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Design, synthesis, and docking studies of new 1,3,4-thiadiazole-2-thione derivatives with carbonic anhydrase inhibitory activity

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Abstract—A new series of 1,3,4-thiadiazole-2-thione derivatives have been prepared and assayed for the inhibition of three physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isozymes, the cytosolic human isozymes I and II, and the transmembrane, tumor-associated hCA IX. Against hCA I the investigated thiones, showed inhibition constants in the range of 2.55–222 μM, against hCA II in the range of 2.0–433 μM, and against hCA IX in the range of 1.25–148 μM. Compound **5c**, 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(5-nitro-2-oxoindolin-3-ylidene)semicarbazide showed interesting inhibition of the tumor-associated hCA IX with K_I value of 1.25 μM, being the first non-sulfonamide type inhibitor of such activity. This result is rather important taking into consideration the known antitumor activity of thiones. In addition, docking of the tested compounds into CA II active site was performed in order to predict the affinity and orientation of these compounds at the isozyme active site. The results showed similar orientation of the target compounds at CA II active site compared with reported sulfonamide type CAIs with the thione group acting as a zinc-binding moiety.

1. Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1)[1–4] constitute interesting targets for the design of pharmacological agents useful in the treatment or prevention of a variety of disorders such as among others, glaucoma, acid–base disequilibria, epilepsy, and other neuromuscular diseases, altitude sickness, edema, and obesity.[5,6] A quite new and unexpected application of the CA inhibitors (CAIs) is with regard to their potential use in the management (imaging and treatment) of hypoxic tumors,[7–14] since at least two CA isozymes of the 15 presently known in humans,[1–5] CA IX and XII, are predominantly found in tumor cells and absent (or are present in very limited amount) in normal tissues.[6,15–18]

Most of the potent CAIs investigated up to now belong to the aromatic/heterocyclic sulfonamide or sulfamate classes,[1–5,19,20] although compounds incorporating other zinc-binding groups have also been investigated.[1–5,21,22] Indeed, such derivatives directly bind by means of the deprotonated sulfonamide/sulfamate moiety to the catalytically critical Zn(II) ion of the enzyme active site, also participating in a multitude of polar and hydrophobic interactions with amino acid residues of the active site cavity.[20,23–28] Typically, clinically used sulfonamide/sulfamate CAIs show potencies in the low nanomolar range against the physiologically relevant isozymes, such as CA I, II, V, and IX among others.[1–8]

Some years ago, Supuran et al.[29] have investigated the interaction of a small series of heterocyclic mercaptans with isozymes CA I, II, and IV. They suggested that the SH moiety of such derivatives may act as a zinc-binding function in the design of CAIs, although the potency of these derivatives was lower than that of the corresponding sulfonamides or sulfenamides reported in the same work.[29] In a newer work,[30] he extended these investigations, reporting the first inhibition study of the tumor-associated isozyme CA IX with a series of heterocyclic mercaptans incorporating 1,3,4-thiadiazole and 1,2,4-triazole moieties. The

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investigated derivatives showed a micromolar range inhibition against CA I, II, and IX.[30] Besides the interest in investigating novel zinc-binding functions in the design of CAIs, thiols may be important for their interaction with tumors from at least two other points of view: it is well documented that oxidative stress is a feature of many tumors expressing CA IX,[31] probably due to an imbalance between reduced and oxidized thiols such as glutathione, cysteine, etc., present in these tissues.[32,33] On the other hand, the activity of protein disulfide isomerase (PDI) enzyme which is critical for the proper folding of proteins is highly influenced by the presence of this type of compounds,[32,33] and many biomedical applications are presently envisaged for thiols that may enhance the catalytic activity of PDI.[34] Thus, thiols as those described here may interact with such multiple biological targets, which make them attractive candidates for in vivo studies in order to design novel approaches for the management of tumors.

2. Results and discussion

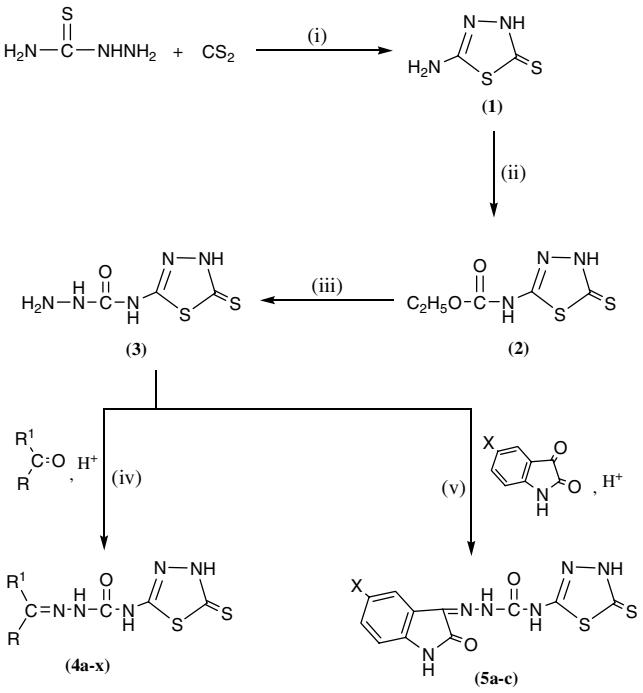
2.1. Chemistry

It has been shown earlier by Supuran et al. that thiol carrying heterocycles can lead to the development of novel types of carbonic anhydrase inhibitors.[29,35–37] However the previously investigated heterocyclic thiols showed moderate inhibitory activities with no preferential inhibition against the tumor-associated isozyme CA IX.[30] Considering the 5-amino-3*H*-1,3,4-thiadiazole-2-thione **1** system as a scaffold; we synthesized a new class of semicarbazone derivatives of such system to be tested against the cytosolic as well as the tumor-associated CA isozymes I, II, and IX.

The synthesis of our target compounds **4a**–**x** and **5a**–**c** is outlined in Scheme 1. The starting compound, 5-amino-3*H*-1,3,4-thiadiazole-2-thione **1**, was prepared according to the method reported by Cho et al.[38] depending on the reaction between thiosemicarbazide and carbon disulfide in alkaline medium followed by acidification. It was converted to its carbamate derivative **2** by the reaction with ethylchloroformate in pyridine.[39]

The preparation of the key intermediate, 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)semicarbazide **3**, was achieved through the neat reaction by stirring the carbamate derivative **2** with a large excess of hydrazine hydrate (80%) for 48 h at room temperature.

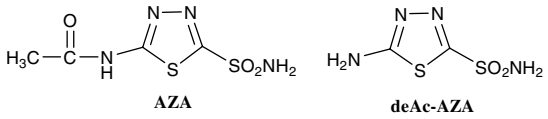
The target compounds, 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(α-substituted/α,α-disubstituted methylene)semicarbazides **4a**–**x**, were obtained by condensing the semicarbazide intermediate **3** with different aldehydes and ketones in slightly acidic medium (pH 5). Similarly, the derivatives 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(2-oxoindolin-3-ylidene)semicarbazides **5a**–**c** were prepared by refluxing the semicarbazide **3** with the appropriate isatin derivative.



Scheme 1. Synthetic pathway of for intermediates **1**–**3** and target compounds **4a**–**x**, **5a**–**c**. Reagents and conditions: (i) KOH, ethanol, reflux 6 h; (ii) $ClCOOC_2H_5$/pyridine, stir 1 h below 40 °C; (iii) hydrazine hydrate (80%), 60 °C (1 h), stir 48 h at room temperature; (iv) ethanol, reflux 1 h, stir overnight; (v) ethanol, reflux 3–5 h, refrigerate overnight.

2.2. Carbonic anhydrase inhibition

Derivatives **4a**–**x** and **5a**–**c** and the intermediate **3** were investigated for their inhibitory activities against three physiologically relevant CA isozymes, the cytosolic, ubiquitous hCA I and II, as well as the tumor-associated, transmembrane isozyme hCA IX (Table 1). The reported inhibitory data for the potent CA inhibitor acetazolamide (**AZA**) as well as its deacetylated derivative **(deAc-AZA)** were also mentioned[30] for comparison reasons.



The following should be noted regarding data presented in Table 1: (i) Derivatives **4a**–**x** and **5a**–**c** inhibit the slow cytosolic isozyme hCA I, showing inhibition constants in the range of 2.55–222 μM. The most potent hCA I inhibitor was **4i**, with a K_I of 2.55 μM, which is more potent than the deacetylated acetazolamide (**deAc-AZA**), with a K_I of 8.6 μM and 10 times less potent than acetazolamide (**AZA**) itself. This result is quite good, taking into account that most reported thiol type CA inhibitors showed inhibition constants in a higher range except for one derivative reported recently by Supuran et al.[30] Good hCA I inhibitory properties were also shown by the derivatives **4a**, **4f**, **4o**–**q**, **4u** as well as the isatin containing derivative **5b** which showed K_I values in the

Table 1. Inhibition data for derivatives **3**–**5** investigated in the present paper and standard sulfonamide CAIs (acetazolamide, **AZA** and its deacetylated derivative, **deAC-AZA**), against isozymes hCA I, II, and IX

Compound	R	R[1]	X	K_I[a] (μM)		
				hCA I[c]	hCA II[c]	hCA IX[d]
AZA[b]	—	—	—	0.25	0.012	0.025
deAc-AZA[b]	—	—	—	8.6	0.060	0.041
3	—	—	—	7.1	9.2	9.3
4a	H	C_6H_5	—	3.00	354	23
4b	H	4-(OH)C_6H_4	—	8.03	3.83	7.15
4c	H	3-(OCH_3)C_6H_4	—	8.54	2.67	7.13
4d	H	4-(OCH_3)C_6H_4	—	26.16	350	24
4e	H	3-(OCH_3)-4-(OH)C_6H_3	—	51.63	386	26
4f	H	2-(Cl)C_6H_4	—	3.28	412	29
4g	H	4-(Cl)C_6H_4	—	18.74	13.46	118.22
4h	H	3-(Br)C_6H_4	—	71.62	235.75	10.10
4i	H	4-(Br)C_6H_4	—	2.55	360	22
4j	H	4-(F)C_6H_4	—	30.77	3.83	7.53
4k	H	2-(NO_2)C_6H_4	—	222.82	433	24
4l	H	4-(NO_2)C_6H_4	—	7.11	2.15	10.00
4m	H	2-[$N(CH_3)_2$]C_6H_4	—	125	9.25	3.35
4n	CH_3	C_6H_5	—	9.09	3.69	7.44
4o	CH_3	4-(CH_3)C_6H_5	—	4.32	3.68	148.9
4p	CH_3	4-(OH)C_6H_4	—	3.34	350	21
4q	CH_3	4-(OCH_3)C_6H_4	—	4.31	346	20
4r	CH_3	4-(Cl)C_6H_4	—	144.07	3.89	8.55
4s	CH_3	4-(Br)C_6H_4	—	42.25	11.25	6.85
4t	CH_3	4-(NO_2)C_6H_4	—	8.62	3.54	7.54
4u	C_6H_5	C_6H_5	—	4.34	2.83	8.43
4v	H	3-Pyridyl	—	8.64	2.07	5.05
4w	H	2-Furyl	—	73.25	13.25	5.75
4x	H	$CH_2CH(CH_3)_2$	—	8.53	8.85	8.83
5a	—	—	H	7.89	8.36	9.40
5b	—	—	Br	3.71	7.97	7.18
5c	—	—	NO_2	5.95	2.15	1.25

[a] Errors in the range of 5–10% of the reported value (from three different assays).
[b] From Ref. 12.
[c] Human cloned isozyme, by the CO_2 hydration method.
[d] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.

range of 3.0–4.3 μM, of the same order of magnitude as deacetylated acetazolamide (**deAc-AZA**) (K_I of 8.6 μM). Thus, conversion of **3** to its semicarbazone derivatives is thought to be beneficial for enhancing the hCA I inhibitory properties of these derivatives, whereas introduction of other moieties (such as 2-nitrobenzaldehyde, 4-dimethylaminobenzaldehyde, and 4-chloroacetophenone) led to compounds (**4k**, **4m**, and **4r**) with greatly diminished hCA I inhibitory properties, with K_I-s in the range of 125–222 μM. It is at this point difficult to explain the relatively good hCA I inhibitory properties of **4g**, **4i**, and **4q**, which present a similar substitution pattern as the less active derivatives **4r**, **4s**, and **4d** in the prepared library.

(ii) The rapid cytosolic isozyme hCA II was inhibited by compounds **4a**–**x** and **5a**–**c** with inhibition constants in the range of 2.0–433 μM, at least two orders of magnitude higher than those of the sulfonamides (**AZA**) and (**deAc-AZA**), with K_I-s in the range of 12–60 nM (Table 1). However, this inhibition range is rather better than the reported data for other thiol type CA inhibitors.[30] Compounds **4b**, **4c**, **4j**, **4l**, **4n**, **4o**, **4r**, **4t**, **4u**, **4v**, and **5c** show moderate activity with K_I values of 2.0–3.8 μM while other derivatives showed either comparable activity with the parent semicarbazide **3** (compounds **4g**, **4m**, **4s**, **4w**, **4x**, **5a**, and **5b**) or highly diminished activity to be very weak inhibitors for hCA II. Although the assigning of a structure–activity relationship is not possible for this isozyme, it seems that semicarbazones obtained from benzaldehyde derivatives (**4a**–**4m**) are of lower activity than other compounds in the series. (iii) Against the tumor-associated isozyme hCA IX, our target compounds **4a**–**x** and **5a**–**c** again showed modest inhibitory activity, with inhibition constants in the range of 1.25–148 μM, being about one order of magnitude lower than those of other reported thiols[30] (Table 1). The tested compounds show inhibition pattern against this isozyme which generally differs from those against the other tested isozymes (CA I and CA II). It is interesting here to report that compound **5c** which shows the highest inhibitory activity against isozyme IX (K_I = 1.25 μM) is the first reported thiol derivative showing such inhibition against that tumor-associated isozyme. We think that, more efforts should be done in order to optimize compounds of this type in order to produce inhibitors of more selectivity against such important isozyme.

2.3. Docking studies

The orientation of compounds carrying thione moiety as a zinc-binding group into CA active site is still unknown

due to lack of the crystallographic data regarding this type of compounds complexed with CA isozymes. We tried to predict such orientation by docking the synthesized compounds **4a**–**x** and **5a**–**c** into human CA II active site using the dock tool of Molecular Operating Environment program (MOE-Dock).[40]

The X-ray crystallographic structure of carbonic anhydrase isozyme II complexed with brinzolamide (PDB: 1A42) was used for the docking calculations after ligand removal and enzyme adjustment. Using the processed CA II crystal structure and the 'Site Finder' tool of the program, the enzyme was searched for its active site. We performed three docking procedures for each ligand and the best configuration of each of the ligand–receptor complexes was selected based on energetic grounds. The binding free energy dG, the total energy of ligand–enzyme complex (E_{total}), the interaction electrostatic (E_{ele}), and van der Waals (E_{vdw}) energies between the ligand and the enzyme (E_{vdw}) were calculated (Table 2).

Table 2. Free energy of binding and other interaction energies between ligands and CA II (kcal/mol)

Compound	dG	E_{ele}	E_{vdw}	E_{total}
4a	−8.61	−9.68	−8.11	6855
4b	−9.62	−14.14	−20.34	6756
4c	−9.41	−27.57	−15.33	6668
4d	−8.16	−14.64	−9.83	6691
4e	−8.84	−7.63	−11.94	6844
4f	−8.65	−8.82	−14.20	6752
4g	−9.26	−25.80	−12.53	6772
4h	−8.67	−10.08	−13.23	6958
4i	−8.54	−24.23	−17.58	6837
4j	−10.52	−8.54	−13.33	6969
4k	−8.37	−9.15	−12.23	6869
4l	−12.32	−26.40	−17.68	6752
4m	−9.15	−31.69	−12.56	6891
4n	−9.16	−6.79	−9.76	6979
4o	−9.80	−23.79	−19.61	6680
4p	−8.38	−25.72	−16.21	6836
4q	−8.71	−25.73	−13.19	6934
4r	−10.35	−7.22	−13.45	6979
4s	−9.58	−11.72	−15.131	6761
4t	−11.40	−15.46	−17.32	6849
4u	−10.24	−11.59	−17.64	6697
4v	−11.48	−21.04	−17.32	6674
4w	−8.96	−21.35	−11.34	6750
4x	−9.90	−29.43	−12.36	6842
5a	−9.51	−11.24	−15.58	7051
5b	−8.91	−17.53	−13.21	7023
5c	−11.84	−23.71	−10.58	6994

The energy data showed a rough correlation between the binding free energy (dG) values of the target compounds and their inhibitory activity against CA II. The most active compounds **4l** and **5c** showed the highest dG values of −12.32 and −11.84 kcal/mol, respectively. On the other hand, compounds which showed weak CA II inhibitory activity (**4a**, **4d**–**f**, **4h**, **4i**, **4k**, **4p**, and **4q**) were found to have lower dG values in the range of −8.16 to −8.84 kcal/mol. Other derivatives showed dG values between −8.91 and −11.48 kcal/mol but generally with fair correlation with their inhibitory activity. Regarding the electrostatic and van der Waals energy values, all derivatives showed high E_{ele} and E_{vdw} values indicating the importance of these types of interactions for enzyme binding.

The saved pose for the ligand–enzyme complex of each molecule was subjected to detailed 3D analysis for its interactions at the enzyme active site. The following notes were observed regarding the docked structures (Figs. 1 and 2): (i) The thione group of the target compounds replaces the hydroxyl ion/water molecule coordinated to zinc atom in the native enzyme. The zinc ion remains in its stable tetrahedral geometry being coordinated, in addition to the thione group, with imidazole nitrogens of His 94, His 96, and His 119. This interaction resembles that observed between the ionized sulfonamide group and the zinc atom in the reported

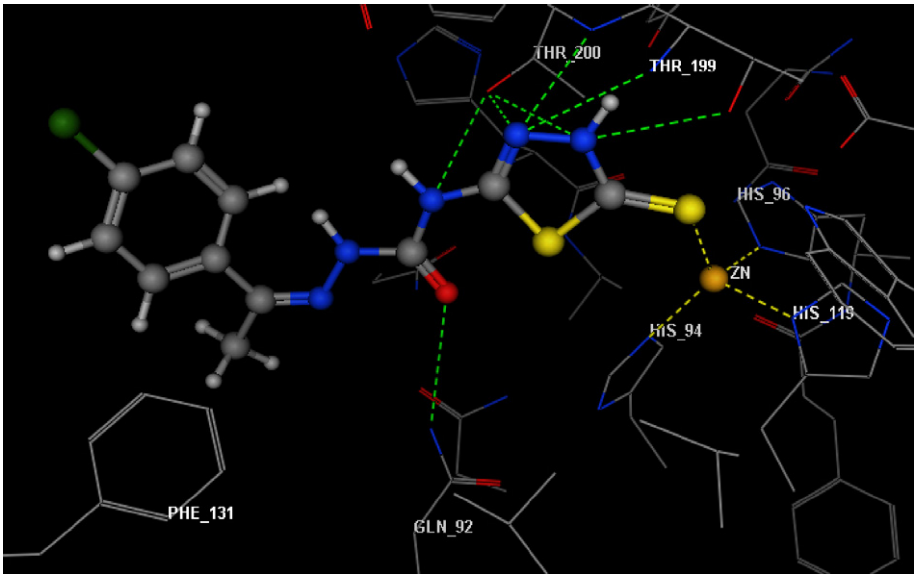


Figure 1. 3D docked structure of **4r** (ball and stick) at hCA II active site; hydrogen bond (green), metal coordination (yellow).

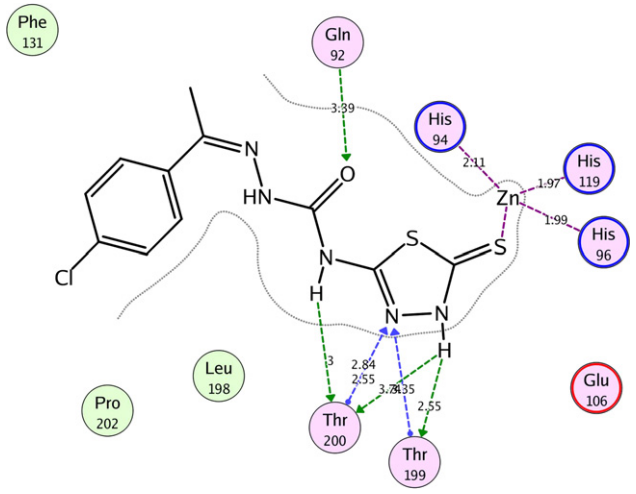


Figure 2. Simplified structure of **4r** docked at hCA II active site; side chain hydrogen bond (green), backbone hydrogen bond (blue), metal coordination (purple).

enzyme–sulfonamide complexes,[20,23–28] Figure 3 shows the orientation of **4l** and brinzolamide in the active site.

The Zn—S distances are in the range of 1.78–2.83 Å which is comparable with the reported data (1.95–2.10 Å).[41] Although there is no clear relationship between the Zn—S bond distance of the docked compounds and their activity, but it was noted that compound **4l** showed the shortest bond distance in agreement with its highest inhibitory activity (Fig. 2).

(ii) The amino acid Thr 200 was found to play an important role in hydrogen bond interactions with the docked compounds. Thr 200 participates in two hydrogen bonds with both thiadiazole nitrogen atoms (Figs. 1 and 2). In the first one, the oxygen of hydroxyl group of Thr 200 forms a hydrogen bond with the thiadiazole N^3 proton with bond distances in the range of 1.77–3.79 Å. In the second one, the hydroxyl group of Thr 200 donates a hydrogen bond to N^2 atom of thiadiazole with bond distances of 2.55–3.78 Å. (iii) Other hydrogen bond interactions were observed in certain derivatives between the thiadiazole nitrogens, semicarbazone moiety, and ring substituents with the amino acid residues, Asn 62, His 64, Ala 65, Asn 67, Gln 92, Thr 199, and Pro 201. (iv) Derivatives containing the indoline moiety (**5a**–**c**) showed additional hydrogen bond interaction, where the NH of the indoline nucleus donates a hydrogen bond to the carbonyl oxygen of Asn 67 (distance of 3.06–3.80 Å). (v) Interactions other than hydrogen bonding including hydrophobic and van der Waals were also observed. The most common amino acid residues to participate in such interactions were Phe 131 and Leu 198 (Fig. 2).

3. Conclusion

We report here the synthesis of a new series of 1,3,4-thiadiazole-2-thione derivatives. The synthesized compounds were tested for their inhibitory activities against three CA isozymes. The results revealed that some derivatives showed inhibition constants in the low micromolar range especially against CA IX presenting the first non-sulfonamide compounds showing such activities. In addition, the binding mode of the tested compounds inside the CA II active site was predicted using a docking technique. The data obtained showed similar orientation of the synthesized derivatives in the CA II active site compared to that of the reported data regarding similar CA inhibitors.

4. Experimental

4.1. General

Melting points were determined using an electrothermal apparatus (Stuart Scientific, England) and are

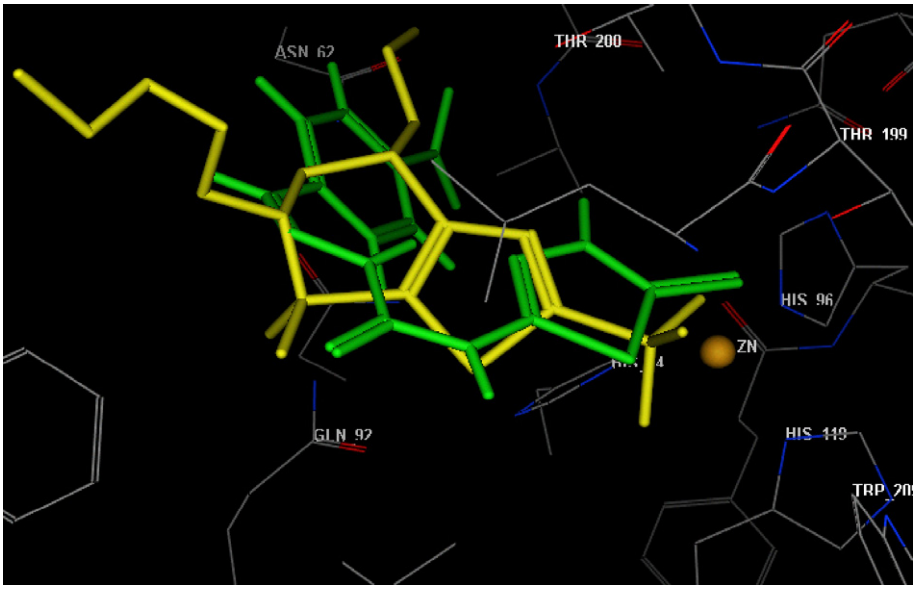


Figure 3. Orientation of **4l** (green) and brinzolamide (yellow) at hCA II active site.

uncorrected. IR spectra were recorded as KBr disk using Shimadzu IR 200-91527 spectrophotometer (Shimadzu Corp., Kyoto, Japan) and the data are given in υ_{max} (cm^{-1}). ^1H NMR (60 MHz) spectra were carried out on Varian EM-360L, 60 MHz (Varian, Palo Alto, CA, USA), where ^1H NMR (300 MHz) and ^{13}C NMR spectra were recorded on Joel JNM-EX 300, 300 MHz (Joel, Tokyo, Japan) using DMSO-d_6 as a solvent and the chemical shifts are given in δ (ppm). Mass spectra were performed on Joel, JMS-600 spectrometer at an ionization voltage of 70 eV (Joel, Tokyo, Japan). Elemental analyses were performed on 'Analytischer Funktionstest vario EL Fab.-Nr. 11982027' (Germany). All reactions were monitored by thin-layer chromatography (TLC) using silica gel 60 GF_{245} precoated sheets 20×20 cm, layer thickness 0.2 mm (E-Merck, Germany) and were visualized by UV-lamp at wavelength (λ) 254 nm. All chemicals and solvents were of reagent grade, and the latter were distilled and dried before use.

4.2. Chemistry

4.2.1. Synthesis of 5-amino-3*H*-1,3,4-thiadiazole-2-thione (1). Was previously reported.[38]

4.2.2. Synthesis of ethyl *N*-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-yl)carbamate (2). Was previously reported.[39]

4.2.3. Synthesis of 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)semicarbazide (3). Hydrazine hydrate 80% (6 ml, 0.125 mol) was added carefully to ethyl *N*-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-yl)carbamate (**2**) (5 g, 0.025 mol). The reaction mixture was warmed to 60 °C for 1 h and allowed to cool with stirring to room temperature for 48 h (controlled by TLC). The formed paste was diluted with anhydrous ethanol where a heavy white precipitate was appeared. The precipitate was filtered off to yield 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)semicarbazide (**3**) (4 g, 84%). The crude product was recrystallized from methanol to give the analytical sample mp 210–212 °C. IR (KBr), cm^{-1}: 3545–3180 (NH and NH_2), 1678 (C=O), 1474, 1060 (C=S). Anal. Calcd for $C_3H_5N_5OS_2$: C, 18.84; H, 2.64; N, 36.62. Found: C, 19.15; H, 2.41; N, 36.43.

4.2.4. General procedure for the synthesis of 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(α-substituted/α,α-disubstituted methylene)semicarbazides (4a–x). A solution of the appropriate carbonyl compound (0.005 mol) in ethanol was added to a stirred hot suspension of 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)semicarbazide (**3**) (1.0 g, 0.005 mol) in ethanol. The reaction mixture was acidified with concentrated hydrochloric acid until pH 4–6. The mixture was refluxed for 1 h, allowed to attain ambient temperature, and stirred overnight. Few drops of water were added to augment precipitation of the product. The precipitated product was filtered off, washed with ethanol, dried, and crystallized from the appropriate solvent.

4.2.4.1. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(α-phenyl methylene)semicarbazide (4a). Yield, 78%; mp 242–244 °C (aq ethanol); IR (KBr): 3550–3410 (NH), 1680 (C=O), 1557 (C—N), 1487, 1055 (C=S). ^1H NMR (DMSO-d_6): 7.39–7.40 (m, 3H, H-2, 4, 6 of phenyl ring), 7.90–7.91 (m, 2H, H-3, 5 of phenyl ring), 7.98 (s, 1H, Ph—CH=N), 11.37 (br s, 2H, NH—CO—NH), 13.94 (br s, 1H, cyclic NH). ^{13}C NMR (DMSO-d_6): 183.50 (C=S), 153.60 (C=N of thiadiazole ring), 152.55 (C=O), 143.81 (CH=N), 133.87, 127.50, 128.41, 129.81, 128.40 and 127.50 (C-1 to C-6 of phenyl ring, respectively). Anal. Calcd for $C_{10}H_9N_5OS_2$: C, 43.00; H, 3.25; N, 25.07. Found: C, 42.90; H, 3.68; N, 25.13.

4.2.4.2. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-hydroxy phenyl)methylene]semicarbazide (4b). Yield, 71%; mp 245–247 °C (aq ethanol); IR (KBr): 3390 (OH), 3390–3155 (NH), 1673 (C=O), 1550 (C—N), 1466, 1060 (C=S). ^1H NMR (DMSO-d_6): 7.25–7.45 (d, 2H; $J \sim 8.0$ Hz; H-3, 5 of phenyl ring), 8.25–8.45 (d, 2H; $J \sim 8.0$ Hz; H-2, 6 of phenyl ring), 8.55 (s, 1H, Ph—CH=N), 10.60 (br s, 1H, OH), 12.00 (br s, 2H, NH—CO—NH), 14.20–15.30 (br s, 1H, cyclic NH). Anal. Calcd for $C_{10}H_9N_5O_2S_2$: C, 40.67; H, 3.07; N, 23.71. Found: C, 40.48; H, 2.53; N, 23.72.

4.2.4.3. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(3-methoxy phenyl)methylene]semicarbazide (4c). Yield, 61%; mp 223–225 °C (ethanol); IR (KBr): 3435–3300 (NH), 1671 (C=O), 1553 (C—N), 1472, 1052 (C=S), 1264, 1026 (Ph—*O*—*C*H_3). ^1H NMR (DMSO-d_6): 4.25 (s, 3H, OCH_3), 7.35–8.10 (m, 4H, H-2, 4, 5, 6 of phenyl ring), 8.50 (s, 1H, Ph—CH=N), 11.80 (s, 1H, C=N—NH), 12.10 (s, 1H, CO—NH—thiadiazole), 14.90 (br s, 1H, cyclic NH). Anal. Calcd for $C_{11}H_{11}N_5O_2S_2$: C, 42.71; H, 3.58; N, 22.64. Found: C, 42.20; H, 3.38; N, 22.13.

4.2.4.4. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-methoxy phenyl)methylene]semicarbazide (4d). Yield, 85%; mp 244–246 °C (ethanol); IR (KBr): 3580–3165 (NH), 1668 (C=O), 1568 (C—N), 1492, 1051 (C=S), 1251, 1026 (Ph—*O*—*C*H_3). ^1H NMR (DMSO-d_6): 3.78 (s, 3H, OCH_3), 6.93–6.96 (d, 2H; $J \sim 8.0$ Hz; H-3, 5 of phenyl ring), 7.83–7.86 (d, 2H; $J \sim 8.0$ Hz; H-2, 6 of phenyl ring), 7.91 (s, 1H, Ph—CH=N), 11.23 (br s, 2H, NH—CO—NH), 13.80–14.75 (br s, 1H, cyclic NH). ^{13}C NMR (DMSO-d_6): 183.50 (C=S), 154.00 (C=N of thiadiazole ring), 152.50 (C=O), 143.80 (CH=N), 126.00, 129.13, 113.89, 160.62, 114.30 and 129.90 (C-1 to C-6 of phenyl ring, respectively), 55.25 (OCH_3). Anal. Calcd for $C_{11}H_{11}N_5O_2S_2$: C, 42.71; H, 3.58; N, 22.64. Found: C, 42.40; H, 3.45; N, 22.47.

4.2.4.5. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-hydroxy-3-methoxyphenyl)methylene]semicarbazide (4e). Yield, 81%; mp 253–255 °C (ethanol); IR (KBr): 3410 (OH), 3410–3385 (NH), 1698 (C=O), 1560 (C—N), 1479, 1073 (C=S), 1273, 1029 (Ph—*O*—*C*H_3). ^1H NMR (DMSO-d_6): 4.00 (s, 3H, OCH_3), 7.00–7.25 (d, 1H; $J \sim 8.0$ Hz, H-2 of phenyl ring), 7.40–7.55 (d, 1H; $J \sim 8.0$ Hz, H-3 of phenyl ring), 7.80 (s, 1H, H-6 of phenyl ring), 8.20 (s, 1H,

Ph—CH=N), 9.60 (br s, 1H, OH), 11.50 (br s, 2H, NH—CO—NH), 13.90–14.75 (br s, 1H, cyclic NH). Anal. Calcd for $C_{11}H_{11}N_5O_3S_2$: C, 40.61; H, 3.41; N, 21.52. Found: C, 40.34; H, 3.39; N, 21.62.

4.2.4.6. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(2-chlorophenyl)methylene]semicarbazide (4f). Yield, 72%; mp 241–243 °C (DMF/water); IR (KBr): 3545–3415 (NH), 1691 (C=O), 1565 (C—N), 1477, 1066 (C=S). ^{1}H NMR (DMSO-d_6): 7.50–7.90 (m, 3H, H-4, 5, 6 of phenyl ring), 8.60–8.85 (m, 2H, H-3 of phenyl ring and Ph—CH=N), 11.85 (br s, 2H, NH—CO—NH), 12.2–14.55 (br s, 1H, cyclic NH). Anal. Calcd for $C_{10}H_8ClN_5OS_2$: C, 38.28; H, 2.57; N, 22.32. Found: C, 38.50; H, 2.24; N, 22.44.

4.2.4.7. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-chlorophenyl)methylene]semicarbazide (4g). Yield, 76%; mp 245–247 °C (DMF/water); IR (KBr): 3620–3335 (NH), 1667 (C=O), 1559 (C—N), 1474, 1048 (C=S). ^{1}H NMR (DMSO-d_6): 7.90–8.20 (d, 2H, H-2, 6 of phenyl ring), 8.50–8.80 (m, 3H, H-3, 5 of phenyl ring and Ph—CH=N), 11.00–15.00 (br s, 1H, cyclic NH), 12.35 (br s, 2H, NH—CO—NH). Anal. Calcd for $C_{10}H_8ClN_5OS_2$: C, 38.28; H, 2.57; N, 22.32. Found: C, 38.18; H, 2.29; N, 22.12.

4.2.4.8. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(3-bromophenyl)methylene]semicarbazide (4h). Yield, 77%; mp 243–245 °C (DMF/water); IR (KBr): 3620–3335 (NH), 1667 (C=O), 1559 (C—N), 1474, 1048 (C=S). ^{1}H NMR (DMSO-d_6): 7.70–8.70 (m, 4H, H-2, 4, 5, 6 of phenyl ring), 8.85 (s, 1H, Ph—CH=N), 12.00–14.85 (br s, 3H, NH—CO—NH and cyclic NH). MS (EI): *m/z* 357 [2.7%, M^+], *m/z* 359 [2.6$C_{10}H_8BrN_5OS_2$: C, 33.53; H, 2.25; N, 19.55. Found: C, 33.40; H, 2.11; N, 19.29.

4.2.4.9. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-bromophenyl)methylene]semicarbazide (4i). Yield, 88%; mp 249–251 °C (DMF/water); IR (KBr): 3545–3410 (NH), 1676 (C=O), 1564 (C—N), 1471, 1065 (C=S). ^{1}H NMR (DMSO-d_6): 7.70–7.95 (d, 2H; $J \sim 8.0$ Hz, H-2, 6 of phenyl ring), 8.00–8.20 (d, 2H; $J \sim 8.0$ Hz, H-3, 5 of phenyl ring), 8.25 (s, 1H, Ph—CH=N), 11.25–12.85 (br s, 3H, NH—CO—NH and cyclic NH). Anal. Calcd for $C_{10}H_8BrN_5OS_2$: C, 33.53; H, 2.25; N, 19.55. Found: C, 33.60; H, 2.19; N, 19.31.

4.2.4.10. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-fluorophenyl)methylene]semicarbazide (4j). Yield, 90%; mp 233–235 °C (ethanol); IR (KBr): 3440–3330 (NH), 1697 (C=O), 1560 (C—N), 1454, 1061 (C=S). ^{1}H NMR (DMSO-d_6): 7.60–7.90 (m, 2H, H-2, 6 of phenyl ring), 8.40–9.00 (m, 3H, H-3, 5 of phenyl ring and Ph—CH=N), 11.25–13.25 (br s, 3H, NH—CO—NH and cyclic NH). Anal. Calcd for $C_{10}H_8FN_5OS_2$: C, 40.39; H, 2.71; N, 23.55. Found: C, 40.72; H, 2.45; N, 23.44.

4.2.4.11. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(2-nitrophenyl)methylene]semicarbazide (4k). Yield, 91%; mp 246–248 °C (acetonitrile); IR (KBr): 3550–3415 (NH), 1680 (C=O), 1560 (C—N), 1521, 1331 (NO_2), 1472, 1067 (C=S). ^{1}H NMR (DMSO-d_6): 7.70–9.00 (m, 4H, H-2-6 of phenyl ring), 8.75 (s, 1H, Ph—CH=N), 11.95 (br s, 2H, NH—CO—NH), 13.50–14.50 (br s, 1H, cyclic NH). Anal. Calcd for $C_{10}H_8N_6O_3S_2$: C, 37.03; H, 2.49; N, 25.91. Found: C, 36.55; H, 2.25; N, 25.98.

4.2.4.12. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-nitrophenyl)methylene]semicarbazide (4l). Yield, 92%; mp 256–258 °C (DMF/water); IR (KBr): 3510–3230 (NH), 1670 (C=O), 1552 (C—N), 1501, 1329 (NO_2), 1471, 1063 (C=S). ^{1}H NMR (DMSO-d_6): 8.65 (s, 1H, Ph—CH=N), 8.85 (s, 4H, H-2, 3, 5, 6 of phenyl ring), 12.30–15.00 (br s, 3H, NH—CO—NH and cyclic NH). Anal. Calcd for $C_{10}H_8N_6O_3S_2$: C, 37.03; H, 2.49; N, 25.91. Found: C, 37.26; H, 2.16; N, 25.55.

4.2.4.13. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-*N*,*N*-dimethylaminophenyl)methylene]semicarbazide (4m). Yield, 84%; mp 250–251 °C (DMF/water); IR (KBr): 3645–3430 (NH), 1686 (C=O), 1553 (C—N), 1475, 1056 (C=S). Anal. Calcd for $C_{12}H_{14}N_6OS_2$: C, 44.70; H, 4.38; N, 26.07. Found: C, 44.44; H, 3.81; N, 25.91.

4.2.4.14. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-methyl-α-phenyl methylene]semicarbazide (4n). Yield, 74%; mp 236–238 °C (DMF/water); IR (KBr): 3435–3175 (NH), 1680 (C=O), 1558 (C—N), 1469, 1064 (C=S). ^{1}H NMR (DMSO-d_6): 2.35 (s, 3H, CH_3), 7.75–8.00 (m, 3H, H-2, 4, 6 of phenyl ring), 8.35–8.60 (m, 2H, H-3, 5 of phenyl ring), 11.10 (s, 1H, C=N—NH), 11.30–12.55 (br s, 1H, CO—NH—thiadiazole), 14.10–15.50 (br s, 1H, cyclic NH). Anal. Calcd for $C_{11}H_{11}N_5OS_2$: C, 45.03; H, 3.78; N, 23.87. Found: C, 45.25; H, 3.30; N, 23.94.

4.2.4.15. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-methyl-α-(4-methylphenyl)methylene]semicarbazide (4o). Yield, 81%; mp 244–246 °C (DMF/water); IR (KBr): 3435–3175 (NH), 1686 (C=O), 1555 (C—N), 1466, 1066 (C=S). ^{1}H NMR (DMSO-d_6): 2.30 (s, 3H, Ph—C(CH_3)=N), 2.45 (s, 3H, 4-CH_3-Ph), 7.60-7.80 (d, 2H; $J \sim 8.0$ Hz, H-3, 5 of phenyl ring), 8.35–8.50 (d, 2H; $J \sim 8.0$ Hz, H-2, 6 of phenyl ring), 11.10 (s, 2H, NH—CO—NH), 11.50–14.05 (br s, 1H, cyclic NH). Anal. Calcd for $C_{12}H_{13}N_5OS_2$: C, 46.89; H, 4.26; N, 22.78. Found: C, 47.01; H, 3.94; N, 22.56.

4.2.4.16. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-hydroxy phenyl)-α-methyl methylene]semicarbazide (4p). Yield, 88%; mp 249–250 °C (DMF/water); IR (KBr): 3545 (OH), 3545–3410 (NH), 1671 (C=O), 1557 (C—N), 1481, 1061 (C=S). ^{1}H NMR (DMSO-d_6): 2.30 (s, 3H, CH_3), 6.85–7.00 (d, 2H; $J \sim 8.0$ Hz, H-3, 5 of phenyl ring), 7.90–8.06 (d, 2H; $J \sim 8.0$ Hz, H-2, 6 of phenyl ring), 9.95 (br s, 1H, OH), 10.40 (br s, 1H, C=N—NH), 11.10 (br s, 1H, CO—NH—thiadiazole), 13.70–14.50 (br s, 1H, cyclic NH). Anal. Calcd for $C_{11}H_{11}N_5O_2S_2$: C, 42.71; H, 3.58; N, 22.64. Found: C, 42.22; H, 3.92; N, 22.37.

4.2.4.17. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-methyl-α-(4-methoxyphenyl)methylene]semicarbazide (4q). Yield, 87%; mp 243–244 °C (DMF/water); IR (KBr): 3545–3165 (NH), 1669 (C═O), 1560 (C─N), 1473, 1056 (C═S), 1252, 1024 (*Ar─O─C*H_3). ^{1}H NMR (DMSO-d_6): 2.30 (s, 3H, CH_3), 3.9 (s, 3H, OCH_3), 7.00–7.15 (d, 2H; $J \sim 8.0$ Hz, H-3, 5 of phenyl ring), 8.05–8.20 (d, 2H; $J \sim 8.0$ Hz, H-2, 6 of phenyl ring), 10.55 (br s, 1H, C═N─NH), 11.30 (br s, 1H, CO─NH─thiadiazole), 13.45–14.90 (br s, 1H, cyclic NH). Anal. Calcd for $C_{12}H_{13}N_5O_2S_2$: C, 44.57; H, 4.05; N, 21.66. Found: C, 44.13; H, 3.64; N, 21.68.

4.2.4.18. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-chlorophenyl)-α-methyl methylene]semicarbazide (4r). Yield, 82%; mp 246–248 °C (DMF/water); IR (KBr): 3545–3415 (NH), 1691 (C═O), 1565 (C─N), 1477, 1066 (C═S). ^{1}H NMR (DMSO-d_6): 2.40 (s, 3H, CH_3), 8.00–8.15 (d, 2H; $J \sim 8.0$ Hz, H-2, 6 of phenyl ring), 8.60–8.75 (d, 2H; $J \sim 8.0$ Hz, H-3, 5 of phenyl ring), 10.60–12.00 (br s, 3H, NH─CO─NH and cyclic NH). Anal. Calcd for $C_{11}H_{10}ClN_5OS_2$: C, 40.30; H, 3.07; N, 21.36. Found: C, 40.22; H, 2.61; N, 21.46.

4.2.4.19. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(4-bromophenyl)-α-methyl methylene]semicarbazide (4s). Yield, 76%; mp 249–251 °C (DMF/water); IR (KBr): 3545–3415 (NH), 1691 (C═O), 1565 (C─N), 1477, 1066 (C═S). ^{1}H NMR (DMSO-d_6): 2.55 (s, 3H, CH_3), 8.10–8.30 (d, 2H; $J \sim 8.0$ Hz, H-2, 6 of phenyl ring), 8.55–8.75 (d, 2H; $J \sim 8.0$, H-3, 5 of phenyl ring), 11.25 (br s, 2H, NH─CO─NH), 12.00–14.00 (br s, 1H, cyclic NH). Anal. Calcd for $C_{11}H_{10}BrN_5OS_2$: C, 35.49; H, 2.71; N, 18.81. Found: C, 35.59; H, 2.52; N, 18.71.

4.2.4.20. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-methyl-α-(4-nitrophenyl)methylene]semicarbazide (4t). Yield, 91%; mp 272–274 °C (DMF/water); IR (KBr): 3510–3230 (NH), 1670 (C═O), 1552 (C─N), 1501, 1329 (NO_2), 1471, 1063 (C═S). Anal. Calcd for $C_{11}H_{10}N_6O_3S_2$: C, 39.05; H, 2.98; N, 24.84. Found: C, 38.96; H, 2.60; N, 24.59.

4.2.4.21. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α,α-diphenyl methylene]semicarbazide (4u). Yield, 70%; mp 221–224 °C (DMF/water); IR (KBr): 3445–3145 (NH), 1658 (C═O), 1551 (C─N), 1460, 1056 (C═S). ^{1}H NMR (DMSO-d_6): 7.70–8.60 (m, 10H, aromatic), 10.05 (s, 1H, C═N─NH), 11.70–12.70 (br s, 1H, CO─NH─thiadiazole), 14.05–15.70 (br s, 1H, cyclic NH). Anal. Calcd for $C_{16}H_{13}N_5OS_2$: C, 54.07; H, 3.69; N, 19.70. Found: C, 53.72; H, 4.39; N, 19.58.

4.2.4.22. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(3-pyridyl)methylene]semicarbazide (4v). Yield, 75%; mp 242–244 °C (DMF/water); IR (KBr): 3445–3280 (NH), 1681 (C═O), 1559 (C─N), 1481, 1043 (C═S). ^{1}H NMR (DMSO-d_6): 7.70–8.00 (m, 1H, H-5 of pyridyl ring), 8.55 (s, 1H, H-2 of pyridyl ring), 8.85–9.20 (m, 2H, H-4, 6 of pyridyl ring), 9.60 (s, 1H, Ph─CH═N), 10.70–13.80 (br s, 1H, cyclic NH), 12.30 (br s, 2H, NH─CO─NH). Anal. Calcd for $C_9H_8N_6OS_2$: C, 38.56; H, 2.88; N, 29.98. Found: C, 38.62; H, 2.62; N, 29.58.

4.2.4.23. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-[α-(2-furyl)methylene]semicarbazide (4w). Yield, 71%; mp 235–237 °C (DMF/water); IR (KBr): 3445–3250 (NH), 1658 (C═O), 1559 (C─N), 1470, 1053 (C═S). ^{1}H NMR (DMSO-d_6): 7.05 (m, 1H, C-4 of furan ring), 7.55 (d, 1H, C-3 of furan ring), 8.25–8.45 (m, 2H, C-5 of furan ring and furan-*CH*═N), 11.30–15.00 (br s, 3H, NH─CO─NH and cyclic NH). Anal. Calcd for $C_8H_7N_5O_2S_2$: C, 35.68; H, 2.62; N, 26.01. Found: C, 35.65; H, 2.48; N, 25.84.

4.2.4.24. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(4-methylpentan-2-ylidene)semicarbazide (4x). Yield, 64%; mp 220–223 °C (ethanol); IR (KBr): 3440–3135 (NH), 1680 (C═O), 1549 (C─N), 1470, 1063 (C═S). ^{1}H NMR (DMSO-d_6): 0.90–1.15 (d, 6H; $J \sim 8.0$ Hz, *C*H_3─CH─*C*H_3), 2.00 (s, 1H, CH_3─C═N), 2.15–2.45 (m, 3H, CH─CH_2), 11.20–13.00 (br s, 3H, NH─CO─NH and cyclic NH). Anal. Calcd for $C_8H_{13}N_5OS_2$: C, 39.54; H, 5.53; N, 25.62. Found: C, 39.49; H, 5.10; N, 25.54.

4.2.5. General procedure for the synthesis of 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(2-oxoindolin-3-ylidene)semicarbazides (5a–c). A solution of the appropriate indoline derivative (0.005 mol) in ethanol/DMF mixture (2:1) was added to a stirred hot suspension of 4-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)semicarbazide (**3**) (1.0 g, 0.005 mol) in ethanol. The reaction mixture was carefully acidified with glacial acetic acid until acidic (litmus paper). After refluxing for 3–5 h (TLC monitored) the solvent was reduced to about half its original volume under vacuum. An equal volume of water was added and the product was allowed to stand in a refrigerator overnight. The produced precipitate was filtered off, washed with ethanol, dried, and crystallized from the appropriate solvent.

4.2.5.1. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(2-oxoindolin-3-ylidene)semicarbazide (5a). Yield, 71%; mp 262–264 °C (DMF/water); IR (KBr): 3530–3200 (NH), 1716 (cyclic C═O), 1665 (N─*CO*─N), 1453, 1069 (C═S). ^{1}H NMR (DMSO-d_6): 7.30–8.25 (m, 4H, H-4-7 of indoline ring), 8.55 (s, 1H, indoline NH), 12.00 (s, 1H, C═N─NH), 13.50 (s, 1H, CO─NH─thiadiazole), 13.00–15.50 (br s, 1H, thiadiazole NH). MS (EI): *m/z* 320 [12.4%, M^+], *m/z* 161 [100%, M─$C_8H_7N_3O$]. Anal. Calcd for $C_{11}H_8N_6O_2S_2$: C, 41.24; H, 2.52; N, 26.23. Found: C, 40.86; H, 2.45; N, 25.95.

4.2.5.2. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(5-bromo-2-oxoindolin-3-ylidene)semicarbazide (5b). Yield, 78%; mp 264–265 °C (DMF/water); IR (KBr): 3485–3160 (NH), 1720 (cyclic C═O), 1671 (N─*CO*─N), 1493, 1057 (C═S). ^{1}H NMR (DMSO-d_6): 7.45–8.80 (m, 4H, H-4, H-6, H-7 of indoline ring and indoline NH), 12.25 (s, 1H, C═N─NH), 13.45 (s, 1H, CO─NH─thiadiazole), 11.70–15.80 (br s, 1H, thiadiazole NH). Anal. Calcd for $C_{11}H_7BrN_6O_2S_2$: C, 33.09; H, 1.77; N, 21.05. Found: C, 33.55; H, 2.07; N, 20.53.

4.2.5.3. 4-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-1-(5-nitro-2-oxoindolin-3-ylidene)semicarbazide (5c). Yield, 65%; mp >300 °C (DMF/water); IR (KBr): 3485–3110 (NH), 1720 (cyclic C=O), 1688 (N—*CO*—N), 1508, 1326 (NO_2), 1455, 1079 (C=S). ^{1}H NMR (DMSO-d_6): 7.55 (d, 1H, H-7 of indoline ring), 8.45–8.95 (m, 3H, H-4, H-6 of indoline ring and indoline NH), 11.00–14.50 (br s, 3H, NH—CO—NH and thiadiazole NH).

4.3. Carbonic anhydrase inhibition assay

4.3.1. Isozyme preparation. Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lidskog et al.[42] Cell growth conditions were those described by Behravan et al.[43] and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[44] Enzyme concentrations were determined spectrophotometrically at 280 nM, utilizing a molar absorptivity of 49 mM^{-1} cm^{-1} for CA I and 54 mM^{-1} cm^{-1} for CA II, respectively, based on Mr = 28.85 kDa for CA I and 29.30 kDa for CA II, respectively[45,46].

The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[47] was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene)). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *E. coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4M urea and 2% Triton X-100. The homogenate thus obtained was extensively centrifuged in order to remove soluble and membrane associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione, and 1 mM oxidized glutathione. Active hCA IX was extensively dialyzed into a solution of 10 mM Hepes (pH 7.5), 10 mM Tris–HCl, 100 mM Na_2SO_4, and 1 mM $ZnCl_2$. The amount of protein was determined by spectrophotometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate.

4.3.2. Evaluation of carbonic anhydrase inhibitory activity. An SX.18MV-R Applied Photophysics stopped-flow instrument was used for measuring the initial velocities for the CO_2 hydration reaction catalyzed by different CA isozymes, by following the change in absorbance of a pH indicator. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength—this anion is not inhibitory for hCA I, hCA II, and hCA IX, but it is for hCA IV case in which it has been replaced by 10 mM $NaNO_3$, which is much less inhibitory), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction were used to determine the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitors were prepared at a concentration of 1–3 mM (in DMSO–water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above.

4.4. Docking studies

All molecular modeling calculations and docking studies were performed using 'Molecular Operating Environment (MOE) version 2006.02', Chemical Computing Group Inc., 1010 Sherbrooke Street West, Suite 910, Montreal, H3A 2R7, Canada. The program operated under 'Windows XP' operating system installed on an Intel Pentium IV PC with a 2.8 MHz processor and 512 RAM.

The target compounds were built using the builder interface of the MOE program and subjected to energy minimization tool using the included MOPAC 7.0. The produced model was subjected to Systematic Conformational Search where all items were set as default with RMS gradient of 0.01 kcal/mol and RMS distance of 0.1 Å.

The X-ray crystallographic structure of hCA II complexed with brinzolamide (1A42) was obtained from the Protein Data Bank. The enzyme was prepared for docking studies where: (i) The ligand molecule was removed from the enzyme active site. (ii) Hydrogen atoms were added to the structure with their standard geometry. (iii) MOE Alpha Site Finder was used for the active sites search in the enzyme structure and dummy atoms were created from the obtained alpha spheres.

The obtained ligand–enzyme complex model was then used in calculating the energy parameters using MMFF94x force field energy calculation and predicting the ligand–enzyme interactions at the active site.

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