Send Orders for Reprints to reprints@benthamscience.net

Medicinal Chemistry, **2023**, *19*, **485-494**

RESEARCH ARTICLE

Synthesis and Molecular Docking of New N-Acyl Hydrazones-Benzimidazole as hCA I and II Inhibitors

Kaan Küçükoğlu[1], Ulviye Acar Çevik[2,*], Hayrunnisa Nadaroglu[3], Ismail Celik[4], Ayşen Işık[5], Hayrani Eren Bostancı[6], Yusuf Özkay[2] and Zafer Asım Kaplancıklı[2]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Selçuk University, Konya, Turkey; [2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Anadolu University, Eskişehir, 26470, Turkey; [3]Department of Food Technology, Erzurum Vocational Training School, Ataturk University, Erzurum, 25240, Turkey; [4]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Erciyes University, Kayseri, 38039, Turkey; [5]Department of Biochemistry, Faculty of Science, Selçuk University, Konya, Turkey; [6]Department of Biochemistry, Faculty of Pharmacy, Sivas Cumhuriyet University, Sivas, Turkey

Abstract: ***Background*:** The carbonic anhydrases (CAs) which are found in most living organisms is a member of the zinc-containing metalloenzyme family. The abnormal levels and activities are frequently associated with various diseases therefore CAs have become an attractive target for the design of inhibitors or activators that can be used in the treatment of those diseases.

***Methods*:** Herein, we have designed and synthesized new benzimidazole-hydrazone derivatives to investigate the effects of these synthesized compounds on CA isoenzymes. Chemical structures of synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS. The synthetic derivatives were screened for their inhibitory potential against carbonic anhydrase I and II by *in vitro* assay.

***Results*:** These compounds have IC_{50} values of 5.156-1.684 μM (hCA I) and 4.334-2.188 μM (hCA II). Inhibition types and Ki values of the compounds were determined. The Ki values of the compounds were 5.44 ± 0.14 μM-0.299 ± 0.01 μM (hCA I) and 3.699 ± 0.041 μM-1.507 ± 0.01 μM (hCA II). The synthetic compounds displayed inhibitory action comparable to that of the clinically utilized reference substance, acetazolamide. According to this, compound **3p** was the most effective molecule with an IC_{50} value of 1.684 μM. Accordingly, the type of inhibition was noncompetitive and the Ki value was 0.299 ± 0.01 μM.

***Conclusion*:** According to the *in vitro* test results, detailed protein-ligand interactions of the compound **3p**, which is more active against hCA I than standard azithromycin (AZM), were analyzed. In addition, the cytotoxic effects of the compounds on the L929 healthy cell line were evaluated.

ARTICLE HISTORY

Received: August 29, 2022
Revised: November 05, 2022
Accepted: November 16, 2022

DOI:
10.2174/1573406419666221222143530

Keywords: Hydrazone, benzimidazole, carbonic anhydrase I, carbonic anhydrase II, MTT, molecular docking.

1. INTRODUCTION

Benzimidazole scaffold, which is an N-heterocyclic compound, is the most common in medicinal chemistry. Since heterocycles are used in pharmaceutical, bioinformatics, and drug design, regularly, such scaffolds are often called 'privileged' [1]. This heterocyclic is formed by the fusion of a benzene ring to the 4 and 5 positions of an imidazole ring [2]. Some drugs containing benzimidazole ring are marketed like anti-cancer drugs nocodazole and velipralib, anti-protozoal albendazole, phosphodiesterase inhibitor adibenden, analgesic benzitramide, hypotensive diabezole, antiviral maribavir and antihistamine lerisetron [1, 3]. Moreover, new benzimidazole analogues continue to be synthesized and their biological activities are tested worldwide these biological activities contain antileukemic [4, 5], antimicrobial [6, 7], antiulcer [8, 9], diuretic [10], analgesic [11], calcium channel blocker [12], anti-Alzheimer [13], anticancer [14] activities.

The n-Acylhydrazone skeleton which is composed of an amide and an imine group has the ability to interact with hydrogen-bond acceptor and donor sites and could be interacted with a wide range of amino-acid residues. Although there are not many drugs with the N-acyl hydrazone group, [15] it is considered to have potential chemical, therapeutic, biological, and industrial properties [16]. In the search for biological activities of compounds containing N-acyl hydrazone groups, they have been found to exhibit antiprotozoal [17], anti-inflammatory [18, 19], antitrypanosomal [20], antiviral [21], antituberculosis [22], antitumoral [23], antileishmanial [24], and antihypertensive [25] activities.

*Address correspondence to this author at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Anadolu University, Eskişehir, 26470, Turkey; E-mail: uacar@anadolu.edu.tr

The carbonic anhydrases (CAs) which are found in most living organisms are a member of the zinc-containing metalloenzyme family. Their duty is to catalyze the conversion of CO_2 and H_2O to HCO_3^- and H^+ [26, 27]. The type of CAs found in mammals is α-class. Many biochemical processes such as respiration, calcification, pH and bicarbonate homeostasis, signal transduction, lipogenesis, and ureagenesis can be counted among the pivotal physiological events in which these enzymes participate [28, 29]. Moreover, abnormal levels and activities are frequently associated with various diseases so CAs have become an attractive target for the design of inhibitors or activators that can be used in the treatment of those diseases [30-32]. Now, CA inhibitors have been used as anticonvulsants [33], diuretics [34], antiglaucoma [35], or anti-obesity drugs [36]. Among the CAs, CA II, CA IV, and CA XII are CA isoenzymes that are antiglaucoma drug targets [37, 38]. Glaucoma is an optic neuropathy and one of the leading causes of global irreversible blindness in the world [39, 40]. Laser treatment, incisional surgery, and drug therapy are treatment options for glaucoma [41, 42]. Although drug therapy is an essential part of the treatment for glaucoma, systemic side effects such as neurological, psychiatric, and gastrointestinal side effects with currently used drugs create a big problem [43], so there is an urgent need for innovative drug development for glaucoma therapy. Acetazolamide, ethoxzolamide, and dichlorphenamide are clinically important CA inhibitors (Fig. **1**).

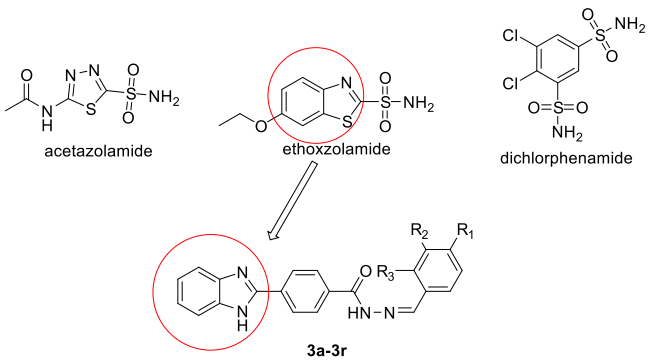


Fig. (1). General structure of acetazolamide, ethoxzolamide and dichlorphenamide and synthesized compounds.

Considering above mentioned problems and the logic explained, the design and synthesis of novel *N*-acyl hydrazones containing benzimidazole ring were considered to develop new hCA I (human carbonic anhydrase) and hCA II inhibitor agents. Antimicrobial effects of compounds **3a**, **3b**, **3e**, **3g**, **3k**, **3m**, **3n**, and **3p** have been reported in previous studies [44]. In this study, the effects of carbonic anhydrase were investigated.

2. MATERIALS AND METHODS

2.1. Chemistry

Synthesis of sodium metabisulfite salt of benzaldehyde derivative:

Ethanol was used to dissolve 5g (0.03 mol) of methyl 4-formyl benzoate. Drop by drop, ethanol-dissolved sodium metabisulfite (6.84 g, 0.036 mol) was added to the benzaldehyde solution. The reaction's components were mixed for an hour at room temperature once the dripping process was finished. It was filtered to obtain the precipitated product.

Synthesis of methyl 4-(1*H*-benzimidazole-2-yl)benzoate (**1**):

The sodium metabisulfite salt of the benzaldehyde derivative (7.09 g, 0.026 mol) was added after the benzene-1,2-diamine (0.022 mol) had been dissolved in dimethylformamide (DMF). The reaction was completed, and the result was precipitated by adding the reaction's components to iced water. The precipitated product was filtered off and crystallized from ethanol.

Synthesis of 4-(1*H*-benzimidazole-2-yl)-benzohydrazide derivatives (**2**):

Compound **1** (0.018 mol), excess of hydrazine hydrate (5 mL), and ethanol (15 mL) were all put into the same vial. Refluxing the mixture for 12 hours. Following the completion of the reaction, the mixture was poured into iced water, the product was filtered.

Synthesis of target compounds **3a-3r**:

The compound **2** and appropriate benzaldehyde derivatives in ethanol were refluxed. The precipitated product is filtered off.

2.1.1. N-(1H-benzimidazole-2-yl)-N'-benzylidenebenzo hydrazide (3a)

Yield: 74%. M.p. 279.5°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.22-7.27 (2H, m, Aromatic CH), 7.46 (3H, s, Aromatic CH), 7.57 (1H, d, *J*= 6.06 Hz, Aromatic CH), 7.70-7.77 (3H, m, Aromatic CH), 8.11 (2H, d, *J*=7.50 Hz, 1,4-disubstituebenzene), 8.33 (2H, d, *J*=7.59 Hz, 1,4-disubstituebenzene), 8.50 (1H, s, Aromatic CH), 12.00 (1H, s, NH), 13.12 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 110.99, 113.04, 118.58, 121.36, 123.57, 125.72, 127.77, 128.34, 128.68, 130.43, 133.48, 134.68, 135.54, 147.40, 149.56, 150.70, 162.98. $[M+H]^+$ calcd for $C_{21}H_{16}N_4O$: 341.1383; found: 341.1397.

2.12. 4-((2-(4-(1H-benzimidazole-2-yl)benzoyl)hydrazine ylidene)methyl)benzoic acid (3b)

Yield: 78%. M.p. 338.3°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.23-7.26 (2H, m, Aromatic CH), 7.61-7.66 (2H, m, Aromatic CH), 7.87 (2H, d, *J*= 8.37 Hz, Aromatic CH), 8.03 (2H, d, *J*= 7.83 Hz, Aromatic CH), 8.10 (2H, d, *J*= 8.64 Hz, Aromatic CH), 8.33 (2H, d, *J*=8.16 Hz, Aromatic CH), 8.54 (1H, s, Aromatic CH), 12.15 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 125.76, 126.63, 127.83, 127.98, 128.74, 129.18, 129.28, 131.33, 131.41, 132.19, 133.59, 134.38, 138.84, 146.13, 148.33, 150.66, 163.12, 167.38. $[M+H]^+$ calcd for $C_{22}H_{16}N_4O_3$: 385.1285; found: 385.1295.

2.1.3. 4-(1H-benzimidazole-2-yl)-N'-(4-(diethylamino) benzylidene)benzohydrazide (3c)

Yield: 72%. M.p. 175.5°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.12 (6H, s, -CH_3), 3.36 (4H, s, -CH_2), 6.72 (2H, s, Aromatic CH), 7.24 (2H, s, Aromatic CH), 7.54 (3H, s, Aromatic CH), 7.70 (1H, s, Aromatic CH), 8.07 (2H, s, Aromatic CH), 8.30 (3H, s, Aromatic CH), 11.64 (1H, s, NH),

13.10 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 12.92, 44.20, 111.52, 111.99, 119.53, 120.93, 121.74, 122.52, 123.49, 126.75, 128.64, 128.95, 129.35, 133.20, 135.50, 135.63, 149.38, 150.64, 162.38. $[M+H]^+$ calcd for $C_{25}H_{25}N_5O$: 412.2140; found: 412.2132.

2.1.4. 4-(1H-benzimidazole-2-yl)-N'-(4-isopropyl benzylidene)benzohydrazide (3d)

Yield: 76%. M.p. 280.9°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.22 (6H, d, *J*= 6.81 Hz, -CH_3), 2.89-2.98 (1H, m, -CH), 7.22-7.27 (2H, m, Aromatic CH), 7.35 (2H, d, *J*=7.95 Hz, Aromatic CH), 7.57 (1H, dd, J_1= 6.39 Hz, J_2=1.29 Hz, Aromatic CH), 7.66-7.72 (3H, m, Aromatic CH), 8.09 (2H, d, *J*=8.37 Hz, Aromatic CH), 8.32 (2H, d, *J*=8.43 Hz, Aromatic CH), 8.45 (1H, s, Aromatic CH), 11.93 (1H, s, NH), 13.11 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 24.15, 33.87, 112.01, 119.58, 122.45, 123.50, 126.80, 127.33, 127.72, 128.78, 132.43, 133.41, 134.65, 135.53, 144.25, 148.49, 150.70, 151.26, 162.88. $[M+H]^+$ calcd for $C_{24}H_{22}N_4O$: 383.1863; found: 383.1866.

2.1.5. 4-(1H-benzimidazole-2-yl)-N'-(4-chlorobenzylidene) benzohydrazide (3e)

Yield: 76%. M.p. 322.4°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.07-7.10 (1H, m, Aromatic CH), 7.20 (1H, d, *J*=8.31 Hz, Aromaric CH), 7.45 (2H, d, *J*=8.28 Hz, Aromaric CH), 7.78-7.81 (3H, m, Aromatic C-H), 7.89-7.90 (3H, m, Aromatic C-H), 7.98-8.00 (3H, m, Aromatic C-H). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm):112.68, 114.05, 118.19, 118.43, 119.12, 121.11, 123.14, 128.38, 130.92, 131.80, 132.38, 134.83, 149.62, 151.71, 153.68, 156.20, 193.17. $[M+H]^+$ calcd for $C_{21}H_{15}N_4OCl$: 375.1005; found: 375.1007.

2.1.6. 4-(1H-benzimidazole-2-yl)-N'-(4-methylthio benzylidene)benzohydrazide (3f)

Yield: 78%. M.p. 290.4°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.52 (3H, s, -CH_3), 7.25-7.27 (2H, m, Aromatic CH), 7.33 (2H, d, *J*=8.37 Hz, 1,4-disubstituebenzene), 7.53-7.58 (1H, m, Aromatic CH), 7.68 (3H, d, *J*=8.37 Hz, Aromatic CH), 8.09 (2H, d, *J*=8.37 Hz, 1,4-disubstituebenzene), 8.32 (2H, d, *J*=8.40 Hz, 1,4-disubstituebenzene), 8.44 (1H, s, Aromatic CH), 11.95 (1H, s, NH), 13.12 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 13.71, 113.14, 118.56, 121.39, 125.05, 125.71, 127.10, 127.74, 127.89, 129.12, 131.07, 133.33, 133.43, 133.53, 134.58, 144.19, 147.01, 150.70, 162.87. $[M+H]^+$ calcd for $C_{22}H_{18}N_4OS$: 387.1263; found: 387.1274.

2.1.7. 4-(1H-benzimidazole-2-yl)-N'-(4-trifluoromethyl benzylidene)benzohydrazide (3g)

Yield: 81%. M.p. 335.5°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.23-7.25 (2H, m, Aromatic CH), 7.64-7.66 (2H, m, Aromatic CH), 7.83-7.85 (2H, m, Aromatic CH), 7.97-7.99 (2H, m, Aromatic CH), 8.10-8.12 (2H, m, Aromatic CH), 8.32-8.35 (2H, m, Aromatic CH), 8.55 (1H, s, Aromatic CH), 12.16 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 105.78, 113.20, 114.05, 118.24, 119.31, 120.99, 122.61, 123.41, 133.31, 135.23, 136.28, 144.02, 149.57, 150.78, 151.69, 153.66, 190.35. $[M+H]^+$ calcd for $C_{22}H_{15}N_4OF_3$: 409.1279; found: 409.1271.

2.1.8. N'-([1,1'-biphenyl]-4-ylmethylene)-4-(1H-benzimidazole-2-yl) benzohydrazide (3h)

Yield: 69%. M.p. 307.4°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.09 (2H, dd, J_1=3.30 Hz, J_2=9.06 Hz, Aromatic CH), 7.19 (1H, s, Aromatic CH), 7.22 (1H, s, Aromatic CH), 7.36-7.38 (4H, m, Aromatic CH), 7.44 (2H, dd, J_1=1.59 Hz, J_2=8.34 Hz, Aromatic CH), 7.78 (1H, s, Aromatic CH), 7.81 (1H, s, Aromatic CH), 7.87-7.90 (4H, m, Aromatic CH), 7.93 (2H, d, *J*=8.25 Hz, Aromatic CH).^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 125.76, 126.11, 126.54, 127.17, 127.78, 127.93, 128.40, 128.50, 128.58, 128.66, 129.28, 129.88, 130.63, 133.47, 133.90, 134.57, 139.79, 146.92, 149.09, 150.66, 162.98. $[M+H]^+$ calcd for $C_{27}H_{20}N_4O$: 417.1727; found: 417.1710.

2.1.9. 4-(1H-benzimidazole-2-yl)-N'-(4-ethoxybenzylidene) benzohydrazide (3i)

Yield: 79%. M.p. 270.1°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.36 (3H, t, *J*=6.90 Hz, CH3), 4.09-4.12 (2H, m, -CH2), 7.13 (3H, d, *J*=8.88 Hz, Aromatic C-H), 7.43-7.47 (2H, m, Aromatic C-H), 7.81 (2H, s, Aromatic CH), 8.04-8.07 (3H, m, Aromatic C-H), 8.15 (3H, d, *J*=8.79 Hz, Aromatic C-H). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 15.53, 64.36, 115.87, 117.31, 119.49, 121.84, 122.20, 123.70, 129.42, 129.82, 134.75, 136.27, 138.81, 145.57, 150.31, 153.74, 156.50, 161.45, 194.29. $[M+H]^+$ calcd for $C_{23}H_{20}N_4O_2$: 385.1670; found: 385.1659.

2.1.10. 4-(1H-benzimidazole-2-yl)-N'-(4-(benzyloxy) benzylidene)benzohydrazide (3j)

Yield: 70%. M.p. 287.0°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ= 5.18 (2H, s, -CH_3), 7.12 (2H, d, *J*=8.64 Hz, Aromatic CH), 7.30-7.41 (4H, m, Aromatic CH), 7.47 (2H, d, *J*=7.35 Hz, Aromatic CH), 7.67-7.72 (4H, m, Aromatic CH), 8.11 (2H, d, *J*=8.13 Hz, Aromatic CH), 8.32 (2H, d, *J*=7.95 Hz, Aromatic CH), 8.43 (1H, s, Aromatic CH), 11.91 (1H, s, NH).^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 69.81, 115.66, 115.99, 116.25, 122.55, 122.93, 123.66, 127.11, 127.45, 128.28, 128.43, 128.83, 128.96, 129.26, 132.31, 135.36, 137.18, 138.26, 139.32, 148.55, 160.48, 162.66. $[M+H]^+$ calcd for $C_{28}H_{22}N_4O_2$: 447.1803; found: 447.1816.

2.1.11. 4-(1H-benzimidazole-2-yl)-N'-(4-methoxy benzylidene)benzohydrazide (3k)

Yield: 71%. M.p. 276.6°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.81 (3H, s, -OCH_3), 7.03 (2H, d, *J*=8.67 Hz, Aromatic CH), 7.28-7.31 (2H, m, Aromatic CH), 7.66-7.69 (4H, m, Aromatic CH), 8.10 (2H, d, *J*=8.43 Hz, 1,4-disubstituebenzene), 8.33 (2H, d, *J*=8.34 Hz, 1,4-disubstituebenzene), 8.44 (1H, s, Aromatic CH), 11.91 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 55.75, 114.84, 115.43, 115.66, 123.51, 127.06, 127.25, 128.38, 128.82, 129.25, 132.31, 135.17, 138.76, 145.41, 148.45, 150.38, 161.37, 162.68. $[M+H]^+$ calcd for $C_{22}H_{18}N_4O_2$: 371.1504; found: 371.1503.

2.1.12. 4-(1H-benzimidazole-2-yl)-N'-(4-fluoro benzylidene)benzohydrazide (3l)

Yield: 68%. M.p. 303.9°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.22-7.27 (2H, m, Aromatic CH), 7.30-7.36 (2H, m, Aromatic CH), 7.57 (1H, dd, J_1= 6.54 Hz, J_2=1.29 Hz, Aro-

matic CH), 7.71 (1H, dd, J_1= 7.02 Hz, J_2=1.50 Hz, Aromatic CH), 7.80-7.85 (2H, m, Aromatic C-H), 8.09 (2H, d, *J*=8.31 Hz, 1,4-disubstituebenzene), 8.32 (2H, d, *J*=8.31 Hz, 1,4-disubstituebenzene), 8.49 (1H, s, Aromatic CH), 12.01 (1H, s, NH), 13.12 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 112.01, 116.30, 116.59, 119.58, 122.46, 123.51, 126.82, 127.75, 128.81, 129.76, 133.48, 134.53, 135.53, 144.26, 147.32, 150.68, 162.97. $[M+H]^+$ calcd for $C_{21}H_{15}N_4OF$: 359.1301; found: 359.1303.

2.1.13. 4-(1H-benzimidazole-2-yl)-N'-(4-cyano benzylidene)benzohydrazide (3m)

Yield: 66%. M.p. 297.1°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.23-7.26 (2H, m, Aromatic CH), 7.64-7.66 (2H, m, Aromatic CH), 7.94 (4H, s, Aromatic CH), 8.11 (2H, d, *J*=8.01 Hz, 1,4-disubstituebenzene), 8.32 (2H, d, *J*=8.43 Hz, 1,4-disubstituebenzene), 8.53 (1H, s, Aromatic CH), 12.23 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 112.35, 119.14, 122.06, 124.48, 125.76, 127.09, 127.87, 129.26, 129.74, 132.19, 133.55, 134.38, 138.95, 139.17, 145.38, 147.71, 150.62, 163.33. $[M+H]^+$ calcd for $C_{22}H_{15}N_5O$: 366.1335; found: 366.1349.

2.1.14. 4-(1H-benzimidazole-2-yl)-N'-(4-nitrobenzylidene) benzohydrazide (3n)

Yield: 78%. M.p. 333.2°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.23-7.26 (2H, m, Aromatic CH), 7.64 (1H, m, Aromatic CH), 8.01 (2H, d, *J*=9.03 Hz, 1,4-disubstituebenzene), 8.11 (2H, d, *J*=8.31 Hz, 1,4-disubstituebenzene), 8.30-8.35 (5H, m, Aromatic CH), 8.58 (1H, s, Aromatic CH), 12.29 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 123.48, 125.72, 127.43, 127.92, 129.44, 130.14, 133.61, 134.16, 136.11, 136.23, 140.99, 144.81, 147.05, 148.32, 150.62, 155.13, 163.25.

2.1.15. 4-(1H-benzimidazole-2-yl)-N'-(3-nitrobenzylidene) benzohydrazide (3o)

Yield: 73%. M.p. 310.1°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.23-7.26 (2H, m, Aromatic CH), 7.64 (2H, s, Aromatic CH), 7.74-7.79 (1H, m, Aromatic CH), 8.11 (2H, d, *J*=8.16 Hz, 1,4-disubstituebenzene), 8.17 (1H, d, *J*=7.50 Hz, Aromatic CH), 8.27 (1H, d, *J*=7.92 Hz, Aromatic CH), 8.33 (2H, d, *J*=8.19 Hz, 1,4-disubstituebenzene), 8.59 (2H, s, Aromatic CH), 12.26 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 121.43, 122.83, 122.99, 123.40, 124.83, 126.85, 127.93, 128.58, 128.92, 130.98, 133.65, 133.93, 134.23, 136.59, 139.24, 146.01, 148.69, 150.63, 163.23.

2.1.16. 4-(1H-benzimidazole-2-yl)-N'-(4-methylbenzylidene) benzohydrazide (3p)

Yield: 72%. M.p. 325.8°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.36 (3H, s, -CH_3), 7.22-7.25 (2H, m, Aromatic CH), 7.29 (2H, d, *J*=7.98 Hz, Aromatic CH), 7.57 (1H, dd, J_1= 6.48 Hz, J_2=1.35 Hz, Aromatic CH), 7.65 (2H, d, *J*=7.95 Hz, Aromatic CH), 7.70 (1H, dd, J_1= 7.23 Hz, J_2=1.98 Hz, Aromatic CH), 8.08 (2H, d, *J*=8.34 Hz, 1,4-disubstituebenzene), 8.31 (2H, d, *J*=8.43 Hz, 1,4-disubstituebenzene), 8.45 (1H, s, Aromatic CH), 11.92 (1H, s, NH), 13.11 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 15.54, 115.35, 116.29, 117.31, 120.72, 121.04, 122.86, 128.36, 128.97, 130.92, 132.37, 133.52, 134.89, 149.59, 153.79, 156.18, 160.15, 193.17. $[M+H]^+$ calcd for $C_{22}H_{18}N_4O$: 355.1545; found: 355.1553.

2.1.17. 4-(1H-benzimidazole-2-yl)-N'-(2-methylbenzylidene) benzohydrazide (3r)

Yield: 81%. M.p. 282.3°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.36 (3H, s, -CH_3), 7.20-7.31 (4H, m, Aromatic CH), 7.57 (1H, dd, J_1= 6.48 Hz, J_2=1.35 Hz, Aromatic CH), 7.64-7.71 (3H, m, Aromatic CH), 8.08 (2H, d, *J*=8.34 Hz, 1,4-disubstituebenzene), 8.31 (2H, d, *J*=8.43 Hz, 1,4-disubstituebenzene), 8.45 (1H, s, Aromatic CH), 11.92 (1H, s, NH), 13.11 (1H, s, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ(ppm): 20.35, 123.79, 125.63, 125.72, 125.84, 127.26, 127.63, 127.72, 127.86, 127.94, 132.78, 133.37, 133.46, 134.56, 137.46, 146.07, 147.64, 148.14, 150.99, 163.73. $[M+H]^+$ calcd for $C_{22}H_{18}N_4O$: 355.1544; found: 355.1553.

2.2. hCA Inhibition Assay

Purification of hCA I and hCA II by affinity chromatography was performed as described in the previous work [45-47, 56].

2.3. Hydratase Activity

The Wilbur-Anderson method, as modified by Wilber *et al*. [47, 48], was used to calculate CA activity. With the help of a bromothymol blue indicator and a measurement of the passing time, the pH changes were calculated using this approach, which causes the hydration of CO_2 to release H^+ ions. The equation (to-tc/tc), where to and tc are the times for pH change of the enzymatic and nonenzymatic processes, respectively, was used to compute the enzyme unit (EU).

2.4. Inhibition Assay

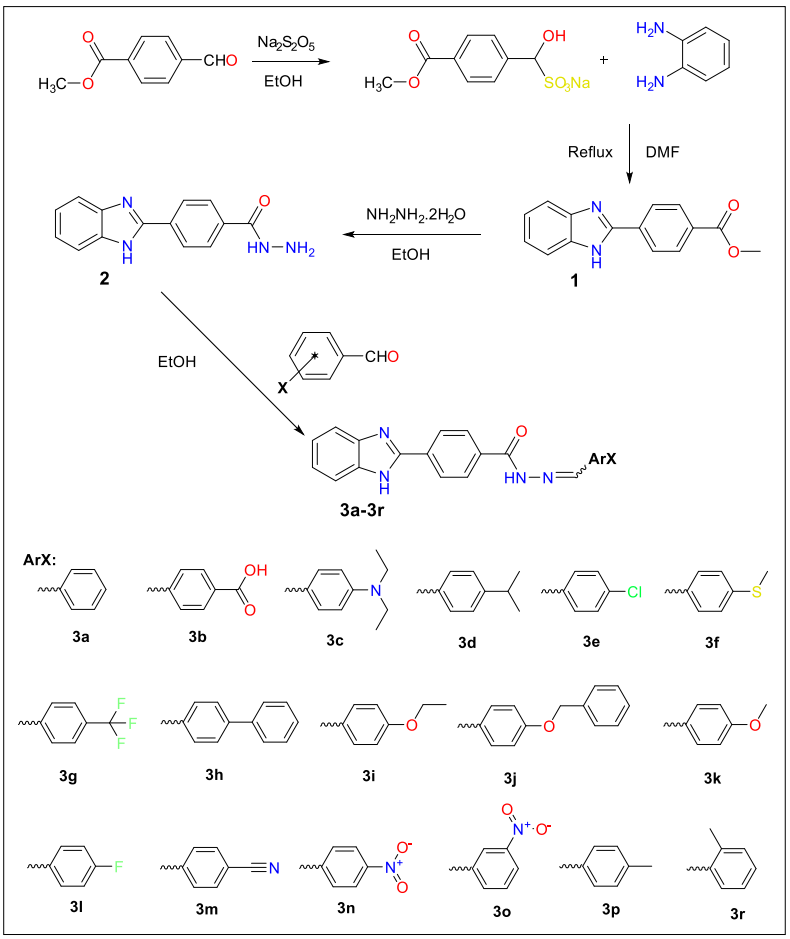
Investigated were the inhibitory effects of compounds **3a-3r** and AZM on the hydratase activity of the isoenzymes hCA I and hCA II. While keeping the concentration of the substrate constant, IC_{50} values for the various compounds were computed. Enzyme activities in the absence of inhibitors in the medium were taken as 100% activity. By assessing the hydratase activity in the presence of various inhibitor concentrations, the activity % values of enzymes were calculated. Utilizing graphs of activity %-[I] for each inhibitor, the IC_{50} value was determined [48-50]. The Cheng-Prusoff equation was used to derive inhibition constants using the nonlinear least squares method [51-53].

2.5. Cytotoxicity Assay

The effect of the compounds between **3a-3r** on the viability of the L929 cell line was analyzed by MTT assay as described in the previous work [54-56]. Cell L929 was obtained from ATCC and multiplied in Hepokur Lab.

2.6. Molecular Docking

All stages of molecular docking studies protein preparation, ligand preparation, active site grid generation, and ligand docking were carried out using Schrödinger Suite software 2021.1 version. 3D structures of target proteins hCA I (PDB ID: 3W6H, Resolution: 2.96 Å) [54] and hCA II (PDB ID: 4G0C, Resolution: 2.00 Å) [55] were obtained from the



Scheme 1. General procedure for synthesis of the final compounds **3a-3r.**

protein data bank (PDB). Target proteins were created using the 'Protein Preparation Wizard' default parameters after water and other heteroatoms other from Zn^{2+} were eliminated. The "LigPrep" tool was used to create the 3D minimizing structures of the compounds **3a-3r** at pH:7.2. With the "Receptor Grid Generation" module based on the cocrystal ligand AZM, the active site coordinates file for both target proteins hCA I (x: 33.6, y: -1.33, z: 9.01) and hCA II (x: -4.98, y: 3.81, z: 14.7) were generated as 20*20*20 Å3. To validate the molecular docking work, re-docking was performed with Glide SP and the cocrystal ligand AZM [56]. Then, molecular docking of all compounds was performed with Glide SP ligand docking tools. 2D protein-ligand interactions diagram 'Ligand Interaction' module and 3D interactions were made in Maestro v12.8 interface.

3. RESULTS AND DISCUSSION

3.1. Chemistry

As shown in Scheme **1**, the target molecules were synthesized in four steps. First, the aldehyde part of the methyl 4-formylbenzoate compound was treated with sodium metabisulfite in ethanol to obtain the sodium disulfide addition product of the aldehyde. In the second step, as a result of the condensation reaction of benzaldehyde sodium metabisulfite product and o-phenylenediamine under reflux and methyl 4-(1*H*- benzimidazol-2-yl)benzoate (**1**) was obtained. In the next step, compound **1** was treated with hydrazine hydrate in ethanol to obtain the 4-(1*H*-benzimidazol-2-yl)benzohydrazide (**2**). The hydrazide derivative compound (**2**) and appropriate benzaldehyde derivatives in ethanol were refluxed and obtained target compounds **3a-3r**. The structures of the target compounds were confirmed via ^{1}H NMR, ^{13}C NMR, and HRMS spectroscopy.

Methyl protons from the -C_2H_5 protons of compound **3c** were observed at 1.12 ppm, and -CH_2 protons at 3.36 ppm. Methyl protons from the -isopropyl group of compound **3d** were observed at 1.22 ppm as duplet, and -CH protons at 2.89-2.98 ppm as a multiplet. The protons of the -CH_3 group of thiomethyl substituent of compound **3f** were observed at 2.52 ppm as a singlet. The methoxy group in the 4th position of the phenyl ring of compound **3k** was observed as a singlet at 3.81 ppm. The signals belonging to aromatic protons were found at 6.72-8.59 ppm. The ^{13}C NMR spectra showed peaks around 165 ppm due to the carbonyl group (C=O). All of the derivatives' ^{13}C NMR spectra revealed carbon values in the expected locations, and the HRMS analysis supported the mass with the target compounds' estimated values.

3.2. *In vitro* hCA Activity

The compounds **3a-3r** were tested for their *in vitro* inhibitory effects on hCA I and hCA II isoenzymes and the results

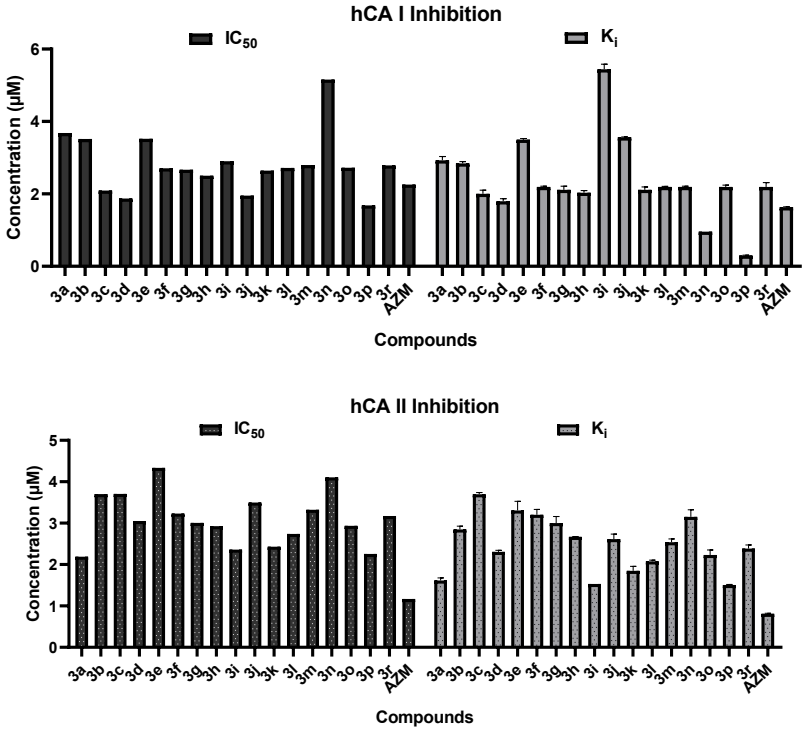


Fig. (2). IC_{50} and K_i values (μM) of the new N-acyl hydrazones compounds **3a-3r** and standard acetazolamide (AZM) with hCA I and hCA II.

are presented in Fig. (**1**). In this work, acetazolamide was used as a reference compound. Compounds **3a-3r** showed hCA I inhibitory activity with IC_{50} values ranging from 1.684 to 5.156 μM. In these series, compounds **3p**, **3d**, **3j**, and **3c** were the only compounds that showed better inhibitory activity against hCA I isoenzyme than the reference compound AZM with the IC_{50} values of 1.684, 1.87, 1.952, and 2.093 μM, respectively.

These series of N-acyl hydrazones exhibited inhibitory effects on hCA II isoenzyme with IC_{50} values ranging from 2.188 to 4.334 μM and none of the compounds had better inhibitory effects compared to AZM (IC_{50} = 1.17 μM). Compounds **3a**, **3i**, **3k**, and **3n** showed more inhibitory activity on hCA II than hCA I, but the other tested compounds showed more inhibitory effects on hCA I than hCA II, except compound **3l**. 4-Fluoro derivative **3l** indicated fairly close inhibitory properties on hCA I and hCA II isozymes with the IC_{50} values of 2.716 and 2.738 μM, respectively.

The inhibition constants (Ki) of compounds **3a-3r** for hCA I were established between 0.299 and 5.44 μM and are shown in Fig. (**2**). In these compounds, 4-methylphenyl derivative **3p** (Ki = 0.299 μM) and a 4-nitrophenyl derivative **3n** (Ki = 0.956 μM) had lower Ki values than that of AZM (Ki = 1.63 μM) representing their inhibitory activity against hCA I isoenzyme. On the other hand, all the tested compounds had greater Ki values ranging between 1.507 and 3.699 μM compared to AZM (Ki = 0.812 μM) on hCA II isoenzyme.

The IC_{50} and Ki values obtained from activity tests revealed that all the compounds tested towards hCA I and II isoforms showed noncompetitive type enzyme inhibition. According to *in vitro* assay, compounds **3p**, **3d**, **3j**, and **3c** indicate significant hCA I inhibitory activity, even though the sulfonamide group, which is an important pharmacophore for hCA inhibitory activity, is not available in their structures. 4-methyl substituents on the phenyl ring at the 4th position were found a generally useful modification in increasing hCA I inhibitory activity. In compound **3r**, where the methyl group is in the 2nd position, it is seen that the activity decreases significantly. It was determined that compound **3d**, which has an isopropyl structure instead of methyl in the 4th position, also showed similar activity to compound **3p**.

3.3. Cytotoxicity Assay

The cytotoxic bioactivity of synthetic compounds was assessed *in vitro* using the MTT test against the L929 cell line for preliminary screening. The target compounds were administered to the fibroblast cells at a constant dose of 100 M to assess their cytotoxic potential. After the cells had been treated for 48 hours, cell viability percentages were calculated. Preliminary anti ınflammatory effect results of compounds **3a-3r** against L929 fibroblast are presented in Fig. (**3**). As a result of the maximum dose applied, all compounds showed 75% more viability. So as a result of this IC_{50} values of compounds were not calculated because they were greater than 100 μM.

3.4. Molecular Docking

A molecular docking study was carried out to detect and show the interaction of the synthesized compounds with hCA I and II. In order to compare the synthesized com-

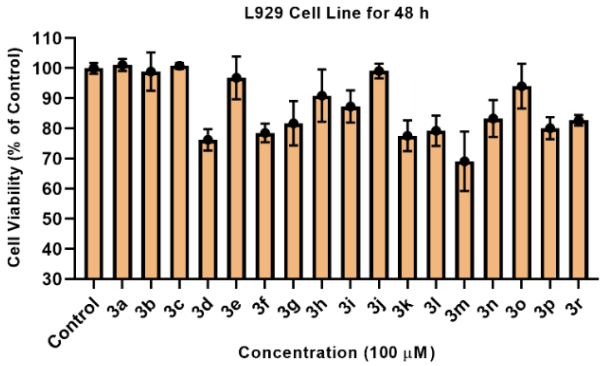


Fig. (3). Cell Viability (%) of L929 fibroblast cell line against compounds (**3a-3r**) for 48h. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

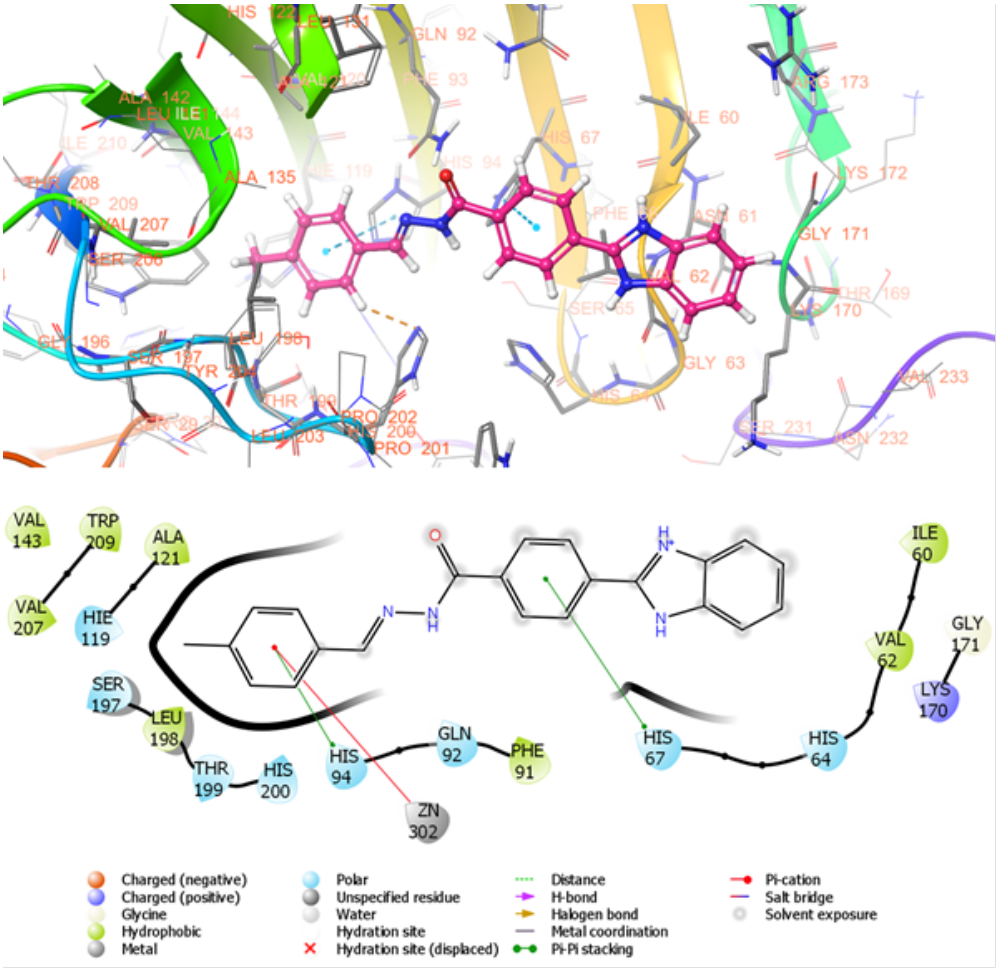


Fig. (4). Binding poses and protein-ligand interaction diagram of most active compounds **3p** against hCA I obtained from Glide SP molecular docking. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

pounds and to validate the molecular docking study, self-docking was performed on the cocrystal ligand AZM, which is located in the hCA I (PDB ID: 3W6H) [54] and II (PDB ID: 4G0C) [55] crystal three-dimensional structures. The RMSD value of AZM between the natural interaction pose and docking pose was measured as 1.36 Å for 3W6H and 0.17 Å for 4G0C. As given in Table **1**, the compounds gave docking scores between -3.369 and -6.713 kcal/mol, and glide emodel docking scores of -38.264 and -71.940 kcal/mol against the hCA I enzyme. Glide docking scores between -3.121 and -6.547 kcal/mol, and glide emodel binding energies of -39.199 and -70.942 kcal/mol against the CA II enzyme were formed. The standard compound and cocrystal ligand AZM gave -7.893 kcal/mol Glide binding energy and -70.865 Glide emodel binding energy to hCA I enzyme, while they gave -7.097 kcal/mol and -67.269 kcal/mol binding energies to hCA II enzyme, respectively.

According to the *in vitro* test results, detailed protein-ligand interactions of the compound **3p**, which is more active against hCA I than standard AZM, were analyzed. As given in Fig. (**4**), compound **3p** formed one H bond with His67 (2.22 Å), π-π stacking interactions with His67 (4.83 Å) and His94 (4.75 Å), and pi-cation interactions with Zn^{2+} (4.25 Å). In addition, hydrophobic interactions with Ile60, Val62, Phe91, Ala121, Val143, Leu198, Val207 and Trp209, positively charged interactions with Lys170, polar van der Waals interactions with His64, His67, Gln92, His94, Hie119, Ser197, Thr199, and His200. The other active compound 3d, two H bonds with Trp5 (2.66 Å) and His67 (2.49 Å), π-π stacking with His64 (5.01 Å) and His200 (4.86 Å and 5.08 Å), and residues His94 (5.86 Å) and Lys170. (4.49 Å) with π-cation interactions. Compound 3j formed π-π stacking with His94 (4.86 Å), hydrophobic interactions with Leu198, Pro202, Tyr204, Ala135, Tyr20 and Ala121, and polar van der Waals interactions with His64, Gln92, His94, His200 and Thr199.

Table 1. Molecular docking binding energies (kcal/mol) of compounds 3a-3r and reference acetazolamide (AZM) with hCA I and hCA II.

-	hCA I		hCA II	
Comp.	**Glide gscore**	**Glide emodel**	**Glide gscore**	**Glide emodel**
3a	-3.708	-44.053	-3.819	-39.199
3b	-6.713	-71.940	-6.547	-70.942
3c	-3.657	-50.887	-3.022	-44.450
3d	-4.536	-38.264	-3.482	-45.824
3e	-3.955	-39.943	-3.250	-48.468
3f	-3.369	-45.741	-3.233	-50.193
3g	-3.771	-47.527	-3.137	-48.827
3h	-3.878	-50.963	-3.453	-53.708
3i	-3.243	-45.768	-3.121	-47.667
3j	-3.692	-50.278	-3.473	-53.466
3k	-3.887	-46.158	-3.199	-46.962
3l	-3.887	-46.158	-3.199	-46.962
3m	-4.926	-47.682	-3.823	-44.852
3n	-4.224	-51.377	-3.103	-50.201
3o	-3.969	-50.551	-3.301	-51.546
3p	-4.430	-44.340	-4.213	-47.914
3r	-4.295	-49.790	-3.514	-45.492
AZM	-7.893	-70.865	-7.097	-67.269

CONCLUSION

In this paper, new N-acyl hydrazones containing benzimidazole ring compounds **3a-3r**, were synthesized and evaluated for their ability to inhibit hCA I and hCA II isoforms. Despite the absence of a sulfonamide group, which is an important functional group for carbonic anhydrase enzyme inhibitory activity, in the structures of these compounds, it is attractive that enzyme inhibition activity is observed. In this study, we have shown that the sulfonamide group is not a must for carbonic anhydrase inhibiting activity. Among them, a 4-methylphenyl derivative **3p** was the strongest compound on hCA I isozyme according to IC_{50} and Ki values. According to the result, we showed that built-in guanidine or =NNH-CO moieties may also play a crucial role in the process of inhibition. These compounds may serve as a promising candidate for further studies to develop new hCA inhibitory compounds. According to the *in vitro* test results, detailed protein-ligand interactions of the compound **3p**, which is more active against hCA I than standard AZM, were analyzed by molecular docking.

LIST OF ABBREVIATIONS

CAs = Carbonic Anhydrases

DMF = Dimethylformamide

EU = Enzyme Unit

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals and humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available within the article.

FUNDING

Ankara University-Scientific Research Unit supported this study by supplying the Schrödinger software purchased under grant project number BAP-21B0237004.

CONFLICT OF INTEREST

The authors confirm that this article's content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank Ankara University-Scientific Research Unit for supplying the Schrödinger software.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Tahlan, S.; Kumar, S.; Narasimhan, B. Pharmacological significance of heterocyclic 1*H*-benzimidazole scaffolds: A review. *BMC Chem.,* **2019**, *13*(1), 101. http://dx.doi.org/10.1186/s13065-019-0625-4 PMID: 31410412

[2] Kamanna, K. *Synthesis and pharmacological profile of benzimidazoles. Chemistry and applications of benzimidazole and its derivatives*; IntechOpen: London, UK, **2019**, pp. 51-69.

[3] Alaqeel, S.I. Synthetic approaches to benzimidazoles from o-phenylenediamine: A literature review. *J. Saudi Chem. Soc.,* **2017**, *21*(2), 229-237. http://dx.doi.org/10.1016/j.jscs.2016.08.001

[4] Kamal, A.; Praveen Kumar, P.; Sreekanth, K.; Seshadri, B.N.; Ramulu, P. Synthesis of new benzimidazole linked pyrrolo[2,1-c][1,4]benzodiazepine conjugates with efficient DNA-binding affinity and potent cytotoxicity. *Bioorg. Med. Chem. Lett.,* **2008**, *18*(8), 2594-2598. http://dx.doi.org/10.1016/j.bmcl.2008.03.039 PMID: 18378445

[5] Garuti, L.; Roberti, M.; Malagoli, M.; Rossi, T.; Castelli, M. Synthesis and antiproliferative activity of some benzimidazole-4,7-dione derivatives. *Bioorg. Med. Chem. Lett.,* **2000**, *10*(19), 2193-2195. http://dx.doi.org/10.1016/S0960-894X(00)00429-7 PMID: 11012027

[6] Rajiv, D.; Sonwane, S.K.; Srivastava, S.K.; Srivastava, S.D. Conventional and greener approach for the synthesis of some novel substituted-4-oxothiazolidine and their 5-arylidene derivatives of 2-methylbenzimidazole: Antimicrobial activities. *J. Chem. Pharm. Res.,* **2010**, *2*(1), 415-423.

[7] Hosamani, K.M.; Seetharamareddy, H.R.; Keri, R.S.; Hanamanthagouda, M.S.; Moloney, M.G. Microwave assisted, one-pot synthesis of 5-nitro- 2-aryl substituted-1*H*-benzimidazole libraries: Screening *in vitro* for antimicrobial activity. *J. Enzyme Inhib. Med. Chem.,* **2009**, *24*(5), 1095-1100. http://dx.doi.org/10.1080/14756360802632716 PMID: 19772484

[8] Patil, A.; Ganguly, S.; Surana, S. Synthesis and antiulcer activity of 2-[5-substituted-1-H-benzo(d) imidazol-2-yl sulfinyl]methyl-3-substituted quinazoline-4-(3H) ones. *J. Chem. Sci.,* **2010**, *122*(3), 443-450. http://dx.doi.org/10.1007/s12039-010-0052-5

[9] Bariwal, J.B.; Shah, A.K.; Kathiravan, M.K.; Somani, R.S.; Jagtap, J.R.; Jain, K.S. Synthesis and antiulcer activity of novel pyrimidylthiomethyl- and pyrimidylsulfinylmethyl benzimidazoles as potential reversible proton pump inhibitors. *Indian J. Pharm. Educ. Res.,* **2008**, *42*(3), 225-231.

[10] Radha, Y.; Manjula, K.M.; Reddy, B.M.; Rao, B.V. Synthesis and biological activity of novel benzimidazoles. *Indian J. Chem.,* **2011**, *50B*, 1762-1773.

[11] Demirayak, S.; Karaburun, A.C.; Kayagil, I.; Uçucu, U.; Beis, R. Synthesis and analgesic activities of some 2-(benzazolylacetyl) amino-3-ethoxycarbonylthiophene derivatives. *Phosphorus Sulfur Silicon Relat. Elem.,* **2005**, *180*(8), 1841-1848. http://dx.doi.org/10.1080/104265090889503

[12] Law, C.S.W.; Yeong, K.Y. Benzimidazoles in drug discovery: A patent review. *ChemMedChem,* **2021**, *16*(12), 1861-1877. http://dx.doi.org/10.1002/cmdc.202100004 PMID: 33646618

[13] Acar Cevik, U.; Saglik, B.; Levent, S.; Osmaniye, D.; Kaya Cavuşoglu, B.; Ozkay, Y.; Kaplancikli, Z. Synthesis and AChE-inhibitory activity of new benzimidazole derivatives. *Molecules,* **2019**, *24*(5), 861. http://dx.doi.org/10.3390/molecules24050861 PMID: 30823470

[14] Blagosklonny, M.V. Analysis of FDA approved anticancer drugs reveals the future of cancer therapy. *Cell Cycle,* **2004**, *3*(8), 1033-1040. http://dx.doi.org/10.4161/cc.3.8.1023 PMID: 15254418

[15] Maia, R.C.; Tesch, R.; Fraga, C.A.M. Acylhydrazone derivatives: A patent review. *Expert Opin. Ther. Pat.,* **2014**, *24*(11), 1161-1170. http://dx.doi.org/10.1517/13543776.2014.959491 PMID: 25213630

[16] Carvalho, S.A.; Feitosa, L.O.; Soares, M.; Costa, T.E.M.M.; Henriques, M.G.; Salomão, K.; de Castro, S.L.; Kaiser, M.; Brun, R.; Wardell, J.L.; Wardell, S.M.S.V.; Trossini, G.H.G.; Andricopulo, A.D.; da Silva, E.F.; Fraga, C.A.M. Design and synthesis of new (E)-cinnamic N-acylhydrazones as potent antitrypanosomal agents. *Eur. J. Med. Chem.,* **2012**, *54*, 512-521. http://dx.doi.org/10.1016/j.ejmech.2012.05.041 PMID: 22727447

[17] Gorantla, V.; Gundla, R.; Jadav, S.S.; Anugu, S.R.; Chimakurthy, J.; Rao, N.S.K.; Korupolu, R. New anti-inflammatory Hybrid N-acyl hydrazone-linked isoxazole derivatives as COX-2 inhibitors: rational design, synthesis and biological evaluation. *Chem. Select.,* **2017**, *2*(26), 8091-8100.

[18] Osmaniye, D.; Sağlık, B.N.; Levent, S.; Özkay, Y.; Kaplancıklı, Z.A. Design, synthesis and biological evaluation of new *N* -acyl hydrazones with a methyl sulfonyl moiety as selective COX-2 inhibitors. *Chem. Biodivers.,* **2021**, *18*(11), e2100521. http://dx.doi.org/10.1002/cbdv.202100521 PMID: 34411436

[19] Hernandes, M.Z.; Rabello, M.M.; Leite, A.C.L.; Cardoso, M.V.O.; Moreira, D.R.M.; Brondani, D.J.; Simone, C.A.; Reis, L.C.; Souza, M.A.; Pereira, V.R.A.; Ferreira, R.S.; McKerrow, J.H. Studies toward the structural optimization of novel thiazolylhydrazone-based potent antitrypanosomal agents. *Bioorg. Med. Chem.,* **2010**, *18*(22), 7826-7835. http://dx.doi.org/10.1016/j.bmc.2010.09.056 PMID: 20961766

[20] Ali, O.; Abdel-Rahman, A.; Amer, H. Synthesis and antiviral valuation of sugar uracil-1-ylmethylhydrazones and their oxadiazoline derivatives. *Synthesis,* **2007**, *2007*(18), 2823-2828. http://dx.doi.org/10.1055/s-2007-983878

[21] Ferreira, M.L.; Gonçalves, R.S.B.; Cardoso, L.N.F.; Kaiser, C.R.; Candéa, A.L.P.; Henriques, M.G.M.O.; Lourenço, M.C.S.; Bezerra, F.A.F.M.; de Souza, M.V.N. Synthesis and antitubercular activity of heteroaromatic isonicotinoyl and 7-chloro-4-quinolinyl hydrazone derivatives. *ScientificWorldJournal,* **2010**, *10*, 1347-1355. http://dx.doi.org/10.1100/tsw.2010.124 PMID: 20623095

[22] Zhang, D.; Ma, Y.; Liu, Y.; Liu, Z.P. Synthesis of sulfonylhydrazone- and acylhydrazone-substituted 8-ethoxy-3-nitro-2H-chromenes as potent antiproliferative and apoptosis inducing agents. *Arch. Pharm.,* **2014**, *347*(8), 576-588. http://dx.doi.org/10.1002/ardp.201400082 PMID: 24866448

[23] Coimbra, E.S.; Nora de Souza, M.V.; Terror, M.S.; Pinheiro, A.C.; da Trindade Granato, J. Synthesis, biological activity, and mechanism of action of new 2-pyrimidinyl hydrazone and N-acylhydrazone derivatives, a potent and new classes of antileishmanial agents. *Eur. J. Med. Chem.,* **2019**, *184*, 111742. http://dx.doi.org/10.1016/j.ejmech.2019.111742 PMID: 31605866

[24] Alencar, A.K.N.; Pereira, S.L.; Montagnoli, T.L.; Maia, R.C.; Kümmerle, A.E.; Landgraf, S.S.; Caruso-Neves, C.; Ferraz, E.B.; Tesch, R.; Nascimento, J.H.M.; de Sant'Anna, C.M.R.; Fraga, C.A.M.; Barreiro, E.J.; Sudo, R.T.; Zapata-Sudo, G. Beneficial effects of a novel agonist of the adenosine A 2A receptor on monocrotaline-induced pulmonary hypertension in rats. *Br. J. Pharmacol.,* **2013**, *169*(5), 953-962. http://dx.doi.org/10.1111/bph.12193 PMID: 23530610

[25] Supuran, C.T.; Scozzafava, A. Carbonic anhydrases as targets for medicinal chemistry. *Bioorg. Med. Chem.,* **2007**, *15*(13), 4336-4350. http://dx.doi.org/10.1016/j.bmc.2007.04.020 PMID: 17475500

[26] Scozzafava, A.; Passaponti, M.; Supuran, C.T.; Gülçin, İ. Carbonic anhydrase inhibitors: Guaiacol and catechol derivatives effectively inhibit certain human carbonic anhydrase isoenzymes (hCA I, II, IX and XII). *J. Enzyme Inhib. Med. Chem.,* **2015**, *30*(4), 586-591. http://dx.doi.org/10.3109/14756366.2014.956310 PMID: 25373500

[27] Supuran, C.T. Carbonic anhydrases: Novel therapeutic applications for inhibitors and activators. *Nat. Rev. Drug Discov.,* **2008**, *7*(2), 168-181. http://dx.doi.org/10.1038/nrd2467 PMID: 18167490

[28] Thiry, A.; Dogné, J.M.; Supuran, C.; Masereel, B. Carbonic anhydrase inhibitors as anticonvulsant agents. *Curr. Top. Med. Chem.,* **2007**, *7*(9), 855-864. http://dx.doi.org/10.2174/156802607780636726 PMID: 17504130

[29] Supuran, C.T.; Capasso, C. Biomedical applications of prokaryotic carbonic anhydrases. *Expert Opin. Ther. Pat.,* **2018**, *28*(10), 745-754. http://dx.doi.org/10.1080/13543776.2018.1497161 PMID: 29973089

[30] Bozdag, M.; Altamimi, A.S.A.; Vullo, D.; Supuran, C.T.; Carta, F. State of the art on carbonic anhydrase modulators for biomedical purposes. *Curr. Med. Chem.,* **2019**, *26*(15), 2558-2573.

http://dx.doi.org/10.2174/0929867325666180622120625 PMID: 29932025

[31] Angeli, A.; Vaiano, F.; Mari, F.; Bertol, E.; Supuran, C.T. Psychoactive substances belonging to the amphetamine class potently activate brain carbonic anhydrase isoforms VA, VB, VII, and XII. *J. Enzyme Inhib. Med. Chem.,* **2017**, *32*(1), 1253-1259. http://dx.doi.org/10.1080/14756366.2017.1375485 PMID: 28936885

[32] Di Fiore, A.; De Simone, G.; Alterio, V.; Riccio, V.; Winum, J.Y.; Carta, F.; Supuran, C.T. The anticonvulsant sulfamide JNJ-26990990 and its S,S-dioxide analog strongly inhibit carbonic anhydrases: Solution and X-ray crystallographic studies. *Org. Biomol. Chem.,* **2016**, *14*(21), 4853-4858. http://dx.doi.org/10.1039/C6OB00803H PMID: 27151329

[33] Carta, F.; Supuran, C.T. Diuretics with carbonic anhydrase inhibitory action: A patent and literature review (2005 – 2013). *Expert Opin. Ther. Pat.,* **2013**, *23*(6), 681-691. http://dx.doi.org/10.1517/13543776.2013.780598 PMID: 23488823

[34] Supuran, C.T.; Altamimi, A.S.A.; Carta, F. Carbonic anhydrase inhibition and the management of glaucoma: A literature and patent review 2013-2019. *Expert Opin. Ther. Pat.,* **2019**, *29*(10), 781-792. http://dx.doi.org/10.1080/13543776.2019.1679117 PMID: 31596641

[35] Scozzafava, A.; Supuran, C.T.; Carta, F. Antiobesity carbonic anhydrase inhibitors: A literature and patent review *Expert Opin. Ther. Pat.,* **2013**, *23*(6), 725-735. http://dx.doi.org/10.1517/13543776.2013.790957 PMID: 23607332

[36] Alp, C.; Maresca, A.; Alp, N.A.; Gültekin, M.S.; Ekinci, D.; Scozzafava, A.; Supuran, C.T. Secondary/tertiary benzenesulfonamides with inhibitory action against the cytosolic human carbonic anhydrase isoforms I and II. *J. Enzyme Inhib. Med. Chem.,* **2013**, *28*(2), 294-298. http://dx.doi.org/10.3109/14756366.2012.658788 PMID: 22380772

[37] Masini, E.; Carta, F.; Scozzafava, A.; Supuran, C.T. Antiglaucoma carbonic anhydrase inhibitors: A patent review. *Expert Opin. Ther. Pat.,* **2013**, *23*(6), 705-716. http://dx.doi.org/10.1517/13543776.2013.794788 PMID: 23627893

[38] Kolko, M.; Horwitz, A.; Thygesen, J.; Jeppesen, J.; Torp-Pedersen, C. The prevalence and incidence of glaucoma in Denmark in a fifteen year period: A nationwide study. *PLOS One,* **2015**, *10*(7), e0132048. http://dx.doi.org/10.1371/journal.pone.0132048 PMID: 26182236

[39] Tham, Y.C.; Li, X.; Wong, T.Y.; Quigley, H.A.; Aung, T.; Cheng, C.Y. Global prevalence of glaucoma and projections of glaucoma burden through 2040: A systematic review and meta-analysis. *Ophthalmology,* **2014**, *121*(11), 2081-2090. http://dx.doi.org/10.1016/j.ophtha.2014.05.013 PMID: 24974815

[40] Storgaard, L.; Tran, T.L.; Freiberg, J.C.; Hauser, A.S.; Kolko, M. Glaucoma clinical research: Trends in treatment strategies and drug development. *Front. Med.,* **2021**, *8*, 733080. http://dx.doi.org/10.3389/fmed.2021.733080 PMID: 34589504

[41] John, M.E.C.f.C.D. *Communications, S*; Agency for Healthcare Research and Quality: Rockville, MD.US, **2007** .

[42] Arbabi, A.; Bao, X.; Shalaby, W.S.; Razeghinejad, R. Systemic side effects of glaucoma medications. *Clin. Exp. Optom.,* **2022**, *105*(2), 157-165. http://dx.doi.org/10.1080/08164622.2021.1964331 PMID: 34402741

[43] Arslan, O.; Nalbantoglu, B.; Demir, N.; Ozdemir, H.; Kufrevioglu, O.I. A new method for the purification of carbonic anhydrase isozymes by affinity chromatography. *Turk. J. Med. Sci.,* **1996**, *26*(2), 163-166.

[44] Özkay, Y.; Tunalı, Y.; Karaca, H.; Işıkdağ, İ. Antimicrobial activity and a SAR study of some novel benzimidazole derivatives bearing hydrazone moiety. *Eur. J. Med. Chem.,* **2010**, *45*(8), 3293-3298. http://dx.doi.org/10.1016/j.ejmech.2010.04.012 PMID: 20451306

[45] Demir, N.; Demir, Y.; Nadaroglu, H. Carbonic anhydrase from bovine bone. *Prep. Biochem. Biotechnol.,* **2001**, *31*(1), 33-47. http://dx.doi.org/10.1081/PB-100103370 PMID: 11321162

[46] Demir, Y.; Demir, N.; Yildirim, S.; Nadaroğlu, H.; Karaosmanoğlu, M.; Bakan, E. The activities of carbonic anhydrase and alkaline phosphatase in ancient human bones. Purification and characterization of outer peripheral, cytosolic, inner peripheral, and integral CA. *Prep. Biochem. Biotechnol.,* **2001**, *31*(3), 291-304. http://dx.doi.org/10.1081/PB-100104910 PMID: 11513093

[47] Wilbur, K.M.; Anderson, N.G. Electrometric and colorimetric determination of carbonic anhydrase. *J. Biol. Chem.,* **1948**, *176*(1), 147-154. http://dx.doi.org/10.1016/S0021-9258(18)51011-5 PMID: 18886152

[48] Rickli, E.E.; Ghazanfar, S.A.S.; Gibbons, B.H.; Edsall, J.T. Carbonic anhydrases from human erythrocytes. Preparation and properties of two enzymes. *J. Biol. Chem.,* **1964**, *239*(4), 1065-1078. http://dx.doi.org/10.1016/S0021-9258(18)91392-X PMID: 14165909

[49] Altintop, M.D.; Ozdemir, A.; Kucukoglu, K.; Turan-Zitouni, G.; Nadaroglu, H.; Kaplancikli, Z.A. Synthesis and evaluation of new thiadiazole derivatives as potential inhibitors of human carbonic anhydrase isozymes (hCA-I and hCA-II). *J. Enzyme Inhib. Med. Chem.,* **2015**, *30*(1), 32-37. http://dx.doi.org/10.3109/14756366.2013.873038 PMID: 24666301

[50] Borras, J.; Scozzafava, A.; Menabuoni, L.; Mincione, F.; Briganti, F.; Mincione, G.; Supuran, C.T. Carbonic anhydrase inhibitors. *Bioorg. Med. Chem.,* **1999**, *7*(11), 2397-2406. http://dx.doi.org/10.1016/S0968-0896(99)00190-X PMID: 10632049

[51] Akocak, S.; Lolak, N.; Vullo, D.; Durgun, M.; Supuran, C.T. Synthesis and biological evaluation of histamine Schiff bases as carbonic anhydrase I, II, IV, VII, and IX activators. *J. Enzyme Inhib. Med. Chem.,* **2017**, *32*(1), 1305-1312. http://dx.doi.org/10.1080/14756366.2017.1386660 PMID: 29072105

[52] Küçükbay, H.; Buğday, N.; Küçükbay, F.Z.; Berrino, E.; Bartolucci, G.; Del Prete, S.; Capasso, C.; Supuran, C.T. Synthesis and carbonic anhydrase inhibitory properties of novel 4-(2-aminoethyl) benzenesulfonamide-dipeptide conjugates. *Bioorg. Chem.,* **2019**, *83*, 414-423. http://dx.doi.org/10.1016/j.bioorg.2018.11.003 PMID: 30419497

[53] Takaoka, Y.; Kioi, Y.; Morito, A.; Otani, J.; Arita, K.; Ashihara, E.; Ariyoshi, M.; Tochio, H.; Shirakawa, M.; Hamachi, I. Quantitative comparison of protein dynamics in live cells and *in vitro* by in-cell 19F-NMR. *Chem. Commun.,* **2013**, *49*(27), 2801-2803. http://dx.doi.org/10.1039/c3cc39205h PMID: 23440262

[54] Fisher, S.Z.; Aggarwal, M.; Kovalevsky, A.Y.; Silverman, D.N.; McKenna, R. Neutron diffraction of acetazolamide-bound human carbonic anhydrase II reveals atomic details of drug binding. *J. Am. Chem. Soc.,* **2012**, *134*(36), 14726-14729. http://dx.doi.org/10.1021/ja3068098 PMID: 22928733

[55] Friesner, R.A.; Murphy, R.B.; Repasky, M.P.; Frye, L.L.; Greenwood, J.R.; Halgren, T.A.; Sanschagrin, P.C.; Mainz, D.T. Extra precision glide: Docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J. Med. Chem.,* **2006**, *49*(21), 6177-6196. http://dx.doi.org/10.1021/jm051256o PMID: 17034125

[56] Küçükoğlu, K.; Acar Çevik, U.; Nadaroglu, H.; Celik, I.; Işık, A.; Bostancı, H.E.; Özkay, Y.; Kaplancıklı, Z.A. Design, synthesis and molecular docking studies of novel benzimidazole-1,3,4-oxadiazole hybrids for their carbonic anhydrase inhibitory and antioxidant effects. *Med. Chem. Res.,* **2022**, *31*(10), 1771-1782. http://dx.doi.org/10.1007/s00044-022-02943-6