Received: 1 February 2018 | Revised: 16 May 2018 | Accepted: 25 June 2018

DOI: 10.1002/jbt.22194

WILEY

1H-indazole molecules reduced the activity of human erythrocytes carbonic anhydrase I and II isoenzymes

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Abstract

Carbonic anhydrase (CA) is an important metabolic enzyme family closely related to many physiological and pathological processes. Currently, carbonic anhydrase inhibitors are the target molecules in the treatment and diagnosis of many diseases. In present study, we investigated the inhibitory effects of some indazole molecules on the CA-I and CA-II isoenzymes isolated from human erythrocytes. We showed that human CA-I and CA-II activities were reduced by of some indazoles at low concentrations. IC_{50} values, K_i constants, and inhibition types for each indazole molecule were determined. The indazoles showed K_i constants in a range of 0.383 ± 0.021 to 2.317 ± 0.644 mM, 0.409 ± 0.083 to 3.030 ± 0.711 mM against CA-I and CA-II, respectively. Each indazole molecule exhibited a noncompetitive inhibition effect. Bromine- and chlorine-bonded indazoles were found to be more potent inhibitory effects on carbonic anhydrase isoenzymes. In conclusion, we conclude that these results may be useful in the synthesis of carbonic anhydrase inhibitors.

KEYWORDS

carbonic anhydrase, human erythrocytes, indazole, inhibition

1 | INTRODUCTION

Carbonic anhydrase isoenzymes (CAs; EC 4.2.1.1) are metaloenzymes commonly found in all living organisms.[1] CAs are encoded by five distinct gene families, consisting of α-, β-, γ-, δ-, and ζ-CA. In recent studies, the presence of a new family of η-ca family in *Plasmodium falciparum*, a malaria pathogen, as well as these five families, has been shown and taken as a sixth-generation family in the literature.[2,3] The active sites of α-CA, β-CA, and δ-CA have Zn^{+2} ion, γ-CA probably has iron(II) ion (Fe^{+2}) but is also active when bound to Zn^{+2} or cobalt(II) ions (Co^{+2}). ζ-CA class use cadmium(II) ion (Cd^{+2}) or Zn^{+2} to catalyze physiological reactions. There are no significant amino acid sequence similarities between these families.[4,5] Up to now, 16 CA isoenzymes have been characterized. From these, CA I, II, III, VII, and XIII are cytosolic isoenzymes; CA IV, IX, XII, XIV, and XV are cell membrane–bound isoenzymes; CA VA and VB are mitochondrial isoenzymes; CA VI is the only secreted isoenzyme. There are also three noncatalytic cytosolic forms called CA-related proteins (CARP): CARP VIII, CARP X, and CARP XI. These isoenzymes differ in their catalytic activity, sensitivity to subcellular localization, inhibitors, and activators.[6]

The CAs catalyze the conversion between carbon dioxide (CO_2) and bicarbonate (HCO_3^-), which is a simple reaction in all organisms. Thanks to this reaction, CAs play a role in many physiological and pathological processes related to pH control, ion transport, and fluid secretion.[1] CA enzymes have been found to be associated with many physiological processes such as cell differentiation and proliferation,[7] pH homeostasis,[8] neurotransmission, epilepsy,[9] Alzheimer,[10] glaucoma,[11] obesity,[12] and cancer[13] in recent years.

CA-I and CA-II are two main isoforms found in mammalian red blood cells.[14] These isoenzymes are mainly involved in the regulation of respiration and acid–base balance. CA-I is involved in retinal and cerebral edema, whereas CA-II is involved in glaucoma and epilepsy.[15] In addition, CA-I and CA-II isoenzymes have been identified in relation to cancer.[16,17] In recent years, CA isoenzymes have been used as targets in drug design because of the association of these isoforms with many diseases. CA inhibitors are often lacking in selectivity and have numerous undesirable side effects. One way to prevent this is to design new inhibitors that inhibit specific CA isoenzymes. Therefore, identification of new CA inhibitors is very important.

In this study, we aimed to investigate the inhibitory effect of some indazoles (1H-indazole, 4-bromo-1H-indazole, 6-bromo-1H-indazole, 7-bromo-1H-indazole, 4-chloro-1H-indazole, 6-chloro-1H-indazole, 7-chloro-1H-indazole, 4-fluoro-1H-indazole, 6-fluoro-1H-indazole, 7-fluoro-1H-indazole) (Figure 1) on CA-I and CA-II isoenzymes isolated from human erythrocytes. Indazole derivatives are important heterocyclic organic molecule with a very broad range of

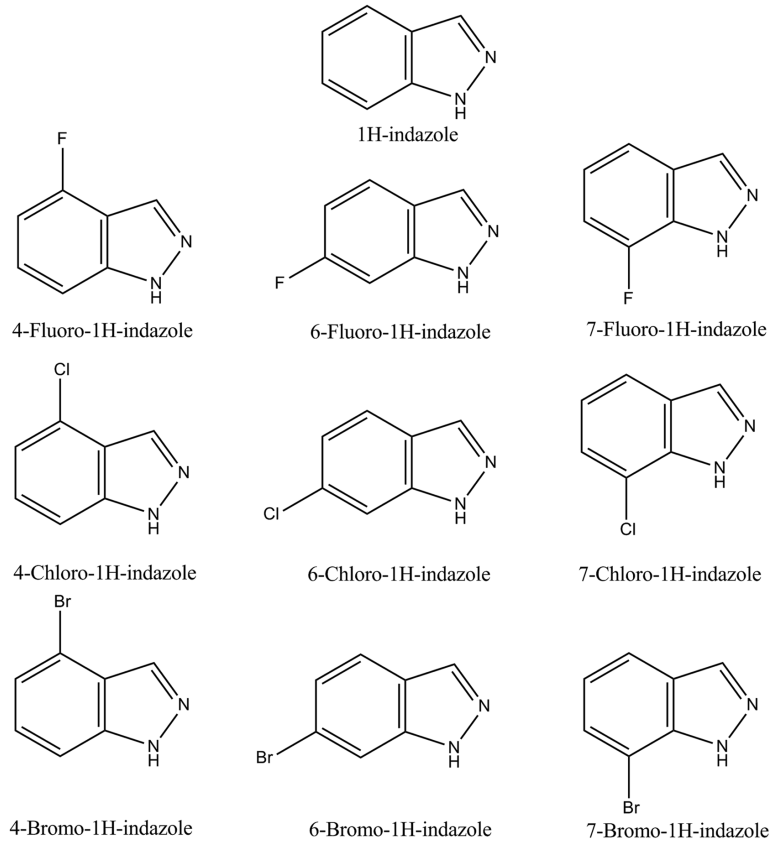

FIGURE 1 The molecular structures of 1H-indazoles used in this study

biological activities. For this purpose, first CA I and II isoenzymes were purified from human erythrocytes using Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography method, and enzyme purity was controlled by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Then whether the inhibitory effects of indazole derivatives on the activity of these isoenzymes were investigated. IC_{50} values and K_i constants were calculated for each molecule which had inhibition effect and also the types of inhibition were determined that help elucidating the mechanism of inhibition.

2 | MATERIAL AND METHODS

2.1 | Chemicals and instruments

Sepharose-4B, indozole molecules, and chemical substances used for electrophoresis were obtained from Sigma-Aldrich (Taufkirchen, Germany). L-tyrosine was obtained E. Merck (Darmstadt Germany). Other chemical substances used were obtained either from Sigma-Aldrich or E. Merck.

2.1.1 | Purification of carbonic anhydrase isoenzymes (hCA-I and hCA-II from human erythrocytes

Erythrocytes were purified from fresh human blood following low-speed centrifugation (1500 rpm for 15 min) and red blood cells were isolated, washed twice with 0.9% NaCl, and hemolyzed with 1.5 volumes of ice-cold water. Ghost and intact cells were removed by high-speed centrifugation (20,000 rpm for 30 min) at 4°C, and the pH of the hemolysate was adjusted to 8.7 with solid Tris. A sepharose-4B-L-tyrosine affinity chromatography column was prepared according to our previous studies.[18] The pH-adjusted human erythrocyte hemolysate (50 mL) was applied to the Sepharose 4B-L-tyrosine-sulfanilamide affinity column preequilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with

TABLE 1 Purification results of CA-I and CA-II from human erythrocyte

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification fold
Erythrocyte hemolysate	140	50	18.1	905	7000	7.735	100	1
hCA-I	482	10.5	0.75	7.875	5061	642.7	72.3	83.1
hCA-II	980	6	0.19	1.14	5880	5157.9	84	666.8

TABLE 2 The IC_{50} values, K_i constants, and inhibition types determined for indazole molecules having inhibitory effects on hCA I and II isoenzymes

1H-Indazole compound	hCA-I			hCA-II		
	IC_{50}(mM)	K_i(mM)	Inhibition type	IC_{50}(mM)	K_i(mM)	Inhibition type
1H-Indazole	1.902	2.317 ± 0.644	Noncompetitive	2.265	3.030 ± 0.711	Noncompetitive
7-Bromo-1H-indazole	1.296	1.099 ± 0.527	Noncompetitive	0.812	1.122 ± 0.359	Noncompetitive
6-Bromo-1H-indazole	0.619	0.684 ± 0.126	Noncompetitive	0.748	0.841 ± 0.062	Noncompetitive
4-Bromo-1H-indazole	0.403	0.383 ± 0.021	Noncompetitive	0.700	0.935 ± 0.098	Noncompetitive
7-Chloro -1H-indazole	0.469	0.533 ± 0.054	Noncompetitive	1.082	0.558 ± 0.215	Noncompetitive
6-Chloro-1H-indazole	0.560	0.817 ± 0.215	Noncompetitive	1.083	2.645 ± 0.796	Noncompetitive
4-chloro-1H-indazole	1.502	2.128 ± 0.148	Noncompetitive	0.683	0.409 ± 0.083	Noncompetitive

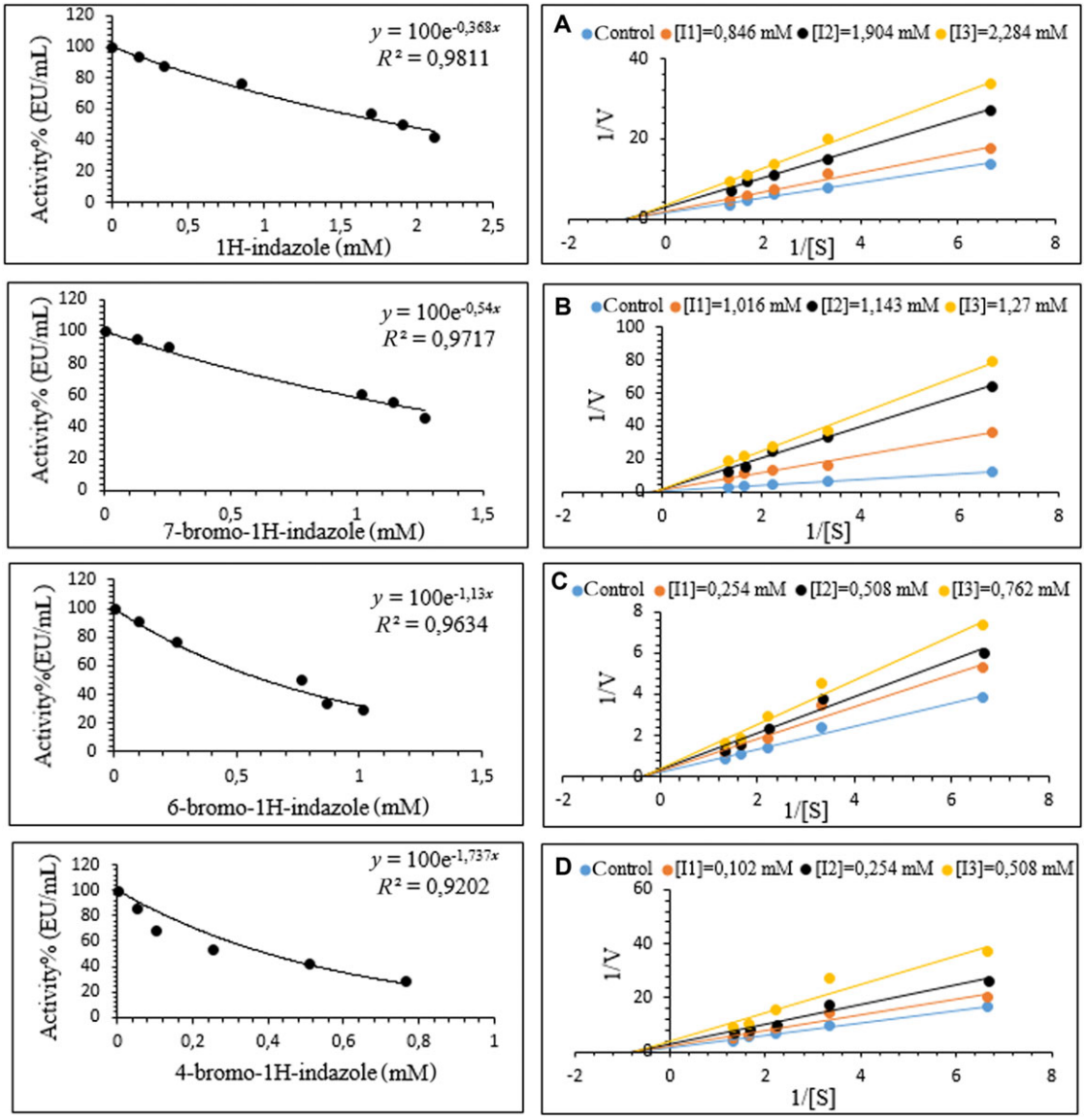

FIGURE 2 IC_{50} graphs and Lineweaver–Burk graphs of 1H-indazole (A), 7-bromo-1H-indazole (B), 6-bromo-1H-indazole (C), and 4-bromo-1H-indazole d (D) for hCA-I

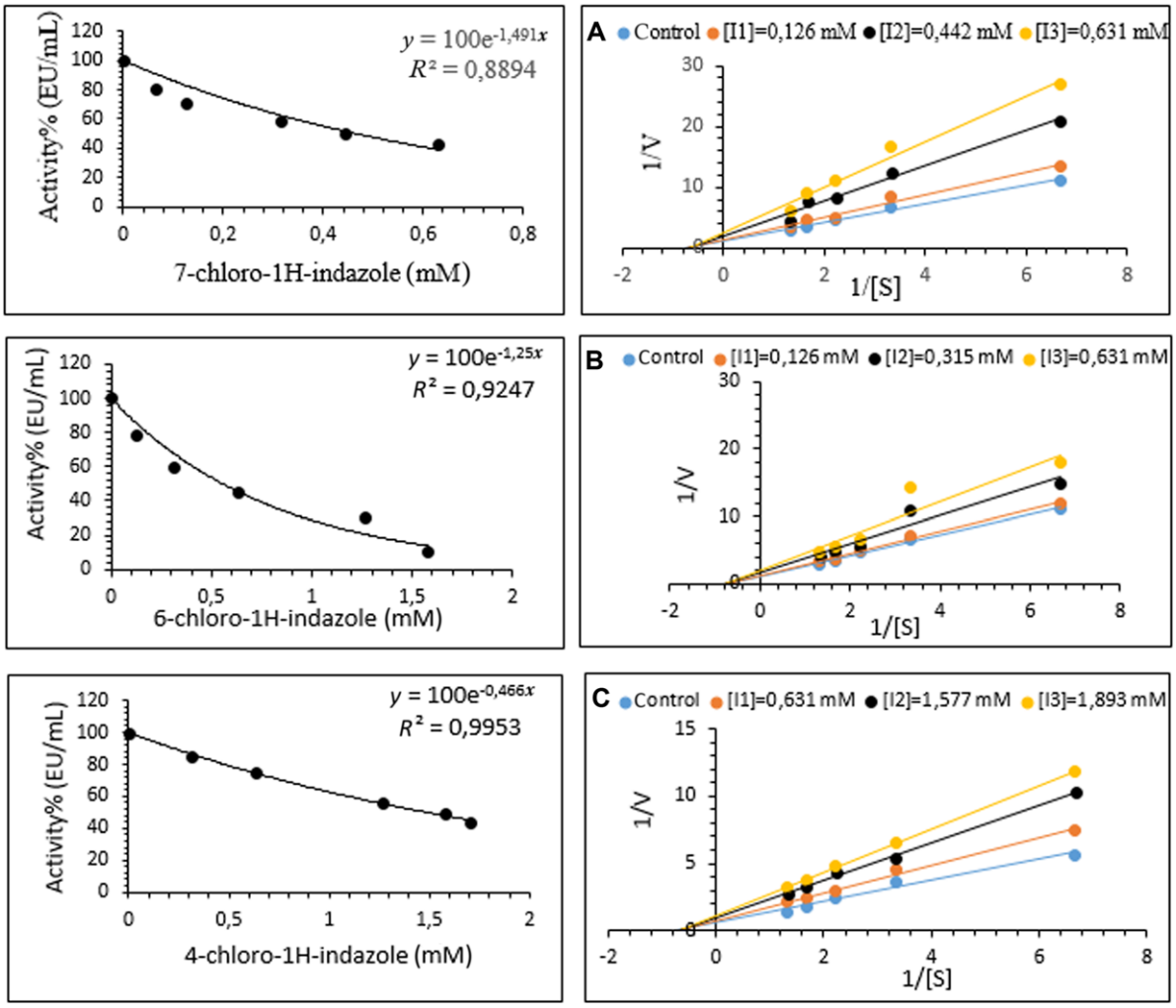

FIGURE 3 IC_{50} graphs and Lineweaver–Burk graphs of 7-chloro-1H-indazole (A), 6-chloro-1H-indazole (B), and 4-chloro -1H-indazole (C) for hCA-I

25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase isozymes (hCA-I and hCA-II) were eluted with 1.0 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6), respectively. During purification procedures of hCA-I and hCA-II, the absorbency at 280 nm was measured to monitor protein elution by affinity chromatography. CO_2-hydratase activity was determined in eluted fractions, and the active fractions were collected. CO_2-hydratase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson.[19] Protein determination was performed using the Bradford method[20] in all purification steps. SDS–PAGE was performed to control of enzyme purity.[21]

2.2 | In vitro inhibition studies

In inhibition studies, CA activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate to a 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer according to the method described by Verpoorte et al.[22] The inhibitory effects of some indazole molecules (1H-indazole, 4-bromo-1H-indazole, 6-bromo-1H-indazole, 7-bromo-1H-indazole, 4-chloro -1H-indazole, 6-chloro-1H-indazole, 7-chloro-1H-indazole, 4-fluoro-1H-indazole, 6- fluoro-1H-indazole, 7-fluoro-1H-indazole) on CA enzyme activity purified from human erythrocyte were tested in triplicate at each concentration used. Control activity in the absence of indazole molecules was assumed to be 100%. For each molecule, a percent activity versus inhibitor concentration graph was drawn. K_i constants of the indazole molecules calculated by measuring enzyme activity at three different indazole concentrations with five different substrate concentrations. Lineweaver–Burk curves were used for determination of K_i constants and inhibition types.[23]

3 | RESULTS

CA isozymes (CA-I and CA-II) were purified from human erythrocytes by Sepharose-4B-L-tyrosine affinity chromatography. The purification results are shown in Table 1. Additionally, purity of enzymes was controlled using SDS–PAGE. Purified CA-I and CA-II isozymes had single bands at 29 kDa. After the purification steps, the in vitro inhibition effects of 1H-indazole, 4-bromo-1H-indazole, 6-bromo-1H-indazole, 7-bromo-1H-indazole, 4-chloro-1H-indazole,

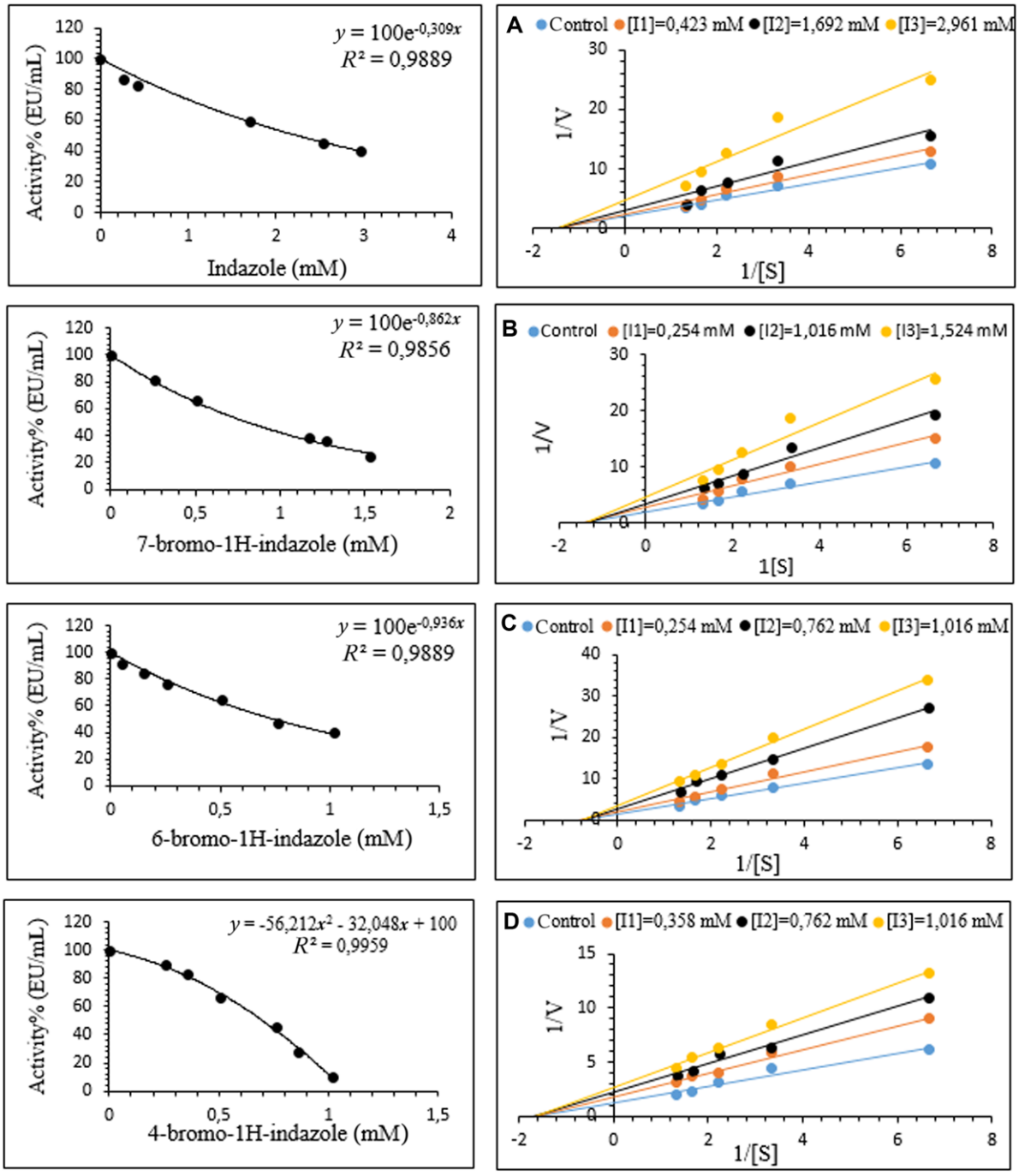

FIGURE 4 IC_{50} graphs and Lineweaver–Burk graphs of 1H-indazole (A), 7-bromo-1H-indazole (B), 6-bromo-1H-indazole (C), and 4-bromo-1H-indazole (D) for hCA-II

6-chloro-1H-indazole, 7-chloro-1H-indazole, 4-fluoro-1H-indazole, 6-fluoro-1H-indazole, and 7-fluoro-1H-indazole on the purified isoenzymes were investigated. Both the IC_{50} and K_i values of the indazole compounds were determined via Activity% – [Indazole], and Lineweaver–Burk graphs (1/V – 1/[S]), respectively. The IC_{50} values were found to be 1.902, 1.296, 0.619, 0.403, 0.469, 0.560, and 1.502 mM for hCA-I for 1H-indazole, 7-bromo-1H-indazole, 6-bromo-1H-indazole, 4-bromo-1H-indazole, 7-chloro-1H-indazole, 6-chloro-1H-indazole, and 4-chloro-1H-indazole, respectively, and 2.265, 0.812, 0.748, 0.700, 1.082, 1.083, and 0.683 mM for hCA-II for 1H-indazole, 7-bromo-1H-indazole, 6-bromo-1H-indazole, 4-bromo-1H-indazole, 7-chloro-1H-indazole, 6-chloro-1H-indazole, and 4-chloro-1H-indazole, respectively. (Table 2). The K_i constants were obtained from the Lineweaver–Burk graphs (1/V – 1/[S]) (Figures 2–5), and the inhibition type was found for each indazole. K_i constants were found to be 2.317 ± 0.644 mM, 1.099 ± 0.527 mM, 0.684 ± 0.126 mM, 0.383 ± 0.021 mM, 0.533 ± 0.054 mM, 0.817 ± 0.215 mM, and 2.128 ± 0.148 mM for hCA-I for 1H-indazole, 7-bromo-1H-indazole, 6-bromo-1H-indazole, 4-bromo-1H-indazole, 7-chloro-1H-indazole, 6-chloro-1H-indazole, and 4-chloro-1H-indazole, respectively. On the

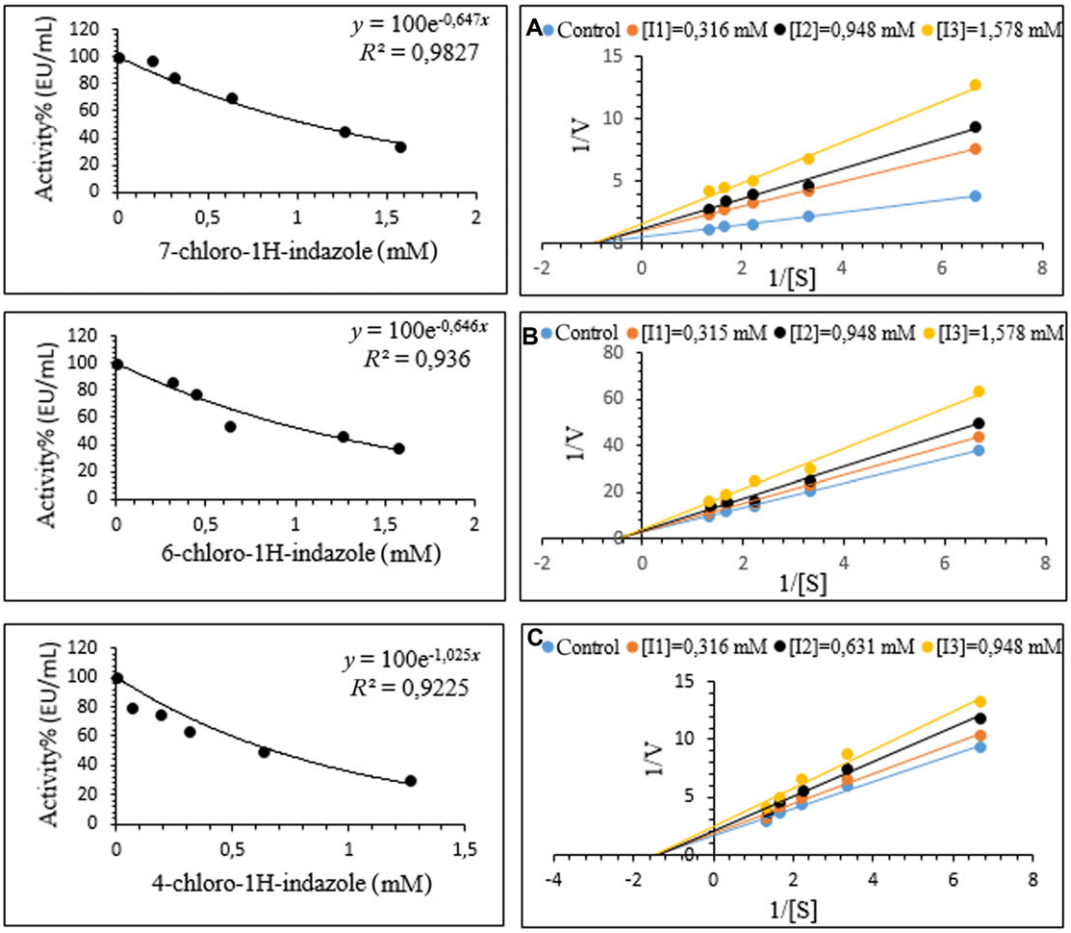

FIGURE 5 IC_{50} graphs and Lineweaver–Burk graphs of 7-chloro-1H-indazole (A), 6-chloro-1H-indazole (B), and 4-chloro -1H-indazole (C) for hCA-II

other hand, K_i constants were found to be 3.030 ± 0.711 mM, 1.122 ± 0.359 mM, 0.841 ± 0.062 mM, 0.935 ± 0.098 mM, 0.558 ± 0.215 mM, 2.645 ± 0.796 mM, and 0.409 ± 0.083 mM for hCA-II for 1H-indazole, 7-bromo-1H-indazole, 6-bromo-1H-indazole, 4-bromo-1H-indazole, 7-chloro-1H-indazole, 6-chloro-1H-indazole, and 4-chloro-1H-indazole, respectively (Table 2). All indazole molecules with inhibitory effect exhibited noncompetitive inhibition on both isoenzymes.

4 | DISCUSSION

The importance of CA inhibitors in the treatment and diagnosis of diseases has been revealed as a result of inhibition studies on the CA enzyme for glaucoma disease treatment. In these inhibition studies, besides clarification of the catalytic mechanisms of CA enzyme, the distribution of this enzyme to tissues and the vital functions in these tissues have been understood, and, as a result, the inhibitors and activators of the enzyme have been accelerated. To date, a wide variety of enzyme inhibitors have been synthesized and these inhibitors have been used primarily as medicines in the treatment of glaucoma. It is also currently used in clinics as an antitumor, analgesic, epilepsy, and neurological disorder medication, antiulcer, diuretic, antibiotic, positron emission tomography, and magnetic resonance imaging diagnostic material.[24] For this reason, it has become very important to know the mechanism of inhibition of CA enzyme and to synthesize new inhibitors.

In this study, we investigated whether the inhibition effect of some indazole derivatives on CA I and II isoenzymes isolated from human erythrocytes. Indazole is a heterocyclic compound formed by the coupling of benzene and pyrazole rings. The indazole derivatives have a wide range of biological activity.[25] The indazoles possess nitric oxide synthase inhibitor activity,[26] anti-inflammatory,[27] antitumor,[28] anti-HIV,[29] antimicrobial, antiparasitic,[30] and serotonin 5-HT3 receptor antagonist activities.[31] There are three tautomers, 1H-indazole, 2H-indazole, and 3H-indazole, according to position of the heteroatoms in the ring of the indazole molecule.[32] Among these, 1H-indazole exhibits a wide range of biological and pharmaceutical activities.[33]

In our study, the inhibitory effect of indazole derivatives was determined by two different values, IC_{50} and K_i. IC_{50} values were calculated by determining percent activity at various inhibitor concentrations, with substrate concentrations kept constant, and then determining the concentration of inhibitor causing 50% inhibition graphically.

We obtained IC_{50} values were found as 1.902, 1.296, 0.619, 0.403, 0.469, 0.560, and 1.502 mM for hCA-I for 1H-indazole, 7-bromo-1H-indazole, 6-bromo-1H-indazole, 4-bromo-1H-indazole,

7-chloro-1H-indazole, 6-chloro-1H-indazole, 4-chloro-1H-indazole, respectively, and 2.265, 0.812, 0.748, 0.700, 1.082, and 1.083, 0.683 mM for hCA-II for 1H-indazole, 7-bromo-1H-indazole, 6-bromo-1H-indazole, 4-bromo-1H-indazole, 7-chloro-1H-indazole, 6-chloro-1H-indazole, and 4-chloro-1H-indazole, respectively. On the other hand, 4-fluoro-1H-indazole, 6-fluoro-1H-indazole, and 7-fluoro-1H-indazole did not have any inhibition effect on hCA-I and hCA-II. According to these results, 4-bromo-1H-indazole had a strong inhibitory effect for hCA I, whereas 4-chloro-1H-indazole had a strong inhibitory effect for hCAII. Accordingly, it was observed in our study that bromine and chlorinated indazole molecules on CA I and II isoenzymes have more effective inhibition than indazole. To determine the K_i constant, Lineweaver–Burk graphs were drawn and K_i constants and inhibition types were determined from these graphs. The K_i constant indicates the binding affinity of the inhibitor to the enzyme. Accordingly, 4-bromo-1H-indazole (K_i: 0.383 ± 0.021 mM) had a high binding affinity for hCA-I and 4-chloro-1H-indazole (K_i: 0.409 ± 0.083 mM) had high binding affinity for hCA-II. It had been determined that all of the indazole molecules having the inhibitory effect exhibited competitive inhibition effect on the HCA-I and hCA-II enzymes. So these indazole molecules may show its inhibitory effect by a decreasing turnover rate or catalytic activity of the hCA-I and hCA-II isoenzymes.

In conclusion, we observed that the indazoles reduced the hCA-I and hCA-II activities at low concentrations. These results can help us to synthesize new CA inhibitors that are indazole derivatives.

ACKNOWLEDGMENTS

The author thank to Ahi Evran University Research Fund Accounting for their support to carry out this work (project number: FEF.A4.16.003).

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How to cite this article: Alim Z. 1H-indazole molecules reduced the activity of human erythrocytes carbonic anhydrase I and II isoenzymes. *J Biochem Mol Toxicol*. 2018;32:e22194. https://doi.org/10.1002/jbt.22194