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Investigation of the effects of some sulfonamides on acetylcholinesterase and carbonic anhydrase enzymes

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Abstract

Human carbonic anhydrase I and II isoenzymes (hCA I and II) and acetylcholinesterase (AChE) are important metabolic enzymes that are closely associated with various physiological and pathological processes. In this study, we investigated the inhibition effects of some sulfonamides on hCA I, hCA II, and AChE enzymes. Both hCA isoenzymes were purified by Sepharose-4B-L-Tyrosine-5-amino-2-methylbenzenesulfonamide affinity column chromatography with 1393.44 and 1223.09-folds, respectively. Also, some inhibition parameters including IC_{50} and K_i values were determined. Sulfonamide compounds showed IC_{50} values of in the range of 55.14 to 562.62 nM against hCA I, 55.99 to 261.96 nM against hCA II, and 98.65 to 283.31 nM against AChE. K_i values were in the range of 23.40 ± 9.10 to 365.35 ± 24.42 nM against hCA I, 45.87 ± 5.04 to 230.08 ± 92.23 nM against hCA II, and 16.00 ± 45.53 to 157.00 ± 4.02 nM against AChE. As a result, sulfonamides had potent inhibition effects on these enzymes. Therefore, we believe that these results may contribute to the development of new drugs particularly in the treatment of some disorders.

KEYWORDS
acetylcholinesterase, carbonic anhydrase, enzyme inhibition, sulfonamides

1 | INTRODUCTION

Carbonic anhydrases (CAs; E.C.4.2.1.1) catalyze carbon dioxide (CO_2) conversion to bicarbonate (HCO_3^-) and proton (H^+) release reversibly.[1–5] CAs are found in eukaryotic and prokaryotic cells and they are encoded by seven distinct gene families, α-, β-, γ-, δ-, ζ-, η-, and θ-CAs.[6–10] Only the α-CA family is found in mammals. In mammalian, when CO_2 in the blood plasma passes into red blood cells by diffusion, it is rapidly converted to carbonic acid by CA enzyme.[11–13]

Carbonic acid is separated into proton and bicarbonate ions and passes back into the blood and provides a natural buffer environment by balancing blood pH at 7.4. Thus, the carbonic anhydrase enzyme regulates the acidity of the chemical environment to prevent damage to body functions.[14–16] Up to now, 16 different α-CA isoenzymes have been characterized in mammals by means of their amino acid sequence, catalytic activity, biochemical properties, subcellular localization, and sensitivity to inhibitors and activators. These isoenzymes are grouped as cytoplasmic CAs (CAs I, II, III, VII, and XIII), membrane-bound CAs (CAs IV, IX, XII, XIV, XV), mitochondrial CAs (CA V), secretory CAs (CA VI), and CA-related proteins (CA-RPs: CAs VIII, X, and XI). CA-RPs have not performed CO_2 hydration activity and physiological function.[17–20]

Due to these important physiological functions, numerous studies have been carried out on CAs. CA I and CA II are the most studied isoenzymes.[21,22] CA I is expressed in erythrocytes and the gastrointestinal tract, whereas CA II is expressed in almost all tissues. CA I and CA II are involved in important metabolic functions, such as gas exchange and ion transport.[23,24]

Metal complexes forming anions, sulfonamides, and their esters are the prominent carbonic anhydrase inhibitors

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(CAIs).[25,26] The sulfonamide group is the most important and widest compound structure used for CA inhibition due to its zinc-binding function.[27] Currently, numerous sulfonamide derivatives have been developed as CAIs, which clinically used in the treatment of glaucoma, epilepsy, cancer, obesity, and ulcer.[28,29]

For the treatment of Alzheimer's disease (AD), one of the most successful methods developed so far is acetylcholinesterase (AChE; E.C.3.1.1.7) inhibition.[30–32] AChE, which consists of multiple subunits, is a membrane-bound enzyme and is present in the cholinergic neurons, brain, and muscles. This enzyme terminates nerve conduction by hydrolyzing acetylcholine (ACh) to acetic acid and choline (Ch) in the cholinergic synapses of the nervous system, somatic system, and central nervous system. The AChE inhibitors are clinically used for the treatment of AD, which increase cholinergic functions in cholinergic synapses.[33–36] ACh is an important neurotransmitter in the brain-related regions.[37,38] Reduction of ACh is maintained at a relatively high level.[39] Today, tacrine, donepezil, rivastigmine, and galantamine are commonly used as AChEIs in AD.[40] Therefore, the identification of novel inhibitors of the AChE contributes significantly to the development of new drugs for the treatment of AD. In recent studies, it has been found that some newly synthesized sulfonamide derivative molecules had inhibition effects on both CA isoenzymes and AChE activities at nanomolar levels.[41]

In light of these information, in this study, we investigated the effects of some sulfonamides (Figure 1), including 2-chloro-4-sulfamoylaniline **(1)**, 3-amino-4-chlorobenzenesulfonamide **(2)**, 3,4-diaminobenzenesulfonamide **(3)**, 3,5-dichlorosulfanilamide **(4)**, 4-amino-3-methylbenzenesulfanilamide **(5)**, 5-amino-1-naphthalenesulfonamide **(6)**, 5-amino-2-methylbenzenesulfonamide **(7)** on hCA I, hCA II, and AChE enzymes.

2 | MATERIALS AND METHODS

2.1 | Chemicals

CNBr-activated Sepharose-4B, L-tyrosine, sulfonamides, and other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). Standard protein markers for electrophoresis were obtained from Thermo Fisher Scientific (Waltham, MA).

2.2 | Preparation of affinity column chromatography

CNBr-activated Sepharose-4B was used as the colon matrix. L-Tyrosine was covalently attached to this colon material.[42,43] This process was performed as follows: the CNBr-activated Sepharose-4B was transferred to a beaker, washed with 250 mL of cold $NaHCO_3$ buffer (0.1 M, pH 10). In 20 mL of the same buffer, 80 mg of L-tyrosine was added to the dissolved cold solution by adding to the beaker. The addition of L-tyrosine solution and stirring with baguette were observed in less than 90 seconds, after which the suspension was stirred for 4 hours at 4°C in a magnetic stirrer and kept at 4°C for 16 hours. At the end of this period, the suspension was washed with distilled water until the wash water did not give absorbance was at 280 nm so that unreacted L-tyrosine was completely removed. The washing process was repeated with 100 mL of $NaHCO_3$ buffer (0.2 M, pH 8.8) and the L-tyrosine inserted gel was taken up in 40 mL of the same buffer. Here L-tyrosine forms the extension arm of the affinity gel.[44,45] Then, the diazolized 5-amino-2-methylbenzenesulfonamide is coupled to tyrosine to form the moiety that specifically binds the enzyme. This process was performed as follows: 25 mg of 5-amino-2-methylbenzenesulfonamide was dissolved in 10 mL of 1 M HCl at 0°C and 5 mL of solution at 0°C in 75 mg $NaNO_2$ was added dropwise to the inhibitor solution. After a 10-minute reaction, the 5-amino-2-methylbenzenesulfonamide, which was diazed, was added

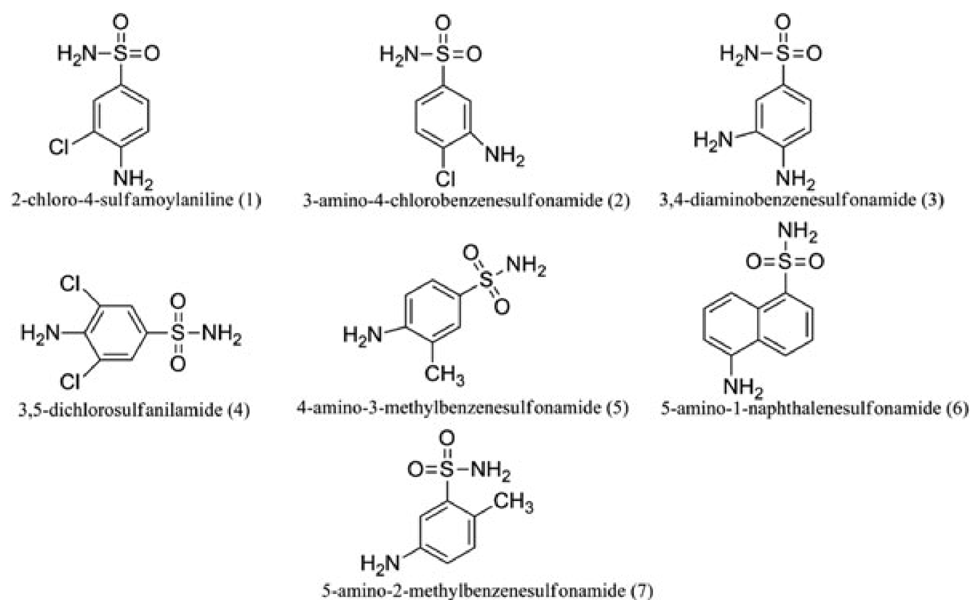

FIGURE 1 The molecular structures of sulfonamides used in this study

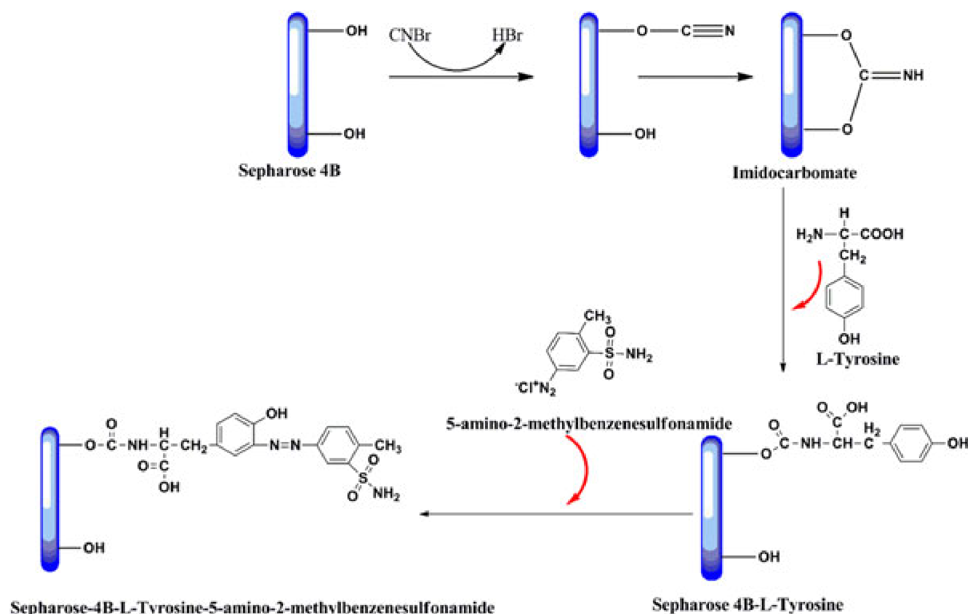

FIGURE 2 Schematic representation of the preparation steps of the Sepharose-4B-L-tyrosine-5-amino-2-methylbenzenesulfonamide affinity column chromatography

to the suspension of 40 mL of Sepharose-4B-L-tyrosine. The pH was adjusted to 9.5 with 1 M NaOH and stirred slowly for 3 hours at room temperature. It was then washed with 1 L of distilled water and 200 mL of phosphate buffer pH 6.8 and stored in the same buffer. The Sepharose-4B-L-tyrosine-5-amino-2-methylbenzenesulfonamide affinity gel prepared in this way (Figure 2) was packed into the column. The column was equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7) buffer solution.[46]

2.3 | Purification of CA isoenzymes from human erythrocyte

Using fresh human blood, erythrocyte hemolysate was prepared as in previous studies, and the pH of the hemolysate was adjusted to 8.7 with solid Tris to facilitate the binding of CA isoenzymes to the affinity ligand.[47] This erythrocyte hemolysate (35 mL) was adjusted to the pre-equilibrated Sepharose-4B-L-tyrosine-5-amino-2-methylbenzenesulfonamide affinity column. After all of the erythrocyte hemolysate was passed through the column, the column was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7) buffer solution to remove proteins that were not bound to the affinity column. After washing was complete, firstly CA I was eluted with 1.0 M NaCl/25 mM Na_2HPO_4 (pH 6.3) buffer. Then CA II was eluted from the column using 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6) buffer. The elution of the CA I and CA II isoenzymes was monitored at 280 nm during the purification procedures. The elution graph is shown in Figure 3. Activity measurements were made for CA I and CA II isoenzymes using the hydratase activity measurement method as determined by Wilbur-Anderson, and active fractions were collected. The collected active enzyme solutions were dialyzed overnight

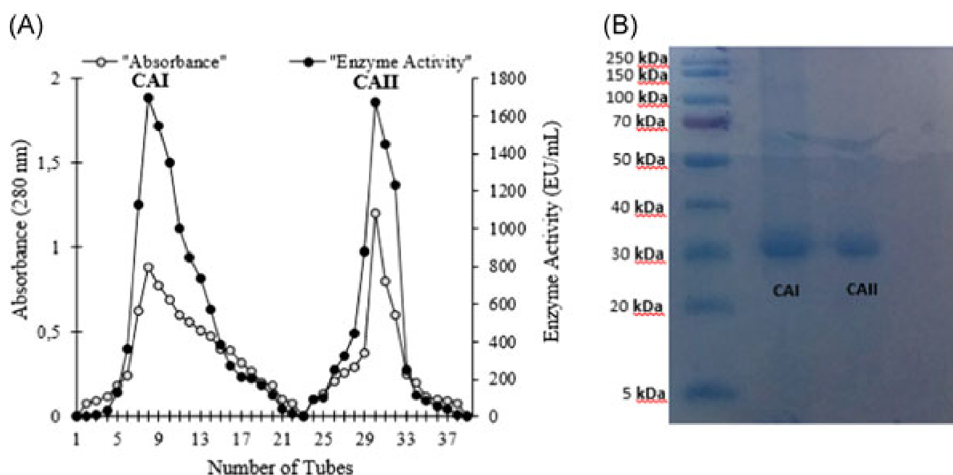

FIGURE 3 The elution graph (A) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) photograph (B) of hCA I, and hCA II isoenzymes

against 0.05 M Tris-SO_4 (pH 7.4) buffer. After the dialysis procedure, CA I and CA II isoenzymes were stored in 1 mL tubes and stored at −80°C for further kinetic processing.[48] Protein determination during purification was carried out using the Bradford method.[49] Bovine serum albumin was used as standard protein as described previously.[50,51] Both CA isoenzymes purity was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [52] as described in detail (Figure 3).[53,54]

2.4 | Inhibition effects of sulfonamides on CA isoenzymes

Esterase activity method was used for CA isoenzymes in vitro studies. The essence of the esterase activity measurement is as follows: the CA enzyme uses *p*-nitrophenylacetate (PNA) as the substrate. The CA enzyme hydrolyzes PNA to *p*-nitrophenol, at 348 nm. In the esterase activity method, absorbance change at 348 nm is measured at 25°C for 3 minutes.[55,56] Activity measurements were made for at least five different concentrations of each sulfonamide molecule to determine the inhibitory effect of some sulfonamides **(1-7)** on CA I and CA II isoenzymes. Each measurement was repeated three times. Control activity was considered to be 100% and inhibitory concentration graphs against activity (%) were plotted for each inhibitor.[57] From these graphs, half maximal inhibitor concentrations (IC_{50} values) against CA activity for each sulfonamide were determined. In addition, to determine the K_i values and inhibition types, which help to elucidate the inhibition mechanisms of sulfonamides activity measurements, were made at three different inhibitors and five different substrate concentrations for each sulfonamide.[58] Lineweaver-Burk graphs were drawn according to these measurement results.[59] From these graphs, K_i values and inhibition types were determined for each sulfonamide.[60]

2.5 | Inhibition effects of some sulfonamides on AChE

AChE catalyzes the hydrolysis of neurotransmitter ACh to choline and acetate. In this study, AChE activities were measured according to the spectrophotometric method of Ellman et al[61] as described previously.[62] Acetylthiocholine iodide was used as substrates of the reaction. The rate of formation of thiocholine was measured by the reaction of thiocholine with 5,5′-dithio-bis(2-nitro-benzoic) acid producing a yellow color resulting from the formation of 5-thio-2-nitrobenzoic acid. The increase in the optical density of the system was measured against the curves for 10 to 15 minutes at 412 nm.[63] In inhibition studies, activity measurements were made for at least five different concentrations of each sulfonamide molecule to determine the inhibitory effect of some sulfonamides **(1-7)** on AChE. Each measurement was repeated three times. Control activity was considered to be 100% and inhibitory concentration graphs against activity (%) were plotted for each inhibitor.[64] From these graphs, inhibitor concentrations (IC_{50} values) against AChE activity of for each sulfonamide molecule were determined. In addition, to determine the K_i values and inhibition types, which help to elucidate the inhibition mechanisms of sulfonamide molecules activity measurements, were made at three different inhibitors and five different substrate concentrations for each sulfonamide. Lineweaver-Burk graphs were drawn according to these measurement results.[59] From these graphs, the K_i values and inhibition types were determined for each sulfonamide molecule.[65]

3 | RESULTS

In this study, we investigated the effects of some sulfonamides on AChE, hCA I, and hCA II enzymes. For this purpose, hCA I and hCA II isoenzymes were purified from human erythrocytes and AChE was purchased (CAS No: 9000-81-1). When purifying the hCA I and hCA II isoenzymes, the affinity gel was prepared using a new ligand, as opposed to the method previously used.[66,67] 5-Amino-2-methylbenzenesulfonamide was connected as a ligand into CNBr-activated Sepharose-4B-L-tyrosine. Using the Sepharose-4B-L-tyrosine-5-amino-2-methylbenzenesulfonamide affinity column chromatography. Human erythrocyte hCA I and hCA II isoenzymes were obtained with a yield of 46.43% and 32.14%, and a specific activity of 1830.98 and 1607.14 EU/mg proteins. Both enzymes were purified as 1393.44 and 1223.09-folds, respectively. Purification results were summarized in Table 1. The purification coefficient obtained with Sepharose-4B-L-tyrosine-5-amino-2-methylbenzenesulfonamide affinity column was found to be higher than the purification coefficient obtained in the Sepharose-4B-L-tyrosine-sulfanilamide affinity column. Therefore, we can say that Sepharose-4B-L-tyrosine-5-amino-2-methylbenzenesulfonamide affinity

TABLE 1 Summary scheme of hCA I and hCA II isoenzymes purified using Sepharose-4B-L-tyrosine-5-amino-2-methylbenzenesulfonamide affinity chromatography

Purification steps	Activity, EU/mL	Total volume, mL	Protein, mg/mL	Total protein, mg	Total activity, EU	Specific activity, EU/mg	Yield, %	Purification fold
Hemolysate	400	35.0	304.30	10650.46	14.0	1.314	100	1.000
Affinity chromatography-hCA I	650	10.0	0.36	3.55	6.5	1830.9	46.4	1393.4
Affinity chromatography-hCA II	900	5.0	0.56	2.80	4.5	1607.1	32.1	1223.1

Abbreviation: hCA I and II, human carbonic anhydrase isoenzymes I and II.

TABLE 2 Inhibition effects of sulfonamides (**1-7**) on hCA I and hCA II isoenzymes

	hCA I			hCA II		
Compounds	IC_{50}, nM	K_i, nM	Inhibition type	IC_{50}, nM	K_i, nM	Inhibition type
1	562.62	365.35 ± 24.42	Competitive	261.96	128.80 ± 57.76	Competitive
2	559.98	152.23 ± 179.87	Competitive	257.76	230.08 ± 92.23	Competitive
3	113.00	82.58 ± 21.26	Noncompetitive	127.25	59.81 ± 27.73	Noncompetitive
4	55.14	23.40 ± 9.10	Competitive	91.19	95.99 ± 20.90	Competitive
5	119.28	63.80 ± 6.90	Competitive	59.29	128.43 ± 28.23	Competitive
6	116.61	117.71 ± 54.60	Competitive	78.35	61.80 ± 23.87	Competitive
7	117.98	174,45 ± 61.46	Noncompetitive	55.99	45.87 ± 5.04	Noncompetitive
AZA[a]	60.78	67.70 ± 2.55	–	55.00	58.60 ± 2.56	–

Abbreviations: AZA, acetazolamide; hCA I and II, human carbonic anhydrase isoenzymes I and II.
[a]AZA used as a standard inhibitor for hCA I, and hCA II isoenzymes.

column chromatography is a novel and efficient affinity technique for the purification of hCA I and hCA II isoenzymes when compared the Sepharose-4B-L-tyrosine-sulfanilamide affinity column chromatography.[68–70]

After purification, the inhibitory effects of some sulfonamides **(1-7)** on hCA I and hCA II isoenzymes were investigated. The sulfonamides used in the study are phenyl-substituent primary sulfonamides. Therefore, they showed inhibition effect at nanomolar levels on hCA I and hCA II isoenzymes. The inhibitory effects of sulfonamides **1-7** were determined by IC_{50} and K_i values. IC_{50} values of sulfonamides **1-7** for hCA I were found in ranging of 55.14 to 562.62 nM for hCA II (Table 2). Also, 3,5-dichlorosulfanilamide (**4**), which posses two chlorine (Cl^-) and one amine ($-NH_2$) groups, had strong inhibition effect on hCA I, and 5-amino-2-methylbenzenesulfonamide (**7**) had the most inhibition effect on hCA II isoenzymes. Chlorine (Cl−) groups can coordinate the Zn^{2+} ions with a distorted tetrahedral geometry like bromide (Br^-) and azide (N_3^-). This coordination, which favors inhibition properties, is inevitable.[71] To determine the inhibition types and K_i constants of s sulfonamides **1-7**, Lineweaver-Burk graphs were drawn.[72,73] K_i values of sulfonamides **1-7** were found between 23.40 ± 9.10 to 365.35 ± 24.42 nM for cytosolic hCA I isoenzyme. On the other hand, acetazolamide (AZA), which used as the reference inhibitor, had IC_{50} and K_i values of 60.78 and 67.70 ± 2.55 nM for hCA I, respectively. These results clearly

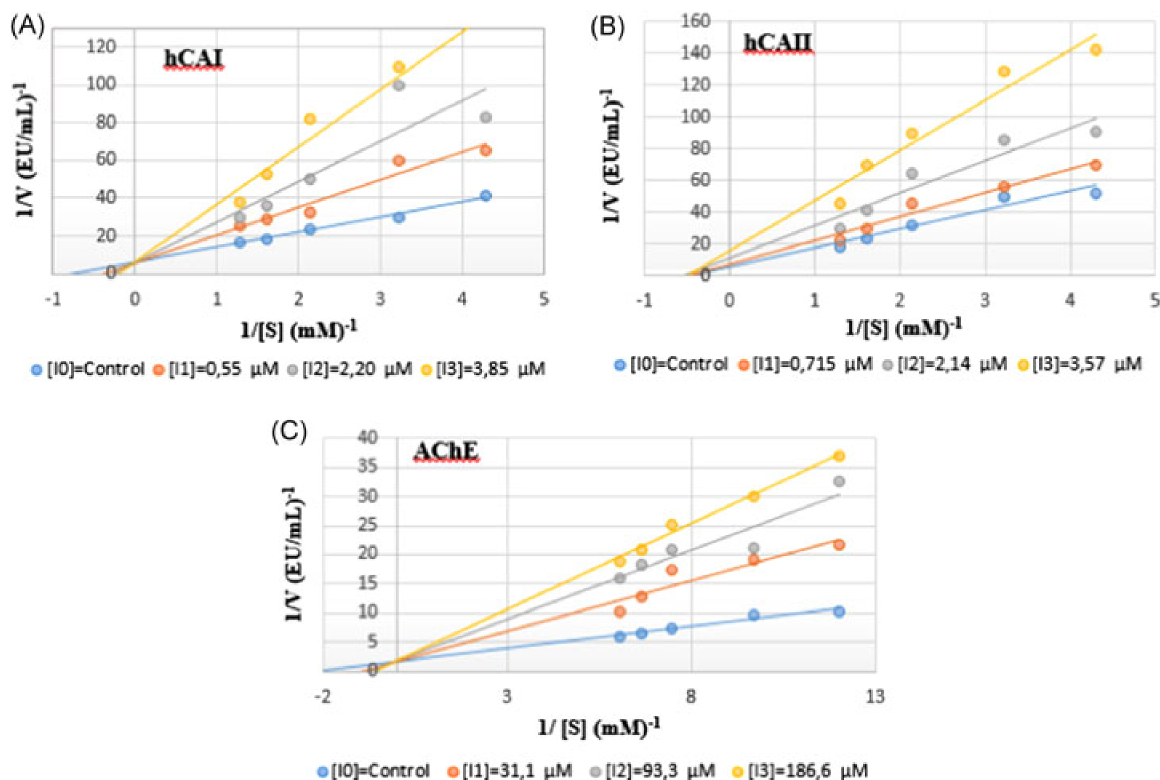

FIGURE 4 Lineweaver-Burk graphs for sulfonamides with the lowest K_i values of hCA I (**4**), hCA II (**7**) isoenzymes and acetylcholinesterase (AChE) (**6**)

TABLE 3 Inhibition effects of sulfonamides (**1-7**) on acetylcholinesterase (AChE) enzyme

Compounds	IC_{50}, μM	K_i, μM	Inhibition type
1	0.184	0.027 ± 0.004	Noncompetitive
2	0.158	0.063 ± 0.008	Competitive
3	0.283	0.057 ± 0.018	Noncompetitive
4	0.098	0.089 ± 0.019	Competitive
5	0.472	0.157 ± 0.004	Noncompetitive
6	0.164	0.016 ± 0.045	Competitive
7	0.149	0.033 ± 0.003	Noncompetitive
Tacrine[a]	0.410	0.390 ± 0.079	–

[a]Tacrine (TAC) was used as a standard inhibitor for acetylcholinesterase (AChE) enzyme.

showed that 5-amino-2-methylbenzenesulfonamide (**7**) had similar inhibitory effect to AZA against hCA I (Figure 4; Table 2).

Also, similar inhibition results of sulfonamides **1-7** were observed against cytosolic dominant hCA II. All sulfonamides **1-7** inhibited hCA II with K_i values ranging from 45.87 ± 5.04 to 230.08 ± 92.23 nM. Also, AZA, which was used as a positive standard inhibitor for CA isoenzymes, showed the inhibition with K_i values of 58.60 ± 2.56 nM for hCA II.

The inhibitory effect of sulfonamides **1-7** on AChE was determined by IC_{50} and K_i values. IC_{50} values of sulfonamides **1-7** were found as 184.42, 158.89, 283.31, 98.65, 472.72, 164.31, 149.92 nM, respectively. On the other hand, K_i constants of sulfonamides **1-7** were calculated as 27.76 ± 4.03, 63.38 ± 8.08, 57.76 ± 18.37, 89.93 ± 19.98, 157.00 ± 4.02, 16.00 ± 45.53, and 33.30 ± 3.01 nM, respectively (Table 3). According to these results, the sulfonamides we used in the study, showed a stronger inhibition effect on AChE than tacrine (IC_{50}: 410.00 nM; K_i: 390.01 ± 79.05 nM).

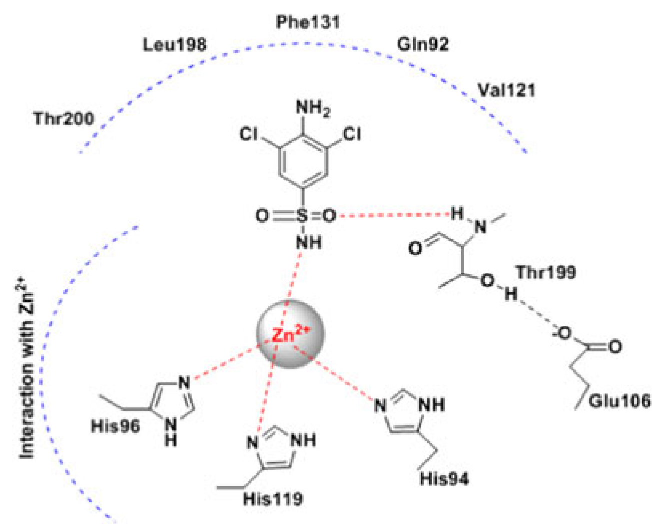

FIGURE 5 Possible schematic representation of the interaction of 3,5-dichlorosulfanilamide with the CA II active site

4 | DISCUSSION

K_i constant refers to the binding affinity of the inhibitor to the enzyme. The small K_i value indicates that the inhibitor is bound to the enzyme with a strong affinity.[74,75] According to this, 3,5-dichlorosulfanilamide sulfonamide (**4**) had a potent binding affinity for hCA I, and 5-amino-2-methylbenzenesulfonamide (**7**) had high binding affinity for hCA II. All sulfonamides except of sulfonamides **3** and **7** showed competitive inhibition. The sulfonamides **3** and **7** molecules showed noncompetitive inhibition. According to these results, sulfonamides **1**, **2**, **4**, **5**, and **6** are believed to reduce the catalytic activity of CA I and CA II isoenzymes by interacting with zinc ion in the active site of CA I and CA II and by making Van der Waals interactions with amino acids in their active site. For example, the possible interaction of the 3,5-dichlorosulfanilamide (**4**) with the active site of CA II is shown schematically in Figure 5. On the other hand, sulfonamides **3** and **7** bind to the outside of the active site of the hCA I, and hCA II isoenzymes and cause inhibition. The second class is connected to the active site without directly interacting with zinc in the active site.[76] Sulfonamides are the most important carbonic anhydrase enzyme inhibitor group and in the class of inhibitors that bind to the catalytic zinc ion in the active site of the enzyme.[77–79]

Primary sulfonamides contain two hydrogen atoms in the sulphonyl groups and secondary sulfonamides contain one hydrogen atom in the sulphonyl groups.[80,81] They are specific inhibitors of CA enzymes, whereas secondary sulfonamides are generally weak CAIs. Secondary and tertiary sulfonamides cannot be bound to zinc in the active site of the CA enzyme due to structural barriers. These sulfonamides are thought to bind to the inlet of the active site of CA. On the other hand, benzenesulfonamides, which contain the sulfamide and phenyl ring, are important aromatic CAIs.[82–84] They can interact with the catalytic Zn^{2+} in the active site of the CA and provide for many Van der Waals interactions with the residues Val121, Gln92, Phe131, Leu198, and Thr200 in the active site.[1] Therefore, benzenesulfonamides had a more precise and explicit inhibition mechanism. In many studies to date, it has been shown that some molecules that exhibit an inhibitory effect on CA activity may also be an AChEIs.[85]

In this study, we also investigated the effect of some sulfonamides on AChE activity in which we determined the inhibitory effect on hCA I and hCA II isoenzymes. The sulfonamides **1-7** we used in our study showed a strong inhibition effect on AChE activity. The sulfonamides **1**, **3**, **5**, and **7** showed a noncompetitive inhibition effect on AChE, while sulfonamides **2**, **4**, and **6** molecules showed a competitive inhibition effect. AChEIs are considered to be the most effective group in reducing symptoms of AChE and improving cholinergic deficiency. Nowadays, studies are underway to determine new and specific AChEIs for use in the treatment of AD. The results clearly showed that the used sulfonamides had advantage when compared with the tacrine, donepezil, rivastigmine, and galantamine because they have no side effects. However, recently, the usage of some of these drugs has been limited because of their side effects such as gastrointestinal disturbance and hepatotoxicity.[86,87]

5 | CONCLUSIONS

In conclusion, the inhibitory effects of some sulfonamides on hCA I, hCA II, and AChE enzymes were evaluated together. The sulfonamides we used in our study showed inhibition effects on hCA I, hCA II, and AChE activities at low concentrations. We believe that these results may be useful in the synthesis of new CA and AChE inhibitors and in the development of drugs for the treatment of some diseases.

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