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SYNTHESIS OF BENZIMIDAZOLE-SUGAR DERIVATIVES: A POTENTIAL TREATMENT FOR ALZHEIMER'S DISEASE

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ABSTRACT. In this study, a new series of chiral benzimidazole-glycosyl thiourea derivatives (**5**-**10**) were synthesized and evaluated for their inhibitory effects against hCA I, hCA II, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The structures of the benzimidazole-glycosyl thiourea compounds were determined by FTIR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis. The K_I values for the CA isoenzymes of the synthesized materials ranged from 6.03 to 42.98 nM. K_I values for cholinesterase enzymes were shown to be between 5.32 and 39.14 nM. It was observed that compound **10** is the best hCA I inhibitor (K_I:6.03 nM), compund **9** is the best hCAII inhibitor (K_I:17.29 nM) and compound **8** is the best AChE (K_I:5.32 nM) and the best BChE (K_I:18.73 nM) inhibitor.

Keywords: *Benzimidazole; amino acid; glycosyl thiourea; carbonic anhydrase; cholinesterase*

INTRODUCTION

In recent years, Alzheimer's disease (AD) has started to appear widely in the population, especially in developed countries. This disease is an important public health problem and is stated as a neurodegenerative condition manifested by abnormal behavior and intellectual decline [1,2] one of the most valid hypotheses is the "cholinergic hypothesis". The cholinergic hypothesis; suggests that disfunction of ACh-containing neurons in the brain contributes significantly to the cognitive decline observed in old age and those with AD [3].

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Changes have been observed in the amount of acetylcholine (ACh), which acts as a neuro mediator in the brain tissues of patients with AD [4]. Inhibitors of AChE/BChE, which hydrolyze and control the concentrations of these neuro mediators, have therefore become a valid option in the treatment of AD [2,5,6]. Because of this situation, many researchers around the world, have tried to identify new inhibitor candidates for these enzymes that appear as targets in AD treatment. AChE regulates the concentration of ACh, which is known to have a critical role in cognition and memory in humans [5]. Because of the loss of cholinergic neurons in AD, the inhibition of cholinesterases, which can hydrolyze ACh, has been adopted as a treatment strategy to increase this decrease in the amount of ACh. Since they act as AChE inhibitors, drugs such as neostigmine, tacrine and donepezil are used in the treatment of AD [5,7,8].

CAs are a class of metalloenzymes that catalyze the rapid hydration of CO_2 into bicarbonate and proton [9]. Since CAs contribute to important biological processes (Ensuring intracellular and extracellular ion balance, regulation of intracellular pH, calcification, resorption, etc.) in living organisms, they are found in the structure of many tissues [10-12]. Since they can regulate these biological processes, CA inhibitors and activators are used in glaucoma, edema, cancer, epilepsy, some cognitive disorders, etc. They are candidate drug molecules to be used in the treatment of diseases [13-18].

Oxidative stress has been cited as one of the causes for AD pathology. An increase in the concentration of intracellular reactive nitrogen and oxygen species can cause oxidative damage to many biomolecules. Modifications that occur in proteins due to oxidative damage can also change the catalytic activities of enzymes [19]. For example, a reduction in CA activity and a number of highly nitrated and/or carbonylated proteins, including hCA II, were observed in the hippocampus of individuals with AD and human brain samples from mild cognitive brain samples [20-21]. Moreover, the fact that hCA II isoenzyme has been identified among a large number of amyloid plaques suggests that it may play a pivotal role in plaque development or may occur together with plaque formation [22]. The high levels of hCA II found in the central [20,23] and peripheral systems [24] also raise the possibility that hCA II expression may represent a biomarker for AD [12]. In addition, promising preclinical evidence of the use of CAIs (CA inhibitors) in amyloidosis models has also been reported lately [25].

Benzimidazole is the heterocyclic compound formed by the fusion of imidazole and benzene. Benzimidazole derivatives are of broad interest due to their diverse biological activities. Several benzimidazole derivatives have been published to exhibit marked antifungal, antibacterial, antioxidant, antiparasitic, antihelmintics, antiproliferative, anti-HIV activities [26-31]. Moreover benzimidazole derivatives are considered as a new promising molecules for Alzheimer inhibitör [32-35].

As excellent intermediates, glucosyl isothiocyanates have been used to prepare various carbohydrate compounds of biological and pharmaceutical interest [36, 37]. In recent years, there have been many studies on synthesizing glycosyl isothiocyanates and their transformation into glycosyl thiourea derivatives. Furthermore, sugar thioureas have synthetic applications in neoglycoconjugate synthetic strategies such as neoglycoproteins, glycodendrimers, glycoclusters and pseudooligosaccharides [38]. In addition, studies have shown that glycosyl derivatives, just like benzimidazoles, have anticholinesterase activity [39-41].

There is a limited number of publications on the synthesis and biological activity of glycosyl thioureas with benzimidazole structure. So, in this paper, a new series of chiral benzimidazole-glycosyl thiourea derivatives (**5-10**) were synthesized and the structures of the compounds were verified by FTIR, ^{1}H-NMR, ^{13}C-NMR, and elemental analysis. After synthesizing and characterizing chiral benzimidazole-glycosyl thiourea derivatives (**5-10**), the inhibitory effects on two hCA isoenzymes (hCA I/hCA II) and commercially obtained AChE and BChE enzymes were investigated in this study.

RESULTS AND DISCUSSION

Chemistry

The syntheses of the chiral benzimidazole compounds were made according to the literature (**4a**-**c**) [42]. The three different amino acids (L-isoleucine, D-phenylglycine, L-phenylalanine) were reacted with sodium bicarbonate and di-*tert*-butyl dicarbonate in H_2O/THF solvent system in order to obtain *N*-Boc protected amino acids (**1a-c**). Then the amide derivatives (**2a-c**) were obtained from compounds **1a-c** by using *o*-phenylenediamine (OFD) and *N*,*N*'-dicyclohexylcarbodiimide (DCC) under N_2 atm for 24 hours [43]. Compounds **3a-c** were synthesized by the reaction of chiral amides (**2a-c**) with acetic acid at 70 ^{o}C. In the last step, *N*-protected benzimidazole derivatives (**3a-c**) were reacted with phosphoric acid in THF at room temperature for 24 h obtaining benzimidazole derivatives(**4a-c**) (Scheme 1).

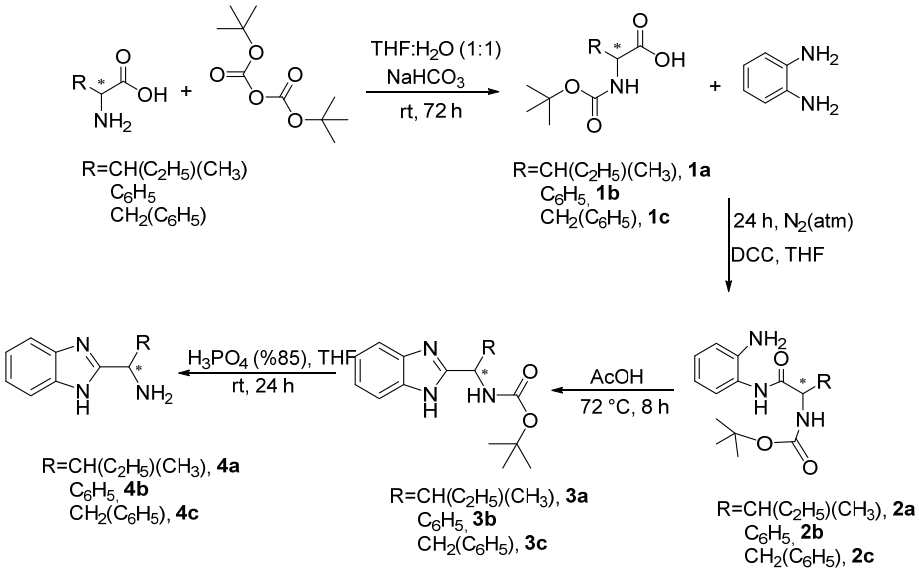
The second part of the study follows the synthesis of glycosylated isothiocyanates. Selected sugars were reacted with sodium acetate and acetic anhydride to obtain monosaccharide acetates [44]. Glycosyl bromides were synthesised following the reaction of acetylated monosaccharides with HBr-AcOH (33%) solution [45]. This follows the synthesis of glycosyl isothiocyanates by the reaction of glycosyl bromides with lead(II) thiocyanate in dry xylene under N_2 atmosphere [46].

To obtain the biologically active target compounds, benzimidazole-glycosyl thiourea derivatives (**5-10**), benzimidazole derivatives (**4a-c**) were reacted with gylcosyl isothiocyanates in acetone at room temperature for 24 hours (Scheme 2). The yield of the reactions ranged from 45 % to 73 %.

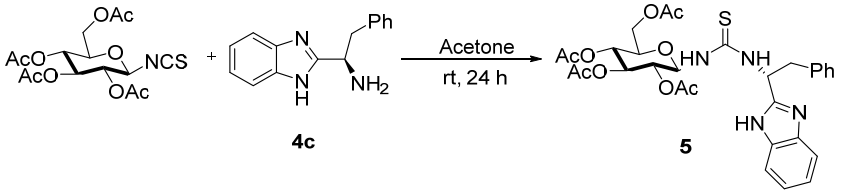
The molecular structures of the newly synthesized thiourea compounds were proved by FTIR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis methods. Accordingly, the FTIR results observed for the synthesized compounds showed C-N stretching vibrational bands at 1224-1260 cm^{-1}, C=S stretching stretching vibrational bands at 1360-1380 cm^{-1}, C=O stretching stretching vibrational bands at 1748-1770 and N-H stretching stretching vibrational bands at 3330-3350 cm^{-1}.

In the ^{1}H-NMR spectral data, the signals observed at 1.74-2.11 ppm ascertain the presence of acetyl groups with 12 protons. The sugar protons' signals have been observed at 4.00-6.10 ppm for the synthesised benzimidazole-glycosyl thiourea derivatives (**5-10**). The signals observed at 6.85-8.81 ppm belong to aromatic protons in all structures.

In the ^{13}C-NMR of the target compounds (**5-10**), the carbon-sulfur double bond signals were observed at about 183 ppm, and the carbon-oxygen double bond signals at about 170 ppm. The carbons in sugar have shown signals at 60-80 ppm, and the signal at about 20 ppm indicates the presence of –CH_3 carbons in the acetyl groups.



Scheme 1. Synthesis of benzimidazole derivatives (**4a-c**)



Scheme 2. Synthesis of benzimidazole-glycosyl thiourea compound

Biochemical Studies

This study reported on the synthesis of benzimidazoles (**5-10**). This study also determined the inhibition effects of the derivatives **5-10** on hCA I and hCA II enzymes activity.

It has been determined that AChE has essential functions in both cognition and memory. These cholinergic enzymes (AChE/BChE) catalyze the hydrolysis of ACh, causing a decrease in the level of neuronal communication of nerve cells with each other. This can lead to decreased brain function and, ultimately, to AD. Therefore, balancing the intracellular level of ACh can be used in treating AD [10-12, 14]. Numerous scientific studies have shown that inhibitors of cholinesterase enzymes (ChEIs) can be used in the treatment of neurodegenerative diseases. Currently, new ChEIs are being tried to be determined because of the low bioavailability of drugs used to treat AD and their gastrointestinal disturbances. In addition, studies conducted in recent years have shown that it can be used in CAIs, especially in the treatment of AD [11-17].

In this study, both CA and cholinesterase inhibitory potentials of **5-10** derivatives were investigated. As a result of this study, predictive models were also proposed for the substances with the most effective inhibitory effect for their interactions with hCA II and AChE enzymes, which are the primary targets in AD treatment (Figure 1).

In this study, the inhibitory effects of **5-10** benzimidazole derivatives (Figure 2) are reported for the first time using the p-nitrophenyl acetate substrate (esterase activity) of these hCA isozymes.

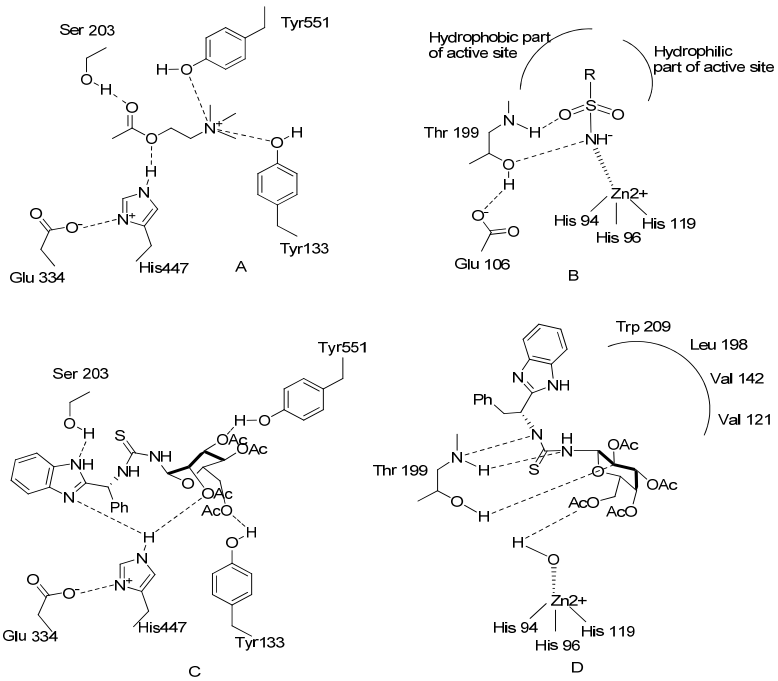


Figure 1. A: The interaction image of AChE enzyme and ACh [47], B: The binding image of hCA II enzyme and sulfonamide molecule [48, 49]. C: Estimated interaction image of AChE and compound 9. D: Estimated interaction image of hCA II and compund 8.

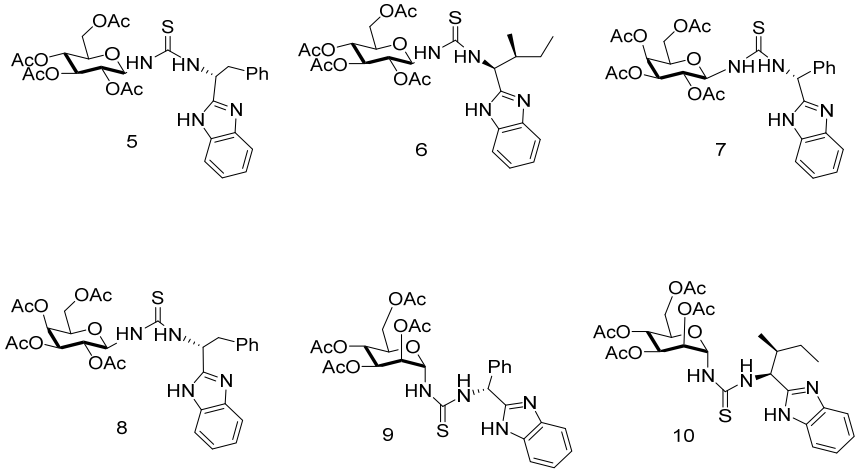


Figure 2. Benzimidazole-sugar compounds **5-10** Inhibition data for hCA I and hCA II measured using the esterase activity of these benzimidazole derivatives are shown below (Table 1):

Table 1. hCA I, hCA II, AChE and BChE inhibition data benzimidazole derivatives **5-10**

K_I / nM				
Inhibitor	hCA I	hCA II	AChE	BChE
5	7.57 ± 0.15	42.98 ± 0.65	16.59 ± 0.11	39.14 ± 0.43
6	6.65 ± 0.13	30.03 ± 0.51	8.71 ± 0.09	24.36 ± 0.27
7	11.33 ± 0.24	34.45 ± 0.54	11.73 ± 0.10	33.08 ± 0.35
8	13.82 ± 0.28	27.53 ± 0.32	5.32 ± 0.07	18.73 ± 0.19
9	9.56 ± 0.19	17.29 ± 0.29	12.20 ± 0.11	35.17 ± 0.38
10	6.03 ± 0.10	21.44 ± 0.30	8.36 ± 0.09	25.34 ± 0.27
Acetazolamide (AZA)	250 ± 0.52	12.0 ± 0.21	-	-
Donepezil	-	-	13.82 ± 0.18	2176 ± 23.1

(i) In inhibition experiments with the human CA I enzyme for benzimidazole derivative **5-10**, K_I values in the range of 6.03 ± 0.10-13.82 ± 0.28 nM were obtained. This study determined that compounds **5**, **6** and **10** followed a better inhibition profile than **7-9**. It has been determined that the **5-10** benzimidazole compounds examined show similar values with many previous studies (bromophenols, sulfonamide derivatives of amino acids, polyphenolic compounds, etc.) [7,12-18, 50].

(ii) It was observed that the K_I values obtained in inhibition experiments with benzimidazole derivative **5-10** for the hCA II enzyme were lower than that of the hCA I enzyme (Table 1). When the structure-activity evaluation for hCA II was made with the benzimidazole derivatives tested: we determined that compounds **5** and **6**, which contain methyl and benzyl groups as functional groups, are less effective than the other compounds. The best hCA II inhibitor among **5-10** derivatives is **9** containing the phenyl group in its structure, and the K_I of this substance was determined as 17.29 ± 0.29 nM.

(iii) Derivative **5** (K_I=16.59 ± 0.11 nM) showed the weakest inhibitory effect in the **5-10** agents against the AChE enzyme. When we look at the results of the inhibition obtained with the AChE enzyme for compounds **5-10**, compound **8** shows the best inhibition (K_I=5.32 ± 0.07 nM) and the compound **5** has the lowest inhibition value (K_I=16.59 ± 0.11 nM). This shows that the AChE enzyme interacts with the sugar group. Molecules **7** (K_I=11.33 ± 0.24 nM) and **9** (K_I =12.20 ± 0.11 nM) contain the same functional groups. The difference between these molecules is the position of the acetyl and thiourea groups in the molecule, and this effect has little effect on the inhibition values.

Molecules **6** (K_I=8.71 ± 0.09 nM) and **10** (K_I=8.36 ± 0.09 nM) contain the same functional groups. The difference between these molecules is the position of the acetyl, ethyl and thiourea groups in the molecule, and this effect has little effect on the inhibition values. Considering the K_I results obtained for benzimidazole derivatives **5-10** and AChE, it shows that the position of different functional groups in the molecule does not have a large effect on the inhibition values in general. The values obtained for AChE (5.32 ± 0.07-16.59 ± 0.11 nM) with the tested substances were lower and more effective when compared to the reference molecule donepezil (13.82 ± 0.18 nM).

(iv) Similar to the AChE enzyme, the weakest inhibitory effect of the six tested substances was determined in compound **5** (K_I=39.14 ± 0.43 nM) in the BChE enzyme. Among these six substances tested, the most potent inhibitor for the BChE enzyme appears to be compound **8** (K_I=18.73 ± 0.19 nM). The results of this study are essential in terms of showing that the BChE enzyme interacts with molecules derived from sugar groups, such as the AChE enzyme. Compound **7** (K_I=33.08 ± 0.35 nM) and **9** (K_I=35.17 ± 0.38 nM) contain the same functional groups. The difference between these molecules is the configuration of the acetyl and thiourea groups within the molecule, and this effect had little effect on the inhibition values. Molecules **6** (K_I=24.36 ± 0.27 nM) and **10** (K_I=25.34 ± 0.27 nM) contain the same functional groups. The difference between these molecules is the position of the acetyl, ethyl and thiourea groups in the molecule, and this effect has little effect on the inhibition values. According to these K_I results obtained for the BChE and its **5-10** benzimidazole derivatives, the position of the groups in the molecule generally shows that it does not have a significant effect on the inhibition value. Moreover, when the values obtained for the BChE (18.73 ± 0.19-39.14 ± 0.43 nM) in our study are compared to the reference molecule donepezil (2176 ± 23.1 nM), it is seen that they are much more effective.

In the inhibition studies carried out by different groups in the last twenty years, it has been discovered that they can show inhibition effects as well as sulfonamides, which are known as potent inhibitors of CA isoenzymes in the multi-bear functional group [12-18]. While this enables the detection of CA inhibitors that do not contain sulfonamide functional groups, it may also be advantageous in treating patients allergic to sulfonamide-derived drugs [13-18]. In some studies conducted in the last decade, it has been stated that hCA II inhibitors have positive effects in studies on AD models and can be used in the treatment of this disease [11,23]. In this study, both hCA isoenzymes and cholinesterase inhibition profiles of newly synthesized benzimidazole derivative (**5-10**) molecules were determined and their potential to be used in neurodegenerative diseases was tried to be determined.

CONCLUSIONS

It was determined that the synthesized benzimidazole-thiourea derivatives **5-10**, showed a compelling inhibition profile of both hCA isoenzymes and cholinesterase enzymes due to the functional groups (ethyl, acetyl, phenyl, benzyl and benzimidazole) in the structures. The findings indicate that these derivatives have inhibition values close to sulfonamides, so that they may be a new CAI class. In addition, inhibition values close to donepezil, the reference molecule, were obtained and determined to be an influential cholinesterase inhibitor group. It was determined by the esterase method that these benzimidazole derivatives obtained were effective CAIs even at nanomolar concentrations. The results of the study are essential in that these benzimidazole derivatives show that other hCA isoforms have the potential to produce strong CAI groups.

If we look at the findings, it shows that chiral benzimidazole-glycosyl thiourea derivatives (**5**-**10**) are potent inhibitors of the enzymes tested. As a result, it has been determined that this new inhibitor group we have identified has the potential to be a drug precursor or drug in treating AD. Comparison of benzimidazole derivatives (**5**-**10**) tested on CAs and cholinesterase enzymes here with current drug inhibitors and other compounds studied in the literature will help further expand the objectives for understanding the structure-activity relationship. These substances can also be used as building blocks in the determination of more effective drugs compared to known drug molecules.

EXPERIMENTAL SECTION

Synthesis of Benzimidazole compounds

The synthesis was essentially according to the amide formation procedure. L-isoleucine, D-phenylglycine and L-phenylalanine were selected as the starting amino acids.

Synthesis of Glycosyl Isothiocyanate

The glycosyl isothiocyanate derivatives can be easily synthesized from the corresponding sugars [51-53]. D-glucose, D-galactose and D-mannose were selected as the starting sugars.

General procedure for the synthesis of benzimidazole-containing gylcosyl thiourea derivatives (5-10)

The chiral benzimidazole compound (10 mmol) was dissolved in 15 mL of acetone under inert atmosphere. Glycosyl isothiocyanate (10 mmol) was added to the solution, and it was stirred at room temperature for 24 hours.

After the termination of the reaction, acetone was evaporated under reduced pressure. The remaining solid was crystallized from the CH_2Cl_2 / Hexane system to give the pure product.

_N_-[(_R_)-1-(1_H_-benzo[d]imidazol-2-yl)-2-phenylethyl)]-_N_-(2,3,4,6-tetra-_O_-acetyl-β-D-glucopyranosyl)thiourea (5)

White solid, 73 % Yield, M.P. = 137-140 °C, IR (NaCl, cm^{-1}): 3330, 3029, 2942, 1752 1534, 1439, 1368, 1226, 1038, 739. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ= 1.94 (s, 3H, OCH_3); 1.95 (s, 3H, OCH_3); 1.97 (s, 3H, OCH_3); 1.99 (s, 3H, OCH_3); 3.28 (d, *J*= 8.0 Hz, 1H, CH-C$\underline{H}_{2a}$-Ar); 3.42 (dd, *J*= 4.0 Hz, 1H, CH-C$\underline{H}_{2b}$-Ar); 3.86 (t, *J*= 12.0 Hz, 1H, H_5); 4.14-4.08 (m, 2H, H_{6b}, H_4); 4.27 (dd, *J*=8.0 Hz, 1H, H_{6a}); 4.90 (m, 1H, H_3); 5.02 (t, *J*=8.0 Hz, 1H, H_2); 5.28 (q, *J*= 6.2 Hz, 1H, NH-CH-CH_2); 5.85 (bs, 1H, NH); 6.08 (s, 1H, H_1); 6.94 (m, 5H, Ar-H); 7.19 (m, 2H, Ar-H); 7.46 (m, 2H, Ar-H); 7.83 (bs, 1H, NH); 8.81 (bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ= 182.42; 171.40; 170.38; 170.00; 169.67; 136.28; 129.34; 128.89; 128.00; 127.14; 126.52; 124.51; 83.25; 72.36; 70.72; 68.82; 67.08; 60.99; 56.63; 38.24; 20.80; 20.66; 20.57; 20.50. Anal. Calcd for $C_{30}H_{34}N_4O_9S$: C, 57.50; H, 5.47; N, 8.94; S, 5.12. Found: C, 55.30; H, 5.41; N, 8.91; S, 5.12.

_N_-[(1S,2S)-1-(1_H_-benzo[d]-imidazol-2-yl)-2-methylbutyl]-_N_-(2,3,4,6-tetra-_O_-acetyl-β-D-glucopyranosyl)thiourea (6)

White solid, 45 % Yield, M.P. = 178-180 °C, IR (NaCl, cm^{-1}): 3350, 2963, 1752, 1541, 1366, 1226, 1038, 733. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ= 0.80 (app. t, *J*= 6.5 Hz, 3H, CH_2CH_3); 0.87 (d, *J*= 6.4 Hz, 3H, $CHCH_3$); 1.18-1.21 (m, 2H, CHC$\underline{H}_2CH_3$); 1.96 (s, 3H, OCH_3); 2.00 (s, 3H, OCH_3); 2,01 (s, 3H, OCH_3); 2.02 (s, 3H, OCH_3); 3.85-3.82 (m, 1H, $CH_3C\underline{H}CH_2$); 4.12 (app. d, *J*= 9.8 Hz, 1H, H_5); 4.29 (app. d, *J*= 9.8 Hz, 1H, H_{6a}); 4.88 (s, 1H, H_4); 5.02 (t, *J*= 8 Hz, 2H, H_{6b}+H_3); 5.32 (t, *J*= 9.4 Hz, 1H, NH-C$\underline{H}$-CH); 5.48 (s, 1H, H_2); 5.68 (s, 1H, H_1); 7.23 (s, 2H, Ar-H); 7.41 (s, 1H, Ar-H); 7.56 (s, 2H, Ar-H); 8.12 (bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ= 183.86; 170.88; 170.62; 169.83; 169.49; 154.20; 122.96; 122.92; 122.90; 122.86; 82.74; 73.44; 72.78; 70.96; 68.25; 61.73; 57.60; 25.48; 20.66; 20.53; 20.49; 20.33; 15.79; 10.55. Anal. Calcd for $C_{27}H_{36}N_4O_9S$: C, 54.72; H, 6.12; N, 9.45; S, 5.41. Found: C, 55.69; H, 6.14; N, 9.45; S, 5.38.

_N_-[(_R_)-1-(1_H_-benzo[d]imidazol-2-yl)(phenyl)methyl]-_N_-(2,3,4,6-tetra-_O_-acetyl-β-D-galactopyranosyl)thiourea (7)

White solid, 54 % Yield, M.P: = 138-140 °C, IR (NaCl, cm^{-1}): 3334, 3060, 1748, 1591, 1455, 1430, 1370, 1225, 1050, 914. ^{1}H NMR (400 MHz,

$CDCl_3$, ppm): δ= 1.91 (s, 3H, OCH_3); 1.96 (s, 3H, OCH_3); 2.02 (s, 3H, OCH_3); 2.11 (s, 3H, OCH_3); 4.07 (d, *J*= 8.6 Hz, 3H, H-6a+H-6b+H-5); 4.20-4.16 (m, 1H, H-2); 5.15 (s, 1H, NH-CH-Ar); 5.23 (t, *J*= 9.0 Hz, 1H, H-3); 5.44 (dd, *J*= 9.6 Hz, *J*= 3.0 Hz, 1H, H-4); 5.65 (s, 1H, H-1); 6.94 (bs, 1H, NH); 7.15-7.21 (m, 4H, Ar-H); 7.35 (d, *J*= 7.1 Hz, 3H, Ar-H); 7.45 (s, 2H, Ar-H); 8.35 (bs, 1H, NH); 8.59 (bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ= 183.59; 171.12; 170.70; 170.44; 170.13; 169.75; 153.02; 137.97; 128.89; 128.85; 128.42; 128.29; 127.71; 127.45; 122,95; 83.12; 72.16; 70.98; 68.36; 67.29; 60.90; 56.80; 20.70; 20.59; 20.53; 20.48. Anal. Calcd for $C_{29}H_{32}N_4O_9S$: C, 56.85; H, 5.26; N, 9.14; S, 5.23. Found: C, 56.89; H, 5.23; N, 9.11; S, 5.21.

***N*-[(*R*)-1-(1*H*-benzo[d]imidazol-2-yl)-2-phenylethyl]-*N*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)thiourea (8)**

White solid, 48 % Yield, M.P. = 142-145 °C, IR (NaCl, cm^{-1}): 3330, 3030, 1770, 1520, 1380, 1260, 740. ^1H NMR (400 MHz, $CDCl_3$, ppm): δ= 1.95 (s, 3H, OCH_3); 1.96 (s, 3H, OCH_3); 2.02 (s, 3H, OCH_3); 2.05 (s, 3H, OCH_3); 3.41-3.48 (m, 2H, CH-CH_2-Ar); 4.02-4.06 (m, 3H, H-6a+H-6b+H-5); 4.12-6.12 (5H, H-1+H-2+H-3+H-4+NH-CH-CH_2); 5.60 (bs, 1H, NH); 6.85 (bs, 1H, NH); 7.05 (app d, *J*= 8.2 Hz, 1H, Ar-H); 7.13 (app d, *J*= 7.2 Hz, 2H, Ar-H); 7.17 (s, 1H, Ar-H); 7.19-7.23 (m, 3H, Ar-H); 7.49 (s, 2H, Ar-H); 7.90(bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ=183.44; 171.08; 170.52; 170.03; 169.70; 155.89; 153.43; 136.43; 129.13; 128.60; 126.90; 122.99; 82.92; 72.35; 70.87; 68.35; 67.15; 61.17; 53.88; 38.79; 20.63; 20.59; 20.48; 20.47. Anal. Calcd for $C_{30}H_{34}N_4O_9S$: C, 57.70; H, 5.47; N, 8.94; S, 5.12. Found: C, 57.74; H, 5.48; N, 8.96; S, 5.11.

***N*-[(*R*)-1-(1*H*-benzo[d]imidazol-2-yl)(phenyl)methyl]-*N*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)thiourea (9)**

White solid, 71 % Yield, M.P. = 151-154 °C, IR (NaCl, cm^{-1}): 3335, 2960, 1752, 1550, 1360, 1225, 1032, 743. 1H NMR (400 MHz, CDCl3, ppm): δ= 1.93 (s, 3H, OCH_3); 1.94 (s, 3H, OCH_3); 1.98 (s, 3H, OCH_3); 2.00 (s, 3H, OCH_3); 3.94 (app d, *J*= 9.2 Hz, 1H, H-5); 4.02 (d, *J*= 11.4 Hz, 1H, H-6a); 4.08-4.13 (m, 1H, H-6b); 4.31 (dd, *J*= 12.2 Hz, *J*= 3.6 Hz, 1H, H-4); 5.06-5.25 (3H, H-2+H-3+NH-CH-Ar); 5.34 (s,1H, H-1); 5.93 (bs, 1H, NH); 7.17-7.21 (m, 4H, Ar-H); 7.32 (d, *J*= 3.8 Hz, 2H Ar-H); 7.41 (s, 3H Ar-H); 7.86 (bs, 1H, NH); 8.23 (bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ=183.58; 170.89; 170.80; 169.81; 169.41; 129.06; 128.99; 128.55; 127.87; 127.59; 123.13; 80.46; 69.69; 69.18; 69.04; 66.05; 61.95; 56.93; 20.74; 20.66; 20.64; 20.47. Anal. Calcd for $C_{29}H_{32}N_4O_9S$: C, 56.85; H, 5.26; N, 9.14; S, 5.23 Found: C, 56.80; H, 5.24; N, 9.15; S, 5.25.

_N_-[(1_S_,2_S_)-1-(1_H_-benzo[d]imidazol-2-yl)-2-methylbutyl)]-_N_-(2,3,4,6-tetra-_O_-acetyl-α-D-mannopyranosyl)thiourea (10)

White solid, 53 % Yield, M.P. = 149-151 °C, IR (NaCl, cm^{-1}): 3340, 2965, 1752, 1534, 1367, 1224, 1052, 749. 1H NMR (400 MHz, CDCl3, ppm): δ= 0.94 (d, *J*= 6.7 Hz, 6H, 2xCH_3); 1.29-1.37 (m, 2H, CH-C<u>H</u>$_2$-CH_3); 1.64-1.69 (m, 1H, CH_3-C<u>H</u>-CH_2) 1.97 (s, 6H, 2XOCH_3); 2.02 (s, 3H, OCH_3); 2.13 (s, 3H, OCH_3); 3.42-3.46 (m, 1H, H-5); 4.00 (s, 1H, H-6a); 4.08 (s, 1H, H-6b); 4.38 (d, *J*= 9.5 Hz, 1H, H-4); 5.12-5.41 (3H, H-3+H-1+NH-C<u>H</u>-CH-CH_3); 5.68 (s, 2H, H-1+ NH); 7.21-7.23 (m, 2H, Ar-H); 7.41 (s, 2H, Ar-H); 7.67 (s, 1H, Ar-H); 8.52 (bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ= 183.19; 170.69; 169.95; 169.89; 169.39; 156.97; 133.05; 122.77; 119.96; 118.69; 111.59; 80.76; 70.05; 69.02; 68.65; 65.63; 61.74; 58.38; 33.81; 24.83; 20.77; 20.69; 20.61; 20.56; 15.72; 10.90. Anal. Calcd for $C_{27}H_{36}N_4O_9S$: C, 54.72; H, 6.12; N, 9.45; S, 5.41 Found: C, 54.70; H, 6.12; N, 9.43; S, 5.40.

Purification of human erythrocytes CA isozymes

Erythrocyte cells were purified using fresh human blood from Atatürk University Research Hospital Blood Center. Plasma and buffy coat were removed by centrifugation for ten minutes using a cooled centrifuge at 2000 rpm by taking 10 mL from the blood collected. The erythrocytes were separated and washed three times with 0.9% NaCl and hemolyzed with two volumes of ice-cold water. It was centrifuged again at 10000 rpm for 30 minutes. The pH of the resulting hemolysate was adjusted to 8.7 with solid Tris. The hemolysate was applied to the prepared Sepharose 4BL-aniline-sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl / 22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA-I and hCA-II) isoenzymes were washed with 1 M NaCl / 25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa / 0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4°C [14-17].

Biological activities for hCA I, hCA II, AChE and BChE

The Ellman method investigated the inhibitory activities between the synthesized benzimidazole derivatives (**5-10**) and cholinesterase enzymes [54]. Ellman's method is an in vitrobiological assay that is based on the reaction of thiocholine with DTNB to form 5-mercapto-2-nitrobenzoicacid (yellow compound). The yellow color can be quantified by its absorbance at 412 nm. First, 1 mg of each inhibitor was dissolved in 1 mL DMSO and then diluted to various concentrations with deionized water. To determine the cholinesterase inhibition activity, six serial dilutions of the inhibitors were measured. The

reaction system was composed of 5-60 µL inhibitor sample, 200 µL buffer (1 M, pH 8.0: Tris-HCl buffer for the AChE assay and phosphate buffer for the BChE assay), 50 µL DTNB (0.5 mM), 50 µL acetylthiocholine iodide/S-butyrylthiocholine chloride (10 mM) and 10 µL enzyme (0.28 units/mL for the AChE assay and 0.32 units/mL for the BChE assay). The reaction was initiated upon addition of the enzyme. The reaction system was prepared at room temperature in a quartz cuvette. The blank reading was composed of all chemicals except the inhibitör. The absorbance of the reaction mixture was measured at 412 nm within 5 minutes from the start of the reaction on a ThermoScientific Evolution 200 Series UV-VIS spectrophotometer. The absorbance for each reaction mixture was measured three times within 5 minutes of adding the enzyme, and the results are reported as mean ± standard deviation.

Carbonic anhydrase (hCA I and II) activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25 ^{o}C using a spectrophotometer (CHEBIOS UV–VIS) according to the method described by Verpoorte et al [55]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M Tris–SO4 buffer (pH 7.4), 1 mL of 3 mM 4-nitrophenylacetate, 0.5 mL H2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of synthesized compounds (**5**-**10**) and acetazolamide were examined. Donepezil for cholinesterases and acetazolamide for hCA isoenzymes were used as reference inhibitors. Each substance tested was assayed in at least three replicates for each concentration. Measurements were made for all these derivatives at different concentrations. The activity in the measurement (control) without inhibitor was accepted as 100%. % Activity-[Inhibitor] graphs are drawn for the substances tested. Measurements were made for all these derivatives (**5**-**10**) at different concentrations. The IC_{50} values determined for benzimidazole derivatives are shown in Table 1. Stock solutions of all substances used in the study were prepared as 1 mg/mL (using dimethylsulfoxide). The prepared stock solutions were diluted with distilled water at different concentrations. Six serial dilutions of these derivatives using distilled water were measured to determine the inhibitory activity of all the enzymes tested. This study used in the previous work [56]. The prepared stock solutions were diluted with distilled water at different concentrations**.**

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