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# Pyrazole[3,4-*d*]pyridazine derivatives: Molecular docking and explore of acetylcholinesterase and carbonic anhydrase enzymes inhibitors as anticholinergics potentials

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ABSTRACT

Recently, the pyridazine nucleus has been widely studied in the field of particular and new medicinal factors as drugs acting on the cardiovascular system. Additionally, a number of thienopyridazines have been claimed to possess interacting biological macromolecules and pharmacological activities such as NAD(P)H oxidase inhibitor, anticancer, and identified as a novel allosteric modulator of the adenosine A1 receptor. The literature survey demonstrates that coumarin, 1,2-pyrazole benzothiazole, and 1,3- thiazole scaffolds are the most versatile class of molecules. In this study, a series of substituted pyrazole[3,4-*d*]pyridazine derivatives **(2a–n)** were prepared, and their structures were characterized by Mass analysis, NMR, and FT-IR. These obtained pyrazole [3,4-*d*]pyridazine compounds were very good inhibitors of the carbonic anhydrase (hCA I and II) isoenzymes and acetylcholinesterase (AChE) with $K_i$ values in the range of 9.03 ± 3.81–55.42 ± 14.77 nM for hCA I, 18.04 ± 4.55–66.24 ± 19.21 nM for hCA II, and 394.77 ± 68.13–952.93 ± 182.72 nM for AChE, respectively. The possible inhibition mechanism of the best-posed pyrazole[3,4-*d*]pyridazine and pyrazole-3-carboxylic acid derivatives and their interaction with catalytic active pocket residues were determined based on the calculations.

## 1. Introduction

The pyrazoles are essential parts of many heterocyclic compounds. They have extensive workspace due to biologic activities and medicinal properties [1,2]. These molecules have potential applications such as antibacterial, antifungal, antibiotics, insecticide, pesticide, biosensors, etc., [3–7]. Pyrazole molecules are known to exhibit specific properties such as antiviral, antitumor, antidepressant, anti-inflammatory and antioxidant activities [8–11]. In the medical sector, the pyrazoles are used in the structure of some drugs such as Viagra, Celebrex, and Zerbaxa [12–14]. Additionally, pyrazole-pyridazine derivatives were used in agricultural products and drug researches due to their diverse biological activities [15]. Furthermore, substituted pyrazoles were used in the polymer structures in terms of optoelectronic properties [16]. The commonly used methods for synthesis of the pyrazole-pyridazine derivatives are reported the cyclocondensation of hydrazine hydrate or monosubstituted hydrazines and 1,3-dicarbonyl compounds [17,18]. In this study, the synthesis of pyrazole-pyridazine was performed using various hydrazines and substituted pyrazole. The synthesized molecules were characterized by IR, NMR, and Mass analysis.

Carbonic anhydrase (CA) isozymes catalyze the reversible hydration of carbon dioxide ($CO_2$) to produce bicarbonate anion ($HCO_3^-$) and a proton ($H^+$) [19,20]. Hence, these enzyme superfamily members including sixteen isoenzymes are primer controllers of pH outside and within the cell. Diversity in subcellular localization and catalytic activity, carbonic anhydrase isoenzymes show the main range of

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biological aims with several therapeutic potentials [21,22]. In particular, inhibitor compounds of hCA I and II isozymes in human cells are utilized as factors for treating glaucoma and edema. Membrane-bound brain-associated hCA IV isoenzyme is involved in pH regulation in neuron cells and glial cells analogously to the tumor-associated CA IX isoform. This plays an important role in progression, acidification of tumors, and metastasis in various cancers. A new approach to therapy of obesity and epilepsy is evaluated by inhibition behavior of special isozymes of CA [23,24]. Indeed, selective inhibitor compounds of CA isoforms in pathogenic microorganisms is gaining enhancing consideration as a new method of therapy of infectious disease [25].

Acetylcholine (ACh) is the main player as far as Alzheimer's disease (AD) is interesting which is synthesized by choline acetyltransferase (CAT). This area of the brain cell is affected in the pathophysiological phenomenon of the AD [26,27]. This reasons, finally, a marked difference in ACh and CAT synthesis. CAT amounts are decreased 58–90% in the AD, which raises the severity of dementia [28]. Acetylcholinesterase (AChE) is the enzyme accountable for the degradation of the ACh, which is markedly decreased in the AD. Therefore, these enzymes cholinergic synapses waiting for ACh and choline acetate is a key enzyme responsible for the hydrolysis. AChE enzyme, both three-dimensional structure of the active site amino acid sequence of the enzyme is related to many insects, and nerve transmission in the backbone is highly conserved [29]. Organic compounds with the potential to inhibit AChE enzyme or AChE inhibitors (AChEIs) are also very noteworthy due to their significance in the therapy of multiple neurodegenerative diseases. It is expected that AD, an extensive shape of dementia, will increase its prevalence globally and will be a significant problem for human health in the coming periods [30].

Herein, the facile synthesis to condensation reaction of pyrazole-3-carboxylic acid compounds (**1a**,**b**) and pyrazole[3,4-*d*]pyridazine (**2a–n**) derivatives have been reported, and their inhibition potential of hCA I, and II isoenzymes were investigated. The most suitable and potent AChE inhibition effects of these molecules are also studied.

## 2. Experimental

### 2.1. Materials and equipment

All chemicals used in this study were purchased from Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany) companies. Bruker DRX-400 MHz FT-NMR spectrometer was employed to obtain the structure of molecules at 1 H (400 MHz) and 13 C (100 MHz) NMR spectra. The infrared spectra were recorded on a Perkin Elmer Precisely Spectrum. Samples were used as pellet form, and the measuring range was from 4.000 to 450 $cm^{-1}$. An LC/MS spectrometer (Thermo Scientific TSQ-Quantum Access) was used to measure the mass spectrum. Melting points of these molecules were determined on an Electrothermal Gallenkamp apparatus and are uncorrected. The possible impurities and reaction steps of resulting compounds were tested by thin layer chromatography (TLC) using an aluminum TLC plate, silica gel coated with a fluorescent indicator of F254, Merck Millipore. Camag TLC devices envisaged these synthesized compounds. The synthesis of molecules has been described in previous studies [1].

### 2.2. Biological evaluation

#### 2.2.1. hCA isoenzyme purification and inhibition studies

Both hCA isoenzymes from human erythrocytes were purified via a simple single-step by method Sepharose-4B-L-Tyrosine-sulphanilamide affinity gel chromatography. For this purpose, the human erythrocyte samples were centrifuged at 13000 rpm for 25 min. Then, the solution was filtered to remove precipitate. hCA isoenzymes were isolated from the serum, which its pH was adjusted to 8.7 by adding solid Tris. Affinity column was equilibrated by buffer solution (25 mM Tris-HCl/0.1 M $Na_2SO_4$) at the pH 8.7. The serum was loaded to affinity gel and washed with buffer solution (25 mM Tris-HCl/22 mM $Na_2SO_4$ at the pH 8.7. hCA isoenzymes were taken from the column in fractions of 2 mL. The hCA isoenzymes activity were measured by following the change at absorbance a specific (348 nm) of p-nitrophenylacetate (PNA) to p-nitrophenolate ion over a period of 3 min at room temperature (25 °C) using a spectrophotometer (Thermo Scientific, UV–Vis Spectrophotometer). There was 0.4 mL of 0.05 M Tris-SO4 buffer (pH 7.4), 03 mL of 3 mM PNA, 0.2 mL of $H_2O$, and 0.1 mL of enzyme solution in a test tube content of this reaction. Esterase activity assays were identified from a series of experiments at three different pyrazole derivatives. CA inhibitory effects of pyrazole-3-carboxylic acid compounds (**1a**,**b**) and pyrazole[3,4-*d*]pyridazine derivatives (**2a–n**) were studied by Verpoorte et al. [31] and previous studies [32–35]. Enzyme activity was measured at 348 nm by UV–Vis spectrophotometer with p-nitrophenyl acetate (PNA) substrate.

#### 2.2.2. AChE inhibition study

AChE inhibitory effects of pyrazole-3-carboxylic acid compounds (**1a**,**b**) and pyrazole[3,4-*d*]pyridazine derivatives (**2a–n**) were studied by per Ellman et al. [36] and previous studies [37–39]. It was studied at 412 nm spectrophotometrically using acetylthiocholine iodide as a substrate for the enzymatic reaction. 5,5′-Dithio-bis(2-nitro-benzoic) acid was utilized for the measurement of the AChE activity [40].

### 2.3. Computational studies

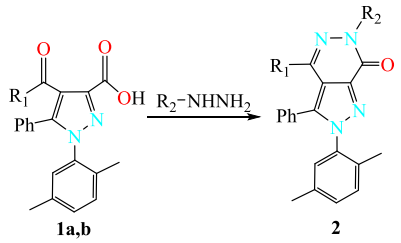
Computational studies were achieved using Small Drug Discovery Suites software (Schrödinger 2017-2, LLC, USA). 2D structures of pyrazole-3-carboxylic acid compounds were drawn, and 3-Dimensional structures of the compounds were achieved using Maestro 11.4. The 3-Dimensional structures were prepared by obtaining accurate protonation state, and molecular geometries at physiological pH with the LigPrep module [15]. It was reached to X-Ray crystal structures of targeted receptors (h CA I PDB code: 4WR7, h CA II PDB code: 5AML, and AChE PDB code: 4M0E) from RCSB Protein Data Bank. The receptors were chosen for molecular docking process due to their low resolution (1.5 Å, 1.36 Å, and 2 Å, respectively). Afterward, the receptor structures prepared with the protein preparation wizard as reported on our previous studies. The bond orders and charges were primarily allocated. Missing side chains and hydrogen atoms were secondarily appended to the structure of the receptor. Amino acids were thirdly ionized at physiological pH with Propka module. Water molecules that were formed less than three contacts with the receptor were fourthly fended off. Lastly, energy minimization was performed with OPLC force field. Prepared receptors were used at detection of the binding pocket and molecular docking process.

In order to designate active catalytic pocket of the receptors and druggability of catalytic active pocket, top-ranked potential protein binding pocket of the receptors were identified with SiteMap module. Following designation catalytic active pocket, for verifying the reliability of molecular docking process, docking validation studies were carried out as reported on our previous studies. Following docking validation, prepared compounds, ligands, were docket into the catalytic active pocket of the receptor using Glide module as reported at our previous studies. Grid box was generated by selecting co-crystallized ligand into the predicted catalytic active pocket. The molecular docking calculation was performed on Standard Precision (SP) and Extra Precision (XP) settings [15].

## 3. Results and discussion

### 3.1. Chemistry

The pyrazole[3,4-*d*]pyridazine compounds are the most commonly studied subject in heterocyclic chemistry due to their biologic activities [17]. Therefore, pyrazole[3,4-*d*]pyridazine compounds were prepared,




| 1 | $R_1$ |
|---|---|
| a | -Ph |
| b | -OEt |

| 2 | $R_1$ | $R_2$ |
|---|---|---|
| a | -Ph | -Ph |
| b | -Ph | -H |
| c | -Ph | -Ph$(CH_3)_2$(2,5) |
| d | -Ph | -Ph$(CH_3)_2$(3,4) |
| e | -Ph | -Ph$(NO)_2$(2,4) |
| f | -Ph | -Ph$(Cl)_3$(2,4,6) |
| g | -Ph | -Ph$(CF_3)$(4) |

| 2 | $R_1$ | $R_2$ |
|---|---|---|
| h | -OEt | -Ph |
| i | -OEt | -H |
| j | -OEt | -Ph$(CH_3)_2$(2,5) |
| k | -OEt | -Ph$(CH_3)_2$(3,4) |
| l | -OEt | -Ph$(NO)_2$(2,4) |
| m | -OEt | -Ph$(Cl)_3$(2,4,6) |
| n | -OEt | -Ph$(CF_3)$(4) |

**Scheme1.** Synthesis of pyrazole[3,4-*d*]pyridazines (**2a–n**).

and spectroscopic methods confirmed their structures. Initially, pyrazole-3-carboxylic acid (**1a**,**b**) compounds were prepared by our research group. Pyrazole[3,4-*d*]pyridazine (**2a–n**) derivatives were directly obtained by cyclization reaction synthesized **1a** and **b** compounds and various hydrazines as shown Scheme1. The yields of **2a–n** compounds were between 25 and 65%. All of the synthesized **2a–n** compounds had two or three important rings. The structures of obtained compounds were characterized via spectroscopic methods.

### 3.2. Biochemistry results

Patients with AD have decreased cerebral generation of ACh and impaired cortical cholinergic function. Thus, reinforcement of cholinergic activity therapeutically can reduce cognitive decline in dementia [41,42]. AChEIs including tacrine, donepezil, rivastigmine, and galantamine, which increase ACh levels in the brain cells via the inhibition of AChE enzyme, are considered first-line pharmacotherapy for dementia. Evidence obtained is that ChEIs affect the development of the cognitive concept. However, the majority of these surveys were conducted in North America and European Caucasians [43,44]. In this work, effects of pyrazole-3-carboxylic acids (**1a** and **b**) and pyrazole [3,4-*d*]pyridazine (**2a–n**) derivatives against hCA I, hCA II, and AChE enzymes were studied. The compounds in hCA I, hCA II and AChE enzymes were studied as *in vitro* and these effects are evaluated with the data in Table 1.

CA I isoenzyme, which is seen as a symptom of hemolytic anemia, is evaluated as a result of the presence of abnormal levels in blood [45]. The slow cytosolic isoform of hCA I was inhibited by the pyrazole-3-carboxylic acids (**1a** and **b**) and pyrazolyl[3,4-*d*]pyridazine (**2a–n**) derivatives, with $K_i$ values ranging between 9.03 ± 3.81 and 55.42 ± 14.77 nM. Furthermore, (**2 g**) and (**2f**) have $K_i$ values of 9.03 ± 3.81 and 10.32 ± 2.64, respectively. These values correspond to the most powerful properties of hCA I isoenzyme inhibition. The $K_i$ values of acetazolamide (AZA), a drug used standard and clinically, are 178.11 ± 40.03 nM (Table 1).

In addition, CA II isozyme is often related to various diseases for instance osteoporosis, renal tubular acidosis, and glaucoma [46]. The hCA II isozyme was also efficiently inhibited by the pyrazole-3-carboxylic acids (**1a** and **b**) and pyrazole[3,4-*d*]pyridazine (**2a–n**) derivatives evaluated here. These molecules appeared to be able to inhibit hCA II, with $K_i$ values ranging from 18.04 ± 4.55 nM to 66.24 ± 19.21 nM. The results obtained in this study indicates better values compared to clinically used drug acetazolamide ($K_i$ of 194.12 ± 68.83 nM). All the investigated pyrazole-3-carboxylic acids (**1a** and **b**) and pyrazole[3,4-*d*]pyridazine (**2a–n**) derivatives demonstrated marked inhibition against hCA II. However, the compounds of (**2g**) and (**2m**) exhibited very good inhibition properties against cytosolic hCA II with $K_i$ values of 18.04 ± 4.55 and 19.24 ± 5.23 nM, respectively (Table 1).

The inhibitory effects of the synthesized pyrazole-3-carboxylic acids (**1a** and **b**) and pyrazole[3,4-*d*]pyridazine (**2a–n**) derivatives on AChE are shown in Table 2. The AChE inhibition profiles of the compounds evaluated here were quite interesting. Overall, the pyrazole-3-carboxylic acids (**1a** and **b**) and pyrazole[3,4-*d*]pyridazine (**2a–n**) derivatives had excellent inhibitory activity with $K_i$ values ranging from 394.77 ± 68.13 nM to 952.93 ± 182.72 nM. Moreover, Tacrine, as a standard AChE inhibitor, was used. The $K_i$ values of 1005.02 ± 198.66 nM toward AChE were obtained. According to experimental data, the inhibition of AChE of pyrazole-3-carboxylic acids (**1a** and **b**) and pyrazole[3,4-*d*]pyridazine (**2a–n**) derivatives is much better than standard drug. The compounds of (**2i**) and (**2l**) showed excellent inhibitions profile against AChE with $K_i$ values of 394.77 ± 68.13 and 463.86 ± 93.07 nM, respectively (Table 1).

$HCO_3^-$ and $CO_2$ conducts the pH buffering among intracellular and extracellular spaces. The adjustment of both parts is under the control of CAs, which catalyzes the reversible conversion of $H_2O$ and $CO_2$ into $HCO_3^-$ and $H^+$ [47]. Hence, with this vital evidence, brain CA records as a significant aim for management of epileptic seizure syndromes. Diverse isoenzymes of CAs are available in the mammalian brain; hCA II isozyme is extensively expressed in myelin sheaths, and oligodendrocytes as well as choroid plexus, which is engaged in several neurological disturbances like epileptogenesis [48]. In this paper, evaluation of the effects of pyrazole-3-carboxylic acids (**1a**,**b**) and pyrazole[3,4-*d*] pyridazine (**2a–n**) derivatives on AChE, and hCA I and II isoenzymes was an important purpose, and inhibition results are present in Table 1. Compounds for the isoforms of HCA I and II are (**2g**), (**2f**) and (**2j**). However, when these compounds are compared, it is seen that the best inhibitor is (**2g**) and (**2f**). (**2j**) shows a weaker inhibitory property compared to others. This was also the case for AChE and gave the best inhibitory property (**2i**) and (**2l**). Similarly, the compound showing a weaker inhibitory property is (**2a**). In addition, for hCA II, (**2g**) and (**2m**) is the best inhibitor (Fig. 4).

### 3.3. Computational studies

Computational tools have become essential to understand drug-receptor interaction and inhibition mechanism. Therefore, interactions and a binding score of the best-posed compounds were analyzed with a computational method. The results obtained on the AChE, hCA I and II receptors were examined using pyrazole-3-carboxylic acid and pyrazole [3,4-*d*]pyridazine derivatives to compare with TAC and AZA reference inhibitors. The reference inhibitors, pyrazole-3-carboxylic acid and pyrazole[3,4-*d*]pyridazine derivatives were similarly docked into the catalytic active pocket of receptors. The exposure viewer module tested the interactions between the receptors and the best-exposed ligands. The studies on molecular docking revealed that all the pyrazole[3,4-*d*] pyridazine and pyrazole-3-carboxylic acid derivatives exhibited higher binding affinity towards hCA I, hCA II, and AChE, and also most of the compounds exactly fit into the active catalytic pocket. The glide docking results are presented in Table 2. The glide docking scores were well suited on a large scale with results of *in vitro* studies, but the theoretical values were not compared with the experimental affinity values for each compound. Herewith, the elucidating for binding mechanisms and interactions between receptors and ligands were the main objectives of this section of the present study. In this study, four parameters including Glide score, docking score, non-bonded interactions, and H-bonds were considered for docking analysis. The reliability of the docking process presented in Fig. 1. The figure shown that docking process carried out at high accuracy due to re-docked and co-crystallized ligands are well located in the active catalytic pocket.

Computational studies have shown that the compounds could easily fit into the active catalytic pocket of hCA I. However, it has been shown only the most active compound **2g** as seen in Fig. 2a. The compound

**Table 1**
Human carbonic anhydrase isoenzymes (CA I, and II), and AChE enzymes inhibition study values of pyrazole-3-carboxylic acids and pyrazole[3,4-*d*] pyridazine derivatives (**1a**,**b,** and **2a–n**).

| Compounds | $IC_{50}$ (nM) | | | | | | $K_i$ (nM) | | |
|---|---|---|---|---|---|---|---|---|---|
| | hCA I | $r^2$ | hCA II | $r^2$ | AChE | $r^2$ | hCA I | hCA II | AChE |
| 1a | 24.84 | 0.9724 | 34.12 | 0.9816 | 894.12 | 0.9602 | 28.42 ± 5.08 | 40.16 ± 11.27 | 674.03 ± 105.73 |
| 1b | 36.03 | 0.9919 | 47.03 | 0.9704 | 1022.16 | 0.9811 | 42.66 ± 11.45 | 52.63 ± 15.62 | 904.72 ± 205.84 |
| 2a | 20.88 | 0.9504 | 29.15 | 0.9815 | 1105.94 | 0.9915 | 24.63 ± 9.23 | 38.53 ± 8.43 | 952.93 ± 182.72 |
| 2b | 41.02 | 0.9815 | 49.14 | 0.9618 | 791.05 | 0.9699 | 40.28 ± 7.33 | 54.84 ± 15.82 | 643.84 ± 97.36 |
| 2c | 45.92 | 0.9581 | 53.82 | 0.9861 | 704.17 | 0.9851 | 51.37 ± 13.70 | 66.24 ± 19.21 | 583.61 ± 137.41 |
| 2d | 33.14 | 0.9632 | 36.04 | 0.9982 | 916.06 | 0.9506 | 37.60 ± 11.36 | 44.36 ± 9.25 | 730.73 ± 193.61 |
| 2e | 38.15 | 0.9811 | 44.26 | 0.9655 | 1001.51 | 0.9821 | 43.95 ± 13.55 | 42.18 ± 14.62 | 817.32 ± 103.83 |
| 2f | 8.84 | 0.9409 | 15.34 | 0.9738 | 816.94 | 0.9593 | 10.32 ± 2.64 | 23.11 ± 7.35 | 721.54 ± 137.83 |
| 2g | 9.41 | 0.9714 | 12.94 | 0.9903 | 834.25 | 0.9924 | 9.03 ± 3.81 | 18.04 ± 4.55 | 703.47 ± 176.07 |
| 2h | 24.66 | 0.9395 | 29.80 | 0.9525 | 702.93 | 0.9592 | 29.64 ± 10.44 | 34.58 ± 10.24 | 563.85 ± 96.90 |
| 2i | 30.18 | 0.9916 | 36.26 | 0.9705 | 506.62 | 0.9905 | 43.71 ± 9.56 | 50.23 ± 14.06 | 394.77 ± 68.13 |
| 2j | 51.73 | 0.9791 | 59.93 | 0.9625 | 806.44 | 0.9958 | 55.42 ± 14.77 | 53.34 ± 12.66 | 638.05 ± 136.07 |
| 2k | 29.15 | 0.9814 | 35.83 | 0.9816 | 1082.48 | 0.9641 | 30.25 ± 6.82 | 39.14 ± 6.56 | 876.12 ± 205.87 |
| 2l | 35.81 | 0.9731 | 42.51 | 0.9506 | 551.05 | 0.9815 | 41.39 ± 9.26 | 40.72 ± 15.84 | 463.86 ± 93.07 |
| 2m | 12.60 | 0.9548 | 20.94 | 0.9773 | 795.33 | 0.9690 | 14.66 ± 3.52 | 19.24 ± 5.23 | 683.08 ± 157.41 |
| 2n | 14.03 | 0.9942 | 18.04 | 0.9930 | 626.01 | 0.9882 | 13.20 ± 2.95 | 22.51 ± 6.08 | 547.06 ± 150.38 |
| AZA* | 171.13 | 0.9425 | 181.55 | 0.9709 | – | – | 178.11 ± 40.03 | 194.12 ± 68.83 | – |
| TAC** | – | – | – | – | 1116.74 | 0.9830 | – | – | 1005.02 ± 198.66 |

* 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.

**Table 2**
Glide scores (kcal/mol) of pyrazole-3-carboxylic acids (**1a**,**b**) and pyrazole[3,4-*d*]pyridazine (**2a–n**) derivatives into the active catalytic pocket of hCA I, and hCA II, and AChE.

| Compounds | hCA I | | hCA II | | AChE | |
|---|---|---|---|---|---|---|
| | Glide XP | | Glide XP | | Glide XP | |
| | Docking score | Glide score | Docking score | Glide score | Docking score | Glide score |
| 1a | −3.520 | −3.520 | −4.033 | −4.033 | −7.078 | −7.078 |
| 1b | −3.701 | −3.701 | −7.293 | −7.293 | −5.776 | −5.776 |
| 2a | −4.507 | −4.507 | −5.098 | −5.098 | −8.481 | −8.481 |
| 2b | −4.160 | −4.422 | −5.181 | −5.443 | −6.270 | −6.881 |
| 2c | −4.215 | −4.215 | −5.658 | −5.658 | −8.218 | −8.218 |
| 2d | −4.378 | −4.378 | −4.527 | −4.527 | −8.730 | −8.730 |
| 2e | −4.678 | −4.678 | −4.474 | −4.474 | −7.658 | −7.658 |
| 2f | −4.942 | −4.942 | −4.215 | −4.215 | −8.378 | −8.378 |
| 2g | −4.985 | −4.985 | −4.252 | −4.252 | −9.009 | −9.009 |
| 2h | −3.978 | −3.978 | −3.909 | −3.909 | −8.786 | −8.786 |
| 2i | −4.555 | −4.731 | −4.344 | −4.520 | −7.797 | −7.976 |
| 2j | −4.525 | −4.525 | −4.571 | −4.571 | −8.083 | −8.083 |
| 2k | −4.704 | −4.704 | −4.155 | −4.155 | −9.083 | −9.083 |
| 2l | −4.641 | −4.641 | −4.497 | −4.497 | −6.007 | −6.007 |
| 2m | −4.205 | −4.205 | −4.499 | −4.499 | −8.638 | −8.638 |
| 2n | −4.329 | −4.329 | −4.093 | −4.093 | −8.547 | −8.547 |
| AZA* | −7.497 | 8.331 | −7.337 | −8.178 | – | – |
| TAC** | – | – | – | – | −8.977 | −8.978 |

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

and reference inhibitor, **AZA**, occupy same binding pocket (data not shown).

It has been found that compound **2g** has the best scores with −4.985 kcal/mol towards hCA I. The best-scored compound is also most active inhibitor for hCA I as for the results of *in vitro* studies. The compound formed a hydrogen bond with Hie64 residue into the active catalytic pocket of hCA I and was also surrounded by hydrophobic amino acid residues including Phe91, Leu198, Pro202, and Tyr204 (Fig. 3a). To confirm of similarity between **2g** and reference inhibitor, **AZA** was docked into the active catalytic pocket of hCA I. Glide score of the best-scored pose of **AZA** was detected as −7.497 kcal/mol towards hCA I. In contrary to *in vitro* experiment, the score showed that **AZA** was theoretically more effective inhibitor than **2g**. **AZA** formed a hydrogen bond with Act304 and formed a salt bridge with Zn301. Additionally, the reference inhibitor formed π-π interaction with His200. It was detected that best-posed **2g** and **AZA** were surrounded by very similar hydrophobic amino acid residues (Fig. 3d). **2g** showed pretty similar interactions with former reported inhibitors [27,49,50].

**1b** with −7.293 kcal/mol score was determined as the best-scored compound towards hCA II with the computational study. However, *in vitro* studies revealed that **2g** was the most active inhibitor of hCA II. For that reason, the interaction and binding mechanism of **2g** with −4.252 kcal/mol score with active pocket residues of hCA II were analyzed in detail. **2g** well located into the active catalytic pocket of the receptor (Fig. 2b). **2 g** formed a hydrogen bond with Gln92 residue, and exhibited hydrophobic interactions Leu60, Ile91, Pro202, Leu204, Val207, and Trp209 with amino acid residues (Fig. 3b). **AZA** acts as a standard inhibitor for HCAs. Thr199 and Asn67 are involved in the

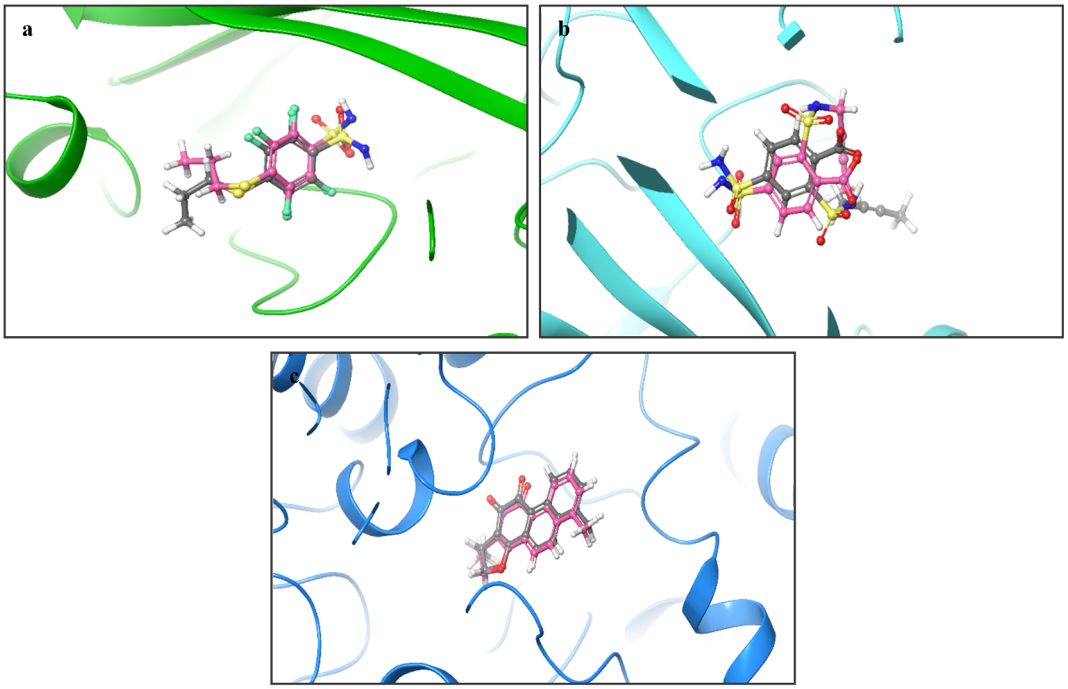



**Fig. 1.** Validation of docking. Receptors are shown in the ribbon model; blue: ACh Ereen: hCA I and cyan: hCA II. Disposed along poses of the crystalline ligand is represented by the gray ball and the ball is placed ligands magenta and bars with modeling is shown.

formation of hydrogen bonds with amino acid residues. In addition, it forms a salt bridge with Zn265 for the hCA II isoenzyme. (Fig. 3e). Best-posed **2g** and **AZA** were surrounded with polar binding pocket. It has been noticed that Gln92 amino acid is a key residue for hCA II isoenzyme. The results agreed with formerly reported inhibitors [49,51,52].

According to docking results, almost all of pyrazole[3,4-*d*]pyridazine derivatives exhibited high binding affinity ranges between −5.776 and −9.083 kcal/mol into AChE receptor as **TAC** standard inhibitor. While **2k** has the best binding affinity towards AChE, **2i** is the most active inhibitor for AChE *in vitro* studies and exhibited well binding affinity with −7.976 kcal/mol towards the receptor (Table 2). Thus, it was analyzed interaction and binding mechanism between compound **2i** and catalytic active pocket residues of AChE. **2i** well positioned into the active catalytic pocket of the receptor (Fig. 2c). **2i** formed a hydrogen bond with Phe295 and Arg296 residue and with a

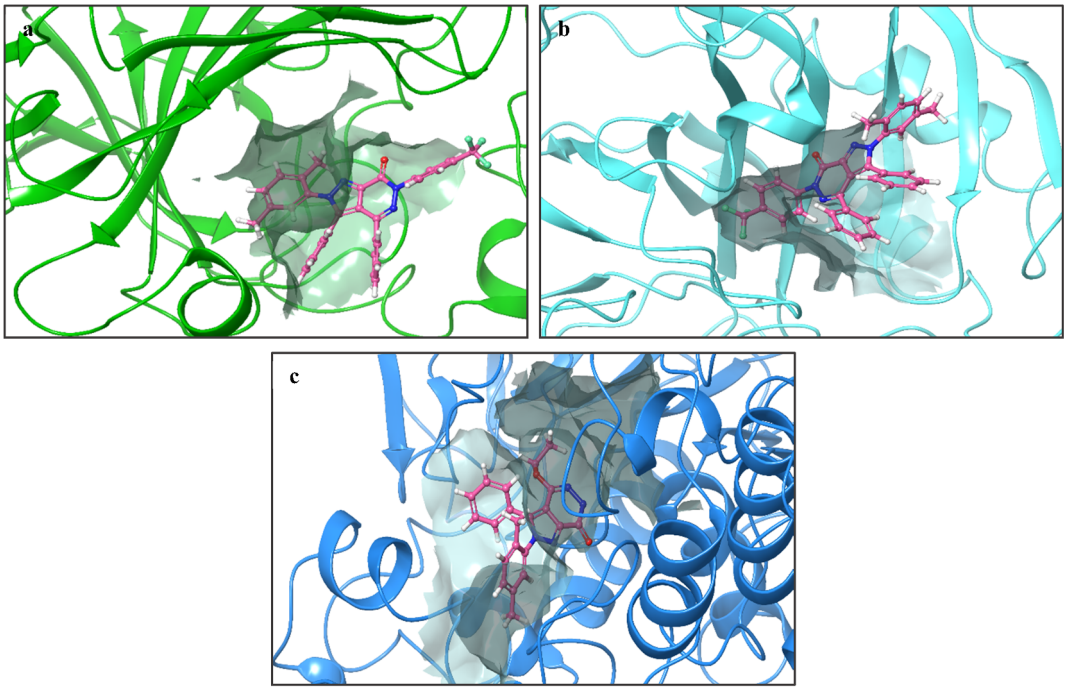



**Fig. 2.** The lowest energy conformation of ligands into AChE, hCA I and hCA II receptors, respectively. (a) Best-pose of **2g** into the catalytic active pocket of hCA I, (b) Best-pose of **2g** into the catalytic active pocket of hCA II, (c) Best-pose of **2i** into the catalytic active pocket of AChE. Receptors were shown in the ribbon model; green: hCA I, cyan: hCA II, blue: AChE. Ligands represent the magenta color with carbon balls and rods. Catalytic active pockets are represented as solid surfaces.

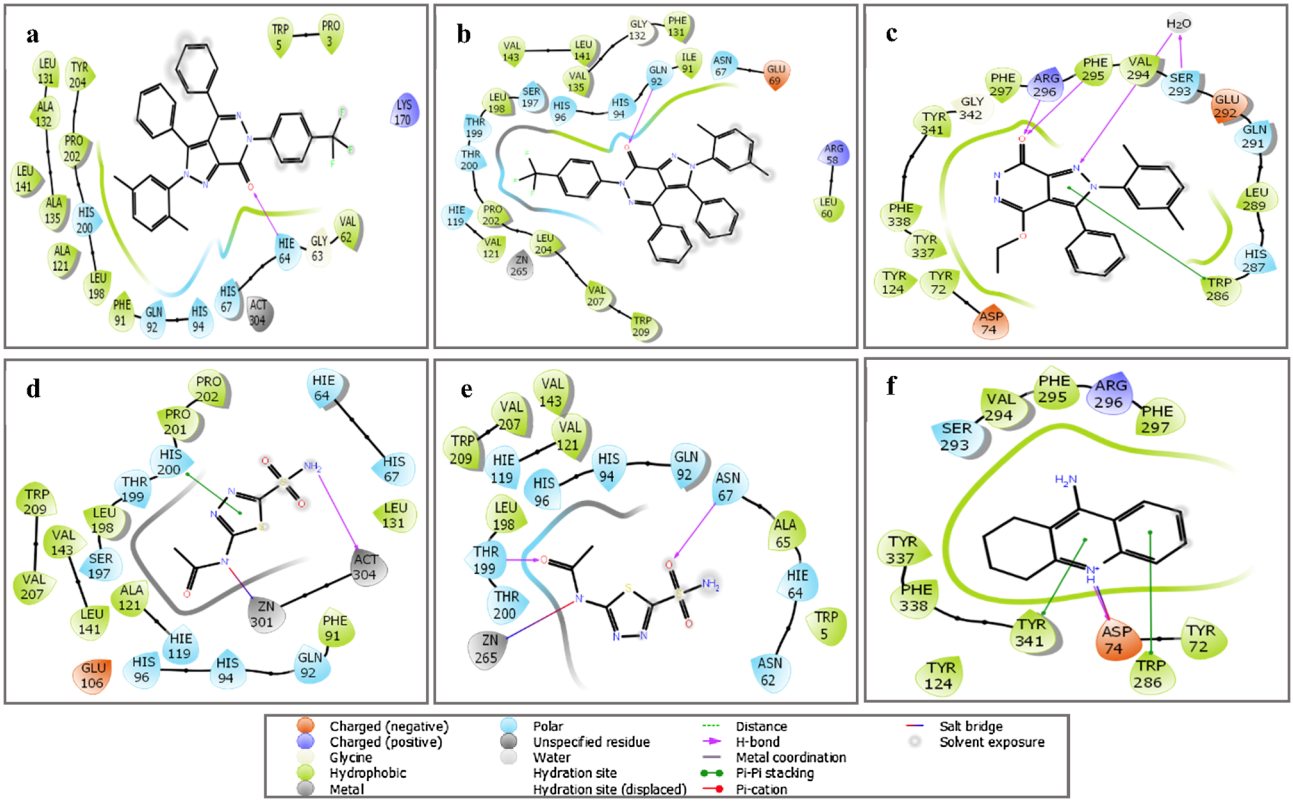



**Fig. 3.** 2D interaction profile between receptor and ligand; (a) **2g**-hCA I, (b) **2g**-hCA II, (c) **2i**-AChE, (d) **AZA**-hCA I, (e) **AZA**-hCA II, (f) **TAC**-AChE.

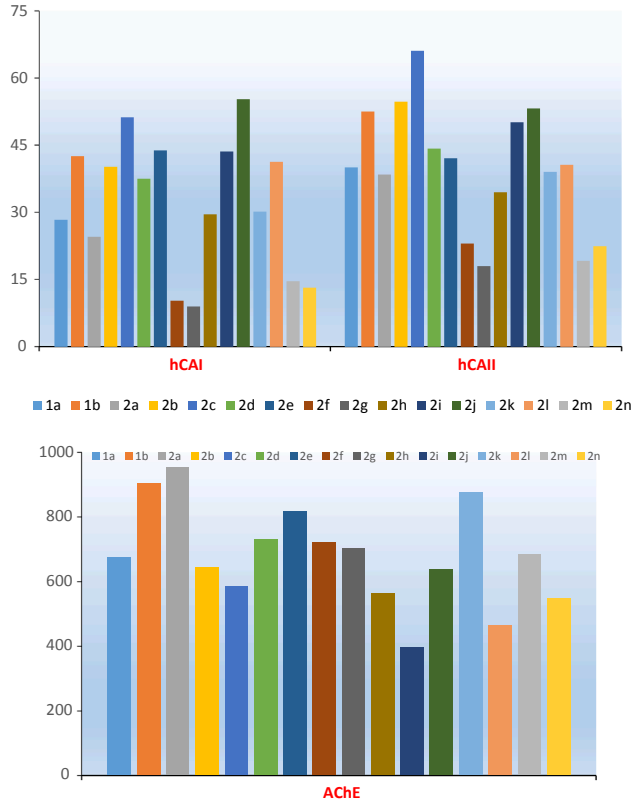



**Fig. 4.** $IC_{50}$ and Ki values of pyrazole-3-carboxylic acids (**1a** and **b**) and pyrazole[3,4-*d*]pyridazine (**2a–n**) against human carbonic anhydrase I and II isoenzymes and acetylcholinesterase (AChE) enzyme.

HOH715 water molecule, as well. Furthermore, a π–π stacking interaction between the **2i** compound and Trp286 amino acid residue were observed. Compound **2i** was surrounded by several residues of hydrophobic amino acid including residues of Tyr72, Val294, Leu289, Phe195, Tyr337, Phe338, Trp286, Phe297, and Tyr341 (Fig. 3c). The binding pocket of **TAC** and **2i** is very similar to one another due to the hydrophobic amino acid residues. (Phe195, Val294, Try72, Tyr337, Phe297, Phe338, and Tyr341). Compound **2i** shown pretty similar interaction as former reported inhibitors [53–55].

## 4. Conclusion

Finally, a series of pyrazole-3-carboxylic acids (**1a** and **b**) and pyrazole[3,4-*d*]pyridazine (**2a–n**) derivatives were synthesized. These compounds were analyzed for AChE inhibition properties and hCAs I and II isoforms. Pharmacophores have gained significant importance in many different synthetic drug designs due to their various biological activities and the scaffolds of various bioactive natural compounds. Among the scaffolds of these compounds, thiazole and coumarin have recently attracted attention as the leading pharmacophore since they have a wide variety of pharmacological activities in the field of medicinal chemistry. Some of the biological properties have anticholinesterase activity (AChE and BChE), carbonic anhydrase I, II and IX, and potential antioxidant inhibition and aflatoxigenic activities. These molecules exhibited inhibition behavior at nanomolar concentration level against those enzymes. The discovery of novel inhibitors of AChE, one of the cholinesterase enzymes, is particularly important for Alzheimer's disease. To see this inhibition in the nanomolar scale from pyrazole derivatives made the study important. According to docking results, hCA I, hCA II, and AChE enzymes inhibited by all compounds through interactions together with H-bonds, electrostatic and hydrophobic interactions.

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## Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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