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Celecoxib Derivatives Containing Pyrazole Linked-Sulfonamide Moiety: Carbonic Anhydrase I–II and Acetylcholinesterase Inhibition Profiles, Molecular Docking Studies

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Heterocyclic pyrazole compounds have cytotoxic, anticancer, antimicrobial, anti-inflammatory properties, as well as their derivatives containing sulfonamide moiety, show superior effects on inhibiting various enzymes. Pre-synthesized celecoxib-derived compounds were studied for their inhibitory effects on human carbonic anhydrase (hCA I and hCA II) isoforms and acetylcholinesterase (AChE). The compound containing 2,3 dimethoxyphenyl functional groups from the celecoxib derivative containing this sulfonamide moiety showed a strong inhibitory effect with K_i values at 21.70 ± 2.50 nM, 4.70 ± 2.20 nM, and 4.58 ± 0.80 nM for hCA I, hCA II, and AChE, respectively. In addition, these compounds were evaluated against acetazolamide (AZA) and tacrine (TAC), which are used as standard inhibitors for the studied enzymes. The compound obtained as a result of the reaction of pyrazole compounds with propionic anhydride and showing the best inhibition effect had higher inhibitory activity than the standard inhibitors we used. In addition, molecular docking analyses to the strongest inhibitor were performed to identify possible binding mechanisms with the active sites of all three enzymes. Based on both in vitro and molecular docking analysis results, this compound was determined as a potential inhibitor of AChE, hCA I, and hCA II isoenzymes.

Introduction

Pyrazoles, which are structural parts in drug discovery studies, are aromatic heterocyclic compounds and contain N–N bonds in a five-membered ring. Due to the pyrrole and pyridine-like structures of nitrogen atoms bonded to each other, they react with both acids and bases.[1] This scaffold, represented by the molecular formula $C_3H_4N_2$, is very important pharmacologically, with almost all kinds of biological activities such as angiotensin-converting enzyme (ACE)[2] and aldose reductase (AR)[3] inhibitors, anti-microbial,[4] anti-inflammatory,[5] anticancer, and antiviral.[6] Carbonic anhydrase (CA) inhibitory properties of benzenesulfonamide structures of these compounds, which are called alkaloids due to their important pharmacological effects on humans, have been discovered recently.[7]

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Carbonic anhydrase inhibition is one of the most frequently targeted enzyme classes, with compounds acting as inhibitors for new drug discovery. CA (EC 4.2.1.1) is a metalloenzyme from the lyase group that is common in prokaryotic and eukaryotic organisms. This enzyme, which is zinc-dependent, plays a role in the conversion of water and carbon dioxide to protons (H^+) and bicarbonates (HCO_3^-).[8] They are essential for almost all life forms as they play a role in physiological and pathological processes such as electrolyte secretion, tumor formation, and pH balance.[9] Of these enzymes encoded by four different gene families, eight different CA have been classified to date, and of these isoforms, only α-CAs with 16 isoenzymes (hCA I–hCA XVI) exist in humans.[10] Among these isoenzymes, only twelve are known to be catalytically active. They differ in their subcellular localization and have various kinetic and inhibitory properties.

According to their cell distribution, these isoforms are grouped membrane-bound (CA IV, CA IX, CA XII, CA XIV, and CA XV), cytosolic (CA I, CA II, CA III, CA VII, and CA XIII), mitochondrial (CA VA and VB), and secretory (CA VI).[11] Of the cytosolic isoforms, hCA I and hCA II are widely expressed in erythrocytes, gastrointestinal tract, eyes, and kidney cells, and these isoforms are considered off-target for tumor-associated, transmembrane hCA IX and XII.[12] hCAs have become drug targets to treat conditions associated with pathologies such as glaucoma diuretics, Alzheimer's, hemolytic anemia, osteoporosis, epilepsy, and the importance of these isoenzyme inhibitors is emphasized in scientific studies.[13] In addition, sulfonamides are the most important and leading CA inhibitor[14] group besides many biological activities such as anticancer,[15] anti-inflammatory,[16] and antibacterial.[17] Although sulfonamides are potent CAIs, most of them lack isoform selectivity and do not

specifically goal the tumor-associated isoforms CA IX and CA XII. Today, there are CA inhibitors such as Acetazolamide (AZA), Zonisamide, Dorzolamide, and Diclofenamide which have undesirable side effects and the non-selectivity of these inhibitors necessitates the identification of new CA inhibitors.[11]

Acetylcholinesterase (AChE), a membrane-bound enzyme, is the most important enzyme associated with neural mechanisms found in the brain, cholinergic neurons, and striated muscles.[18] With the excessive secretion of AChE in the human body, acetylcholine is hydrolyzed to acetate and choline, reducing neurotransmitter levels and as a result, communication between nerve cells is cut off.[19] Although there is no definitive treatment for Alzheimer's disease, which causes problems such as cognitive dysfunction, memory loss, and speech insufficiency, which are the most common in the elderly, drug development studies on AChE inhibition related to the disease continue rapidly.[20] Currently, four AChE inhibitors (tacrine, donepezil, rivastigmine, and galantamine) with different clinically approved pharmacokinetic profiles, which often have side effects, have been licensed in the treatment of this disorder.[21]

In our research laboratory, celecoxib derivatives (**1**, **4**, **7**), compounds formed by the reaction of these pyrazoles with propionic anhydride (**2**, **5**, **8**), and sulfonamide salts (**3**, **6**, **9**) were synthesized, evaluated their aldose reductase inhibition effects, and already published.[3] In the present study, we investigated the inhibitory properties of these celecoxib derivatives containing pyrazole linked-sulfonamide moiety compounds we had presented before against hCAs and AChE.

Results and Discussion

Chemistry

The synthesis of Celecoxib derivatives compounds **1**–**9** is reported by Bayrak in the previous study. The experimental details, data, and spectral analysis of these compounds are presented in the study.[3] The synthesis of celecoxib derivatives containing a sulfonamide moiety is outlined in Figure 1. Using the same procedure in our previous publication, firstly, aromatic pyrazole compounds (**1**, **4**, and **7**) were reacted with propionic anhydride in the presence of NEt_3, to obtain **2**, **5**, and **8**.[22] Secondly, **3**, **6**, and **9**, derivatives of Valdecoxib and Parecoxib sodium compounds, containing both methyl and $N(Na)CO_2Et$ functionalities, were synthesized by the reaction of these compounds (2,5,8) with $NaHCO_3$ in methanol.

Enzyme inhibition results

α-CAs with esterase or thioesterase activity, commonly found in mammals, have important roles in many physiological and biochemical processes.[23] In the clinic, abnormal expression and/or activation of some isoenzymes of α-CAs are observed in some diseases such as cancer, congestive heart failure, and epilepsy. For this reason, specific inhibitors are used for the inhibition of hCAs in the treatment of certain diseases.[24]

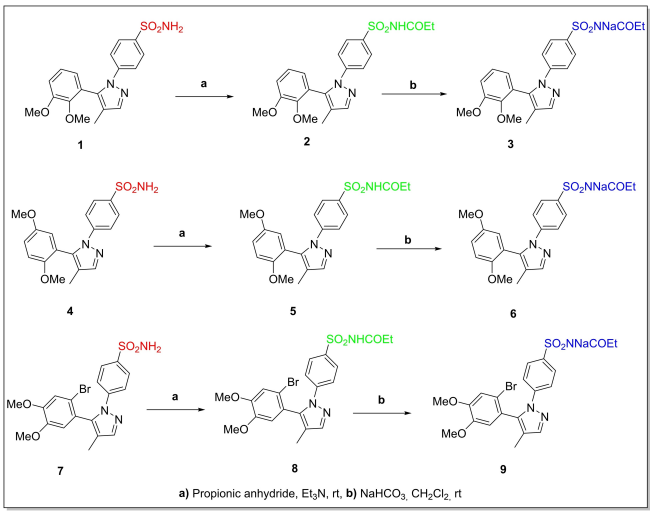

Figure 1. Synthesis of compounds (**1**–**9**).

Although the important biochemical change in the brain of AD patients is the decrease in ACh level, this decrease occurs by hydrolysis by AChE, the basic enzyme of nerve impulse transmission. In this sense, AChE inhibition is seen as the most effective way to treat this disease.[25] For all these reasons, this study aims to examine the in vitro effects of synthesized 9 different celecoxib derivatives containing pyrazole linked-sulfonamide moiety hCAs and AChE. The results are presented in Table 1.

The inhibitory effect of pyrazole sulfonamide derivatives on the ubiquitous cytosolic isoform hCA I, a marker for hemolytic anemia, was investigated.[26] It was determined that these compounds have IC_{50} values ranging from 8.34 nM to 2566.66 nM and K_i values ranging from 21.70 ± 2.50 nM to 6335.25 ± 1402.90 nM. Compound **2** with 2.3 dimethoxyphenyl functional group with IC_{50} value of 8.34 nM and K_i value of 21.7 ± 2.50 nM was determined to be the most effective compound on hCA I enzyme. In this compound, the shift of the methoxy group to the 5 position (compound **5**) caused a very serious decrease in activity (K_i: 968.71 ± 218.74 nM), while a 40-fold increase in activity (K_i: 23.24 ± 8.35 nM) was observed when its sulfonamide salt (compound **6**) was formed. Compounds **1**, **3**, **4**, and **5** showed a low inhibitory effect compared to AZA (K_i: 26.54 ± 3.11 nM), which is known to be an effective CA inhibitor. Shifting the 3-methoxy group in compound 1 to the 5-position to produce compound 4 results in an approximately 40-fold reduction in activity. K_i values of other **6**, **7**, **8**, and **9** aromatic pyrazole compounds were found as 23.24 ± 8.35 nM, 159.27 ± 61.69 nM, 324.95 ± 145.52 nM, and 46.01 ± 19.36 nM, respectively. Of these, compound **9**, a derivative of parecoxib sodium from bromo compounds containing 4,5-dimethoxyphenyl functional group, showed good inhibitory activity compared to the other two bromo compounds and showed only 1.7 times lower activity when compared to AZA. Compound **6** showed good inhibition activity relative to AZA. This compound, which is a derivative of valdecoxib and parecoxib sodium compounds, contains both methyl and $N(Na)CO_2Et$ functionalities. When the

Table 1. Inhibition data of human CA isoforms I, II, and AChE with the synthesized celecoxib derivatives containing pyrazole linked-sulfonamide moiety **1**–**9**, and AZA, TAC as standard inhibitors.

Compounds	hCA I IC_{50} (nM)	R^2	hCA II IC_{50} (nM)	R^2	AChE IC_{50} (nM)	R^2	hCA I K_i (nM)	hCA II K_i (nM)	AChE K_i (nM)
1	624.88	0.996	493.23	0.997	5.44	0.978	157.64 ± 14.72	671.50 ± 144.05	6.35 ± 2.47
2	8.34	0.981	5.90	0.980	4.87	0.969	21.70 ± 2.50	4.70 ± 2.20	4.58 ± 0.80
3	1540.00	0.965	11.14	0.983	4.91	0.987	3907.25 ± 1862.28	7.79 ± 2.14	7.96 ± 1.8
4	2566.66	0.992	33.59	0.969	623.20	0.980	6335.25 ± 1402.90	83.43 ± 18.60	1274 ± 537
5	786.21	0.995	12.55	0.986	5.64	0.959	968.71 ± 218.74	7.29 ± 1.82	9.80 ± 1.48
6	10.76	0.963	7.96	0.993	6.28	0.967	23.24 ± 8.35	17.12 ± 6.35	5.01 ± 0.60
7	84.40	0.980	103.83	0.973	26.05	0.969	159.27 ± 61.69	79.87 ± 31.19	7.30 ± 1.76
8	157.03	0.959	99.01	0.987	5.55	0.997	324.95 ± 145.52	94.20 ± 36.61	19.90 ± 5.30
9	16.25	0.967	13.95	0.994	20.90	0.987	46.01 ± 19.36	7.86 ± 1.92	5.37 ± 1.13
AZA[a]	29.17	0.986	24.59	0.979			26.54 ± 3.11	21.73 ± 2.42	
TAC[b]					28.68	0.981			23.12 ± 2.05

[a] AZA and
[b] TAC were used as standard inhibitors for hCA I, hCA II, and AChE

results were evaluated, the inhibitory property of the molecule was greatly reduced by the displacement of methoxy moiety attached to the phenyl group in compound **2** (compared to compound **5**), which has the highest inhibitory activity. This shows that small structural changes between pyrazole compounds lead to dramatic differences in their inhibitory activities.

The inhibitory effect of celecoxib-derived compounds on the CA II isozyme, which has recently been found to be associated with neurodegeneration and aging processes, which is generally associated with people suffering from diseases such as osteoporosis, glaucoma, and renal tubular acidosis, was investigated.[12] hCA II is effectively inhibited by many of the pyrazole compounds reported here. The effective inhibitors are compounds **2**, **3**, **5**, **6**, and **9** with K_i values in the range of 4.70 ± 2.20–17.12 ± 6.35 nM. **2** and **5** from these are compounds obtained by the reaction of pyrazole compounds with propionic acid, while **3**, **6**, and **9** are sulfonamide salts formed by the reaction of these compounds with $NaHCO_3$ in methanol. The shift of the methoxy group of compound **2** to the 5 position (compound **5**) indicates a 1.5-fold reduction in inhibitory activity. In addition, a 1.6-fold decrease in activity is observed in the formation of sodium salt of compound **2**. The introduction of a 2-bromine atom in compound **6** and the shift of the 2-methoxy group to the 4-position (compound **9**) led to an improvement in activity.

Among the aromatic pyrazole compounds (**1**, **4**, and **7**), compound **1** containing the 2,3- dimethoxyphenyl functional group has the lowest inhibition potential. The shift of the 2-methoxy group in compound **4** to the 4-positions to give compound **7** resulted in a very small increase in inhibition activity.

Compound **8** (K_i: 94.20 ± 36.61 nM) containing the 4,5-dimethoxyphenyl functional group exhibited a 4.3 fold lower inhibitory effect compared to the reference inhibitor, AZA (K_i: 21.73 ± 2.42 nM). The sulfonamide salt (compound **9**) formed by the reaction of this compound with $NaHCO_3$ in methanol showed 2.8 times better inhibition activity than AZA. Compound **2** (K_i: 4.70 ± 2.20 nM), which has the best inhibition potential among all compounds, appears to be more effective on the hCA II isoform than the hCA I (K_i: 21.70 ± 2.50) isoform.

Efforts to develop drugs for AChE inhibition have become the focus for the treatment of Alzheimer's disease. It has been shown that pyrazole derivative compounds have an inhibitory effect on the AChE enzyme.[27] The AChE was generally effectively inhibited by the celecoxib derivatives presented here. The best inhibitory effects were followed using compounds **1**, **2**, **3**, **5**, **6**, **7**, **8**, and **9**, which had inhibition constants ranging between 4.58 ± 0.80 nM and 19.90 ± 5.30 nM (better than the reference inhibitor TAC, which has a K_i of 23.12 ± 2.05 nM against AChE). Among them, compound **2**, which contains 2,3 dimethoxyphenyl functional groups, has the best inhibition effect (K_i: 4.58 ± 0.80 nM), as in the other two enzymes (hCA I, HCA II), as seen in Figure 2. Looking at the sodium salt form (compound **3**) of this compound, an approximate 1.8-fold decrease in activity was observed. Again, the shift of both methoxy groups in the same compound to the 4 and 5 positions (compound **8**) showed itself with a 4.5-fold decrease in activity.

The K_i values of the following compounds also ranged from 5.01 ± 0.60 nM to 19.90 ± 5.30 nM, and all of them showed a higher inhibition effect than the reference inhibitor. Small structural differences between the aromatic pyrazole compounds led to marked changes in their inhibitory activities. For example, a very large difference in inhibitory potential was observed for the two isomeric methoxyphenyl substituted compounds **1** and **4**. While the compounds in this group had the functional group 2,3-dimethoxyphenyl (compound **1**) and 4,5-dimethoxyphenyl (compound **7**) no great difference was observed in the inhibitory activity, but the activity decreased dramatically when the methoxy groups were shifted to the 2 and 5 positions (compound **4**). One of the synthesized parecoxib sodium derivatives, bromo compound with 4,5-dimethoxyphenyl functional group (compound **9**), showed similar activity with 2,5-dimethoxyphenyl (compound **6**) but when the methoxy groups were changed to 2 and 3 positions (compound **3**), the activity decreased 1.6 times. According to K_i values, compound **2** shows superior activity against standard drugs used against all three enzymes.

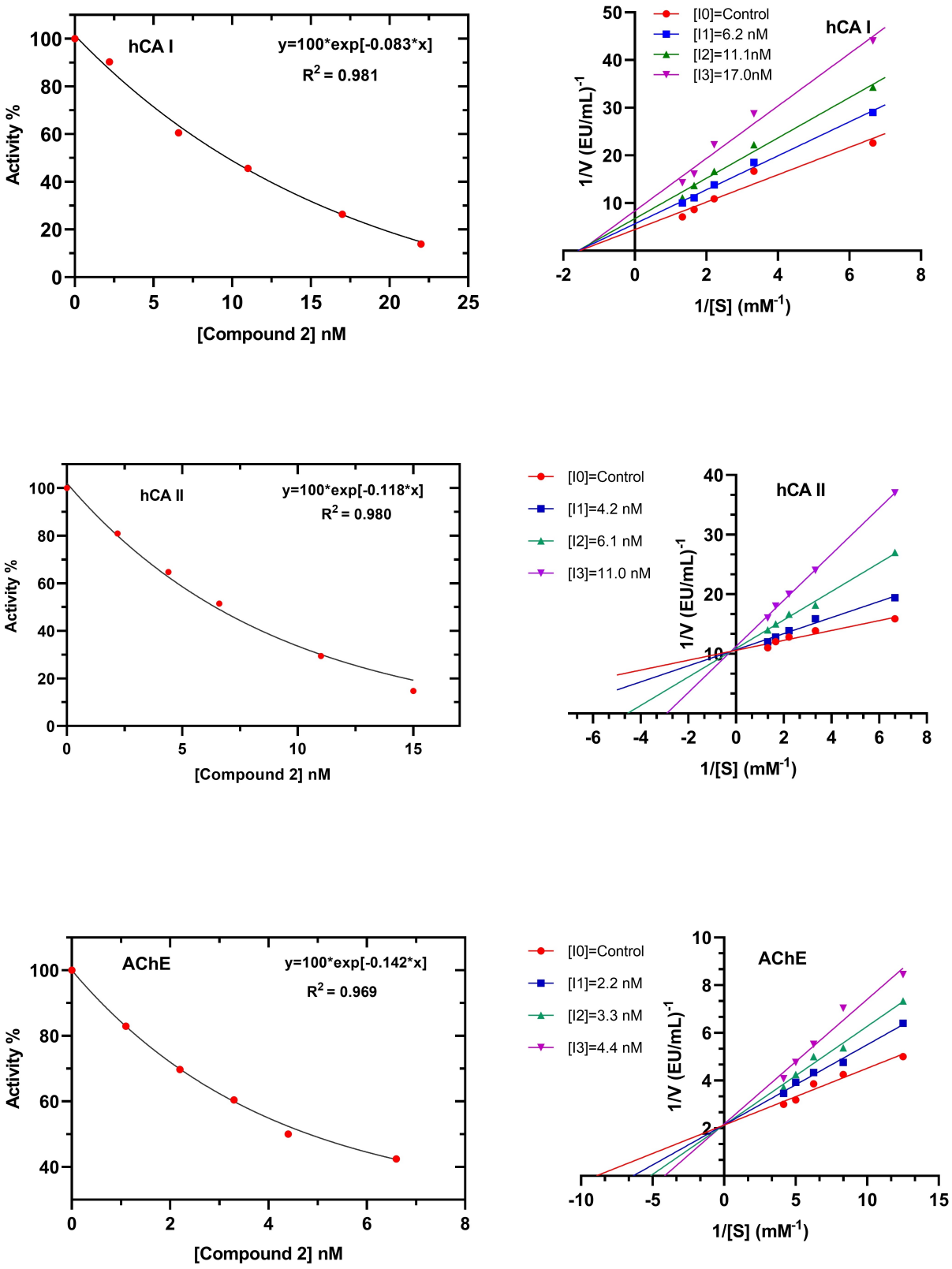

Figure 2. IC_{50} values and K_i constants of compound **2** with the most potent inhibitory effect for hCA I, hCA II, and AchE.

The selectivity index (SI) values for all compounds are summarized in Table 2. These values against hCA I, hCA II, and AChE for compound **2** were determined to be 1.22, 4.62, and 5.04 fold, respectively.

Table 2. Selectivity index values of the synthesized compounds (**1**–**9**).

Compounds	K_i (hCA II)/ K_i (hCA I)	K_i (AZA)/ K_i (hCA I)	K_i (AZA)/ K_i (hCA II)	K_i(TAC)/ K_i (AChE)
1	4.25	0.16	0.03	3.64
2	0.21	1.22	4.62	5.04
3	$1.99*10^{-3}$	$6.79*10^{-3}$	2.78	2.90
4	0.01	$4.18*10^{-3}$	0.26	0.01
5	$7.52*10^{-3}$	0.02	2.98	2.35
6	0.73	1.14	1.26	4.61
7	0.50	0.16	0.27	3.16
8	0.28	0.08	0.23	1.16
9	0.17	0.57	2.76	4.30

Molecular docking studies

In the present study, it has presented *in vitro* inhibition studies against hCA I, hCA II, and *ee*AChE enzymes of some celecoxib derivatives (**1**–**9**), which contain a pyrazole ring and a sulfonamine, a sulfonamide or a sodium sulfonamide subunits, previously synthesized by our group. Among these compounds, compound **2** showed better activity against all three enzymes than the standards and other compounds. Therefore, molecular docking studies were carried out to examine the interactions of celecoxib derivative **2** and standards with related enzymes. In docking studies, the ligands were flexible, and the enzymes were rigid. The docking processes for hCA I, hCA II, and *ee*AChE were validated, and RMSD values were detected to be less than 2 Å. The 2D interactions and best binding poses of **2** are visualized in Figures 3 and 4.

Docking into hCA I and hCA II

Compound **2** and acetazolamide which is a standard for metabolic hCA I and hCA II were docked with hCA I (PDB ID: 4WR7) and hCA II (PDB ID: 5AML) enzymes. For both enzymes, while the best binding energy values of **2** were calculated to be −7.5 and −7.4 kcal/mol, respectively, that of acetazolamide were −6.0 and −6.1 kcal/mol (Table 3). In the interactions with hCA I of **2**, it was observed that the pyrazole and sulfonamide structures play an important role in the interactions (Figure 3). According to this, the sulfonamide formed a C–H bond with His94 (2.77 Å) residue and it also formed two π-sulfur interactions with Phe91 (5.15 Å) and His94 (5.68 Å) residues.

The ethyl group of sulfonamide formed four π-alkyl interactions with His200 (4.11 Å), His119 (5.30 Å), His96 (5.06 Å), and His94 (4.67 Å) residues. While the pyrazole ring formed two π-alkyl interactions with Ala135 (5.28 Å) and Pro202 (4.19 Å), it formed an alkyl interaction with Pro202 (4.76 Å). Additionally, compound **2** formed two π-alkyl and an alkyl interaction with His67 (4.79 Å), Leu198 (4.29 Å), and Val62 (4.05 Å) residues, respectively. In the interactions with hCA II of **2**, It has been observed that the sulfonamide group and 2,3-dimethoxy phenyl ring attached to the pyrazole ring is more effective than the pyrazole ring in interactions. The sulfonamide group formed three conventional hydrogen bonds with Thr195 (2.66 Å), Thr196 (2.54 Å and 7.78 Å), Gln89 (2.30 Å), and also a C–H bond with His91 (2.67 Å). The 2,3-dimethoxy phenyl ring formed a conventional hydrogen bond with Asn64 (2.17 Å) a π-alkyl with İle88 (5.46 Å), a π-π T-shaped with Phe127 (5.36 Å), an alkyl Leu57 (5.09 Å), a C–H bond with Glu66 (3.55 Å). Additionally, the pyrazole ring formed a π-π T-shaped with Phe127 (4.70 Å).

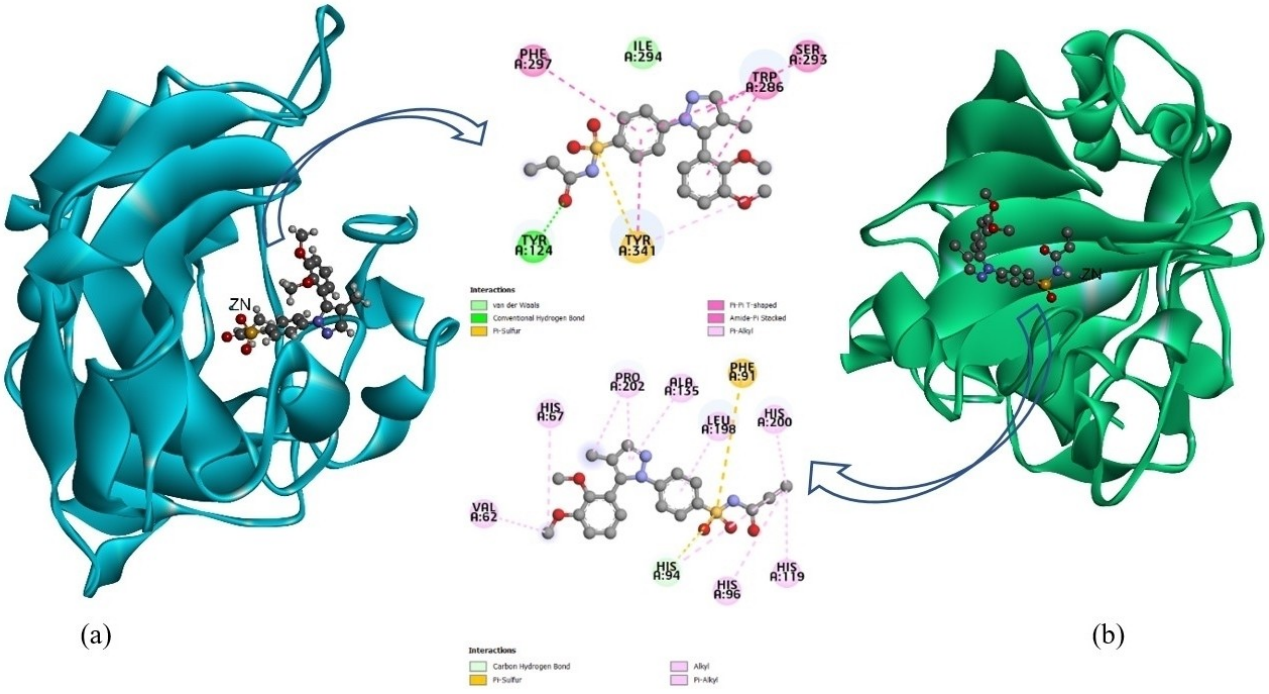

Figure 3. The best binding poses and 2D interactions of compound **2** with the active site of hCA I (a) and hCA II (b).

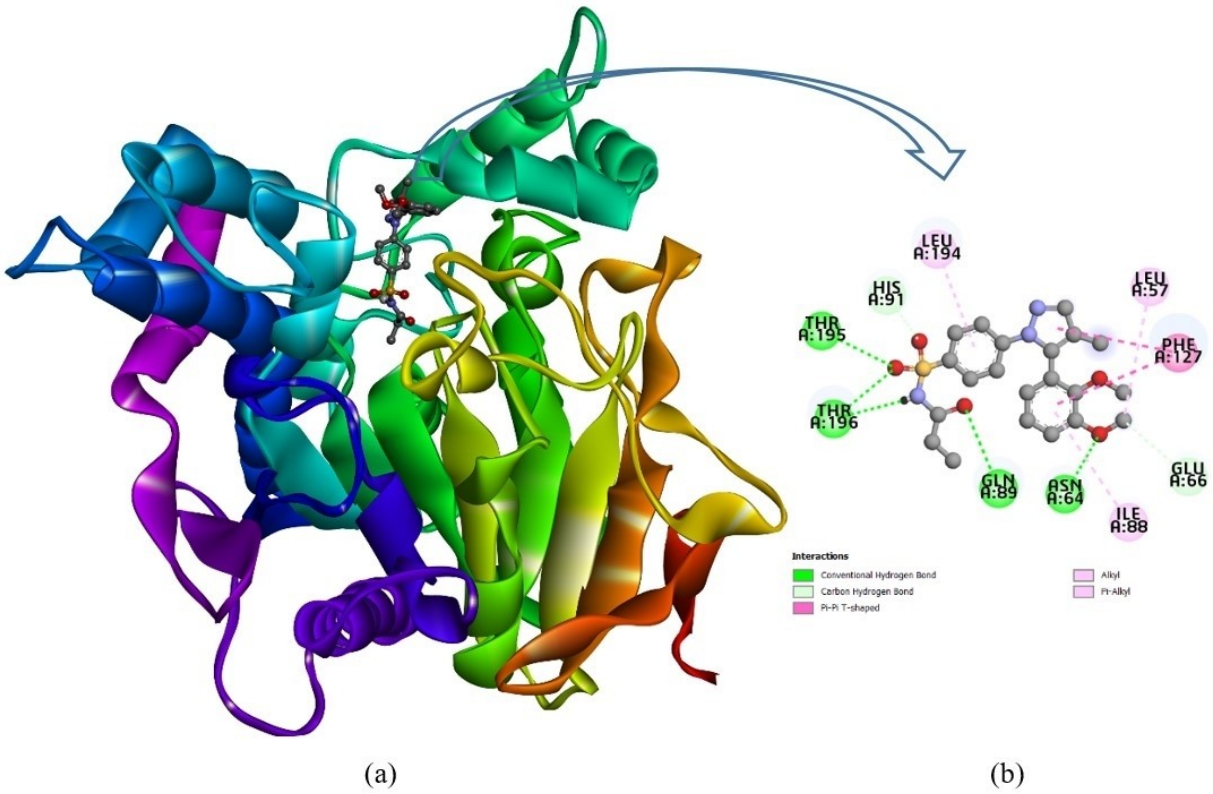

Figure 4. The best binding pose (a) and 2D interactions (b) of compound **2** with the active site of *ee*AChE.

Table 3. The best binding energies of compound **2**, acetazolamide, and tacrine with the active sites of hCA I, hCA II, *ee*AChE, and *h*AChE-CAS.

Compounds	hCA I	hCA II	*ee*AChE	*h*AChE-CAS
2	−7.5	−7.4	−9.2	−8.1
AZA	−6.0	−6.1		
TAC			−7.7	−9.0

Docking into *ee*AChE and *h*AChE

AChE enzyme purchased commercially and obtained from electric eel was used in *in vitro* studies. For this reason, the docking studies were performed on *ee*AChE (PDB ID: 1 C2B) using the blind docking method, which we previously mentioned in a study presented by our group.[28] Compound **2** and tacrine, a standard for AChE inhibition, were docked in the same conditions. The best binding energies are −9.2 and −7.7 kcal/mol, respectively (Table 3). These values are consistent with the results obtained from *in vitro* studies. *Homo sapiens* AChE was used to obtain a more accurate result in new drug discovery.[28–29] Therefore, we also examined their interactions with *h*AChE *(Homo sapiens* AChE).

AChE enzyme has structurally three different active parts. They are catalytic active site (CAS), mid-gorge, and peripheral anionic site (PAS). Tacrine bonds to the CAS region of *h*AChE. For this reason, a PDB file (PDB ID:5XN1) containing tacrine as a co-crystallized ligand was selected for the docking study. In the docking procedure applied to the CAS region of *h*AChE. The binding energies are founded to be −8.1 and −9.0 kcal/mol, respectively (Table 3). In this case, it can be thought that compound **2** will show better inhibition constant against *h*AChE. In the docking study with *ee*AChE of **2**, it was observed that **2** interacted with the PAS and mid-gorge regions. According to this, in the PAS regions, its sulfonamide group formed a conventional hydrogen bond Tyr124 (2.17 Å) and π-sulfur with Tyr314 (4.00 Å). The benzene ring attached to the sulfonamide group formed a π-π stacked with Tyr341 (5.22 Å), a π-π T-shaped with Trp286 (5.28 Å). The 2,3-dimethoxy phenyl ring attached to the pyrazole ring formed a π-π T-shaped with Trp286 (4.91 Å) and a π-alkyl with Tyr341 (5.30 Å). In the interactions with the mid-gorge, while the pyrazole ring formed an amide-π stacked with Ser293 (5.26 Å), the benzene ring attached to the sulfonamide group formed a T-shaped with Phe297 (5.93 Å) (Figure 4).

Conclusions

In the present study, some celecoxib derivatives synthesized, containing pyrazole linked-sulfonamide moiety, tested against the cytosolic CA isozymes hCA I and hCA II, and cholinesterase (AChE) enzyme. All compounds were screened for inhibitory potential in vitro using the Ellman and Verporte methods. Compound **2** with the strongest inhibitory effect, containing 2,3 dimethoxyphenyl groups, inhibited these enzymes even more than the reference drugs. Molecular docking studies of the

compound were performed on the 3D crystallographic structures of hCA I, hCA II and AChE and the best binding poses were determined. According to the ligand-receptor interactions of the compound, it showed good binding affinity for all three enzymes. The binding energy values of compound **2** were calculated as −7.5 kcal/mol for hCA I, −7.4 kcal/mol for hCA II and −9.2 kcal/mol for AChE. As a result, this compound stands out as a promising candidate for further studies.

Experimental Section

General information

Purified AChE from electric eel (*Electrophorus electricus*), dimethylsulfoxide (DMSO), silica gel 60 PF, all commercially available reagents, and other chemicals were obtained from (Sigma-Aldrich, St. Louis, MO), Acros Organics, (Merck, Darmstadt, Germany).

Synthesis of celecoxib derivatives containing pyrazole linked-sulfonamide moiety

Celecoxib-derived compounds (**1**–**9**) were synthesized according to the protocol described in our previous study[3] (Figure 1).

hCA isoenzyme inhibition studies

To investigate the inhibitory effects of celecoxib derivatives containing pyrazole-linked sulfonamide moiety on cytosolic hCA I and II isoenzymes (I and II) from human erythrocytes, as in previous studies, hCAs were isolated at one-step by Sepharose-4B-L-tyrosine sulfanilamide affinity chromatography method.[30] Quantitative protein determination in the eluates from the column was performed according to the Bradford method.[31] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied to control its purity hCAs. This method was carried out under denaturing conditions by loading the purified enzyme and protein markers in a system consisting of 3% stacking gel and 10% separation gel as explained by Laemmli.[32] The hCAs isoenzymes were dialyzed against pH 7.4 Tris-SO4 buffer for 24 hours and then stored at −80 °C for use in experimental studies. Then the activities of these isoenzymes Verpoorte et al. were carried out by esterase activity measurement method.[33] The conversion of *p*-nitrophenyl acetate, which was used as a substrate in inhibition studies, to *p*-nitrophenol was monitored by measuring absorbance at 348 nm and 25 °C. In order to determine the inhibition activities of each synthesized compound, at least five concentrations were determined and measured, and the percent activity values at these concentrations were calculated by assuming the control activity of the enzyme as 100%. Inhibitor concentrations were plotted against percent activity and IC_{50} values were determined from these plots. Acetazolamide (AZA) was used as the reference inhibitor.

Inhibiton Assay on AChE

The inhibitory effects of previously synthesized inhibitor molecules on AChE activity were measured using a modification of Ellman's spectrophotometric method[34] as previously described.[35] The basis of this method is to form 5-mercapto-2-nitrobenzoic acid by the reaction of thicholine and 5,5′-dithio-bis-2-nitro-benzoic acid (DTNB). While acetylthiocholine iodide (AChI) was used as the substrate, DTNB was used to determine the activity. The yellow color intensity of the compound obtained as a result of this reaction was measured spectrophotometrically at 412 nm. In the present work, purified AChE from an electric eel (*Electrophorus electricus*), AChI and DTNB were obtained from Sigma-Aldrich. IC_{50} values of synthesized compounds were determined by plotting % activity-inhibitory at least five concentrations [compounds **1**–**9**] graphs. K_i values were calculated using three different inhibitor concentrations and inhibition types were determined as in previous studies.[36] Lineweaver-Burk curves were plotted and calculations were performed. Tacrine (TAC) molecule was used as a reference inhibitor against this enzyme.

Molecular Docking Studies

The docking studies were carried out on the 3D crystallographic structures of hCA I, hCA II, *ee*AChE, and *h*AChE enzymes (PDB ID: 4WR7, 5AML, 1C2B, 7XN1, respectively), which were downloaded from the Protein Data Bank (www.rcsb.org).[37] The 2D structures of ligands were drawn on ChemDraw Professional 16.0 and they were optimized with Avogadro software.[38] The PDB files were created on the UCSF Chimera (1.17.1) program[39] deleting all co-crystallized ligands and adding polar hydrogens and gasteiger partial charges. The grid boxes were created with AutoDock Vina.[40] The ligands were flexible, and the enzymes were rigid. The grid box coordinates were arranged as X: 14.9725 Å, Y: 36.5028 Å, Z: 17.143 Å for 4WR7; X: −4.84674 Å, Y: 3.53719 Å, Z:14.6506 Å; X: 20 Å, Y:83 Å, Z: 19 Å for 1 C2B; X: 49.2202 Å, Y: −45.5023 Å, Z: −30.4724 Å for the CAS of 7XN1. The grid box sizes were set at 23×19×18 $Å^3$ for 4WR7; 20×20×20 $Å^3$ for 5AML; 54×66×75 $Å^3$ for 1 C2B, 20×20×20 $Å^3$ for the CAS of 7XN1. The docking results were visualized with Discovery Studio Visualizer 2021.[41]

Supporting Information Summary

The data that support the findings of this study are available in the supplementary material of this article. Additional references cited within the Supporting Information.[42,43]

Conflict of Interests

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.

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