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Synthesis, biological evaluation and molecular docking of novel pyrazole derivatives as potent carbonic anhydrase and acetylcholinesterase inhibitors

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ABSTRACT

A series of substituted pyrazole compounds (**1–8** and **9a**, **b**) were synthesized and their structure was characterized by IR, NMR, and Mass analysis. These obtained novel pyrazole derivatives (**1–8** and **9a**, **b**) were emerged as effective inhibitors of the cytosolic carbonic anhydrase I and II isoforms (hCA I and II) and acetylcholinesterase (AChE) enzymes with K_i values in the range of 1.03 ± 0.23–22.65 ± 5.36 µM for hCA I, 1.82 ± 0.30–27.94 ± 4.74 µM for hCA II, and 48.94 ± 9.63–116.05 ± 14.95 µM for AChE, respectively. Docking studies were performed for the most active compounds, **2** and **5,** and binding mode between the compounds and the receptors were determined.

1. Introduction

Pyrazoles are important class of heterocyclic compounds, which have rapidly increased in importance due to their wide range of properties. Their biologic activities, have guided researchers towards the synthesis of substituted pyrazole derivatives using existing methods [1]. Substituted pyrazole compounds are significant target molecules for organic synthesis chemists in terms of potential applications such as medicine [2], pharmacology [3], agriculture [4] and material science [5]. Some known applications are antibacterial [6], antifungal [7], antibiotic [8], pesticide [9], and sensors [10]. Moreover, some molecules having pyrazole structure exhibited excellent properties such as antiviral [11], antioxidant [12], anti-inflammatory [13], antitumor [14] and antidepressant [15] activities. On the other hand, pyrazole-containing compounds are widely commercialized such as Tartrazine [16], Fomepizole [17], Ceftolozane [18], Celebrex [19], Rimonabant [20] and Viagra [21]. Furthermore, substituted pyrazole molecules were used to ligand as a catalyst in the cross coupling reaction [22] and also was used α-Helix mimetic as inhibitors of protein-protein interactions [23]. The cyclocondensation of hydrazines with 1,3-dicarbonyl compounds, *α,β*-unsaturated aldehydes ketones and 2,3-furandiones generally were used for synthesis of the pyrazoles [1,6]. The similar method was used to syntheses of pyrazole-based molecules.

Dementia disease is a main health concern in old people worldwide, which can affect multitude aspects of a functioning and person's life. Indeed, there is no cure for most kinds of dementia disease, but various drugs are utilized in its office [24,25]. The acetylcholinesterase inhibitors (AChEI) were developed as an effect of the cholinergic assumption of cognitive reduction [26]. Indeed, the effectiveness of these treatments has been investigated in a great number of randomized controlled tests among global, functional, neuropsychiatric domains, and cognitive [27]. AChEI compounds are demonstrated for the symptomatic treatment of mild-to-moderate Alzheimer's disease (AD). They inhibit AChE, which is accountable for the separation of acetylcholine (ACh), a neurotransmitter molecule that associated with memory function [28,29].

Two genetically distinguished human carbonic anhydrase (hCA) isoforms designated hCA I, and II isoenzymes are present in RBC cells. Both isoforms exhibit 60% sequence homology, several particular activities and several immunologic specificities [30]. While, CA II isoform is expressed in an extensive diversity of tissues and cells, CA I isoform has a more restricted cellular expression. Also, hCA II isoform can produce NO molecule, owing to the structural similarity between nitrite and bicarbonate [31]. The hCA II isoform is a zinc ions (Zn^{2+}) and catalysis reversible interconversion of carbon dioxide (CO_2) and bicarbonate (HCO_3^-). It is found in platelets, vascular smooth muscle, and erythrocytes. Also, hCA II isoenzyme contributes to vascular adjustment and could be a source of good hCA II-mediated generation of nitric oxide (NO) from nitrite [32]. Since nitrite exhibits similar to sp^2 geometry as HCO_3^-, it is hypothesized that hCA II isoform acts as a

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nitrous anhydrase factor, wherein nitrite binds hCA II active site and is dehydrated, analogously to HCO_3^- [33,34]. CA isoforms are zinc-containing metallocatalists that perform the reversible hydration of CO_2 to HCO_3^- and a H^+. This simple reaction is essential for many physiological mechanisms including electrolyte secretion, respiration, pH regulation, bone resorption, tumorigenesis, biosynthetic reactions, and calcification, which require HCO_3^- as a substrate. The hCA isoenzymes are interesting therapeutic targets where their inhibition could be utilized to treat a range of disorders including anemia, oxidative stress, glaucoma, edema, cancer, epilepsy, osteoporosis, obesity, and sterility [35–38].

In this study, we have performed a facile synthesis of well-defined novel pyrazole derivatives (**1–8** and **9a, b**). We also investigated their inhibition potential towards hCA I, and II isoenzymes as well as towards AChE inhibition.

2. Results and discussion

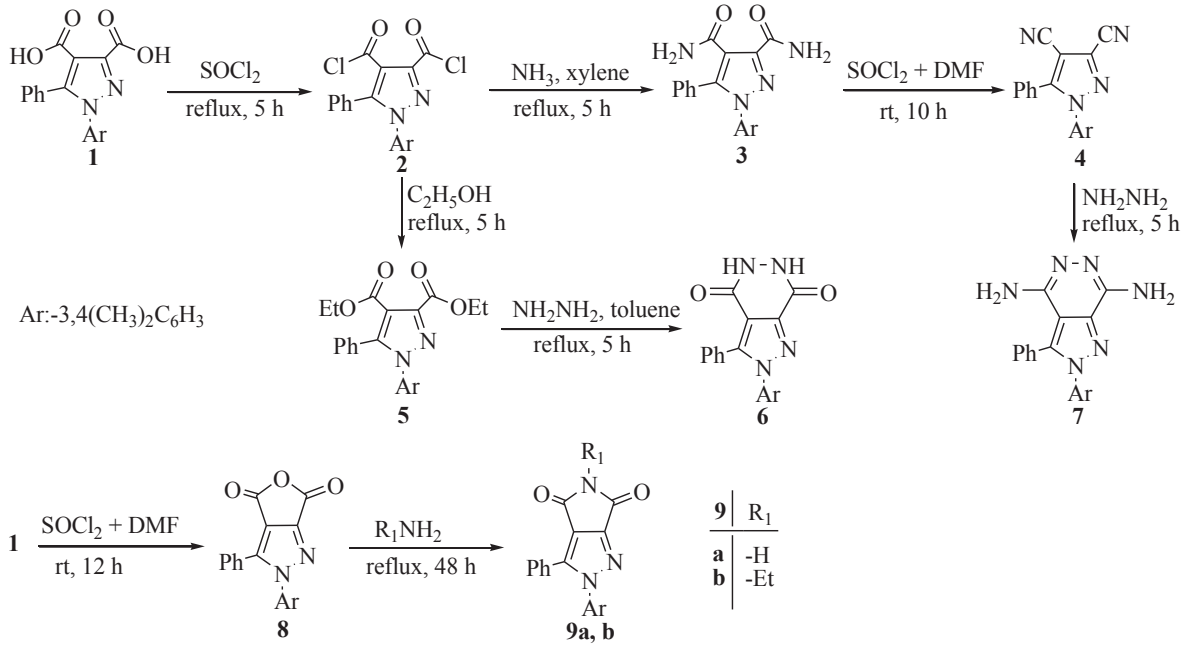
2.1. Chemistry

The starting material pyrazole-3,4-dicarboxylic acid (**1**) was previously synthesized by our research group [39]. Pyrazole-3,4-dicarbonyl chloride was prepared *via* heating compound **1** with excess thionyl chloride. The novel pyrazole derivatives; pyrazole-3,4-dicarboxyamide, pyrazole-3,4-dicarbonitrile, pyrazole-3,4-dicarboxylate, pyrazolo[3,4-*d*]pyridazine-4,7-dione, pyrazolo[3,4-*d*]pyridazine-4,7-diamine and furo[3,4-*c*]pyrazole-4,6-dione were prepared by reactions of carboxylic acid and/or chloride with various O- and/or *N*-nucleophiles in Scheme 1.

All new compounds were confirmed by spectroscopic methods. Pyrazole-3,4-dicarboxyamide **3** was synthesized through reaction of **2** and ammonia in xylene for 5 h. Its yield was 84%. The structure of **3** was clarified by ^{13}C NMR spectroscopy in which characteristic pyrazolo-carbodiamide (C═O) peaks were observed at δ 167.2 and 160.6 ppm. Pyrazolo-carbonitrile **4** was obtained by treating pyrazolo-carbodiamide with a mixture of DMF/$SOCl_2$ at cold for 2 h. The characteristic FT-IR signal of the nitrile group(s) bands in **4** were observed at 2194, 2186 cm^{-1}. Also, characteristic ^{13}C NMR peaks for **4** were seen at δ 114.8, 111.2 ppm. The pyrazole-3,4-dicarboxylate **5** was easily synthesized using Schotten-Baumann method. The structure of **5** was seen dicarbonyl carbon signals at δ 159.9 and 156.0 ppm in ^{13}C NMR spectroscopy and the characteristic FT-IR absorption of the ester structure at 1721 cm^{-1} was observed. Pyrazolo[4,3-*d*]pyridazine-4,7-dione **6** was obtained from cyclization reaction of **5** and anhydrous hydrazine (Scheme 1). Groups of amine as a nucleophile were attacked to ester groups. The related product was occurred at 62% yield. The structure of **6** was clarified ^{13}C NMR spectroscopy in which characteristic hydrazide (C═O) peaks were seen at δ 168.9 and 160.2 ppm and −NH peaks were observed at δ 8.5 and 8.3 ppm in ^{1}H NMR spectroscopy. Pyrazolo[4,3-*d*]pyridazine-4,7-diamine **7** was synthesized from cyclization reaction of pyrazole-3,4-dicarbonitrile **5** and anhydrous hydrazine. Compound **7** was clarified ^{1}H NMR spectroscopies in which characteristic amine peaks were observed at δ 4.4 and 4.1 ppm. The furo[3,4-*c*]pyrazole-4,6-dione furo[3,4-*c*]pyrazole-4,6-dione **8** was obtained with dehydration of pyrazole-3,4-dicarboxylic acid **1** and DMF/$SOCl_2$ in cold medium. Characteristic ^{13}C NMR signals of the −C═O carbonyl groups in **8** were observed at δ 167.5 and 159.9 ppm. Also, yield of **8** was occurred at 68% (see experimental). **9a**,**b** were synthesized with refluxing of corresponding compound **8** and ammonia with ethyl amine as nucleophiles in ethanol for two days (Scheme 1). Theirs yields were obtained at 65%. ^{13}C NMR spectrum revealed the signals of −C═O carbonyl groups in the range of δ 167.9 and 161.2 ppm. All synthesized compounds **1–8** and **9a**,**b** were confirmed by spectroscopic methods which was agreement with our previous findings and literature reports [6].

2.2. Biochemical results

In this study, we obtained the effects of novel pyrazole compounds (**1–8** and **9a**, **b**) derivatives against hCA I, hCA II, and AChE enzymes. Inhibition act of the CA isoforms has pharmacologic applications in several fields, such as antiglaucoma, diuretics, antiobesity, anticonvulsant, and anticancer agents/diagnostic tools, also, it can be emerging for designing anti-infective, i.e., antibacterial, antifungal, and antiprotozoan factors with a new process of action [40]. For a long time it has been considered that the pharmacologic effects of CA activation or inhibition are mostly due to effects on pH regulation in tissues or cells where the enzymes are present. We report the inhibition effects of these derivatives on the activity of hCA I, hCA II AChE enzymes under *in vitro* conditions. The following results are presented in Table 1:



Scheme 1. Synthesis of **1–8** and **9a-b** derivatives.

Table 1
Human carbonic anhydrases I, and II isoenzymes (hCA I, hCA II), and acetylcholinesterase (AChE) Enzyme inhibition values of substituted pyrazole compounds (**1–8** and **9a, b**).

Compounds	IC_{50} (μM)						K_i (μM)		
	hCA I	r^2	hCA II	r^2	AChE	r^2	hCA I	hCA II	AChE
1	3.54	0.9704	5.52	0.9683	85.94	0.9723	4.88 ± 0.83	6.95 ± 1.01	65.36 ± 11.47
2	0.83	0.9935	1.36	0.9052	103.65	0.9593	1.03 ± 0.23	1.82 ± 0.30	79.74 ± 21.15
3	13.93	0.9465	18.63	0.9693	145.26	0.9205	16.46 ± 2.66	18.02 ± 2.63	116.05 ± 14.95
4	9.26	0.9401	14.28	0.9890	64.04	0.9793	12.54 ± 1.46	17.73 ± 3.74	55.93 ± 10.42
5	5.04	0.9880	7.88	0.9424	60.26	0.9688	6.58 ± 0.95	8.93 ± 1.72	48.94 ± 9.63
6	18.25	0.9366	24.44	0.9882	74.98	0.9816	22.65 ± 5.36	27.94 ± 4.74	62.04 ± 15.04
7	6.66	0.9835	9.02	0.9758	86.05	0.9911	8.03 ± 1.83	11.62 ± 2.41	66.04 ± 14.72
8	6.01	0.9535	10.21	0.9395	100.82	0.9582	6.95 ± 1.25	14.88 ± 3.60	84.05 ± 16.03
9a	10.15	0.9905	14.05	0.9692	93.72	0.9105	13.77 ± 2.80	14.88 ± 2.52	80.52 ± 25.88
9b	16.22	0.9693	21.58	0.9355	78.03	0.9924	18.83 ± 4.05	24.80 ± 5.04	72.07 ± 18.72
AZA*	21.13	0.9665	28.55	0.9789	–	–	28.11 ± 10.33	34.12 ± 8.23	–
TAC**	–	–	–	–	166.74	0.9498	–	–	125.02 ± 18.63

* Acetazolamide (AZA) was used as a standard inhibitor for both human carbonic anhydrases I, and II isoenzymes (hCA I, hCA II).
** Tacrine (TAC) was used as a standard inhibitor for acetylcholinesterase (AChE) enzyme.

Abnormal levels of CA I enzyme in the blood are used as a marker for hemolytic anemia [41]. The slow cytosolic isoform hCA I was inhibited by the investigated novel pyrazole compounds (**1–8** and **9a**, **b**) derivatives, with K_i values ranging between 1.03 ± 0.23 and 22.65 ± 5.36 nM. Furthermore, 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarbonyl dichloride (**2**) and 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxylic acid (**1**) which demonstrated the most powerful hCA I isoenzyme inhibition properties with K_i values of 1.03 ± 0.23 and 4.88 ± 0.83 μM. In addition, it is known that the molecules, that had affinity against CA isoenzymes. The standard and clinically used drug acetazolamide (AZA) demonstrated a K_i value of 28.11 ± 10.33 μM (Table 1). Thus, the investigated compounds showed better inhibitory profiles compared to AZA, a clinically used CA inhibitor.

In addition, CA II is often associated with several diseases such as glaucoma, osteoporosis, and renal tubular acidosis. The hCA II was also efficiently inhibited by the novel pyrazole compounds (**1–8** and **9a**, **b**) investigated here. These compounds appeared to strongly inhibit hCA II, with K_i values ranging from 1.82 ± 0.30 nM to 27.94 ± 4.74 μM. These values are better than those of the clinically used drugs acetazolamide (K_i of 34.12 ± 8.23 μM). All the investigated these novel pyrazole compounds (**1–8** and **9a**, **b**) derivatives demonstrated marked inhibition against hCA II, but the compounds of 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarbonyl dichloride (**2**) and 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxylic acid (**1**) showed excellent inhibitions profile against cytosolic hCA II with K_i values of 1.82 ± 0.30 and 6.95 ± 1.01 μM, respectively (Table 1).

Anti-cholinesterases or AChEIs inhibit cholinesterase, increasing the length and level of ACh action. A diversity of usages of AChEI compounds is popular in agriculture and medicine [42,43]. AChEI molecules traditionally utilized for medical aims include carbamates and organophosphates. In diseases such as AD and myasthenia gravis, in which cholinergic function is defective, organophosphates/carbamates provide therapeutic options due to high effectiveness as AChEI compounds [44]. These inhibitors compounds are also utilized as pesticides for the deletion of insects that pose a threat to agriculture, gardening, and public health [45]. The inhibitory effects of the synthesized novel pyrazole compounds (**1–8** and **9a**, **b**) on AChE are shown in Table 1. The AChE inhibition profiles of the compounds evaluated here were quite interesting. Overall, the novel pyrazole compounds (**1–8** and **9a**, **b**) had excellent inhibitory activity with K_i values ranging from 48.94 ± 9.63 μM to 116.05 ± 14.95 μM. Furthermore, tacrine, used as a standard AChE inhibitor in this study, demonstrated K_i value of 125.02 ± 18.63 μM toward AChE. As these results show, the inhibition of AChE of novel pyrazole compounds (**1–8** and **9a**, **b**) is much better than standard drug. The compounds of 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxylate (**5**) and 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarbonitrile (**4**) showed excellent inhibitions profile against AChE with K_i values of 48.94 ± 9.63 and 55.93 ± 10.42 μM, respectively (Table 1).

2.3. Docking studies

We performed docking studies to identify the best pose of the most active compounds that interact with the receptors and possible inhibition mechanism after *in vitro* experimental results. We firstly identified the catalytic site on hCA I and II, and AChE receptors. The catalytic site was generated using SiteMap module and Dscore was calculated 1.061, 0.945, and 1.113 for hCA I and II, and AChE receptors, respectively. The catalytic sites were considered as druggable site. Following catalytic site generating process, we assessed the correct binding mode between the receptor and co-crystallized ligand with re-docking procedure performing an effective docking process. Docking validation provides useful in the assessment of docking method. The accuracy of docking process has been shown that co-crystallized and docked ligands are closely docked into the receptors as seen in Fig. 1.

Most active inhibitors and reference inhibitors were docked into catalytic site of all receptors with both Glide docking process and IFD process. AZA was used as a reference inhibitor for hCA I and II receptors and TAC was used as a reference inhibitor for AChE receptor. IFD process against the catalytic site of the receptors resulted in multiple docking poses for each syntheses compound. Pose viewer module was used analyzing the interactions between best-scored compounds and the receptors. The results of Glide docking and IFD of the best-scoring compounds and reference inhibitors are presented in Table 2. The results clearly shown that most of active compounds possessed high binding affinity against hCA I and II, and AChE receptors.

We focus on explanation for interactions and binding mechanisms between most active compounds and the receptors. So we considered IFD score, hydrogen bonds and non-bonded interactions for docking results analysis. We firstly analyzed 2D interaction diagram of most active compounds and reference inhibitors on the receptors. According to 2D interaction diagram, compound **2** formed π-π interaction with His200 residue as seen Fig. 2a. The complexed compound displayed interactions with 35 residues of hCAI. Superimposed pose of compounds **2** has shown that the compound well placed into the catalytic site of hCAI as seen in Fig. 3a. In detailed binding mode of the compound has also shown that formed halogen bonds with His122, Ala142, and Thr199 residues and aromatic hydrogen bond with His94 and Pro202 residues. We considered as key residues His94, Thr199, and

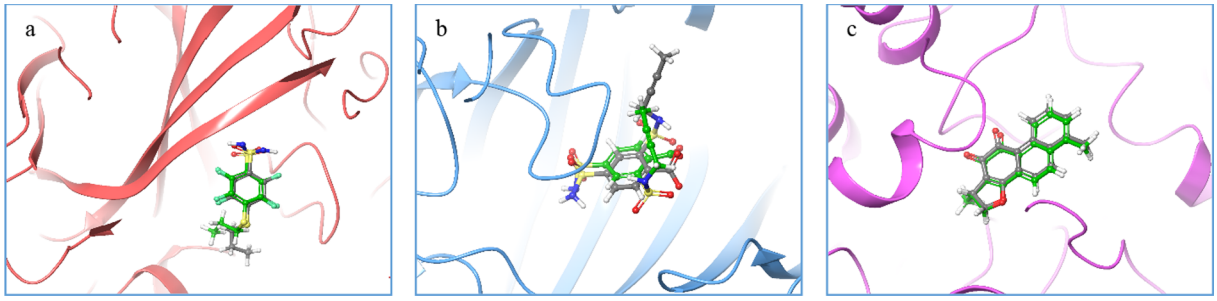


Fig. 1. Docking validation. Receptors are depicted in the ribbon model which hCA I with red color, hCA II with blue color, and AChE with purple color. The poses of co-crystallized ligands are represented in grey color ball and stick modeling while that of docked ligands is represented in green color ball and stick modeling.

His200 residues of hCA I, because previously syntheses inhibitors and reference inhibitor interacted with these residues [46–48].

2D interaction diagram of compound **2** there was no any hydrogen bound or non-bonded interactions with residues of hCAII, but the compound was located into the cavity creating from hydrophobic, polar and metal residues. The residues were reasonably similar with residues of hCAII which surrounding AZA as seen Fig. 2b and d. Furthermore, the residues in binding pocket of compound **2** are closely similar to residues which surrounding sulfonamide derivatives [49] and EMAC1011g compound [50]. The complexed compound displayed interactions withjust25 residues of hCA II, on the contrary, hCA I. In Fig. 3b, it can be seen that the compound formed two halogen bonds with Asn62 and Asn67 residues. According to these results, we observed that the chloride group of compound **2** has been contributed to inhibition of hCA I and II enzymes.

The compound **5** formed π-π interaction with Trp286 and Phe297 residues and also hydrogen bonds with Tyr72 and Phe295 residues by courtesy of water molecules in crystal structure. The aromatic ring of TAC formed π-π interaction with Trp286 residue as compound **5** as seen Fig. 2c and f. In previous studies shown that Tyr72 and Trp286 residues play a key role on inhibition of AChE enzyme [51,52]. The complexed compound displayed interactions with 27 residues of AChE. Superimposed pose of compounds **5** has shown that the compound well placed into the catalytic site of AChE as seen in Fig. 3c. This figure also shown that these compounds interacted with the key residues into the catalytic site of the receptors.

3. Conclusion

We synthesized novel substituted pyrazole derivatives, which have two or three important rings. Binding free energy of most active compounds was calculated with induced fit docking process. After docking process, inhibition mechanism of most active compounds is identified by detecting binding mode of the compounds in the catalytic site of hCA I, hCA II and AChE receptors. Key residues, which are responsible from inhibition each receptor was detected. These compounds were investigated for their hCAs I and II isoforms, and AChE inhibition properties. These molecules have shown inhibition against those enzymes at nanomolar concentration level and also, the docking results revealed that all compounds inhibited hCAI, hCA II, and AChE enzymes through interactions including hydrophobic interactions, electrostatic interactions, and H-bonds. Inhibition of AChE and hCA I, and II isoforms can have a significant role in discovery and drug design as well as in toxicology and medicine. Additionally, these molecules are drug candidates as anticholinergic and antiepileptic.

4. Experimentals

4.1. Materials and equipment

All the used chemical materials were purchased from Merck and Sigma Aldrich companies. The obtained compounds were done checking the purity and follow up of the reactions tested in each step by thin layer chromatography (TLC) SiO_2 using a DC Alufolien Kieselgel 60 F 254 Merck. Camag TLC device was used to visualize in reactions conditions. ^1H and ^{13}C NMR spectra on a Bruker DRX-400 high performance digital FT-NMR spectrometer were used to characterizations of chemical structures. The infrared spectra were recorded on a Perkin Elmer Precisely Spectrum one spectrometer using pressed disc in the range of 400–450 cm^{-1}. The mass spectrum was measured on Thermo Scientific TSQ-Quantum Access LC/MS spectrometer. Melting points of synthesized compounds were performed on an Electrothermal Gallenkamp apparatus and are uncorrected.

4.2. 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxylic acid (1)

To synthesize compound **1**, procedure existed in literature was followed [39].

4.3. 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarbonyl dichloride (2)

To synthesize compound **2**, procedure existed in literature was followed [39].

Table 2
Glide and IFD scores (kcal/mol) of the pyrazole derivatives in the catalytic sites of hCA I and II, and AChE.

Compounds	hCA I		hCA II		AChE	
	Glide Score	IFD Score	Glide Score	IFD Score	Glide Score	IFD Score
2	−3.597	−6.776	−4.896	−5.774	–	–
5	–	–	–	–	−6.749	−9.534
AZA*	−8.331	−9.016	−8.178	−9.560	–	–
TAC**	–	–	–	–	−8.978	−9.579

* Acetazolamide (AZA) was used as a standard inhibitor for human carbonic anhydrase isoenzymes I, and II (hCA I, and II).
** Tacrine (TAC) was used as a standard inhibitor for acetylcholinesterase (AChE) enzyme.

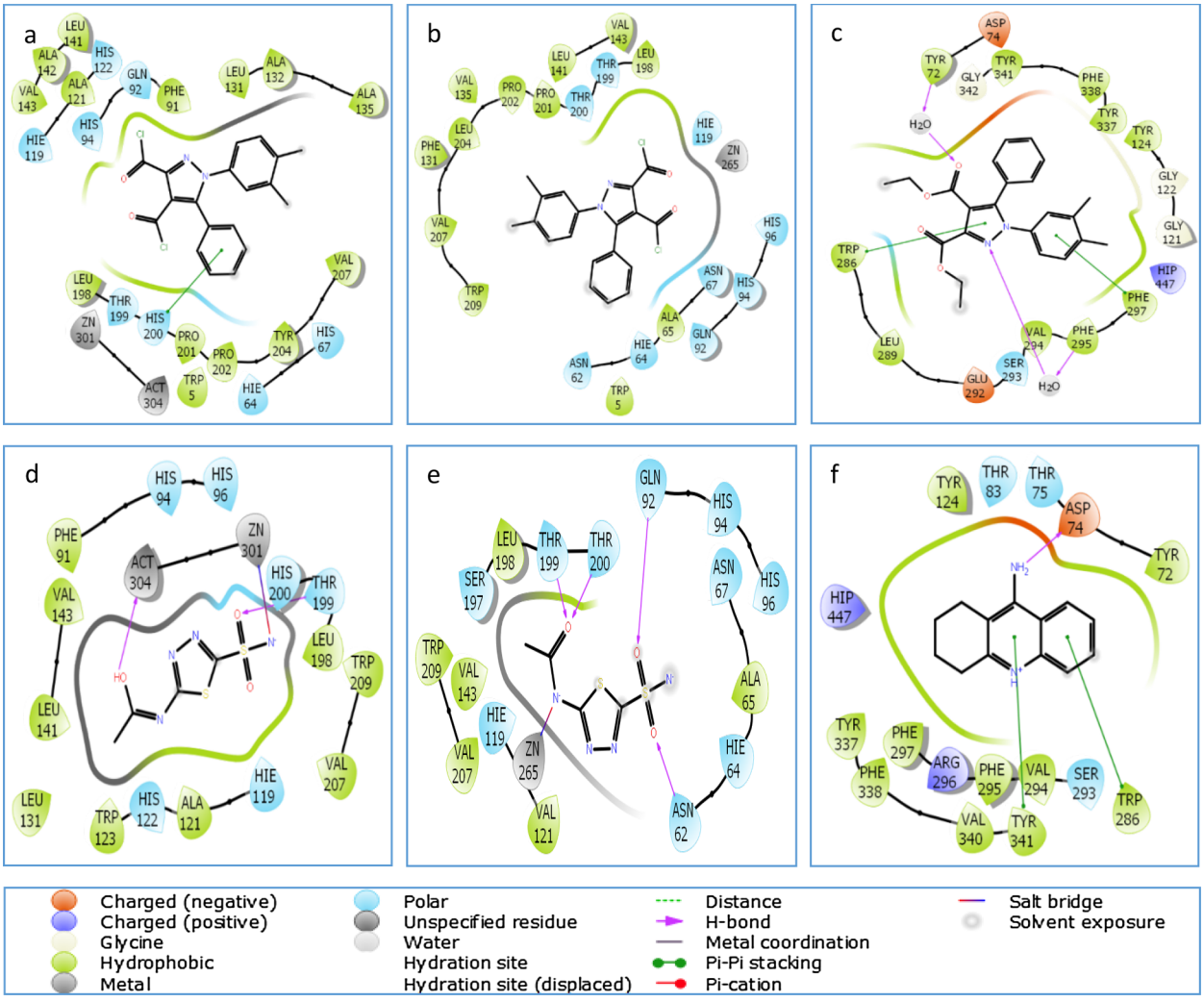


Fig. 2. 2D receptor–ligand interaction profile; (a) **2**-hCA I, (b) **2**-hCA II, (c) **5**-AChE, (d) **AZA**-hCA I, (e) **AZA**-hCA II, and (f) **TAC**-AChE.

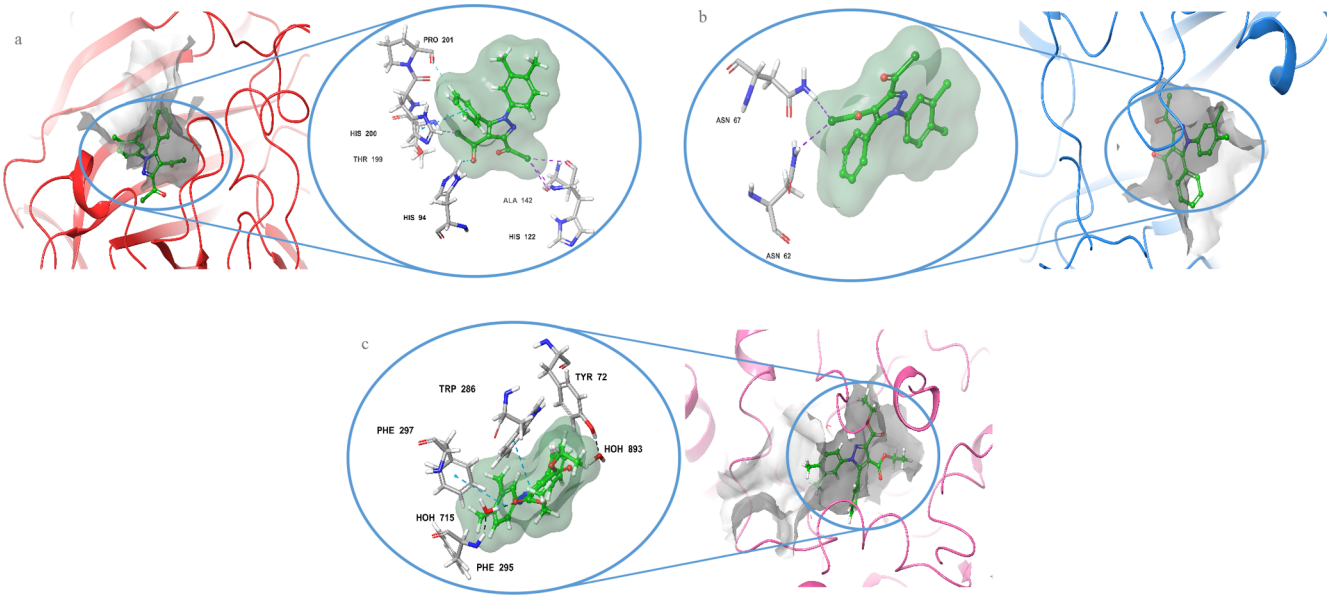


Fig. 3. The lowest energy conformation of compounds into hCAI, hCA II, and AChE receptors. (a) Best-pose of **2** into catalytic active site of hCAI, (b) Best-pose of **2** into catalytic active site of hCA II, and (c) Best-pose of **5** into catalytic active site of AChE. Receptors are depicted in the ribbon model which hCAI with red color, hCA II with blue color, and AChE with purple color. Catalytic active sites are represented as solid surface. Compounds are represented in ball and stick modeling which green color carbon and residues are represented in ball and stick modeling which grey color carbon.

4.4. 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxamide (3)

Pyrazole-3,4-dicarbonyl dichloride **2** (0.373 g, 1 mmol) was dissolved in xylene (10 mL). Ammonia as nucleophile (1 mmol) was added to solution. The reaction mixed was refluxed in xylene for 5 h. After evaporation, the oily residue was treated with dry ether and the formed crude product was crystallized from ethanol. Yield: 84%. mp 211–213 °C; FT-IR (ν, cm^{-1}): 3256, 3210, 3062, 2936, 1662, 1654, 1598, 1520, 1498, 1443, 1315; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.7–6.5 (m, 8H, Arom.), 5.5 (s, 2H), 5.0 (s, 2H), 2.0 (s, 3H), 1.6 (s, 3H), ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 167.2, 160.6, 144.5, 141.4, 140.4, 135.5, 131.0, 130.7, 130.0, 129.6, 128.5, 127.5, 126.8, 125.1, 121.6, 120.2, 111.9, 21.1, 17.9. (+)ESI-HRMS *m/z*: calculated for [$C_{19}H_{18}N_4O_2 + H^+$]: 335.3782; observed 335.3789.

4.5. 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarbonitrile (4)

Pyrazole-3,4-dicarboxamide **3** (0.167 g, 0.5 mmol) was added in xylene (5 mL). $SOCl_2$ (0.146 mL, 2 mmol) and DMF (2 mL) were added to solution in 0 °C. Reaction mixed was stirred at room temperature for (10) hours. The ice water was added to the mixture. Precipitated solid was filtered, dried. It was purified from ethanol. Yield: 42%. mp 198 °C. FT-IR (ν, cm^{-1}): 3064, 2932, 2194, 2186, 1597, 1442, 1315; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.9–6.6 (m, 8H, Arom.), 2.0 (s, 3H), 1.7 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 145.9, 136.2, 134.1, 130.7, 129.6, 128.9, 127.1, 121.6,120.6, 115.0, 114.8, 111.2, 21.8, 16.5. (+) ESI-HRMS *m/z*: calculated for [$C_{19}H_{14}N_4 + H^+$]: 240.3426; observed 240.3431.

4.6. Diethyl 1-(3,4-dimethylphenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxylate (5)

Pyrazole-3,4-dicarbonyl dichloride **2** (0.373 g, 1 mmol) was dissolved ethanol (15 mL). The prepared solution was heated with a catalytic amount of pyridine for (5) hours [53]. It was cooled to room temperature. It was acidified via hydrochloric acid (12%). Precipitated solid was filtered, dried. It was crystallized from ethanol. Yield: 85%. mp 124 °C. FT-IR (ν, cm^{-1}): 3062, 2954, 1721, 1592, 1521, 1490, 1442, 1315; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.9–6.5 (m, 8H, Arom.), 3.7 (m, 2H), 3.4 (m, 2H), 2.0 (s, 3H), 1.6 (s, 3H), 1.3 (t, J = 5.2 Hz 3H), 1.2 (t, J = 5.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 159.9, 156.0, 144.9, 140.1, 135.5, 134.1, 133.1, 132.0, 130.0, 128.6, 127.5, 126.1, 125.1, 124.0, 123.3, 110.8, 41.3, 40.2, 21.1, 17.6, 12.4, 11.7. (+)ESI-HRMS *m/z*: calculated for [$C_{23}H_{24}N_2O_4 + H^+$]: 393.4551; observed 393.4556.

4.7. 2-(3,4-dimethylphenyl)-3-phenyl-5,6-dihydro-2H-pyrazolo[3,4-d] pyridazine-4,7-dione (6)

Pyrazole-3,4-dicarboxylate **5** (0.196 g, 0.5 mmol) was added in anhydrous toluene (10 mL). Anhydrous hydrazine (0.016 g, 0.5 mmol) was added to solution. The reaction mixture was refluxed for (5) hours. Precipitated solid was filtered. It was crystallized from hexane. Yield: 62%. mp 264–266 °C. FT-IR (ν, cm^{-1}): 3226, 3064, 2956, 1652, 1650, 1596, 1528, 1496, 1447, 1328; ^{1}H NMR (400 MHz, DMSO) δ (ppm): 8.5, 8.3 (s, 2H), 7.8–6.5 (m, 8H, Arom.), 2.2 (s, 3H), 1.6 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ (ppm): 168.9, 160.2, 145.2, 143.2, 140.1, 139.0, 133.1, 131.7, 130.7, 129.6, 128.2, 127.5, 126.4, 125.8, 125.1, 119.1, 111.9, 21.1, 16.5. (+)ESI-HRMS *m/z*: calculated for [$C_{19}H_{16}N_4O_2 + H^+$]: 333.3635; observed 336.3641.

4.8. 2-(3,4-dimethylphenyl)-3-phenyl-2H-pyrazolo[3,4-d]pyridazine-4,7-diamine (7)

Pyrazole-3,4-dicarbonitrile **4** (0.15 g, 0.5 mmol) was added dry ethanol (10 mL). Anhydrous hydrazine (0.016 g, 0.5 mmol) was added to solution. The reaction mixture was refluxed for (5) hours. Precipitated solid was filtered and it was washed with diethyl ether. The product was crystallized by ethanol. Yield: 72%. mp > 300 °C. FT-IR (ν, cm^{-1}): 3264, 3258, 3062, 2936, 1597, 1521, 1496, 1442, 1321; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.0–6.8 (m, 8H, Arom.), 4.4 (s, 2H), 4.1 (s, 2H), 2.3 (s, 3H), 1.8 (s, 2H); ^{13}C NMR(100 MHz, $CDCl_3$) δ (ppm): 164.7, 159.2, 145.2, 141.4, 141.1, 137.2, 134.1, 131.7, 131.4, 130.0, 129.3, 128.9, 128.6, 127.1, 123.7, 22.1, 16.5. (+)ESI-HRMS *m/z*: calculated for [$C_{19}H_{18}N_6 + H^+$]: 331.4042; observed 333.3641.

4.9. 2-(3,4-dimethylphenyl)-3-phenyl-2H-furo[3,4-c]pyrazole-4,6-dione (8)

Pyrazole-3,4-dicarboxylic acid **1** (0.17 g, 0.5 mmol) was added in DMF (0.2 mL) and $SOCl_2$ (0.7 mL). The solution was mixed at 0 °C for four hours. Then, it was stirred to room temperature for (12) hours. The ice water was added to the mixture. The product was crystallized by ethanol. Yield: 68%. mp 181–183 °C. FT-IR (ν, cm^{-1}):3062, 2928, 1676, 1596, 1524, 1498, 1442, 1316; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.0–6.5 (m, 8H, Arom.), 2.2 (s, 3H), 1.6 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 167.5, 159.9, 146.6, 141.1, 136.1, 131.7, 130.3, 129.3, 128.9, 127.8, 127.1, 126.1, 124.4, 123.7, 121.3, 119.5, 110.8, 20.8, 16.2. (+)ESI-HRMS *m/z*: calculated for [$C_{19}H_{14}N_2O_3 + H^+$]: 319.3326; observed 319.3329.

4.10. 2-(3,4-dimethylphenyl)-3-phenylpyrrolo[3,4-c]pyrazole-4,6(2H,5H)-dione (9a)

Pyrazole-4,6-dione **8** was (0.16 g, 0.5 mmol) was dissolved ethanol (20 mL). Ammonia as nucleophile (0.009 g, 0.5 mmol) was added to solution. Reaction was refluxed for (2) days. It was cooled room temperature. The solvent was evaporated. The residue compound was washed with diethyl ether. Precipitated solid was filtered, dried. It was crystallized by ethanol. Yield: 65%. mp 215–217 °C. FT-IR (ν, cm^{-1}):3274, 3068, 2927, 1658, 1589, 1517, 1496, 1442, 1313; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.3 (s, 1H), 7.9–6.5 (m, 8H, Arom.), 2.1 (s, 3H), 1.5 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 167.9, 161.2, 144.2, 138.3, 134.8, 134.5, 132.4, 131.0, 130.7, 129.3, 128.9, 128.2, 126.4, 125.4, 124.0, 122.7, 110.1, 23.9, 17.9. (+)ESI-HRMS *m/z*: calculated for [$C_{19}H_{15}N_3O_2 + H^+$]: 318.3421; observed 318.3425.

4.11. 2-(3,4-dimethylphenyl)-5-ethyl-3-phenylpyrrolo[3,4-c]pyrazole-4,6(2H,5H)-dione (9b)

Pyrazole-4,6-dione **8** was (0.16 g, 0.5 mmol) was dissolved ethanol (20 mL). Ethylamine as nucleophile (0.023 g, 0.05 mmol) was added to solution. Reaction was refluxed for (2) days. It was cooled room temperature. The solvent was evaporated. The residue compound was washed with diethyl ether. Precipitated solid was filtered, dried. It was crystallized by ethanol. Yield: 65%. mp 224 °C. FT-IR (ν, cm^{-1}): 3310, 3064, 2926, 1662, 1594, 1524, 1498, 1444, 1321; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.0–6.5 (m, 8H), 3.0 (m, 2H, Arom.), 2.1 (s, 3H), 1.7 (s, 3H), 1.1 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 163.7, 161.2, 143.5, 140.1, 137.2, 133.8, 131.7, 130.3, 130.0, 129.6, 128.9, 128.5, 127.5, 126.8, 125.8, 124.4, 109.0, 33.6, 23.5, 19.3, 11.4. (+)ESI-HRMS *m/z*: calculated for [$C_{21}H_{19}N_3O_2 + H^+$]: 346.3986; observed 346.3989.

4.12. Biochemical studies

4.12.1. hCA isoenzymes purification and inhibition studies

In this paper, both CA isozymes were purified by Sepharose-4B-L-Tyrosine sulfanilamide as affinity chromatography in a single phase [54]. The column chemical material of affinity chromatography containing Sepharose-4B-L-Tyrosine-sulfanilamide was created comprising to a prior procedure [55,56]. The protein molecules flow in the column eluates was spectrophotometrically obtained at 280 nm as illustrated

formerly. CA isozymes' inhibitory activity evaluation of novel pyrazole (**1–8** and **9a**, **b**) derivatives was obtained using the spectrophotometric style of Verpoorte et al. [57] as explained in former studies [58–60]. In this study, changes in absorbance were recorded during 3 min at 348 nm using p-nitrophenylacetate was utilized as a substrate which converted by both isoenzymes to the p-nitrophenolate ion molecule [61,62].

4.12.2. AChE inhibition study

The inhibitory effects of novel pyrazole (**1–8** and **9a**, **b**) derivatives on AChE activity were performed according to the spectrophotometric method of Ellman et al. [63] and described previously [64,65]. Acetylthiocholine iodide (AChI) substrate was utilized for the inhibition act and enzymatic reaction. 5,5′-Dithio-bis(2-nitro-benzoic) acid (DTNB) compound was utilized in this study for the measurement of the AChE activity. Briefly, 750 µL of sample solution dissolved in deionized water at different concentrations with 100 µL of Tris/HCl buffer (1.0 M, pH 8.0), and 50 µL of AChE solution were mixed and incubated for 64 min at 21 °C [66]. Then reaction was initiated by the addition of 50 µL of AChI. Then, 50 µL of DTNB (0.5 mM) was added. The hydrolysis of these substrates was monitored spectrophotometrically by formation of the yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of thiocholine with DTNB, released by enzymatic hydrolysis of AChI, with an absorption maximum at a wavelength of 412 nm [67].

4.13. Docking studies

The x-ray crystal structures of hCA I, hCAII, and AChE (PDB code: 4WR7, 5AML, and 4M0E, respectively) were taken in pdb format from RCSB Protein Data Bank with resolution of 1.5 Å, 1.36 Å, and 2 Å, respectively. Then, the crystal structures of the receptors were used for molecular docking studies. Molecular docking studies were performed using Small Drug Discovery Suites package (Schrödinger 2017-2, LLC, USA). The receptors preparation was performed according to our previous study. Briefly, the 3D crystal structures were repaired and prepared via protein preparation wizard in Maestro 11.4. Bond order and charges were assigned and then all missing hydrogen atoms were added to protein structure. Missing side chains were filled using Prime module of the program. Amino acids were ionized by setting physiological pH with the help of Propka software. Water molecules that were formed less than 3 contacts with the protein or ligand were removed. Then, energy minimization has also been performed using OPLC force field. Single binding site of the prepared receptors was identified using SiteMap module in Maestro 11.4.3D structures of synthesized compounds were produced with Maestro 11.4 by sketching 2D structures. 3D structure of ligands was created using LigPrep module in Maestro 11.4. In order to obtain correct molecular geometries and protonation state at pH 7.0 ± 2.0, Epik module and OPLS-2005 force field were used.

Molecular docking was performed to identify binding affinity and possible interactions between synthesized compounds and receptors. Briefly, selecting crystallized inhibitors at the binding site using the Receptor Grid Generation platform before the docking process generated grid box. Docking calculations were set as Extra Precision (XP) by keeping the ligand flexible. After the docking process, the types of interactions and interacted residues for best-scored compound results were analyzed. In order to identification accuracy of the docking process, docking validation was performed with re-docking procedure by extracting inhibitor complexed in the crystal structure of the receptor, before docking of synthesized compounds. Following Glide docking, induced fit docking (IFD) studies were performed using IFD module in Maestro 11.4. In the IFD experiment, three ligands, which exhibited the most effective inhibition *in vitro* experiment, were docked on the receptors. Briefly, centroid of the residues was generated around the selected residues in the catalytic site. After that, side chains were automatically trimmed based on B-factor; closest residues to the ligand were refined within 3.4 Å of ligand pose in prime refinement. Then flexible receptor-docking simulations were performed in Maestro 11.4 [68].

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Conflict of Interest

The authors declare that they have no conflict of interest.

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