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ARTICLE

Evaluation of some thiophene-based sulfonamides as potent inhibitors of carbonic anhydrase I and II isoenzymes isolated from human erythrocytes by kinetic and molecular modelling studies

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Abstract

Background Thiophene(s) are an important group in therapeutic applications, and sulfonamides are the most important class of carbonic anhydrase (CA) inhibitors. In this study, inhibition effects of some thiophene-based sulfonamides on human erythrocytes carbonic anhydrase I and II isoenzymes (hCA-I and hCA-II) were investigated. Thiophene-based sulfonamides used in this study showed potent inhibition effect on both isoenzymes at very small concentrations.

Materials and methods We report on the purification of the carbonic anhydrase I and II isoenzymes (hCA-I and hCA-II) using affinity chromatography method. The inhibition effect of the thiophene-based sulfonamides was determined by IC_{50} and K_i parameters. A molecular docking study was performed for each molecule.

Results Thiophene-based sulfonamides showed IC_{50} values of in the range of 69 nM to 70 µM against hCA-I, 23.4 nM to 1.405 µM against hCA-II. K_i values were in the range of 66.49 ± 17.15 nM to 234.99 ± 15.44 µM against hCA-I, 74.88 ± 20.65 nM to 38.04 ± 12.97 µM against hCA-II. Thiophene-based sulfonamides studied in this research showed non-competitive inhibitory properties on both isoenzymes. To elucidate the mechanism of inhibition, a molecular docking study was performed for molecules 1 and 4 exhibiting a strong inhibitory effect on hCA-I and hCA-II. The compounds inhibit the enzymes by interacting out of catalytic active site. The sulfonamide and thiophene moiety played a significant role in the inhibition of the enzymes.

Conclusion We hope that this study will contribute to the design of novel thiophene-based sulfonamide derived therapeutic agents that may be carbonic anhydrase inhibitors in inhibitor design studies.

Keywords Thiophene · Sulfonamide · Carbonic anhydrase · Inhibition · Molecular modelling

Introduction

Sulfonamides are a crucial class of drugs having the general formula $R\text{-}SO_2NH_2$ [1, 2] in the field of pharmacology because of their biological activities such as antibacterial [3], antiviral [4], anticancer [5], anti-obesity [6], hypoglycemic [7], diuretic [8], anti-neuropathic pain [9], carbonic anhydrase inhibition and they have been used extensively in the treatment of human diseases [1, 2]. Sulfonamides are considered the most important class of carbonic anhydrase inhibitors [10]. Sulfonamide groups are ideal ligands for the CA active site for two reasons: first, the sulfonamides combine the positively charged zinc ion with the negative charge of deprotonated nitrogen ion. Second, a proton in the coordinated nitrogen atom is compatible with Thr199, a hydrogen bond acceptor that forms a tight bond with hydrogen [11]. Currently, most of the CA inhibitors used in the clinic for

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the treatment of various diseases are sulfonamides [12]. For example, methazolamide, acetazolamide, zonisamide [13], dorzolamide, brinzolamide, diclofenamide, ethoxzolamide and indisulam are sulfonamide derivative CA inhibitors [13, 14].

Carbonic anhydrase (CA) (EC 4.2.1.1) enzymes are metalloenzymes commonly found in virtually all living organisms, which recyclables the hydration/dehydration of CO_2/HCO_3^-. Apart from the reversible hydration reaction of CO_2 to bicarbonate, α-CAs also catalyze a variety of other reactions such as hydration from cyanate to carbamic acid or hydration from syamide to urea, hydration of aldehyde to gem-diols, and hydrolysis of carboxylic esters or sulfonic esters [15]. CA-I and CA-II are the two main isoforms of α-CAs found in the cytosol of mammalian red blood cells. The most important function of these isoenzymes takes place in the respiratory event by catalyzing the conversion of the metabolic product CO_2 molecule into HCO_3^- compound in the tissue capillaries and the conversion of HCO_3^- to CO_2 in the pulmonary capillary. Studies have shown that CA-I, CA-II and most of other CA isoenzymes have important roles in various physiological processes such as H^+ production, acid–base homeostasis, pH balance, metabolic acidosis, and have demonstrated the association of abnormal levels or activities of these isoenzymes with human diseases. Therefore, CA isoenzymes are considered as potential therapeutic targets that can be employed in the treatment of most of diseases such as glaucoma, edema, obesity, cancer, epilepsy, osteoporosis [16–18]. Since CA isoenzymes were associated with many disorders, CA inhibitors are target molecules in drug design studies. Nowadays, studies for the identification of new and specific inhibitors of CA isoenzymes are continuing rapidly.

In this study, we investigated the inhibitory effect of some thiophene-based sulfonamide molecules (**1-8**) (Fig. 1) on CA-I and CA-II isoenzymes isolated from human erythrocytes. We observed that the thiophene-based sulfonamide molecules which selected had a very strong inhibitory effect on hCA-I and hCA-II isoenzymes at nM and μM levels. We believe that our results would contribute the structure–activity relationships when determining new thiophene-based sulfonamide-derived CA inhibitors.

Materials and methods

Chemicals

Thiophene-based sulfonamide molecules: (5-(2-thienylthio) thiophene-2-sulfonamide (**1**), 5-chloro-3-methyl-1-benzothiophene-2-sulfonamide (**2**), 5-(2-aminoethyl)thiophene-2-sulfonamide (**3**), 4,5-dichlorothiophene-2-sulfonamide (**4**), 5-bromothiophene-2-sulfonamide (**5**), 5-(aminomethyl)

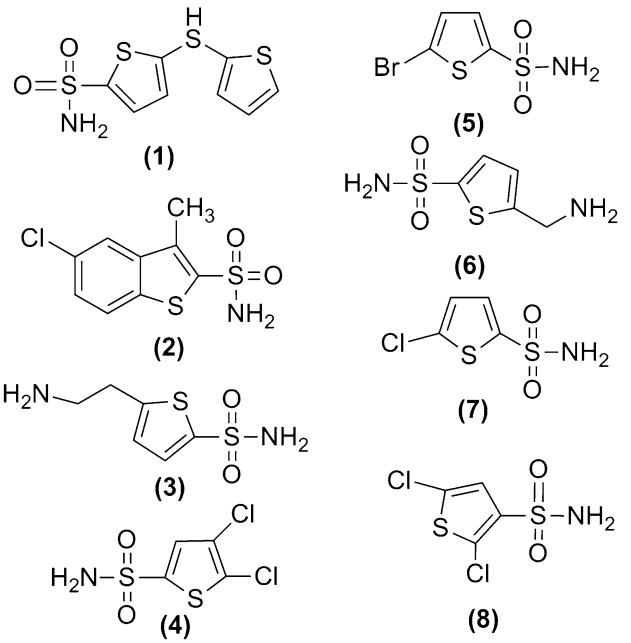

Fig. 1 The molecular structure of thiophene-based sulfonamides used in this study

thiophene-2-sulfonamide (**6**), 5-chlorothiophene-2-sulfonamide (**7**), 2,5-dichlorothiophene-3-sulfonamide (**8**)) and all chemicals used in purification processes and kinetic studies were purchased from Sigma-Aldrich Co. (Steinheim, Germany).

Purification process of CA I and CA II from human erythrocytes

Fresh human erythrocyte suspension was obtained from the University Hospital Blood Center of Ataturk University and all purification procedures of CA-I and CA-II isoenzymes from human erythrocytes were performed in the Biochemistry research laboratory of the Faculty of Science at Ataturk University. Preparation of hemolysate and purification process was performed according to our previous study [16]. 30 ml of the erythrocyte suspension obtained from the blood center were hemolyzed 1,5 volumes with distilled ice water. The resulting hemolysate was centrifuged at 20000 rpm and 4 °C for 30 min to remove ghost and intact cells. After centrifugation, the precipitate was discarded and the erythrocyte hemolysate (40 mL) in the upper phase was taken for use in purification processes. The pH of the erythrocyte hemolysate was adjusted to 8.7 with solid Tris before loading on the affinity column. CA-I and CA-II isoenzymes were purified from human erythrocytes using affinity chromatography method. CNBr-activated Sepharose-4B-L-tyrosine-sulfanilamide was used as a filler in the affinity column. The affinity column was equilibrated using 25 mM Tris–HCl/0.1 M

Na_2SO_4 (pH 8.7) buffer solution. Erythrocyte hemolysate was loaded onto the equilibrated column. After the passage of the hemolysate through the column was completed, the column was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7) buffer to remove unbound proteins. After the washing process was finished, the hCA-I and hCA-II isoenzymes were eluted with 1.0 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6), respectively [16, 17]. During the purification, the elution of CA-I and CA-II was monitored by measuring the absorbance at 280 nm. CA-I and CA-II activities in the eluates were measured by the hydratase activity measurement method determined by Wilbur-Anderson [18]. The active enzyme fractions were collected and dialyzed against 0.05 M Tris-SO_4 (pH 7.4) buffer for overnight. After dialysis, CA-I and CA-II isoenzymes were stored at -80 °C in small fractions for use in subsequent kinetic studies. The amount of protein in the hemolysate and the purified hCA-I and hCA-II enzyme solutions were determined using the Bradford method [19] (Bradford 1976). The purity control of the isoenzymes was performed by SDS-PAGE determined by Laemmli [20].

In vitro ınhibition studies

Biological activity studies were carried out in Kırşehir Ahi Evran University, Faculty of Arts and Sciences, Biochemistry research laboratory. The activity of CA isoenzymes in inhibition studies was measured using esterase activity described by Verpoorte et al. [21]. In this method, the CA enzyme hydrolyzes p-nitrophenyl acetate used as substrate to p-nitrophenol and p-nitrophenolate. The formation of p-nitrophenylate from p-nitrophenyl acetate was monitored at 25 °C for 3 min using the absorbance at 348 nm [18–21]. To determine the IC_{50} value of each thiophene-based sulfonamide, activity measurements were performed using at least five different concentrations of thiophene-based sulfonamides. In the absence of thiophene-based sulfonamide molecules, the control activity of the CA-I and CA-II isoenzymes is assumed to be 100%. Measurement results are plotted as Activity %/[Inhibitor Concentration] graphs. IC_{50} value is the concentration of inhibitor that halves the activity of the enzyme. The IC_{50} values were calculated for each molecule from Activity %/[Inhibitor Concentration] graphs equations. To determine the inhibition type of thiophene-based sulfonamide molecules, activity measurements were performed at three different inhibitor concentrations and five different substrate concentrations (0.15, 0.30, 0,45, 0,60, 0.75 mM). Lineweaver–Burk graphs (1/V-1/[S]) [21] were created using the current results. For hCA-I and hCA-II, K_i values and inhibition types of each molecule were derived from these graphs. In this study, the sulfonamide molecules, which examined the effect on hCA-I and hCA-II isoenzymes, showed a non-competitive inhibitory effect for these isoenzymes. In the case of non-competitive inhibition, the V_{max} of enzymes decreases, while the K_M value is not affected by the non-competitive inhibitor. Therefore, the maximum speed (V^Imax) in the presence of a non-competitive inhibitor was calculated using following equation:

$$V^I_{max} = \frac{V_{max}}{1 + \frac{[I]}{K_i}}.$$

Molecular modelling study

Protein target selection and processing

The X-ray crystal structure of hCA-I and hCA-II isoenzymes (PDB ID: 4WR7 and 5AML, respectively) was selected for molecular docking studies. The structures have low resolution and also have co-crystallized ligand which can be used in the study of docking validation. The structures were downloaded from RCSB Protein Data Bank and prepared using the protein preparation wizard module of Maestro, because the structures were not suitable for immediate use in molecular modelling calculations [22]. The protein preparation workflow which detailed described in previous studies was incrementally introduced as a summary. (I) Bond order and charges have been assigned and then missing hydrogen atoms have been added to crystal structure. (II) Missing side chains have been filled using prime module of the program. Amino acids have been ionized by setting physiological pH with the help of Propka software. (III) Water molecules that were formed less than three contacts with the protein or ligand have been removed. (IV) Energy minimization and geometry optimization have also been performed using the OPLS force field [23, 24].

Binding site prediction

Binding sites of prepared receptors have been predicted using SiteMap module of Mastro [25]. SiteScore and Dscore of the sites were calculated using the default parameter of top-ranked potential protein-binding site. The allosteric site and druggable site properties of predicted binding sites were determined by analyzing SiteScore and Dscore, respectively. The binding sites were also used at target selection and docking hits evaluation [26].

Ligands preparation

Suitable structures for molecular docking studies of the compounds were prepared using the LigPrep module of Maestro [27]. Their 2D structures were sketched and subsequently, 3D structures of the cephalosporin drugs were produced.

Briefly, their correct molecular geometries and protonation state at pH 7.0 ± 2.0 were prepared using the Epik module and OPLS-2005 force field of Maestro [28].

Molecular docking study

To identify binding affinity and possible inhibition mechanism of the most active compounds were carried out Glide docking and Induced-fit docking studies. Glide docking studies were performed to detect binding site where the most active compounds are best fitted using Glide module of Maestro [29]. Validation of the Glide docking method has been performed with a re-docking procedure by extracting co-crystallized ligand in the crystal structure of the receptor before the drugs docked into binding sites of the receptor. Then, a grid box was generated by selecting the predicted binding site using the Receptor Grid Generation module of Maestro. Following grid generation, the most active compounds were docked into all predicted binding site by setting Extra Precision (XP). After the docking process, XP scores were evaluated for detection of the best suitable binding site for the compounds [30, 31].

After detection of the best suitable binding site for the compounds, they were docked into the suitable binding site for each compounds using Induced-fit docking module of Maestro [32]. Validation of Induced-fit docking method was performed with the re-docking procedure by extracting co-crystallized ligand in the crystal structure of receptors, before the drugs docked into the suitable binding site. For this purpose, centroid of the residues was generated around the selected residues in the binding site of the receptors. After that, side chains were automatically trimmed based on B-factor, closest residues to the ligand were refined within 3.4 Å of ligand pose in prime refinement. Reliability of Induced-fit docking method was evaluated on the basis of RMSD value which calculated with Atom pair method in Superposition panel of Maestro, between co-crystallized ligand and re-docked ligand. The most active compounds were docked into their suitable binding site with the same procedures. After the docking process, the binding affinity of the compounds was determined by analyzing the Docking score and the Emodel score [33].

Results

In the present study, hCA-I and hCA-II were purified from human erythrocyte using CNBr-activated Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography method with a yield of 57.1% and 39.47%, 2774.44 and 3661.6 purification fold, a specific activity of 2543 EU/mg and 3409 EU/mg, respectively. The purification results are summarized in Table 1. The purity of the isoenzymes was checked by SDS-PAGE and the isoenzyme's purity is demonstrated in Fig. 2. Then, the in vitro inhibition impacts of **1–8** molecules on the purified isoenzymes were determined. The inhibitory effects of thiophene-based sulfonamides **1–8** were determined by IC_{50} and K_i values. IC_{50} values of thiophene-based sulfonamides **1–8** for hCA I were found as 69 nM, 125 nM, 25 µM, 153 nM, 240 nM, 70 µM, 188 nM, 374 nM, respectively. K_i constants of **1–8** were found as 66.49 ± 17.15 nM, 182.79 ± 32.48 nM, 221.47 ± 16.13 µM, 85.98 ± 7.89 nM, 151.75 ± 34.97 nM, 234.99 ± 15.44 µM, 553.80 ± 24.81 nM, 504.50 ± 91.37 nM for hCAI, respectively. IC_{50} values of thiophene-based sulfonamides **1–8** for hCA-II were found as 26 nM, 68 nM, 990 nM, 23.4 nM, 37 nM, 1.405 µM,

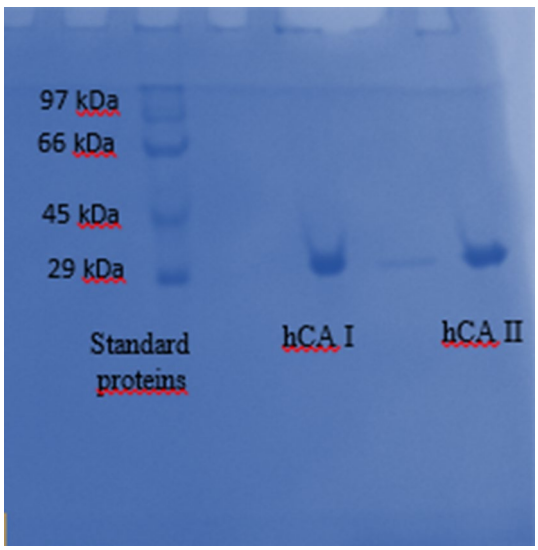

Fig. 2 SDS-PAGE photo showing the purity of isoenzymes

Table 1 Purification table of hCA-I and hCA-II isoenzymes isolated from human erythrocytes

Purification Steps	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification fold
Erythrocyte Hemolysate	380	40	408	16,320	15,200	0.931	100	1.00
hCA-I	620	14	0.240	3.36	8680	2583	57.1	2774.44
hCA-II	750	8	0,220	1.76	6000	3409	39.47	3661.6

44.8 nM, 176 nM, respectively. For hCA-II, K_i constants of **1–8** were determined as 159.92 ± 90.33 nM, 406.73 ± 34.95 nM, 1.408 ± 1.166 µM, 74.88 ± 20.65 nM, 233.01 ± 19.62 nM, 38.04 ± 12.97 µM, 182.28 ± 84.63 nM, 138.89 ± 38.29 nM, respectively. Thiophene-based sulfonamides studied in this research showed noncompetitive inhibitory properties on both isoenzymes. To clarify this inhibition mechanism, we conducted molecular docking studies for compound **1**, which had a strong inhibitory effect for hCA-I, and compound **4**, which had a strong inhibitory effect for hCA-II. And, we showed that molecular docking results confirmed the mechanism of the inhibition (Table 2).

Discussion

Thiophenes were highly stable heterocyclic compounds with aromatic properties. In medical chemistry, thiophene derivatives are very important for their therapeutic applications and they are found in the structure of a large number of compounds that exhibit pharmacological properties [34]. Sulfonamides are the most important group of carbonic anhydrase inhibitors [14–16]. In this study, the inhibition effects of some thiophene-based sulfonamide derivatives on hCA-I and hCA-II isoenzymes isolated from human erythrocytes were investigated due to both the pharmacological importance of the thiophene group and the strong effect of the sulfonamide group in CA inhibition. Inhibition results in our study differed in molecules by the position of the sulfonamide group in the benzene ring, the number and position of the thiophene group, the number and position of chlorine and bromine ions, and the number of carbon atoms in the aliphatic chain associated with the amino group.

In this study, we determined IC_{50} and K_i values for each molecule to evaluate the inhibitory effect of thiophene-based sulfonamide molecules (**1–8**) on pure hCA-I and hCA-II isoenzymes. IC_{50} is the concentration of inhibitor that halves enzyme activity. A low IC_{50} value indicates strong inhibition. K_i is constant indicating the binding affinity of the inhibitor to the enzyme. The small K_i value indicates that the inhibitor has a high affinity for the enzyme. In this study, IC_{50} values of **1–8** for hCA-I were found as 69 nM, 125 nM, 25 µM, 153 nM, 240 nM, 70 µM, 188 nM, 374 nM, respectively. According to these results, **1** (5-(2-thienylthio)thiophene-2-sulfonamide) had the strongest inhibitory effect for hCA-I (Fig. 3), while **6** (5-(aminomethyl)thiophene-2-sulfonamide) had the weakest inhibitory effect. K_i constants of **1–8** were found as 66.49 ± 17.15 nM, 182.79 ± 32.48 nM, 221.47 ± 16.13 µM, 85.98 ± 7.89 nM, 151.75 ± 34.97 nM, 234.99 ± 15.44 µM, 553.80 ± 24.81 nM, 504.50 ± 91.37 nM for hCA-I, respectively. According to K_i results, **1** had the highest affinity for hCA-I and **6** had the lowest affinity. IC_{50} values of thiophene-based sulfonamides **1–8** for hCA-II were found as 26 nM, 68 nM, 990 nM, 23.4 nM, 37 nM, 1.405 µM, 44.8 nM, 176 nM, respectively. According to these results, **4** (4,5-dichlorothiophene-2-sulfonamide) had the strongest inhibitory effect for hCA-II (Fig. 4), while **6** (5-(aminomethyl) thiophene-2-sulfonamide) had the weakest inhibitory effect. Furthermore, **1** is a more potent inhibitor for hCA-II than hCA-I. **1** contains two thiophene groups unlike other molecules and we thought that these groups cause strong inhibition. **3** and **6** containing aminoethyl thiophene and these molecules showed weak inhibition on both isoenzymes with respect to other molecules. Other molecules (**2, 4, 7, 8**) containing chlorine showed strong inhibition at nM concentrations on both isoenzymes. For hCA-II, K_i constants of **1–8** were determined as 159.92 ± 90.33 nM, 406.73 ± 34.95 nM, 1.408 ± 1.166 µM, 74.88 ± 20.65 nM, 233.01 ± 19.62 nM, 38.04 ± 12.97 µM, 182.28 ± 84.63 nM, 138.89 ± 38.29 nM, respectively. According to these values, **4** had the highest affinity for hCA-II and **6** had the lowest affinity. The reason why thiophene-based sulfonamides

Table 2 Results of in vitro inhibition studies

Compounds	hCA I			hCA II		
	IC_{50}	K_i	Inhibition type	IC_{50}	K_i	Inhibition type
1	69 nM	66.49 ± 17.15 nM	Noncompetitive	26 nM	159.92 ± 90.33 nM	Noncompetitive
2	125 nM	182.79 ± 32.48 nM	Noncompetitive	68 nM	406.73 ± 34.95 nM	Noncompetitive
3	25 µM	221.47 ± 16.13 µM	Noncompetitive	990 nM	1.408 ± 1.166 µM	Noncompetitive
4	153 nM	85.98 ± 7.89 nM	Noncompetitive	23.4 nM	74.88 ± 20.65 nM	Noncompetitive
5	240 nM	151.75 ± 34.97 nM	Noncompetitive	37 nM	233.01 ± 19.62 nM	Noncompetitive
6	70 µM	234.99 ± 15.44 µM	Noncompetitive	1.405 µM	38.04 ± 12.97 µM	Noncompetitive
7	188 nM	553.80 ± 24.81 nM	Noncompetitive	44.8 nM	182.28 ± 84.63 nM	Noncompetitive
8	374 nM	504.50 ± 91.37 nM	Noncompetitive	176 nM	138.89 ± 38.29 nM	Noncompetitive
AZA*	60.78 nM	67.70 ± 2.55 nM	–	55 nM	58.60 ± 2.56 nM	–

AZA acetazolamide, *hCA-I and II* human carbonic anhydrase isoenzymes I and II

*AZA used as a standard inhibitor for hCA-I and hCA-II isoenzymes. These values were obtained from in our previous study [17]

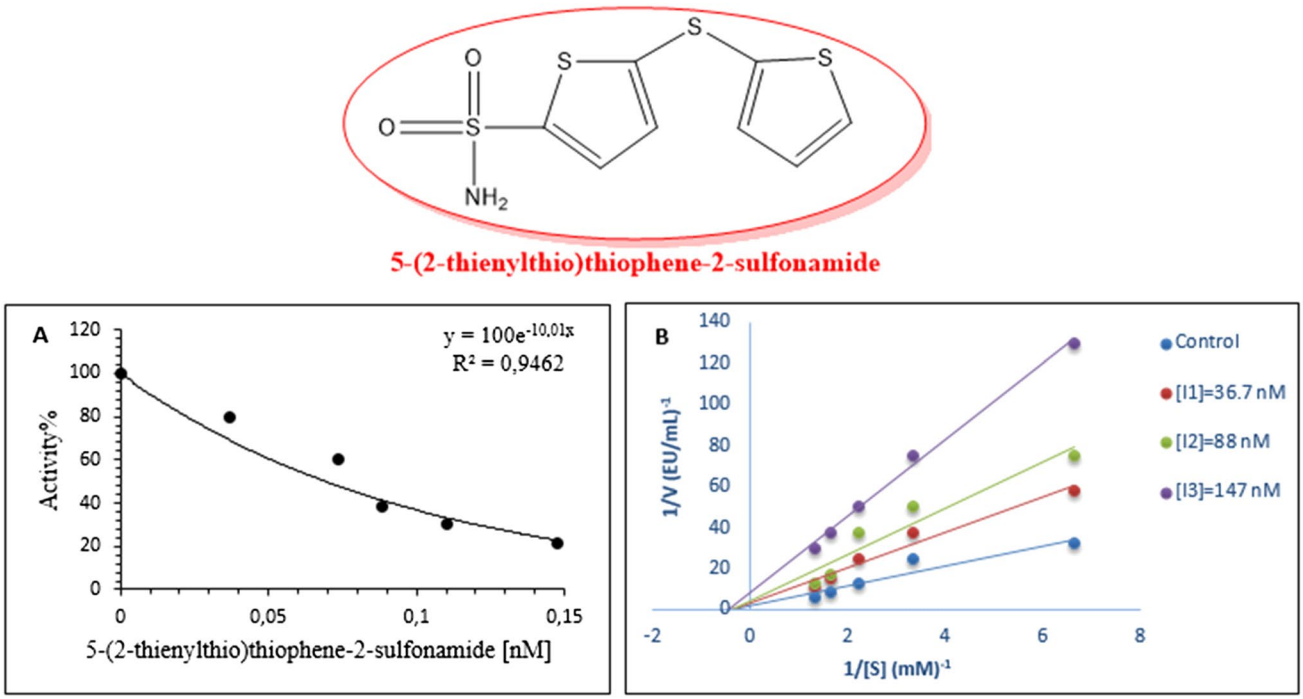

Fig. 3 IC_{50} graph **a** and Lineweaver–Burk graph **b** of 5-(2-thienylthio)thiophene-2-sulfonamide for hCA I

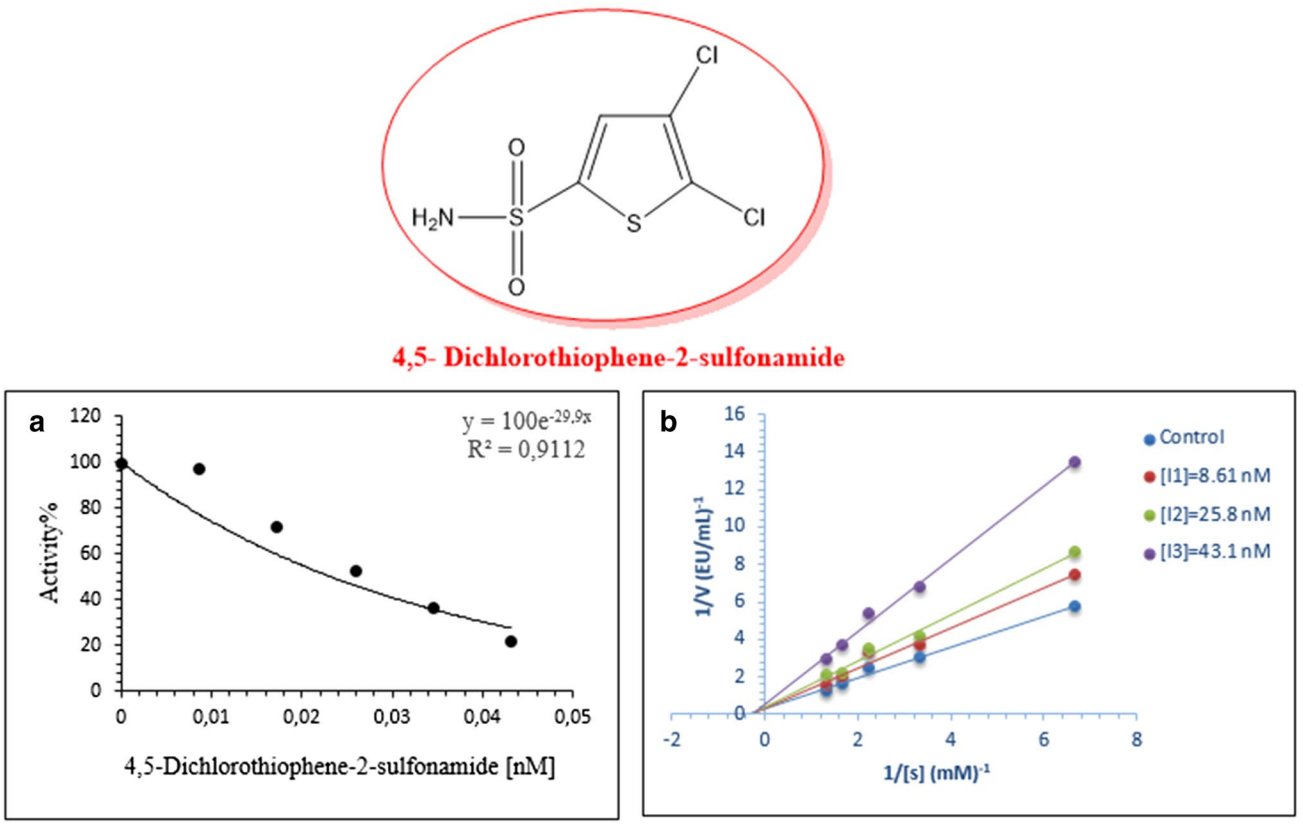

Fig. 4 IC_{50} graph **a** and Lineweaver–Burk graph **b** of 4,5-dichlorothiophene-2-sulfonamide for hCAII

Table 3 Physically and pharmaceutically properties of the compounds

Compound	[a]rtwFG	[b]MW	[c]DHB	[d]AHB	[e]logPo/w	[f]logHERG	[g]logBB	[h]CaCo	[i]PMDCK	[j] % Hum. Oral Abs.
1	0	279.405	3.600	4.500	1.510	−4.745	−0.683	429.863	829.433	82.916
2	0	261.741	2.000	4.500	1.501	−3.982	−0.384	575.997	1128.161	82.916
3	0	206.277	4.000	5.500	−0.902	−4.499	−1.033	26.169	16.109	47.037
4	0	232.099	2.000	4.500	0.450	−3.369	−0.300	427.713	1892.992	76.671
5	0	242.105	2.000	4.500	0.281	−3.464	−0.425	427.180	919.010	75.676
6	0	192.250	4.000	5.500	−1.212	−4.281	−0.893	27.329	17.616	45.559
7	0	197.654	2.000	4.500	0.208	−3.418	−0.432	427.778	850.861	75.256
8	0	232.099	2.000	4.500	0.480	−3.231	−0.196	523.675	2447.391	78.420

[a]Reactivity/toxicity (0–2)

[b]Molecular weight (< 500 Da)

[c]Number of hydrogen bond donors (< 5)

[d]Number of hydrogen bond acceptors (< 10)

[e]Octanol/water partition coefficient (recommended range: − 2.0 to 6.5)

[f]I C_{50} value for blockage of HERG K^+ channels (acceptable range: above − 5)

[g]Blood/Brain partition coefficient (recommended range: − 3.0 to 1.2)

[h]Cell permeability in nm/s (< 25 is poor and > 500 is great)

[i]Cell permeability in nm/sec (< 25 is poor and > 500 is great)

[j]Percentage of human oral absorption (< 25% is poor and > 80% is high)

used in our study have different rates of inhibition effect on hCA-I and hCA-II isoenzymes is that CA isoenzymes have different catalytic activities and inhibitory sensitivities. All the thiophene-based sulfonamides we used in the study showed noncompetitive inhibition on both isoenzyme activities. Accordingly, these molecules reduce the catalytic activity of isoenzymes to linkages outside the active site of the isoenzymes. To clarify this inhibition mechanism, we carried out molecular docking studies of most active compounds against hCA-I and hCA-II enzymes. According to the in vitro study, while compound **1** exhibited potent inhibitory effect to hCA-I enzyme, compound **4** exhibited potent inhibitory effect to hCA-II. Therefore, we have focused on compound **1** and compound **4** at the molecular

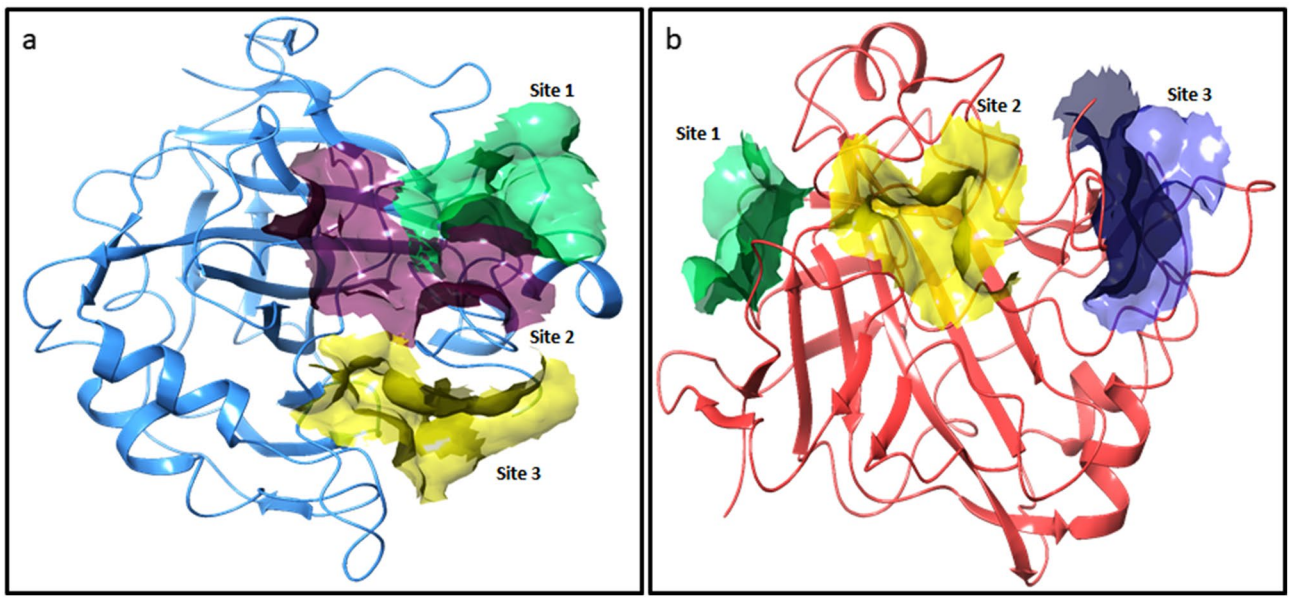

Fig. 5 In this figure have illustrated predicted allosteric sites. Allosteric sites were represented with colored surface. According to Dscore, Site3 is the best suitable binding site for hCA I and Site2 is the best suitable binding site for hCA II

Table 4 IFD Glide scores (kcal/mol) of the most active compounds in the catalytic sites of hCA-I, and hCA-II

Compounds	hCA-I		hCA-II	
	Docking score	Emodel	Docking score	Emodel
1	−6.892	−55.991	–	–
4	–	–	−6.112	−41.034
AZA*	−9.016	−88.005	−9.560	−99.052

*Acetazolamide (AZA) was used as a standard inhibitor for human erythrocytes carbonic anhydrase isoenzymes I, and II (hCA-I, and II)

docking study. As the beginning, we have calculated the physical and pharmaceutical properties of the compounds. These properties provide valuable information about drug-likeness properties of the compounds by comparing with physical and pharmaceutical properties those of 95% of known drugs. The physical and pharmaceutical properties of the compounds are presented in Table 3. Thiophene-based sulfonamides used in this study are non-toxic compounds due to the absence of reactive groups in their backbones. Human ether-a-go–go-related gene potassium (hERG K^+) channel plays a vital role in the regulation and maintenance

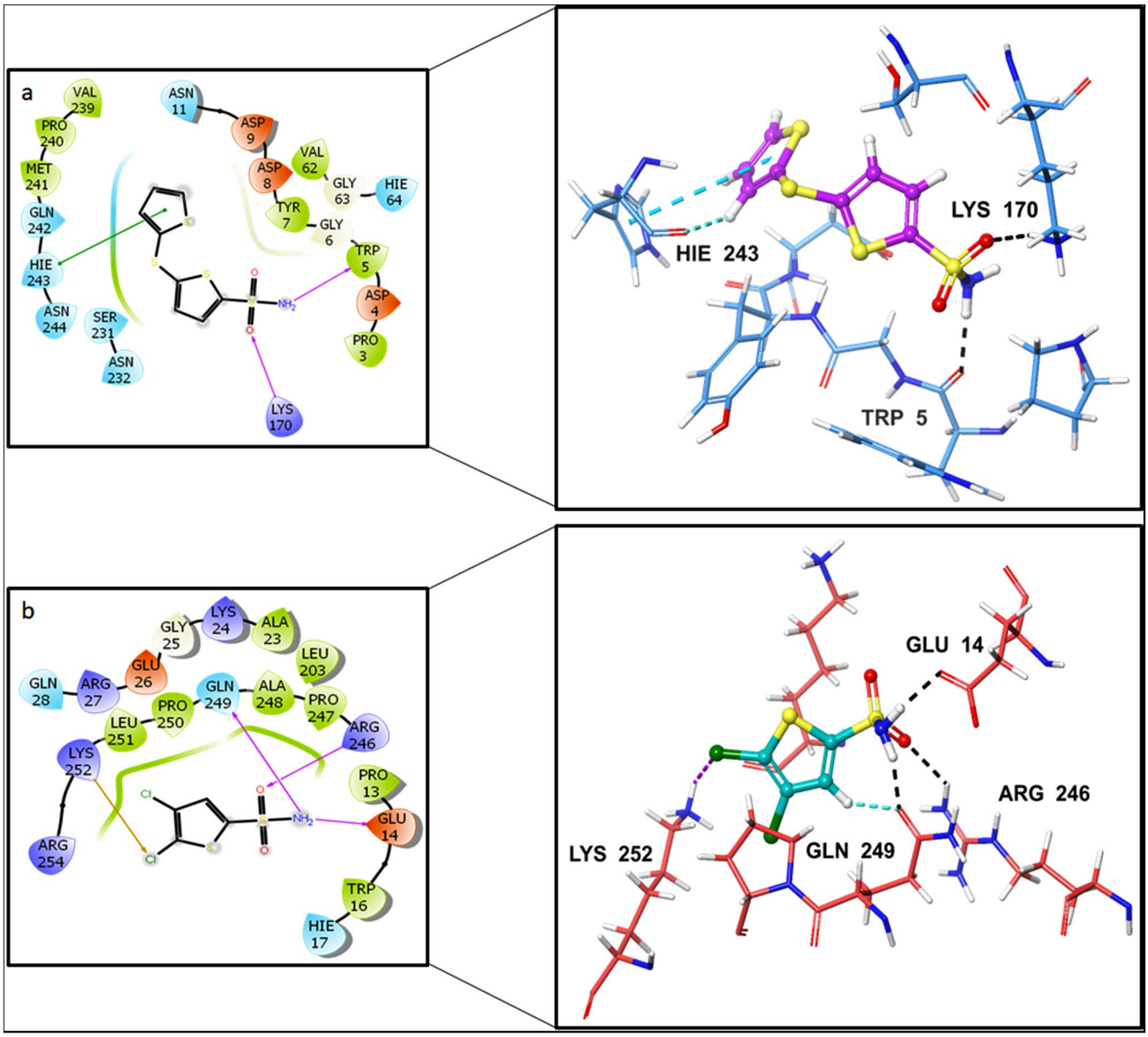

Fig. 6 2D and 3D detailed interaction mode of the most active compounds. **a** Compound **1**- hCA I and **b** Compound **4**—hCA II. Hydrogen bond was represented with purple arrow and black dashed-line. π-π stacking interaction was represented with green line and cyan dashed line. Halogen bond was represented with orange arrow and purple dashed line. Aromatic hydrogen bond was represented with turquoise dashed line

of normal cardiac rhythm and its blockage lead to cardiac failure [33]. The compounds had acceptable IC_{50} value for the blockage of hERG K^+. Blood/Brain partition coefficient and percentage of human oral absorption values of the compounds were within the recommended ranges. All compounds except compound **3** and **6** exhibited well cell permeability for CaCo and MDCK cell lines. The cell permeability results indicated that the compounds could be actively transported by intestinal cells. Besides the compounds satisfied Lipinski's rule of five [35] due to molecular weight, number of hydrogen bond donors and acceptors, and Octanol/water partition coefficient. In vitro inhibition studies shown that the compounds inhibited as non-competitively both hCA I and hCA II enzymes. Thus, we predicted potential allosteric binding sites of the enzymes. The predicted allosteric binding sites are illustrated in Fig. 5. According to these results, hCA-I and hCA-II enzyme had three allosteric binding sites. The sites were used for detection of the best suitable binding site for the compounds **1** and **4**.

Compounds **1** and **4**, which were most active compounds were docked using Glide docking method into the predicted sites of hCA-I and hCA-II enzymes, respectively. Compound **1** docked into hCA-I enzyme did not give any docking pose. However, Compound **4** docked into hCA-II enzyme gave poses for Site2 and Site3. The poses had Glide Emodel scores with -27.623 and -29.330 kcal for Site2 and Site3, respectively. According to the score, we detected Site2 as best suitable binding site for compound **4**. It was not any best suitable binding site for Compound **1** with glide docking method. Therefore, we used the Induced-fit docking method for detection of the best suitable binding site for Compound **1**. The compound was docked into the predicted allosteric binding sites of hCA-I. After docking process, pose with highest Emodel scores in negative direction was chosen as best-pose. The best-poses of compound **1** had -47.595, -55.991, and -48.440 kcal of Emodel score for Site1, Site2, and Site3, respectively. According to the scores, we detected Site2 as best suitable binding site for the compound. The compound **4** was docked into the Site2 of hCA-II which its best suitable binding sites and pose with highest Glide Emodel scores in negative direction was chosen as best-pose. Docking and Emodel scores of the compounds best-posed are presented in Table 4. These two most active compounds exhibited slightly weak binding affinity compared to standard inhibitor of hCAs due to its Docking and Emodel scores. However, the scores have indicated that they can act a good inhibitory effect on hCAs. Because their scores were compatible with scores of reported hCAs inhibitors [37–39]. Best-pose of the compounds analyzed on the basis of interaction modes for detection their possible inhibition mechanism. Their 2D and detailed interaction mode are presented in Fig. 6 . Compound **1** constructed hydrogen bonds with Trp5 and Lys170 residues through the sulfonamide moiety and – stacking interaction with Hie243 residue (Histidine with hydrogen on the epsilon nitrogen) through the thiophen-2-ylthio. The compound also formed an aromatic hydrogen bond with Hie243 residue as seen in detailed binding mode (Fig. 6a). The compound was surrounded by mostly hydrophilic residues, however, the thiophen ring bounded sulfonamide slightly interacted hydrophobic residues as seen in Fig. 7a. Compound **4** constructed hydrogen bonds with Glu14, Arg246, and Gln249 residues through the sulfonamide moiety and halogen bond with Lys252 residue (Fig. 6b). Moreover, the

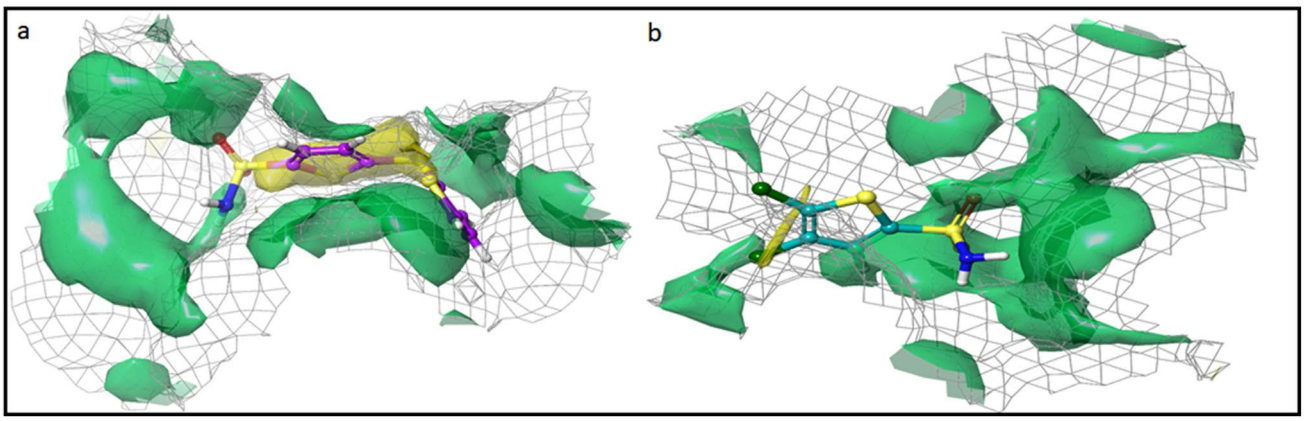

Fig. 7 Binding site analyses. **a** Compound **1**-hCA I and **b** Compound **4**-hCA II. The binding site is represented as grey mesh, the hydrophilic site is represented as green surface, and hydrophobic site is represented as yellow surface

compound formed aromatic hydrogen bonds with Gln249 residue as seen in detailed binding mode.

Zn ion within a hydrophobic pocket of the active site is responsible from catalytic activity of carbonic anhydrases, because the ion constructs coordination with a bicarbonate ion. The metal ion is within the active site including His64 residues for most CAs and the majority of their inhibitors bind to the enzymes active site residues or coordinate with Zn ion [36]. However, some inhibitors exhibit different inhibition mechanism, unlike known inhibitors. D'Ambrosio et al. [40] have expressed that 2- benzylsulfonyl benzoic acid forms have inhibited by binding completely different mode from any other CAs inhibitors. Our molecular docking results were quite parallel with their findings, because they have revealed that the compound 3 constructed strong van der Waals contacts with residues Gly6, Tyr7, Gly8, Phe231 and Glu239, while the benzysulfonyl moiety interacted with Phe231 and Asn232. As seen in Fig. 2, compound **1** has interacted similar binding pocked residues with the proposed site. Supuran [41] has referred to this binding site as out of the active site binding. The inhibition mechanism discovered by D'Ambrosio et al. [40] and after the discovery same inhibition mechanism never observed. In this study, we have confirmed this mechanism for the first time.

Consequently, we determined that some thiophene-based sulfonamides had a very strong inhibitory effect on hCA-I and hCA-II isoenzymes. In addition, interactions of the most active compounds (1 and 4) provided detailed information about their possible inhibition mechanism. Moreover, our molecular docking results confirmed the new inhibition mechanism at out of the active site. Therefore, we hope that this study will contribute to new CA inhibitor design studies.

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Author contributions Z. Alim carried out purification and inhibition studies of hCA-I and hCA-II isoenzymes. Analysis of the results was done by Z. Alim and Z. Koksal. Molecular modeling studies were carried out by M. Karaman. The writing and language correction of the article were done by Z. Alim, Z. Koksal and M. Karaman.

Compliance with ethical standards

Conflict of interest The authors declare that no conflicts of interest exist.

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