REPUBLIC OF TURKEY
YILDIZ TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

EFFECTS OF SOME ANGIOTENSIN CONVERTING ENZYME-INHIBITOR DRUGS USED FOR HEART DISEASES ON ESTERASE ACTIVITY OF HUMAN CARBONIC ANHYDRASE I AND II

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ISTANBUL, 2017

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ACKNOWLEDGEMENTS

I would like to thank,

My advisor Prof. Dr. Barbaros NALBANTOĞLU, who guides and supports me in conducting MSc thesis, I have benefited greatly from his experiences and knowledge.

Assoc. Dr. Murat ŞENTÜRK from Chemistry Department at Ağrı İbrahim Çeçen University for helping and supporting me I'm really grateful to him.

I would like to thank all the staff in the the Central Research Laboratories at Ağrı İbrahim Çeçen University, I was able to participate and work in their laboratories.

Prof.Dr. Emine KARAKUŞ the head of Chemistry Department, at Yıldız Technical University.

My supportive family; my parent, big brother and sisters have always been proud and supportive of me, and everyone who has made this achievement possible, I'm grateful to you all.

December, 2017

Mohammed Salah Malallah AL-TAMEEMI

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LIST OF SYMBOLS

A	Angstrom
h	hour
I	Inhibitor
L	Litre
M	Molarity
m	milli
n	nano
S	Substrate
α	Alpha
β	Beta
γ	Gamma
δ	Delta
ζ	Zeta
η	Eta
μ	Micro
ΔG	Gibbs free energy
×g	Times of earth gravitational force

LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
AG	Anchoring Group
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
CA	Carbonic Anhydrase
CAIs	Carbonic Anhydrase Inhibitors
CNS	Central Nervous System
Cys	Cysteine
DNA	Deoxyribonucleic Acid
E.C	Enzyme Commission number
EU	Enzyme Unit
GI Tract	Gastro Intestinal Tract
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
hCA-I	human Carbonic Anhydrase I
hCA-II	human Carbonic Anhydrase II
His	Histidine
IC_{50}	The Inhibitor Concentration, which reduces the maximum rate to half.
Ile	Isoleucine
Kcal	Kilocalorie
K_{cat}	constant of turnover rate of ES complex to P and E
kDa	kilo Dalton
K_m	Michaelis constant
Leu	Leucine
Lys	Lysine
PDB	Protein Data Bank
PAGE	Polyacrylamid Gel Electrophoresis
PER	Ammonium Persulphate
pH	-log of the hydrogen ion concentration
Phe	Phenylalanine
pKa	-log10 of the acid dissociation constant (Ka) of a solution
Pro	Proline
Pyr	Pyroglutamic acid
RNA	Ribonucleic Acid
Ser	Serine
SDS	Sodium Dodecyl Sulfate

SG	Sticky Group
TEMED	N, N, N ', N'-tetramethylenediamine
Thr	Threonine
Tpr	Tetratricopeptide repeat
Tris	Trihydroxymethylaminomethane
Trp	Tryptophan
Tyr	Tyrosine
UCSF	University of California, San Francisco
UV / Vis	Ultraviolet-Visible
Val	Valine
ZBF	Zinc Binding Functions
ZBG	Zinc Binding Group

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ABSTRACT

EFFECTS OF SOME ANGIOTENSIN CONVERTING ENZYME-INHIBITOR DRUGS USED FOR HEART DISEASES ON ESTERASE ACTIVITY OF HUMAN CARBONIC ANHYDRASE I AND II

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Effect of ACE-inhibitor drugs (Cilazapril, Perindopril, Ramipril, Trandolapril, and Zofenopril calcium) used for heart diseases were investigated in vitro on esterase activity of human carbonic anhydrase I and II (hCA-I and hCA-II) purified from human erythrocytes by using Sepharose-4B-L-tyrosine-sulfanilamide affinity gel. hCA-I was purified 94.62 fold with a specific activity of 704.35 EU/mg protein and 67.5 % yield, hCA-II was purified 470.19 fold with a specific activity of 3500 EU/mg protein and 54.69% yield. Afterwards purity of isozymes was controlled by SDS-PAGE. In order to determine the effect of these drugs, absorbances were determined at 348 nm by hydrolysis of p-nitrophenylacetate which is the substrate. IC_{50} values were calculated by drawing % Activity-[Inhibitor] graphs. IC_{50} values of these drugs were between 0.090 - 0.226 μM for hCA-I esterase activity and between 0.195 -0.201 μM for hCA-II esterase activity. All of these drugs showed inhibition effect on both hCA-I and hCA-II. Trandolapril and Perindopril showed the best inhibition effect on hCA-I and hCA-II, respectively. Molecular docking studies were used in order to understand the interaction of these drugs with the domains of hCA-I and hCA-II. All of these drugs interact with amino acids in the binding pocket of hCA-I and hCA-II except Zofenopril calcium in case of hCA-II interacts with H_2O in active site. In the light of the results, it can be concluded that in vivo investigation of these drugs on carbonic anhydrase may be important. These drugs may be lead to increase tissue CO_2 concentrations and lower

tissue pH, resulting in vascular dilation and increased blood flow, which means that the patient could be suffering from the onset of a new disease during using these drugs.

Key words: ACE-inhibitor drugs, hCA-I, hCA-II, esterase activity, $IC_{50,}$ molecular docking.

ÖZET

İNSAN KARBONİK ANHİDRAZ I VE II'NİN ESTERAZ AKTİVİTESİ ÜZERİNE KALP HASTALIĞINDA KULLANILAN BAZI ANJİYOTENSİN DÖNÜŞTÜRÜCÜ ENZİM-İNHİBİTÖR İLAÇLARININ ETKİLERİ

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Kimya Anabilim Dalı

Yüksek Lisans Tezi

Tez Danışmanı: Prof. Dr. Barbaros NALBANTOĞLU

Kalp hastalıkları için kullanılan ACE inhibitörü ilaçlarının (Silazapril, Perindopril, Ramipril, Trandolapril ve Zofenopril kalsiyum) etkileri, insan eritrositlerinden Sepharose-4B-L-tyrosine-sulfanilamide afinite jeli kullanılarak saflaştırılan karbonik anhidraz I ve II'nin (hCA-I ve hCA-II) esteraz aktivitesi üzerine in vitro olarak araştırılmıştır. hCA-I, 704.35 EU/ mg protein spesifik aktivite ve % 67.5 verim ile 94.62 kat saflaştırıldı. hCA-II, 3500 EU/ mg protein spesifik aktivite ve % 54.69 verimle 470.19 kat saflaştırıldı. Daha sonra izoenzimlerin saflığı SDS-PAGE ile kontrol edildi. Bu ilaçların etkisi substrat olan p-nitrofenil asetatın hidrolizi ile oluşan ürünün 348 nm'de absorbansının okunmasıyla belirlendi. IC_{50} değerleri, % Aktivite-[İnhibitör] grafikleri çizilerek hesaplandı. Bu ilaçların IC_{50} değerleri, hCA-I esteraz aktivitesi için 0.090-0.26 μM, hCA-II esteraz aktivitesi için 0.195-0.201 μM arasındaydı. Bu ilaçların tümü hem hCA-I hem de hCA-II üzerinde inhibisyon etkisi gösterdi. Trandolapril ve Perindopril sırasıyla hCA-I ve hCA-II üzerinde en iyi inhibisyon etkisini gösterdi. Bu ilaçların hCA-I ve hCA-II ile etkileşimini anlamak için moleküler yerleştirme çalışmaları yapıldı. Bu ilaçların tümü, hCA-I ve hCA-II'nin bağlanma bölgesindeki amino asitler ile etkileşirken, sadece Zofenopril kalsiyum hCA-II'nin aktif bölgesinde H_2O ile etkileşmektedir. Bu sonuçlar ışığında, bu ilaçların karbonik anhidraz üzerinde in vivo araştırmasının önemli olabileceği sonucuna varılabilir. Bu ilaçların kullanımı nedeniyle doku CO_2 konsantrasyonu arttırılarak ve doku pH düşürülerek vasküler

genişleme ve artmış kan akışı ortaya çıkabilir. Bu durum da, bu ilaçların kullanımı sırasında hastanın yeni bir hastalığın başlangıcından muzdarip olabileceği anlamına gelebilir.

Anahtar Kelimeler: ACE inhibitörü ilaçlar, hCA-I, hCA-II, esteraz aktivitesi, IC_{50}, moleküler yerleştirme.

CHAPTER 1

INTRODUCTION

1.1 Literature Review

Carbonic anhydrase (CA) is one of the most important enzymes due to their hydration activity of CO_2 to HCO_3^- and H^+. In 1930, carbonic anhydrase enzyme had been discovered by Meldrum, Roughton, Stadie, and O'Brien unaware of each other. Meldrum and Roughton purified the carbonic anhydrase from human erythrocyte at a high purity and in the late of 1930, the enzyme was purified from bovine erythrocytes. In 1944, Keilin and Martin discovered the presence of zinc in carbonic anhydrase, and they understood the specific role of zinc in catalysis activity. Thus, the enzyme had been mentioned as a first metalloenzyme to be identified. CA enzymes have been purified and characterized from other sources. CA-II isozyme is one of the most efficient enzymes with a number of turnover $1.3\text{-}1.9\text{x}10^6$ s^{-1} in physiological conditions It was found that human CA-II gene is similar to CA-I and CA-III genes more than 8 chromosomes. At the present time six distinct and unrelated CA gene families (α, β, γ, δ, ζ and η-CAs) have been identified [1-8].

Several studies have been carried out on the activities of CA-I and CA-II isozymes with medicines, chemically synthesized compounds, and sulfonamide derivatives. CA inhibitors, especially aromatic and heterocyclic sulfanilamides have been used clinically for many years in the treatment of many diseases such as glucocorticoid, acid-base imbalance, gastric and duodenal ulcer. In addition, inhibitors are also administered as antitumor agents [9-11].

The last research was done by Iranian researchers; they worked on captopril and enalapril which are ACE inhibitor drugs used for heart diseases. The inhibition effect against esterase activity of human CA-II purified from human erythrocyte was studied.

Both captopril and enalapril can be considered as inhibitors of CA esterase activity at micromolar concentrations [12].

1.2 Objective of the Thesis

Five drugs (Cilazapril, Perindopril, Ramipril, Trandolapril, Zofenopril calcium) from ACE inhibitors used for heart diseases were investigated in order to determine their effect on esterase activity of human CA-I and CA-II purified from human erythrocytes.

1.3 Hypothesis

Investigation of the effects of drugs on hydratase or esterase activities of hCA-I and hCA-II is important for drugs design which are used to treated different diseases by inhibiting or activating some enzymes that responsible of these diseases. At the same time, the drugs may inhibit or activate other enzymes that belong to the same group lead to occur a disease as an unknown side effect of used drugs. In this study, the investigated drugs had been chosen in order to the fact both ACE and CA as called metalloenzymes. The findings of this study may be lead to consider these drugs as new inhibitors or activators for hCA-I and hCA-II by in vitro esterase activity. Other in vivo investigations are also required in order to understand inhibition or activation of hCA-I and hCA-II means an abnormal level of CO_2 and HCO_3^- in the body tissues that may gradually lead to occur of different diseases.

CHAPTER 2

GENERAL INFORMATION

2.1 Carbonic Anhydrase (CA)

Carbonic anhydrase (CA, EC 4.2.1.1) is ubiquitous (metalloenzyme) zinc enzyme found in all living systems (animals, plants, algae, and bacteria). CA plays important role in biological systems by catalyzing the reversible hydration of CO_2 to HCO_3^- and H^+. Six distinct and unrelated CA gene families (α, β, γ, δ, ζ and η-CAs) have been identified [7], [8]. CA isoforms in animals are involved in a number of physiological functions and homeostasis such as respiration and gas exchange, for example "transport of CO_2 and HCO_3^- between body tissues and respiratory surfaces and it can change hemoglobin's affinity for oxygen by controlling the movement of CO_2 gas between air and liquid compartments", pH homeostasis, bone resorption and calcification, electrolyte transport in various epithelia, and involve in biosynthesis of important biomolecules such as urea, glucose, lipids, pyrimidines, detoxification of metabolic wastes and tumorigenicity [13], [14]. The enzyme also catalyzes hydration of various aldehydes as well as hydrolysis of esters in other living cells such as algae, plants and some bacteria [15]. CA isoforms are also fundamental for photosynthesis [16], [17].

2.2 Carbonic Anhydrase Families

2.2.1 α-Carbonic Anhydrases

The α-carbonic anhydrases are a monomeric or dimeric molecule found in animals, some fungi, bacteria, algae and green plants [18]. In mammals, sixteen different α-carbonic anhydrase isozymes were isolated; fifteen from their present in humans but the CA XV isozyme is not expressed in humans, with very different subcellular localization and tissue distribution (Table 2.1) [9-17]. From these fifteen isozymes, twelve of them

(CAI, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV) have catalytic activity, the remaining three isozymes (CARP VIII, CARP X, and CARP XI) are known as CA-related proteins and they do not have the catalytic activity [19-26]. Many of these isozymes are important targets for inhibitors or activators used in clinical applications.

Table 2.1 The α-CA isozymes and their subcellular localization (h: human, m: mouse).

Isozyme	**Subcellular localization**	**Tissue/ organ localization**
hCA-I	Cytosol	Erythrocytes, GI tract
hCA-II	Cytosol	Erythrocytes, eye, GI tract, bone, osteoclasts, kidney, lung, testis, brain
hCA-III	Cytosol	Skeletal muscle, adipocytes
hCA-IV	Membrane bound	Kidney, lung, pancreas, brain, capillaries colon, heart muscle.
hCA-VA	Mitochondria	Liver
hCA-VB	Mitochondria	Heart, skeletal muscle, pancreas, kidney, spinal cord, GI tract
hCA-VI	Secreted (saliva /milk)	Salivary, mammary glands
hCA-VII	Cytosol	CNS
hCA-VIII	Cytosol	CNS
hCA-IX	Transmembrane	Tumors, GI mucosa
hCA-X	Cytosol	CNS
hCA-XI	Cytosol	CNS
hCA-XII	Transmembrane	Renal, intestinal, reproductive epithelia, eye, tumors
hCA-XIII	Cytosol	Kidney, brain, reproductive tract
hCA-XIV	Transmembrane	Kidney, brain, liver
mCA-XV	Membrane bound	Kidney

α-CAs catalyze a variety of reactions (Figure 2.1), such as the reversible hydration of CO_2 to bicarbonate (reaction 1), the hydration of cyanate to carbamic acid or the hydration of cyanamide to urea (reactions 2 and 3). The aldehyde hydration to gem-diols (reaction 4); the hydrolysis of carboxylic or sulfonic (reactions 5 and 6), as well as other less investigated hydrolytic processes, such as those described by equations 7– 9 [20], [21].

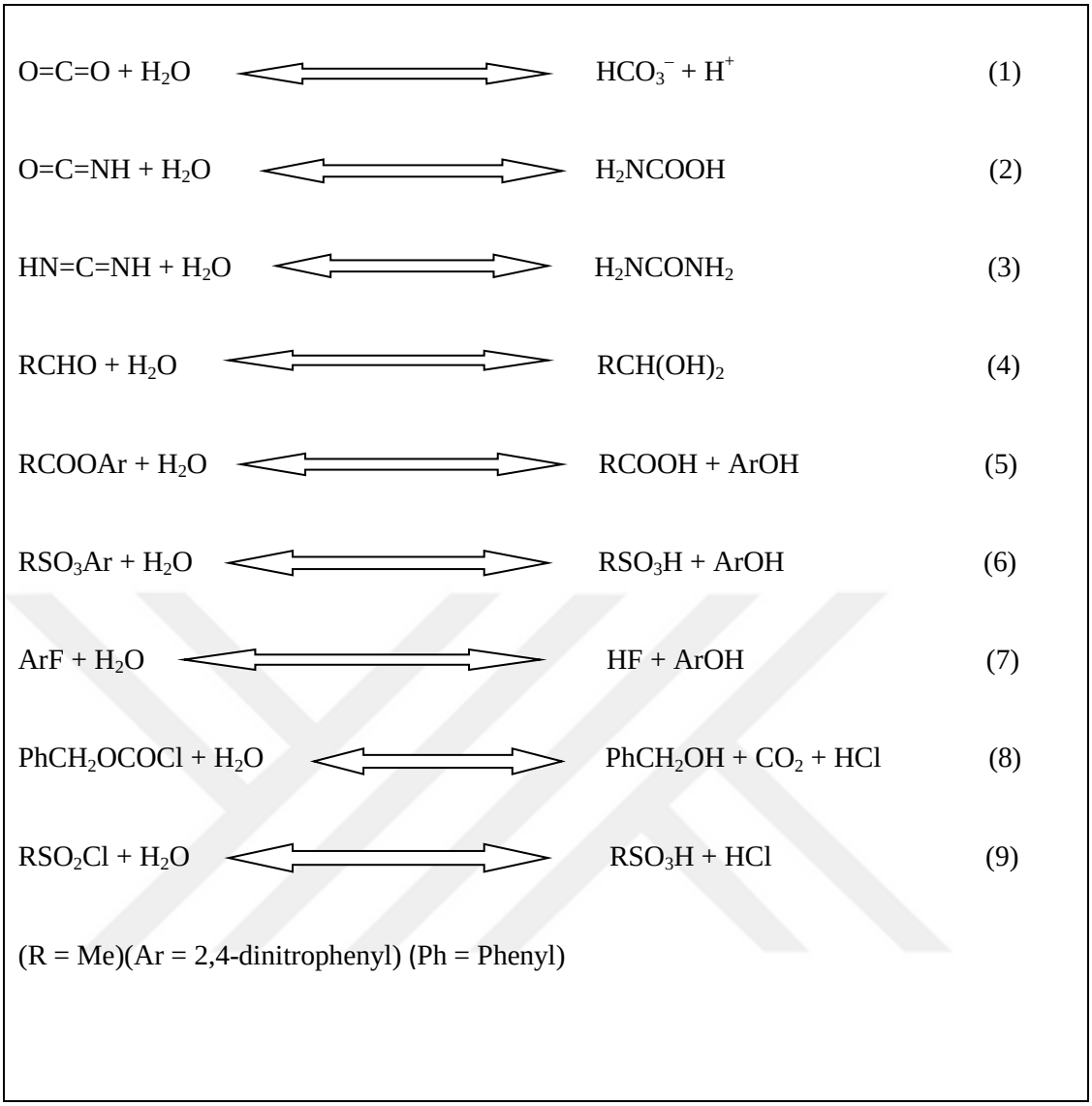


Figure 2.1 Reactions catalyzed by α-CAs.

2.2.2 ß-Carbonic Anhydrases

The ß-carbonic anhydrases are dimers, tetramers or octamers, generally formed by 2–6 monomers of a molecular weight of 25–30 kDa, and are expressed mainly in fungi, bacteria, archaea, algae, and chloroplasts of monocotyledons, dicotyledons, and some prokaryotes [21], [27-31].

2.2.3 y̓-Carbonic Anhydrases

The y̓-carbonic anhydrases are homotrimer with the approximate molecular weight of 70 kDa that has been described in bacteria, archaea and plants [22], [32], [33].

2.2.4 η-Carbonic Anhydrases

η-Carbonic anhydrases were found recently in some species of the Plasmodium genus. These groups are previously ascribed to α family, with a number of unique features. These features include their metal ion coordination pattern [8].

2.2.5 δ- and ζ-Carbonic Anhydrases

Delta and Zeta families are present in several classes of marine phytoplankton. The δ-class has been described in diatoms, and its prototype is the CA TWCA1 from the marine diatom Thalassiosira weissflogii. The ζ-CAs is probably monomers and has three slightly different active sites on the same protein molecule [34-36].

2.3 Zinc and Catalytic Site of Carbonic Anhydrase

Zn^{2+} is essential for catalysis in carbonic anhydrase located at the core of the enzyme active site. Generally exhibit a distorted tetrahedral geometry with only three O/N/S ligands (L) bound to the zinc ion (Figure 2.2 and Table 2.2), the fourth ligand being a water molecule that is an activated nucleophile for the catalytic process [37].

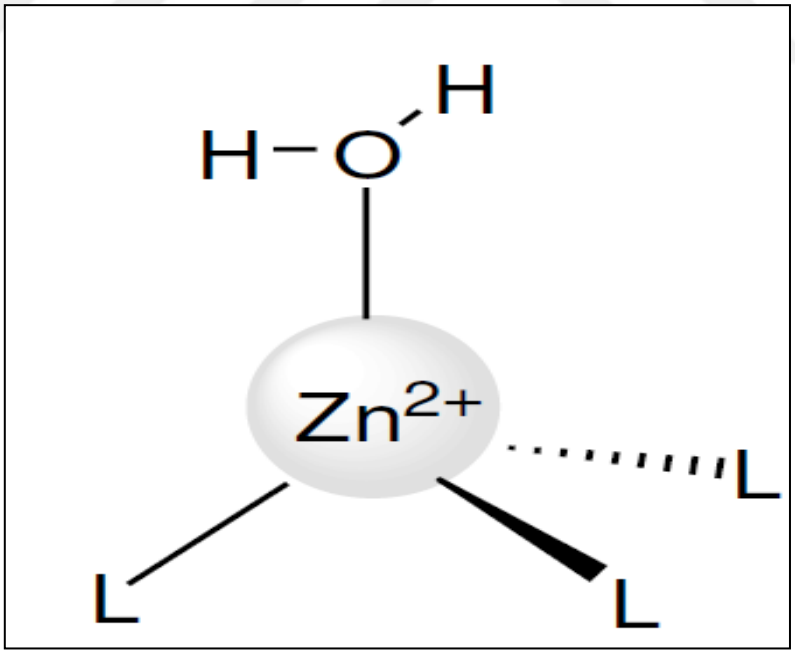


Figure 2.2 Tetrahedral geometry catalytic zinc binding sites of carbonic anhydrase isozymes [37].

Table 2.2 Ligands that represented in the carbonic anhydrase isozymes, by three key amino acid residues [38].

CA Classes	**Ligands**
α-CA	His, His, His
ß-CA	His, Cys, Cys
ɣ-CA	His, His, His
η-CA	His, His, His
δ-CA	His, Cys, Cys
ζ-CA	His, His, Gly

This Zinc-coordinated water molecule is crucial for the catalysis promoted by the metal center, as it can be either involved in the catalytic process as hydroxide ion or activated via the polarizing effect of the neighboring amino acids of the active site [37], [39], [40]. Moreover, H_2O can be displaced by the substrate (S) or expanded upon interaction with the substrate (Figure 2.3). The presence of water molecules in the coordination sphere is usually a distinguishing feature that allows to differentiate a catalytic zinc site from a structural one [41].

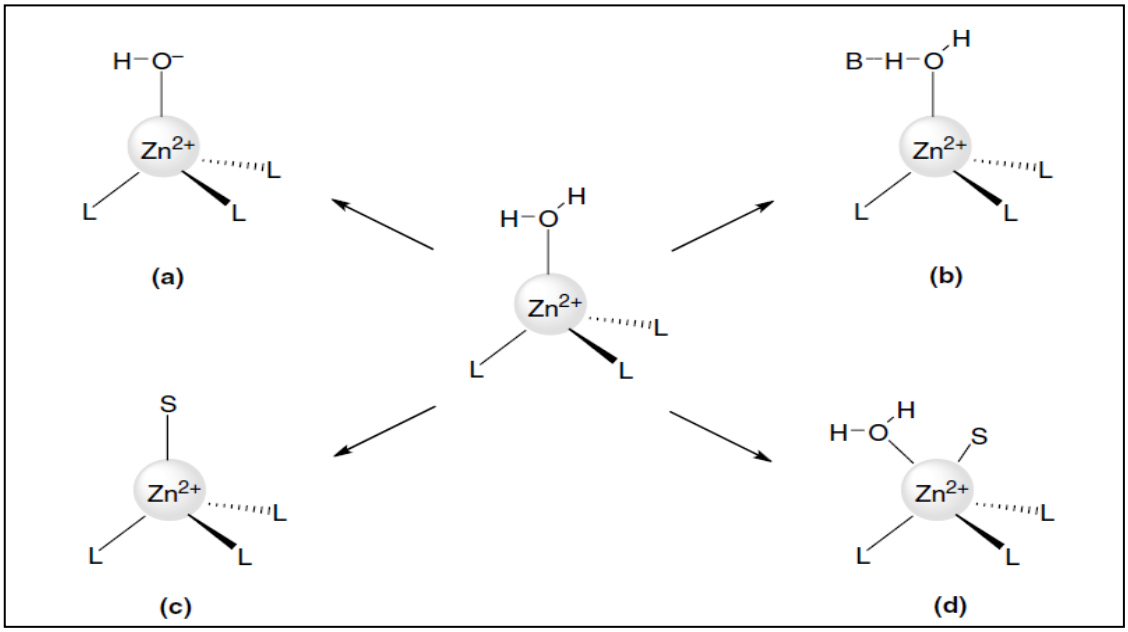


Figure 2.3 Role of the zinc-coordinated water molecule in catalysis: ionization (a), polarization (b), displacement (c), and expansion (d) [37], [39], [40].

In α-carbonic anhydrase X-ray crystallographic showed that the Zn^{2+} is located at the bottom of a 15A° deep active site cleft (Figure 2.4), interconnected by three histidine residues (His94, His96, and His119) and a water molecule/hydroxide ion [19-26], [42-47].

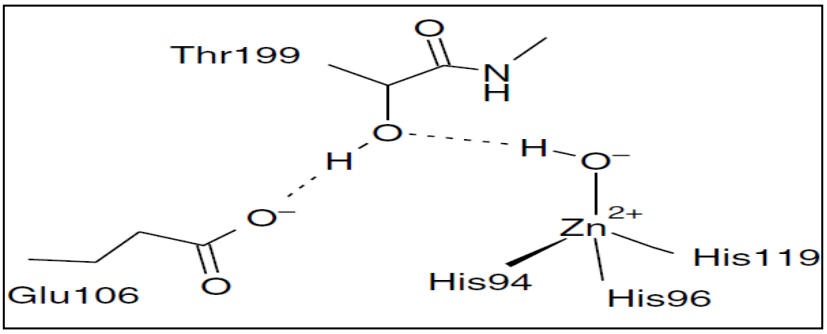


Figure 2.4 The Zn^{2+} coordination in the hCA-II active site, with the three histidine ligands (His94, His96, and His119) and the "gate keepers" (Thr199 and Glu106) [21].

2.3.1 Catalytic Mechanisms of Carbonic Anhydrase

Zn^{2+} is a major metal in catalytic mechanism for all classes, but other transition metals in (Table 2.3) have been demonstrated to bind to the catalytic site as physiologically-relevant metal cofactors or displacers of the native cofactor.

Table 2.3 Metals as physiologically-relevant cofactors of CA.

CA Family	Metals as physiologically-relevant CA cofactors	Ref.
α-CA	Zn^{2+}	[48]
ß -CA	Zn^{2+}	[28]
ɣ–CA	Fe^{2+} Zn^{2+}	[49],[50]
η-CA	Zn^{2+}	[8]
δ-CA	Zn^{2+}	[35]
ζ-CA	$Cd^{2+}Zn^{2+}$	[37]

Through a metal-hydroxide [Lig3M^{2+} (OH)] mechanism all CA isozymes catalyze the reversible hydration of CO_2 to HCO_3^- and H^+ [14], [47], [51].

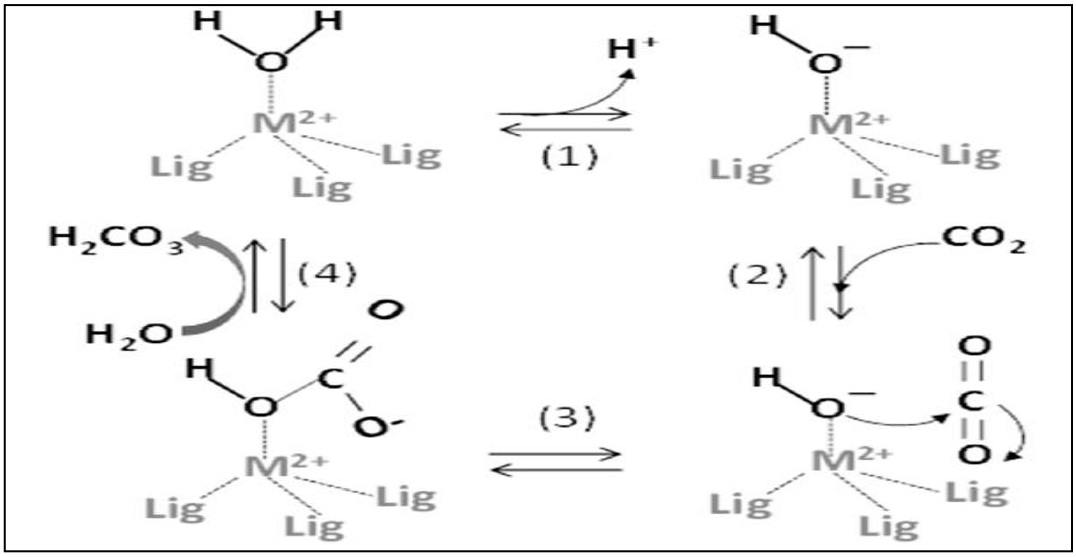


Figure 2.5 The reversible hydration of carbon dioxide to bicarbonate catalyzed by carbonic anhydrases by means of metal (M)-hydroxide mechanism [52].

This mechanism can be explained as in Figure 2.5 in four steps:

- The proton that released from the zinc-coordinated water cause to generates the zinc-coordinated OH^-
- CO_2 molecule binds to the active site and is positioned for optimal interaction with the zinc-coordinated OH^-.
- The hydroxide ion attacks the carbonyl of CO_2, producing HCO_3^-.
- The releasing of HCO_3^- regenerates the enzyme.

In I, II, IV, VII, IX, and XII–XIV isozymes the proton shuttle process is assisted by a histidine residue placed at the entrance of the active site (His64). By accepting a proton from the zinc-coordinated water, the imidazole moiety of this residue becomes protonated, the same proton may be transferred to the environments well as by a cluster of histidines, which lead to ensuring a very efficient proton transfer process for the most efficient CA isozyme, CA-II [21].

The process can be represented by equations:

$$\text{E-Zn}^{2+}\text{-OH}^- + \text{CO}_2 \Leftrightarrow \text{E-Zn}^{2+}\text{-HCO}_3^- \overset{\text{H}_2\text{O}}{\Leftrightarrow} \text{E-Zn}^{2+}\text{-OH}_2 + \text{HCO}_3^-$$

$$\text{E-Zn}^{2}\text{-OH}_2 \Leftrightarrow \text{E-Zn}^{2+}\text{-OH}^- + \text{H}^+$$

The rate-limiting step in catalysis is the second reaction, that is the proton transfer that regenerates the zinc hydroxide species of the enzyme [19-26], [46], [47]. From the previous explain it can be understood why the CA-II is one of the most active enzymes known (with a $k_{cat}/K_m = 1.5\times10^8\ M^{-1}\ s^{-1}$), close to the limit of diffusion control, and has important consequences for the design of inhibitors with clinical applications [19-21], [46], [47].

2.3.2 Inhibition Mechanisms for Carbonic Anhydrase

Understanding of the catalytic and inhibition mechanisms of carbonic anhydrases was helpful for the design of potent inhibitors. Modifications of the nature of the organic scaffold and tail moiety of the inhibitors to obtain tighter binding and eventually, isozyme-specific CA inhibitors have allowed to develop two general but complementary approaches [21], [53]:

- The ‘‘ring’’ approach, which consists of exploring a great variety of ring systems (aromatic or heteroaromatic) to which a sulfonamide ZBF is attached. and is used for the discovery of the topical antiglaucoma agents dorzolamide and brinzolamide.
- The ‘‘tail’’ approach, which consists of attaching different tails to the scaffolds of well-known aromatic/heterocyclic sulfonamides to modulate the physicochemical properties such as water solubility and enzyme binding capacity of these pharmacological agents.

The most successful one was ‘‘the tail approach’’ [19], [21], [54-57].

2.3.2.1 CA Inhibition by the Zinc Binders

These CA inhibitors bind in deprotonated form, as anions, to the Zn^{+2} ion from the enzyme active site, which is in a tetrahedral geometry, with the Zinc Binding Group (ZBG) (SO_2NH- moieties for the three main classes of inhibitors including sulfonamides, sulfamates, and sulfamides which are in fact sulfonamide isosters) directly interacting with the metal ion .The ZBG also interact with two other conserved residues in all α-CAs, acting as ‘‘gate keepers’’, i.e. Thr199 (hydrogen bonded through its OH group with the water molecule/hydroxide ion coordinated to the zinc in the uninhibited enzyme, and with the ZBG, as shown in Figure 2.6, in the enzyme-inhibitor

adducts) and Glu106, which is hydrogen bonded to Thr199 through its carboxylate moiety [54], [57-68].

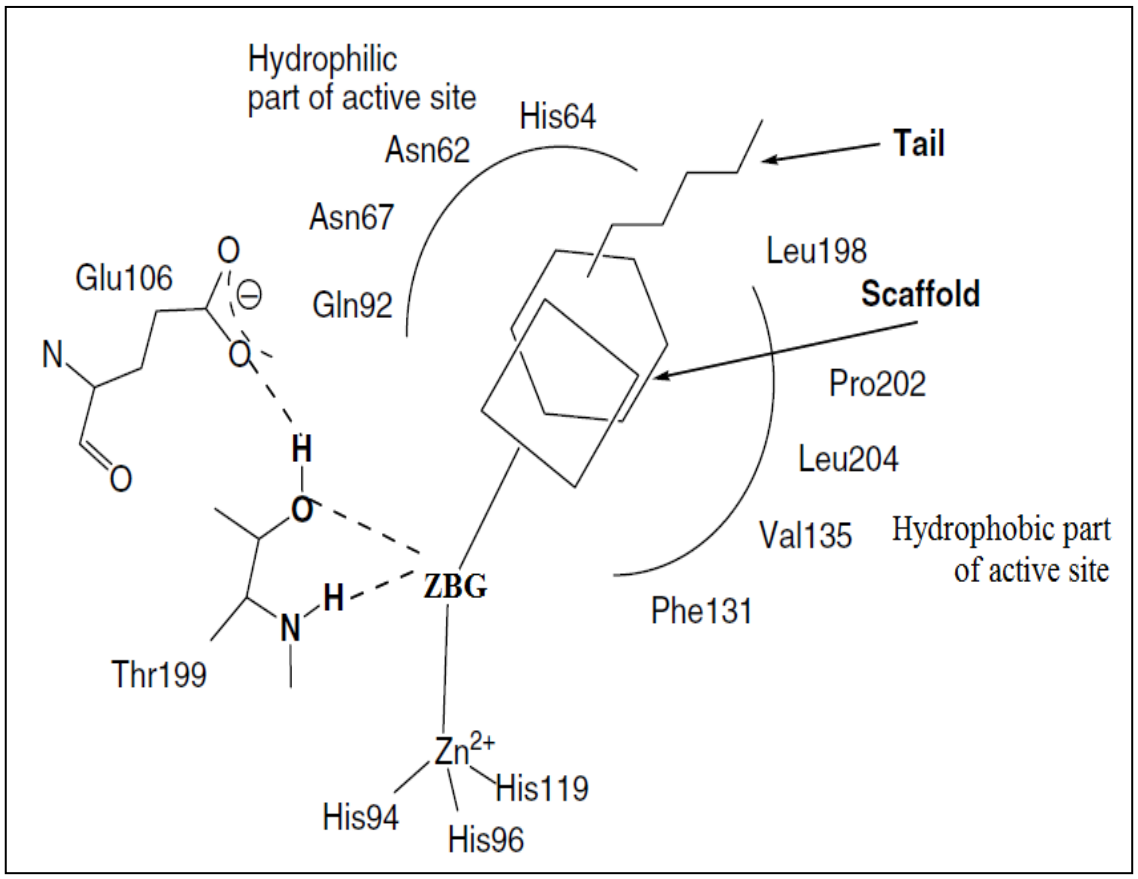


Figure 2.6 CAIs belonging to the zinc binders (sulfonamides, sulfamates, carboxylates, hydroxamates, phosphonates).

The ZBG is coordinated to the metal ion and participates in strong interactions with the gate keeper residues Thr199–Glu106. The scaffold may occupy either the hydrophilic or hydrophobic (or both) halves of the active site, whereas the tails generally are orientated toward the exit of the cavity where the most variable amino acid residues among the different mammalian isoforms are found [19], [21], [54], [68], [69].

2.3.2.2 CA Inhibition by Anchoring to the Zinc-Coordinated Water/Hydroxide Ion

These CA inhibitors are characterized by a presence of an anchoring group (AG) which is attached to a scaffold possibly incorporating tails which can interact with the two halves of the active site (as for the zinc binders). The main difference between the zinc binders and the inhibitors anchoring to the zinc nucleophile consist in the fact that the

inhibitor is not in a direct contact with the metal ion. As reported in the last year's by several groups, the AGs belong to a variety of chemical functionalities. They include phenolic OH [70-78], primary amine [79], COOH [77], COOMe [76], and SO_3H moieties [81]. The scaffolds of these compounds can be of the aromatic, aliphatic, heterocyclic, or sugar type [80-89], and many of them display efficient inhibitory activity against CAs belonging to the α- and β-CA classes (Figure 2.7 and Figure 2.8) [68], [71-89]

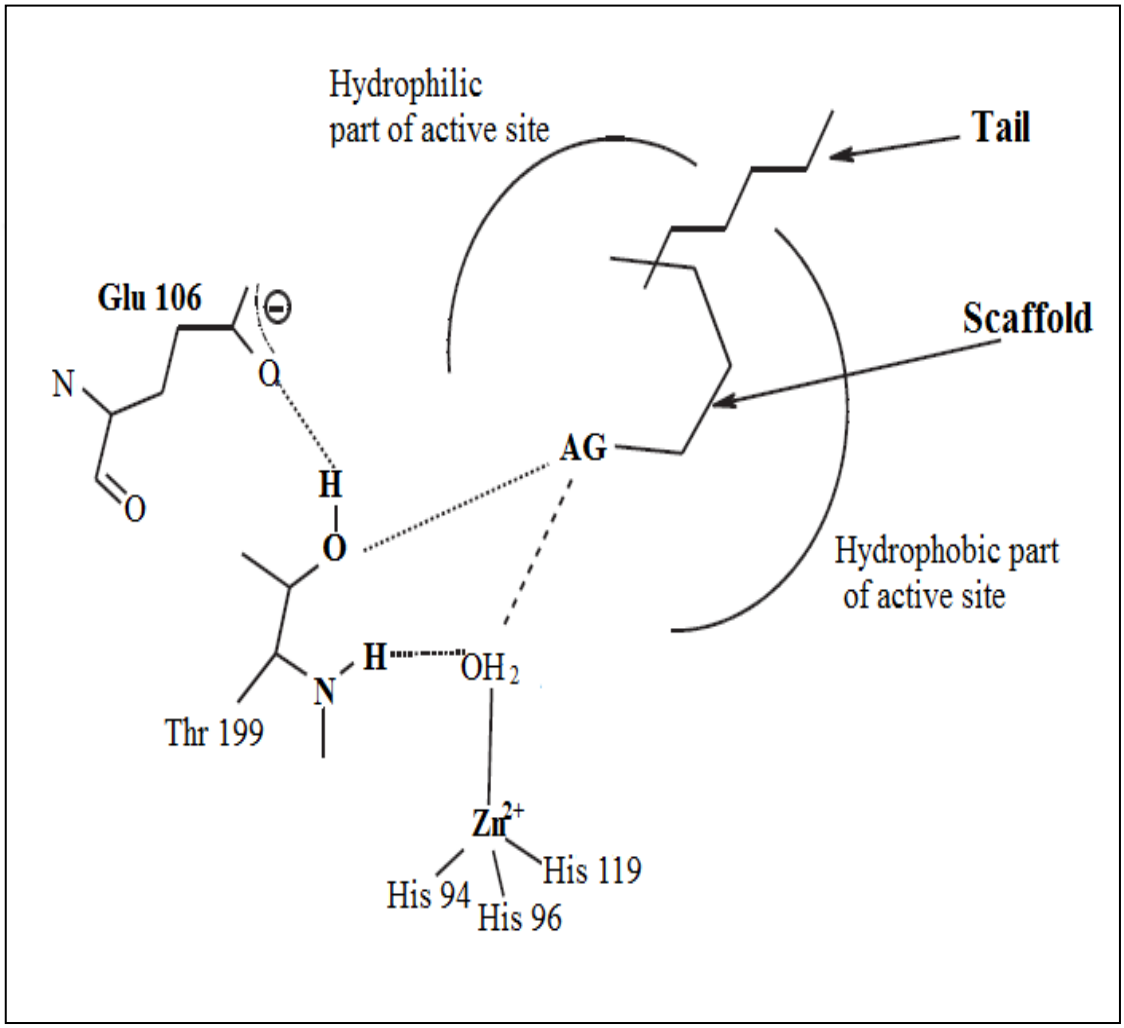


Figure 2.7 Compounds which anchor to the Zinc-coordinated water molecule/hydroxide ion.

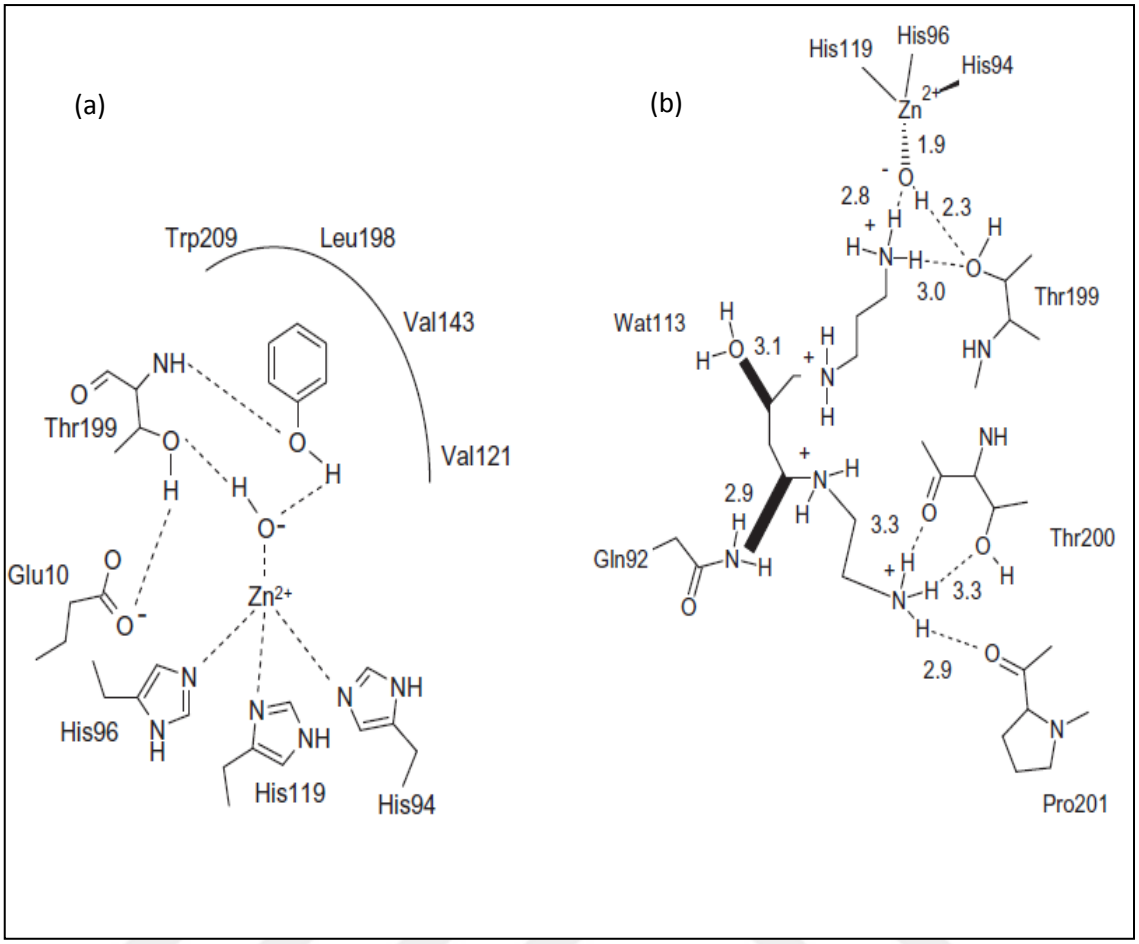


Figure 2.8 The first compounds which inhibit CAs by anchoring to the zinc-coordinated water molecule: Phenol (a), Spermine (b) [70], [89].

2.3.2.3 CA Inhibition by Occlusion of the Active Site Entrance

These CA inhibitors bind basically at the entrance of the active site cavity. Compounds acting by this mechanism of inhibition possess a sticky group (SG) attached to a scaffold which can be aromatic, heterocyclic, or aliphatic (Figure 2.9). They may also incorporate a tail, which can extend away from the active site, since, as mentioned above, these compounds bind in a rather external region of the cavity. These CA inhibitors bind even further away from the metal ion compared to the zinc binders or the compounds anchoring to the zinc coordinated water molecule [90], [91].

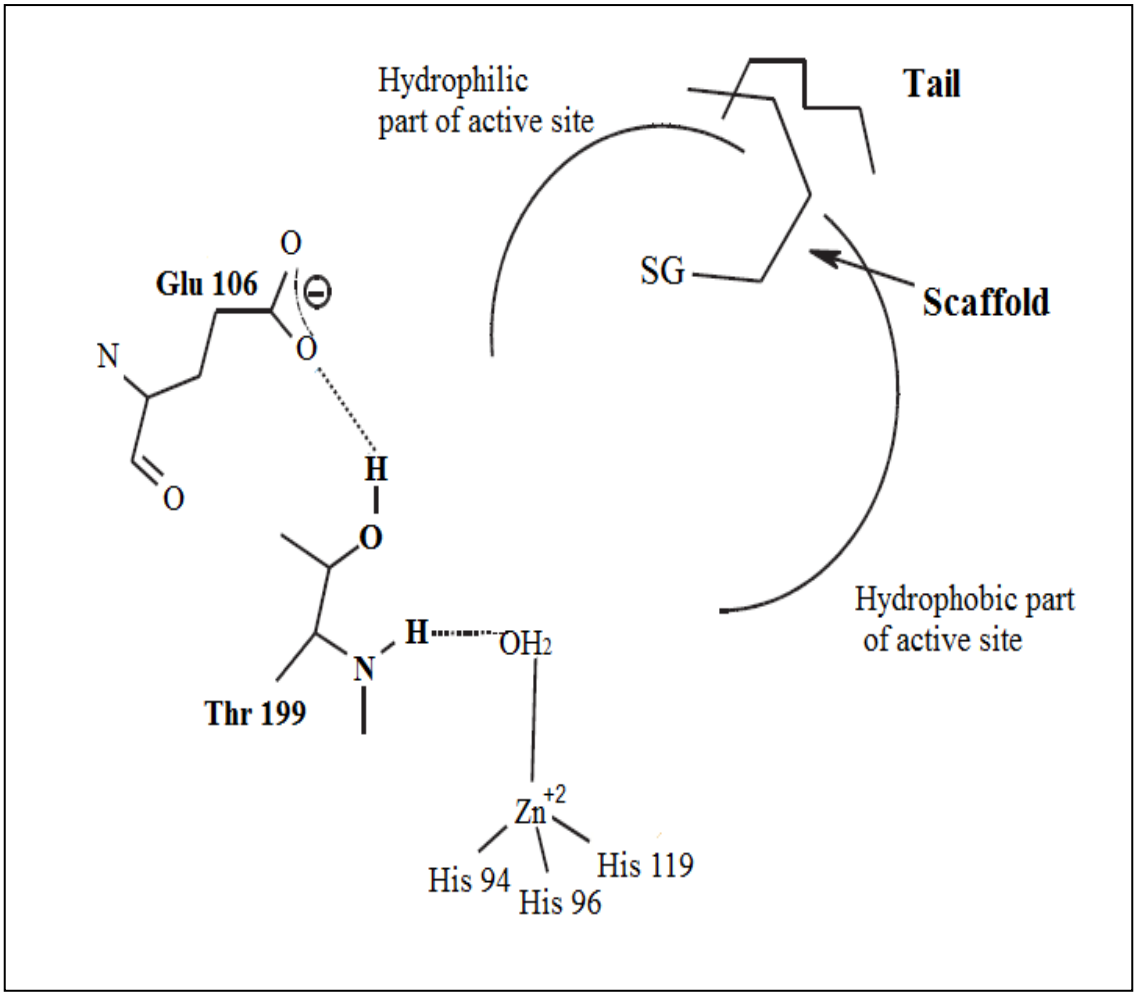


Figure 2.9 Compounds which inhibit the CAs by occluding the entrance to the active site.

SG represents a sticky group, of the phenol, carboxylic acid or amide type, the scaffold binds at the entrance of the active site cavity, occluding it, and a tail may be also present, which will interact with residues on the surface of the protein [21], [68],[90], [91].

2.3.2.4 CA Inhibition out of the Active Binding Site

This mechanism discovered recently when the 2-(benzylsulfonyl)-benzoic acid was co-crystallized with hCA-II. The electronic density of the inhibitor was observed in an adjacent binding pocket to the active site, where other inhibitors were never observed earlier [68], [92]. This compound blocking the proton shuttle of the enzyme, which assisted by a histidine residue placed at the entrance of the active site (His64), proton shuttle which is indeed the transfer of a proton from the zinc-coordinated water molecule to the environment, with generation of the zinc hydroxide species of the

enzyme [21], [68], [92]. Interfering with this process by2-(benzylsulfonyl)-benzoic may block the entire catalytic cycle, blocking the proton shuttle through a network of hydrogen bonds shown in detail in Figure 2.10 and Figure 2.11 [68], [92].

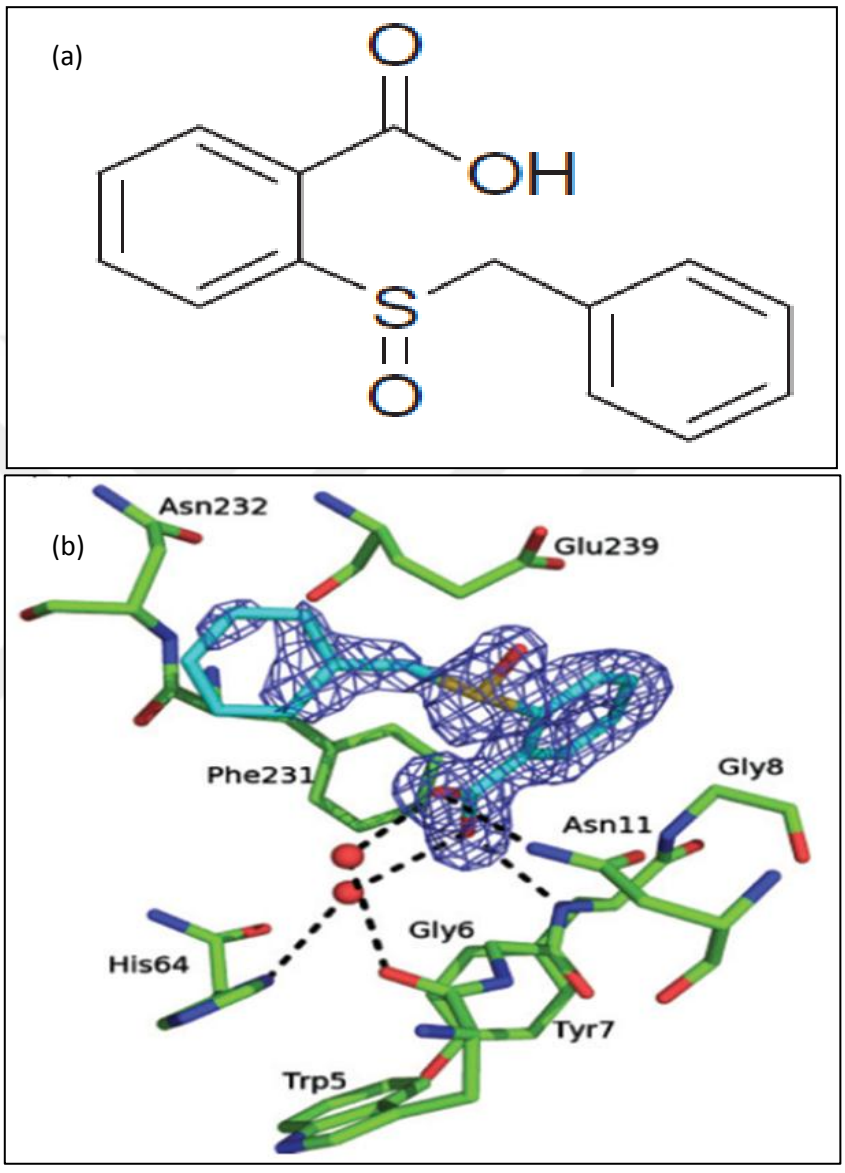


Figure 2.10 Chemical structure of 2-(benzylsulfonyl)-benzoic acid (a). Interactions between the inhibitor 2-(benzylsulfonyl)-benzoic acid and amino acid residues from the binding pocket (b) [68], [92].

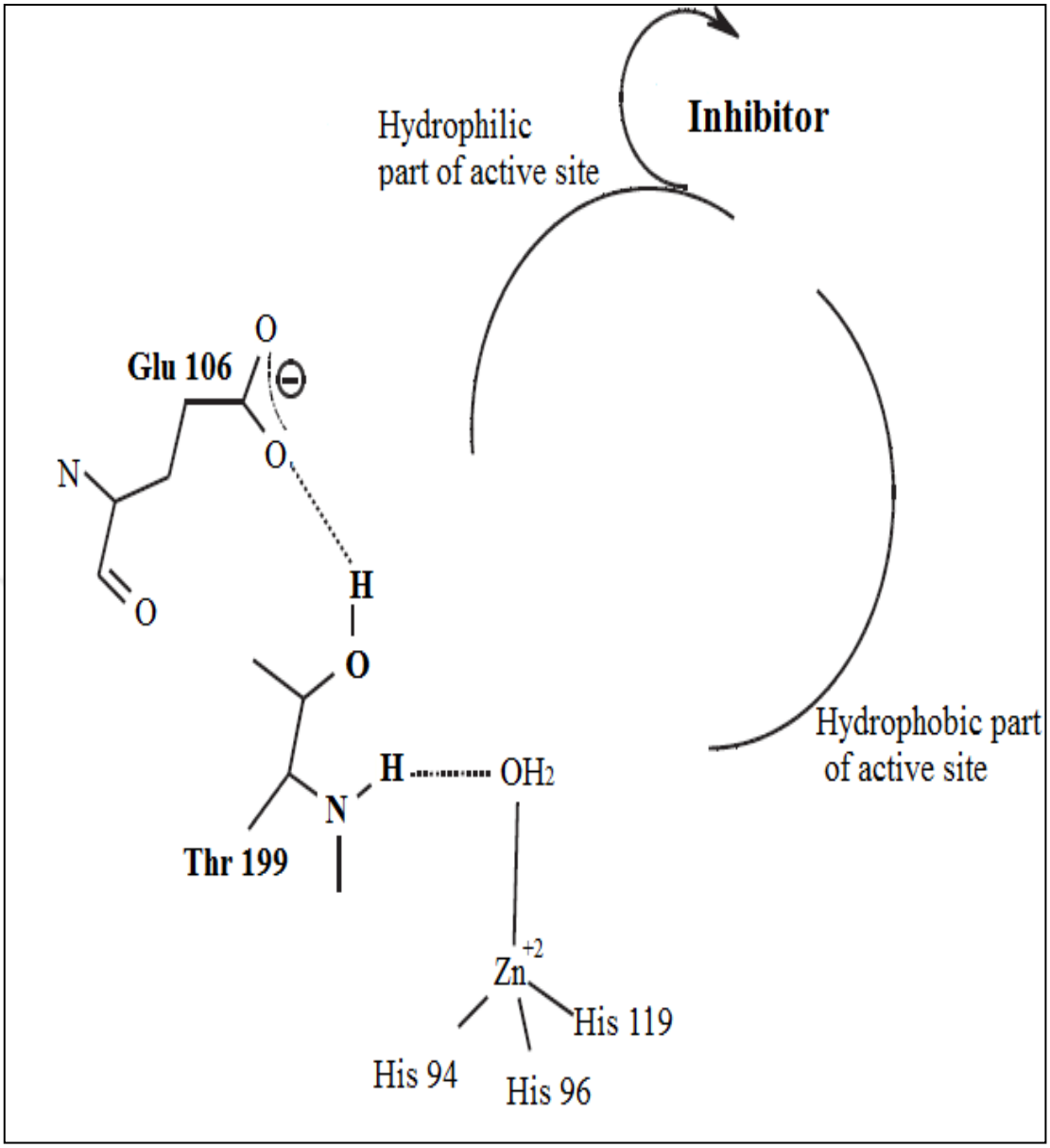


Figure 2.11 CA inhibition mechanism out of the active site [68], [92].

2.3.2.5 CA Inhibitors with an Unknown Mechanism

In the last period relevant evidence accumulated on other classes of compounds, which show various levels of CA inhibitory properties, but for which the precise mechanism of action is not known due to the fact that they were not yet crystallized in adducts with the enzyme. This is most of the time due to solubility problems for some of these derivatives, but it should be also stressed that many classes of CAIs only recently started to be investigated [93-99].

2.4 Metalloenzyme as a Target for Drugs

At the end of the nineteenth and the beginning of the twentieth century, drug target is an old concept that was suggested by Ehrlich and Langley who developed the idea that compounds display biological activity by binding to cellular constituents [100].

Drug targets are:

(i) enzymes, (ii) substrates, metabolites, and proteins, (iii) receptors, (iv) ion channels, (v) transport proteins, (vi) DNA/RNA and the ribosome, (vii) targets for monoclonal antibodies, (viii) various physicochemical mechanisms, and (ix) unknown mechanisms of action [27], [101].

For a long time, enzymes have been considered as valuable drug targets for treatment of major human diseases. The human genome contains thousands of enzymes, they play a key role in virtually every physiological/pathological process. At least 66 human enzymes and 20 bacterial, viral, fungal, or parasite enzymes are targets of approved drugs up to 40% of the known drug targets. The relevance of zinc metalloproteins to biomedical sciences and modulation of their activity with small-molecule drugs, designed to interact with a clearly defined ligand binding site, constitutes a challenging area in drug design and discovery [27], [101-103].

2.5 Some Drugs as Inhibitors for α-Carbonic Anhydrases

Sulfonamides are the most important class of CA inhibitors [21], [104-109], with at least 20 such compounds in clinical use for decades or clinical development in the last period (Figure 2.12) [21], [68], [110-119]. They include acetazolamide (1), methazolamide (2), ethoxzolamide (3), sulthiame (4), dichlorophenamide (5), dorzolamide (6), brinzolamide (7), sulpiride (8), zonisamide (9), topiramate (10), saccharin (11), celecoxib (12), chlorothiazide/highceiling diuretics of types (13–19), including the widely prescribed hydrochlorothioazide, furosemide (18), bumethanide (19), compounds in clinical use for many years, as diuretics, antiglaucoma agents, antiepileptics [17], [21], [68], [110-133]. Compound (20), SLC-0111, is in Phase I a clinical trial as an antitumor/antimetastatic agent and was discovered in the author's laboratory [134] (Figure 1. 12). Most of the sulfonamides (1–19) act as potent CA inhibitors and are in clinical use for decades, but their main problem is related to the fact that by inhibiting most of the catalytically active CA isozymes found in vertebrates they show a wide range of side effects [104-109].

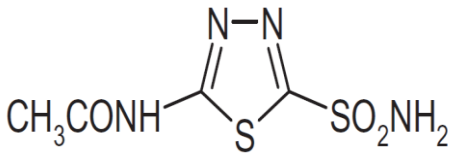
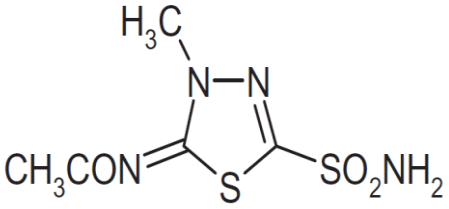
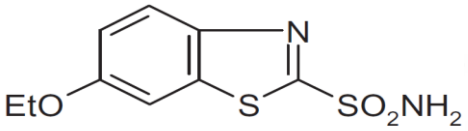
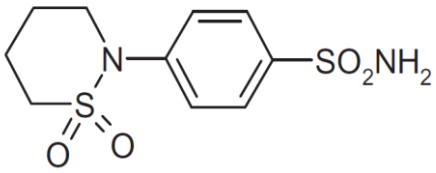
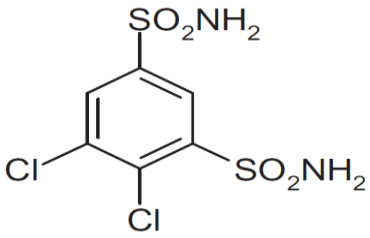
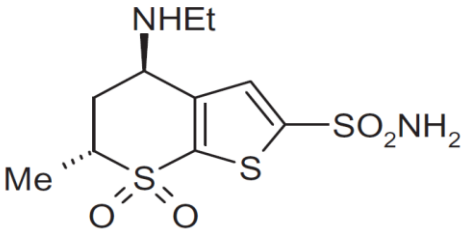
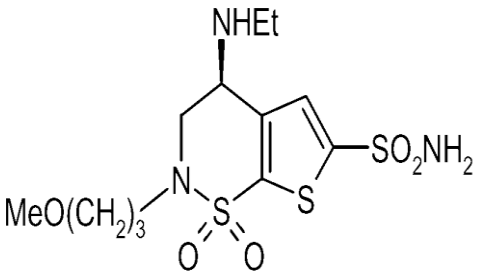
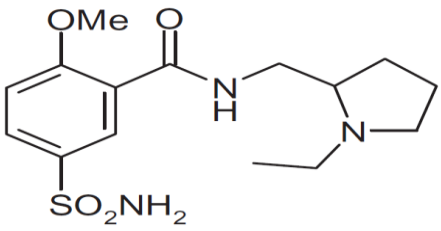
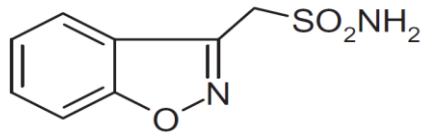
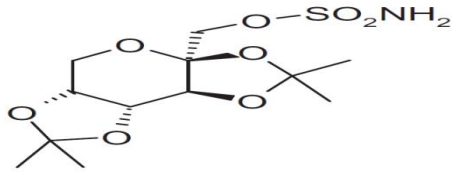
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Figure 2.12 clinically used sulfonamide and sulfamate CA inhibitors.

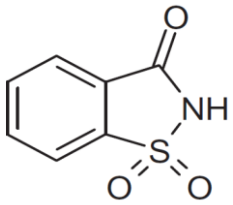
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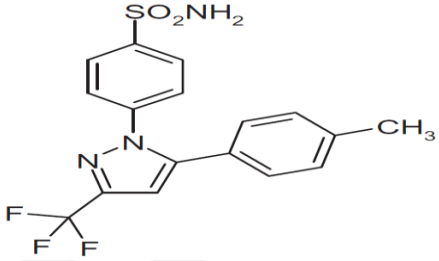
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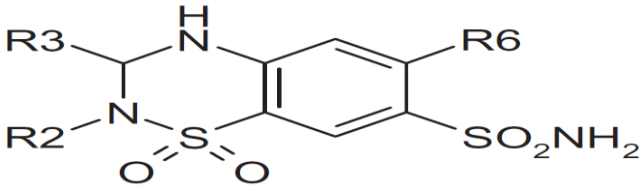
a:R2=R3=H, R6=Cl, Hydrochlorothiazide

b:R2=R3=H, R6=CF_3, Hydroflumethiazide

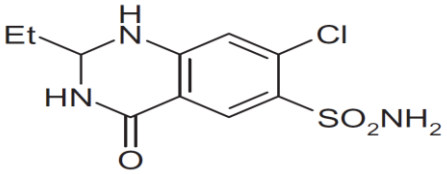
c:R2=H, R3=$PhCH_2$, R6=CF_3, Bendroflumethiazide

d:R2=H, R3=$CHCl_2$, R6=Cl, Trichloromethiazide

e:R2=Me, R3=$CH_2SCH_2CF_3$, R6=Cl, Polythiazide



14



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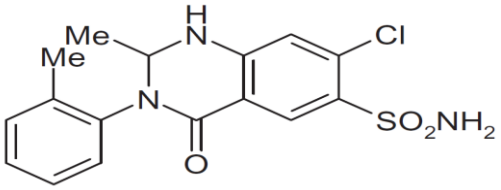
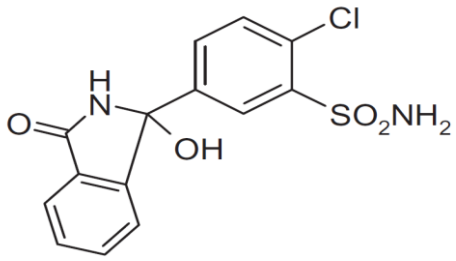
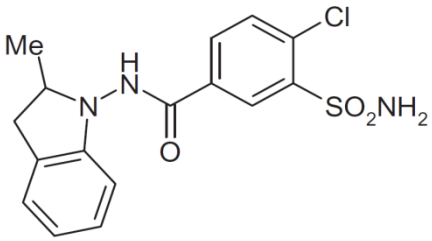


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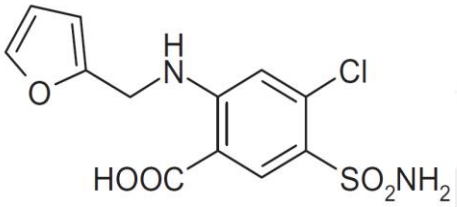
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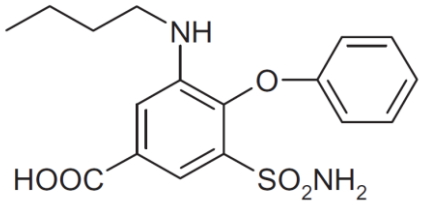
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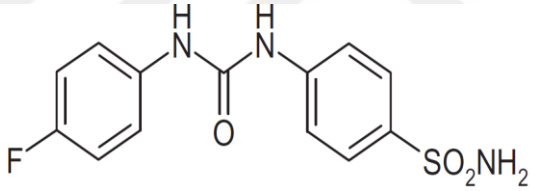
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SLC-0111

Figure 2.12 Cont'd.

Newer generation inhibitors, among which 20 is a rather recent example, were designed in such a way as to act as isoform-selective inhibitors for the transmembrane, tumor-associated isoforms hCA-IX and hCA-XII, they probably will show less side effects compared to the classical CA inhibitors of types (1–19) discussed above. Furthermore, sulfonamides and sulfamates strongly inhibit CAs belonging to most families, not only the α-class enzymes, and this may be exploited in the future for obtaining anti-infective with a new mechanism of action [21], [104-109].

2.6 Angiotensin Converting Enzyme (ACE)

Angiotensin-converting enzyme (ACE, E.C. 3.4.15.1) also known as peptidyl-dipeptidase A or kininase II, in 1956, was first isolated and it is a chloride-dependent metalloenzyme that cleaves a dipeptide from the carboxyl terminus of the decapeptide angiotensin I (AGI), Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu, to form the potent vasopressor angiotensin II (AGII) [135]. Enzyme kinetic experiments with isolated ACE indicated that this enzyme was a zinc metallopeptidase with a catalytic center similar to that of carboxypeptidase A. At that moment, the availability of the three-dimensional structure of the carboxypeptidase A allowed to tackle the development of ACE inhibitors based on the structure of the active site of this enzyme [104].

2.6.1 Angiotensin Converting Enzyme-Inhibitor Drugs for Treatment of Hypertension and Heart Diseases

Hypertension is a major risk factor for myocardial infarction, stroke, and renal failure which is an important disease that affects more than a 25% of the adult population in the world, to prevent these adverse outcomes and to regulate high blood pressure, drugs from a number of different classes are used [136], [137].

A chemical structure classification of ACE inhibitors can be made based on the zinc-interacting group in sulfhydryl-containing ACE inhibitors, dicarboxyl-containing ACE inhibitors, and phosphorus-containing ACE inhibitors [138]. Besides, the therapeutic effect of ACE inhibitors in lowering the high blood pressure, some of these drugs have also been proved to be successful in the treatment of symptomatic or asymptomatic left ventricular systolic dysfunction, postmyocardial infarction, renal failure, and diabetic nephropathy. Because of its effectiveness, but they have important side effects such as a cough and angioneurotic edema, and important drug-drug interactions [138-140].

Historically in the 1970s, By Cushman and Ondetti the first orally active antihypertensive inhibitor developed using “rational drug design”. It was captopril [141], and the target protein was the angiotensin I converting enzyme. The development of ACE inhibitors depends on the structure of the active site of this enzyme. Simultaneously, medical research by researchers from Squibb Institute had evidence that the venom peptides isolated from a Brazilian viper inhibited ACE [142]. The best results were obtained with teprotide (Pyr-Tpr-Pro-Arg-Pro-Gln-Ile-Pro-Pro) and this nonapeptide was the first ACE inhibitor to be studied in hypertensive patients with

promising results in lowering blood pressure. The major problem with this inhibitor was its lack of oral activity. The design of new synthetic venom peptide analogs was based on structure-activity studies and these studies yield a succinyl amino acid derivative, D-2-methylsuccinyl-L-proline, where the replacement of the carboxyl group by a sulfhydryl group gave the potent ACE inhibitor captopril. The assumption of the sulfhydryl group binding to the active site zinc atom was supported by the structural studies with other metalloprotease, the thermolysin [143].

2.6.2 Some Angiotensin-Converting Enzyme Inhibitors that Inhibit Carbonic Anhydrase

In the subject of successful metalloprotein inhibitors, the most widely prescribed inhibitors are the ACE as well as CA inhibitors. Sulfonamide, thiol, hydroxamic acid moiety, carboxylate, phosphonate, phenols, and polyamines are most popularized metal binding groups (MBGs) for use in classical and non-classical metalloenzyme inhibitors [105-107], [144], [145].

Academic researchers worked on captopril and enalapril which are ACE inhibitor drugs used for heart diseases. They tested the inhibiting effect against esterase activity of human carbonic anhydrase II that purified from human erythrocyte. The results of this study were both captopril and enalapril can be considered as inhibitors of hCA-II esterase activity at micromolar concentrations. The results also show that enalapril is approximately 1.5-fold more potent CA inhibitor than captopril. Since enalapril includes no sulfhydryl group, thus, carboxylate was suggested as MBG of the drugs. Overall, although CA inhibitory potencies of captopril and enalapril are much lower than that of the sulfonamide drugs, and the drugs did not cause acute/effective CA enzyme inhibition at the concentrations reached in vivo [12].

For that reasons, in the current study, five drugs (Cilazapril, Perindopril, Ramipril, Trandolapril, Zofenopril calcium) from ACE inhibitors used for hypertension and heart diseases (Table 1.4-1.8) were investigated in order to determine their effect on esterase activity of human carbonic anhydrase I and II purified from human erythrocytes [146].

Table 2.4 ACE inhibitor drug (Cilazapril) for treatment of hypertension and heart diseases.

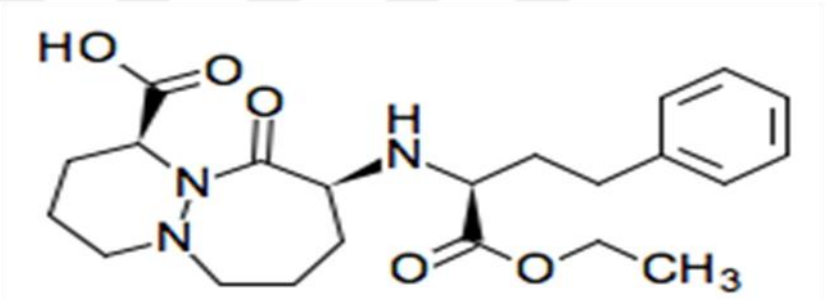
Name	Cilazapril
Formula	$C_{22}H_{31}N_3O_5$
Exact mass	417.2264
Mol weight	417.4986
Structure	
Class	Cardiovascular agent Renin-angiotensin system inhibitor Angiotensin-converting enzyme inhibitor
Target Pathway	ACE, Renin-angiotensin system
Activity	Antihypertensive, Angiotensin-converting enzyme inhibitor

Table 2.5 ACE inhibitor drug (Perindopril) for treatment of hypertension and heart diseases.

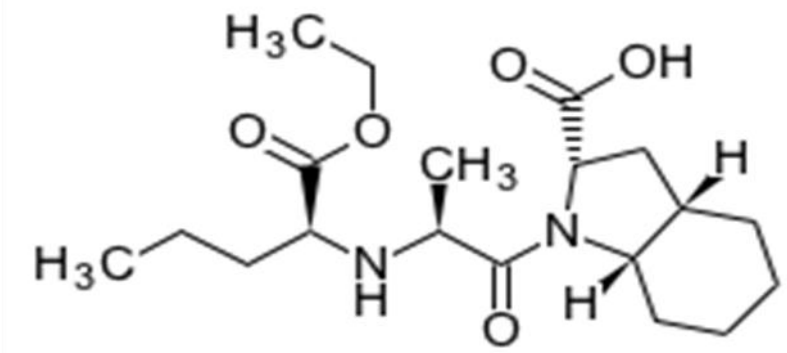
Name	Perindopril
Formula	$C_{19}H_{32}N_2O_5$
Exact mass	368.2311
Mol weight	368.4678
Structure	
Class	Cardiovascular agent Angiotensin-converting enzyme inhibitor Renin-angiotensin system inhibitor Angiotensin-converting enzyme inhibitor
Pathway Target	ACE Renin-angiotensin system
Activity	Antihypertensive, Angiotensin-converting enzyme inhibitor

Table 2.6 ACE inhibitor drug (Ramipil) for treatment of hypertension and heart diseases.

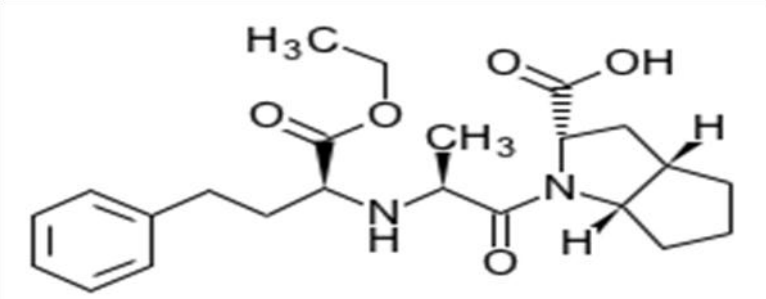
Name	Ramipril
Exact mass	416.2311
Mol weight	416.5106
Structure	
Class	Cardiovascular agent Renin-angiotensin system inhibitor Angiotensin-converting enzyme inhibitor
Target Pathway	ACE , Renin-angiotensin system
Activity	Antihypertensive, Angiotensin-converting enzyme inhibitor

Table 2.7 ACE inhibitor drug (Trandolapril) for treatment of hypertension and heart diseases.

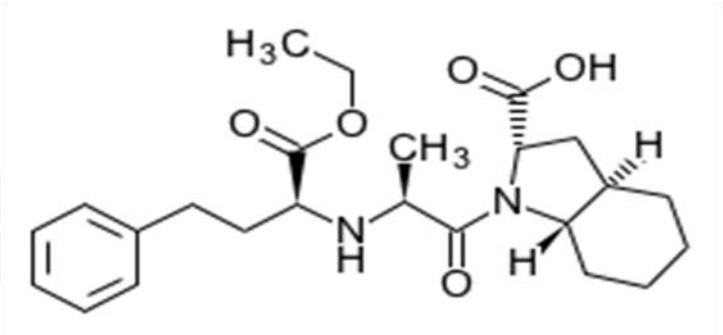
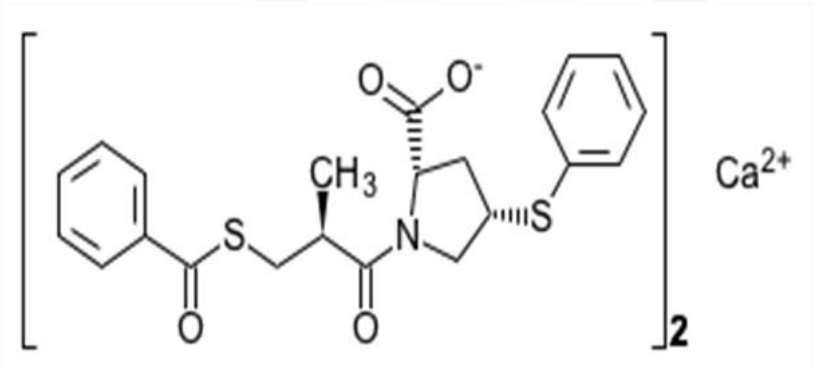
Name	Trandolapril
Formula	$C_{24}H_{34}N_2O_5$
Exact mass	430.2468
Mol weight	430.5372
Structure	
Class	Cardiovascular agent Angiotensin-converting enzyme inhibitor Renin-angiotensin system inhibitor Angiotensin-converting enzyme inhibitor
Target Pathway	ACE Renin- Renin-angiotensin system
Activity	Antihypertensive, Angiotensin-converting enzyme inhibitor

Table 2.8 ACE inhibitor drug (Zofenopril calcium) for treatment of hypertensionand heart diseases.

Name	Zofenopril calcium
Formula	$(C_{22}H_{22}NO_4S_2)_2$. Ca
Exact mass	896.1606
Mol weight	897.1668
Structure	
Class	Cardiovascular agent Renin-angiotensin system inhibitor Angiotensin-converting enzyme inhibitor
Target Pathway	ACE Renin-angiotensin system
Activity	Antihypertensive, Angiotensin-converting enzyme inhibitor

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 The Used Chemical Materials

Sepharose-4B, p-nitrophenylacetate, standard serum albumin, sulfanilamide, N, N, N', N'-tetramethylethylenediamine (TEMED), sodium hydroxide, sodium bicarbonate, trihydroxymethylaminomethane (Tris), sodium sulfate, sodium acetate, sodium perchlorate, 2-mercaptoethanol, bromothymol blue, hydrochloric acid, sulfuric acid, glycine, phosphoric acid, methanol, acetone, ethanol, isopropanol, sodium barbital, acrylamide, N, N'-methylenebisacrylamide, coomassie brilliant blue G250 and R-250 are from E.Merk AG. Carbon dioxide was bought from laboratory equipment office. The drugs were bought from pharmacy. Active ingredients of Inhibace, Coversyl, Blokace Gopten Forte and Zoprotec are Cilazapril, Perindopril, Ramipril, Trandolapril and Zofenopril calcium, respectively.

3.1.2 The Used Tools and Devices

UV / Vis spectrophotometer: Evolution 201 Thermo Scientific
Freezer: Vastel
pH- meter: Hanna Instrument
Automatic pipettes: Isolab
Vortex: Wisemix VS 10, Daihan Scientific Co.
Refrigerated Centrifuge: Thermo Scientific SL16-16R
Pure water device: Firstreem calypso MK 1
Magnetic stirrer: Isolab MS-H280-Pro.
Peristaltic pump: Gilson Miniplus 3

Electrophoresis tank: Thermo Scientific

Power source: Consort EV265

3.1.3 The Used Human Blood

Erythrocytes were purified from fresh human blood which obtained from the Blood Centre of the Research Hospital at Ataturk University. The blood was stored at 4°C until used in the experiments.

3.1.4 The Used Solutions

3.1.4.1 Solutions for Hydratase Activity of Carbonic Anhydrase

1. Veronal buffer 0.025 M (pH 8.2): 0.025 mol of sodium barbital was dissolved in 900 ml of distilled water, treated with 0.1 M HCl to adjust acidity at (pH 8.2) and then the total volume was completed to 1 liter with distilled water.

2. 0.04% bromothymol blue solution: 0.1 g of the indicator was dissolved in 16 ml of 0.01 N NaOH and then the total volume was completed to 250 ml with distilled water.

3. CO_2 solution: it was prepared by passing CO_2 gas through distilled water for half an hour at 0°C.

3.1.4.2 Solutions for Esterase Activity of Carbonic Anhydrase

1. 0.05 M Tris-SO_4 (pH 7.4): 6.055 g of Tris was dissolved in 950 ml of distilled water, treated with 0.1 N H_2SO_4 to adjust acidity at pH 7.4 and then the total volume was completed to 1 liter with distilled water.

2. 3 mM p-nitrophenylacetate solution: 1 ml of acetone was added to 27.2 mg of p-nitrophenylacetate and then the total volume was completed to 50 ml with distilled water.

3. The drug solutions were prepared in Table 3.1.

Table 3.1 Preparation of drug solutions.

Drugs	Imported Company	Active ingredient	Stock preparation
	Deva İlaç	Cilazapril	5 mg solved in 10 ml of distilled water, and diluted 100 times, the final concentration is 11.97 µM
	Servier İlaç	Perindopril	5 mg solved in 11 ml of distilled water, and diluted 100 times, the final concentration is 12.0 µM
	Actavis İlaç	Ramipril	10 mg solved in 20 ml of distilled water, and diluted 100 times, the final concentration is 12.0 µM
	Abbott İlaç	Trandolapril	4 mg solved in 8 ml of distilled water after that bay using filtered to remove the other ingredient that not dissolved in water, and diluted 100 times the final concentration is 11.61 µM
	Ufsa İlaç	Zofenopril calcium	30 mg solved in 30 ml of distilled water, and diluted 100 times, the final concentration is 11.15 µM

3.1.4.3 Solutions for Preparing Affinity Gel

1. 0.2 M $NaHCO_3$ (pH 8.8): 16.8 g of $NaHCO_3$ was dissolved in 950 ml of distilled water, treated with 0.1 N NaOH to adjust acidity at pH 8.8 and the total volume was completed to 1 liter with distilled water.

2. 0.2 M $NaHCO_3$ (pH 10): 16.8 g of $NaHCO_3$ was dissolved in 950 ml of distilled water, treated with 0.1 N NaOH to adjust acidity at pH 10 and the total volume was adjusted to 1 liter with distilled water.

3. Sulfanilamide: 25 mg sulfanilamide in 0ºC was dissolved in 10 ml of 1 M HCl and followed by adding of (5 ml of a solution containing 75 mg of $NaNO_2$ at 0°C) dropwise.

3.1.4.4 Solutions for Enzyme Purification

1. 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7) (used for equilibration of affinity gel): 3.0275 g Tris and 14.2 g Na_2SO_4 in 950 ml distilled water was dissolved and then titrated with 0.1 N HCl to pH 8.7. the total volume was completed to 1 liter with distilled water.

2. 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7) (used for washing affinity gel after application of hemolysate): 3.0275 g Tris and 3.124 g Na_2SO_4 was dissolved in 950 ml distilled water. After the pH was brought to 8.7 with 0.1 N HCl, the total volume was completed to 1 liter with distilled water.

3. 25 mM Na_2HPO_4 /1 M NaCl (pH 6.3) (used for elution hCA-I isozyme): 2.2 g Na_2HPO_4 and 14.625 g NaCl was dissolved in 200 ml of distilled water. After the pH was brought to 6.3 with 0.1 N HCl, the total volume was completed to 250 ml with distilled water.

4. 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6) (used for elution hCA-II isozyme): 9.187 g $NaClO_4$ and 2.04 g $CH_3COONa.3H_2O$ was dissolved in 120 ml of distilled water. After the pH was brought to 5.6 with 0.1 N HCl, the total volume was completed to 150 ml with distilled water.

3.1.4.5 Solution for Quantitative Protein Determination

Coomassie Brilliant Blue: 100 mg of reactive Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 95 % phosphoric acid. The volume of the solution was completed to 1litre with distilled water. This solution must be kept in dark.

3.1.4.6 Solutions for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 1 M Tris-HCl (pH 8.8): 12.11 g Tris was dissolved in 80 ml of distilled water. After pH adjustment, the total volume was completed to 100 ml with distilled water.

2. 1 M Tris-HCl (pH 6.8): 12.11 g Tris was dissolved in 80 ml of distilled water. After pH adjustment, the total volume was completed to 100 ml with distilled water.

3. 30% Acrylamide-0.8% Bisacrylamide: 15 g acrylamide, 0.4 g bisacrylamide in 50 ml of distilled water was dissolved by stirring.

4. 10% ammonium persulfate: 1 g ammonium persulfate was dissolved in 10 ml of distilled water.

5. 10% SDS: 1 g SDS was dissolved in 9 g of distilled water.

6. Running buffer: 1.51 g Tris and 7.51 g glycine was dissolved in 450 ml of distilled water. Then, 5 ml of 10% SDS was added and the pH was adjusted to 8.3. The total volume was completed to 500 ml with distilled water.

7. Loading buffer: 0.5 ml of 1 M Tris-HCl (pH 8), 1 ml of 10% SDS, 1 ml of 100% glycerin and 1 ml of 0.1% bromothymolblue were added in a beaker and the volume was completed to 10 ml with distilled water. Prior to use of this buffer, 50 µl β-mercaptoethanol was added 950 µl of loading buffer.

8. Fixing solution (used to immobilize the proteins carried out on the gel): 50% isopropanol, 10% TCA and 40 % distilled water were mixed

9. Gel dyeing: 50 ml methanol, 10 ml acetic acid were added to the mixture of 0.1 g Coomassie Brilliant Blue R-250 reactive solved in 40 ml of distilled water.

10. Gel washing: 50 ml of methanol, 10 ml of acetic acid and 40 ml of distilled water were mixed.

3.2 Methods

3.2.1 Preparation of Affinity Gel

The affinity gel was prepared with Sepharose-4B matrix activated with CNBr. L-tyrosine was covalently attached to the column material. Subsequently, sulfanilamide was conjunct to tyrosine after diazotization. Here, tyrosine was extended on the affinity gel; on the other hand, sulfanilamide is a specific inhibitor of carbonic anhydrase that specifically binds to the enzyme for purification at a high level [147], [148]. The affinity gel was prepared according to the following procedure (Figure 3.1):

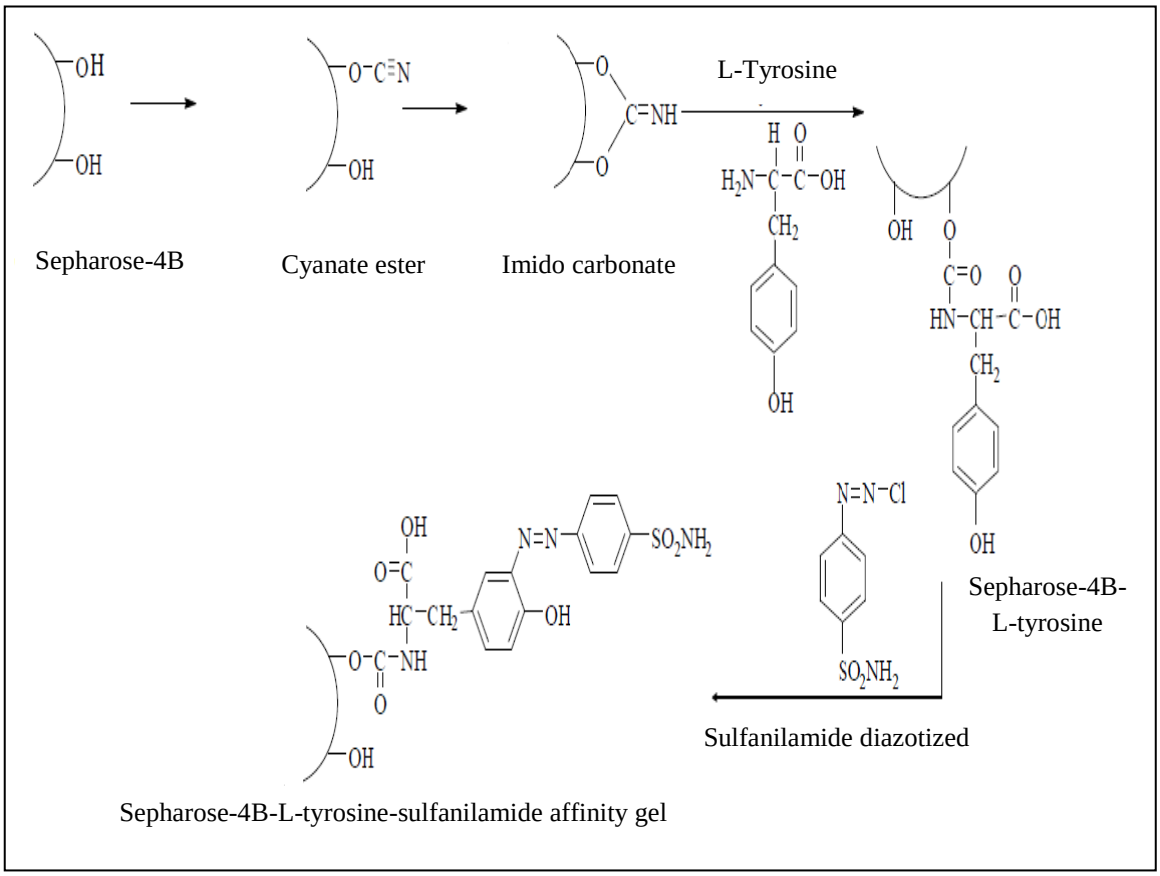


Figure 3.1 Preparation of CNBr Sepharose-4B-L-tyrosine-sulfanilamide affinity gel.

3.2.1.1 Insertion of Tyrosine to the CNBr of Activated Sepharose-4B

Sepharose-4B activated with CNBr was washed with 250 ml of cold 0.1 M $NaHCO_3$ buffer (pH 10) and transferred to a beaker. In 20 ml of the same buffer 80 mg of tyrosine was dissolved, the cold solution was added to the beaker and mixed. Adding of the tyrosine was completed for less than 90 seconds. Then the suspension was stirred in a magnetic stirrer for 2 hours at 4°C. After this mixture was waited for 16 hours at 4°C, the mixture was washed by water until when it does not give absorbance at 280 nm. Last washing was done with 100 ml of 0.2 M $NaHCO_3$ buffer (pH 8.8).The tyrosine attached to the gel was transferred to the same buffer of 40 ml.

3.2.1.2 Coupling of Sulfanilamide

25 mg of sulphanilamide was solved in 10 ml of 1 M HCl at 0^oC, and 75 mg of $NaNO_2$ at 0^oC was solved in 5 mL of 1 M HCl. $NaNO_2$ solution was added drop-drop to the sulfanilamide solution. After 10 minutes of reaction, the diazotized sulfanilamide was added to a suspension of 40 ml of Sepharose-4B-L-tyrosine. The pH of the affinity gel was adjusted to 9.5 with 1 M NaOH and kept for 3 hours at room temperature. Then the affinity gel was washed with 1 liter of distilled water and 200 ml of 0.05 M Tris-SO_4 (pH 7.4) buffer. After that, the same buffer was added to the affinity gel and kept hidden at 4^oC [147]. The open formulas for all step reactions are shown in Figure 3.1.

3.2.1.3 Packing of Affinity Column

The prepared affinity gel was suspended by taking equilibration buffer (Tris-HCl pH 8.7) in it by using water trompe (the air and vacuum were taking). The suspended gel was packed in a cool column of 1x10 cm of a closed system. After the gel precipitation, by using the peristaltic pump the gel was washed with washing and equilibration buffers. Equilibration of the column was understood from both of pH and absorbance at 280 nm of elute and buffer.

3.3 Enzyme Purification Steps

3.3.1 Preparation of Hemolysate

Fresh blood was filled in centrifuge tubes and centrifuged at 2500×g for 15 minutes at 4°C. The plasma and leucocytes were removed. The red cells were isolated and washed three times with 0.154 M NaCl solution (isotonic) and centrifugation at 2500×g for 15 minutes at 4°C after every washing, the supernatant was removed by using the dropper, and erythrocytes was hemolyzed by mixing with five times volume of ice-cold distilled water. The intact cells were removed by centrifugation at 20,000×g for 30 min at 4°C. The upper hemolysate cells were carefully taken it via dropper and stored at 4°C. The pH was adjusted to 8.7 with solid Tris [149], [150].

3.3.2 Sample Application and Elution in the Affinity Column

The column was washed with 16 ml of 25 mM Tris-HCl/0.1 mM Na_2SO_4 (pH 8.7), for balancing the gel. After that 12 ml of hemolysate was added to the column and washed with 18 ml of 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7). The enzyme was bound to the affinity gel other impurities were removed. Then 14 ml 25 mM Na_2HPO_4/1 M NaCl (pH 6.3) was added gradually for a specific elution of hCA-I. The enzyme was collected in 1 ml volume type tubes. Before the elution was not completely finished from the column, 2 ml of 25 mM Tris-HCl/0.1 mM Na_2SO_4 (pH 8.7) was added for washing to balance the column. After the separation of hCA-I, 14 ml gradually of hCA-II enzyme was eluted by using the 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6) solution and the hCA-II was taken up in 1 ml tubes. Before the elution was not completely finished from the column, 25 mM Tris-HCl/0.1 mM Na_2SO_4 (pH 8.7) was added to stop the separation. The flowing rate of the column was adjusted to 20 ml/h by using the peristaltic pump during the operations.

3.4 Enzyme Activity Assay

3.4.1 Hydratase Activity Assay

Hydratase activity was used to calculate enzyme unit (EU) by using the equation (t_0-t_c/t_c) where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively according to the method described by Wilbur and Anderson

[151]. pH= 8.2 is decreased to pH= 6.3 by using the method. The procedure of hydratase activity assay can be explained in Figure 3.2.

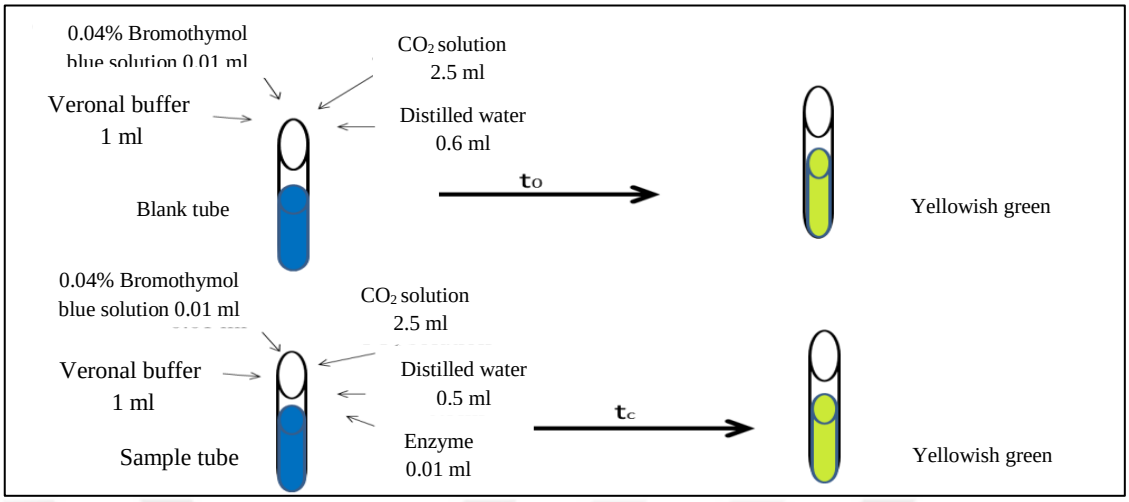


Figure 3.2 The procedure of hydratase activity assay. Time was calculated as soon as the CO_2 was added.

3.4.2 Esterase Activity Assay

This method based on esterase activity of carbonic anhydrase to hydrolysis p-nitrophenylacetate as a substrate to p-nitrophenol or p-nitrophenolate mechanism of reaction (Figure 3.3):

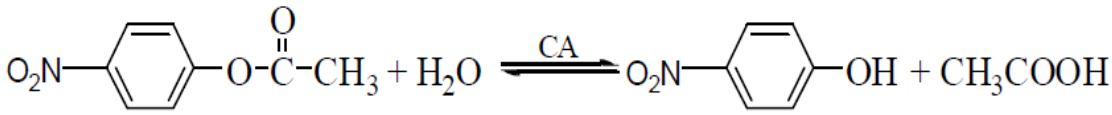


Figure 3.3 Mechanism of the conversion of p-nitrophenylacetate to p-nitrophenol.

At 348 nm both p-nitrophenol and p-nitrophenolate show the same absorbance [152], [153]. At this wavelength, because p-nitrophenylacetate it has very little absorption, it is used as a blank.

The p-nitrophenylacetate substrate solution used in the experiments must be prepared daily. 27.2 mg p-nitrophenylacetate in 1 ml of acetone was rapidly solved and mixed with 49 ml distilled water. The water was slowly added. The concentration of this solution is 3 mM. Acetone is chosen because acetone causes least inhibiting hydrolysis reaction than other relatively organic solvents [153].

The procedure is shown in Table 3.2, and Table 3.3.

Table 3.2 The determination of the hCA-I activity by using 1 ml quartz cuvette.

Used materials	Control (µl)	Sample (µl)
0.05 M Tris-SO_4 pH:7.4	580	580
p-nitrophenylacetate	350	350
Enzyme hCA-I	0	20
25 mM Na_2HPO_4 /1 M NaCl pH= 6.3	20	0
Total volume	950	950

Table 3.3 The determination of the hCA-II activity by using 1 ml quartz cuvette.

Used materials	Control (µl)	Sample (µl)
0.05 M Tris-SO4 pH:7.4	500	500
p-nitrophenylacetate	350	350
Enzyme hCA-II	0	100
0.1 M $NaCH_3COO$ /0.5 M $NaClO_4$ pH: 5.6	100	0
Total volume	950	950

Both absorbance values at the beginning of the reaction and at the ending of the reaction were read at 348 nm at 25°C. The reaction took 3 minutes. The enzyme activity was determined by subtracting the first value from last value. Before measuring the enzyme activity, the spectrophotometer was set to zero by distilled water.

3.5 Protein Determination

3.5.1 Qualitative Protein Determination

The qualitative protein determination is based on the presence of aromatic groups in the structure of tyrosine and tryptophan amino acids which give maximum absorbance at 280 nm on the basis of known Warburg method [154]. Qualitative protein identification was performed on all elutes that were eluted by using Sepharose-4B-L-tyrosine-sulfonamide affinity chromatography. Quartz cuvette was used and the absorbance at 280 nm was read against the curves.

3.5.2 Quantitative Protein Determination

In the purified enzyme solution by affinity chromatography, the protein quantities were determined based on principle the fact that protein conjugate to Coomassie Brilliant Blue G-250. This complex gives absorbance at 595nm. Protein-dye complex remains in solution for a long time. The accuracy of this method is between 1-100 µg [155].

This procedure was followed in the assays: standard bovine serum albumin solution containing 1 mg of protein per ml was added to the tubes 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µl. The volume of all the tubes was completed to 0.1 ml with distilled water. Then 4900 µl Coomassie Brilliant Blue G-250 was added to each tube. Each tube was mixed with the vortex. After 10 minutes, 3 ml protein-dye complex was transferred to the cuvette and the absorbance of the complex was read at 595nm against the blank. A mixture of 100 µl of distilled water and 4900 µl of Coomassie Brilliant Blue G-250 was used as a blank. The standard curve was drawn by using µg serum albumin values against the absorbance values. This procedure was followed in the assay of samples: 10 µl of sample was pipet to a test tube, diluted 10 times by distilled water to 100 µl and then 4900 µl Coomassie Brilliant Blue G-250 was added to the tube. This tube was mixed with the vortex. After 10 minutes, 3 ml sample-dye complex was transferred to the cuvette and the absorbance of the complex was read at 595 nm against the blank. The standard curve was used for quantitative protein determination in the purified enzyme solution.

3.6 Controlling of Enzyme Purity by SDS-Polyacrylamide Gel Electrophoresis

The purity of the hCA-I and-II isozymes was checked by used 3-8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [156].

Electrophoresis plates were thoroughly washed with water, then with alcohol. Dedicated glass plates placed on top of each other and placed in special nylon containers. Vaseline was applied as a thin layer to combining the glass plates from the edge. Then the glass plates were carefully compressed to clamp. When the separation gel was prepared as soon as injected carefully between plates and it must be sure there are no air bubbles in the environment. Then the comb was placed on the top of the plates, and the plates were kept at room temperature to freeze the gel. After freezing, the comb was carefully removed and wells were ready for loading of the samples and controls.

The separation gel was prepared as follows: 15 ml 1 M Tris-HCl (pH 8.8), 10.66 ml 30% acrylamide-0.8% bisacrylamide, 0.61 ml 1% SDS, 0.4 ml of 5% TEMED (N, N, N' ,N'-tetramethylethylenediamine) and 11.94 ml of distilled water were mixed. 0.8 ml of 1.5% ammonium persulfate $(NH_4)_2S_2O_8$ (PER) was added to the mixture solution. The used PER must be freshly prepared. The loading gel was prepared as follows: 1.24 ml from 1 M Tris-HCl (pH 6.8), 1 ml from 30% acrylamide-0.8% of bisacrylamide, 0.1 ml from 1% SDS, 0.1 ml from 5% TEMED and 7.36 ml of distilled water were mixed. Finally, 0.2 ml of the fresh prepared 1.5% PER was added to the mixture solution. Plates were placed in the electrophoresis tank, gels were washed with distilled water and then with execution buffer before marking the blanks. Execution buffer was placed on the top and bottom of electrophoresis tank. From every sample 20 μg had taken and put in separated tubes, after that added buffer to all tubes as 1/1 ratio, the total volume was 100 μl for all tubes, and they were incubated in a boiling water bath for 3 minutes. After that samples were cooled and applied to the gel wells with a very fine injector. Tank covers contain (Anode) cable from the bottom side, (Cathode) cable from the top side. At 80 volts for half an hour, the tank was left. Then, the current is set at 120 volts for 2-3 hours at room temperature. At the end of time, the electric was cut, the gel between the glass plates is carefully removed, and the gel was put 15 minutes in fixing solution (50% isopropanol + 10% TCA + 40% distilled water). Then the gel removed from the fixing solution and placed in the special container, and covered with Coloring solution. It was left on the shaker for about 45 minutes. After that, it was removed from the coloring solution and taken to the washing solution and left for 1-2 days until protein bands became clear. The gel is removed from the washing solution and then picture was taken.

3.7 Determination of the Effects of Drugs on Esterase Activities of hCA-I and hCA-II Isozymes

Absorbances of esterase activity of hCA-I and hCA-II isozymes with the investigated drugs were measured at 380 nm following the procedure in Table 3.4, and Table 3.5. Six different concentrations of the five drugs were used in the range 0.06-0.37 μM. The absorbance value of the esterase reaction without inhibitor was accepted as 100 % activity. % Activity values of the drugs were calculated by using absorbance values with

and without inhibitor. Then, IC_{50} values for these drugs were calculated by using the % Activity-[I] graphs.

Table 3.4 Cuvette ingredient that used for measuring the effect of investigated drugs on esterase activity of hCA-I.

Substrate µl	**Buffer µl**	**hCA-I µl**	**Inhibitor µl**	**Total volume µl**
350	580	20	0	950
550	580	20	5	955
350	580	20	10	960
350	580	20	15	965
350	580	20	20	970
350	580	20	25	975
350	580	20	30	980

Table 3.5 Cuvette ingredient that used for measuring the effect of investigated drugs on esterase activity of hCA-II.

Substrate µl	**Buffer µl**	**hCA-II µl**	**Inhibitor µl**	**Total volume µl**
350	500	100	0	950
350	500	100	5	955
350	500	100	10	960
350	500	100	15	965
350	500	100	20	970
350	500	100	25	975
350	500	100	30	980

3.8 Molecular Docking for Drugs to the hCA-I and hCA-II

Structures of the five investigated drugs with the crystal structure of hCA-I (PDB 2CAB) and of hCA-II (PDB 3KS3) were chosen from the (www.swissdock.ch/ docking). This site is directly connected to the (http://zinc.docking.org/) and (PDB) (www.pdb.org). After the results of the requested dockings were received, the best values of ΔG (-) with full fitness were chosen and analyzed by using UCSF Chimera molecular visualization application (Chimera1.11.2) which was downloaded from (www.cgl.ucsf.edu/chimera/download. html).

CHAPTER 4

RESULTS

4.1 Standard Graph for Quantitative Protein Assay

The Coomassie Brilliant Blue method was used for quantitative protein determination [155]. A standard curve was prepared. The amounts of protein in the hemolysate and in the purified enzyme solution were determined according to this curve. The absorbance values corresponding to the μg protein in the standard solution are shown in Figure 4.1.

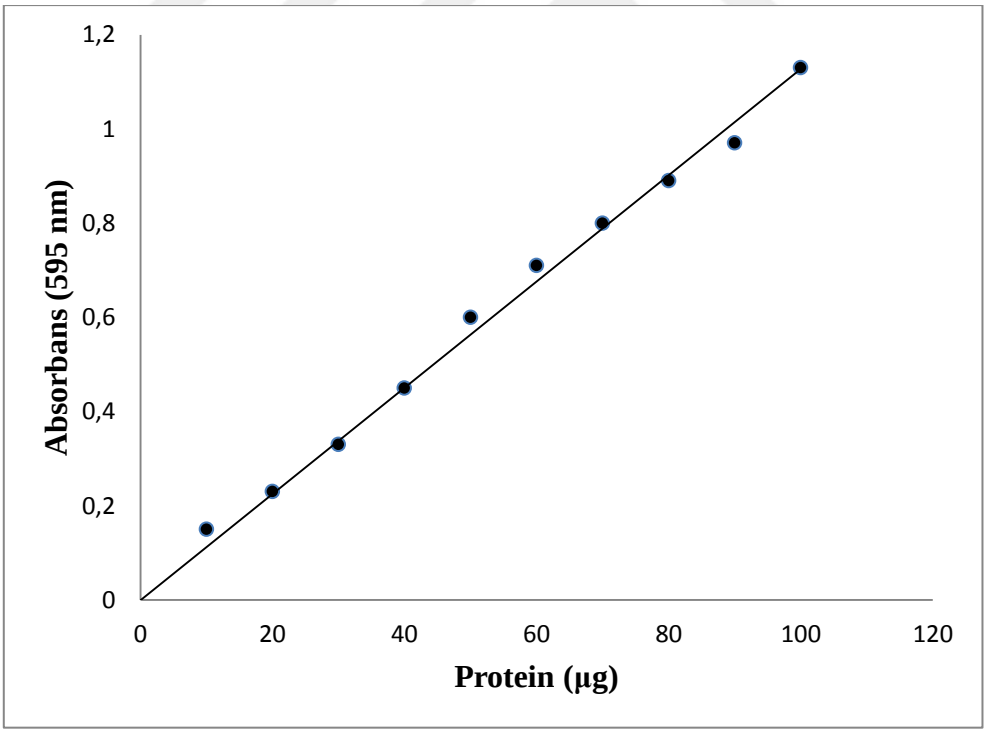


Figure 4.1 Standard graphic of Bradford method used for quantitative protein determination.

4.2 Purification of Human Erythrocyte hCA-I and hCA-II Isozymes by Affinity Chromatography

hCA-I and hCA-II isozymes were purified from hemolysate by using Sepharose-4B-L-tyrosine-sulfanilamide affinity. The results are shown in Table 4.1.

Table 4.1 Purification results of hCA-I and hCA-II isozymes before and after affinity chromatography.

Purification steps	Total Volume (ml)	Protein (mg/ml)	Activity (EU/ml)	Total protein(mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification coefficient
Hemolysate	12	16.12	120	193.44	1440	7.444	100	1
hCA-I	6	0.23	162	1.38	972	704.35	67.5	94.62
hCA-II	4.5	0.05	175	0.225	787.5	3500	54.69	470.18

4.3 SDS-PAGE Results

The purity of the purified hCA-I and hCA-II isozymes by using SDS-PAGE was shown. The protein bands of hCA-I, hCA-II, and controls were photographed (Figure 4.2).

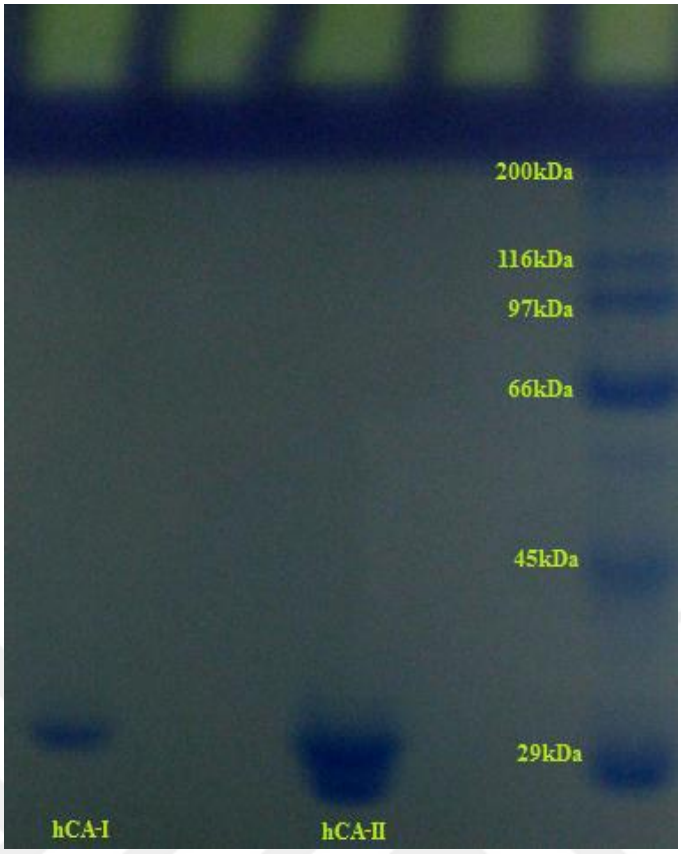
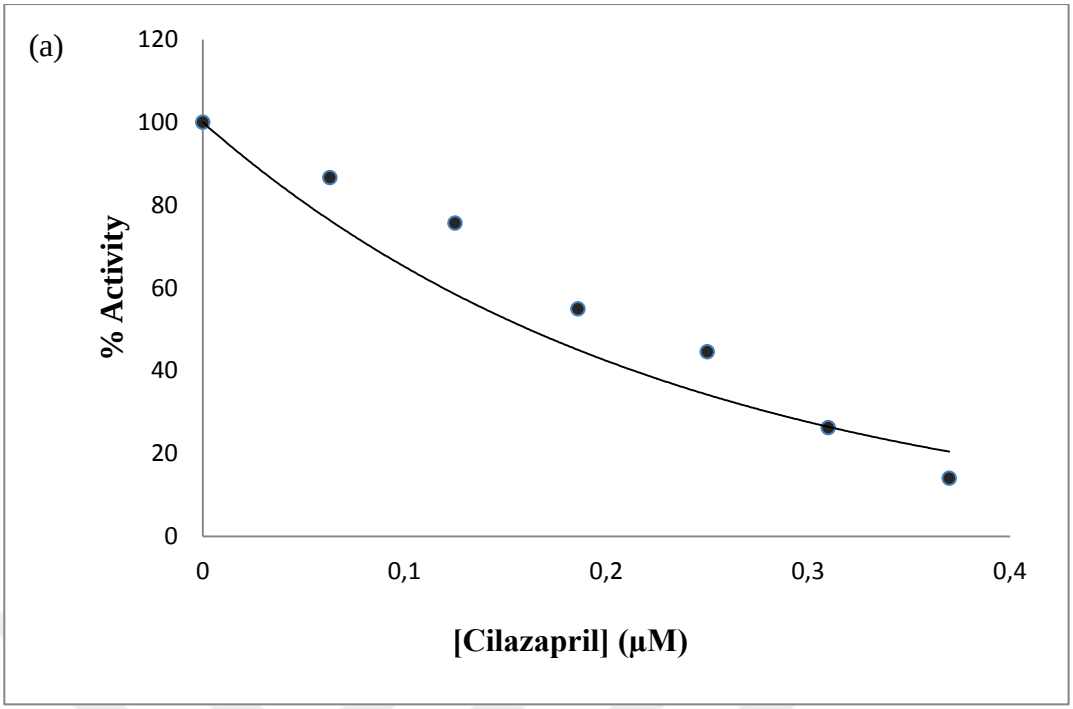


Figure 4.2 SDS-PAGE of the purified hCA-I and hCA-II isozymes.

Controls: porcine myosin (200kDa), β-galactosidase from E. coli (116kDa), rabbit muscle phosphorylase b (97kDa), bovine albumin (66kDa); ovalbumin (45kDa), bovine erythrocytes CA (29kDa).

4.4 The Results of Drugs Effect on Esterase Activity Carried out on hCA-I and hCA-II

Drug concentrations that caused 50% inhibition of esterase activity (IC_{50} values) were calculated by drawing % Activity-[Inhibitor] graphs (Figure 4.3-4.7).



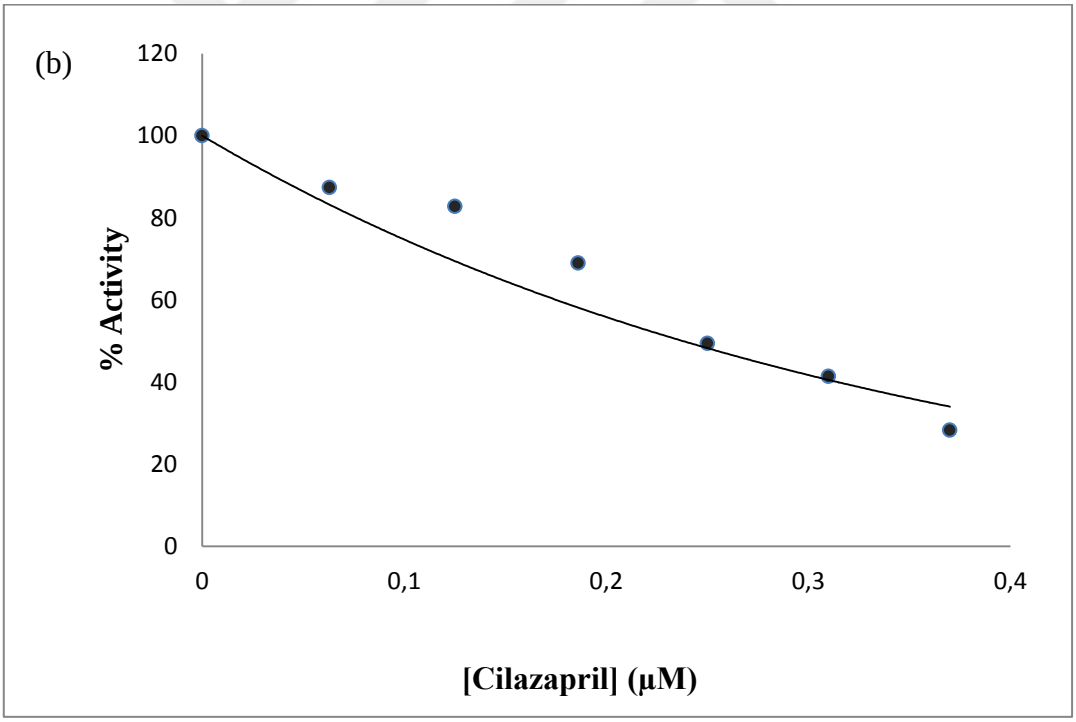
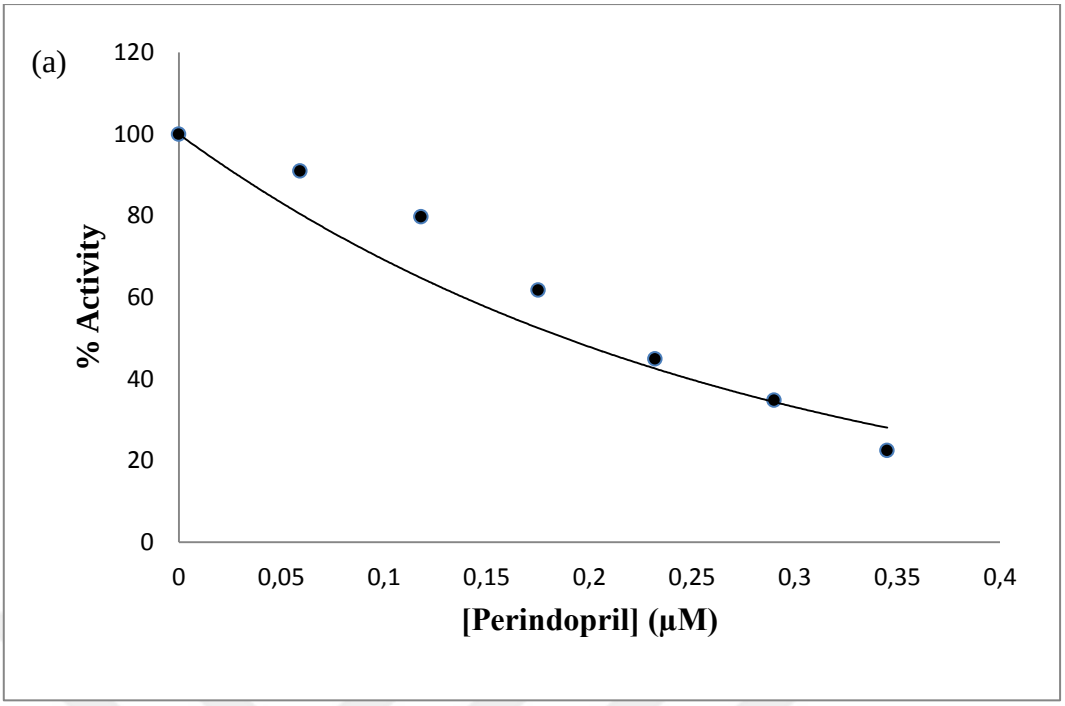


Figure 4.3 % Activity vs [Cilazapril] for hCA-I **(**a**)** and hCA-II (b).



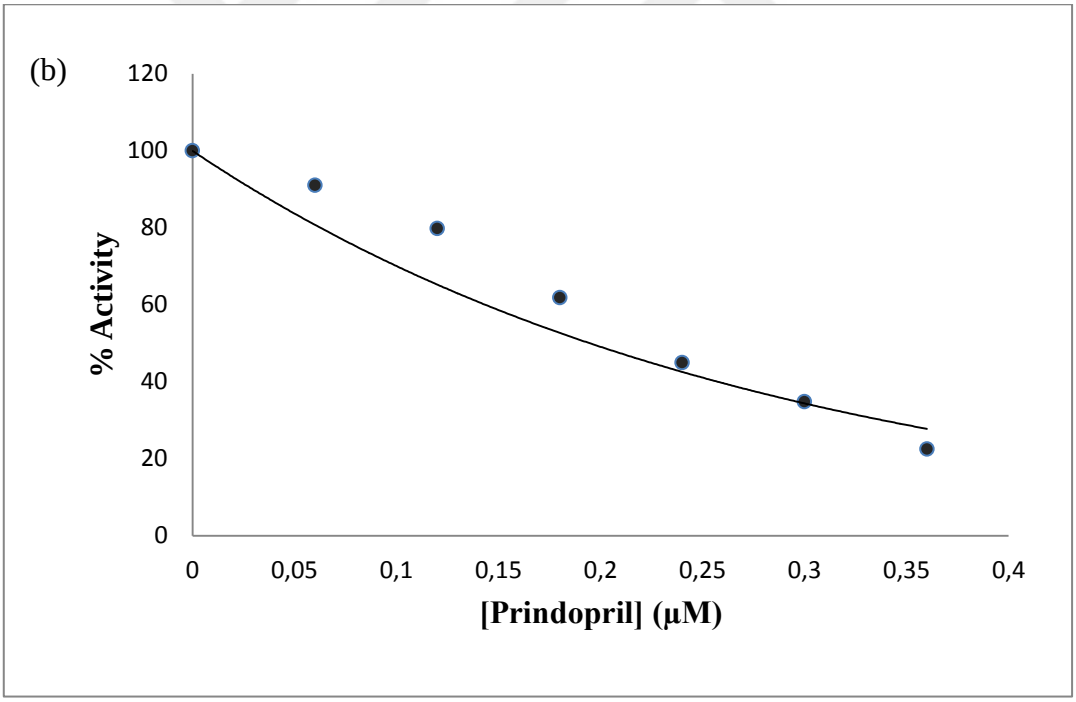
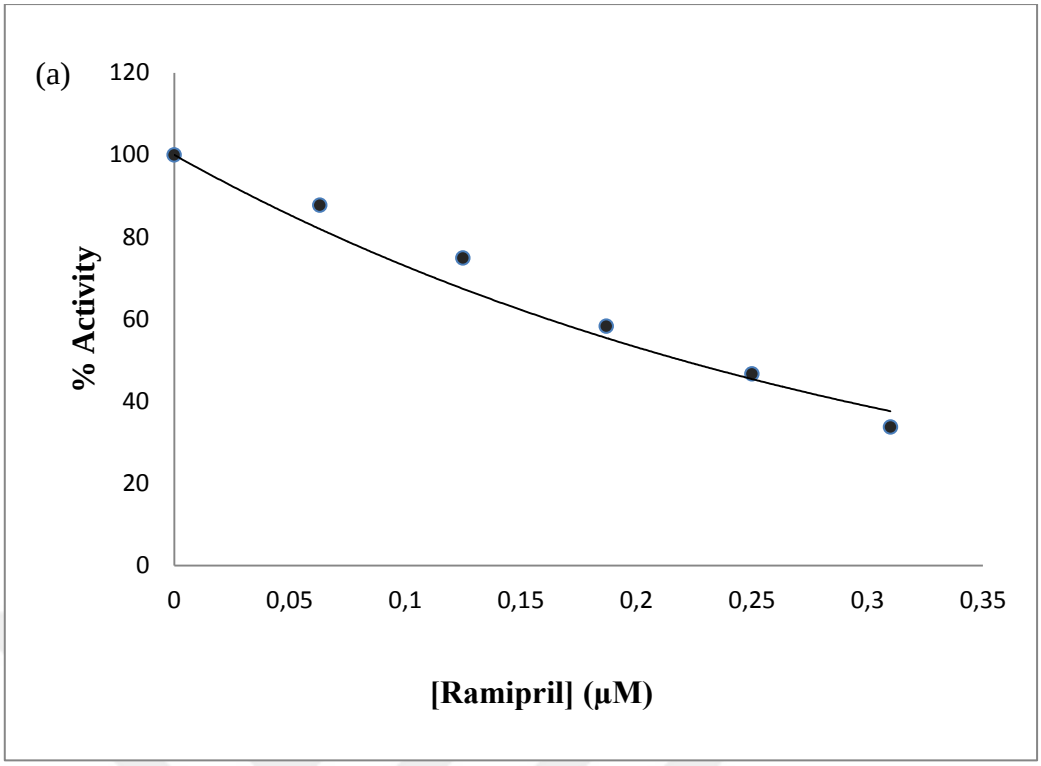


Figure 4.4 % Activity vs [Perindopril] for hCA-I **(a)** and hCA-II (b).



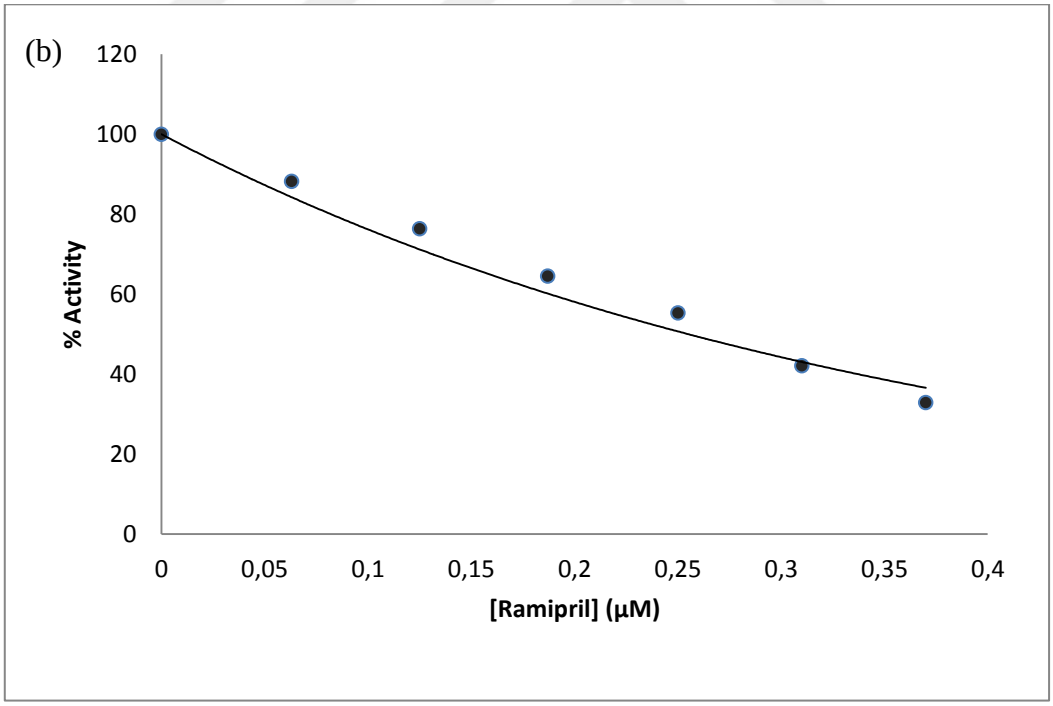
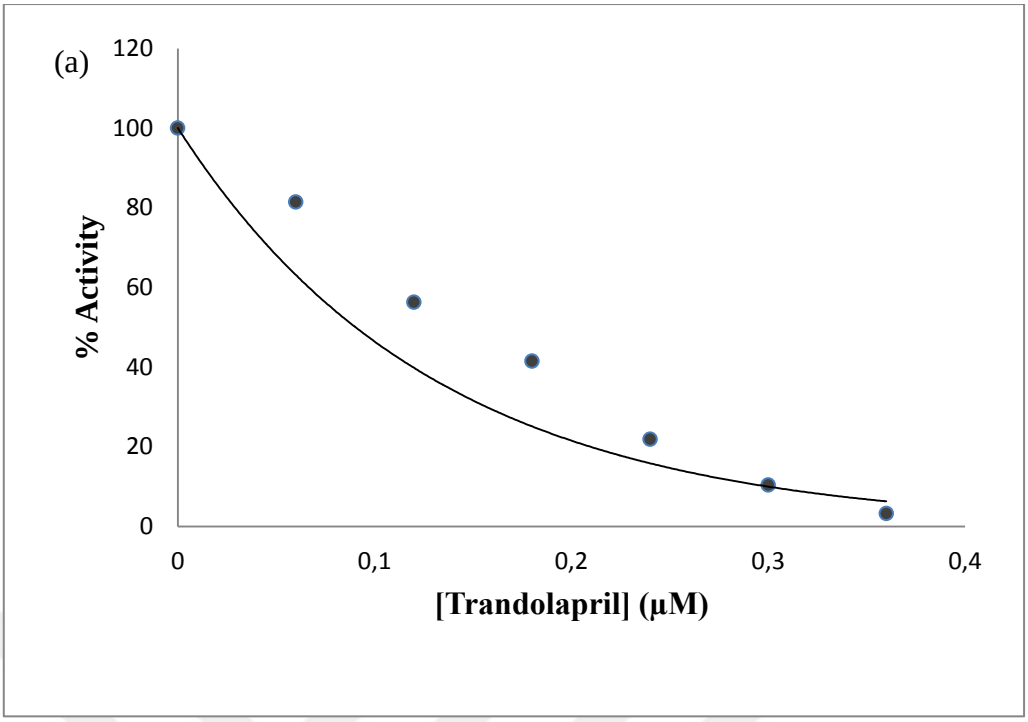


Figure 4.5 % Activity vs [Ramipril] for hCA-I **(**a**)** and hCA-II (b).



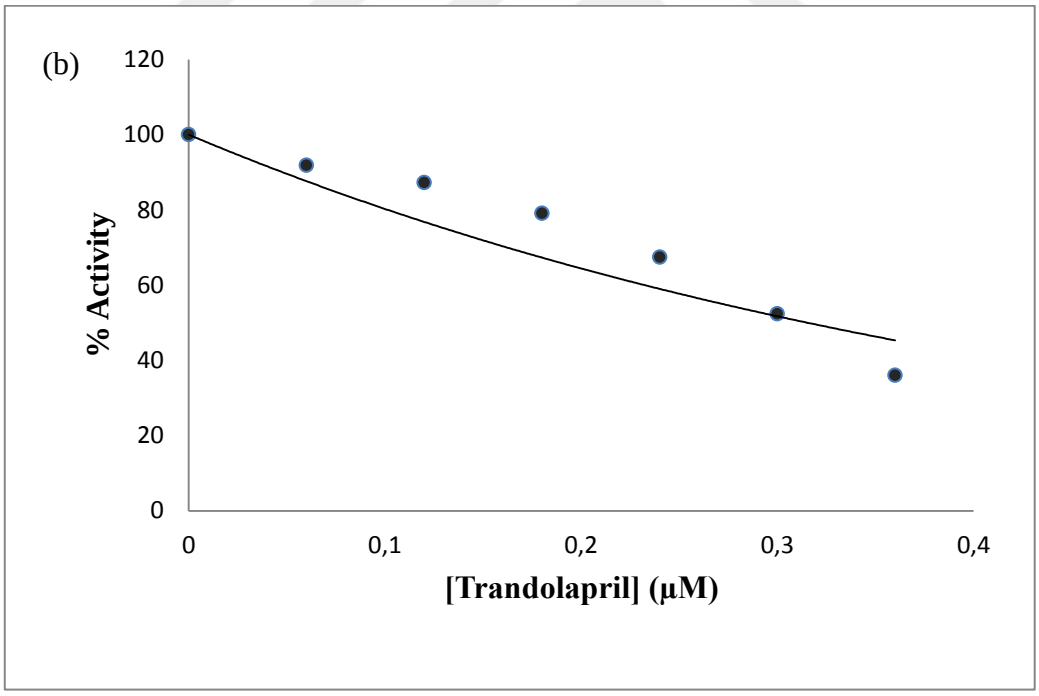
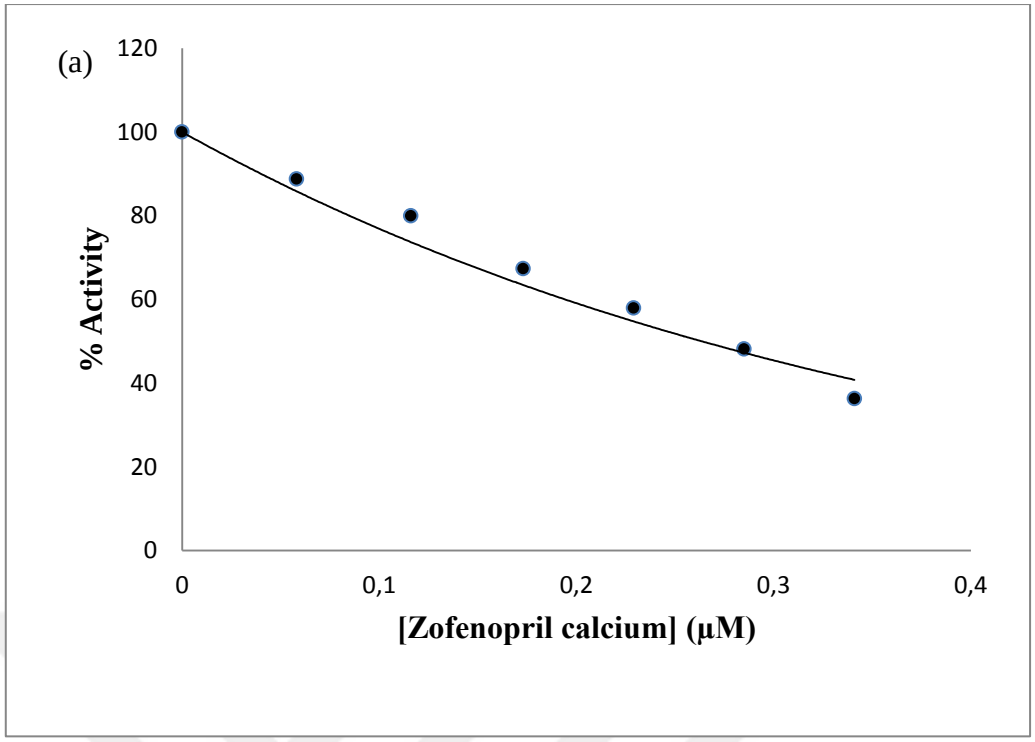


Figure 4.6 % Activity vs [Trandolapril] for hCA-I **(**a**)** and hCA-II (b).



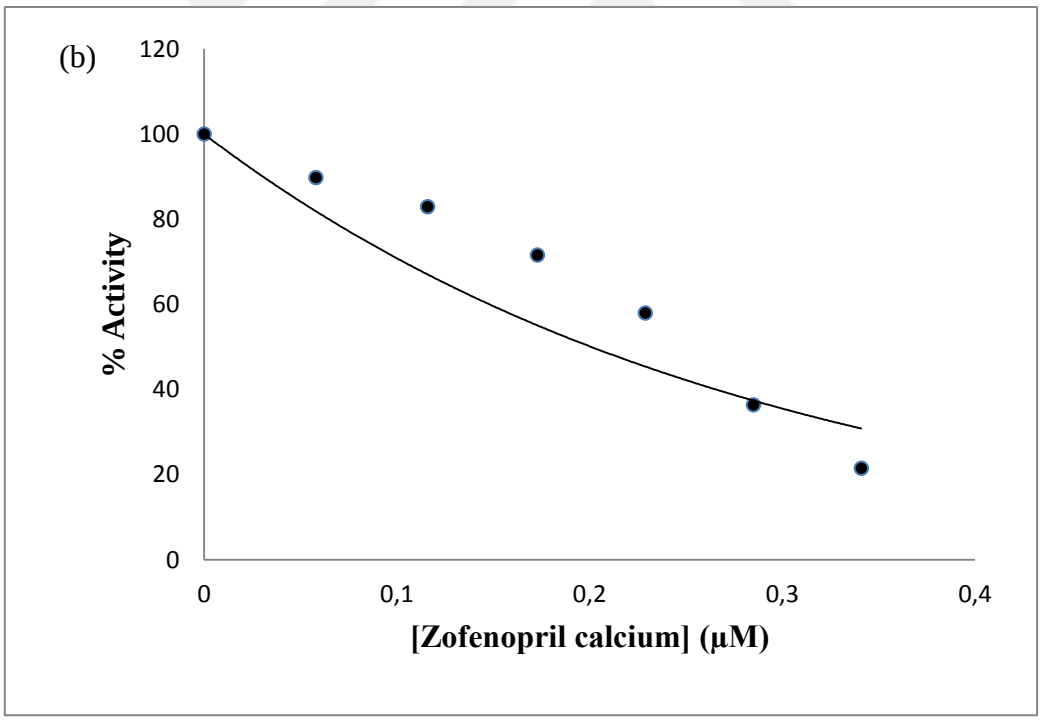


Figure 4.7 % Activity vs [Zofenopril calcium] for hCA-I **(**a**)** and hCA-II (b).

4.5 Results of Molecular Docking for Drugs to the hCA-I and hCA-II

By using UCSF Chimera (Chimera1.11.2), the best values of ΔG with full fitness were analyzed and the results were shown below (Figure 4.8-4.12).

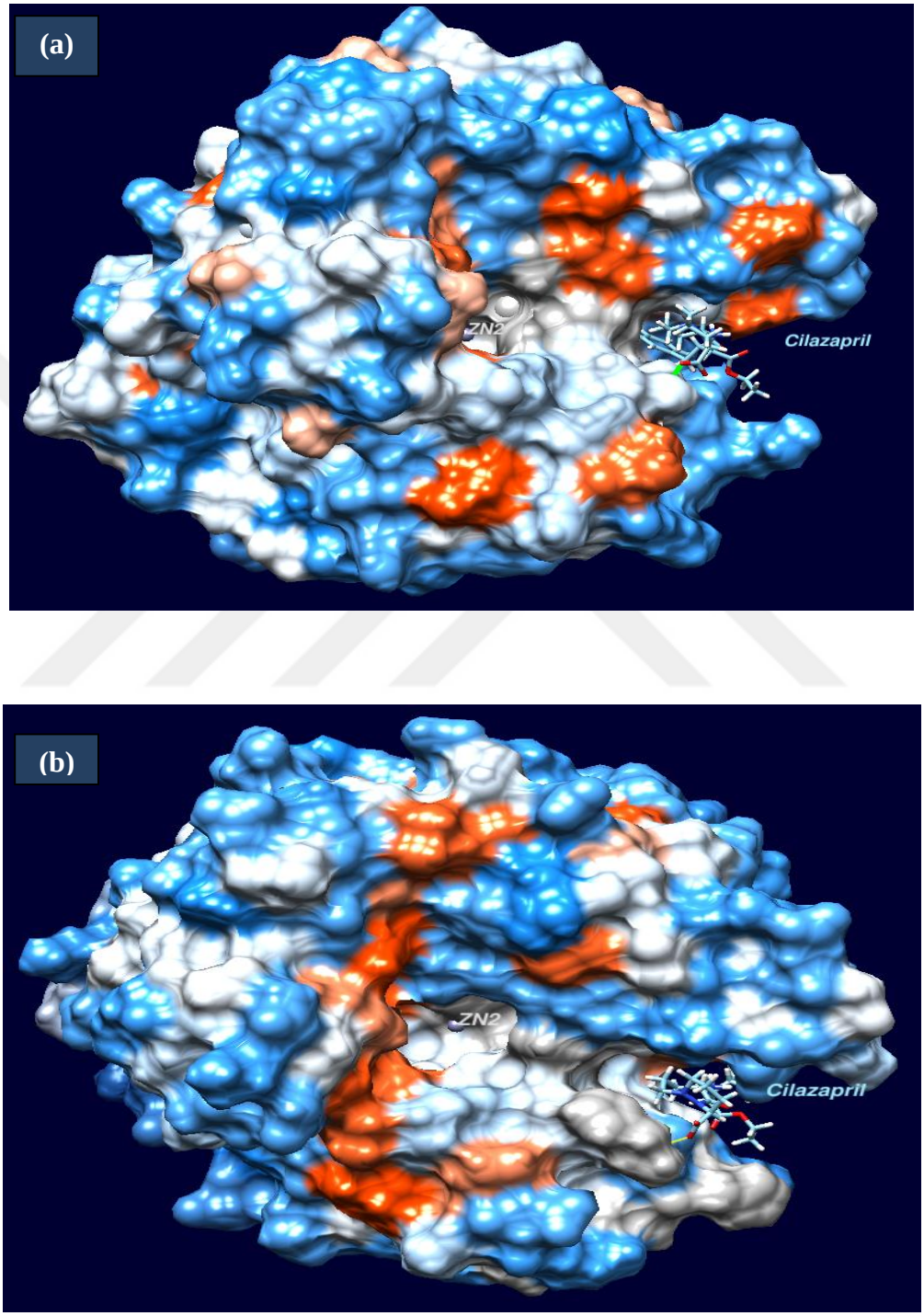


Figure 4.8 Molecular docking of Cilazapril to hCA-I (a) and hCA-II (b).

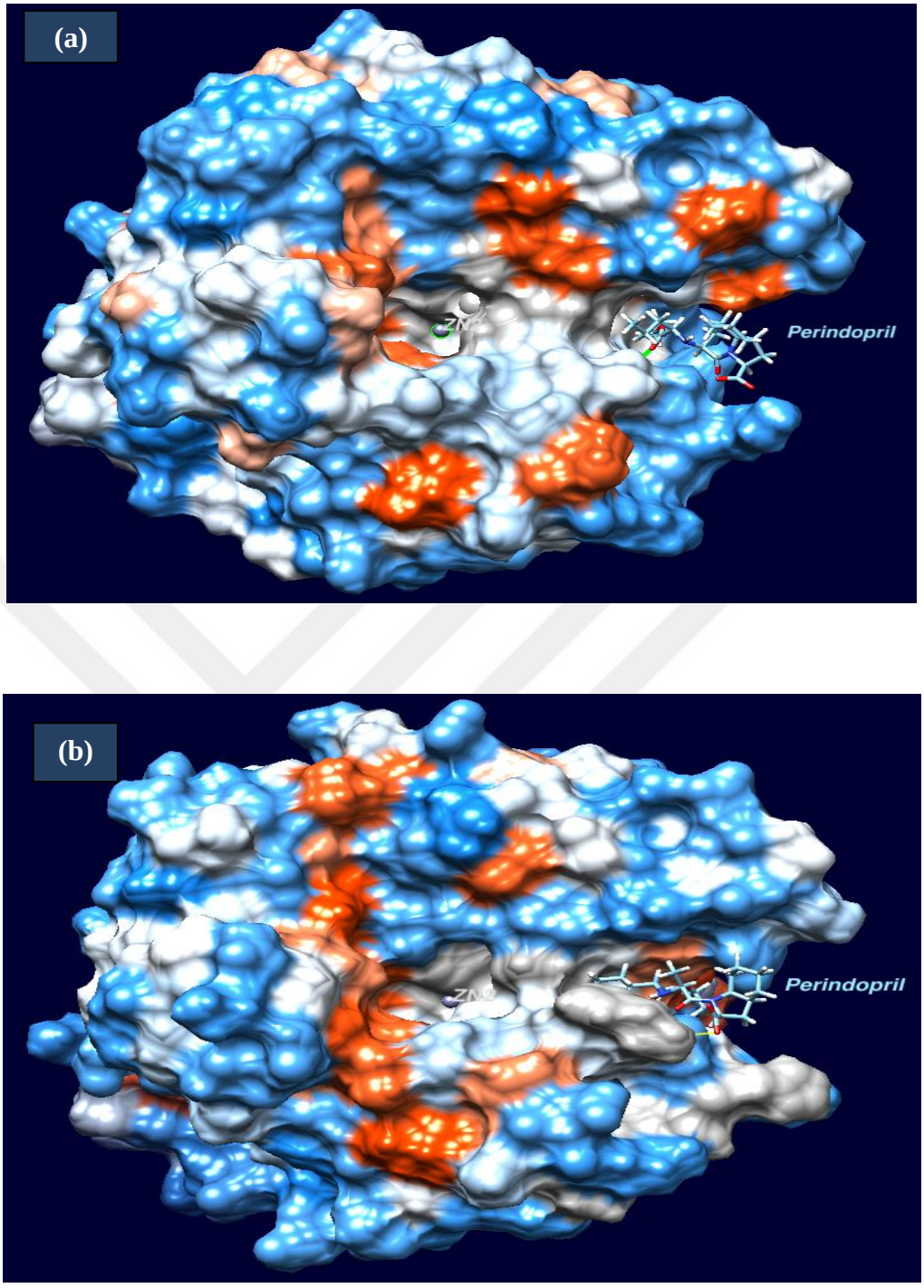
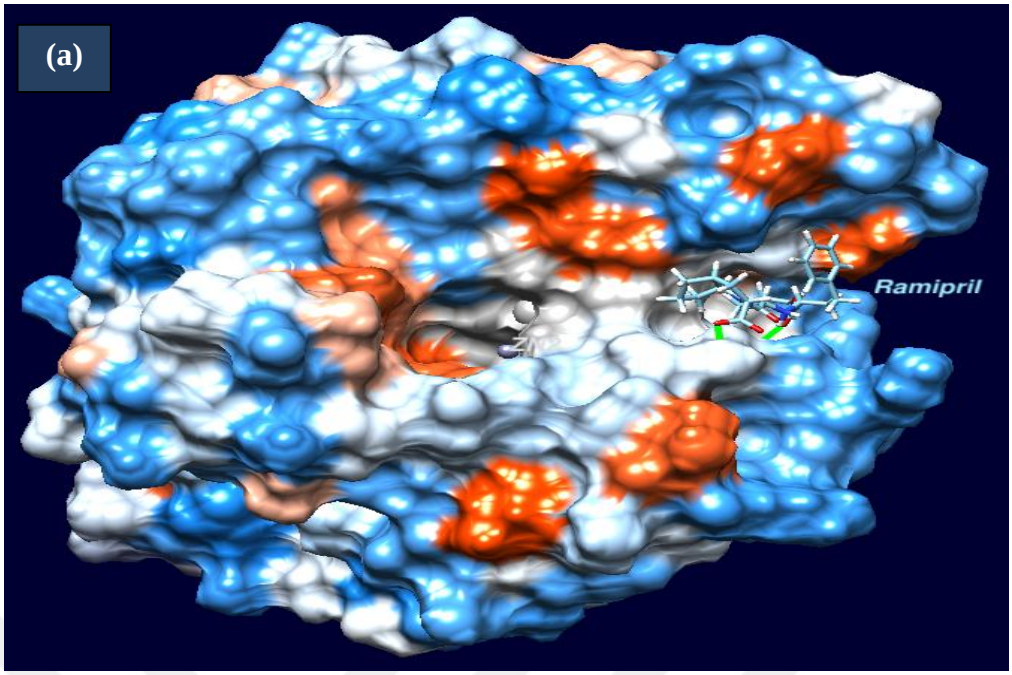


Figure 4.9 Molecular docking of Perindopril to hCA-I (a) and hCA-II (b).



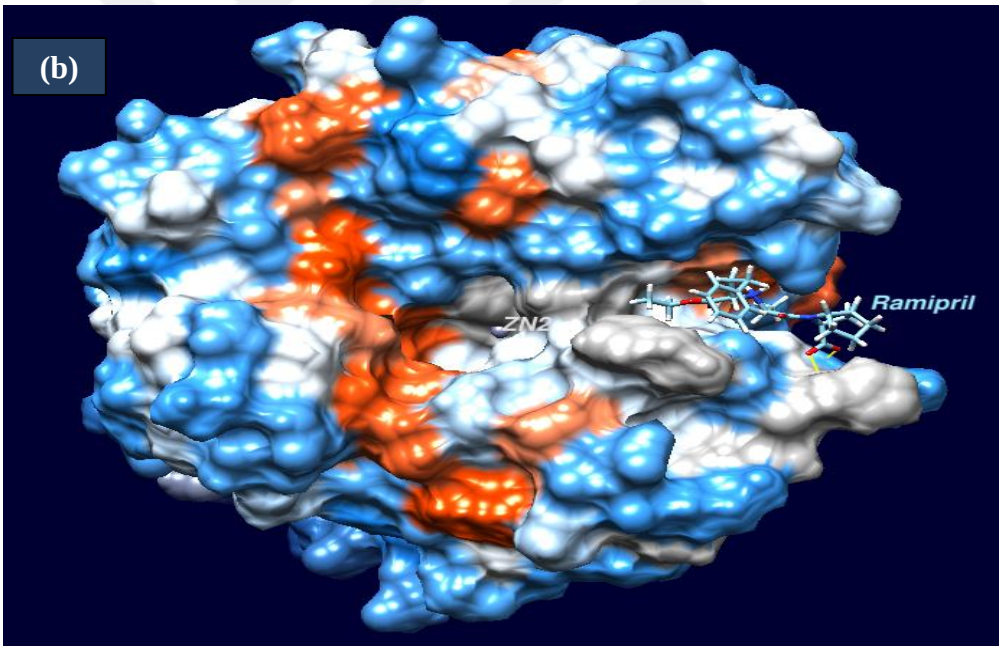


Figure 4.10 Molecular docking of Ramipril to hCA-I (a) and hCA-II (b).

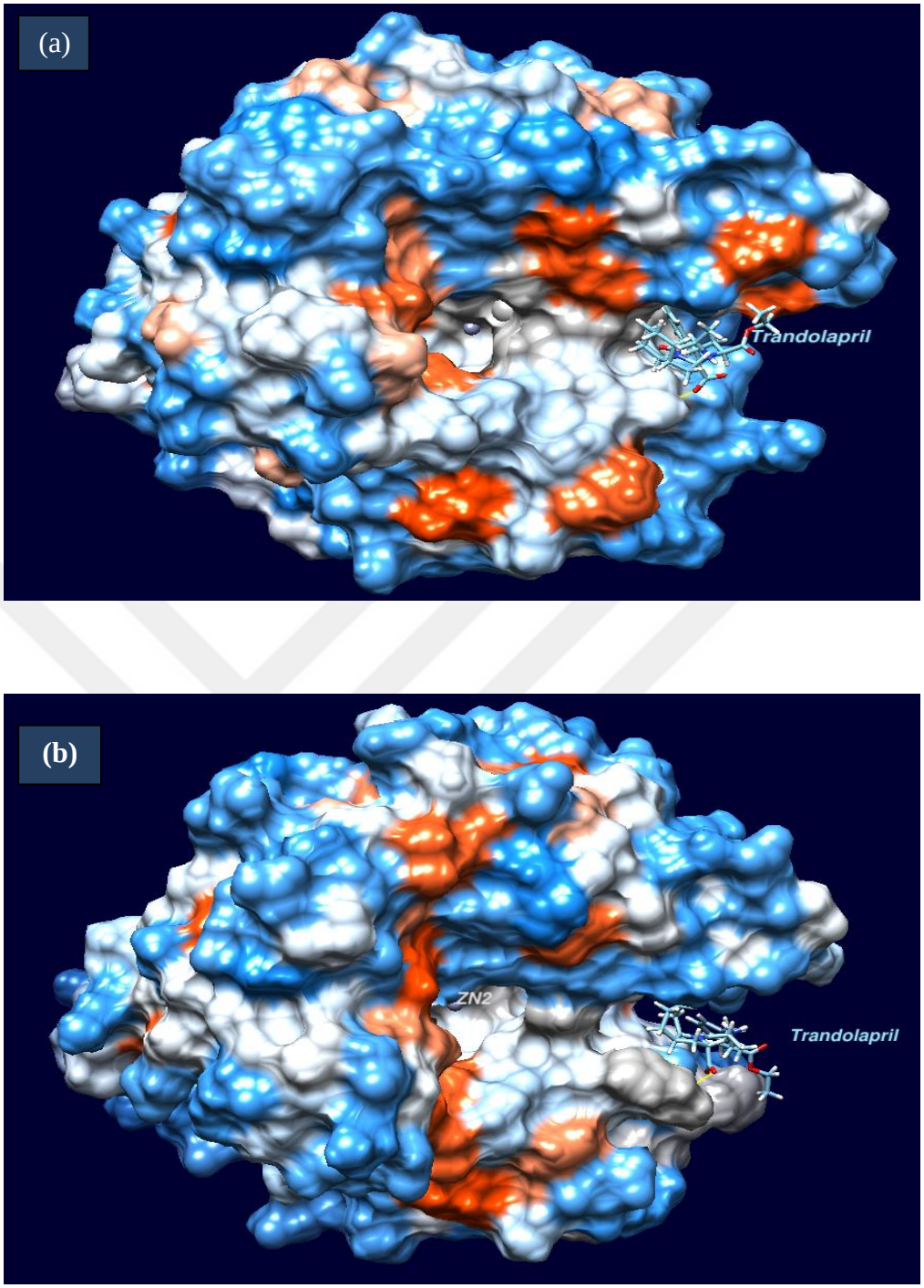


Figure 4.11 Molecular docking of Trandolapril to hCA-I (a) and hCA-II (b).

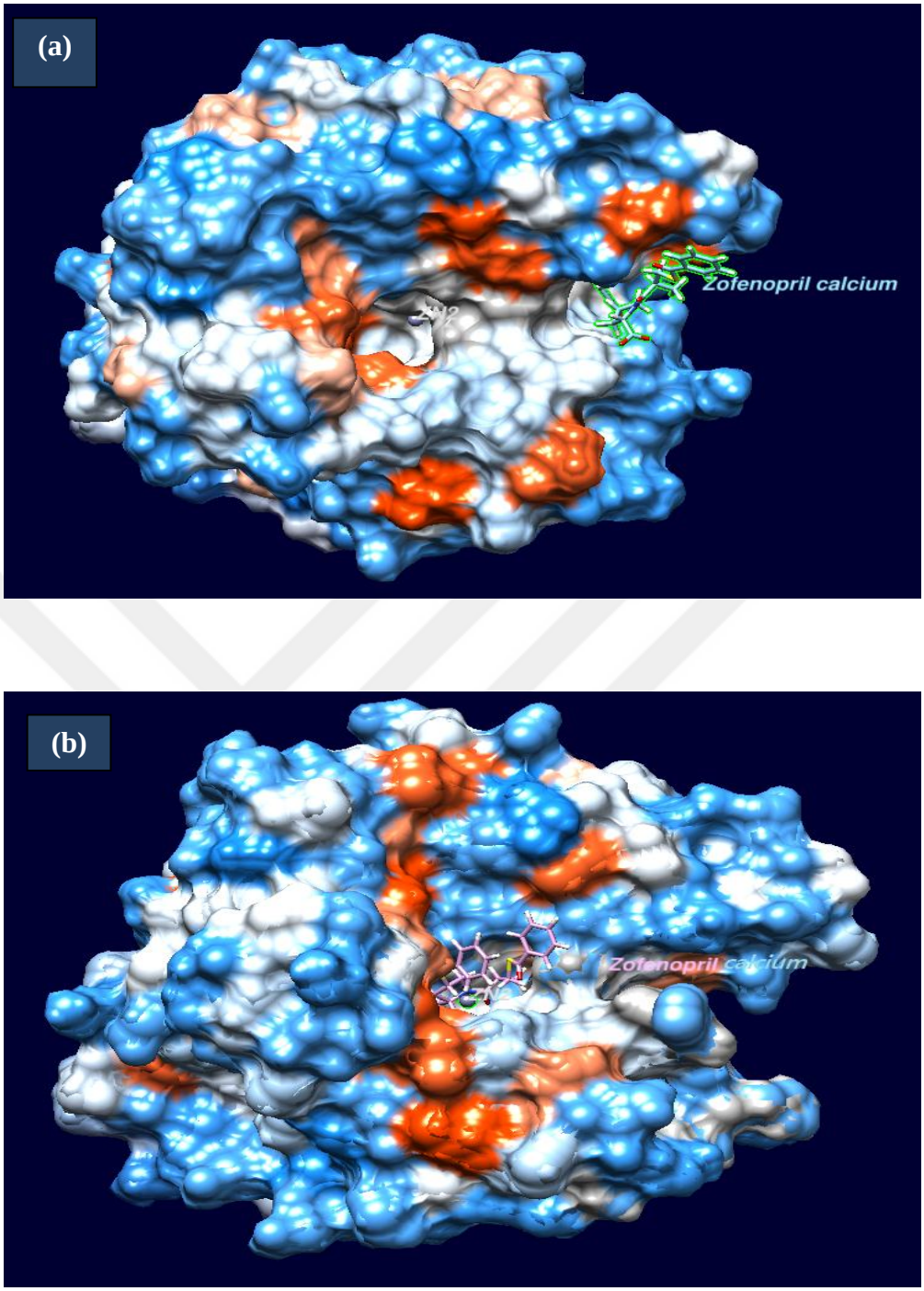


Figure 4.12 Molecular docking of Zofenopril calcium to hCA-I (a) and hCA-II (b).

4.6 Total Results Related to Esterase Activity of hCA-I and hCA-II with Drugs

IC_{50} value of each drug was calculated from the %Activity-[I] graphs. The Full Fitness, Estimated ΔG, and Binding Domain were theoretically found from the results of molecular docking studies (Table 4.2).

Table 4.2 IC_{50}values and results of molecular docking studies; Full Fitness, Estimated ΔG, and Binding Domain of the drugs on esterase activity of hCA-I and hCA-II.

Drug	hCA-I				hCA-II			
	IC_{50} (μM)	**Full Fitness (kcal/mol)**	**Estimated ΔG (kcal/mol)**	**Binding Domain**	**IC_{50} (μM)**	**Full Fitness (kcal/mol)**	**Estimated ΔG (kcal/mol)**	**Binding Domain**
1	0.161	-1221.77	-8.38	Binding pocket	0.238	-1352.24	-8.90	Binding pocket
2	0.160	-1197.50	-9.19	Binding pocket	0.195	-1351.63	-8.84	Binding pocket
3	0.220	-1209.79	-8.76	Binding pocket	0.255	-1337.17	-8.34	Binding pocket
4	0.090	-1197.46	-8.04	Binding pocket	0.316	-1351.96	-8.83	Binding pocket
5	0.226	-1230.61	-9.14	Binding pocket	0.201	-1400.12	-12.24	Active site

* 1; Cılazapril, 2; Perindopril, 3; Ramipril, 4; Trandolapril, 5; Zofenopril calcıum.

CHAPTER 5

DISCUSSION

The hCA-I, and hCA-II are the major isozymes that are present at high concentrations in the cytosol in erythrocytes, and hCA-II has the highest turnover rate of all the CAs, together with CA-IX [157]. There is a fact that CO_2 is a major end product of aerobic metabolism, this CO_2 is released from tissues into the blood and transported to the lungs for exhalation. In the blood, carbon dioxide reacts with water. The product of this reaction is a moderately strong acid, carbonic acid (pK_a = 3.5), which becomes bicarbonate ion by losing a proton [158], this process catalyzed by hCA-I and hCA-II in the blood which they have two activities:

1. Hydratase activity by catalyzing the reversible hydration of carbon dioxide to HCO_3^- and H^+, HCO_3^- in the blood must be dehydrated to form CO_2 for exhalation as the blood passes through the lungs. Conversely, CO_2 must be converted into HCO_3^- for the generation of the aqueous humor of the eye and other secretions [7], [8], [21], [68], [158].

2. Esterase activity, there are several enzymes showing this activity, but only under in vitro conditions, the esterase activity of carbonic anhydrase was determined. Esterases are known to hydrolyse endogenous substrates majority hydrolyse lipid ester substrates [159].

hCA-I and hCA-II have two domains called active site and the binding pocket as shown in Figure 5.1.

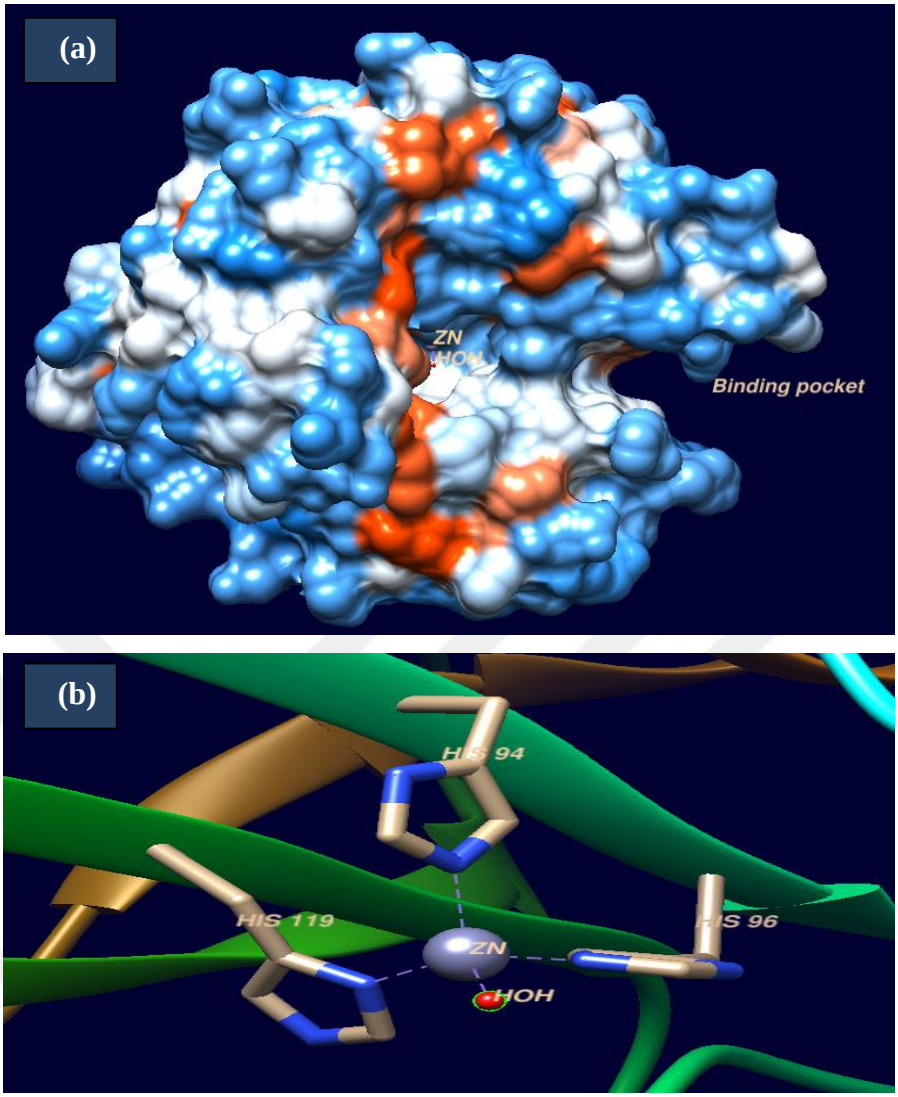


Figure 5.1 The active site and binding pocket of hCA-II (a), amino acids at the active site of hCA-II (b).

In this study, effects of ACE-inhibitor drugs (Cilazapril, Perindopril, Ramipril, Trandolapril, and Zofenopril calcium) used for heart diseases were investigated in vitro on esterase activity of hCA-I and hCA-II which purified from human erythrocytes by using Sepharose 4B L-tyrosine sulfanilamide affinity chromatography. hCA-I was purified 94.62 fold with a specific activity of 704.35 EU/mg protein and 67.5 % yield, hCA-II was purified 470.19 fold with a specific activity of 3500 EU/mg protein and 54.69% yield. Afterwards purity of isozymes was controlled by SDS-PAGE. All of these drugs showed inhibition effect at μM concentration. IC_{50} values were calculated by drawing % Activity-[Inhibitor] graphs. Molecular docking was used in this study to investigate the interactions between these drugs and the amino acids in the domains of hCA-I and hCA-II. These isozymes are important therapeutic targets with the potential

to be inhibited to treat several disorders including edema, glaucoma, obesity, cancer, epilepsy, and osteoporosis [157].

The effects of the five drugs on both hCA-I and hCA-II are discussed by using esterase activity and molecular docking results:

1. Cilazapril, showed the better inhibition effect on esterase activity of hCA-I with IC_{50} = 0.161 µM, than from the effect on the esterase activity of hCA-II with IC_{50} =0.238 µM. This drug interacted with the amino acid residues in the binding pocket.

- In case of hCA-I, the functional groups (COO^-) and (R-O-R) of cilazapril interact side chains of TRP 5, ASN 11 and SER 231 by formed a hydrogen bond (Figure 5.2.a and 5.2.b).
- In case of hCA-II, the functional group (COO^-) of cilazapril interacts side chains of HIS 4 and ASN 11 by formed a hydrogen bond (Figure 5.3.a - 5.3.b).

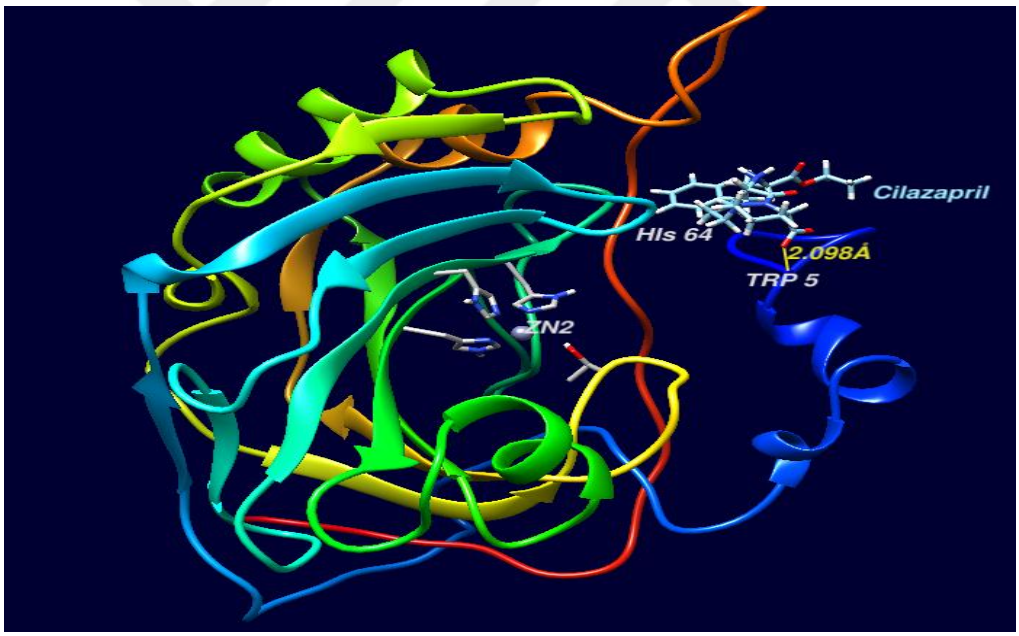


Figure 5.2a The ribbon diagram of hCA-I and cilazapril shows interactions with the residues in the binding pocket.

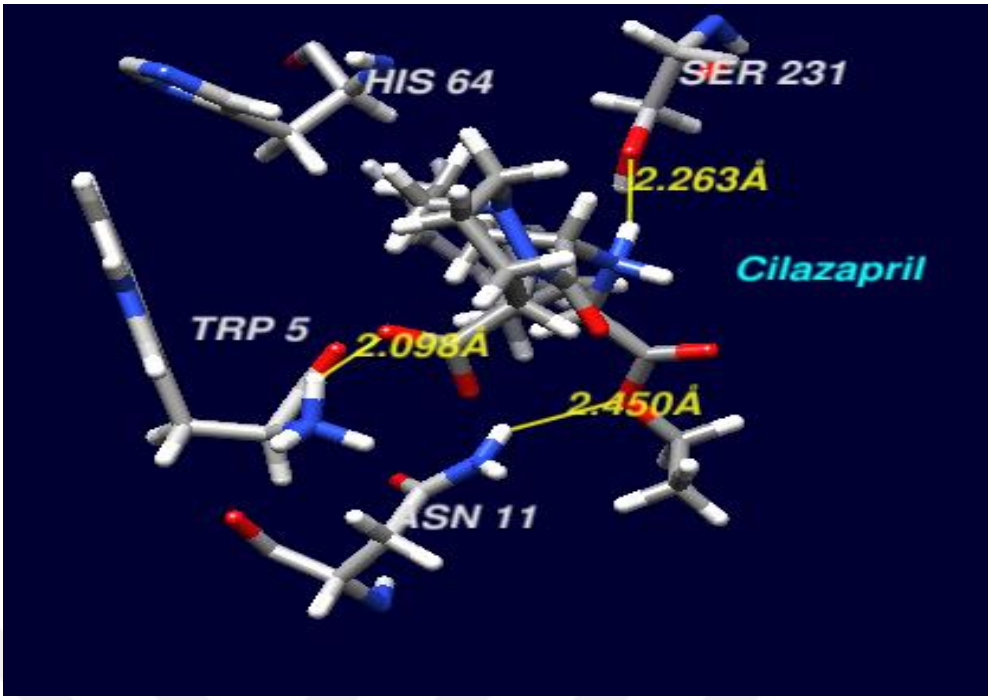


Figure 5.2b Cilazapril interacts with the amino acids in the binding pocket of hCA-I by hydrogen bonds.

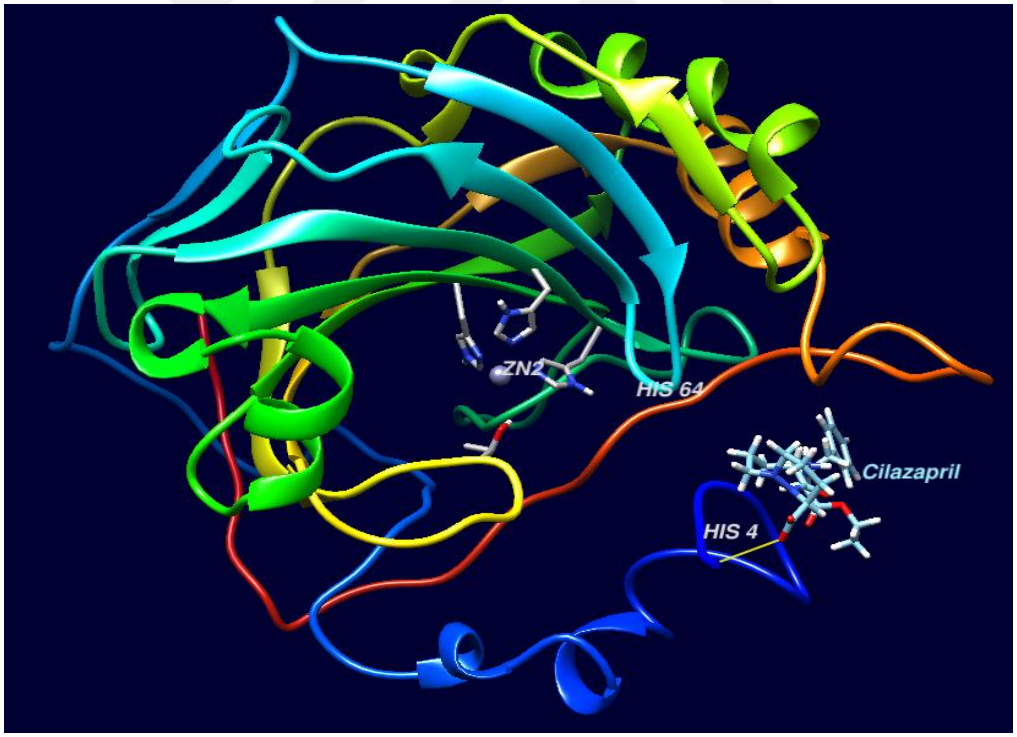


Figure 5.3a The ribbon diagram of hCA-II and cilazapril shows interactions with the residues in the binding pocket.

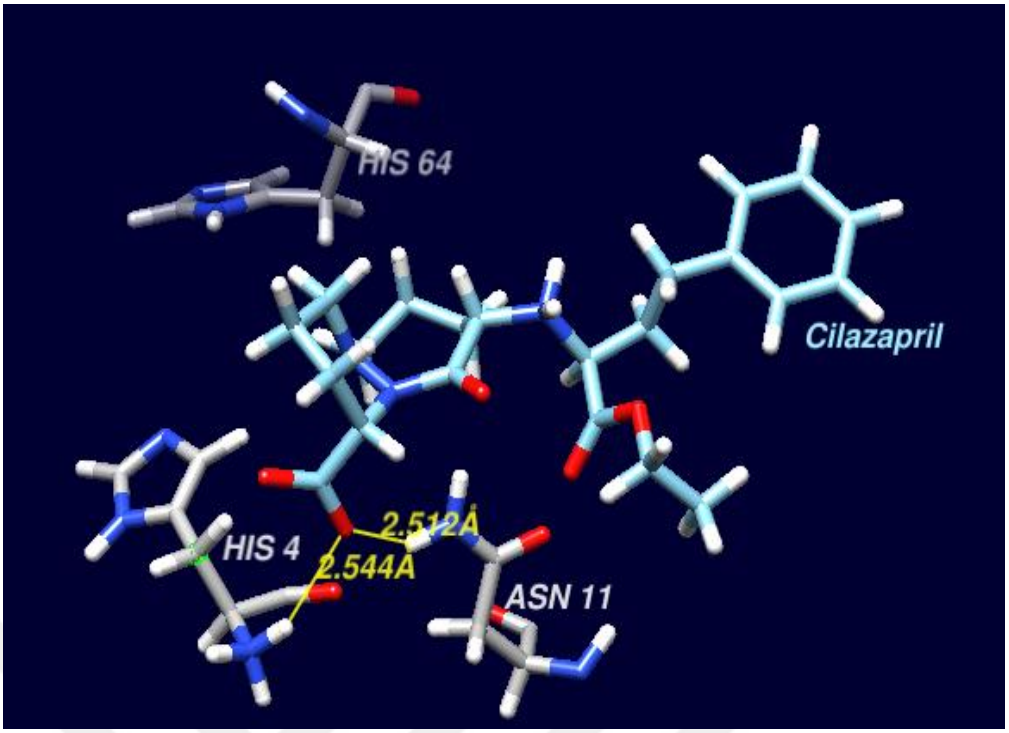


Figure 5.3b Cilazapril interacts with the amino acids in the binding pocket of hCA-II by hydrogen bonds.

2. Perindopril showed the best inhibition effect on esterase activity for hCA-II with IC_{50} = 0.195 µM, but also better inhibition effect on the esterase activity of hCA-I with IC_{50} = 0.160 µM. This drug interacted with the amino acid residues in the binding pocket.

- In case of hCA-I the functional group (COO^-) and (C=O) interacts side chains of LYS 10, ASN 11, and TYR 7 by formed a hydrogen bond (Figure 5.4.a and 5.4.b).
- In case of hCA-II the functional group (COO^-) and (C=O) interact side chains of HIS 4 and TYR 7 by formed a hydrogen bond (Figure 5.5.a and 5.5.b).

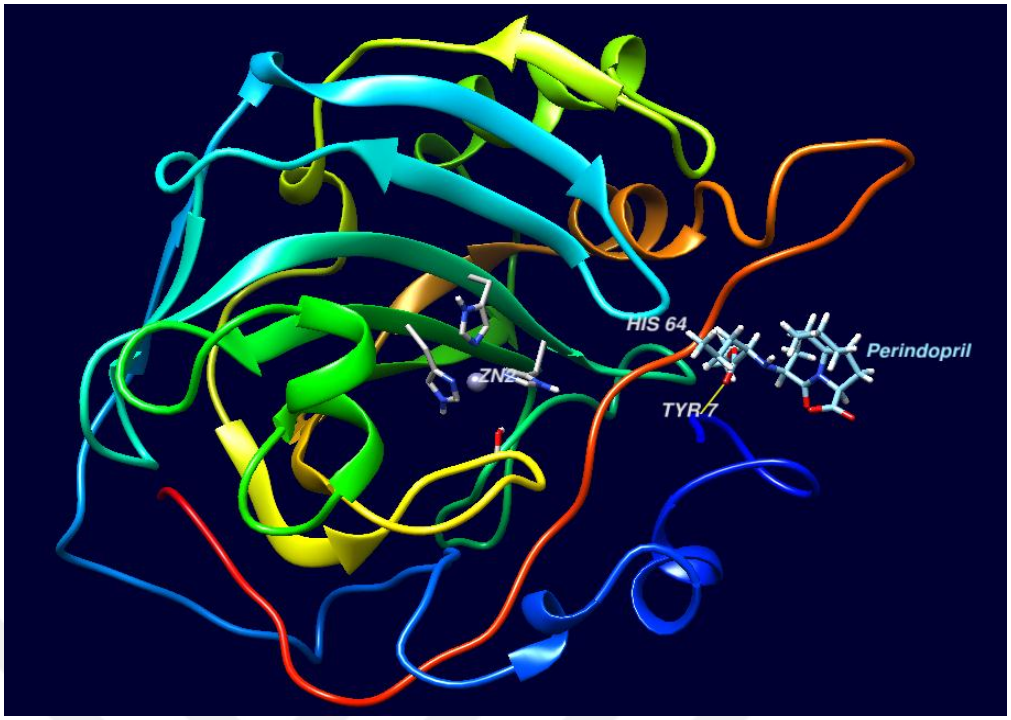


Figure 5.4a The ribbon diagram of hCA-I and perindopril shows interactions with the residues in the binding pocket.

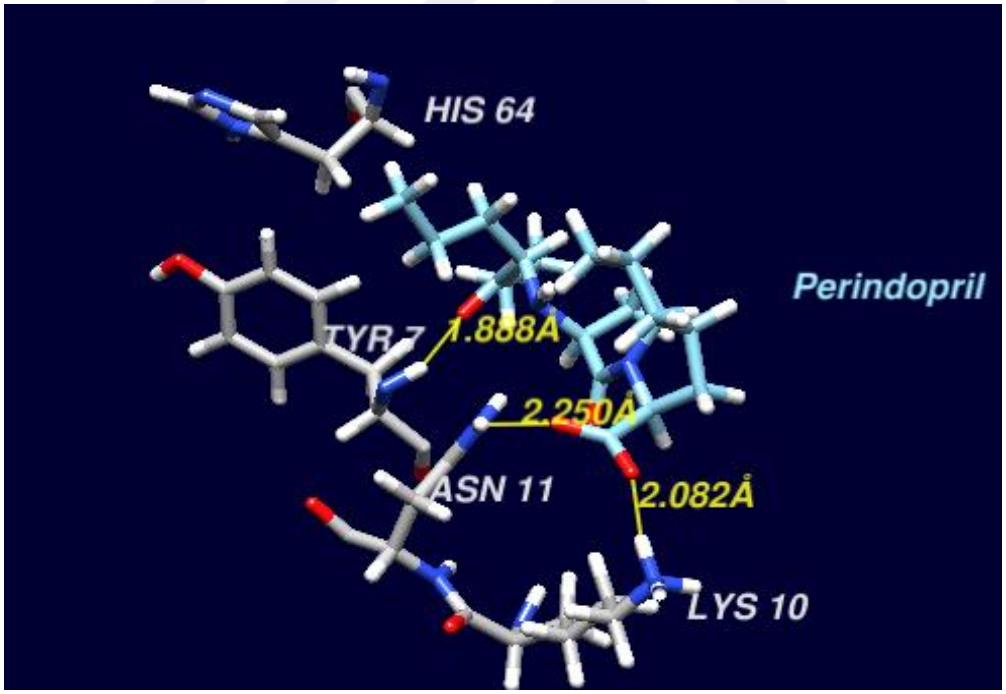


Figure 5.4b Perindopril interacts with the amino acids in the binding pocket of hCA-I by hydrogen bonds.

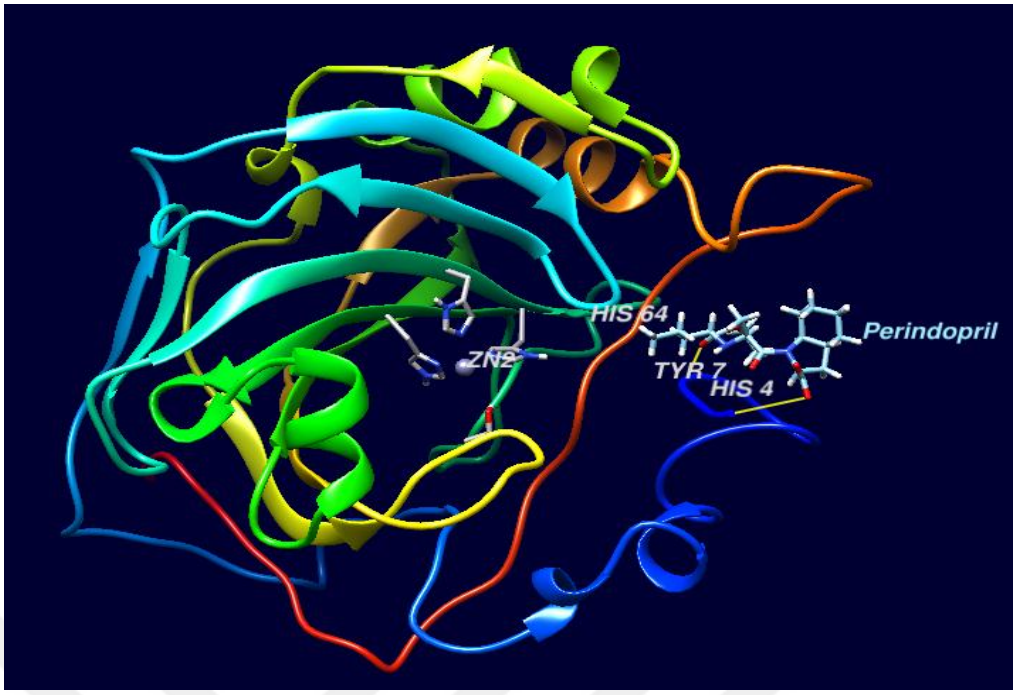


Figure 5.5a The ribbon diagram of hCA-II and perindopril shows interactions with the residues in the binding pocket.

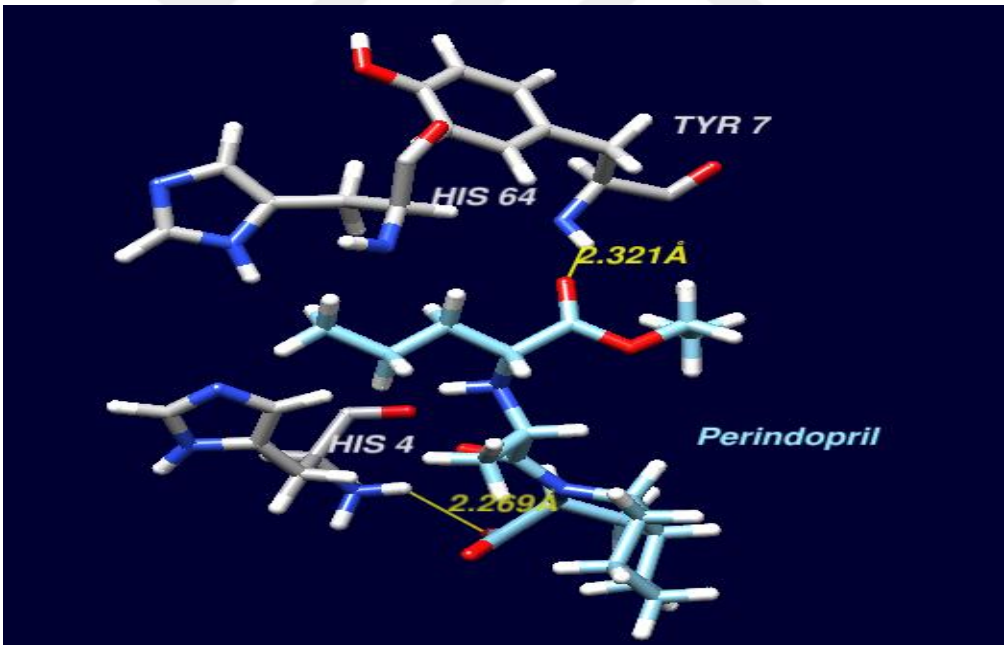


Figure 5.5b Perindopril interacts with the amino acids in the binding pocket of hCA-II by hydrogen bonds.

3. Ramipril showed the better inhibition effect on esterase activity for hCA-I with IC_{50} = 0.220 µM, than from the effect on the esterase activity of hCA-II with IC_{50} = 0.255 µM. This drug interacted with the amino acid residues in the binding pocket.

- In case of hCA-I the functional group (COO-) and (C=O) interact side chains of the TRP 5, and TYR 7 by formed a hydrogen bond (Figure 5.6.a and 5.6.b).
- In case of hCA-II the functional group (COO-) interact side chains of HIS 10, and LYS 9 by formed a hydrogen bond (Figure 5.7.a and 5.7.b).

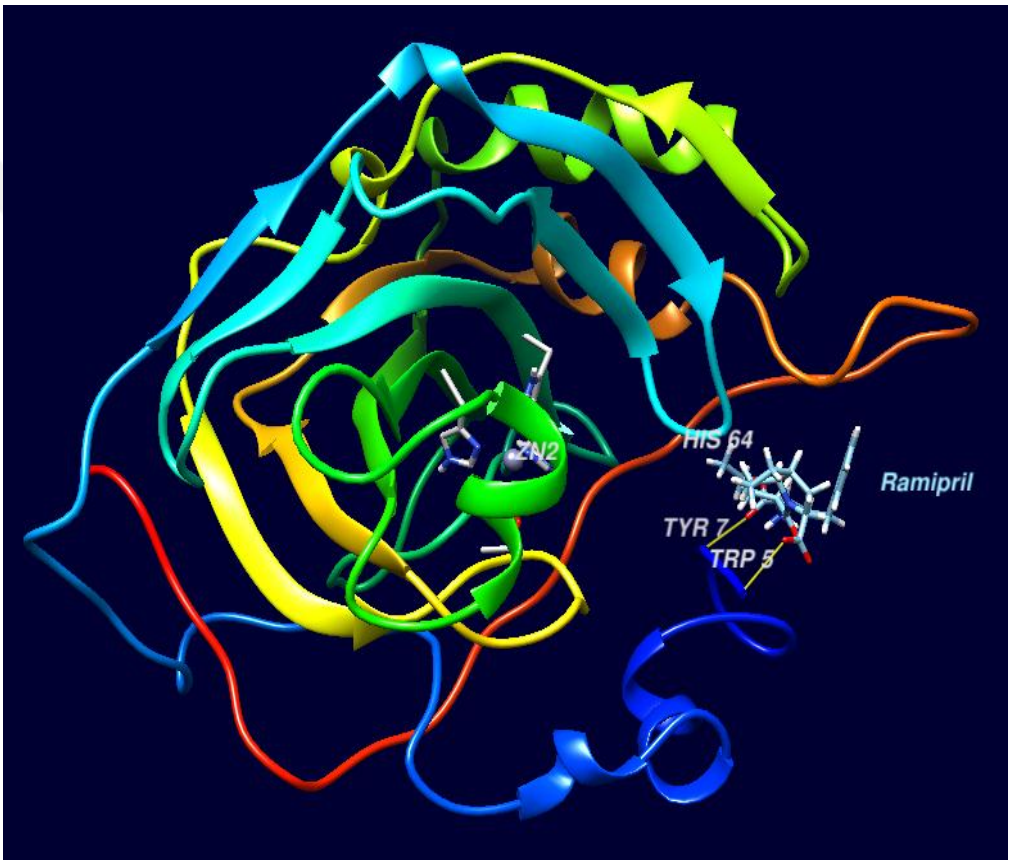


Figure 5.6a The ribbon diagram of hCA-I and ramipril shows interactions with the residues in the binding pocket.

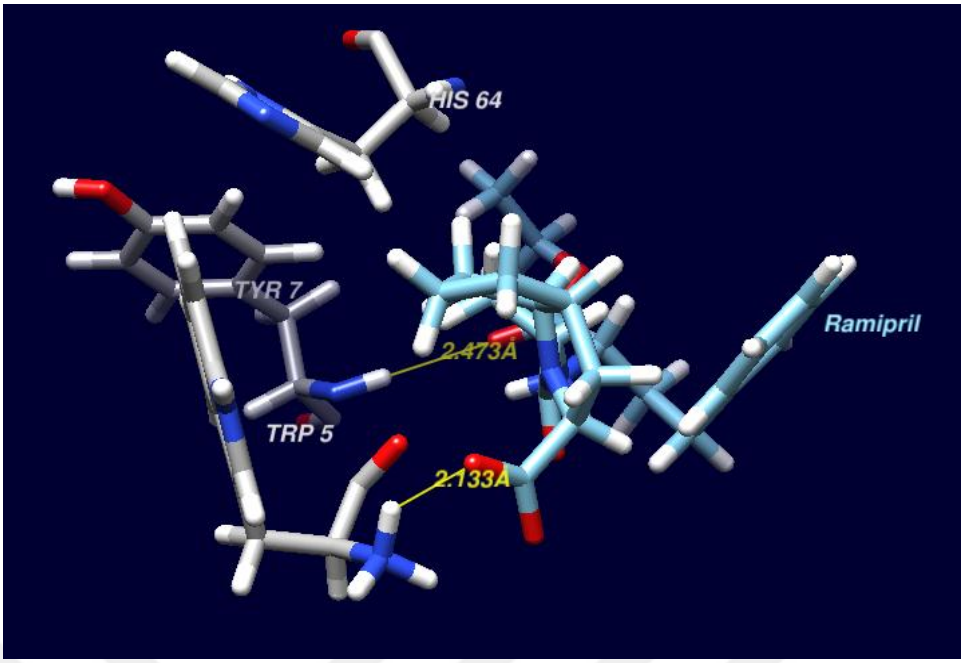


Figure 5.6b Ramipril interacts with the amino acids in the binding pocket of hCA-I by hydrogen bonds.

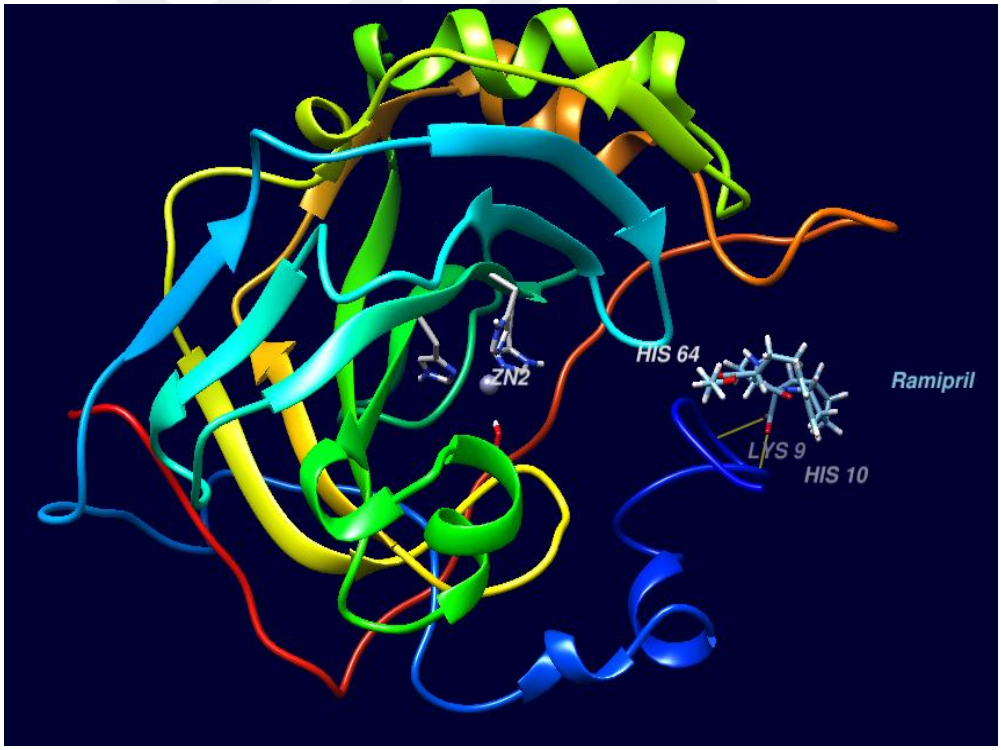


Figure 5.7a The ribbon diagram of hCA-II and ramipril shows interactions with the residues in the binding pocket.

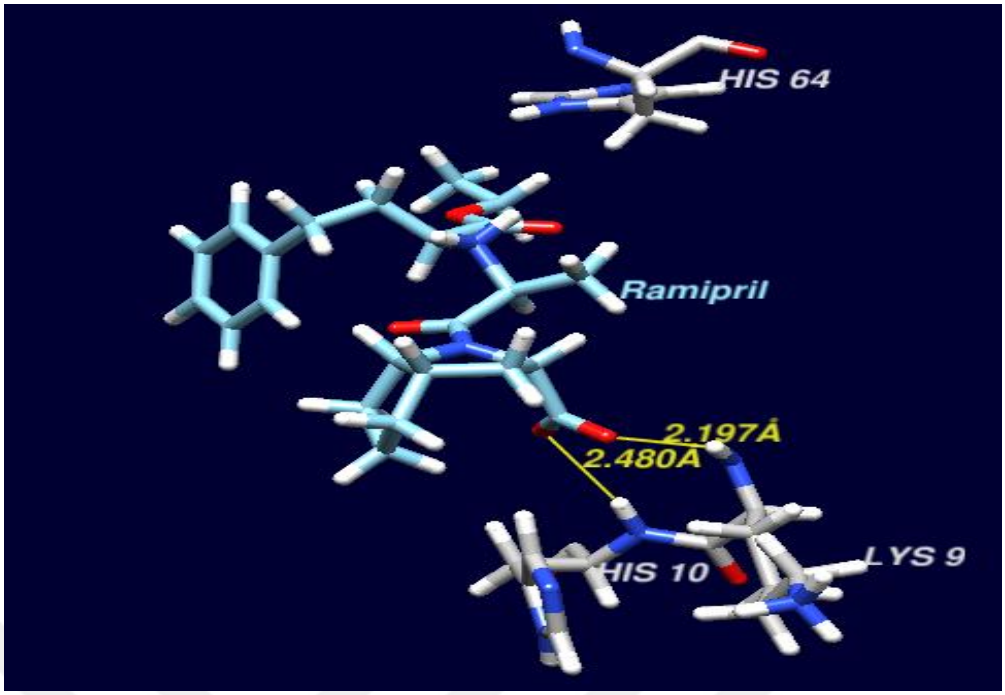


Figure 5.7b Ramipril interacts with the amino acids in the binding pocket of hCA-II by hydrogen bonds.

1. Trandolapril showed the best inhibition effect on esterase activity for hCA-I IC_{50} = 0.090 μM, but the lowest effect on esterase activity of hCA-II with IC_{50} = 0.316 μM. This drug interacted with the amino acid residues in the binding pocket of enzyme.

    - In case of hCA-I the functional group (COO^-) interacts side chain of TRP 5 by form a hydrogen bond (Figure 5.8.a and 5.8.b).
    - In case of hCA-II functional group (COO^-) and (C=O) interact side chain of HIS 4 and ASN 11, by form a hydrogen bond (Figure 5.9.a and 5.9.b).

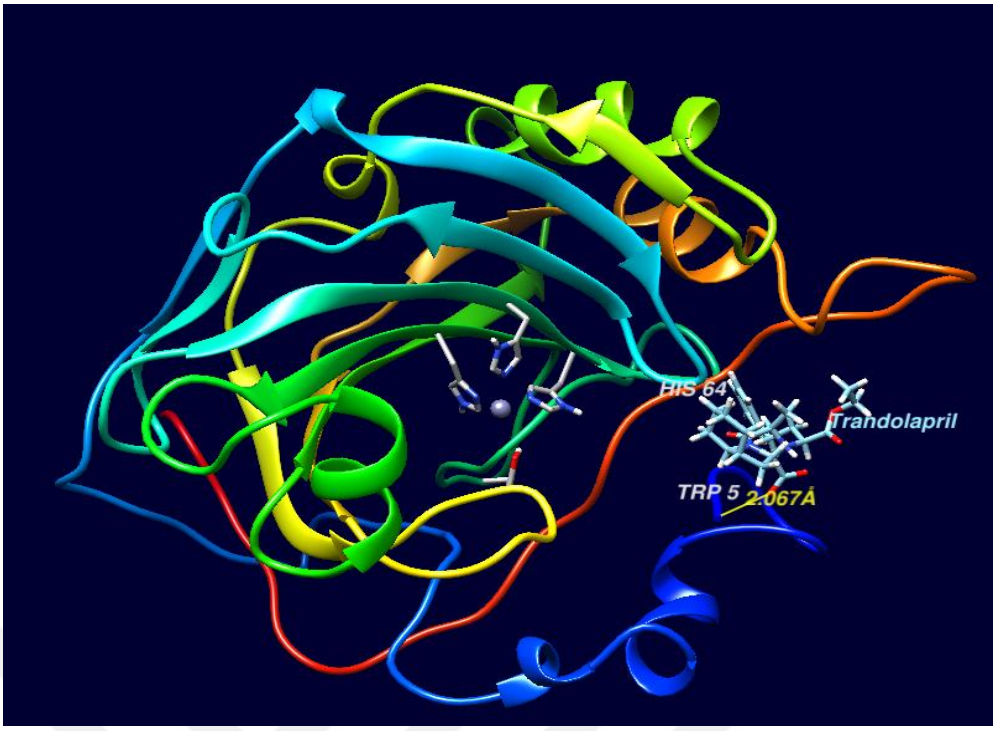


Figure 5.8a The ribbon diagram of hCA-I and trandolapril shows interactions with the residues in the binding pocket.

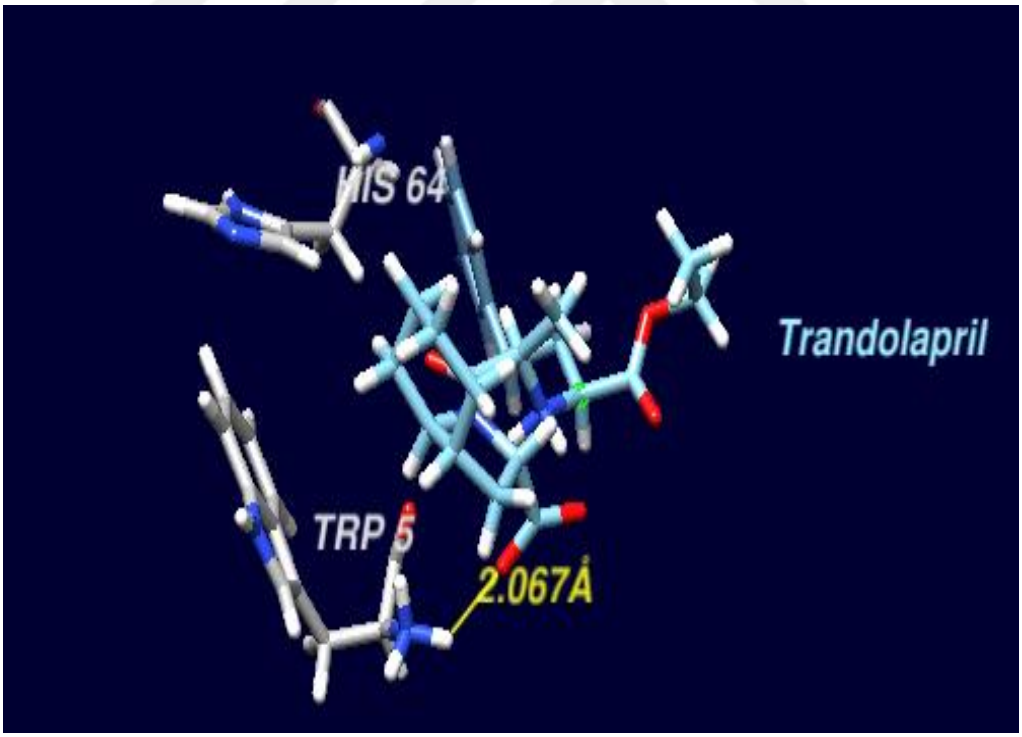


Figure 5.8b Trandolapril interacts with the amino acids in the binding pocket of hCA-I 3by hydrogen bonds.

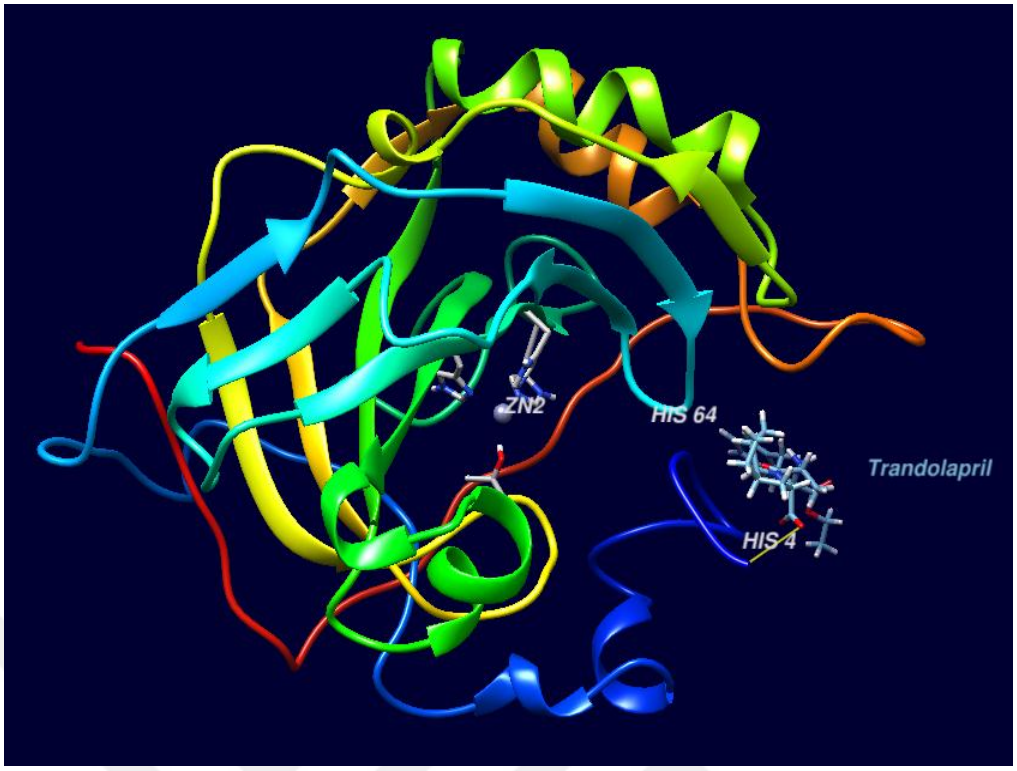


Figure 5.9a The ribbon diagram of hCA-II and trandolapril shows interactions with the residues in the binding pocket.

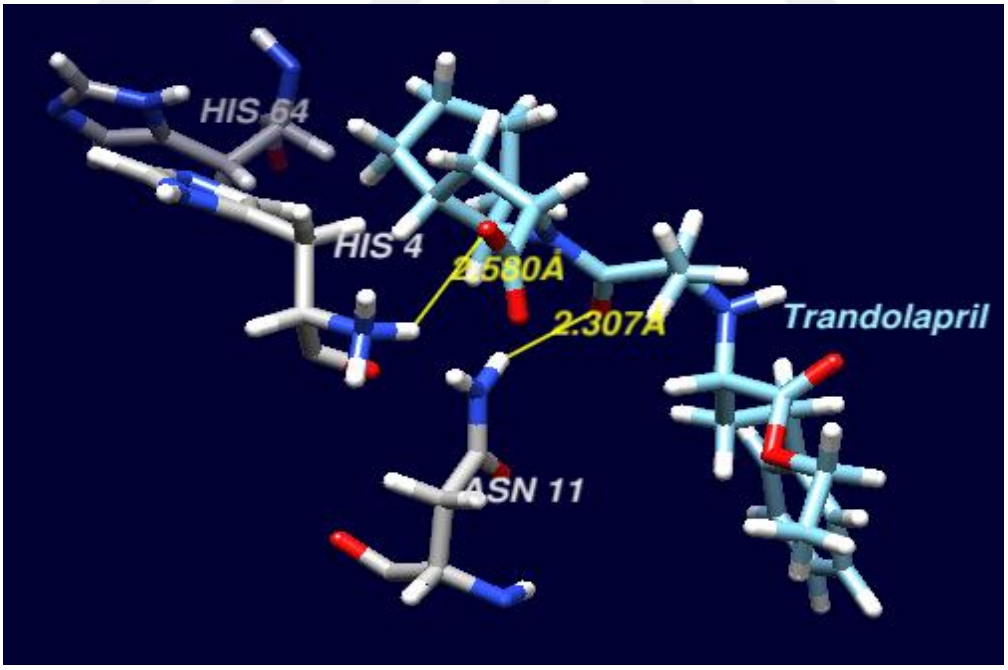


Figure 5.9b Trandolapril interacts with the amino acids in the binding pocket of hCA-II by hydrogen bonds.

2. Zofenopril calcium, showed lower inhibition effect on esterase activity for hCA-I with IC_{50} = 0.226 µM, than from the effect on the esterase activity of hCA-II with IC_{50} =0.201 µM. This drug interacted with the amino acid residues in the binding pocket and H_2O molecule in the active site.
   - In case of hCA-I the functional group (C=O) interacts side chain of the LYS 170 by formed a hydrogen bond (Figure 5.10.a and 5.10.b).
   - In case of hCA-II the functional group (C=O) and (COO-) interact with the H_2O by formed a hydrogen bond (Figure 5.11.a and 5.11.b).

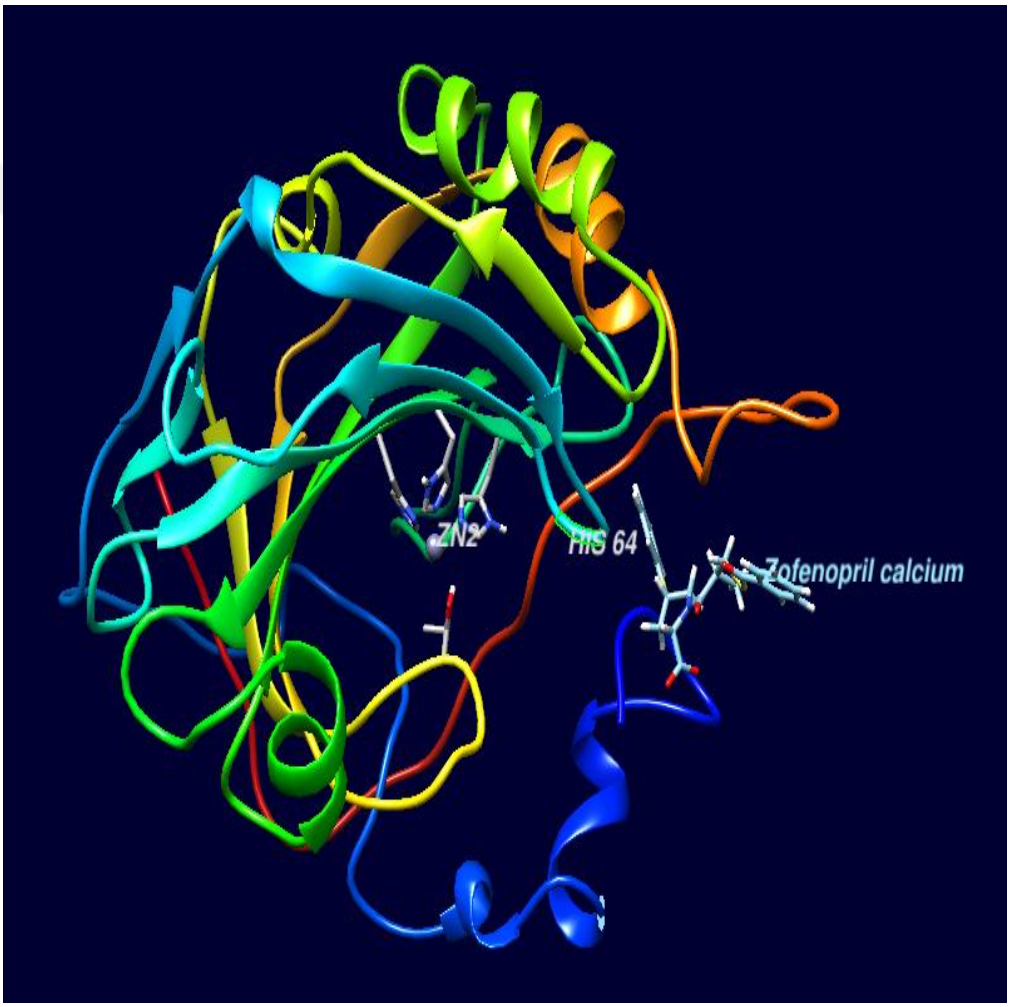


Figure 5.10a The ribbon diagram of hCA-I and zofenopril calcium shows interactions with the residues in the binding pocket.

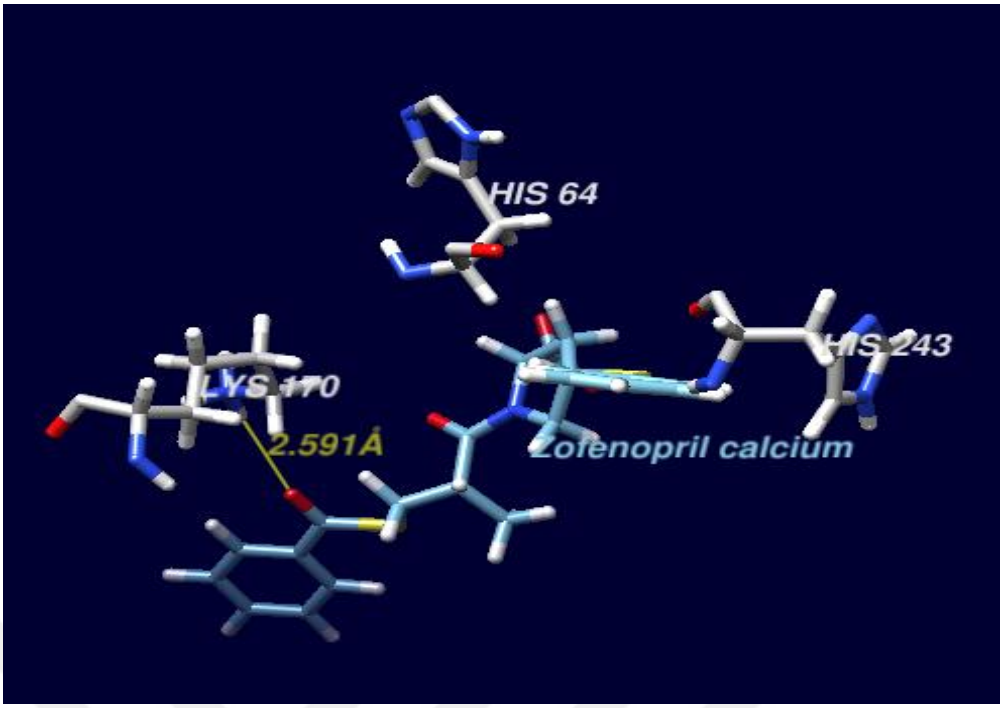


Figure 5.10b Zofenopril calcium interacts with the amino acids in the binding pocket of hCA-I by hydrogen bonds.

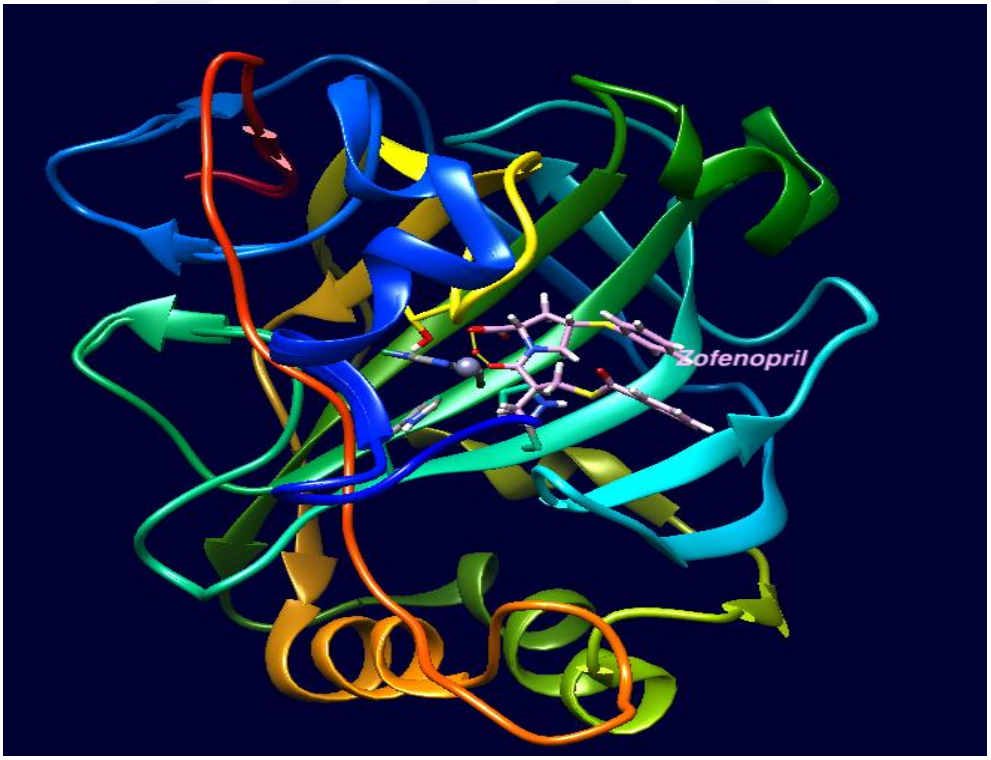
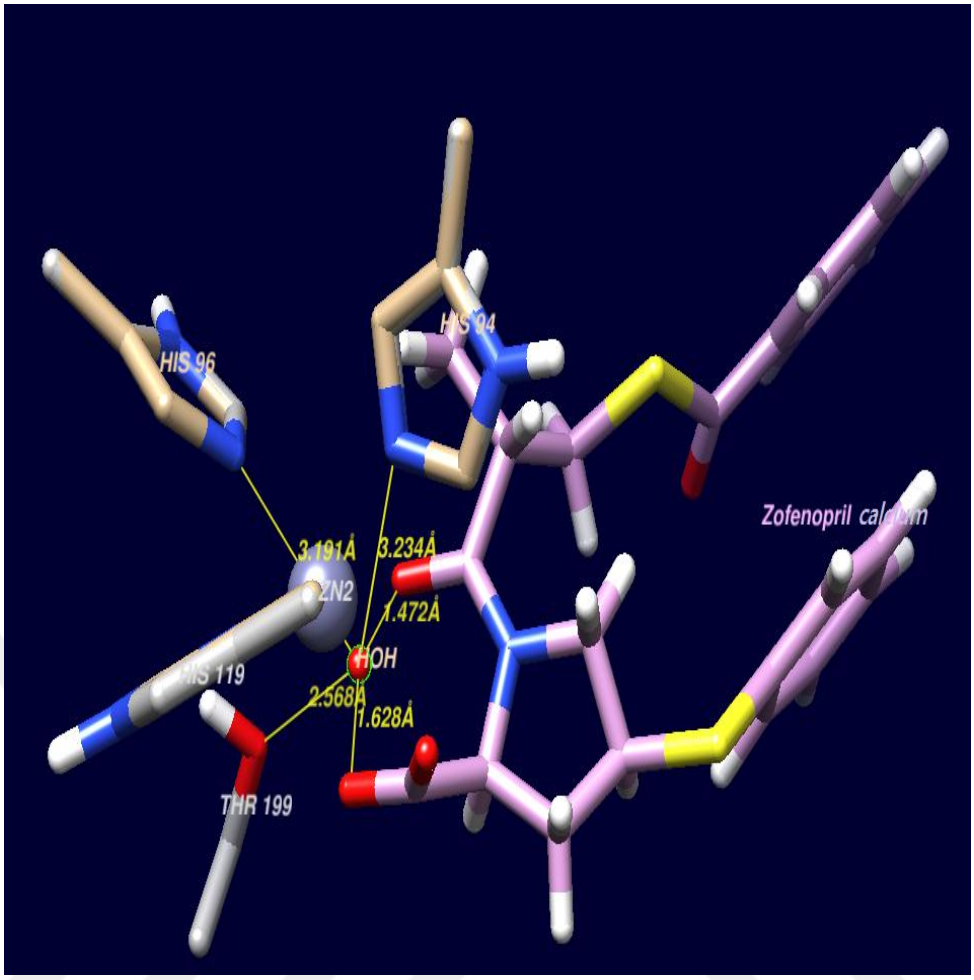


Figure 5.11a The ribbon diagram of hCA-II and zofenopril calcium shows interactions with the H_2O molecule in the active site.



5.11b Zofenopril calcium interacts with the H_2O molecule in the active site of hCA-II by hydrogen bonds.

The previous informations of molecular docking were collected in Table 5.1.

Table 5.1 The functional groups in the drugs and the interacted residues in domains of hCA-I and hCA-II.

Drug Name	hCA-I			hCA-II		
	Functional group	**Residue**	**Domain**	**Functional group**	**Residue**	**Domain**
Cilazapril	COO-	TRP 5	Binding pocket	COO-	HIS 4	Binding pocket
	R-O-R	ASN 11			ASN 11	
		SER 231				
Perindopril	COO^-	LYS 10	Binding pocket	COO^-	HIS 4	Binding pocket
		ASN 11		C=O	TYR 7	
	C=O	TYR 7				
Ramipril	COO^-	TRP 5	Binding pocket	COO-	HIS 10	Binding pocket
	C=O	TYR 7			LYS 9	
Trandolapril	COO^-	TRP 5	Binding pocket	COO^-	HIS 4	Binding pocket
				C=O	ASN 11	
Zofenopril calcium	C=O	LYS 170	Binding pocket	C=O	H_2O	Active site
				COO-		

The inhibition out of the active site was discovered recently when the 2-(benzylsulfonyl)-benzoic acid was co-crystallized with hCA-II, the electronic density of the inhibitor was not observed within the active site but in an adjacent binding pocket to the active site, where other inhibitors were never observed earlier [92]. By blocks the proton shuttle of the enzyme, His64, in its out conformation, interfering thus with the rate determining step of the entire catalytic cycle, which is indeed the transfer of a proton from the zinc-coordinated water molecule to the environment, with generation of the zinc hydroxide species of the enzyme (Figure 5.12. a and 5.12.b) [68], [92].

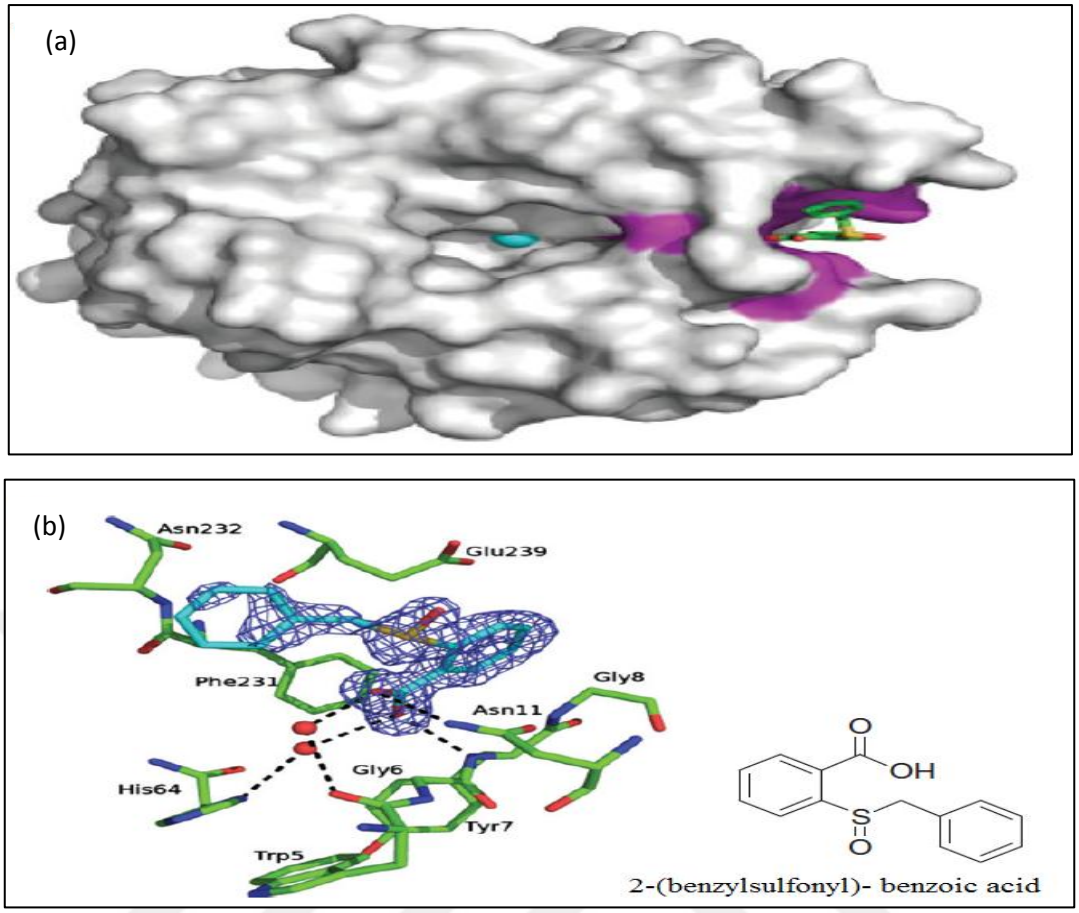


Figure 5.12 The carboxylic acid from 2-(benzylsulfonyl)-benzoic acid complexed with hCA-II (a), Interactions between the inhibitor 2-(benzysulfonyl)-benzoicacid and amino acid residues from the binding pocket (b) [93].

The inhibitor interacts side chain of ASN 11 and TYR 7 in the binding pocket domain [92], and TRP 5 interacts with H_2O by a hydrogen bond, in the same time this H_2O molecule interacts with HIS 64 that cause interaction with proton shuttle mechanism (Figure 5.12.b). In hCA-II, the hydrogen bond network of water molecules connecting the Zn^{2+} – solvent molecule and HIS 64 are responsible for fine tunes the properties of proton transfer during catalysis. The studies showed that residues (TYR 7, LYS 170 and several hydrophilic residues) have been shown to interact with these solvent molecules demonstrated that changes in solvent position can be correlated with changes in the rate of the proton transfer process [160]. Briganti et al.[46], who suggested that the highest catalytic efficiency of hCA-II could be related to a cluster of histidines (HIS 3, HIS 4, HIS 10, HIS 15, and HIS 17) which support HIS 64 in the proton transfer, they form a channel that connect the active site with the surface of the enzyme [160].

In order to understand the magnitude of the effect of some drugs on esterase activity of hCA-I and hCA-II, IC_{50} values of the drugs used in the study were compared to the IC_{50} values of the other drugs that had been shown inhibitory effect on esterase activity of hCA-I and hCA-II (Table 5.2).

Table 5.2 Comparison between drugs that had been shown inhibitory effect on esterase activity of hCA-I and hCA-II in previous studies with IC_{50} values of investigated drugs in this current study.

Drugs from previous studies	**hCA-I**	**hCA-II**	**Ref.**	**Drugs from current study**	**hCA-I**	**hCA-II**
	IC_{50}(µM)	**IC_{50}(µM)**			**IC_{50}(µM)**	**IC_{50}(µM)**
Acetazolamide	x	0.312	[161]	Cilazapril	0.161	0.238
Tenoxicam	0.198	0.11	[162]	Perindopril	0.160	0.195
Fluorometholone acetate	2.18	17.5		Ramipril	0.220	0.255
Dexamethasone	11.7	14		Trandolapril	0.090	0.316
Enalapril	x	60	[12]	Zofenopril calcium	0.226	0.201
Captopril	x	90				

In conclusion, it can be said from our results that:

- All investigated drugs showed inhibitory effect on esterase activity on hCA-I and hCA-II in micromolar concentration in vitro study.
- All of these drugs showed inhibition out of the active site by binding to the amino acid in the binding pocket of hCA-I and hCA-II except zofenopril calcium in case of hCA-II.
- Binding pocket is an important domain for esterase activity on hCA-I and hCA-II.
- TRP 5, TYR 7, LYS 10, ASN 11, and LYS 170 residues in hCA-I, and the HIS 4, TYR 7, LYS 9, HIS 10, and ASN 11 residues in hCA-II play a role in proton shuttle mechanism and the drugs when interacted with these amino acids cause inhibition by changes in the rate of the proton transfer process.
- Zofenopril calcium competes with CO_2 molecule in the active site and interacts with H_2O by forming a hydrogen bond that causes to inhibit the hydratase activity of hCA-II and already inhibit esterase activity.

- IC_{50} values of (Cilazapril, Perindopril, Ramipril, Zofenopril calcium) are less than the IC_{50} value of acetazolamide on esterase activity of hCA-II (Table 5.2).

Finally, investigation of the effects of drugs on hydratase or esterase activities of hCA-I and hCA-II are important for drug design which are used to treated different diseases. Some drugs may be treated for some diseases via inhibiting some enzyme that responsible to occur a disease. But at the same time, the same used drug may be inhibit or activate another enzyme that lead to occur a disease as unknown side effect of used drugs. Regarding the vital physiological roles of CAs and taking the earlier statements into account, it seems plausible that modulation of CA activity to normal levels either by its inhibition or activation offers interesting therapeutic options [12]. Development of isozyme-specific or at least organ-selective inhibitors would be highly beneficial both for obtaining novel types of drugs, devoid of major side effects and for physiological studies in which specific inhibitors may constitute valuable tools for understanding the physiology and physiopathology of these enzymes [19], [53].

In the light of these results, in vivo investigation of these drugs on carbonic anhydrase may be important. These drugs may be lead to increase tissue CO_2 concentrations and lower tissue pH, resulting in vascular dilation and increased blood flow. Deficiency of hCA, on the other hand, is the primary defect in the syndrome of osteopetrosis, renal tubular acidosis, and cerebral calcification [163].

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PUBLISHMENTS

Conference Papers

1. Altameemi, M.S., Şentürk, M., Nalbantoğlu, B., (2017). “Effects of Some ACE-Inhibitor Drugs Used For Heart Diseases On Esterase Activity Of Human Carbonic Anhydrase I And II” .ULUSLARARASI KATILIMLI KONGRE & LAB EXPO, 1 - 5 October 2017, Antalya.