Journal of Molecular Structure 1295 (2024) 136770

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstr

Design, synthesis and biological evaluation of novel ketone derivatives containing benzimidazole and 1,3,4-triazole as CA inhibitors

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ARTICLE INFO

Keywords:
Benzimidazole
1,3,4-Triazole
Carbonic anhydrase
Molecular docking
Cytotoxicity

ABSTRACT

In this study, we synthesized a series of new benzimidazole-triazole (**6a-6k**) derivatives and characterized them by ^{1}H NMR, ^{13}C NMR, and HRMS. These compounds were evaluated for their inhibitory activity against hCA-I and hCA-II. All the compounds exhibited good hCA-I and hCA-II inhibitory activities with IC_{50} values in the range of 1.158 μM to 3.48 μM. Among all these compounds, compound **6j,** with an IC_{50} value of 1.288 μM and 1.6197 μM, is the most active against hCA-I and hCA-II, respectively. Compounds **6a-6k** were also evaluated for their cytotoxic effects on the L929 mouse fibroblast (normal) cell line. Enzyme inhibition kinetics showed all compounds **6a-6k** to inhibit the enzyme by non-competitive. The most active compound **6j** was subjected to molecular docking, which revealed their binding interactions with the enzyme's active site, confirming the experimental findings.

1. Introduction

Carbonic anhydrases (CAs) catalyze the reversible hydration of carbon dioxide to bicarbonate anion and proton on the cell surface (Eq. (1)) [1]. These are a member of the metalloenzyme family because they have the zinc metal ion (Zn^{2+}) in their active site [2–4]. It was known that CA isoenzymes were found in high plants, algae, some bacteria, vertebrates, and mammals [5–7]. Currently, 16 CA isoforms have been discovered, and the α isoform is found in mammals, generally [8–10]. Of these, CA I and CA II are detected in the cytosol of erythrocytes, the gastrointestinal tract, brain, lung, kidneys, and etc. [11,12]. The reaction catalyzed by CAs is involved in many physiological and some pathological processes: pH and CO_2 homeostasis, transport of CO_2 and bicarbonate, secretion of electrolytes, gas exchange, gluconeogenesis, calcification, lipogenesis, ureagenesis, and tumorigenicity [13–22].

Heterocyclic chemistry is of great importance for discovering and developing of novel agents that have potential in the treatment of diseases that are the main cause of death. In medicinal chemistry, many compounds carrying heterocyclic rings are synthesized, and their biological activities are tested. Anyway, many drugs used in the clinics for years include heterocyclic rings [23]. One heterocyclic rings is triazole carrying two carbon and three nitrogen atoms [24]. Triazole can be easily synthesized and act as an amide, ester, carboxylic acid, and other heterocycles (e.g. pyrazole) [25]. The triazole moiety can influence the hydrogen bonding ability, polarity, and lipophilicity of the compounds; therefore it can cultivate their physicochemical properties, toxicology, pharmacokinetics, and pharmacology [26,27]. It is found in the literature compounds bearing triazole ring have cholinesterase inhibitor [28], anti-tubercular [29,30], anti-microbial [31], anti-mycobacterial [32], antitumoral [33], and carbonic anhydrase inhibitor [34] activities.

Since benzimidazole was first synthesized by Hoebrecker using 2-nitro-4-methylacetanilide as the starting material, [35] this heterocycle core and many derivatives have been obtained and evaluated their biological activities commonly. Different modifications created by various substitutions on the benzimidazole core have led to diverse biological activities [36,37]. The benzimidazole derivatives obtained as a result of these modifications have been found to have a wide variety of

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https://doi.org/10.1016/j.molstruc.2023.136770
Received 31 July 2023; Received in revised form 14 September 2023; Accepted 2 October 2023
Available online 3 October 2023

biological activities, for example cholinesterase inhibitor [38], antimycobacterial [39], antifungal [40] anti-inflammatory [41], antimicrobial [42], anticancer [43,44], anti-diabetic [45], antiviral [46], antihypertensive [47], aromatase inhibitor [48].

The molecular hybridization approach is considered to be a useful structural modification approach for the design of novel agents with the potential to exhibit biological activity [49]. In this study, in an effort to specify new CA inhibitory molecules, we report the design, synthesis, and biological evaluation of a novel series of benzimidazole-1,3,4-triazole derivatives that we designed through the hybridization of hCA I and hCA II inhibitory pharmacophores like benzimidazole, triazole rings.

2. Results and discussion

2.1. Chemistry

The benzimidazole-triazole derivatives (**6a-6k**) were synthesized by adopting our previous published work, [50] and the reaction strategy is shown in Scheme 1. In the first step, the methyl 4-formylbenzoate was dissolved in ethanol and sodium metabisulfite in distilled water was added drop by drop in the mixture. In the second step, by condensing 4,5-dimethylbenzene-1,2-diamine with the $Na_2S_2O_5$ adduct of methyl 4-formylbenzoate in DMF, compound **2** was obtained. In the third step, a hydrazide compound was obtained by reacting the ester structure of compound **2** with hydrazine hydrate. In order to synthesize the triazole ring from the hydrazide compound, firstly, the hydrazide compound (**3**) was reacted with methyl isocyanate, the obtained compound was reflux in ethanol with NaOH solution and the product was precipitated with HCl at the end of the reaction. In the last step, the final compounds were obtained by reacting the thiol group in the 2nd position of the triazole ring with various phenacyl bromide derivatives. Structures of synthesized compounds were proved by ^{1}H NMR, ^{13}C NMR and HRMS spectroscopic analysis methods.

2.2. In vitro hCA activity

The in vitro human carbonic anhydrase (hCA) inhibitory activity of compounds **6a-6k** were tested and commercial hCA inhibitory drug acetazolamide (AAZ) was chosen as a positive control. The IC_{50} values of the tested compounds are summarized in Table 1. As shown in Table 1, all the synthesized compounds displayed higher inhibitor activity than that of AAZ (IC_{50} = 2.26 μM), and it was found that their IC_{50} values were between 1.158 and 2.221 μM. Among them, compound **6j** displayed the most inhibitor potency against hCA I isoform with IC_{50} value of 1.158 μM. Furthermore, compounds **6c** and **6d,** having IC_{50} values of 1.397 and 1.347 μM, respectively, were other derivatives with significant inhibitory activity on hCA I isoenzyme.

The results revealed that compounds **6a-6k** did not show more inhibitory properties than AAZ (IC_{50} = 1.17 μM) on hCA II, and their IC_{50} values were between 1.6197 and 3.4769 μM. All the compounds tested exhibited noncompetitive inhibition against both isoforms of hCA.

It was important to note that this series of compounds displayed more inhibitory properties against hCA I than hCA II, except compound **6j**. Although this compound had fairly close IC_{50} values against hCA I (IC_{50} = 2.221 μM) and hCA II (IC_{50} = 2.106 μM), it showed slightly more inhibitory activity on hCA II isozyme.

This series of ketone compounds displayed K_i values varying from 0.812 to 1.788 μM on hCA I and their constants were more significant that of AAZ (K_i = 1.63 μM), except compounds **6f** and **6j** (Table 1). According to these data, a 4-cyano derivative **6c** had the most potent compound on hCA I isoform (K_i = 0.812 μM). Compounds **6d** and **6j** were the other derivatives with more inhibitor activity on hCA I than AAZ and their K_i constants calculated as 1.056 and 1.096 μM, respectively. However, these compounds exhibited no more inhibitory activity than AAZ (K_i = 0.812 μM), the K_i constants of them were changed from 1.231 to 2.785 μM.

2.3. Molecular docking

Binding energies and molecular interaction profile of the compounds were compared with the Acetazolamide which is a standard compounds used for invitro activity and Famotidine which is a co-crystallized ligand with the protein structures of carbonic anhydrase I & II.

Binding affinity outcomes obtained from the docking asessments are as follows:

Molecular interaction profile of compound U2 with Carbonic Anhydrase I & II

Molecular interaction analysis of compound 6j with CAI and CAII:

Compound **6j** had a binding energy of −8 K.cal/mol and mostly had a non-H-bond interaction with the active site residues of hCA I. LEU198 and ALA132 had amide pi-sigma interactions with the benzimidazole and triazole heterocyclic rings of the compound. PRO201 had an amide-pi stacked interaction with one of the phenyl rings adjacent to the sulfanyl group. Dimethyl substituents on the benzimidazole ring had alkyl hydrophobic interactions with the ALA121, VAL143, and HIS94 residues, whereas the ALA135, LEU131, and PRO202 had pi-alkyl interactions with the phenyl rings localized between the heterocyclic compounds and on the extreme positions in this elongated structure of U2 and finally and it had van der waals interactions with the remaining active site amino acids and Zinc ion.

Compound **6j** had three H-bond interactions with CAII, ASN62 (3.42 A^o) through the -NH substituted on the benzimidazole ring, Sulfanyl had another H-bond interaction with GLN92 (5.36 A^o), and then the third hydrogen bond interaction involves one of the nitrogens from the triazole ring with THR200 (3.84 A^o). VAL135 had pi-sigma interaction with the extreme phenyl group, whereas the PHE131 and TRP5 had pi-pi T-shaped interactions and ALA65 and PRO202 had pi-alkyl interactions with aromatic rings. Remaining active site residues nearby, like TYR7, ASN67, GLY132, LEU204, LEU198, and VAL121 had van der waals interactions (Fig. 1).

The binding affinity of compound **6j** with both hCAI and hCAII is better when compared to the Acetazolamide and Famotidine (Fig. 2).

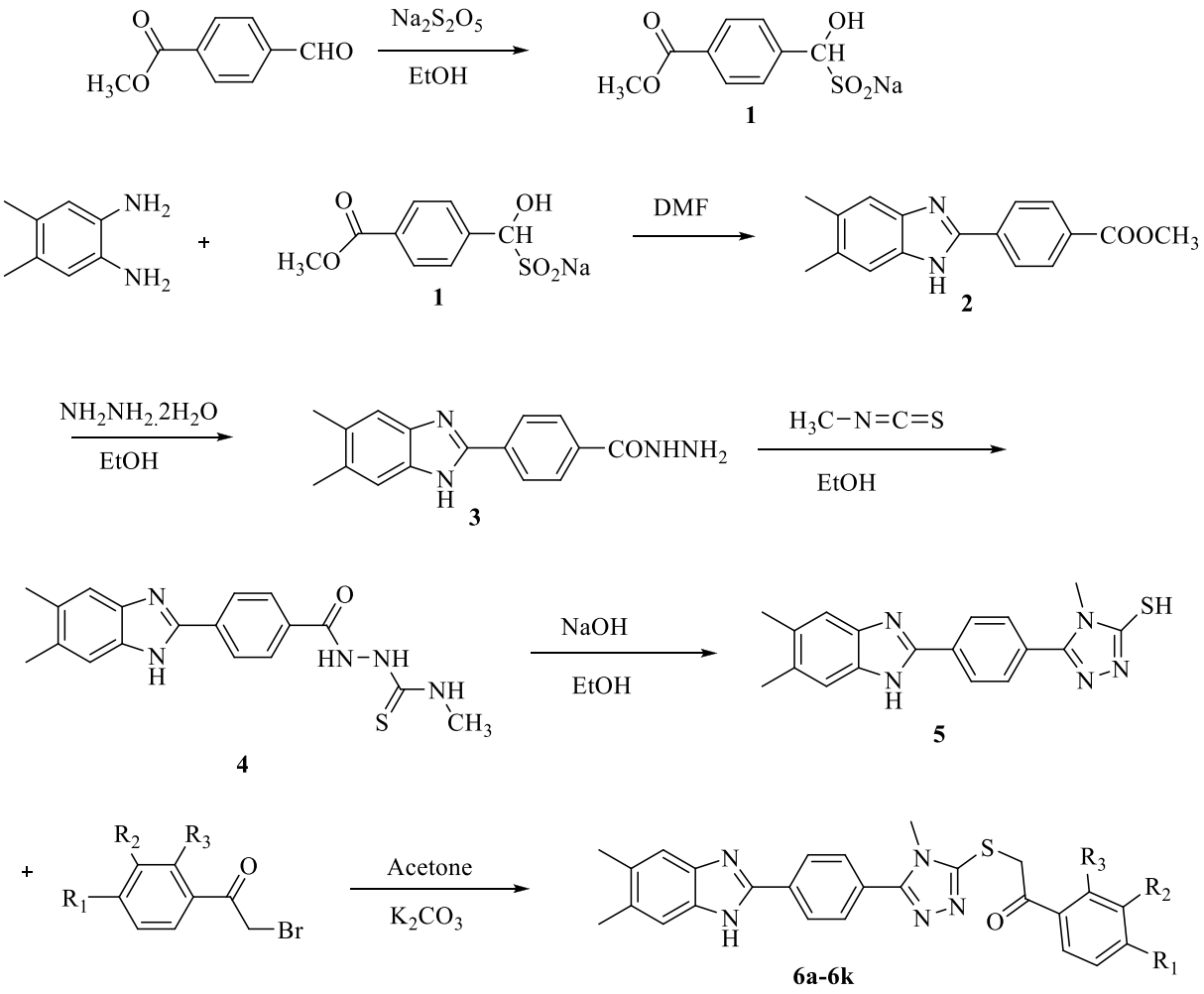
Overall the compound **6j** had a good binding energy and interaction profile towards hCAII and the distinct binding modes of compound **6j** in the human carbonic anhydrase I and II active site pocket can be viewed from the Fig. 3A (CA I), Fig. 3B (CA II).

Interactions of Acetazolamide and Famotidine with human CAI&CAII

Acetazolamide had two hydrogen bonds with THR199 (2.90 A^o) and GLN92 (5.21 A^o) residues of human CA I. The -C = O of the acetamide that was linked to the thiadiazole ring had a H-bond with GLN92 and -NH of the sulfamoyl had another H-bond with THR199. Pi-sulfur interaction with the sulfamoyl group was observed with HIS200 and the same residue is also involved in the pi-pi stacked interaction. There were also pi-alkyl interactions with LEU198, ALA121, metal-acceptor interactions with the Zinc ion and Van der waals interactions with HIS119, TRP209, HIS96, LEU131, HIS67, VAL143, and PHE91.

With human CA II, Acetazolamide had two H-bond interactions with THR200 (4.29 A^o) and THR199 (4.06, 4.19, 3.30 and 3.08 Ao). The acetamide moiety contributes one H-bond with THR 200 through the -C = O group and another H-bond with THR199 contributed by the sulfamoyl group and the same group is also involved in the pi-sulfur interaction with HIS94 and also a metal-acceptor interaction with zinc ion was observed along with the van der waals interactions with the remaining active site residues (Fig. 4).

In CAI, Famotidine displayed two hydrogen bond interactions with THR199 (3.93 and 3.39 A^o) and HIS200 (3.43 A^o). Both the sulfamoyl -*S* = *O* groups had H-bonds with THR199 and THR200. Sulfamoyl and Sulfanyl moieties had pi-sulfur interaction with HIS96 and PHE91



Comp.	R_1	R_2	R_3
6a	-Br	-H	-H
6b	-CH_3	-H	-H
6c	-CN	-H	-H
6d	-Cl	-Cl	-H
6e	-Cl	-H	-H
6f	-H	-H	-H
6g	-F	-H	-H
6h	-OCH_3	-H	-H
6i	-NO_2	-H	-H
6j	-Phenyl	-H	-H
6k	-Cl	-H	-Cl

Scheme 1. General procedure for synthesis of the final compounds **6a-6k.**

Table 1
The IC_{50} and K_i values (μM) of compounds **6a-6k** towards hCA I and II.

Comp.	hCA I Inhibition			hCA II Inhibition		
	IC_{50} (μM)	K_i (μM)	Type of inhibition	IC_{50} (μM)	K_i (μM)	Type of inhibition
6a	1.818	1.463±0.012	Noncompetitive	2.734	2.077±0.021	Noncompetitive
6b	1.779	1.381±0.015	Noncompetitive	2.445	1.846±0.032	Noncompetitive
6c	1.397	0.812±0.01	Noncompetitive	2.458	1.62±0.01	Noncompetitive
6d	1.347	1.056±0.013	Noncompetitive	2.504	1.64±0.02	Noncompetitive
6e	1.422	1.138±0.06	Noncompetitive	2.003	1.538±0.07	Noncompetitive
6f	2.178	1.706±0.03	Noncompetitive	3.4769	2.615±0.021	Noncompetitive
6g	1.555	1.281±0.0	Noncompetitive	1.752	1.392±0.0	Noncompetitive
6h	1.792	1.319±0.0	Noncompetitive	3.4096	2.785±0.0	Noncompetitive
6i	2.221	1.788±0.051	Noncompetitive	2.106	1.615±0.016	Noncompetitive
6j	**1.158**	1.096±0.013	Noncompetitive	**1.6197**	1.231±0.02	Noncompetitive
6k	1.865	1.463±0.011	Noncompetitive	2.002	1.538±0.014	Noncompetitive
Asetazolamide	2.26	1.63±0.011	Noncompetitive	1.17	0.812±0.01	Noncompetitive

residues, respectively. Pi-alkyl interactions were observed with LEU198 and ALA121 and metal-acceptor interactions with zinc ions and then van der waals interactions with remaining active site residues.

The sulfamoyl (*-S = O*) group of famotidine with CAII displays two hydrogen bonding interactions with THR199 (4.16, 4.11 A^o) and THR200 (3.21 A^o). GLN92 (6.27 A^o) had another hydrogen bond interaction via the sulfanyl group. There were also other interactions like pi-sulfur interactions with HIS96 and HIS94 residues through sulfamoyl and thiazole rings, respectively. HIS94 also had a pi-pi T-shaped interaction with the same thiazole ring with pi-sulfur interactions. There are pi-alkyl and alkyl interactions with ALA65, LEU198 residues, and metal-acceptor interactions with zinc ions and the majority of the other residues in the active site interact through the van der waals interactions (Fig. 5).

The binding modes of the compound **6j**, Acetazolamide, and Famotidine have represented in Fig. 6 and the compound **6j** with its elongated structure had a distinct binding mode where it is orienting as a folded conformation in CAI, whereas it has an extended conformation across the binding pocket in the CAII and this can be visualized from the Fig. 3A (CAI) and Fig. 3B (CAII).

The comparison of molecular interactions and binding poses of compound **6j** with the co-crystallized ligand (Famotidine) and standard drug Acetazolamide indicates that they had a comparable and similar H-bond interaction with critical active residues THR200, GLN92 and that might be a holding potential as a promising CA inhibitor and this hit compound could be subjected to futher investigations in the further studies (Table 2).

2.4. ADME properties

Compound **6j** was subjected to analysis using the SWISS ADME webserver (http://www.swissadme.ch/) [51] to determine its ADME properties and drug-likeness. The results indicated that the Lipinski drug likeness of the compound has slight deviation with molecular weight and Log P. The compound was predicted to have no BBB permeation and was not identified as a P-glycoprotein (P-gp) substrate. In terms of metabolism, the compound has the potential to inhibit the CYP2C19 and CYP3A4 isoforms. However, its pharmacokinetic properties revealed low gastrointestinal absorption, and its has poor solubility. These findings suggest that further optimization is required to enhance the compounds' pharmaceutical properties in future studies (Table 3).

2.5. Cytotoxicity assay

Cytotoxicity of compounds **6a-6k** was evaluated against L929 cell line. For preliminary screening, the cytotoxic bioactivity of synthesized compounds was evaluated in vitro against L929 cell line with the MTT assay. Cell viability percentages were calculated after the treatment of cells for 48 h. The preliminary cytotoxic effect results of compounds **6a-6k** against L929 fibroblast are presented in Fig. 7. The data obtained at the end of the study showed that almost all compounds **6a-6k** showed low cytotoxic effects. As a result of the maximum dose applied, all compounds except compounds **6f, 6i**, and **6j** showed 80 % and more viability. It is essential that compound **6j**, which is effective against both hCA I and hCAII enzymes, has a low cytotoxic effect on healthy cell lines.

3. Conclusion

Here, we synthesized new ketone compounds with benzimidazole and 1,3,4-triazole rings and investigated their ability to inhibit hCA I and hCA II isoforms towards the reference compound AAZ. Although these compounds haven't a sulfonamide group, an important pharmacophore for hCA inhibition, compounds **6c, 6d,** and **6j** compounds had remarkable inhibitory activity on the hCA I isoform. On the other hand, it was observed that the synthesized compounds hadn't more inhibitory activity than that of AAZ on hCA II isoform. These compounds (**6c, 6d,** and **6j**) may serve as model compounds to design new hCA I inhibitory agents for further studies. The effects of the compounds on the L929 mouse fibroblast (normal) cell line were studied to determine the site of cytotoxicity. Furthermore, molecular docking studies are used to predict how designed or synthesized compounds interact with the target protein/enzyme.

4. Material and method

Whole chemicals employed in the synthetic procedure were purchased from Sigma-Aldrich Chemicals (Sigma-Aldrich Corp., St. Louis, MO, USA) or Merck Chemicals (Merck KGaA, Darmstadt, Germany). Melting points of the obtained compounds were determined by the MP90 digital melting point apparatus (Mettler Toledo, OH, USA) and were uncorrected. ^1H NMR and ^{13}C NMR spectra of the synthesized compounds were registered by a Bruker 300 MHz and 75 MHz digital FT-NMR spectrometer (Bruker Bioscience, Billerica, MA, USA) in DMSO-d_6, respectively. Splitting patterns were designated as follows: s: singlet; d: doublet; t: triplet; m: multiplet in the NMR spectra. Coupling constants (J) were reported as Hertz. $M + 1$ peaks were determined by the Shimadzu LC/MSMS system (Shimadzu, Tokyo, Japan). All reactions were monitored by thin-layer chromatography (TLC) using Silica Gel 60 F254 TLC plates (Merck KGaA, Darmstadt, Germany).

4.1. Chemistry

Synthesis of sodium metabisulfite salt of benzaldehyde (**1**) derivative:

Methyl 4-formyl benzoate (5 g, 0.03 mol) was dissolved in ethanol. Sodium metabisulfite (6.84 g, 0.036 mol) in ethanol dropped dropwise into the benzaldehyde solution. After the dripping was completed, the reaction contents were stirred at room temperature for one hour. The

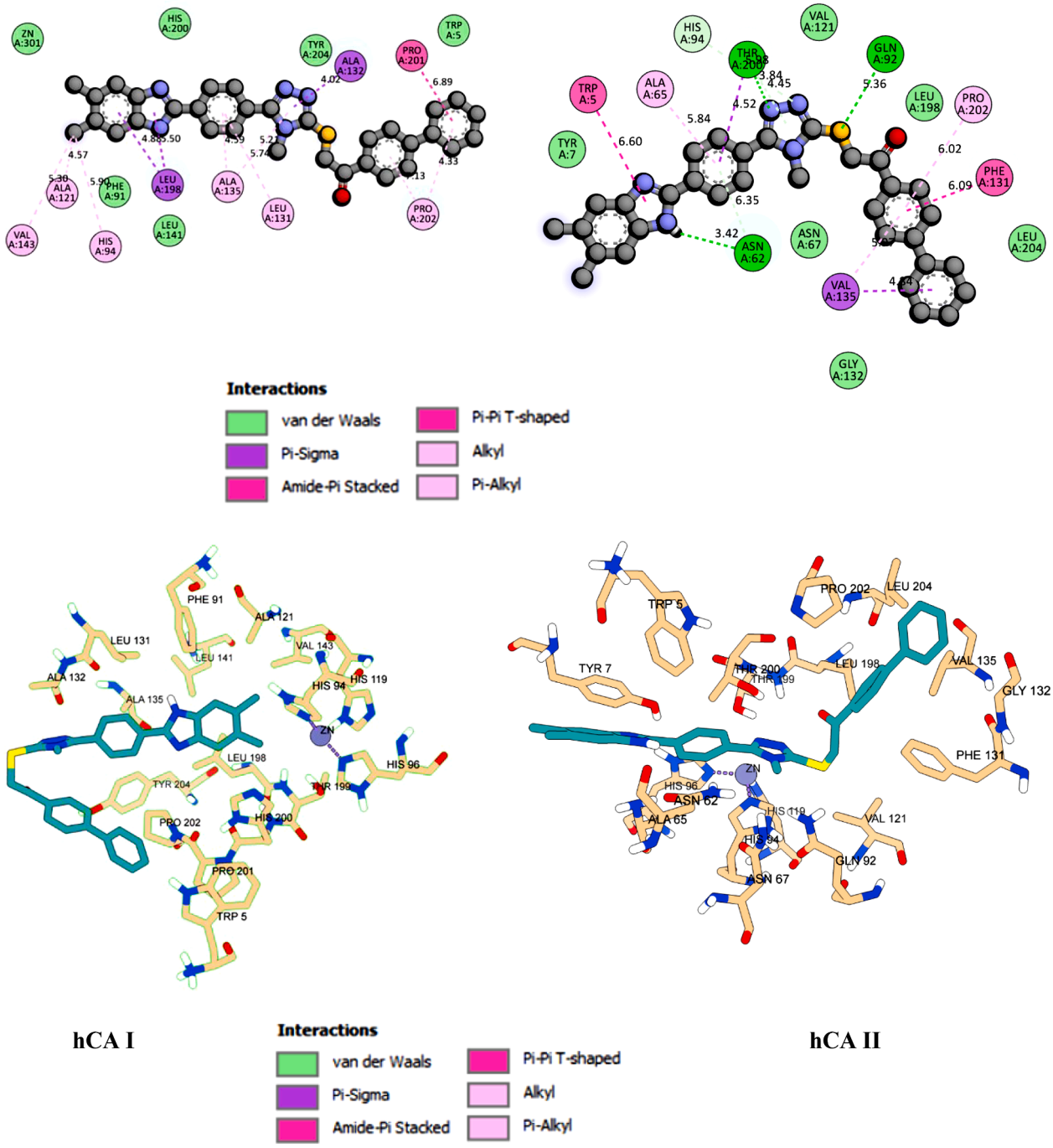


Fig. 1. 2D molecular interactions and binding site of compound **6j** with the critical amino acid residues of the Carbonic Anhydrase I & II proteins. Interactions were displayed as color coded dashed lines, green lines indicated the H–bonds.

precipitated product was filtered off.

Synthesis of 4-(5,6-dimethyl-1*H*-benzo[*d*]imidazol-2-yl)benzoic acid methyl ester (**2**):

4,5-dimethylbenzene 1,2-diamine (3 g, 0.022 mol) was dissolved in DMF and sodium metabisulfite salt of benzaldehyde derivative (7.09 g, 0.026 mol) was added. At the end of the reaction, the product was precipitated by pouring the reaction contents into ice water. The precipitated product was filtered off and crystallized from ethanol.

Synthesis of 2-(5,6-dimethylphenyl)−1*H*-benzo[*d*]imidazole-6-carbohydrazide derivatives (**3**):

Compound **2** (0.018 mol) and excess of hydrazine hydrate (5 mL) were placed in the same vial and ethanol (15 mL) was added. The mixture was reflux for 12 h. When the reaction was completed, the mixture was poured into iced water, and the product was filtered.

Synthesis of *N*-methyl-2-[4-(5,6-dimethyl-1*H*-benzo[*d*]imidazol-2-yl)benzoyl]hydrazine-1-carbo thioamide (**4**):

2-(5,6-Dimethylphenyl)−1*H*-benzo[*d*]imidazole-6-carbohydrazide derivatives (**3**) (2.8 g, 0.01 mol) and methyl isothiocyanate (0.012 mol) in ethanol was refluxed for 2 h. The precipitated product was filtered, washed with ethanol, and dried.

Synthesis of 4-methyl-5[4-(5,6-dimethyl-1*H*-benzo[*d*]imidazol)−2-yl)phenyl)−4H-1,2,4-triazole-3-thiol (**5**):

N-methyl-2-[4-(5,6-dimethyl-1*H*-benzo[*d*]imidazol-2-yl)benzoyl] hydrazine-1-carbothioamide (**4**) (0.001 mol) and NaOH (0.012 mol) in ethanol was refluxed for 2 h. After completion of the reaction, the solution was acidified with HCl 37 %; the precipitate was filtered, washed

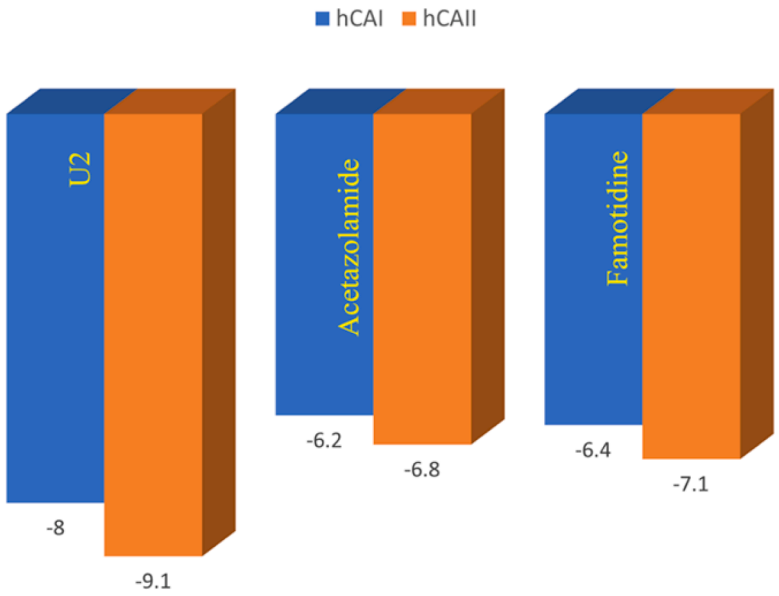


Fig. 2. Binding affinity plot of compound **6j,** Acetazolamide and Famotidine with human carbonic anhydrase I and II.

with water, dried, and then recrystallized from ethanol.

Synthesis of 2-(4-(4-methyl-5-(2-(substitutedphenyl)−2-oxo-ethyl-thio)−4H-1,2,4-triazol-3yl)-phenyl)−5,6-dimethyl-1*H*-benzo[*d*]imid-azole (**6a-6k**):

A solution of 4-methyl-5-[4-(5,6-dimethyl-1*H*-benzo[*d*]imidazol)−2-yl)phenyl)−4*H*-1,2,4-triazole-3-thiol (**5**) (0.001 mol) in acetone (10 ml), an appropriate substituted 2-bromoacetophenone derivative (0.001 mol) and potassium carbonate (0.138 g, 0.001 mol) were refluxed at 40 °C for 12 h. The solvent was evaporated, residue was washed with water, dried, and recrystallized from ethanol.

1-(4-Bromophenyl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo [*d*] imid-azole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio)ethan-1-one (6a):

Yield: 75 %. M.p. 267.3 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.34 (6H, s, -CH_3), 3.71 (3H, s, -CH_3), 4.92 (2H, s, CH2), 7.41 (2H, s, Benz-imidazole C—H), 7.79 (2H, d, *J* = 8.60 Hz, 1,4-disubstituted benzene C—H), 7.87 (2H, d, *J* = 8.44 Hz, 1,4-disubstituted benzene C—H), 7.97 (2H, d, *J* = 8.64 Hz, 1,4-disubstituted benzene C—H), 8.30 (2H, d, *J* = 8.48 Hz, 1,4-disubstituted benzene C—H). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.09, 32.12, 36.85, 114.65, 116.11, 118.75, 120.49, 122.58, 123.48, 124.94, 126.82, 130.64, 132.66, 134.19, 135.44, 137.94, 139.13, 148.03, 151.92, 154.91, 193.98. [*M* + *H*]$^{+}$ calcd for $C_{26}H_{22}N_5OSBr$: 532.0779; found: 532.0801.

1-(4-Methylphenyl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo [*d*] imid-azole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio)ethan-1-one (6b):

Yield: 72 %. M.p. 328.1 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.49 (9H, s, -CH_3), 3.81 (3H, s, -CH_3), 5.01 (2H, s, CH_2), 7.43 (1H, d, *J* = 7.92 Hz, Aromatic C—H), 7.71 (3H, s, Aromatic C—H), 8.00 (1H, d, *J* = 8.24 Hz, Aromatic C—H), 8.15 (2H, d, *J* = 8.32 Hz, Aromatic C—H), 8.45 (3H, d, *J* = 8.44 Hz, Aromatic C—H), 14.20 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.45, 21.69, 32.31, 38.06, 114.11, 115.49, 120.84, 122.16, 123.83, 125.36, 126.05, 127.24, 128.72, 129.05, 129.86, 129.94, 130.98, 136.44, 147.82, 150.25, 154.84, 194.82. [*M* + *H*]$^{+}$ calcd for $C_{27}H_{25}N_5OS$: 468.1855; found: 468.1853.

1-(4-Cyanophenyl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo [*d*] imid-azole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio)ethan-1-one (6c):

Yield: 76 %. M.p. 317.0 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.49 (6H, s, -CH_3), 3.82 (3H, s, CH_3), 5.07 (2H, s, -CH_2), 7.72 (2H, s, Aromatic C—H), 8.12–8.19 (4H, m, Aromatic C—H), 8.25–8.27 (1H, m, Aromatic C—H), 8.44 (3H, d, *J* = 8.56 Hz, Aromatic CH), 14.20 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.44, 31.77, 37.61, 114.23, 116.18, 119.03, 120.77, 121.88, 123.48, 125.57, 128.00, 128.42, 128.73, 129.55, 129.66, 133.33, 138.29, 138.78, 147.75, 151.36, 154.70, 194.61. [*M* + *H*]$^{+}$ calcd for $C_{27}H_{22}N_6OS$: 479.1666; found: 479.1649.

1-(3,4-Dichlorophenyl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo [*d*] imidazole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio) ethan-1-one (6d):

Yield: 71 %. M.p. 302.8 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.49 (6H, s, -CH_3), 4.03 (3H, s, -CH_3), 5.05 (2H, s, CH_2), 7.72–7.73 (2H, m, Aromatic C—H), 7.85–7.93 (1H, m, Aromatic C—H), 8.05–8.07 (1H, m, Aromatic C—H), 8.15–8.17 (1H, m, Aromatic CH), 8.25–8.32 (2H, m, Aromatic CH), 8.45–8.49 (1H, m, Aromatic CH), 8.53–8.55 (1H, m, Aromatic CH). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.45, 32.89, 34.04, 114.10, 115.35, 116.88, 119.66, 120.01, 124.32, 125.36, 125.71, 128.87, 130.40, 131.38, 131.77, 136.47, 136.61, 146.91, 150.39, 154.07, 195.93.

1-(4-Chlorophenyl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo [*d*] imid-azole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio)ethan-1-one (6e):

Yield: 77 %. M.p. 316.8 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.49 (6H, s, -CH_3), 3.82 (3H, s, CH_3), 5.04 (2H, s, -CH_2), 7.70–7.72 (4H, m, Aromatic C—H), 8.11–8.17 (3H, m, Aromatic C—H), 8.45–8.47 (3H, m, Aromatic C—H). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.45, 32.70, 38.01, 114.06, 115.35, 120.56, 121.40, 123.20, 128.78, 129.44, 129.69, 129.96, 130.18, 130.74, 130.86, 134.43, 136.53, 148.03, 151.01, 154.98, 197.32. [*M* + *H*]$^{+}$ calcd for $C_{26}H_{22}N_5OSCl$: 488.1324; found: 488.1306.

2-((5-(4-(5,6-dimethyl-1*H*-benzo [*d*] imidazole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio)−1-phenylethan-1-one (6f):

Yield: 70 %. M.p. 327.3 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.37 (6H, s, -CH_3), 3.62 (3H, s, CH_3), 4.97 (2H, s, -CH_2), 7.52–7.53 (3H, m, Aromatic C—H), 7.57–7.59 (1H, m, Aromatic C—H), 7.98–8.01 (3H, m, Aromatic C—H), 8.03–8.06 (1H, m, Aromatic CH), 8.36–8.38 (3H, m, Aromatic CH), 14.07 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.48, 32.29, 32.57, 114.73, 114.89, 119.36, 120.73, 127.89, 128.02, 128.70, 128.92, 129.32, 129.39, 129.65, 134.04, 134.28, 135.84, 148.33, 151.12, 154.55, 194.89. [*M* + *H*]$^{+}$ calcd for $C_{26}H_{23}N_5OS$: 454.1701; found: 454.1696.

1-(4-Fluorophenyl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo [*d*] imid-azole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio)ethan-1-one (6 g):

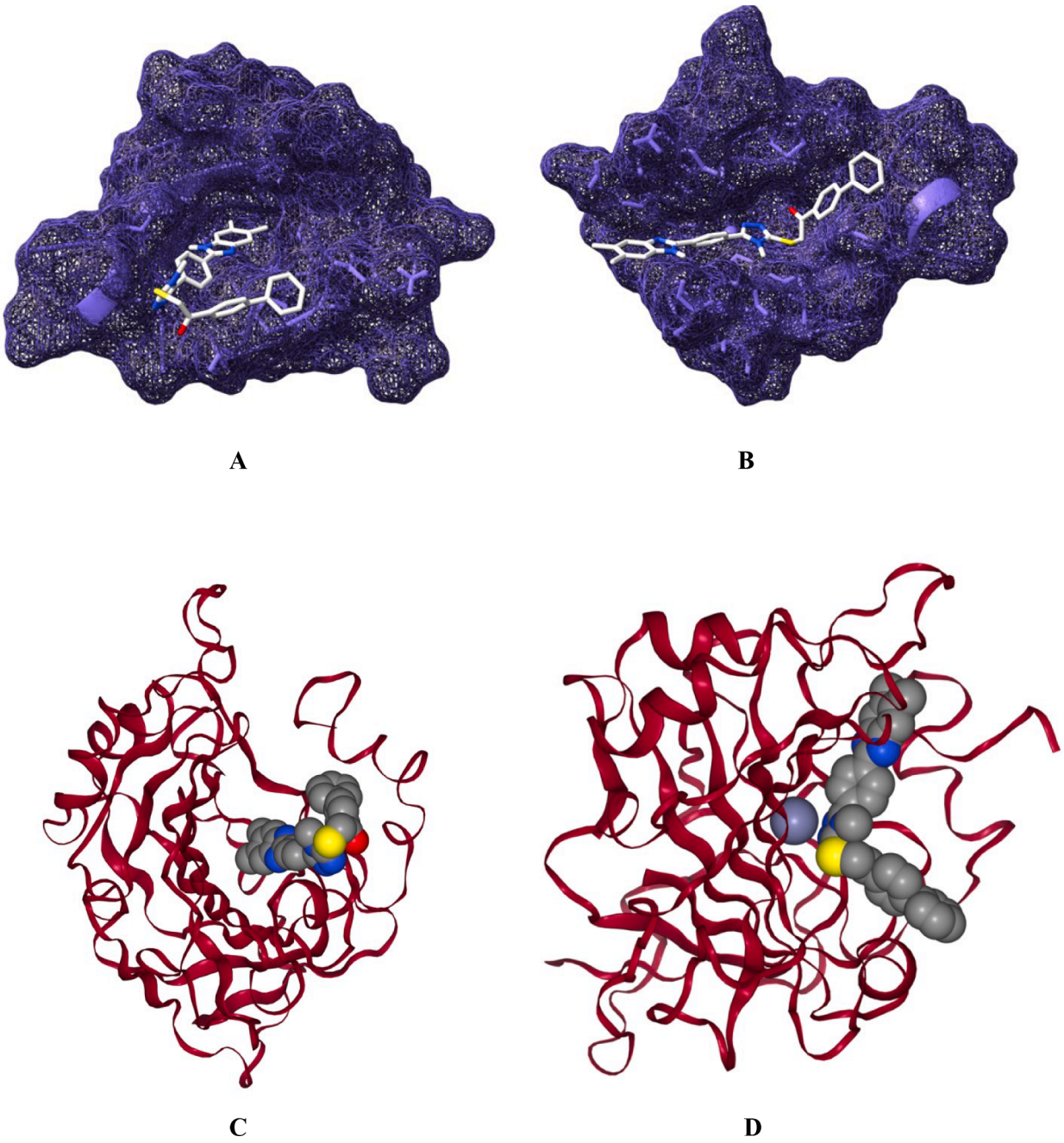


Fig. 3. Binding orientations in active site pocketS (**A, B**) and protein ligand complexes of compound **6j** in the human Carbonic Anhydrase I and II proteins (**C** (CAI), **D**(CAII)).

Yield: 70 %. M.p. 324.7 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.48 (6H, s, -CH_3), 3.69 (3H, s, Aromatic CH), 5.03 (2H, s, -CH_2), 7.47 (1H, t, *J* = 8.88 Hz, Aromatic C—H), 7.69 (3H, s, Aromatic CH), 8.13–8.17 (3H, m, Aromatic C—H), 8.20–8.22 (1H, m, Aromatic C—H), 8.45 (2H, d, *J* = 8.48 Hz, Aromatic CH), 14.19 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.45, 32.63, 34.07, 112.91, 114.10, 119.73, 120.56, 122.23, 125.22, 126.33, 128.64, 129.60, 129.90, 132.24, 133.49, 135.02, 136.21, 147.33, 150.74, 154.77, 195.79.

1-(4-Methoxyphenyl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo [*d*] imidazole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio) ethan-1-one (6 h**):**

Yield: 69 %. M.p. 317.4 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.49 (6H, s, -CH_3), 3.69 (3H, s, CH_3), 3.92 (3H, s, OCH_3), 4.98 (2H, s, CH_2), 7.14 (1H, d, *J* = 8.96 Hz, Aromatic C—H), 7.70 (3H, s, Aromatic C—H), 8.08 (1H, d, *J* = 8.96 Hz, Aromatic C—H), 8.16 (2H, d, *J* = 8.52 Hz, Aromatic C—H), 8.45 (3H, d, *J* = 8.48 Hz, Aromatic C—H), 14.19 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.45, 32.31, 33.03, 56.04, 114.17, 114.54, 117.43, 118.41, 120.49, 122.58, 123.48, 125.08, 126.05, 128.66, 129.92, 131.27, 131.35, 136.24, 147.28, 151.01, 154.42, 194.61. $[M + H]^+$ calcd for $C_{27}H_{25}N_5O_2S$: 484.1810; found: 484.1802.

1-(4-Nitrophenyl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo [*d*] imidazole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio)ethan-1-one (6i):

Yield: 71 %. M.p. 333.6 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.41 (6H, s, -CH_3), 3.63 (3H, s, CH_3), 5.03 (2H, s, -CH_2), 7.60 (3H, s, Aromatic C—H), 8.08 (3H, d, *J* = 8.60 Hz, Aromatic C—H), 8.36 (4H, d, *J* = 8.52 Hz, Aromatic C—H), 14.11 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.46, 32.30, 37.39, 114.44, 115.76, 118.61, 120.84, 121.53, 124.87, 125.64, 128.37, 129.84, 130.43, 131.27, 132.45, 135.48,

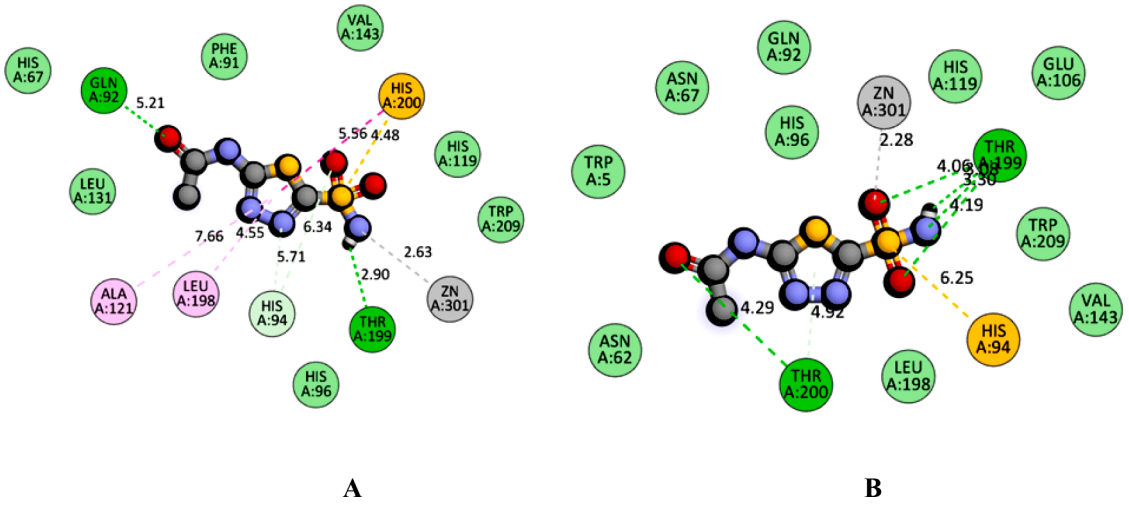


Fig. 4. 2D molecular interactions and binding site of Acetazolamide with the critical amino acid residues of the Carbonic Anhydrase I & II proteins, respectively (A, B). Interactions were displayed as color coded dashed lines, green lines indicated the H–bonds.

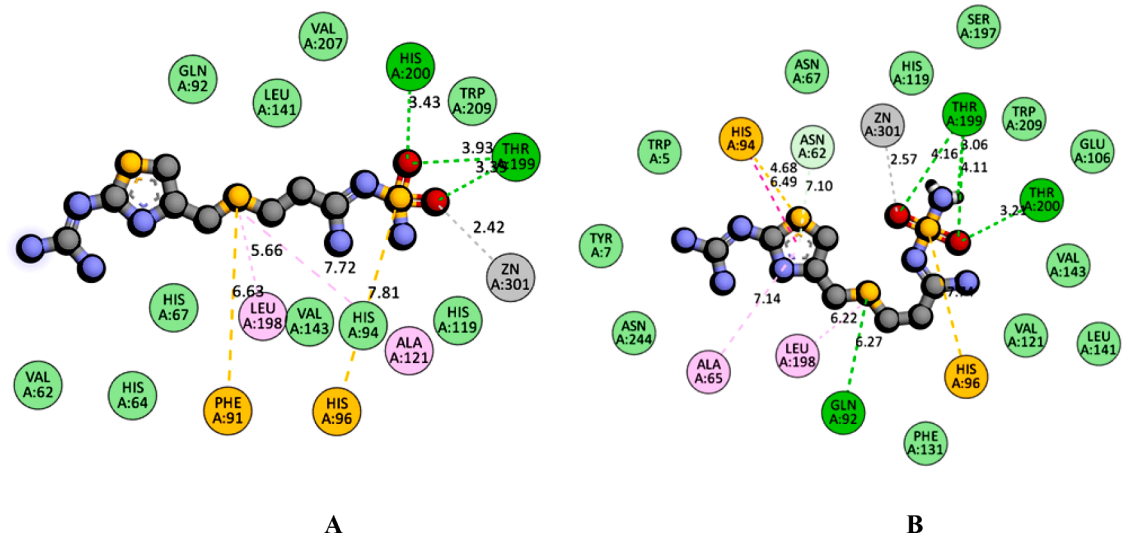


Fig. 5. 2D molecular interactions and binding site of Famotidine with the critical amino acid residues of the Carbonic Anhydrase I & II proteins, respectively (A, B). Interactions were displayed as color coded dashed lines, green lines indicated the H–bonds.

138.29, 147.71, 150.95, 154.84, 194.26. $[M + H]^+$ calcd for $C_{26}H_{22}N_6O_3S$: 499.1540; found: 499.1547.

1-([1,1′-biphenyl]−4-yl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo[*d*]imidazole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio) ethan-1-one (6j):

Yield: 69 %. M.p. 325.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.41 (6H, s, -CH_3), 3.77 (3H, s, CH_3), 5.01 (2H, s, -CH_2), 7.44 (1H, d, J = 7.36 Hz, Aromatic C—H), 7.50 (1H, d, J = 7.72 Hz, Aromatic C—H), 7.64 (3H, s, Aromatic C—H), 7.76 (1H, d, J = 7.20 Hz, Aromatic C—H), 7.86 (1H, d, J = 8.48 Hz, Aromatic C—H), 8.08–8.13 (4H, m, Aromatic C—H), 8.42 (4H, d, J = 8.44 Hz, Aromatic CH), 14.13 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.45, 32.31, 32.70, 114.08, 116.32, 117.50, 121.33, 124.73, 125.64, 127.44, 127.50, 128.75, 129.60, 129.69, 129.90, 130.68, 130.87, 131.13, 132.51, 136.44, 145.59, 147.05, 150.81, 154.77, 193.31.

1-(2,4-Dichlorophenyl)−2-((5-(4-(5,6-dimethyl-1*H*-benzo[*d*]imidazole-2-yl)-phenyl)−4-methyl-4H-1,2,4-triazol-3-yl)thio) ethan-1-one (6k):

Yield: 73 %. M.p. 329.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.42 (6H, s, -CH_3), 3.63 (3H, s, CH_3), 4.83 (2H, s, -CH_2), 7.63 (3H, s, Aromatic C—H), 8.08 (3H, d, J = 8.52 Hz, Aromatic C—H), 8.38 (3H, d, J = 8.52 Hz, Aromatic C—H), 14.13 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ(ppm): 20.45, 31.36, 32.47, 114.27, 114.86, 119.03, 120.70, 123.00, 125.43, 125.98, 128.56, 129.91, 130.98, 132.38, 133.54, 134.95, 136.01, 138.15, 139.68, 146.77, 151.22, 154.35, 194.33.

4.2. *hCA inhibition assay*

4.2.1. *Purification of hCA I and hCA II by affinity chromatography*

Fresh human blood was obtained from the blood center Ataturk University. The blood samples were centrifuged to separate erythrocytes at 2500 rpm for 15 min, and plasma and buffy coat were removed carefully. 0.9 % NaCl solution was used to wash underlying erythrocytes and upper portions were also discarded. The erythrocytes were hemolyzed with distilled water at 0 °C, and it was stirred for half an hour at 4 °C. The hemolysate was centrifuged at 20,000 rpm for 30 min. Then, cell membranes were separated. pH was adjusted to 8.8 by using solid Tris. The hemolysate was recovered to be applied to the column [52,53].

The affinity gel was prepared on Sepharose-4B matrix. Sepharose-4B was activated with CNBr, and L-tyrosine was covalently fitted.

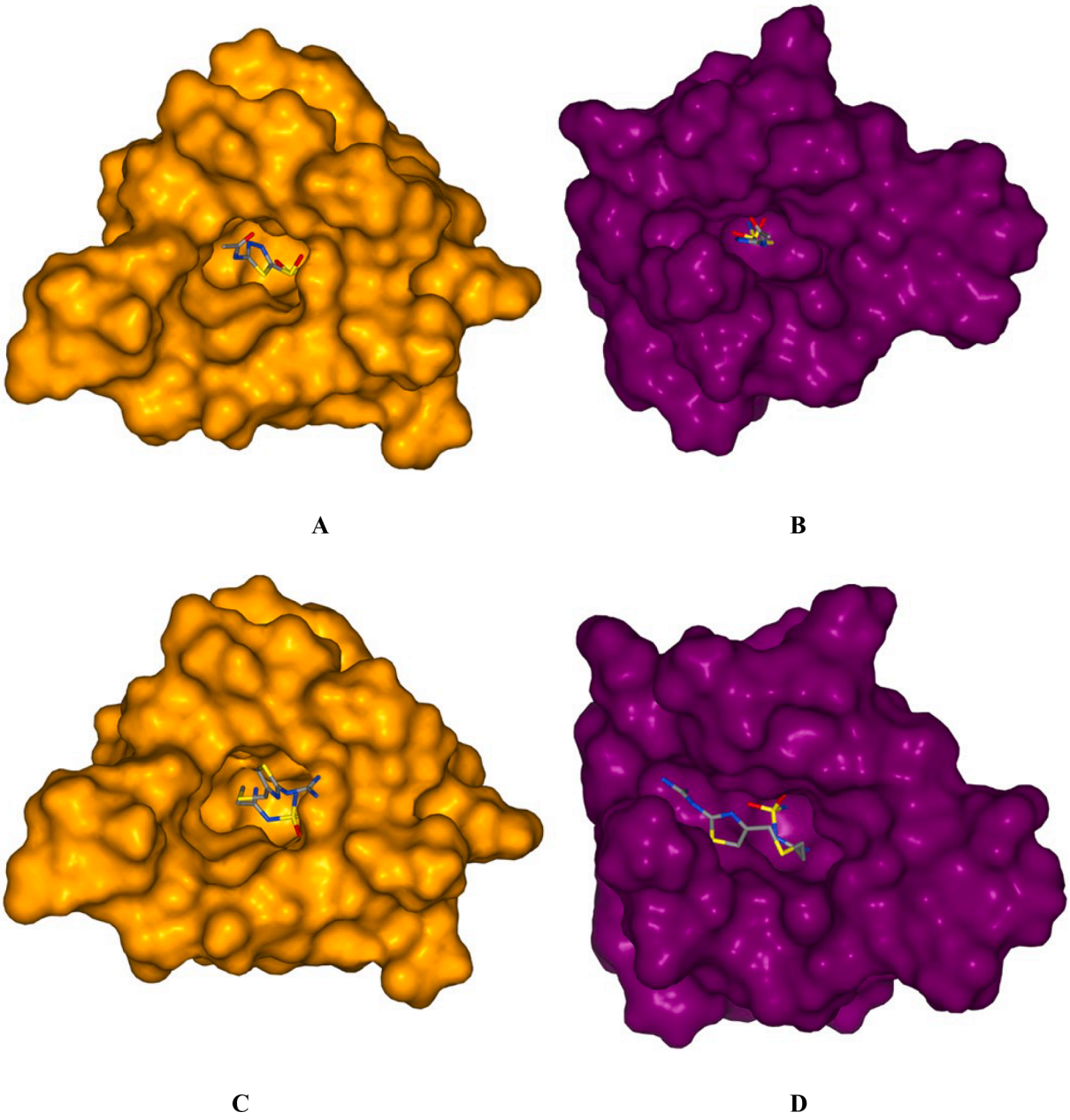


Fig. 6. Binding poses of Acetazolamide (A, B) and Famotidine (C,D) in the active site pocket of the Carbonic Anhydrase I (orange) & II (purple) proteins.

Table 2
Interaction summary of compound **6j,** Acetazolamide and Famotidine.

Compounds	Binding Energy (K.cal/mol)	Human Carbonic Anhydrase I interactions
Compound 6j	−8	ALA132, PRO201, LEU198, ALA135, LEU131, PRO202, ALA121, HIS94, VAL143
Acetazolamide	−6.2	GLN92, THR199, HIS200, HIS94, LEU198, ALA121
Famotidine	−6.4	THR199, HIS200, HIS96, PHE91, ALA121, LEU198
Human Carbonic Anhydrase II interactions		
Compound 6j	−9.1	THR200, GLN92, ASN62, VAL135, PHE131, PRO202, TRP5, ALA65, PRO202
Acetazolamide	−6.8	THR199, THR200, HIS94
Famotidine	−7.1	THR199, THR200, GLN92, HIS96, HIS94, LEU198, ALA65

Table 3
Physico-chemical properties and drug-likeness predictions of compound **6j** with better in vitro and in silico performance using SWISS ADME.

Parameters	Compound 6j
Molecular Weight (g/mol)	42,529.650
Log P o/w	6.06
No.of. H-bond Donors	1
No.of H-bond Acceptors	4
Solubility	Poor
TPSA	101.76 $Å^2$
GI absorption	Low
BBB permeation	No
P-gp substrate	No
Drug likeness (Lipinski)	2 deviations (MW and Log P)
Bioavailability score	0.17
CYP450 isoforms inhibition	CYP2C19/3A4

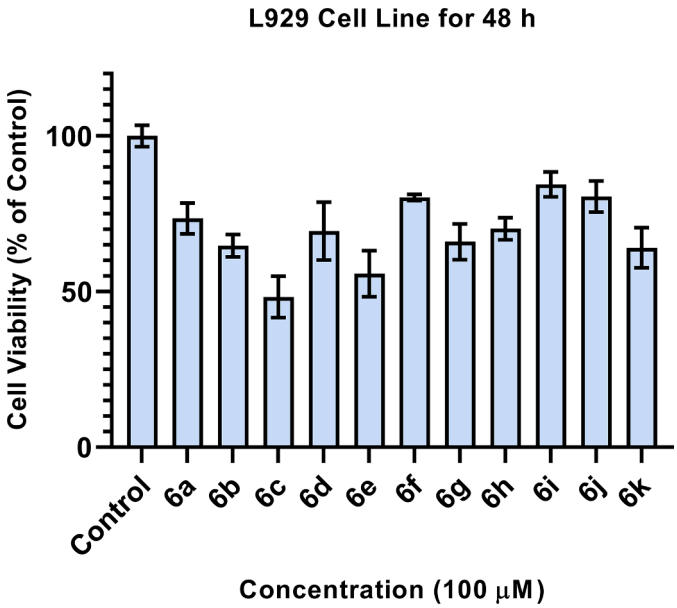


Fig. 7. Cell viability (%) of L929 fibroblast cell line against compounds (**6a-6k**) for 48 h.

Sulfanilamide was coupled to tyrosine with a diazotization reaction as a ligand. The hemolysate was applied to the prepared Sepharose-4B-L-tyrosine-sulfanilamide affinity column equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed by using of 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The human CA isozymes (hCA I and hCA II) were eluted 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 carried out at 4 °C [54].

4.2.2. Hydratase activity

CA activity was stated using the Wilbur-Anderson Method, which by Rickli et al. modified [54,55]. This method, as a result hydration of CO_2 is released H^+ ions and the pH changes were determined by means of bromothymol blue indicator based on the measurement of the elapsed time. Enzyme Unit (EU) was calculated using the equation $(t_o\text{-}t_c/t_c)$ where t_o and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

4.2.3. Inhibition assay

The inhibitory effects of compounds **6a-6k** and AAZ on the hydratase activity of hCA I and hCA II isoenzymes were investigated. IC_{50} values were calculated for the compounds at different concentrations while maintaining a constant substrate concentration. The activities of enzymes in the medium without inhibitors were used as 100 % activity. The activity% values of enzymes were calculated by measuring the hydratase activity in the presence of different concentrations of inhibitors. The IC_{50} value was calculated by utilizing graphs of activity %-[I] for each inhibitor [55–57]. Inhibition constants were calculated by the nonlinear least squares method using the Cheng-Prusoff equation [58–60].

4.3. Molecular docking

Docking simulations were performed by using AutoDock VINA integrated in the PyRx [61] 0.8 virtual screening tool to identify compounds with high binding affinity.

In silico docking simulation studies to evaluate the molecular interactions of compound **6j** were done with the human carbonic anhydrase I & II (PDB: 6G3V and 6G3Q) [62] proteins with a co-crystallized Famotidine as an antagonist ligand. Protein structures were processed to ensure an optimized structure for docking studies and it was executed with the UCSF Chimera Dock Prep module and, that includes the following steps: elimination of water molecules and other ligands, addition of missing atoms and residues, energy minimization and assigning charges and polar hydrogens and then converted to the pdbqt format.

The 2D structure of the ligands was drawn with ChemDraw software and the structures were optimized through energy minimization with MMFF94 force field parameters and conjugate gradient algorithm using the Open Babel module of PyRx and eventually converted the ligands to the AutoDock compatible pdbqt format to carry out the docking explorations.

Post-docking analysis and visualization of binding poses and molecular interactions were done with BIOVIA Discovery Studio 2021 and Chimera X tools.

4.4. ADME properties

Compound **6j** was subjected to analysis using SWISS ADME webserver (http://www.swissadme.ch/) [51] to determine its ADME properties and drug likeness.

CRediT authorship contribution statement

Ulviye Acar Çevik: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Ayşen Işık:** Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Ravikumar Kapavarapu:** Methodology, Software, Investigation, Writing – review & editing. **Kaan Küçükoğlu:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Hayrunnisa Nadaroglu:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Hayrani Eren Bostancı:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Yusuf Özkay:** Conceptualization, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Supervision. **Zafer Asım Kaplancıklı:** Conceptualization, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2023.136770.

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