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Inhibition Effects of Lisinopril and Quinapril on the Activity of Carbonic Anhydrase: in Vitro and Molecular Docking Studies

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Abstract

α-Carbonic anhydrase family (α-CAs) exist in different body tissues and have an essential function in the physiology of cells. In this study, the possible inhibitory effects of Lisinopril and quinapril had investigated on the activities of CA I and II. CA I and II were purified from human erythrocytes using an affinity gel chromatography column (sepharose 4B-tyrosine – sulfanilamide). The purity of isozymes was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).The esterase activity was determined spectrophotometrically at 348 nmusing 4-nitrophenyl acetate as a substrate. The half concentration of inhibition (IC_{50}) values had calculated from graphs of the relation between the %Activity and the concentration of drugs at micromolar (µM), also, Calculated the inhibition constant (K_i) and inhibition types via Lineweaver-Burk plots. Finally, Drugs were docked into proteins (CA I and II) using molecular operating environment software (MOE). Lisinopril showed better inhibition values (IC_{50} and K_i) against the CA I and II than quinapril. The results of this study may be significant in the drug development field for treating disorders related to abnormal activities of these isozymes.

*Keywords:*Carbonic anhydrase, CAI, CAII, Esterase activity, Lisinopril, Quinapril, Enzyme inhibition.

1. Introduction

Carbonic anhydrase (EC.4.2.1.1) is a common rife metalloenzyme in all living systems with different seven distinct and unrelated CA gene families, which are α-, β-, γ-, δ-, ζ-, η-, and Θ-CAs [1]. In human beings, just α-CAs family had found. This family consists of 16 isozymes [1,2], while in human beings, just 15 exist in different body tissues except for α-CAXV isozyme, which isn't found in humans but found in other mammals like mice [1,2]. In the α-CAs family, the metal ion (Zn^{+2}) is bound with four ligands(His 94, His 96, His119, and H_2O) within the active site Figure 1.

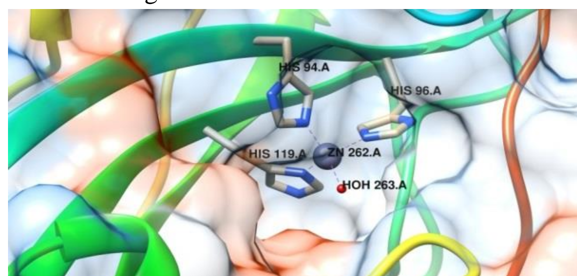
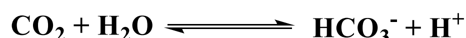


Fig. 1. The active site cavity of CA II, Zn^{+2} coordinated with three histidine residues and H_2O. This structure was downloaded from the protein data bank site with code (1CA2) [3] and represented with molecular docking software (UCSF-chimera) version 1.11.2. (https://www.rcsb.org/)

Generally, these enzymes catalyze the following reversible reaction [1,2]:



$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

In the erythrocyte, catalyzing the former reaction by CA I and II lead to changing haemoglobin's affinity for O_2 to allow gas exchange between tissue and erythrocyte and keep pH balancing of erythrocytes [1,2].

In pharmacology, enzymes represent a fundamental drug target [4], but in treating chronic diseases like hypertension, there is a risk of overlap targets when prolonged administration of medicines. When binding randomly to unpurposedmetalloenzymes, it seems that drugs reduce the bioavailability of medications for targeting the enzyme. Furthermore, the prospective inhibition of unpurposed regulatory enzymes may be the reason to occur un coveted side effects resulting from harmful effects on the normal metabolism. So, modulating metalloenzymes activities by utilizing inhibitor drugs is a significant and challenging area in the drug design[4, 5].

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There are many chemical compounds used to control the activity of metalloenzymes like sulfonamide, thiol, hydroxamic acid moiety, carboxylate, phosphonate, phenols, and polyamines are examples of metalloenzyme inhibitors for angiotensin-converting enzyme (ACE) and carbonic anhydrases (CAs) [5-15].

Carbonic anhydrase inhibitors (CAIs), most of them belong to the diuretic category of drugs. For example, acetazolamide, a classic inhibitor used in the treatment of the following cases; glaucoma by reducing the secretion of homer aqueous, which leads to decreasing the intraocular pressure, before surgery of acute-angle closure glaucoma, used to solubilize uric acid and cystine stones by turned the pH of urine to alkaline, Also, in the treatment of intracranial hypertension idiopathic by reducing cerebrospinal fluid (CSF) production, Furthermore, it used in the treatment of epilepsy, congestive heart failure, and hypoxia [16, 17].

In the last few years, molecular docking studies occupied a considerable place in drug research due to their efficiency in reducing research costs assisted by computer software. It became a crucial tool in drug design by predicting the molecular interactions between drug and receptor, drug affinity to the receptor, and analyzing the binding mode. [18].

Finally, many ACE-inhibitor drugs showed the ability to inhibit carbonic anhydrase's activities in vitro [5, 19]. While the effects of lisinopril and quinapril (ACE-inhibitors) against esterase activity of CA I and II have notmentioned so far, Also, since these two isozymes are excellent models for inhibition studies according to the superb characterization of their structures which consist of a single-chain protein without disulfide bonds, and the well-known catalytic mechanism of activity, as well as, easily purified from erythrocytes in the laboratory [20]. This study aims; to determine the possible effects of lisinopril and quinapril on the activity of CA I and II and study the molecular docking of these ligands with these two isozymes.

2. Experimental

2.1. The used chemical materials

Lisinopril 5 mg, and acuitel 20 mg, were purchased from a local pharmacy. While other chemicals and substrate p-nitrophenyl acetate had purchased from Sigma-Aldrich (MerkKGaA).

2.2. Preparation of drug solutions

The chemical structure and how the stocks had prepared to have explained in Figure 2. and Table 1. respectively.

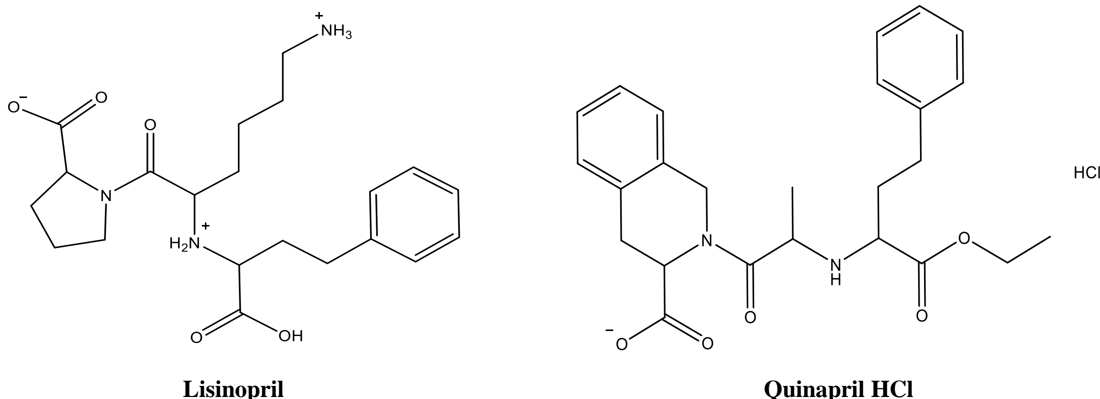

Fig. 2 Chemical structure of the active ingredient of drugs (lisinopril and quinapril) [21, 22]

Table 1: Preparation of drug solutions

Trade Name	Active ingredient	Company	Molecular weight	Stock Concentrations
Lisinopril 5mg	Lisinopril	Bristol Lab.Ltd	405.5	41 mg solved in 20 ml of distilled water; the final concentration is 5.0 mM
Acuitel 20 mg	Quinapril HCl	Pfizer	475.0	48 mg solved in 20 ml of distilled water; the final concentration is 5.0 mM

2.3. Purification of isozymes

The purification method of CA I and II from erythrocytes had described by Nalbantoğlu et al. [22].The procedure can explain as follows [15, 19, 20, 23, 24]: the first step is the preparation of hemolysate from fresh blood. The fresh blood was filled in centrifuge tubes and centrifuged at (2500×g) for fifteen minutes; then, the erythrocytes were

separated and washed three times with (0.154 M) NaCl and centrifuged again at (2500×g) for fourteen minutes. And removed the supernatant using a dropper every time, and erythrocytes were hemolyzed by mixing with five times the volume of ice-cold water. The intact cells were removed using centrifugation at (20,000×g) for thirty minutes. The upper hemolysate cells were carefully taken via a dropper and stored at (4°C). The second step ispurification via affinity gel chromatography column (Sepharose 4B-tyrosine –sulfanilamide). The pH of hemolysate was adjusted to (8.7) using solid Tris (hydroxymethyl) aminomethane. (25 mM) Tris–HCl/0.1 M Na_2SO_4 (pH 8.7) was used to equilibrate the column. Then hemolysate was applied to the column; after that, washed the affinity gel with (25 mM) Tris–HCl/22mM Na_2SO_4 (pH8.7). Then the human CA I, and II isoenzymes were eluted with (1M) NaCl/25 mM Na_2HPO_4 (pH 6.3) and (0.1 M) CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures have performed at (4°C).

2.3.1. Determination of protein

After the purification steps of enzymes, Bradford's method had used to determine protein in the elution by using a spectrophotometer at (595 nm) [25].

2.3.2. Analyze thepurity by using SDS-PAGE.

The purity of isoenzymeshad checked using 3-8% (SDS-PAGE) according to the procedure described by Laemmli[26].

2.3.3. Enzyme activity assay

2.3.2.1. Hydrataseactivity assay

The hydrataseactivity of purified enzymes had monitored via hydration of CO_2according to the method described by Wilbur and Anderson [27]. The enzyme unit (EU) was determined via changes in the pH with time using the following equation (t_0-tc/tc). t_0 represent the required time for pH changing in the tube containing non-enzymatic reaction, and tc represents the time needed for pH changing in the tube containing enzymatic reaction [27, 28**].**

2.3.2.2. Esterase activity assay

Esterase activity has been measured via a Spectrophotometer using the hydrolysis reaction of 4-nitrophenyl acetate as a substrate to 4-nitrophenol or 4-nitrophenolate [15, 19, 20, 22, 28-30]. This chemical reaction takes 3 minutes; Absorbance values at the beginning and end of the reaction have read at 348 nm at 25°C. The enzyme activity had determined by subtracting the first value from the last value. Before measuring the enzyme activity, the spectrophotometer has set to zero by distilled water.

2.3.2.3. Determination of lisinopril and quinapril effects on esterase activity

Absorbance values of investigated drugs on esterase activity had measured at 380 nm. Six different concentrations from the investigated drugs ranged from~50 to ~360 µM. The absorbance values of the esterase reaction in the absence inhibitor had accepted as 100 % activity by using the absorbance values in the existence and absence of drugs. % 100 Activity values of CAI and CAII had calculated. Then, IC_{50} values for these drugs have calculated from the % Activity-[Drug] graphs [15, 19,20, 28-30].

2.3.2.4. Determination of inhibition mode

The inhibition type and constant of inhibition (K_i) were determined as following: five different concentrations from p-nitrophenyl acetate (0.15, 0.45, 0.75, 1.05, 1.35) mM were used in presence and absence of drug [5, 15, 19, 27, 29, 30]. In the absence of an inhibitor, K_m and V_{max}had calculated from the relation between 1/V versus 1/[S], which represents the Lineweaver-Burk equation (the double reciprocal plot), the linear equation [31]:

$$\frac{1}{V} = \frac{1}{[S]} \times \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \quad (1)$$

K_m: Michalis-Menten constant; V: velocity of enzymatic reaction; V_{max}: Maximum Velocity of enzymatic reaction. [S]: p-nitrophenyl acetate concentration .

In the presence of an inhibitor, Three different concentrations of lisinopril and quinapril (100, 200, 400) µM with CA I and (100, 150, 300) µM with CA II were tested triplicate at each used concentration. Inhibition types were determined from plots. When all intercepts cross through the y-axis from the same point, V_{max} is constant, and Km increases, then the inhibition type is competitive; in this case, the equation (1) will be as follows. [31]:

$$\frac{1}{V} = \frac{1}{[S]} \times \frac{\alpha K_m}{V_{max}} + \frac{1}{V_{max}} \quad (2).$$

The (α) represents the amount of decreasing from the K_m value in the presence of an inhibitor, and idiomatically it is called apparent K_m. Then K_i value could be calculated as follows:

$$\boldsymbol{K_m^{app.}} = \boldsymbol{\alpha K_m} = K_m[1 + \frac{[I]}{K_i}] \quad (3)$$

When intercepts cross through the x-axis from the same point means K_m=$K_m^{app.}$While V_{max} is changed, the inhibition mode is Non-competitive. [31].

$$\frac{1}{V_0} = \frac{1}{[S]} \times \frac{K_m}{\frac{V_{max}}{\alpha`}} + \frac{\alpha`}{V_{max}} (4)$$

The (α`) represents the amount of change in the value of V_{max} in the presence of an inhibitor, and idiomatically, it is called apparent V_{max}. ($V_{max}^{app.}$) [31].

$$(V_{max}^{app.}) = \frac{V_{max}}{\alpha`} = \frac{V_{max}}{\left[1+\frac{[I]}{K_i}\right]} (5)$$

Finally, equation (1) for mixed inhibition will be as follows:

$$\frac{1}{V_0} = \frac{1}{[S]} \times \frac{\alpha K_m}{V_{max}} + \frac{\alpha`}{V_{max}} (6)$$

In which both K_m and $V_{max}$are changed [31].

2.4. Molecular docking

CA I and II's crystallographic structures were downloaded from the Protein Data Bank archive site (https://www.rcsb.org) in protein data bank format (PDB), The PDB codes for CA I and II were: 5GMM and 2HD6, respectively. The structures were optimized using molecular operating environment software (MOE-2015). The optimizations included removing water molecules and ligands and adding the polar hydrogen atoms via choosing the protonate 3d structure from (compute list); also adjusted partial charges by selecting the casteiger method. Then, adjusted 5tgn energy minimization by choosing (amber10: EHT and gradient: 0.1). Additional adjustments from (edit list) by choosing (potential fix). Then from (build's sub-list), selected (automatic connect and type) to add bonds and assign types for isozymes structures. The (site finder) choice was selected from the (compute list) to identify possible ligand binding sites (alpha centres) within the optimized structure of CA I and II.Finally, the docking process was performed in a blind manner and into a defined active site using the default triangle matcher placement for the induced fit docking, then scored using London dG and GBVI/WSA dG. Depending on the poses values of (Ligand-protein) binding free energy, the relatively lowest ΔG value had selected.

3. Results

The specific activities of purified enzymes via the affinity gel chromatography technique were equal to 896.35 EU/mg and 6546.67 EU/mg for both human CA I and II, respectively. The SDS-PAGE and purification results have clarified in Table 3 and Figure 3.

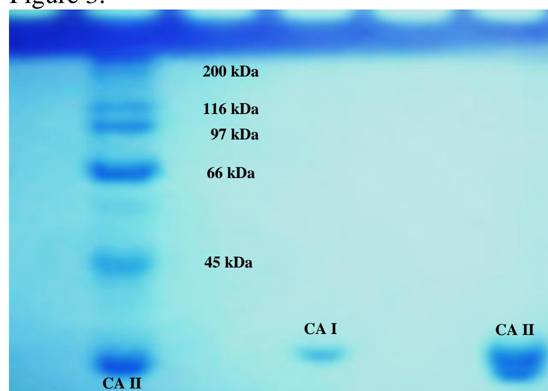

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At the left, controls (29-200) kDa.; at the right, purified isozymes (CA I and CA II)

Table 3: Purification results of human CA I & II before and after affinity chromatography

Purification steps	Total Volume (ml)	Protein concentration (mg/ml)	Activity (EU/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification coefficient
Hemolysate	70	21.5	168	1505	11760	7.81	100	1
CA I	13	0.53	473	6.86	6149	896.35	52.3	114.76
CA II	6	0.15	982	0.9	5892	6546.67	50.1	838.24

IC_{50} values of benazepril and moexipril have been calculated by drawing % Activity-[Drugs] graphs, as shown in Figure 4, and IC_{50} values have been clarified in Table 4, While the molecular docking results shown in Figures (5,6) and Table (5).

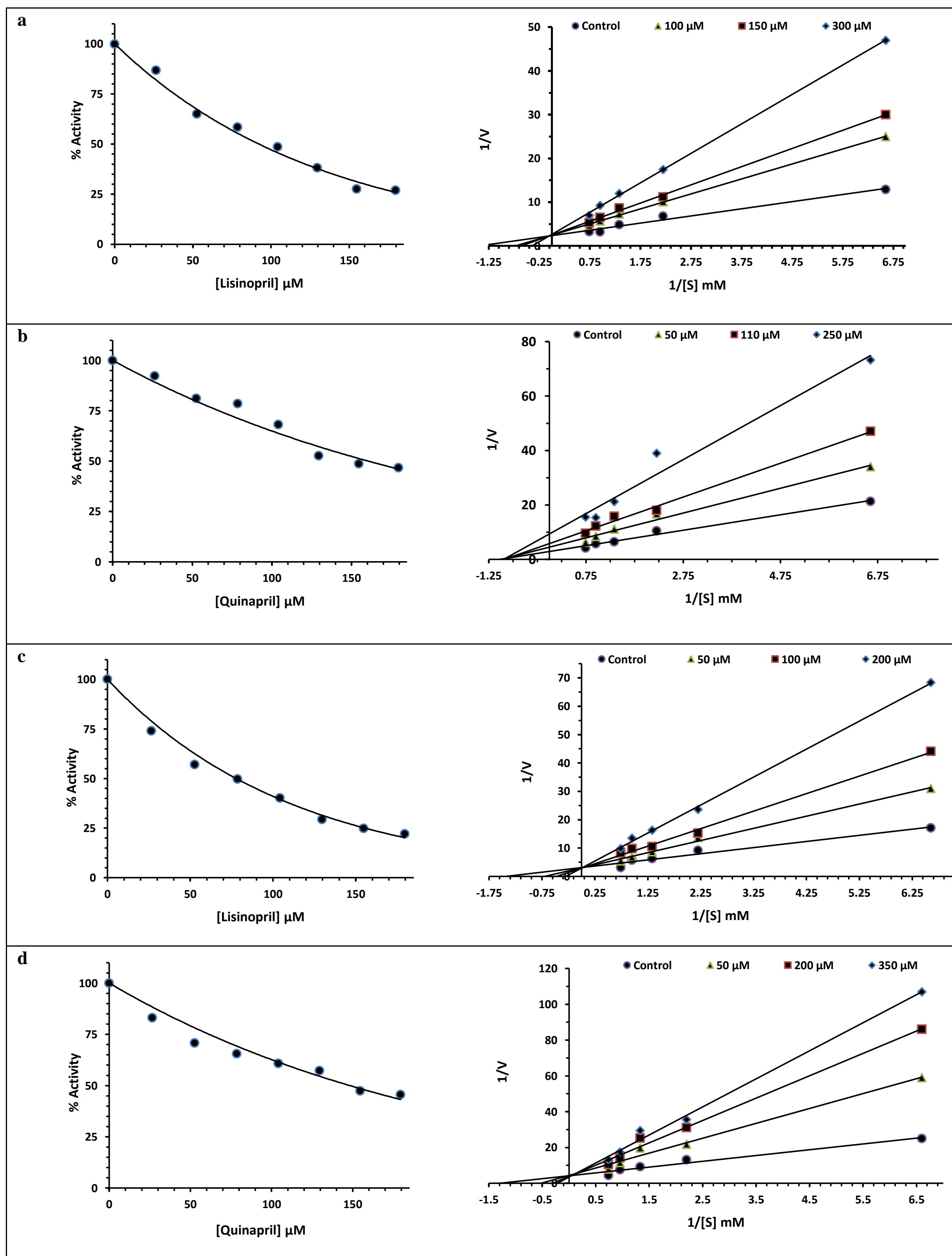

Fig.4. % Activity (esterase)and Lineweaver-Burk plots; (a) Lisinopril with CAI, (b) Quinapril with CAI, (c) Lisinopril with CAII, (d) Quinapril with CAII

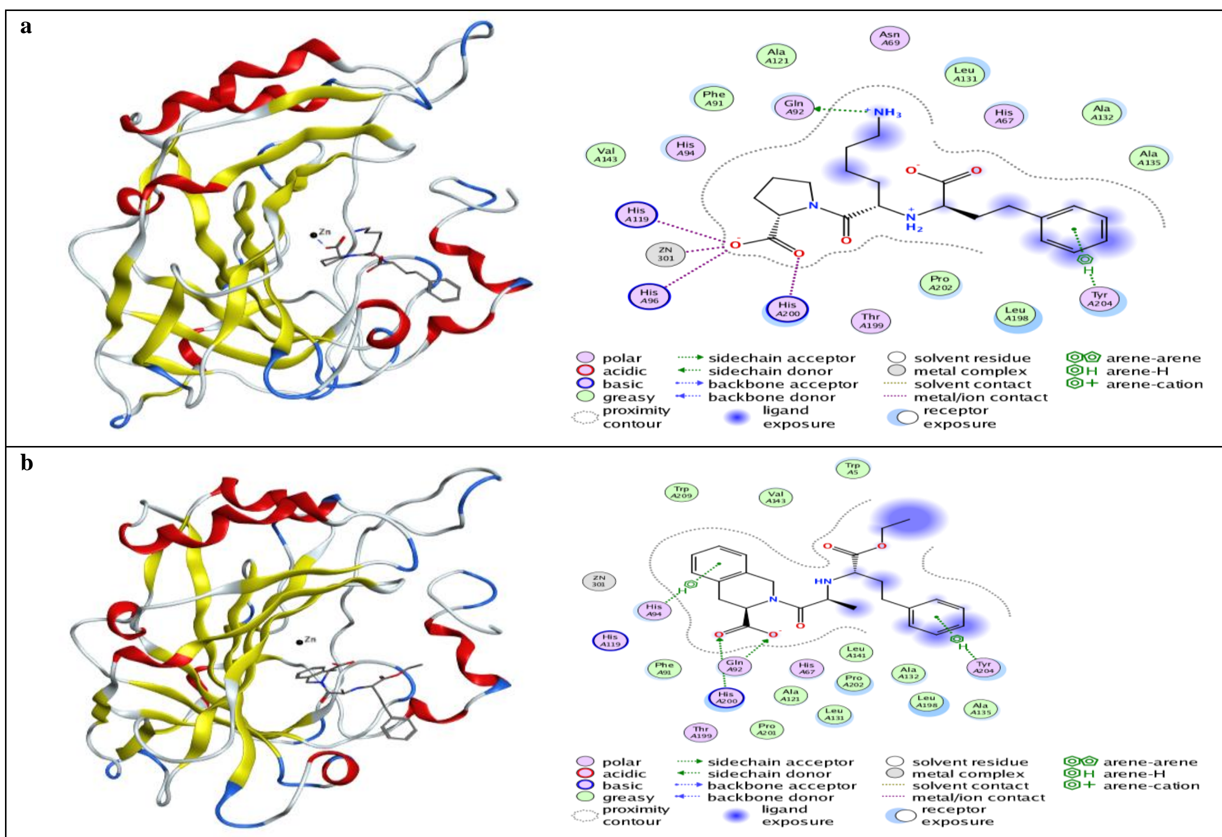

Fig.5: Molecular docking of Lisinopril (a) and Quinapril (b) with CAI using MOE software

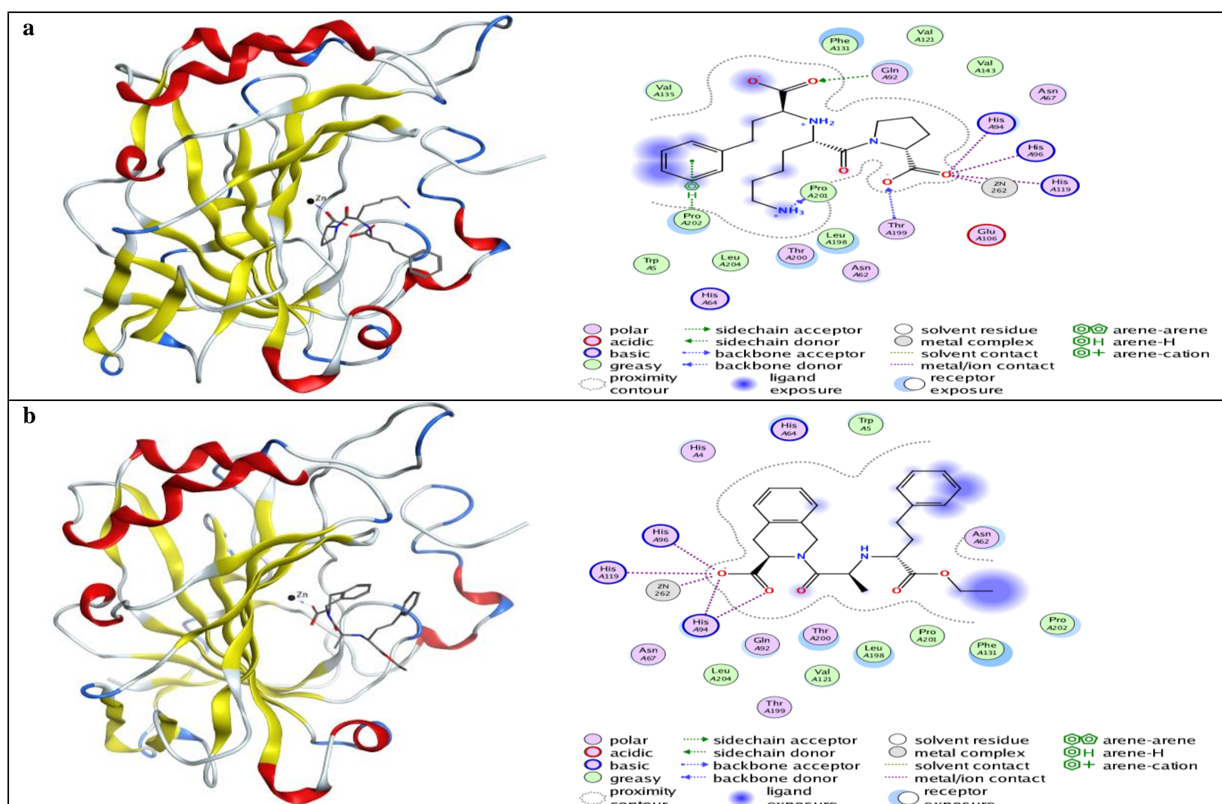

Fig.6: Molecular docking of Lisinopril(a) and Quinapril (b) with CAII using MOE software

Table 4: Results of inhibition effects of Lisinopril and quinapril on CAI and II

Drugs	CA I				CA II			
	IC_{50} (μM)	K_i (μM)	Inhibition type	ΔG_B (kcal/mol)	IC_{50} (μM)	K_i (μM)	Inhibition type	ΔG_B (kcal/mol)
Lisinopril	92.3	53.5 ± 2.9	Competitive	-9.30	77.9	48.3 ± 2.2	Competitive	-9.76
Quinapril	160.8	219.8 ± 8.0	Non-competitive	-6.91	148.3	68.5 ± 4.6	Competitive	-8.49

4. Discussion

Carbonic anhydrase inhibitors (CAIs) and ACE-inhibitors have medical applications in treating abnormal catalytic activities [5-17]. Some of these inhibitors interact with the catalytic zinc ion centre through the thiol moiety like captopril; some interact through carboxylate moiety like lisinopril, while the rest interact through the phosphate group fosinopril [5]. Different inhibitor typeshave been mentioned according to the binding domain from the literature. These types are; zinc-binding inhibitors, inhibitors that anchor zinc coordinate water/hydroxyl ion, inhibitors that occlusion of the active site entrance, inhibitors that bind to residues out of the active site, and inhibitors that bind with unknown mechanism [9].

In this study, lisinopril and quinapril have shown inhibition effects on esterase activity of both human CA I and II. In contrast, lisinopril showed the best inhibition values with both isozymes (Table 4). For further information at the molecular level about interactions between the enzyme and drugs, molecular docking approaches havebeen used.

Lisinopril and quinapril were docked in the blind manner(whole protein) and into defined active sites of both CA I (PDB:2nn7) and CA II (PDB: 2hd6), the binding free energy ΔGB scores were calculated by using MOE. There was an agreement between the experimental results and recorded theoretical ΔGB scores. Lisinopril had a higher affinity (lower free energy score) to binding with the residues located in the active site more than quinapril had done. Which confirmed the strong interactions of lisinopril with the residues conserved in the active site of both CA I and II (Figures 5-aand 6-a). A coordinate bond had formed between the oxygen atom of the carboxylate group in the lisinopril and catalytic zinc ion in the active site of both CA I and II with a mono-dentate style and the interactions mentioned in Table 5. While quinapril, when docked into the active site of both isozymes, just in the case of CAII, the functional group (COO^-) in quinapril interacted with residues and catalytic zinc ion (Figure 6-b). The inhibition abilities of these drugs are imputable to existing functional groups, as mentioned in Table 5. The literature defines small molecules with carboxylic moiety as non-classical CA inhibitors[5].

Table 5: Ligand interactions report between functional groups in lisinopril and quinapril with residues in the active site of CAI and II

Drug	CA I			CA II		
	Ligand (Functional group)	Residue (Receptor)	Interaction	Ligand (Functional group)	Residue (Receptor)	Interaction
Lisinopril	NH_3^+	Gln 92	H-donor	NH_3^+	Pro 201	H-donor
	COO^-	Zn^{2+} 301	Metal	COO^-	Gln 92	H-acceptor
		His 96	Ionic	COO^-	Thr 199	H-acceptor
		His 119	Ionic		Zn^{+2}	Metal
		His 200	Ionic		His 94	Ionic
	Aromatic ring	Tyr 204	Pi-H		His 96	Ionic
					His 119	Ionic
				Aromatic ring	Pro 202	Pi-H
Quinapril	COO^-	His 200	Ionic	COO^-	Zn^{2+}	Metal
		Gln 92	H-acceptor			
	Aromatic ring	His 94	Pi-H			
		Tyr 204			His 94	Ionic
					His 96	Ionic
					His 119	Ionic

Finally, the values of K_i from the current study compared with those from previous studies (Table 6), it is clear that sulphonamide drugs, like acetazolamide, the classic inhibitor for CA I and II, have inhibitory effects potencies more than lisinopril and quinapril.

Table 6: Comparing K_i values from different studies

inhibitor	K_i (μM)		reference
	CA I	CA II	
Lisinopril	53.5 ± 2.9	48.3 ± 2.2	Current study
Quinapril	219.8 ± 8.0	68.5 ± 4.6	
Captopril	-	26 ± 3	[5]
Enalapril	-	17 ± 2	
Acetazolamide	0.734±0.12	0.159±0.04	[32]

5. Conclusion

Lisinopril and quinapril showed inhibition effects against human CA I and II at micromolar concentration. According to the results, lisinopril is more potent than quinapril in inhibition.

In light of these results, in vivo investigations about the effects of these drugs are still necessary to understand the inhibition abilities of these drugs.

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