1. **Introduction**

Amino acid derivatives are molecules that are the focus of interest in modern drug discovery studies and medicinal chemistry studies because of the use of modified peptides as drugs, the ease of synthesizing amino acids with various side chains, their multiple biological activities, their chiral configurations and commercial availability. In the field of medicinal chemistry, amino acid derivatives are widely used as enzyme inhibitors. In this study, the inhibitory effects of some amino acid derivatives on carbonic anhydrase I and II isoenzymes and Acetylcholine esterase activity, which are metabolically important and inhibitors are target molecules for drug design studies, were investigated. The enzyme carbonic anhydrase catalyzes the reversible conversion of CO2 and water to carbonic acid. The carbonic acid then dissociates into bicarbonate and hydrogen ions. This reaction catalyzed by carbonic anhydrase is closely related to many physiological processes such as pH balance, respiration and gas exchange, kidney function, digestion, ion transport, and bone regeneration. To date, eight different CA families (α-CAs,β-CAs,γ-CAs,δ-CAs,ζ-CAs,η-CAs,θ-CAs, and t-CAs) with different structural and functional properties have been identified. Of these, the α-CAs, which have 16 isoenzymes, is found in humans. Of these isoenzymes, CAI and CAII are the most studied isoenzymes and also CAII has the highest catalytic activity. Each of these isoenzymes has different cellular localities and catalytic activities, and their behavior towards inhibitors and activators is different. Due to their role in pH balance, ion transport and other physiological processes, carbonic anhydrase isoenzymes have been associated with many diseases such as glucoma, osteoporosis, neurological and kidney diseases, and cancer. Therefore, carbonic anhydrase inhibitors play a large role in a wide range of biology, medicine, and drug design research. Today, Acetazolamide (AZA), Methazolamide, Dorzo-lamide, Brinzolamide, Diclofenamide, Ethoxazolamide, Indisulam are CA inhibitors used in the treatment of glaucoma. Apart from these, Topiramate and Zonisamide used in the treatment of epilepsy and Bendroflumethiazide used in the treatment of hypertension are carbonic anhydrase inhibitors. Although many drugs that are carbonic anhydrase inhibitors have been identified, it is an important requirement to identify new carbonic anhydrase inhibitors that are specific to isoenzymes and have fewer side effects for the treatment of various diseases. And there are many scientific research studies on this subject. Until today, CA inhibitory properties of many compound groups such as sulfonamides, benzene sulfonamides, sulfonamides with zinc binding groups, Coumarins, Quinolines, Hydrazides, Hydrazones, Thiadiazole Derivatives have been determined. In addition, both proteinogenic and non-proteinogenic amino acids have been widely used to synthesize new CA inhibitors, considering the benefits of their chemical properties.

Acetylcholine esterase hydrolyzes acetylcholine to choline and acetate by cleaving the ester bond of acetylcholine. The main physiological function of acetylcholinesterase is to terminate the action of the neurotransmitter acetylcholine in the cholinergic system [2]. ACh plays a potential role in cognitive functions, particularly memory. Therefore, the predominant therapeutic agents for Alzheimer's disease are acetylcholinesterase (AChE) inhibitors, which improve cholinergic neurotransmission in the synaptic cleft by reducing the degradation of ACh [3-6]. AChE inhibitors have been the primary target of researchers in drug development studies for AD. For this purpose, many AChE inhibitors such as donepezil, rivastigmine and galantamine, tacrine have been developed [1]. These drugs can provide symptomatic relief and improve cognitive impairment in patients [21,22] However, the positive effects of these AChE inhibitors are temporary and do not prevent the progression of the disease [23,24] At the same time, the developed inhibitors have many side effects, which means that new, effective, less toxic AChE inhibitors necessitates the identification of inhibitors. Apart from many classes of compounds, studies have been conducted in which various amino acid derivatives have been evaluated as AChE inhibitors.

In this study, the inhibitory effects of some non-proteinogenic amino acid compounds (hippuric acid (**a**), *N*-(9-Fluorenylmethoxycarbonyl)-D-valine (**b**), *N*-Z-(1-Benzotriazolylcarbonyl) methylamine (**c**), (*S*)-*N*-Z-1-Benzotriazolylcarbonyl-2-phenylethylamine (**d**)) (Figure 1) on both hCA-I, hCA-II and AChE activities were investigated. Molecular modeling studies were carried out for amino acid derivatives that have an inhibitory effect.

metin, taslak, diyagram, çizim içeren bir resim

Açıklama otomatik olarak oluşturuldu

**Figure 1.** Non-proteinogenic amino acid compounds used in this study.

1. **Materials and Methods** 
   1. **Materials**

The amino acids compounds and all other chemicals used in this study were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH, Germany).

* 1. **Biological Activity Studies**
     1. **Inhibition Studies on hCA-I and hCA-II**

The hCAI and hCAII isoenzymes used in the study were isolated using the CNBr-activated Sepharose-4B-L-tyrosine sulfanilamide affinity chromatography method as in our previous studies. In the isolation steps of isoenzymes, isoenzyme activities were determined by hydratase activity measurement method and quantitative protein amounts were determined by Bradford protein determination method. The purity of the isolated isoenzymes was checked by SDS-PAGE. The purified isoenzymes were dialyzed against 50 mM Tris-SO4 (pH 7.4) buffer overnight, then separated into small fractions of 1 mL and stored at -80C until used in biological activity studies. The esterase activity measurement method was used when measuring the activities of hCAI and hCAII in inhibition studies. In this method, p-nitrophenyl acetate was used as the substrate. The principle of the method is based on the hydrolysis of p-nitrophenyl acetate by the enzyme CA to p-nitrophenol and acetic acid. The p-nitrophenol formed as a result of the reaction gives an absorbance at 348 nm. Therefore, the formation of p-nitrophenol from p-nitrophenyl acetate was monitored by measuring the absorbance at 348 nm, 25°C using a spectrophotometer. The enzyme unit was calculated using the absorption coefficient (ε = 5.4 x 103 M-1 cm-1) of p-nitrophenyl acetate at 348 nm. In order to determine the inhibition effects of **a**, **b**, **c**, **d** compounds on hCAI and hCAII activities, at least five different concentrations of **a**, **b**, **c** and **d** compounds were measured. The %Activities of hCAI and hCAII against five different inhibitor concentrations were calculated for molecules that showed inhibitory effects. The same experiment was also performed for the reference inhibitor Acetazolamide. The control activity was accepted as 100% and the graphs of % Activity versus inhibitor concentration were drawn for the molecules showing the inhibition effect and for the reference inhibitor. From these graphs, IC50 values, which express the inhibitor concentration that halves the enzyme activity, were determined.

* + 1. **Inhibiton Studies on AChE**

The AChE enzyme used in the study, electrical eel (Electrophorus electricus) (CAS no. 9000-81-1), was purchased from Sigma-Aldrich. To investigate the inhibitory effect of compounds **a**, **b**, **c** and **d** on AChE activity, AChE activity was analyzed by the spectrophotometric method described by Ellman et al. The Ellman method is a sensitive and reliable method for measuring acetylcholine esterase enzyme activity. Acetylthiocholine iodide is used as substrate in this reaction. AChE catalyzes the conversion of acetylthiocholine to thiocholine and acetic acid. The thiocholine formed as a result of the reaction reacts with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) added to the reaction medium. As a result of this reaction, 5-thio-2-nitrobenzoic acid and choline are formed. 5-thio-2-nitrobenzoic acid is a compound with a yellow color, which can be measured spectrophotometrically at 412 nm. AChE activity was measured at least five concentrations for **a-d** compounds to determine the inhibition effects of **a-d** on AChE activity. The %Activities of AChE against five different inhibitor concentrations were calculated for compounds that showed inhibitory effects. The same experiment was also performed for the reference inhibitor Tacrine. The control activity was accepted as 100% and the graphs of % Activity versus inhibitor concentration were drawn for the molecules showing the inhibition effect and for the reference inhibitor. From these graphs, IC50 values, which express the inhibitor concentration that halves the enzyme activity, were determined.

* 1. **Molecular Docking Studies**

The 3D crystal structures of AChE (PDB code 4EY7), hCA I (PDB code 6G3V), and hCA II (PDB code 3PO6) were received from the Protein Data Bank website(Angeli, Ferraroni, & Supuran, 2018; Cheung et al., 2012; Mader et al., 2011). The 3D molecular structures of compounds were retrieved from PubChem website. Molecular docking calculations were performed with Molegro Virtual Docker using the MolDock Score function(Thomsen & Christensen, 2006). For docking studies, a region on the crystal structures where the inhibitor is placed in the center was selected. Docking protocols were first evaluated with co-crystal ligands via redocking experiments. The best-docked pose was taken into account for each compound in docking studies. The Discovery Studio 2021 Client was used to visualize interaction at the molecular level of the docking experiments.

* 1. **Molecular dynamic simulation**

Molecular dynamics (MD) simulation was carried out using GROMACS 2022.2. The following steps were utilized.

* + 1. **Preparation of enzyme**

The 3-dimensional (3D) models of enzyme, human carbonic anhydrase I (PDB ID: 6G3V) in complex with CA1 and CA2 molecules were exported to .pdb format using Pymol. The dynamic behavior of the complexes was evaluated using molecular dynamic (MD) simulation in the GROMACS package program (version 2022.2) **[1-3]**. Protein topology was constructed by pdb2gmx with the CHARMM27 force field **[4],** and ligand topology was generated using the SwissParam server **[5]**.

* + 1. **Setting up system for simulation**

After applying the force field, the complexes were inserted into the system. They were solvated with the SPC water model **[6]** in a cubic box greater than 1 nm from the edge of the protein with periodic boundary conditions. The system was neutralized by adding Na+ ions, and energy minimization was done for 50,000 steps using the steepest descent algorithm. It was then followed by 100 ps of NVT simulation at 300 K and 100 ps of NPT simulation to equilibrate the whole system. Leapfrog algorithm was employed in the constant-temperature, constant-pressure (NPT) ensemble to separately couple each component like protein, ligand, water molecules, and ions **[7]**. The Berendsen temperature and pressure coupling constants were set to .1 and 2, respectively, to keep the system in a stable environment (300 K temperature and 1 bar pressure) **[8]**. Finally, MD simulation for 100 ns was performed in isothermal and isobaric condition ensemble at 300 K. The pressure coupling with time-constant was set at 1 ps to maintain pressure constant at 1 bar, and LINCS algorithm **[9]** was used to constrain the bond lengths. The Van der Waals and Coulomb interactions were truncated at 1.2 nm, and the PME algorithm **[10]** inbuilt in GROMACS was used to minimize the error from truncation.

* + 1. **Visualization and analysis of simulation**

The trajectory files are visualized through VMD (Visual Molecular Dynamics) 1.9.2. **[11]** and analyzed by indigenously developed tool HeroMDAnalysis **[12, 13]** and Xmgrace 5.1.25 **[14]**.

1. **Results and Discussion**
   1. ***In Vitro* Inhibition Studies**

Due to the function of CA isoenzymes in balancing pH and bicarbonate levels in various tissues, these isoenzymes have become valuable tools in medicinal chemistry and clinical applications. CA inhibitors are of clinical importance in the treatment of various metabolic disorders, from glaucoma to cancer and even epilepsy. To date, various CA inhibitors have received drug approval, but due to their side effects and nonspecificity, there is a need to identify new, more effective and specific CA inhibitors. On the other hand, the most researched group of compounds in drug development studies for the treatment of Alzheimer's disease are AChE inhibitors. Because AChE inhibitors play a role in improving cholinergic synapses in AD disease by increasing Acetylcholine levels in the brain and thus slowing down cognitive decline. AChE inhibitor drugs tacrine, rivastigmine, galantamine, metrifonate, and donepezil have been developed for the treatment of AD. However, these drugs have very serious side effects and cannot provide a complete cure for AD disease. For this reason, today it is very important to identify new AChE inhibitors that have fewer side effects and are more effective against AD disease, and many scientific studies are being carried out in this field. In the field of medicinal chemistry, amino acid derivatives are widely used as enzyme inhibitors. In addition, amino acid derivatives are a group of compounds that attract a lot of attention in prodrug development studies, both due to the important chemical properties of amino acids and their tendency to be transported across cell membranes. In this study, the inhibitory effects of some non-proteinogenic amino acid compounds (hippuric acid (**a**), *N*-(9-Fluorenylmethoxycarbonyl)-D-valine (**b**), *N*-Z-(1-Benzotriazolylcarbonyl) methylamine (**c**), (*S*)-*N*-Z-1-Benzotriazolylcarbonyl-2-phenylethylamine (**d**)) on both hCAI, hCAII and AChE activities were investigated.

Inhibitory effects of **a-d** compounds on hCA-I, hCA-II, and AChE activity were determined by IC50 (inhibitor concentration that halves the activity) values. IC50 value means the inhibitor concentration that reduces the activity of the enzyme by half, and low IC50 value indicates high inhibition power. The **a** molecule had an inhibitory effect on both hCAI and II isoenzymes and AChE activity. Considering the IC50 values (IC50 for hCAI: 43.04 µM, IC50 for hCAII: 55.92 µM, IC50 for AChE: 233.3 µM) it had a stronger inhibitory effect on hCAI and II isoenzymes than AChE. Molecule **c** did not show any inhibitory effect on either CAI and II or AChE activity. While **b** molecule had an inhibitory effect on AChE activity (IC50 value of **b** for AChE: 100 µM), it did not affect the activity of hCAI and II isoenzymes. Molecule **c** had no inhibitory effect on either hCAI and II or AChE activity. While **d** molecule had no inhibitory effect on AChE activity, it had a strong inhibitory effect on hCAI and hCAII activity. The IC50 values of molecule **d** for hCAI and hCA were determined as 0.840 µM and 0.661 µM, respectively. Accordingly, molecule **d** had a stronger inhibitory effect than molecule **a** for hCAI and II isoenzymes. Acetazolamide was used as reference inhibitor for hCAI and hCAII, and tacrine was used as reference inhibitor for AChE. Inhibition results are summarized in **Table 1**. When the results were compared with the reference inhibitors, the molecules that had an inhibitory effect on the enzymes in question had less inhibitory power than the reference inhibitors, but the fact that they showed inhibition effects at the micromolar level may be useful in synthesizing new amino acid derivative inhibitors. Additionally, in our studies, molecular docking and molecular dynamics simulation studies were performed to elucidate the inhibition results.

**Table 2.** In vitro inhibition results

|  |  |  |  |
| --- | --- | --- | --- |
| **Compounds** | **IC50 for hCAI** | **IC50 for hCAII** | **IC50 for AChE** |
| **a** | 43.04 µM | 55.92 µM | 233.3 µM |
| **b** |  |  | 100 µM |
| **c** | - | - | - |
| **d** | 0.840 µM | 0.661 µM | - |
| **AZA** | 0.473 µM | 0.104 µM | - |
| **TAC** | - | - | 0.159µM |

**IC50 grafikleri eklenecek**

* 1. **Molecular Docking Studies**

The binding modes predicted for the **d** into the hCA-I and hCA-II active cavity was displayed in **Figure 2**, and its interactions detail tabulated in **Table 2**. The top-ranked pose of **d** in hCA-I active region generated by Molegro Virtual Docker has -134.271 MolDock Score. It was mediating hydrogen bond interactions with the side chain residues of Gln92. The benzene rings of it formed favorable hydrophobic contacts with Leu198, Val143, His94, His200, Ala135 and Leu131 and Pi-Cation interaction with a catalytic Zn ion. Also, it interacted with Tyr199, Leu141, Tyr204, His67, Ala132, His64, Trp5, Pro201, Ala121, Phe9, Trp209 and His119 via van der Waals.

The binding energy of the compound **d** towards the hCA-II was found to be -152.271 Moldock Score. The compound showed Pi-Pi Stacked interaction with His94, Pi-Pi T-shaped interaction with Phe131, and Pi-Alkyl interactions with Val121, Leu198, Val121, Val143, and Leu198. It binds within the pocket of hCA-II by forming hydrogen bonds with Tyr200, Gln92, Asn62, and Asn67 264, and Pi-Cation interactions with the zinc ion. The docked pose of the compound shows that the 2H-benzotriazole and benzene rings of the ligand are located near the zinc ion. It takes part in the first contact by making a nucleophilic attack in the interaction with carbonic anhydrase and carbon dioxide (Supuran, 2008). It is thought that one of the most important factors of the strong inhibition effect of the compound on the enzyme activity may be this proximity.

In order to obtain more information on the binding mode and interactions of the compound **b** in the active site of the AChE enzyme, molecular docking analysis was enforced. The interaction details and representations of the docking result are depicted in Table 3 and Figure 3. For it the binding affinity is found to be -145.039 MolDock Score. It interacted with the protein via both H-bond and hydrophobic contacts. The carboxyl group of the compound is localized inside the acyl and anionic binding site, making three hydrogen bonds with TYR337 and TYR341. His447 and Trp86 residues mainly contribute to the attachment of the molecule to the catalytic binding site via Pi-Pi Stacked interactions. Also, it formed Pi-Sigma interaction with TYR341 and Pi-Alkyl interaction with Tyr124, Phe338x2 and Phe297. The N-(9-Florenilmetoksikarbonil)-D-valin molecule is stabilized in the AChE binding cavity mostly through van der Waals interactions with residues Trp286, Gly121, Gly122, Ser203, Glu202, Gly448, Ser125, Asp74, and THR83.

**Table 2.** Important interactions of compound **d** in the hCA-I and hCA-II active cavity

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Category | Types | Residues | Distance |
| Carbonic anhydrase II | Hydrogen Bond | Conventional Hydrogen Bond | ASN62 | 2.73 |
| ASN67 | 2.17 |
| GLN92 | 1.96 |
| THR200 | 2.52 |
| Electrostatic | Pi-Cation | ZN1 | 4.81 |
| ZN1 | 4.04 |
| ZN1 | 3.62 |
| Hydrophobic | Pi-Pi Stacked | HIS94 | 4.08 |
| Pi-Pi T-shaped | PHE131 | 4.71 |
| Pi-Alkyl | VAL121 | 4.87 |
| LEU198 | 5.02 |
| LEU198 | 5.05 |
| VAL121 | 5.16 |
| VAL143 | 4.98 |
| LEU198 | 5.15 |
| Carbonic anhydrase I | Hydrogen Bond | Conventional Hydrogen Bond | GLN92 | 2.15 |
| Electrostatic | Pi-Cation | ZN1 | 3.52 |
| Hydrophobic | Pi-Sigma | LEU198 | 3.40 |
| Pi-Pi T-shaped | HIS94 | 5.50 |
| HIS94 | 5.08 |
| HIS200 | 4.35 |
| Pi-Alkyl | LEU131 | 5.06 |
| ALA135 | 3.42 |
| VAL143 | 5.11 |
| LEU198 | 4.73 |

**Table 3.** Important interactions of compound **b** in AChE active site

|  |  |  |  |
| --- | --- | --- | --- |
| Category | Types | Residues | Distance |
| Hydrogen Bond | Pi-Donor Hydrogen Bond | TYR337 | 3.86 |
| TYR341 | 3.79 |
| Hydrophobic | Pi-Sigma | TYR341 | 3.68 |
| Pi-Pi Stacked | TRP86 | 4.34 |
| TRP86 | 4.17 |
| TRP86 | 4.02 |
| TRP86 | 5.51 |
| HIS447 | 5.14 |
| Pi-Alkyl | TYR124 | 4.89 |
| PHE297 | 4.37 |
| PHE338 | 5.03 |
| PHE338 | 4.63 |

diyagram içeren bir resim

Açıklama otomatik olarak oluşturuldu

**Figure 2.** Interaction diagrams for hCA-I and hCA-II binding site with compound **d**

diyagram, çizgi içeren bir resim

Açıklama otomatik olarak oluşturuldu

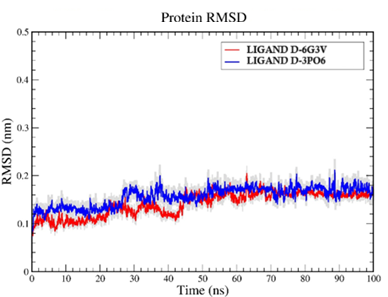
**Figure 3.** Interaction diagrams for AChE binding site with compound **b**

* 1. **Molecular Dynamic Simulation**
     1. **Molecular dynamic simulations of human carbonic anhydrase I in complex with hCA-I and hCA-II**

In order to evaluate the binding of molecules, CA1 (PDB ID: 6G3V) and CA2 (PDB ID: 3PO6) against human carbonic anhydrase, we have carried out MD simulations for a period of 100 ns for two models namely, LIGAND D-CA1 and LIGAND D-CA2. Their simulations were evaluated using various statistical parameters including: Root-Mean-Square-Deviation (RMSD), Root-Mean-Square-Fluctuation (RMSF), h-bond interactions, and its %occupancies over the time.

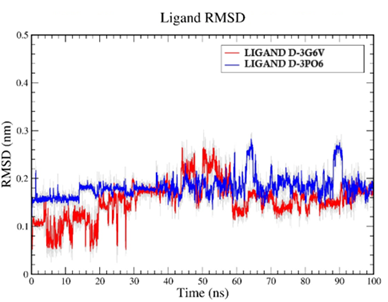
* + 1. **RMSD analysis**

Analyzing the protein-RMSD can give insights into any structural conformation that protein undergoes during the simulation. The multiplot for protein Cα versus time for 2 simulations is shown in figure 4. Both the complexes, LIGAND D-CA1 and LIGAND D-CA2 has attained an equilibrium RMSD value of around 0.15 nm. The approximate value less than 0.2 nm indicates that both the complexes were stable throughout the simulation.



**Figure 4:** Graphical representation of the plots showing protein Cα RMSD (nm) versus time (100 ns) for (A) LIGAND D-CA1 (red in color) and (B) LIGAND D-CA2 (blue in color) complex.

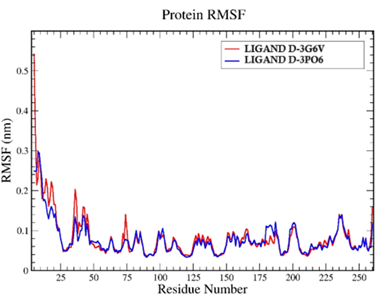
Analysing the ligand-RMSD indicates how stable the ligand is with respect to the protein and its binding pocket. The multiplot for ligand RMSD (nm) versus time for 2 simulations is shown in Figure 5. The ligand, CA2 have displayed a relative better deviation across the simulation. It attained an equilibrium value of 0.19 nm while the ligand CA1 has displayed quite a distress across the simulation while also ending up with the value of 0.19 nm. The RMSD values observed in both the cases has somewhat pointed towards supremacy of ligand CA2 over CA1, in binding the human carbonic anhydrase I.



**Figure 5:** Graphical representation of the plots showing ligand RMSD (nm) versus time (100 ns) for (A) LIGAND D-CA1 (red in color) and (B) LIGAND D-CA2 (blue in color) complex.

* + 1. **RMSF analysis**

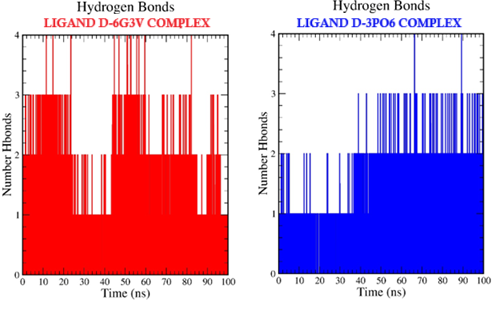
The Protein-RMSF is useful for characterizing local changes along the protein chain. The multiplot for protein-RMSF (nm) versus residue number index is shown in figure 6. Notably, the plot describes fluctuation of less than 0.25 nm for both the ligand-protein complexes. Also, the residues of the binding cavity have displayed even lower fluctuation, this indicated stability of ligand in terms of binding the protein.



**Figure 6:** Graphical representation of the plots showing the protein RMSF (nm) versus residue index number of protein for (A) LIGAND D-CA1 (red in color) and (B) LIGAND D-CA2 (blue in color) complex.

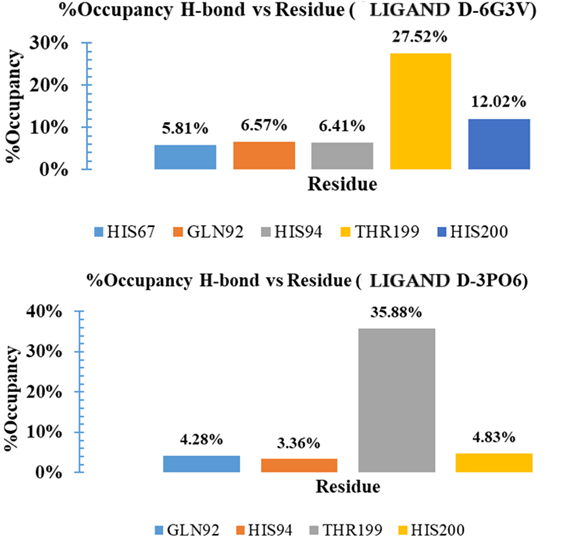
* + 1. **H-bond interaction**

Molecular interactions, particularly the h-bond interactions are distance and angle depend and liable to disrupt under dynamic conditions. Herein, we have analyzed the ligand-protein interactions for both the complexes. The plot for number of hydrogen vs time is being shown for (A) LIGAND D-CA1 (red in color) and (B) LIGAND D-CA2 (blue in color) complex. in figure 7. From the plots, it is obvious that both ligands, CA1 and CA2 have shown an average of 2 stable h-bond contacts during the simulation.



**Figure 7:** Pictorial representation of the number of h-bond contacts formed by ligand, (A) CA1 (red in color) and (B) CA2 (blue in color) complex.

The figure 8, represents the histogram of %occupancies of the h-bond contacts formed by CA1 and CA2, respectively. In case of CA1, the most stable interaction was with the residue THR199 with the occupancy of 27.52%. The other notable h-bond interactions with residues HIS200, GLN92, HIS94 and HIS67 were stable for 12.02, 6.57, 6.41 and 5.81% duration of simulation. In case of CA2, the most stable interaction was with residue THR199, which was stable for 35.88% duration of simulation. The other weak interactions were with residues HIS200, GLN92, and HIS94 with %occupancies of 4.83, 4.28, and 3.36%, respectively. Overall, it can be concluded that both the ligands are equally efficient in binding inside the cavity of human carbonic anhydrase I.



**Figure 8:** Histogram representation of %occupancies of the h-bond protein ligand contacts of (A) CA1 (red in color) and (B) CA2 (blue in color) complex.