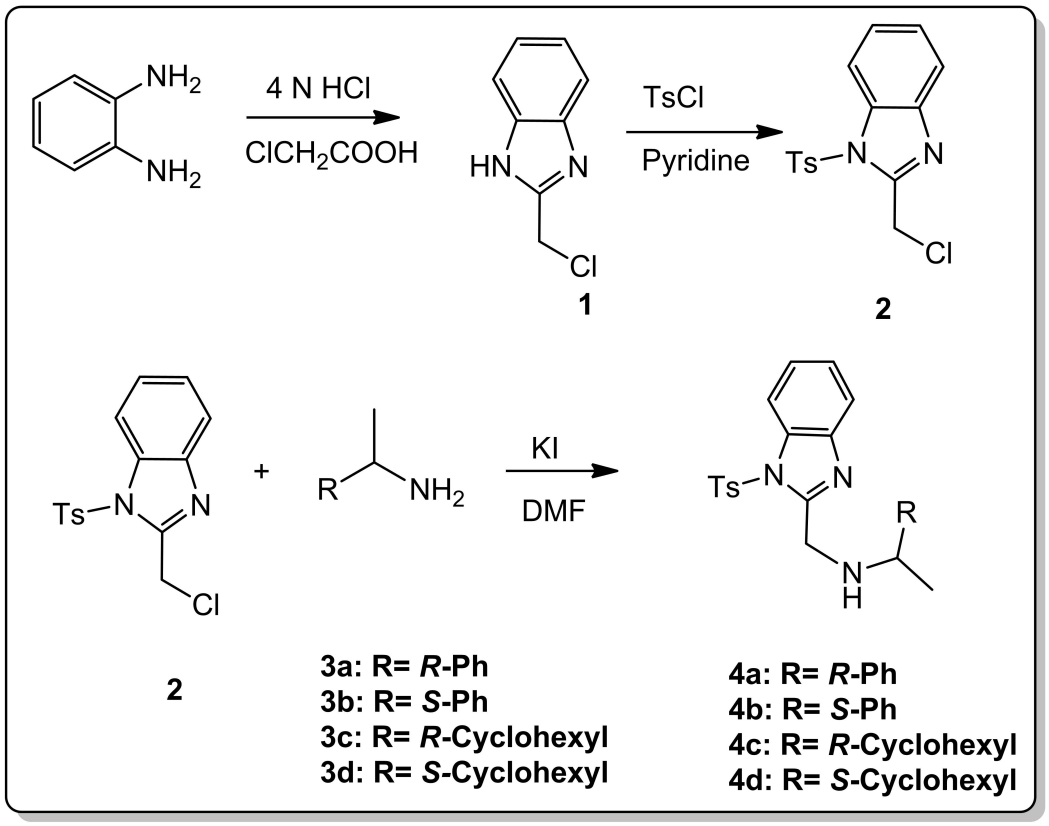
We previously synthesized 2-(Chloromethyl)-1-[(4-methylphenyl)sulfonyl]-1*H*-benzimidazole with the reaction of monochloro acetic acid and 1,2-diaminobenzene in 4 N HCl followed by *N*-tosylation of 2-(chloromethyl)-1*H*-benzimidazole **1** with *p*-toluenesulfonyl chloride in pyridine.[44] We started the synthesis of targeted chiral amine-benzimidazole hybrids **4a**, **4b**, **4c**, and **4d** from commercially available chiral amines with the 2-(chloromethyl)-*N*-tosyl-1*H*-benzimidazole **2** according to our previous method[44] (Scheme 1). The nucleophilic substitution reactions were conducted with one equivalent of 2-(chloromethyl)-*N*-tosyl-1*H*-benzimidazole **2** and five equivalent chiral amines **3a**, **3b**, **3c**, and **3d** in DMF in the presence of KI. The excess amines were used. The reactions resulted in a high yield (87–94%).

^{1}H-NMR spectra of the **4a** and **4b** and **4c** and **4d** are similar as expected (**S1**–**S11**). The methylene proton of benzimidazole unit in **4a** and **4b** resulted in a singlet at 4.17 and 4.16 ppm, respectively (**S1**–**S3**). The methyl proton of the *p*-toluenesulphonyl group resulted in a singlet. The methyl proton on the amine core resulted in a doublet, and the methine proton resulted in a quartet as expected. The aromatic signals are consistent with the structure. While methylene proton on the benzimidazole unit in **4a** and **4b** results in a singlet, it is fascinating that the methylene proton of benzimidazole unit in **4c** and **4d** resulted in two doublet and shifts to a lower field (**S3**, **S6**, **S8**, **S11**). This system was observed in a similar structure.[45,46] The methyl proton of the *p*-toluenesulphonyl group resulted in a singlet, the methyl group on the amine core resulted in a doublet and the methine proton resulted in quintets expected for **4c** and **4d** (**S6**, **S8**). In ^{13}C-NMR spectra of **4a** and **4b**, 4 peaks in the aliphatic region and 14 peaks in the aromatic region were observed. We assume that one ^{13}C peak was overlapped, and as a result we observed 18 peaks instead of 19 peaks (**S2**, **S5**). In the case of **4c** and **4d** 8 peaks in the aliphatic and 6 peaks in the aromatic region were observed (**S7**, **S10**). LCMS data were consistent with the structures (**S3**, **S5**, **S8**, **S10**).

Biological Activity Studies

AChE, hCA-I, and hCA-II inhibition activity

The summary of the IC_{50} value (μM) for hCA isoenzymes is given in Table 1. Acetazolamide (AZA) was used as a reference inhibitor to compare the inhibitory effect of compounds **4a**–**4d** for hCA isoenzymes. IC_{50} values, described as the inhibitor concentration that halves the enzyme activity, were used to determine the inhibition effects of the compounds **4a**–**4d**. We designed these novel chiral benzimidazole amine hybrids to test whether chiral compounds reduce undesirable toxic and ecological impacts and unnecessary excess drug use. The



Scheme 1. The synthesis of chiral amine benzimidazole hybrids.

Table 1. The IC_{50} values for **4a**–**4d** compounds on AChE, hCA-I, and hCA-II.

Compounds	For AChE IC_{50} (nM)	R^2	For hCA-I IC_{50} (μM)	R^2	For hCA-II IC_{50} (μM)	R^2
4a	87.5	0.9928	4.895	0.9896	0.469	0.9753
4b	100	0.9835	1.750	0.8842	0.380	0.7581
4c	26.92	0.9736	0.173	0.9673	0.233	0.9527
4d	100	0.9552	0.620	0.9454	0.635	0.9567
Tacrine[a]	70	0.9875	–	–	–	–
AZA[b]	–	–	0.536	0.9834	0.281	0.9877

[a] Tacrine (TAC) was used as a standard inhibitor for AchE. [b] Acetazolamide (AZA) was used as a standard inhibitor for hCA-I and hCA-II.

structures of compounds **4a** and **4b** are similar but differ in configuration. Compound **4a** was synthesized from (*R*)-(+)-Phenylethylamine, and compound **4b** was synthesized from (*S*)-(-)-Phenylethylamine. **4c** and **4d** were synthesized from (*R*)-(-)-cyclohexylethylamine and (*S*)-(+)-cyclohexylethylamine, respectively. IC_{50} of **4a**–**4d** were found as 4.895 μM, 1.750 μM, 0.173 μM, and 0.620 μM, respectively, for hCA-I. The experimental results show that *S* enantiomer (**4b**) exhibited better inhibition activity than *R* enantiomer (**4a**). The inhibition concentration of the *R* enantiomer (**4a**) is higher by 2.8 fold than the *S* enantiomer (**4b**). In the case of **4c** and **4d**, the *R* enantiomer (**4c**) exhibited better inhibition than the S enantiomer (**4d**). The inhibition concentration of the *S* enantiomer (**4d**) is higher by 3.58 fold than the *R* enantiomer (**4c**). **4c** exhibited the best inhibition activity amongst these four chiral compounds, even better than the reference inhibitor AZA. IC_{50} of **4a**–**4d** were reported as 0.469 μM, 0.380 μM, 0.233 μM, 0.635 μM, respectively, for hCA-II. The same results were obtained for the hCA-II. **4c** exhibited the best inhibition activity amongst these four chiral compounds, even better than the reference inhibitor AZA. In the case of the AChE IC_{50} value, (nM) is given in Table 1. Tacrine (TAC) was used as a reference inhibitor to compare the inhibitory effect of compounds **4a**–**4d** for AChE. IC_{50} of **4a**–**4d** were found to be 87.5 nM, 100 nM, 26.92 nM, 100 nM, respectively, for AChE. The experimental results show that *R* enantiomer (**4a**) exhibited better inhibition activity than *S* enantiomer (**4b**). The inhibition concentration of the *S* enantiomer (**4b**) is higher by 1.15 fold than the *R* enantiomer (**4a**). In the case of **4c** and **4d**, the *R* enantiomer (**4c**) exhibited better inhibition than the S enantiomer (**4d**). The inhibition concentration of the *S* enantiomer (**4d**) is higher by 3.71 fold than the *R* enantiomer (**4c**). **4c** exhibited the best inhibition activity amongst these four chiral compounds, even better than the reference inhibitor TAC. Although *in silico* docking studies showed that the binding affinity is slightly better for *R* derivatives, *in vitro* studies showed that there is high selectivity between enantiomers of four chiral compounds. In the case of **4c** and **4d** *R* enantiomer showed a very high inhibition effect against Human Erythrocytes Carbonic Anhydrase I, II and Acetylcholinesterase than *S* enantiomers. However, in the case of **4a** and **4b** *S* enantiomer showed a better inhibition effect against hCA-I, and hCA-II than *R* enantiomer, *R* enantiomer showed a better inhibition effect against AChE than *S* enantiomers. A kind of reversed enantioselectivity was observed. The *in vitro* inhibition results show that the effect of enantioselectivity was observed clearly in all cases.

In silico docking study

The binding energies (scoring function based) in kcal/mole of all four compounds were observed as negative scores and presented in Table 2. They were successfully docked at the active sites of the CA-I and CA-II, and AChE and docking poses were given (obtained from Discovery Studio Visualizer 4.5) in Figures 1, 2, and 3.

The binding energies of all the compounds for CA-II were found in the range of −8.8 to −8.2 kcal/mole, which was relatively lower than those for the CA-I but still higher than the similar compounds previously studied.[47–48]

The binding energies of all of the compounds for CA-I were found to be approximately -9.6 ± 2 kcal/mole, which is relatively high compared to similar studies.[47–48] Although the inhibition activities of all four ligands differed experimentally, any meaningful differences were not observed through their binding energies.

N3 of benzimidazole and H of core -NH group of all ligands have formed hydrogen bonds with the GLN92 with a length less than 3 Å. There is a hydrophobic interaction (pi-pi T shaped) between the phenyl group of 4a and 4b ligands and HIS94 residue, which plays a crucial role in the inhibitory process of CA-I and CA-II. Similarly, the cyclohexyl group of 4c and 4d ligands and HIS94 residue have shown hydrophobic (pi-alkyl) interactions. There are also electrostatic (pi-cation) interactions between the Zn atom near the activity sites of CA-I (HIS94) and ligands **4a** and **4b**. In the case of cyclohexyl substitution (**4c** and **4d**) formation of zinc complexes near the active sites was not observed.

Table 2. Binding affinities of 4-compounds for CA-I (2NMX), CA-II (3MN04), and AChE (1EEA).

Compounds	Binding affinity (kcal/mol) CA-I (2NMX)	CA-II (3 M04)	AChE (1EEA)
4a	−9.7	−8.8	−11.1
4b	−9.4	−8.5	−10.9
4c	−9.6	−8.5	−11.5
4d	−9.5	−8.2	−11.2

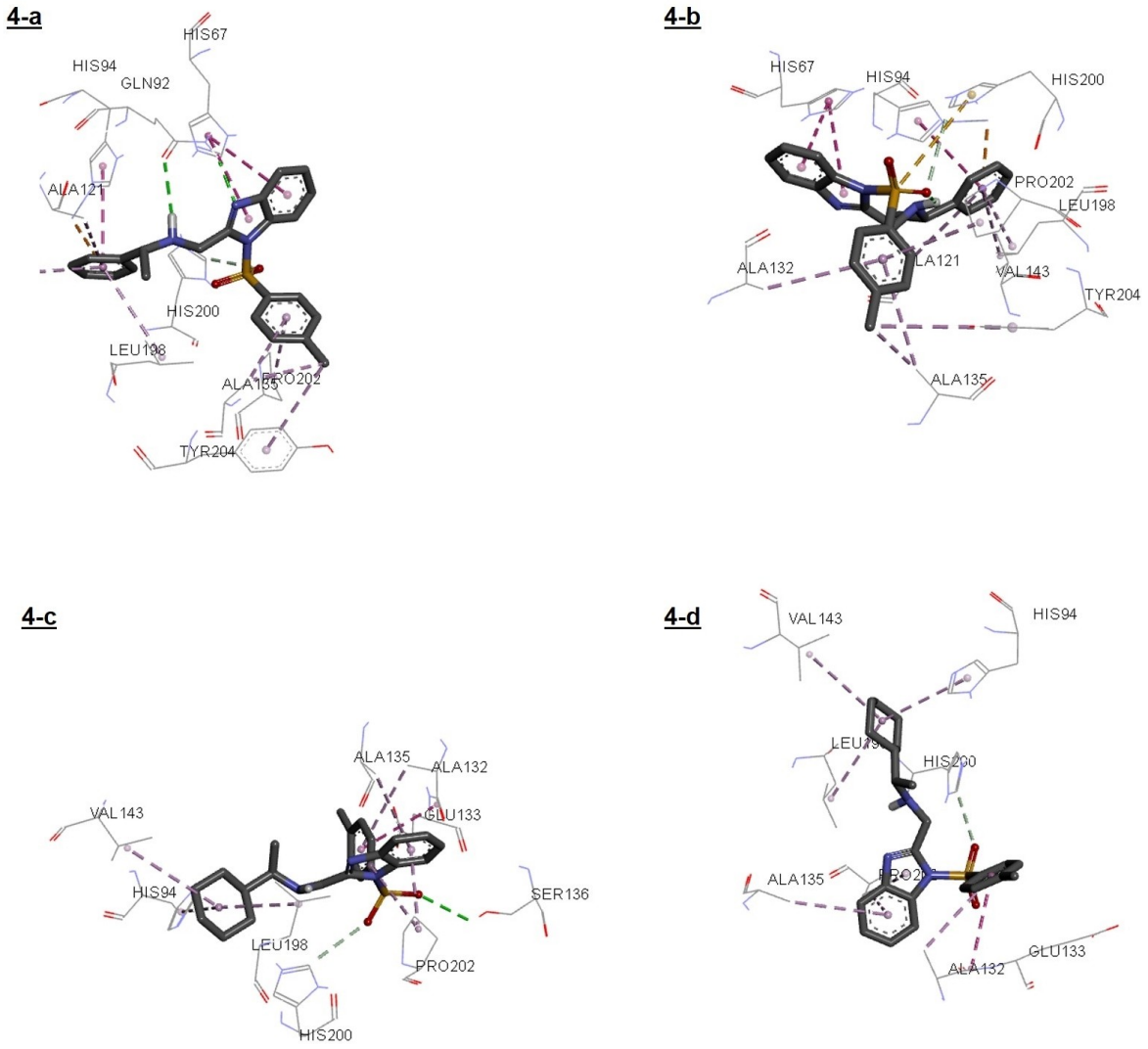


Figure 1. Interaction of CA-I (2NMX) and compounds: **4-a**; **4-b**; **4-c**; **4-d**.

Docking poses of the ligands for CA-II were also presented in Figure 2. Some differences were observed using the type and strength of interactions between the ligands and CA-II active site residues compared to those observed in the case of CA-I. For instance, hydrogen bond formation was only observed between N3 for benzimidazole and GLN92. The formation of zinc complexes in the active sites' neighborhoods was not observed. The hydrophobic interaction (pi-alkyl) between HIS94 and the phenyl or the cyclohexyl substituent of the ligands is relatively longer and weaker. The relatively lower binding energies obtained for CA-II and all ligands will be explained in this discussion.

The highest binding energies for all of the compounds (ligands) were found in the case of AChE. The value is approximate -11.2 ± 3 kcal/mole. These results are consistent with the experimental data since all compounds have shown excellent inhibition properties.

Binding properties using type and strength for interactions between the ligands and AChE were also discussed using docking poses in Figure 3. The residues in active sites such as TYR334, SER200, TRP84, ASP72, and HIS440 were chosen for comparison.[49] Hydrogen bond formation was observed between the ligands and TRP84 (length less than 5 Å). There is a hydrophobic interaction (pi-sigma) between TYR334 and C–H for ligand **4d** (S-cyclohexyl substituent) and **4a** (R-phenyl substituent). Another hydrophobic interaction (pi-pi stacked) was observed between PHE330 and O from all ligands. Pi-alkyl hydrophobic interaction was also found between HIS440 and C of the tosyl group of ligand **4d**. Electrostatic interaction (pi-anion) also formed between ASP72 and the benzimidazole group of **4b** (S-phenyl substituent). Hence results showed that all ligands showed different types but stronger interactions with the residues of active sites on AChE.

All compounds synthesized in this study were found to show the inhibitory effect of CA-I and CA-II, and AChE enzymes according to *in silico* studies. Furthermore, their inhibitory activity is highest for the AChE experimentally.

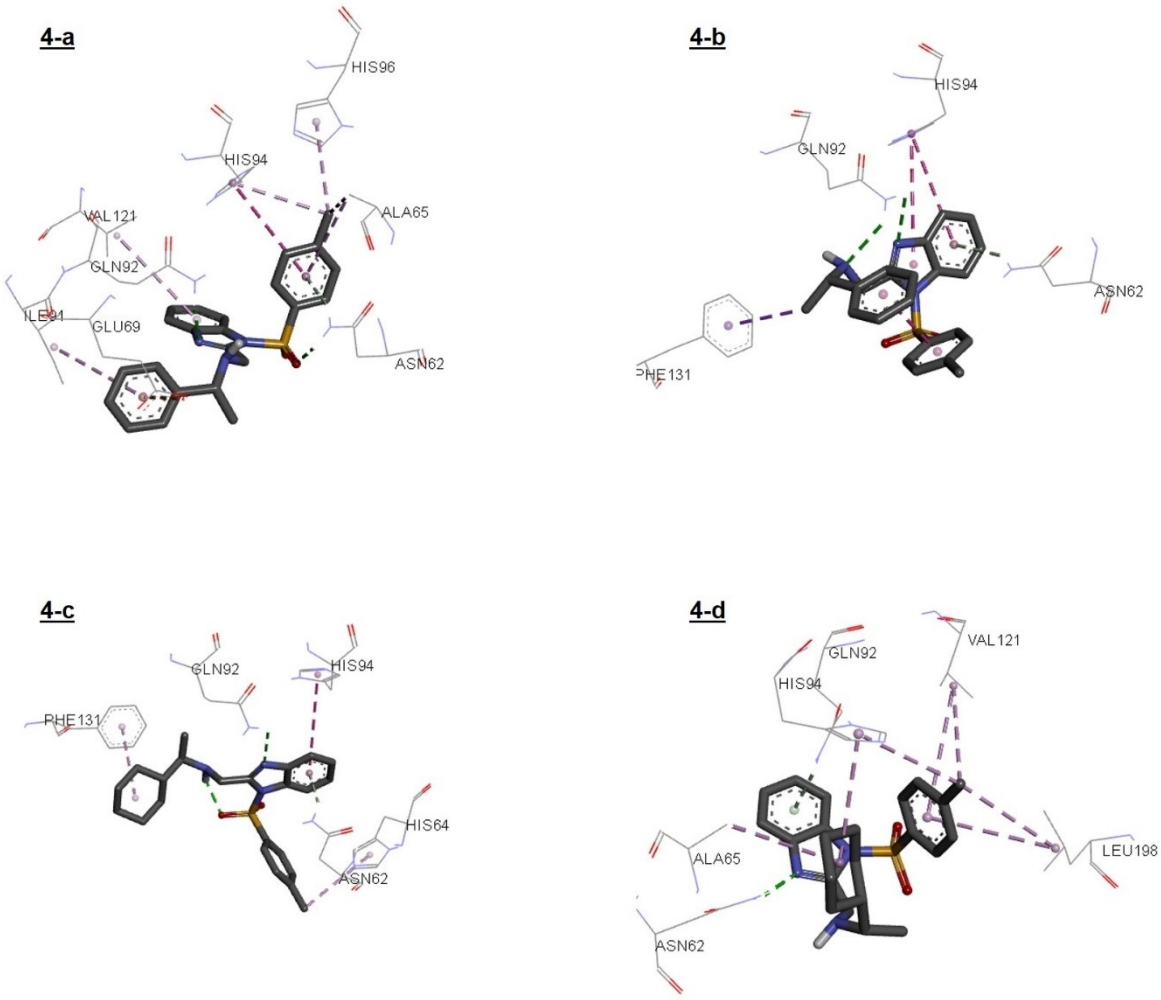


Figure 2. Interaction of CA-II (3 M04) and compounds: **4-a**; **4-b**; **4-c**; **4-d**.

ADME (absorption, distribution, metabolism, and excretion) predictions of all compounds by SwissADME were given as *Supporting Information* (Table 3). According to the data presented in Table 3, there are no violations of Lipinski rules for all compounds, no brain-blood barrier (BBB) permeation, and a negative lop Kp value, which indicates less skin permeation. As a result, they could all be identified as having drug-like potential.

Conclusions

In conclusion, four chiral benzimidazole amine hybrids **4a**, **4b**, **4c**, and **4d** were synthesized, and inhibition properties were investigated against AChE, hCA-I, and hCA-II enzymes. All four molecules showed good inhibition activity against tested enzymes. The **4c** showed the best inhibition activity among the four chiral benzimidazole derivatives tested which was better than the reference inhibitor Tacrine (TAC) and Acetazolamide (AZA). The influence of chirality was observed clearly. In general *R* enantiomers (**4a**, **4c**) are better inhibition effect than the *S*

Table 3. The results of ADME analysis of compounds.

	MW/gmol^{-1}	HBD ($\leq$5)	HBA ($\leq$10)	cLogP ($\leq$5)	Molar Refractivity	Log K_p (cm/s)
4a–4b	405.51	1	4	4.09	115.64	-5.70
4c–4d	411.56	1	4	4.53	117.88	-5.09

*MW: Molecular weight $<$500, HBD: Hydrogen bond donor $\leq$5, HBA: Hydrogen bond acceptor $\leq$10, cLogP: High lipophilicity (expressed as consensus LogP) $<$5, Molar refractivity should be between 40 and 130, LogKp: skin permeability: The more negative log Kp, the less skin permeant is the compound. No alert for medicinal chemistry.

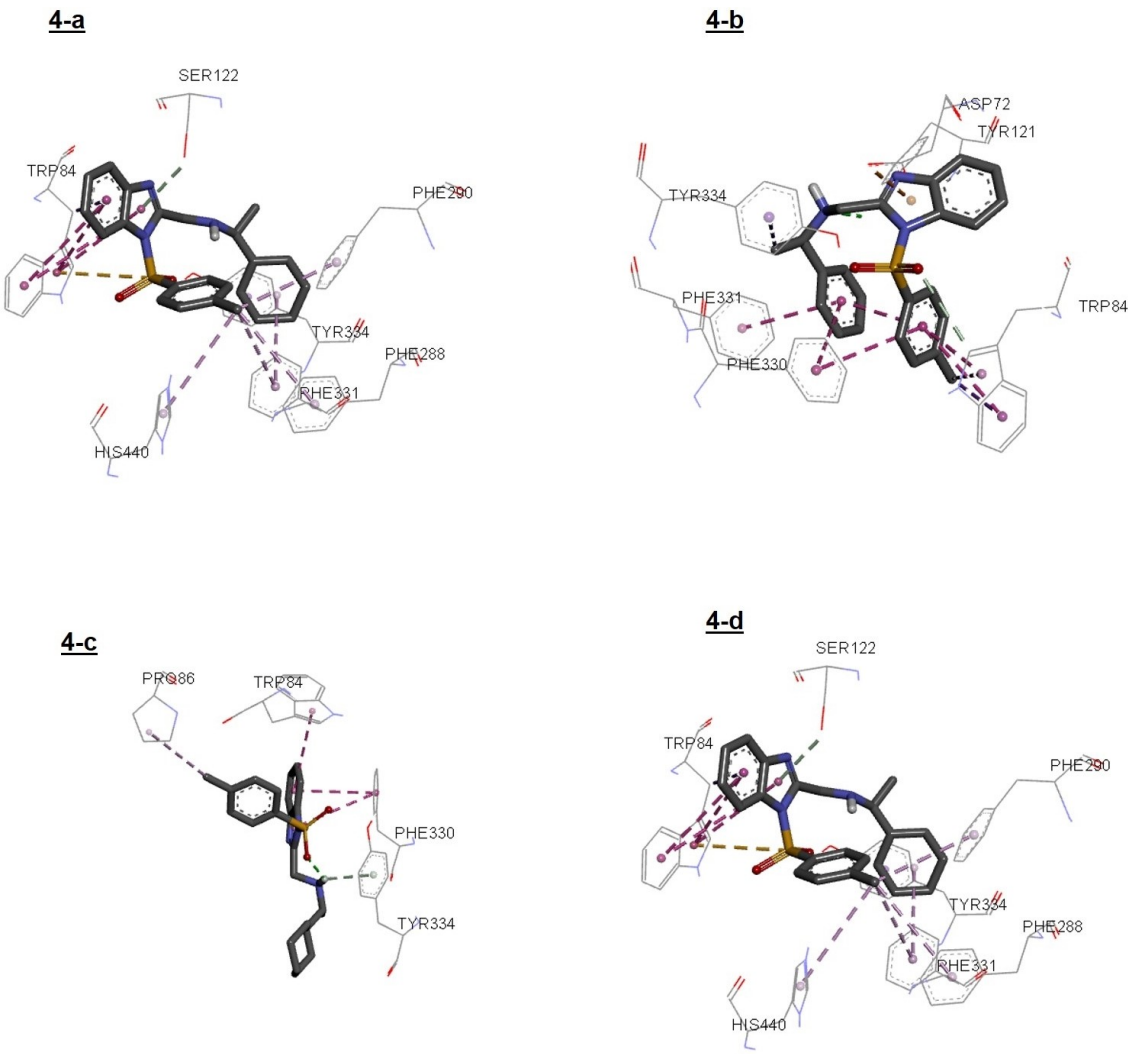


Figure 3. Interaction of AChE (1EEA) and compounds: **4-a**; **4-b**; **4-c**; **4-d**.

enantiomers (**4b**, **4d**) for AChE. Although *R* enantiomer (**4c**) is better inhibition effect than *S* enantiomer (**4d**) for hCA-I and hCA-II, *S* enantiomer (**4b**) is better inhibition effect than the *R* enantiomer (**4a**). The *in vitro* studies demonstrated that chirality alters the biological effects of 1-phenyl-*N*-((1-tosyl-1*H*-benzo[d]imidazol-2-yl)methyl)ethanamine and 1-cyclohexyl-*N*-((1-tosyl-1*H*-benzo[d]imidazol-2-yl)methyl)ethanamine. Molecular docking studies for **4a**, **4b**, **4c**, and **4d** indicated that all compounds interacted with high binding energies with AChE, hCA-I, and hCA-II enzymes. The highest binding energy was observed between **4c** and AChE at −11.5 kcal/mol. The ADME (absorption, distribution, metabolism, and excretion) studies indicated that all chiral benzimidazole derivatives **4a**, **4b**, **4c**, and **4d** have high gastrointestinal absorption and no brain-blood barrier (BBB) permeation, and less skin permeation. In brief, these molecules could be recognized as a drug-like potential for acetylcholinesterase and carbonic anhydrase proteins.

Experimental Section

Materials

All available solvents and reagents were purchased commercially. DMF was dried before use. IR spectra were recorded on a Nicolet-6700 ATR-FT-IR spectrophotometer in the 4000–400 cm^{-1} region, and melting points were measured using a Thermo Fisher Scientific Electrothermal 9100 apparatus. ^{1}H-NMR (300 MHz) spectra and ^{13}C-NMR (100 MHz) spectra were collected on a Bruker Ultrashield spectrometer at room temperature with TMS, Mass spectra were recorded with Thermo Scientific TSQ Quantum Access Max LC/MS spectrometers in methanol/acetonitrile mixture. Optical rotations were recorded using an ANTON PAAR MCP100 model polarimeter. Chiral amines, solvents and reagents were purchased from Sigma-Aldrich.

Synthesis of Novel Chiral Benzimidazole Amine Hybrids

(R)-(+)-1-phenyl-N-((1-tosyl-1H-benzo[d]imidazol-2-yl)methyl)ethanamine (4a)

(*R*)-(+)-Phenylethyl amine (1.210 g, 10 mmol) was added to the solution of 2-(chloromethyl)-1-[(4-methylphenyl)sulfonyl]-1*H*-benzimidazole (0.641 g, 2 mmol), KI (0.91 g, 6 mmol) in dry DMF (12 ml) at room temperature. The mixture was stirred for 18 h at room temperature. Next, the reaction completion was monitored by TLC. When the 2-(chloromethyl)-1-[(4-methyl phenyl)sulfonyl]-1*H*-benzimidazole disappeared, the reaction was quenched by saturated $NaHCO_3$. The aqueous phase was extracted with Et_2O (20 mL ×3) and the organic phase was combined and washed with water three times. The organic phase was dried over anhydrous Na_2SO_4. The product was purified by silica gel column chromatography using AcOEt and hexane (1:4) as eluent to give **4a**; $[\alpha]_D^{20} = +40.82$ (c = 0.022, CH_2Cl_2); IR νmax (KBr) 3321, 3057, 2971, 2920, 1734, 1682, 1595, 1539, 1492, 1450, 1375, 1269, 1229, 1169, 1120, 1086, 1031, 763 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz), δ 1.44–1.46 (d 3H, J = 6), 2.36 (s 3H), 3.19 (bs 1H, NH), 3.93–3.99 (q 1H, J = 6), 4.17 (s 2H), 7.16–7.19(d 2H, J = 9), 7.32–7.36 (m 7H), 7.48–7.50(d 1H, J = 6), 7.68–7.71 (d 2H, J = 9), 7.96–7.99 (d 1H, J = 9); ^{13}C-NMR ($CDCl_3$, 100 MHz), δ 21.15, 23.74, 45.50, 57.23, 112.83, 119.42, 124.17, 126.52,126,40,126,60, 128.05, 129.66, 132.62, 134.41, 141.21, 144.27, 145.42, 152.80; LC/MS (*m/z*): 406.06 [M + H] (calc. 405.15).

(−) (S)-(-)-1-phenyl-N-((1-tosyl-1H-benzo[d]imidazol-2-yl)methyl)ethanamine (4b)

(−) (*S*)-(-)-Phenylethylamine (1.817 g, 15 mmol) was added to the solution of 2-(chloromethyl)-1-[(4-methyl phenyl)sulfonyl]-1*H*-benzimidazole (0.641 g, 2 mmol), KI (1.360 g, 9 mmol) in dry DMF (12 ml) at room temperature. The mixture was stirred for 18 h at room temperature. Next, the reaction completion was monitored by TLC. When the 2-(chloromethyl)-1-[(4-methylphenyl) sulfonyl]-1*H*-benzimidazole disappeared, the reaction was quenched by saturated $NaHCO_3$. The aqueous phase was extracted with Et_2O (20 mL ×3) and the organic phase was combined and washed with water three times. The organic phase was dried over anhydrous Na_2SO_4. The product was purified by silica gel column chromatography using AcOEt and hexane (1:4) as eluent to give **4b**; $[\alpha]_D^{20} = -7.06$ (c = 0.117, CH_2Cl_2); IR ν_{max} (KBr) 3320, 2977, 2926, 1734, 1682, 1622, 1595, 1532, 1493, 1447, 1375, 1271, 1208, 1161, 1120, 1031, 1007, 743 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz), δ 1.43–1.46 (d 3H, J = 9), 2.35 (s 3H), 2.87 (bs 1H, NH), 3.91–3.98 (q 1H, J = 8), 4.16 (s 2H), 7.16–7.18(d 2H, J = 6), 7.25–7.34 (m 8H), 7.68–7.70 (d 2H, J = 6), 7.97–8.01 (m 1H); ^{13}C-NMR ($CDCl_3$, 100 MHz), δ 21,31, 23.74, 45.48, 57.25, 112.65, 119.66, 124,17, 124.53, 126.39, 126,61, 128,05, 129.67, 132.41, 134.42, 141.20, 144.23, 145.44, 152,82; LC/MS (*m/z*): 406.02 [M + H] (calc. 405.15).

(R)-(-)-1-cyclohexyl-N-((1-tosyl-1H-benzo[d]imidazol-2-yl)methyl)ethanamine (4c)

(−)(*R*)-(-)cyclohexylethylamine (1.272 g, 10 mmol) was added to the solution of 2-(chloromethyl)-1-[(4-methylphenyl) sulfonyl]-1*H*-benzimidzole (0.641 g, 2 mmol), KI (0.910g, 6 mmol) in dry DMF (12 ml) at room temperature. The mixture was stirred for 18 h at room temperature. Next, the reaction completion was monitored by TLC. When the 2-(chloromethyl)-1-[(4-methylphenyl)sulfonyl]-1*H*-benzimidazole disappeared, the reaction was quenched by saturated $NaHCO_3$. The aqueous phase was extracted with Et_2O (20 mL ×3) and the organic phase was combined and washed with water three times. The organic phase was dried over anhydrous Na_2SO_4. The product was purified by silica gel column chromatography using AcOEt and hexane (1:4) as eluent to give **4c**; $[\alpha]_D^{20} = -7.45$ (c = 0.115, CH_2Cl_2), m.p. 160–163 °C; IR νmax (KBr) 3301, 3055, 2923, 2851, 1736, 1595, 1491, 1447, 1377, 1271, 1169, 1120, 1085, 1031, 1007, 766 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz), δ 1.05–1.07 (d 3H, J = 6), 1.14–1.28(m 6H), 1.37–1.46(p 2H, J = 6), 1.66–1.76 (q 4H, J = 9 Hz), 2.37 (s 3H), 2.55–2.63 (p 1H J = 6), 4.24–4.29 (d, 1H, J = 15), 4.34–4.39 (d, 1H, J = 15), 7.25–7.28(d 2H, J = 9), 7.31–7.37(m 2 H), 7.66–7.69(q 1H, J = 3), 7.88–7.91 (d 2H, J = 9), 7.97–8.00 (q 1H, J = 3); ^{13}C-NMR ($CDCl_3$, 100 MHz), δ 14.80, 20.77, 25.12, 25.52, 26.63, 28.71, 38.89, 51.29, 123.47, 125.64, 128.34, 128.55, 128.84, 138.80, 152.41; LC/MS (*m/z*): 412.08 [M + H] (calc. 411.20).

(−) (S)-(+)-1-cyclohexyl-N-((1-tosyl-1H-benzo[d]imidazol-2-yl)methyl)ethanamine (4d)

S-(+)-Cyclohexylethylamine (1.908 g, 15 mmol) was added to the solution of 2-(chloromethyl)-1-[(4-methylphenyl) sulfonyl]-1*H*-benzimidzole (0.961 g, 3 mmol), KI (1.365, 9 mmol) in dry DMF (7.5 l) at room temperature. The mixture was stirred for 18 h at room temperature. Next, the reaction completion was monitored by TLC. When the 2-(chloromethyl)-1-[(4-methylphenyl)sulfonyl]-1*H*-enzbimidazole disappeared, the reaction was quenched by saturated $NaHCO_3$. The aqueous phase was extracted with Et_2O (20 ml ×3) and the organic phase was combined and washed with water three times. The organic phase was dried over anhydrous Na_2SO_4. The product was purified by silica gel column chromatography using AcOEt and hexane (1:4) as eluent to give **4d**; $[\alpha]_D^{20} = +7.14$ (c = 0.120, CH_2Cl_2), m.p. 161–163 °C; IR νmax (KBr) 3304, 3056, 2923, 2851, 1736, 1647, 1595, 1492, 1447, 1375, 1226, 1169, 1120, 1087, 1032, 1008, 765 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz), δ 1.03–1.05 (d 3H, J = 6), 1.16–1.29(m 6H), 1.35–1.4 (p 2H, J = 6), 1.65–1.76 (q 4H, J = 10.5 Hz), 2.10 (bs 1H, NH), 2.37 (s 3H), 2.53–2.61 (p 1H J = 6), 4.22–4.27 (d, 1H, J = 15), 4.31–4.37 (d, 1H, J = 18), 7.24–7.27 (d 2H, J = 9), 7.31–7.36 (m 2 H), 7.65–7.68 (q 1H, J = 3 Hz), 7.87–7.90 (d 2H, J = 9), 7.97–8.00 (q 1H, J = 3); ^{13}C-NMR ($CDCl_3$, 100 MHz), δ 14.67, 20.81, 25.11, 25.55, 26.70, 28.91, 40.33, 51.10, 123.67, 125.65, 128.21, 128.42, 128.56, 138.81, 152.60; LC/MS (*m/z*): 412.09. [M + H] (calc. 411.20)

In vitro inhibition studies of 4a, 4b, 4c, and 4d on hCA-I and hCA-II isoenzymes

CA isoenzymes were purified from human erythrocytes in one step using CNBr-activated Sepharose-4B–L-tyrosine sulfanilamide affinity chromatography as in our previous studies.[40,50,51] Quantitative protein determination was performed in the purification steps with the Bradford method.[52] The purity of isoenzymes was checked with the SDS-PAGE method[53] as in our previous studies.[40,54,55] hCA-I and hCA-II isoenzymes purified by affinity chromatography dialyzed against 50 mM Tris-SO4 (pH 7.4) buffer overnight. After dialysis, isoenzymes were stored in small fractions of one milliliter at −80 °C for use in inhibition studies. In inhibition studies, the activities of hCA-I and hCA-II isoenzymes were performed according to the esterase activity measurement method.[56] The basis of this method is based on the hydrolysis of CA isoenzymes to *p*-nitrophenyl acetate to *p*-nitrophenol and acetic acid. Accordingly, *p*-nitrophenyl acetate was used as the substrate in the inhibition studies. The formation of *p*-nitrophenol from *p*-nitrophenyl acetate was monitored by measuring the absorbance at 348 nm, 25 °C using a spectrophotometer. The enzyme unit was calculated using the absorption coefficient ($\varepsilon = 5.4 \times 10^3\ M^{-1}cm^{-1}$) of *p*-nitrophenyl acetate at 348 nm. Inhibitory effects of 4a–4d molecules were determined on the esterase activity of hCA-I and II isoenzymes. For this, hCA-I and hCA-II activities were measured for at least five

concentrations of each **4a**–**4d** molecule. The Activity% values at five different inhibitor concentrations were calculated. The control activity of the enzyme was accepted as 100%, and inhibitor concentrations were graphed against Activity%. IC_{50} values were determined from the equations of these graphs. Acetazolamide (AZA) was used as the reference inhibitor for hCA-I and hCA-II isoenzymes.

In vitro inhibition studies of 4a, 4b, 4c, and 4d on AChE

The AChE (CAS no. 9000-81-1) enzyme was commercially available from Sigma-Aldrich. AChE activity in inhibition studies was performed according to the spectrophotometric method of Ellman et al.[57] In this method, AChE hydrolyzes acetylthiocholine to thiocholine and acetic acid. The thiocholine formed from the reaction reacts with DTNB (Ellman's reagent, 5,5-dithiobis(2-nitrobenzoic acid) used in the reaction medium to create 5-thio-2-nitrobenzoic acid, a yellow compound, and the color intensity of the colored compound measured spectrophotometrically.[58] Accordingly, acetylthiocholine iodide was used as a substrate in inhibition studies. The color intensity of the resulting colored compound was measured at 412 nm. AChE activity was measured for at least five concentrations for **4a**–**4d** compounds to determine the inhibitory effects of **4a**–**4d** on AChE activity. The control activity of the enzyme was accepted as 100%, and inhibitor concentrations were graphed against Activity%. IC_{50} values were determined from the equations of these graphs. Tacrine (TAC) was used as the reference inhibitor for AChE.

In Silico Studies

Minimum energy calculations of synthesized compounds (**4a**, **4b**, **4c**, and **4d**) were performed using Gaussian software by quantum mechanical DFT/B3LYP/6-31G calculations. The crystal structure of CA-I (PDB: 2NMX), CA-II (PDB:3M04), and AChE (PDB:1EEA) were subjected to protein preparation. Water molecules and ligands were deleted during the protein preparation, but Zn atoms remained for CA-I and CA-II. Scoring function-based docking studies were carried out by AutoDock Vina software in triplicate. All the compounds were docked with prepared protein structures CA-I, CA-II, and AChE, and results were compared by employing binding energies and binding sites. Discovery Studio Visualizer 4.5 (trial version) was used to visualize the interactions between ligands and proteins; in addition to the docking and experimental data, pharmacokinetics and drug-like properties of all four synthesized compounds were also evaluated by using online SwissADME web tools (http://www.sib.swiss), which also predicted physicochemical properties such as lipophilicity, solubility, etc. of compounds.[59]

Author Contributions

Synthesis studies were carried out by T. Tunç and N. Demirel, Z. Alım carried out biological activity studies and molecular docking studies were carried out by S. Abdurrahmanoğlu. Analysis of the results and writing of the article were done by T. Tunç, S. Abdurrahmanoğlu, A. Günel, N. Demirel and Z. Alım.

Acknowledgements

The financial support of this work by the Ahi Evran University grant FEF.A4.22.013 is gratefully acknowledged.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: acetylcholinesterase · carbonic anhydrase · chiral benzimidazole · inhibition · molecular docking

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Manuscript received: February 12, 2023
Accepted manuscript online: May 10, 2023
Version of record online: May 10, 2023