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Investigation of the inhibitory effects of isoindoline-1,3-dion derivatives on hCA-I and hCA-II enzyme activities

Hayrunnisa Nadaroglu [a, b, *], Azize Alaylı Gungor [b, c], Özlem Gundogdu [d], Nurhan Horasan Kishali [e, **], Belgin Sever [f], Mehlika Dilek Altintop [f]

[a] Ataturk University, Erzurum Vocational School, Department of Food Technology, Erzurum, Turkey
[b] Department of Nano-Science and Nano-Engineering, Institute of Science and Technology, Ataturk University, 25240 Erzurum, Turkey
[c] Ataturk University, Erzurum Vocational Collage, Department of Chemical Technology, Erzurum, Turkey
[d] Ahi Evran University, Kaman Vocational School, Department of Food Technology, Kırsehir, Turkey
[e] Ataturk University, Faculty of Science, Department of Chemistry, 25240 Erzurum, Turkey
[f] Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Eskişehir, Turkey

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ABSTRACT

Inhibition of carbonic anhydrase (CA) has emerged as a promising approach for the treatment of a variety of diseases such as glaucoma, epilepsy, obesity and cancer. As a result, the design of CA inhibitors (CAIs) is an outstanding field of medicinal chemistry. Due to the therapeutic potential of isoindoline-1,3-diones as CAIs, herein hCA I and hCA II isozymes were purified from human erythrocytes using affinity chromatography and the inhibitory effects of a series of isoindoline-1,3-diones on hydratase activities of these isozymes were investigated. Among these compounds, compound **3a** was found to be the most effective compound on hCA I with an IC_{50} value of 7.02 μM, whereas compound **3c** was the most potent compound on hCA II with an IC_{50} value of 6.39 μM. Moreover, molecular docking studies were carried out for all compounds and acetazolamide (AAZ), the reference agent, in the active sites of hCA I and hCA II. Generally, the compounds showed high affinity through salt bridge formation and metal coordination with Zn^{2+} ion and π-stacking interaction with His94 residue. According to *in silico* Absorption, Distribution, Metabolism and Excretion (ADME) studies, the pharmacokinetic parameters of all compounds were within the acceptable range.

1. Introduction

Carbonic anhydrases (CAs; EC 4.2.1.1) are ubiquitous zinc-containing metalloenzymes and they play a pivotal role in all living metabolism. The CA enzyme is present in 16 isoforms in mammals [1]. These isoforms are found to be specialized in different tissues of the organism. Although CAs are involved in many different reactions, the removal of waste carbon dioxide in the metabolism is their main task (Fig. 1) [2]. Due to their role in the conversion of carbon dioxide to bicarbonate, CAs participate in key biosynthetic reactions such as lipogenesis, gluconeogenesis and ureagenesis. Some CA isoenzymes contain zinc in their active regions, while others catalyze the reaction in the active site without zinc [3].

The regulation and control of enzyme activity is important for all living things. There are many regulations that increase the activity of enzymes, as well as decreasing regulations. In addition, enzyme activity is regulated by covalent modifications, phosphorylation and allosteric effects. One of the most important approaches for the reduction of this type of enzyme activity is the inhibition of CAs [4,5].

Over the past 60 years, heterocyclic and aromatic sulfonamides have been used as CA inhibitors in the treatment of many diseases such as glaucoma, obesity, epilepsy, cancer, altitude sickness. Moreover, in the last years CA inhibitors have emerged as anti-infective agents [6].

In addition to the sulfonamide derivatives such as AAZ, methazolamide, ethoxzolamide, aromatic/heteroaromatic sulfonamide-based Schiff bases have also been identified as CA inhibitors [6,7].

Isoindoline-1,3-dione is a prominent compound in organic

* Corresponding author. Ataturk University, Erzurum Vocational School, Department of Food Technology, 25240 Erzurum, Turkey.
** Corresponding author.
E-mail addresses: hnisa25@atauni.edu.tr (H. Nadaroglu), nhorasan@atauni.edu.tr (N.H. Kishali).

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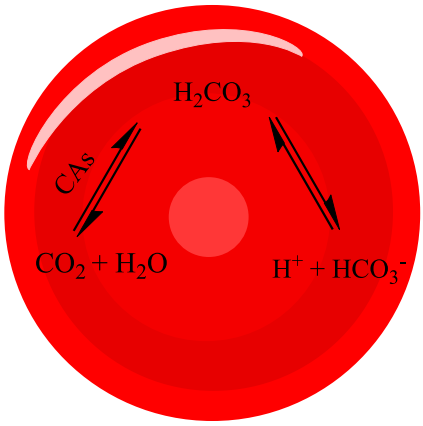


Fig. 1. Removal of waste carbon dioxide in the metabolism.

synthesis for the preparation of diverse biologically active molecules [8–12]. Isoindoline-1,3-dione derivatives exhibit various biological activities, like anticancer [13], antimicrobial [14], antioxidant [15], anti-inflammatory [16] and analgesic [17] activities. The hydrophobic character of isoindoline-1,3-diones increases their potential to cross different biological membranes *in vivo* [18].

In this paper, we synthesized a series of *N*-substituted isoindoline-1,3-diones and investigated their inhibitory effects on hCA I and hCA II isoenzymes. All compounds were docked to the active sites of hCA I and hCA II with AAZ to explore their possible binding modes in the active sites of these enzymes. A computational study for the prediction of ADME properties of all compounds was also performed.

2. Experimental

2.1. Chemistry

All chemicals were commercially available and were used without purification or after distillation and treatment with drying agents. Melting points (M.p.) were determined on a capillary melting apparatus (Buechi 530) and are uncorrected. IR spectra were obtained from solutions in 0.1 mm cells with a PerkinElmer spectrophotometer. The ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on Varian and Bruker spectrometers; δ in ppm. Elemental analyses were performed on a Leco CHNS-932 apparatus. All column chromatography was performed on silica gel (60-mesh, Merck). Preparative layer chromatography (PLC) is preparative thin layer chromatography: 1mmof silica gel 60 PF (Merck) on glass plates.

2.1.1. General procedure for the synthesis of compounds ***2a-c*** and ***3a-c***

A mixture of compound **1** (1 equiv.), Et_3N (3 equiv.), R–NH_2 (1.2 equiv.) and toluene (10 mL) was heated at reflux temperature for 24 h. After that, the solvent was evaporated under reduced pressure and the remaining solid was filtered from a small column. The product **2** was crystallized from EtOAc/Petroleum ether (Yield: 90–95%).

To a magnetically stirred solution of **2** (1 equiv.) in methanol (10 mL) was cooled to 0 °C. Then $SOCl_2$ (1.2 eq.) was added. The mixture was stirred refluxed for 18 h. After that, the reaction mixture was cooled to room temperature and the solvent removed in evaporator, and The solid was extracted by addition of EtOAc (2 × 20 mL) and NH_4Cl (20 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed in an evaporator. The crude product was purification with column chromatography. The product **3** was crystallized from EtOAc/petroleum ether (Yield: 90–95%)

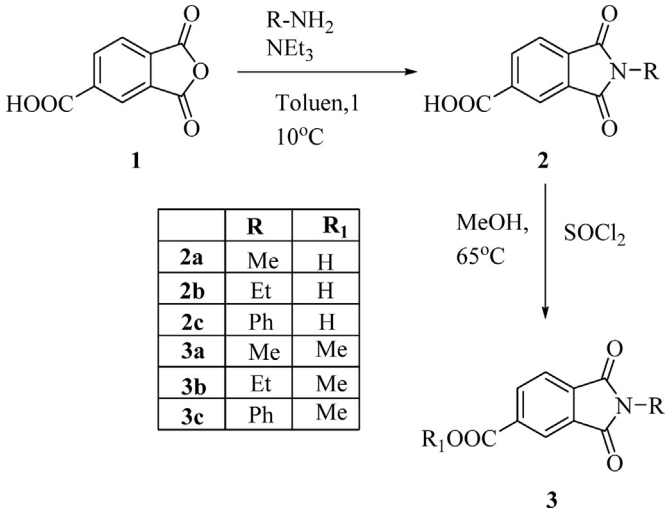
*2.1.1.1. 2,3-Dihydro-2-methyl-1,3-dioxo-1H-isoindole-5-carboxylic acid (**2a**).* M.p: 232–233 °C (lit: 240 °C) [24], IR (KBr): 3775, 3444, 3185, 1773, 1717, 1695, 1607, 1570, 1478, 1449, 1378, 1253, 1214, 1164. ^{1}H NMR (400 MHz, $CDCl_3$): 8.57 (s, 1H), 8.48 (dd, J = 7.7, 1.5 Hz, 1H), 7.97 (d, J = 7.7 Hz, 1H); 3.23 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 169.4 (2C), 167.5, 136.5, 136.2, 134.7, 132.8, 125.1, 123.6, 24.5. Anal. calc. for $C_{10}H_7NO_4$ (205.17): C, 58.54; H, 3.44; N, 6.83; found: C, 58.20; H, 3.66; N, 6.41.

*2.1.1.2. 2,3-Dihydro-2-ethyl-1,3-dioxo-1H-isoindole-5-carboxylic acid (**2b**).* M.p: 158–159 °C, IR (KBr): 3772, 3443, 2982, 2947, 1772, 1702, 1441, 1400, 1385, 1308, 1257, 1199, 1077. ^{1}H NMR (400 MHz, $CDCl_3$): 8.57 (s, 1H), 8.48 (dd, J = 7.7, 1.1 Hz, 1H), 7.97 (d, J = 7.7 Hz, 1H); 3.79 (q, J = 7.3 Hz, 2H), 1.30 (t, J = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 169.8 (2C), 167.3, 136.6, 136.2, 134.7, 132.8, 125.1, 123.6, 33.6, 14.1. Anal. calc. for $C_{11}H_9NO_4$ (219.20): C, 60.28; H, 4.14; N, 6.39; found: C, 60.62; H, 4.70, N, 6.48.

*2.1.1.3. 2,3-Dihydro-1,3-dioxo-2-phenyl-1H-isoindole-5-carboxylic acid (**2c**).* M.p: 259–260 °C (lit: 257–259 °C [25]), IR (KBr): 3445, 3053, 1787, 1720, 1504, 1392, 1296, 1221, 1124, 1104, 1072. ^{1}H NMR (400 MHz, CD_3OD): 8.48 (m, 2H), 8.44 (m, 1H), 7.64–7.33 (m, 5H). ^{13}C NMR (100 MHz, CD_3OD): 166.7, 166.6, 166.3, 135.5, 135.0, 132.1, 131.9, 128.6, 128.4, 127.9, 126.68, 123.90, 123.24. Anal. calc. for $C_{15}H_9NO_4$ (267.24): C, 67.42; H, 3.39; N, 5.24; found: C, 67.62; H, 3.21; N, 5.70.

*2.1.1.4. Methyl 2,3-dihydro-2-methyl-1,3-dioxo-1H-isoindole-5-carboxylate (**3a**).* M.p: 122–123 °C, IR (KBr): 3455, 3078, 2956, 1773, 1738, 1717, 1480, 1438, 1383, 1288, 1261, 1188, 1161. ^{1}H NMR (400 MHz, $CDCl_3$): 8.47 (dd, J = 1.5, 0.7 Hz, 1H), 8.39 (dd, J = 7.7, 1.5 Hz, 1H), 3.98 (s, 3H), 3.21 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 167.7, 167.7, 165.5, 135.7, 135.6, 132.7, 129.1, 124.5, 123.5, 53.1, 24.5. Anal. calc. for $C_{11}H_9NO_4$ (219.20): C, 60.28; H, 4.14; N, 6.39; found: C, 60.01; H, 4.15; N, 5.99.

*2.1.1.5. Methyl 2,3-dihydro-2-ethyl-1,3-dioxo-1H-isoindole-5-carboxylate (**3b**).* M.p: 96–97 °C, IR (KBr): 3457, 3115, 2980, 2960, 1773, 1725, 1459, 1431, 1399, 1380, 1330, 1295, 1256, 1197, 1183. ^{1}H NMR (400 MHz, $CDCl_3$): 8.47 (s, 1H), 8.39 (d, J = 7.8 Hz, 1H), 7.90 (d,



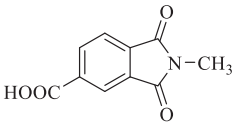
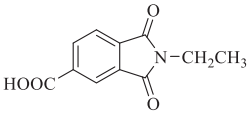
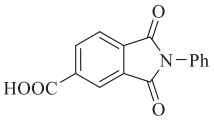
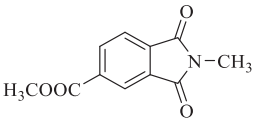
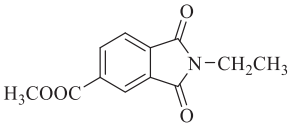
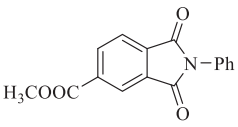
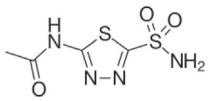
	R	R_1
2a	Me	H
2b	Et	H
2c	Ph	H
3a	Me	Me
3b	Et	Me
3c	Ph	Me

Scheme 1. The synthetic route for the preparation of isoindoline-1,3-dione derivatives.

Table 1
Purification of hCA I and hCA II from human erythrocytes.

Purification steps	Volume	Activity	Total Activity		Protein	Specific Activity	Purification Fold
	mL	(EU/mL)	EU	%	(mg protein/mL)	(EU/mg protein)	
Hemolysate	100	44.67	4467	100	$1.64*10^3$	0.27	–
hCA I	45	59.8	2691	60.2	0.075	797.33	2953.1
hCA II	35	66.8	2338	52.3	0.033	2024.24	7497.2

Table 2
The results obtained from regression analysis graphs for hCA I and hCA II in the presence of the 1,3-dioxoisoindoline derivatives.

Compound	hCA-I Inhibition			hCA-II Inhibition			hCA-I/hCA-II
	IC_{50} (μM)	r^2	Ki (μM)	IC_{50} (μM)	r^2	Ki (μM)	
 $C_{10}H_7NO_4$ (**2a**)	16.95	0.9883	12.77 ± 4.33	14.345	0.9971	10.45 ± 1.78	1.182
 **$C_{11}H_9NO_4$ (2b)**	13.04	0.9990	9.56 ± 4.21	8.97	0.9896	6.33 ± 1.08	1.454
 $C_{15}H_9NO_4$ (**2c**)	16.16	0.9884	10.21 ± 2.26	11.36	0.9923	8.92 ± 2.66	1.423
 $C_{11}H_9NO_4$ (**3a**)	7.02	0.9974	4.13 ± 1.25	9.19	0.9894	6.89 ± 2.33	0.538
 $C_{12}H_{11}NO_4$ (**3b**)	12.85	0.9905	7.21 ± 2.56	26.69	0.9914	27.23 ± 4.18	0.481
 **$C_{16}H_{11}NO_4$ (3c)**	10.74	0.9911	8.42 ± 3.33	6.39	0.9845	4.78 ± 1.44	1.68
 **Acetazolamid**	5.76	0.9875	7.63 ± 1.04	6.27	0.9925	10.45 ± 1.78	0.918

$J = 7.7$ Hz, 1H), 3.98 (s, 3H), 3.76 (q, $J = 7.2$ Hz, 1 H), 1.28 (t, $J = 7.2$ Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 167.3, 167.2, 165.3, 135.6, 135.5, 135.3, 132.5, 124.2, 123.2, 52.8, 33.2, 13.9. Anal. calc. for $C_{12}H_{11}NO_4$ (233.22): C, 61.80; H, 4.75; N, 6.01; found: C 62.22; H 4.21; N 6.31.

*2.1.1.6. Methyl 2,3-dihydro-1,3-dioxo-2-phenyl-1H-isoindole-5-carboxylic acid (**3c**).* M.p: 210–211 °C, IR (KBr): 3444, 2964, 1776, 1730, 1622, 1597, 1505, 1493, 1458, 1424, 1386, 1353, 1293, 1278, 1217, 1112, 1097. ^1H NMR (400 MHz, $CDCl_3$): 8.60 (d, $J = 0.7$ Hz, 1H), 8.50 (dd, $J = 7.7$, 1.5 Hz, 1H), 8.04 (d, $J = 7.7$ Hz, 1H), 7.55–7.43 (m,

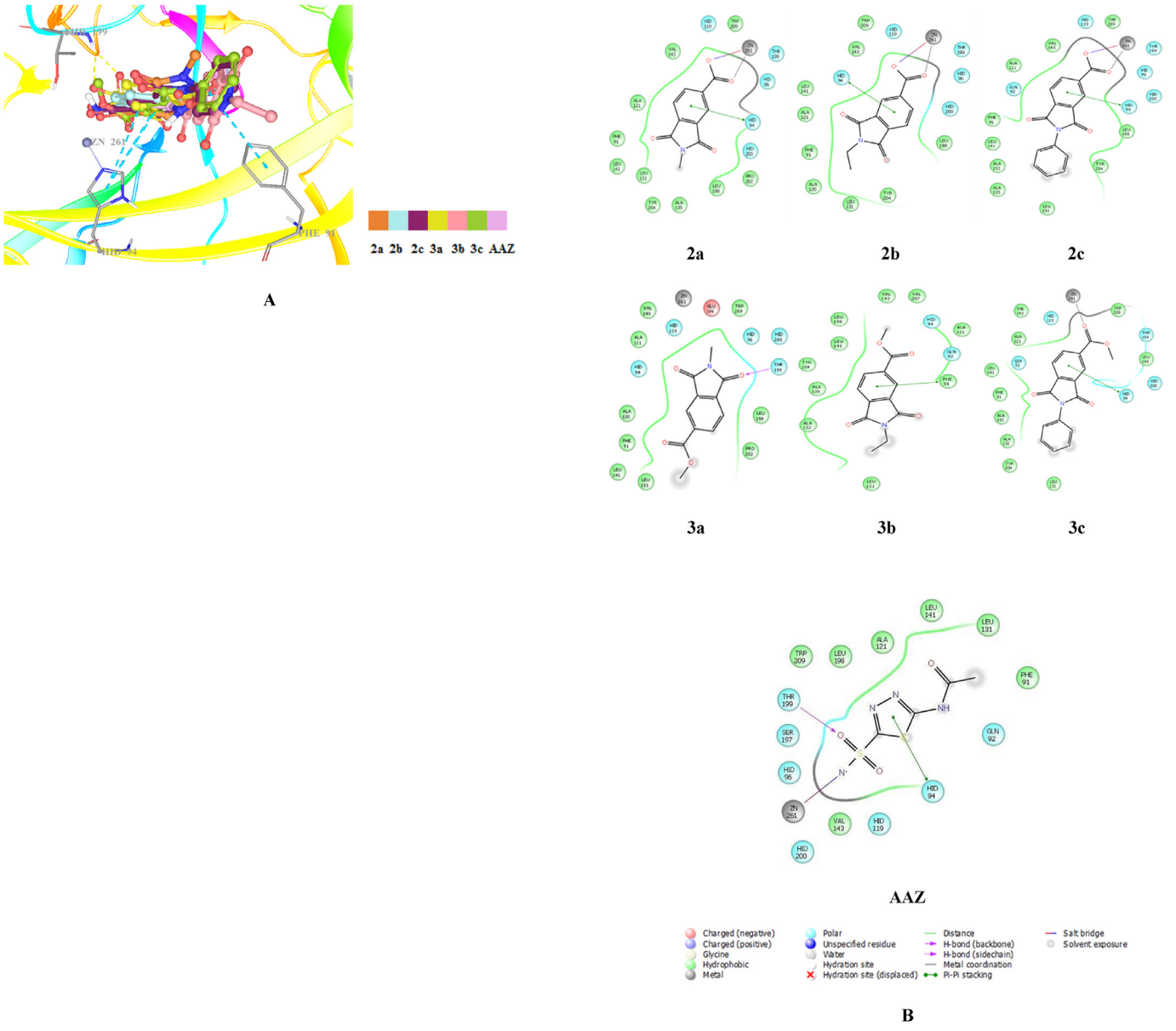


Fig. 2. Docking poses (A) and interactions (B) of all compounds in the active site of hCA I (PDB code: 1AZM). (Ligand custom carbons are colored as given in the figure and the zinc atom is displayed in grey sphere).

5H), 4.01 (s, 3H), 1.55 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 166.7, 166.6, 166.2, 136.2, 136.0, 135.3, 132.5, 129.5, 128.6, 126.7, 125.1, 124.1, 123.2, 53.2. Anal. calc. for $C_{16}H_{11}NO_4$ (281.27): C, 68.33; H, 3.94; N, 4.98; found: C, 68.62, H, 3.81, N, 4.75.

2.2. Biochemistry

Sepharose 4B, sulphanilamide, L-tyrosine, Tris, Na_2SO_4, protein assay reagents, and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from Merck.

2.2.1. Purification of hCA I and hCA II from human erythrocytes

Fresh human blood was obtained from Ataturk University, Blood Center, kept at 4 °C and used within 2–3 days at most.

Purification of CA isozymes (hCA I and hCA II) from human erythrocytes was carried out by affinity chromatography as described previously [19].

2.2.2. Hydratase activity assay

CA activity was determined using the Wilbur-Anderson Method.

2.2.3. Inhibition assays

In order to determine the IC_{50} values for hCA I and hCA II enzymes, the compounds (**2a-c**, **3a-c**) were studied at different concentrations, keeping the substrate concentration constant on hydratase activity. The activities of the enzymes in the uninhibited medium were used as 100% activity. The activity% values were calculated by measuring the hydratase activities of the enzymes in the presence of the inhibitors at different concentrations. The IC_{50} value for each inhibitor was calculated using the plot of Activity%-[I] plotted [20,21].

2.3. Molecular docking studies

The compounds were docked to the active sites of hCA I and hCA II. Ligands were set to the physiological pH (pH = 7.4) at the protonation step and crystal structures of hCA I and hCA II were retrieved from Protein Data Bank server (PDB codes: 1AZM and 6B59, respectively). In molecular docking simulations: Glide/XP docking protocols were applied for the prediction of topologies of compounds in the active sites of target structures by Schrödinger's Maestro molecular modelling package programme (Schrödinger Release 2016-2: Schrödinger, LLC, New York, NY, USA).

2.4. Prediction of pharmacokinetic parameters

The ADME properties of all compounds were *in silico* predicted using QikProp module of Schrödinger's Molecular modelling package (Schrödinger Release 2016-2: QikProp, Schrödinger, LLC, New York, NY, 2016).

3. Results and discussion

3.1. Chemistry

In this study, isoindoline-1,3-dione derivatives were obtained by the reaction of 1,3-dioxo-1,3-dihydroisobenzofuran-5-carboxylic acid (**1**) with methyl, ethyl, phenyl amine (**2a**, **2b**, **2c**), then converted to the methyl ester derivatives (**3a**, **3b**, **3c**) (Scheme 1). The structures of these compounds were approved on the basis of IR, ^{1}H NMR, ^{13}C NMR spectral data and elemental analysis. All spectral data were in good agreement with their structures.

3.2. Biochemistry

hCA I and hCA II isoenzymes were purified in one step using human blood Sepharose 4B-L-tyrosine-sulphanilamide affinity chromatography. hCA I was purified with 2979.1 fold and 60.2% efficiency with 797.33 specific activity and hCA II was purified with 7497.2 fold and 52.3% efficiency with 797.33 specific activity (Table 1).

The inhibitory effects on the activity of hCA I and hCA II isoenzymes of 1,3-dioxoisoindoline derivatives (**2a-c, 3a-c**) were tested *in vitro*. IC_{50} values were determined for all 1,3-dioxoisoindoline derivatives (Table 2). AAZ was used as a standard inhibitor.

The following structure–activity relationships (SAR) were gathered from the inhibition data reported in Table 2. In order to determine the relationship between the structures of isoindoline-1,3-dione derivatives and their hCA-I and hCA-II inhibitory effects,

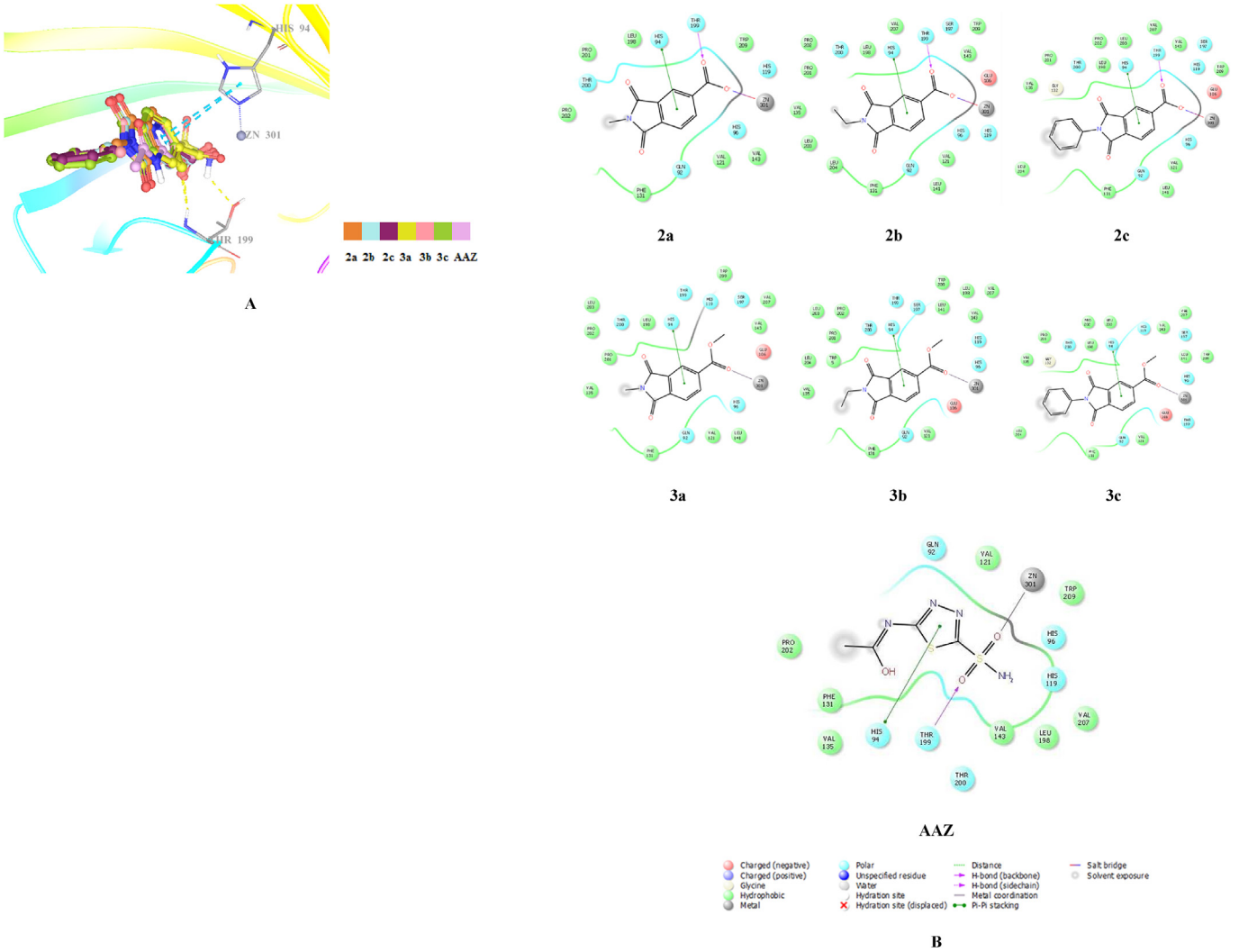


Fig. 3. Docking poses (A) and interactions (B) of all compounds in the active site of hCA II (PDB code: 6B59). (Ligand custom carbons are colored as given in the figure and the zinc atom is displayed in grey sphere).

Table 3
Docking score (kcal/mol), glide gscore (kcal/mol) and glide emodel (kcal/mol) results of all compounds in the active site of hCA I (PDB code: 1AZM) and hCA II (PDB code: 6B59).

Compound	hCA I			hCA II		
	Docking score	Glide gscore	Glide emodel	Docking score	Glide gscore	Glide emodel
2a	−2.91	−2.91	−12.02	−5.46	−5.46	−48.21
2b	−3.48	−3.48	−22.30	−6.14	−6.14	−87.74
2c	−3.28	−3.28	−21.47	−5.62	−5.62	−53.39
3a	−6.99	−6.99	−24.47	−6.09	−6.09	−78.98
3b	−6.34	−6.34	−28.03	−5.29	−5.29	−47.31
3c	−6.32	−6.32	−25.71	−6.38	−6.38	−83.59
AAZ	−7.24	−8.23	−66.57	−6.87	−7.87	−79.31

it is necessary to look at these compounds in two aspects. The first one is *N*-alkyl/aryl group of the phthalimide ring, namely methyl, ethyl and phenyl groups. The second one is esters of corresponding carboxylic acids, for this purpose, two series were prepared for each group bonded to the nitrogen atom. These are 1,3-dioxoisoindoline-5-carboxylic acids (**2a-c**) and their corresponding methyl esters (**3a-c**). The compounds inhibited both hCA I and hCA II. This indicates that all synthesized compounds (**2a**, **2b**, **2c**, **3a**, **3b**, **3c**) have groups that can interact with the active site of the enzyme. In general, the esters were found to be more effective than their corresponding carboxylic acids. The most effective hCA I inhibitor in this series was found as compound **3a**, whereas the most active hCA II inhibitor was found as compound **3c**. Interestingly, among carboxylic acid derivatives (**2a-c**) *N*-ethyl group enhanced hCA inhibitory activity, among esters (**3a-c**) *N*-ethyl group decreased hCA inhibitory activity.

3.3. Molecular docking studies

Molecular docking studies indicated that compounds **2a-c**, **3a-c** showed high affinity in the active sites of hCA I and hCA II when compared with AAZ, the reference agent (Figs. 2 and 3). The carboxylates of compounds **2a**, **2b** and **2c** were engaged with salt bridge formation and metal coordination with Zn261, whereas the ester group of compound **3c** interacted with Zn261 forming a metal coordination in the active site of hCA I. The isoindolines of compounds **2a**, **2b**, **2c** and **3c** displayed π-stacking interaction with His94 in the active site of hCA I (Fig. 2). On the other hand, compound **3b** was found to be capable of forming π-stacking interaction with Phe91 due to the isoindoline scaffold. The imide moiety of compound **3a** formed a hydrogen bond with Thr199. The thiadiazole of AAZ forged a key π-stacking interaction with His94, whereas the sulfonamide moiety of AAZ presented hydrogen bond and salt bridge formation with Thr199 and Zn261, respectively (Fig. 2).

As regards to the active site of hCA II, the carboxylates of compounds **2a**, **2b** and **2c** presented hydrogen bonding and salt bridge formation with Thr199 and Zn301, respectively. However, compounds **3a**, **3b** and **3c** formed metal coordination with Zn301. Besides, the isoindolines of all compounds displayed π-stacking interaction with His94 in the active site of hCA II similar to hCA I. The thiadiazole of AAZ forged a key π-stacking interaction with His94, whereas the sulfonamide moiety of AAZ presented hydrogen bond and metal coordination with Thr199 and Zn301, respectively (Fig. 3).

In general, Emodel scores are used to compare the different conformations of the same ligand, whilst docking scores are used for the comparison of different ligands [22]. The docking score of compound **3a** for hCA I was found as −6.99 kcal/mol, whereas the docking score of compound **3c** for hCA II was found as −6.38 kcal/mol. The docking scores, glide gscores and glide emodel results of all compounds were given in Table 3.

3.4. In silico ADME studies

In order to enlighten the biological, pharmaceutical and drug similarities of these compounds, *in silico* ADME studies were performed. The results given in Table 4 were found to be within the acceptable range making these derivatives as promising drug candidates for further studies.

4. Conclusion

In the present paper, 1,3-dioxoisoindoline derivatives were found to inhibit hCA I and hCA II at high levels. Accordingly, it has been found that 1,3-dioxoisoindoline derivatives have the potential to be used as hCA inhibitors for the development of drugs against various diseases. Furthermore, molecular docking studies showed that these compounds presented proper interactions with Zn^{2+} ion,

Table 4
Predicted ADME properties of compounds **2a-c**, **3a-c.**

Compound	QPlogPo/w*	QPlogS*	QPlogBB*	QPlogKhsa*	Human Oral Absorption%[a]	Rule of Five[b]	Rule of Three[c]
2a	0.46	−1.87	−1.23	−0.69	54.92	0	0
2b	0.96	−2.21	−1.22	−0.60	60.03	0	0
2c	1.91	−3.37	−1.22	−0.28	66.16	0	0
3a	0.46	−2.46	−0.94	−0.64	73.67	0	0
3b	1.09	−2.41	−0.91	−0.52	79.57	0	0
3c	2.11	−3.70	−0.89	−0.13	86.08	0	0

[a] QPlogPo/w: Predicted octanol/water partition coefficient (Recommended range: −2.0 to 6.5), QPlogS: Predicted aqueous solubility, log S. S in mol dm^{-3} is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid (Recommended range: −6.5 to 0.5), QPlogBB: brain/blood partition coefficient (Recommended range: −3 to 1.2), QPlogKhsa: binding to human serum albumin (Recommended range: −1.5 to 1.5), Human Oral Absorption%: Human oral absorption on 0–100% scale (>80% is high, <25% is poor).

[b] Rule of Five: Number of violations of Lipinski's rule of five. The rules are: mol_MW (molecular weight of the molecule) < 500, QPlogPo/w (predicted octanol/water partition coefficient) < 5, donorHB (hydrogen-bond donor atoms) ≤ 5, accptHB (hydrogen-bond acceptor atoms) ≤ 10. Compounds that provide these rules are considered as drug-like molecules.

[c] Rule of Three: Number of violations of Jorgensen's rule of three. The three rules are: QPlogS (predicted aqueous solubility) > −5.7, QPPCaco (predicted apparent Caco-2 cell permeability in nm/s) > 22 nm/s, # Primary Metabolites < 7. Compounds with fewer (and preferably no) violations of these rules are more likely to be orally available agents (Schrödinger Release 2016-2: Schrödinger, LLC, New York, NY, USA).

His94 and Thr199 residues in the active sites of hCA I and hCA II. According to *in silico* ADME studies, the compounds did not violate Lipinski's rule of five and Jorgensen's rule of three. On the basis of these findings, these compounds are expected to be potential orally bioavailable hCA inhibitors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molstruc.2019.07.070.

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