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Microwave-assisted synthesis of 1-substituted-1*H*-benzimidazolium salts: Non-competitive inhibition of human carbonic anhydrase I and II

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Abstract

A series of 1-substituted-1*H*-benzimidazolium *p*-toluenesulfonate salts were synthesized in good yields by the reaction of 1-substituted benzimidazole derivatives and *p*-toluenesulfonic acid under microwave irradiation. Two iodide salts were synthesized by the anion exchange reaction of the corresponding *p*-toluenesulfonate salt and NaI. All compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR, LC-MS spectroscopic methods, and elemental analyses. The crystal structure of 1-methoxyethyl-1*H*-benzimidazolium *p*-toluenesulfonate **2d** showed that cation and anion are interconnected by N−H···O and C−H···O hydrogen bonds. All compounds were examined as inhibitor of human carbonic anhydrase (hCA) I and II, and all of them inhibited hCA I and hCA II. Kinetic investigation results revealed that these compounds inhibit hCA I and hCA II in a non-competitive manner. The iodide salts had higher inhibitory activity than their corresponding *p*-toluenesulfonate salts.

KEYWORDS
benzimidazole, benzimidazolium salt, carbonic anhydrase, inhibitors, microwave chemistry

1 | INTRODUCTION

Heterocyclic compounds are one of the indispensable building blocks for drug design and development for decades. Synthesis and pharmacological investigation of heterocyclic compounds have always been an important research area for organic and medicinal chemists. Structural tunability and diverse pharmacological profiles of this class of compounds provide them an important role in medicinal research. In addition, conformationally restricted structures of the heterocycles make them more selective and bioactive compared to their acyclic analogues.[1] Benzimidazole is a member of the nitrogen heterocycles and is a fused form of imidazole and benzene rings. The therapeutic journey of benzimidazole derivatives began with the discovery of *N*-ribosyl-5,6-dimethylbenzimidazole which serves as an axial ligand to cobalt in the structure of vitamin B12.[2] Many benzimidazole-based commercial drugs were developed thanks to anticancer, antihelmintic, antifungal, and antiulcer properties of benzimidazole derivatives. Additionally, some benzimidazole derivatives performed remarkable antibacterial, antioxidant, anticonvulsant, anti-inflammatory, and anti-HIV activities.[3–7] Benzimidazole derivatives exhibit anticancer effects

by enzyme inhibition or DNA intercalation.[8] Therefore, studies about the inhibitory properties of benzimidazoles on the activities of different enzymes including topoisomerase[8] and protein kinase[9] attracted much attention in recent years.

Besides the neutral benzimidazole derivatives, *N*,*N*-disubstituted benzimidazolium salts were also examined in medicinal applications. Reported studies revealed that these salts perform antimicrobial, anticancer,[10] and anti-inflammatory[11] activities. However, studies about the enzyme interaction properties of the benzimidazolium salts are very rare. In 2008, Tarrago et al.[12] reported amino-functionalized benzimidazolium salts as human prolyl oligopeptidase inhibitors. Our group reported a series of coumarin or benzoxazinone-substituted benzimidazolium salts as human carbonic anhydrase (hCA) inhibitors and anticonvulsant agents,[13,14] and human paraoxonase 1 inhibitors[15] and anthracene-substituted salts as tyrosinase inhibitors.[16] Later, Gülçin et al. investigated the inhibitory properties of phthalimide, vinylbenzyl, and hydroxyethyl-substituted benzimidazolium salts on the activities of hCA and some different enzymes.[17,18]

Carbonic anhydrases (CA, EC 4.2.1.1) are a family of metalloenzymes and most of CAs contain a zinc cation in the active site. Seven genetically different CA families are known: α-, β-, γ-, δ-, ζ-, η-, and θ-CAs, and hCAs comprise 16 α-CA isoenzymes.[19,20] CAs catalyze a highly important physiological reaction, reversible hydration of CO_2 to bicarbonate and a proton, and so they play a critical role in physiological pH control.[21] It is also known that abnormal levels of CA activity are associated with glaucoma,[22] epilepsy,[23] obesity,[24] and most recently cancer.[25] Therefore, inhibition of CAs is an important point in the treatment of these diseases.[22–25] Sulfonamides are the main class of CA inhibitors (CAIs) and they are in clinical use more than 50 years as antiglaucoma drugs.[26] However, this class of inhibitors has some side effects due to promiscuous inhibition of hCA isoforms.[27] Other well-known types of CAIs are phenols,[28] coumarins[29] and most recently carboxylic acids[30] and the research in this area focused on the discovery of selective inhibitors for tumor-associated hCA IX and XII. Supuran and co-workers reported that sulfonamide,[27] coumarin-sulfonamide hybrids,[31] imidazole,[32] and saccharin[33] derivatives inhibit transmembrane tumor-associated CA IX and XII.

As mentioned above, many benzimidazole derivatives were synthesized and examined in medicinal applications. However, according to our literature survey, synthesis of 1-substituted-1*H*-benzimidazolium salts is very scarce and there is no report in the literature about their pharmacological properties. In our previous study, we reported the synthesis and cytotoxicities of four 1-benzyl-1*H*-benzimidazolium *p*-toluenesulfonates complexed with Ag(I).[34] In view of the enzyme inhibitory profile of benzimidazolium salts, we decided to synthesize a new series of 1-substituted-1*H*-benzimidazolium salts in order to investigate their inhibitory properties on the activities of hCA I and II. For this purpose, seven novel and one known 1-substituted-1*H*-benzimidazolium *p*-toluenesulfonate salts and two 1-substituted-1*H*-benzimidazolium iodide salts were synthesized and characterized. Inhibitory properties of nine novel and five previously reported salts were investigated on the activities of hCA I and hCA II.

2 | RESULTS AND DISCUSSION

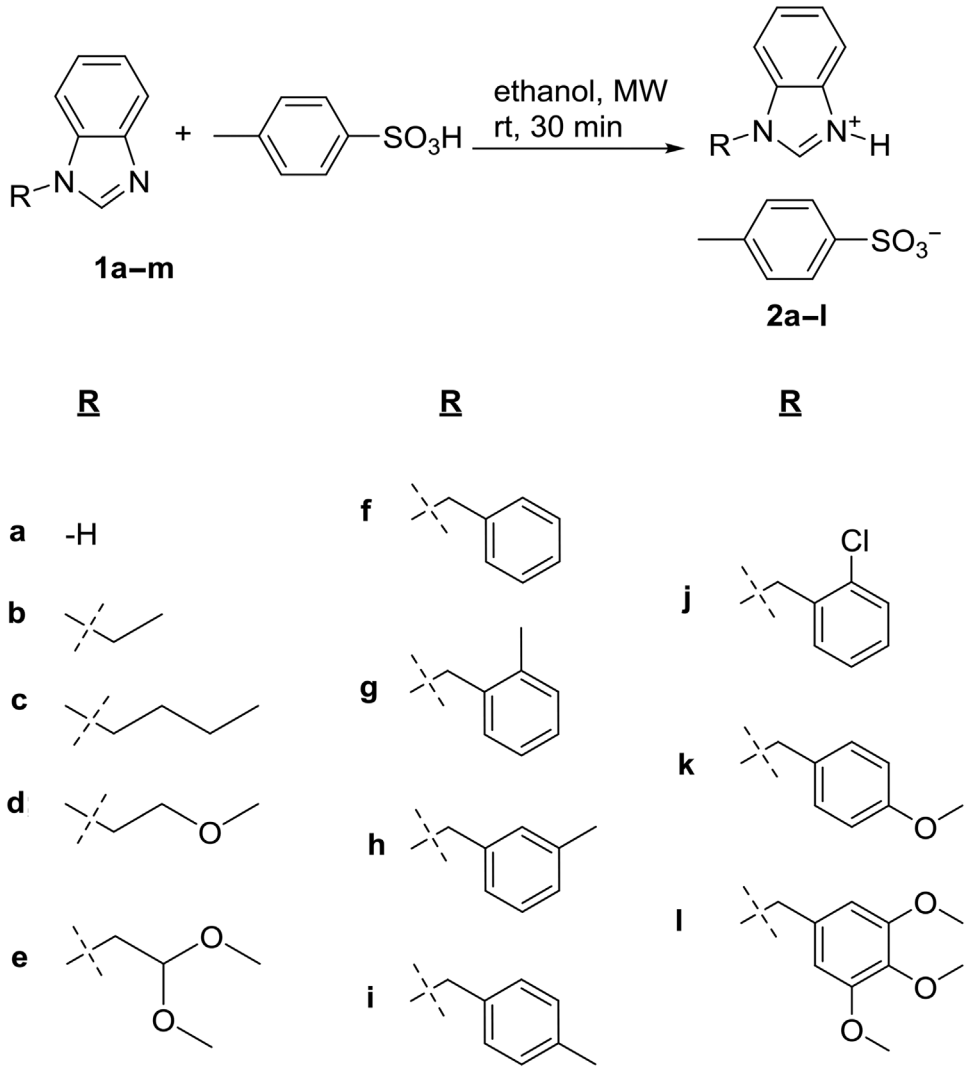
2.1 | Synthesis

In our previous study, we had synthesized four 1-benzyl-1*H*-benzimidazolium *p*-toluenesulfonate derivatives by the reaction of *N*-benzylbenzimidazole derivatives and *p*-toluenesulfonic acid under microwave irradiation.[34] In this study, we expanded this reaction with some other benzyl derivatives and aliphatic groups which also include oxygen heteroatoms. Synthesis and structures of 1-substitue-1*H*-benzimidazolium *p*-toluenesulfonates are outlined in Scheme 1. The compounds **2f**, **2g**, **2i**, and **2j** were available from our previous study.[34] The reaction was carried out under microwave irradiation in ethanol at room temperature for 30 min. Target compounds (**2a**–**e**, **2h**, **2k**, **2l**) were obtained in good yields between 54 and 89%. We carried out the same reaction during 24 h for all substituents with the conventional method but we could not achieve more than 30% yield for any compound. According to our knowledge, only Muskawar et al.[35] reported the synthesis of 1-ethyl-1*H*-benzimidazolium *p*-toluenesulfonate, **2b**. They reacted *N*-ethylbenzimidazole and *p*-toluenesulfonic acid in acetonitrile at 80°C for 36 h and they obtained 96% yield. In this study, we synthesized the same compound by microwave irradiation with 88% yield. If we consider the shortening of reaction time and decrease in temperature, it is clear that the obtained yield is quite satisfactory. All compounds are stable both in solid state and solution and highly soluble in water and organic solvents such as dichloromethane, ethanol, and dimethyl sulfoxide.

After the synthesis and characterization of *p*-toluenesulfonate salts, we made anion exchange reactions with compounds **2d** and **2f** in order to see their reactivity in anion exchange reaction and possible differences in CA inhibition. Compounds **2d** and **2f** reacted with NaI in ethanol at room temperature for 24 h to yield their iodide derivatives, **3a**,**b**, with 77 and 79% yields were obtained, respectively. Synthesis and structures of **3a**,**b** are given in Scheme 2. Compounds **3a** and **3b** are highly stable in solid state and solution, and well soluble in water and organic solvents like their *p*-toluenesulfonate derivatives. Interestingly, we could not synthesize the nitrate derivatives of **2d** and **2f** in the same reaction conditions. In addition, we obtained only *p*-toluenesulfonate salts even after heating at 60°C for 48 h.

2.2 | Spectral characterization

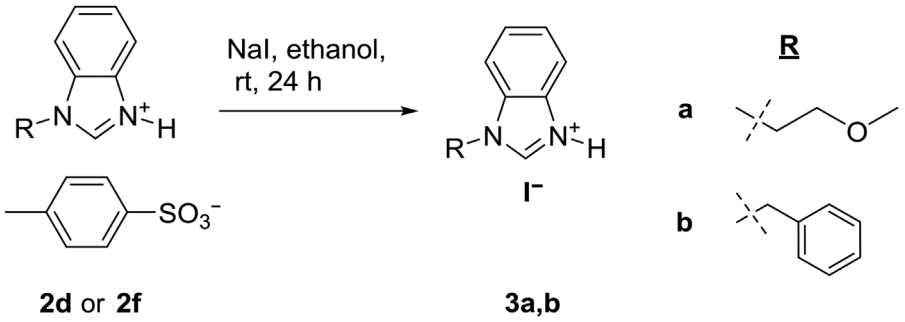
All compounds were characterized by ^{1}H NMR, ^{13}C NMR, LC-MS, infrared spectroscopic methods, and elemental analyses. In ^{1}H NMR spectra of *p*-toluenesulfonate derivatives, signals of acidic NC*H*N hydrogens were observed in the range of 9.39–9.89 ppm for



SCHEME 1 Synthesis and structures of benzimidazolium *p*-toluenesulfonates. **2f**, **2g**, **2i**, and **2j** were available from our previous study[34]

aliphatic substituents and 9.90–10.06 ppm for benzyl-derived substituents. The signals of the hydrogens which are bound to cationic nitrogen were observed at 15.57 and 15.69 ppm only for **2d** and **2e**, respectively. For other compounds, N^+-H resonances were not detected. Other expected resonances depending on substituents were observed in accordance with integrities and coupling patterns. In ^{13}C NMR spectra of *p*-toluenesulfonate derivatives, NCHN resonances were observed in the range of 141.0–142.1 ppm. Other expected resonances depending on substituents were observed as expected from the structures of compounds. In IR spectra of compounds **2b**–**l**, broad signal of the bond between cationic nitrogen and hydrogen was observed in the range of 2395–2604 cm^{-1}. This signal was observed at 2771 cm^{-1} for compound **2a**. Although N^+-H resonances were not detected for some compounds in ^1H NMR spectra, observed signals in IR spectra for all compounds prove the protonation of nitrogen of benzimidazole at 3-position. In addition, elemental analyses results also support that the compounds are in the proposed structures. In order to make further characterization, we measured LC-MS spectra of all compounds and maximal peak intensities are attributable to the cationic part of salts (see Supporting Information for all spectra and Section 4 for the data).

In ^1H NMR spectra of benzimidazolium iodides, signals of acidic NC*H*N hydrogens were observed at 9.87 and 10.12 ppm, respectively. The resonance of the hydrogen which bound to cationic nitrogen was observed at 12.36 ppm for **3a**, while not observed for **3b**. The most important evidence for the formation of iodide salts is the disappearance of the signals from *p*-toluenesulfonate group. Aromatic hydrogens of the *p*-toluenesulfonate group were observed at 7.77 and 7.08 ppm as doublets for **2d**, 7.73 and 7.00 ppm for **2f**.[34] In addition, signals of methyl groups of *p*-toluenesulfonate anion were observed at 2.26 and 2.20 ppm for **2d** and **2f**, respectively. These signals were not observed in the ^1H NMR spectra of **3a** and **3b**. In ^{13}C NMR spectra of **3b**, NCHN resonance was observed at 139.7 ppm. Resonances belonging to the *p*-toluenesulfonate group were not detected. In IR spectra of **3a**,**b**



SCHEME 2 Synthesis and structures of benzimidazolium iodides, **3a** and **3b**

broad signals of N^+-H bonds were observed at 2354 and 2610 cm^{-1}, respectively. Elemental analyses results also confirm the exchange of *p*-toluenesulfonate with iodide for **3a** and **3b**.

Combination of the spectroscopic methods and elemental analyses results clearly indicate that compounds have structures as shown in Schemes 1 and 2. Moreover, suitable crystals for X-ray analysis were obtained for **2d** by the slow diffusion of diethyl ether into the concentrated solution of ethanol.

2.3 | Description of the crystal structure of 2d

The solid-state structure of **2d** was determined by single crystal X-ray crystallography. ORTEP-3 view of the compound with the atom-labeling scheme is depicted in Figure 1, while selected bond lengths and angles are given in Table 1. The compound crystallized as a salt in the monoclinic system $P2_1/n$ with $Z = 4$, and is composed of a 1-(2-methoxyethyl)-1*H*-benzo[*d*]imidazol-3-ium cation and a *p*-toluenesulfonate anion.

The benzimidazole ring system is planar with a r.m.s. deviation of 0.011 Å. The 2-methoxyethyl substituent deviates from the plane of the benzimidazole ring, with the torsion angle of 90.7(2)° for C1–N2–C8–C9. Within the imidazole ring, the N1–C1 [1.311(2) Å] and N2–C1 [1.322(2) Å] bond lengths indicate a delocalized bonding, from N atoms to central C1 atom. The N1–C1–N2 bond angle of 110.62(17)° is clearly larger than the typical values of 104–107° known for NHCs.[36] The remaining bond lengths and angles fall within the range reported for related compounds.[37–39] The benzene ring in the *p*-toluenesulfonate anion makes dihedral angle of 64.92(6)° with the benzimidazole ring of the cation.

In the crystal structure of **2d**, the cations and anions are interconnected through one N–H···O and three C–H···O interactions to form a three-dimensional network. The geometric parameters of these interactions are given in Table 2 and a view of the packed structure is shown in Figure 2. The crystal structure is also stabilized by π···π stacking interactions between the benzimidazole ring systems in the cations at (*x*, *y*, *z*) and (−*x*, −*y*, 2 − *z*) with the corresponding ring-centroid separations being 3.6517(11) Å.

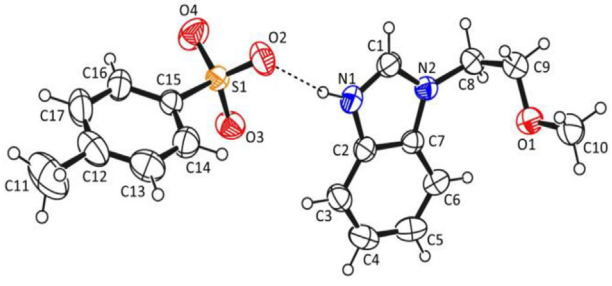


FIGURE 1 A view of **2d** showing the atom-numbering scheme. Displacement ellipsoids are drawn at the 40% probability level and dashed lines show the intermolecular hydrogen bond.

2.4 | CA inhibition

In the present study, we examined the inhibitory properties of **2a**–**l** and **3a**,**b** on the activity of hCA I and hCA II. IC_{50} values (for hydratase activity) and K_i values (for esterase activity) of all compounds are outlined in Table 3. During the enzyme inhibition assay, all compounds were dissolved in water. As seen from Table 3, all compounds inhibited the hCA I and hCA II and IC_{50} values for hydratase activity were found

TABLE 1 Selected geometric parameters for **2d**

Parameter	Value	Parameter	Value
Bond lengths (Å)			
S1—O2	1.4519(15)	N1—C1	1.311(2)
S1—O3	1.4327(14)	N1—C2	1.387(2)
S1—O4	1.4341(16)	N2—C1	1.322(2)
S1—C15	1.7682(19)	N2—C7	1.396(2)
O1—C9	1.396(2)	N2—C8	1.467(2)
O1—C10	1.408(3)		
Bond angles (°)			
O2—S1—O3	110.96(9)	C9—O1—C10	111.97(17)
O2—S1—O4	111.30(10)	N1—C1—N2	110.62(17)
O3—S1—O4	114.87(10)	C1—N1—C2	108.85(16)
O2—S1—C15	106.13(9)	C1—N2—C7	107.95(15)
O3—S1—C15	106.29(9)	N2—C8—C9	112.56(16)
O4—S1—C15	106.67(9)	C8—C9—O1	108.93(17)

TABLE 2 Hydrogen bonding geometry for **2d**

D—H···A	D—H (Å)	H···A (Å)	D···A (Å)	D—H···A (°)
N1—H1A···O2	0.86	1.92	2.764 (2)	167
C8—H8B··· O3^{i}	0.97	2.32	3.240 (2)	159
C1—H1···O2^{ii}	0.93	2.40	3.260 (2)	154
C10—H10B···O3^{iii}	0.96	2.60	3.475 (3)	151

Symmetry codes: ^{i} $-x+1, -y+1, -z+1$; ^{ii} $-x+1, -y+1, -z$; ^{iii} $-x+2, -y+1, -z+1$.

in the range of 134–745 μM for hCA I and 151–541 μM for hCA II. K_i values for esterase activity were determined in the range of 95–665 μM for hCA I and 113–477 μM for hCA II. The kinetic investigation revealed that all compounds inhibit hCA I and hCA II activity in a non-competitive manner (see Supporting Information for the Lineweaver–Burk plots). Hydrolysis of *p*-nitrophenyl acetate is not a physiological reaction and catalyzed under *in vitro* conditions by carbonic anhydrase. According to literature, non-competitive inhibition of esterase activity is very rare. Sarıkaya et al.[40] reported that some phenol derivatives inhibit the esterase activity in a non-competitive manner. Additionally, Beydemir and co-workers reported that some thiadiazole-substituted sulphonamides[41] and some anti-inflammatory agents (fluorometholone acetate and dexamethasone)[42] inhibit esterase activity in a non-competitive manner. The non-competitive inhibition of esterase activity is limited to these reports and our results are new contribution for the non-competitive inhibition of esterase activity.

The enzyme inhibition results revealed that iodide salts, **3a** and **3b** are two- or threefold more active for both hCA I and hCA II than their corresponding *p*-toluenesulfonate derivatives (**2d** and **2f**, respectively). This result clearly indicated that anions of organic salts contribute to the activity. Iodide derivative **3a** was found out as a most active compound for hCA I with IC_{50} of 134 μM for hydratase activity and K_i of 95 μM for esterase activity. When we compare the activities of *p*-toluenesulfonate salts for hCA I in order to understand the effects of substituents on benzimidazole core, some generalization can be made. Substitution on the nitrogen of benzimidazole decreased the inhibitory effect except for **2g** which has very close IC_{50} and K_i values to **2a**. Another significant differentiation was observed for *p*-toluenesulfonate salts containing benzyl derivatives. Electron donating methyl or methoxy-substituted salts, **2g**, **2h**, **2i**, **2k**, **2l**, performed better activity than not substituted benzyl bearing salt, **2f**. On the other hand, chlorine-substituted salt, **2j**, performed lower activity than **2f**. However, according to results observed for hCA II, it is difficult to reach any definite opinion about the effects of substituents.

All compounds examined in this study inhibited the activity of hCA I and hCA II, however, this inhibition level is low compared to the main class of CA inhibitors, sulfonamides and other reported types of inhibitors. In addition, previously reported *N*,*N*-disubstituted benzimidazolium salts which contain coumarin,[13] benzoxazinone,[14] phthalimide,[17] vinylbenzyl,[17] and hydroxyethyl[18] groups performed better CA inhibition in micromolar or submicromolar levels. On the other hand, good solubility of compounds both in water and organic solvents provides an advantage in medicinal evaluation.

Nowadays, research about the CA inhibition focused on the treatment of cancer. Carbonic anhydrase IX is a membrane-bound isoform and upregulated in many solid tumors, so plays a role in tumor progression and acidification.[27,31–33] This fact makes this isoform a target for anticancer agents. On the other hand, hCA II isoform is the main target in the treatment of glaucoma, however, inhibition of promiscuous hCA II by some carbonic anhydrase IX inhibitor anticancer agents causes some side effects.[27,31–33] Therefore, developing of the isoform selective hCA inhibitors gained great importance.

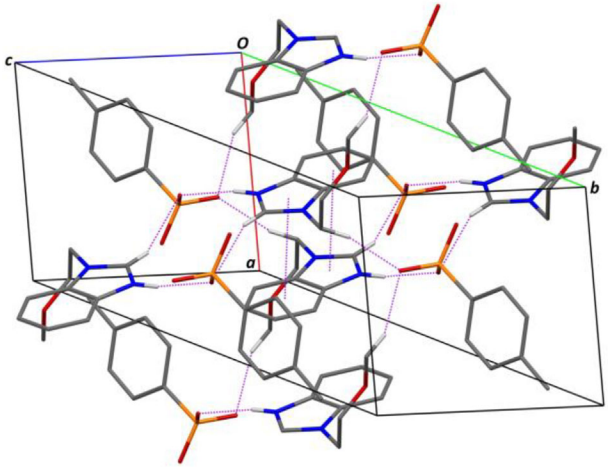


FIGURE 2 A part of the crystal packing of **2d** showing the intermolecular interactions as broken lines. For the sake of clarity, H atoms not involved in hydrogen bonding have been omitted.

3 | CONCLUSION

In summary, a new series of 1-substituted-1*H*-benzimidazolium *p*-toluenesulfonates, and two 1-substituted-1*H*-benzimidazolium iodides were synthesized and fully characterized. Crystal structure of **2d** showed that 1*H*-benzimidazolium cation and *p*-toluenesulfonate anion interconnect to each other by N-H···O and C-H···O hydrogen bonds. Inhibitory activities of all newly synthesized and four previously reported benzimidazolium salts were determined against hCA I and hCA II and all compounds inhibited the activity of hCA I and hCA II. Iodide salts were found as more active than their *p*-toluenesulfonate derivatives. More importantly, all compounds inhibited the esterase activity of hCA I and II in a non-competitive manner which is a rare case in literature.

TABLE 3 IC_{50} and K_i values of compounds for hCA I and hCA II

	hCA I		hCA II	
Compound	IC_{50} (μM) (hydratase)	K_i (μM)[a] (esterase)	IC_{50} (μM) (hydratase)	K_i (μM)[a] (esterase)
2a	182	179	276	220
2b	267	228	358	294
2c	484	350	160	158
2d	542	311	471	431
2e	367	256	147	127
2f	745	421	541	477
2g	171	133	179	136
2h	402	309	333	270
2i	251	221	216	131
2j	730	665	436	285
2k	239	221	248	226
2l	401	347	269	195
3a	134	95	164	144
3b	422	339	227	194

[a]Inhibition type is non-competitive.

Although inhibitory properties of compounds reported in this study against hCA I and hCA II activity is not in the satisfactory levels, in view of the selective inhibition of membrane-bound tumor-associated isoform carbonic anhydrase IX and strong solubility of compounds, these results may be an advantage and therefore, studies about the inhibitory properties of compounds on the activities of other isoforms of hCAs are in progress.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

Alkyl and benzyl halides, *p*-toluenesulfonic acid, *p*-toluenesulfonyl chloride and solvents were purchased from Aldrich Chemical Co. and used as received. Synthesis of benzimidazolium salts was carried out in a CEM Discover System microwave reactor. ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker UltraShield 300 operating at 300 MHz (^{1}H), 75 MHz (^{13}C) and Bruker Ascend™ 400 Avance III HD operating at 400 MHz (^{1}H), 100 MHz (^{13}C) FT spectrometers using $CDCl_3$ and DMSO-d_6 as solvent. Chemical shifts are given in ppm relative to tetramethylsilane (TMS). NMR multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, sex = sextet, br = broad, m = multiplet signal. Coupling constants, *J*, are given in Hz. Melting points were determined in open capillary tubes by Electrothermal-9200 melting point apparatus. IR spectra in the range of 4000–400 cm^{-1} were obtained in ATR Sampling Accessory with Perkin Elmer Spectrum 100 spectrophotometer. The C, H, N, and S elemental analysis data were determined by LECO CHNS-932 elemental analyzer at IBTAM (Inonu University Scientific and Technological Research Central). LC-MS spectra were recorded in an Agilent 1100 LC/MSD SL mass spectrometer equipped with an electrospray ion source at IBTAM.

The ^{1}H NMR, ^{13}C NMR, and LC-MS spectra of the investigated compounds can be found in the Supporting Information. The InChI codes of the investigated compounds together with some biological activity data are also provided as Supporting Information.

4.1.2 | General procedure for the synthesis of 1-substituted-1*H*-benzimidazolium *p*-toluenesulfonates 2a–l

1-Alkyl and 1-benzylbenzimidazole derivatives were synthesized according to previously reported method.[43] Benzimidazolium *p*-toluenesulfonates were synthesized by our previously described method.[34] *p*-Toluenesulfonic acid (0.9 g, 5.2 mmol) and 1-substituted-benzimidazole (5 mmol) were dissolved in ethanol (10 mL). The mixture was stirred under 200 W of microwave irradiation during 30 min without heating. After this period of time, solvent was evaporated under reduced pressure to half of initial volume. Approximately twice the last volume of diethyl ether was added to mixture. Colorless crystals were collected, washed three times with diethyl ether, and dried under reduced pressure. Compounds, **2f**, **2g**, **2i,** and **2j** were available from our previous study.[34]

1*H*-Benzimidazolium *p*-toluenesulfonate 2a

White solid, yield: 1.20 g (82%), mp: 215–216°C. Elemental analysis, calculated for $C_{14}H_{14}N_2O_3S$: C: 57.92, H: 4.86, N: 9.65, S: 11.04; found: C: 58.11, H: 4.94, N: 9.77, S: 10.90. LC-MS, calculated for cationic part, $C_7H_7N_2$, *m/z*: 119.06; found: 119.10. FT-IR (υ_{max}, cm^{-1}): 3184 (C-H aromatic), 2927 (C-H aliphatic), 2891 (C-H aliphatic), 2771 (N^+-H), 1455 (CN). ^{1}H NMR (400 MHz, DMSO-d_6, 298 K): δ N^+-H signal was not detected, 9.60 (s, 1H, NC*H*N), 7.87 (dd, 2H, $H_{Ar\text{-}benzimidazole}$, *J* = 3.1 Hz), 7.59 (dd, 2H, $H_{Ar\text{-}benzimidazole}$, *J* = 3.1 Hz), 7.55 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 7.9 Hz), 7.14 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 7.9 Hz), 2.29 (s, 3H, $H_{CH3\text{-}PhSO3}$). ^{13}C NMR (100 MHz, DMSO-d_6, 298 K): δ 145.7, 141.7 (NCHN), 138.5, 130.9, 128.7, 126.6, 126.0, 114.9, 21.2 (CH_3PhSO_3).

1-Ethyl-1*H*-benzimidazolium *p*-toluenesulfonate 2b

White solid, yield: 1.40 g (88%), mp: 122–123°C. Elemental analysis, calculated for $C_{16}H_{18}N_2O_3S$: C: 60.36, H: 5.70, N: 8.80, S: 10.07; found: C: 60.59, H: 5.88, N: 8.91, S: 9.87. LC-MS, calculated for cationic part, $C_9H_{11}N_2$, *m/z*: 147.09; found: 147.10. FT-IR (υ_{max}, cm^{-1}): 3211 (C-H aromatic), 3191 (C-H aromatic), 2930 (C-H aliphatic), 2477 (N^+-H), 1452 (NC). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ N^+-H signal was not detected, 9.89 (s, 1H, NC*H*N), 7.98–7.93 (m, 1H, $H_{Ar\text{-}benzimidazole}$), 7.86 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.1 Hz), 7.61–7.49 (m, 3H, $H_{Ar\text{-}benzimidazole}$), 7.14 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.1 Hz), 4.56 (q, 2H, $H_{NCH2CH3}$, *J* = 7.3 Hz), 2.32 (s, 3H, $H_{CH3\text{-}PhSO3}$), 1.58 (t, 3H, $H_{NCH2CH3}$, *J* = 7.3 Hz). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 142.5, 141.0 (NCHN), 140.0, 131.5, 130.7, 128.9, 126.7, 126.3, 125.9, 116.3, 111.7, 42.4 (-NCH_2CH_3), 21.3 (CH_3PhSO_3), 14.9 (-NCH_2CH_3).

1-(*n*-Butyl)-1*H*-benzimidazolium *p*-toluenesulfonate 2c

White solid, yield: 1.45 g (84%), mp: 111–112°C. Elemental analysis, calculated for $C_{18}H_{22}N_2O_3S$: C: 62.40, H: 6.40, N: 8.09, S: 9.25; found: C: 62.61, H: 6.44, N: 8.23, S: 9.10. LC-MS, calculated for cationic part, $C_{11}H_{15}N_2$, *m/z*: 175.12; found: 175.10. FT-IR (υ_{max}, cm^{-1}): 3135 (C-H aromatic), 2537 (N^+-H), 1450 (NC). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ N^+-H signal was not detected, 9.89 (s, 1H, NC*H*N), 7.98–7.95 (m, 1H, $H_{Ar\text{-}benzimidazole}$), 7.86 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.1 Hz), 7.59–7.56 (m, 1H, $H_{Ar\text{-}benzimidazole}$), 7.53–7.49 (m, 2H, $H_{Ar\text{-}benzimidazole}$), 7.15 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.1 Hz), 4.50 (t, 2H, $H_{NCH2CH2CH2CH3}$, *J* = 7.4 Hz), 2.32 (s, 3H, $H_{CH3\text{-}PhSO3}$), 1.88 (quin, 2H, $H_{NCH2CH2CH2CH3}$, *J* = 7.5 Hz), 1.33 (sex, 2H, $H_{NCH2CH2CH2CH3}$, *J* = 7.6 Hz), 0.90 (t, 2H, $H_{NCH2CH2CH2CH3}$, *J* = 7.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 142.5, 141.3 (NCHN), 140.0, 131.5, 130.9, 128.9, 126.6, 125.9, 116.3, 111.8, 47.0 ($NCH_2CH_2CH_2CH_3$), 31.4 ($CH_2CH_2CH_2CH_3$), 21.3 (CH_3PhSO_3), 19.7 ($NCH_2CH_2CH_2CH_3$), 13.5 ($NCH_2CH_2CH_2CH_3$).

1-(2-Methoxyethyl)-1*H*-benzimidazolium *p*-toluenesulfonate 2d

White solid, 1.30 g (72%), mp: 149–150°C. Elemental analysis, calculated for $C_{17}H_{20}N_2O_4S$: C: 58.60, H: 5.79, N: 8.04, S: 9.20; found: C: 58.84, H: 5.88, N: 8.07, S: 9.02. LC-MS, calculated for cationic part, $C_{10}H_{13}N_2O$, *m/z*: 177.10; found: 177.10. FT-IR (υ_{max}, cm^{-1}): 3138 (C-H aromatic), 2928 (C-H aliphatic), 2603 (N^+-H), 1452 (N=C). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 15.57 (br, 1H, N^+-*H*), 9.68 (s, 1H, NC*H*N), 7.87–7.81 (m, 1H, $H_{Ar\text{-}benzimidazole}$), 7.77 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.1 Hz), 7.61–7.57 (m, 1H, $H_{Ar\text{-}benzimidazole}$), 7.45–7.39 (m, 2H, $H_{Ar\text{-}benzimidazole}$), 7.08 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.0 Hz), 4.61 (t, 2H, $H_{NCH2CH2OCH3}$, *J* = 4.7 Hz), 3.70 (t, 2H, $H_{NCH2CH2OCH3}$, *J* = 4.6 Hz), 3.19 (s, 3H, $H_{NCH2CH2OCH3}$), 2.26 (s, 3H, $H_{CH3\text{-}PhSO3}$). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 142.3, 141.5 (NCHN), 140.2, 131.6, 131.1, 128.9, 126.6, 126.2, 126.0, 115.9, 112.6, 70.4 ($NCH_2CH_2OCH_3$), 59.0 ($NCH_2CH_2OCH_3$), 47.2 ($NCH_2CH_2OCH_3$), 21.3 (CH_3PhSO_3).

1-(2,2-Dimethoxyethyl)-1*H*-benzimidazolium *p*-toluenesulfonate 2e

White solid, 1.10 g (58%), mp: 143–144°C. Elemental analysis, calculated for $C_{18}H_{22}N_2O_5S$: C: 57.13, H: 5.86, N: 7.40, S: 8.47; found: C: 57.32, H: 5.98, N: 7.62, S: 8.18. LC-MS, calculated for cationic part, $C_{11}H_{15}N_2O_2$, *m/z*: 207.11; found: 207.10. FT-IR (υ_{max}, cm^{-1}): 3046 (C-H aromatic), 2953 (C-H aliphatic), 2604 (N^+-H), 1450 (NC). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 15.69 (br, 1H, N^+-*H*), 9.75 (s, 1H, NC*H*N), 7.87–7.82 (m, 1H, $H_{Ar\text{-}benzimidazole}$), 7.76 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.1 Hz), 7.61–7.56 (m, 1H, $H_{Ar\text{-}benzimidazole}$), 7.46–7.40 (m, 2H, $H_{Ar\text{-}benzimidazole}$), 7.08 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.0 Hz), 4.65 (t, 1H, $H_{NCH2CH(OCH3)2}$, *J* = 4.4 Hz), 4.55 (d, 2H, $H_{NCH2CH(OCH3)2}$, *J* = 4.4 Hz), 3.33 (s, 6H, $H_{NCH2CH(OCH3)2}$), 2.26 (s, 3H, $H_{CH3\text{-}PhSO3}$). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 142.2, 142.0 (NCHN), 140.2, 131.9, 131.1, 128.9, 126.5, 126.2, 125.9, 115.8, 112.8, 55.8 ($NCH_2CH(OCH_3)_2$), 48.7 ($NCH_2CH(OCH_3)_2$), 35.1 ($NCH_2CH(OCH_3)_2$), 21.3 (CH_3PhSO_3).

1-(3-Methylbenzyl)-1*H*-benzimidazolium *p*-toluenesulfonate 2h

White solid, 1.75 g (89%), mp: 114–115°C. Elemental analysis, calculated for $C_{22}H_{22}N_2O_3S$: C: 66.98, H: 5.62, N: 7.10, S: 8.13; found: C: 66.70, H: 5.88, N: 7.20, S: 8.08. LC-MS, calculated for cationic part, $C_{15}H_{15}N_2$, *m/z*: 223.12; found: 223.10. FT-IR (υ_{max}, cm^{-1}): 3134 (C-H aromatic), 2569 (N^+-H), 1442 (NC). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ N^+-H signal was not detected, 9.98 (s, 1H, NC*H*N), 7.98–7.91 (m, 1H, H_{Ar}), 7.83 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.1 Hz), 7.50–7.39 (m, 3H, H_{Ar}), 7.21–7.13 (m, 1H, H_{Ar}), 7.14–7.04 (m, 5H, H_{Ar} and $H_{Ar\text{-}CH3PhSO3}$), 5.65 (s, 2H, $H_{NCH2Ph\text{-}3\text{-}CH3}$), 2.30 (s, 3H, $H_{CH3\text{-}PhSO3}$). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 142.4, 141.5 (NCHN), 140.1, 139.2, 132.9, 131.6, 130.9, 129.8, 129.1, 128.9, 128.5, 126.7, 126.4, 125.9, 125.0, 116.3, 112.5, 51.0 ($NCH_2Ph\text{-}3\text{-}CH_3$), 21.3 ($CH_3PhSO_3$ and $NCH_2Ph\text{-}3\text{-}CH_3$ overlapped).

1-(4-Methoxybenzyl)-1*H*-benzimidazolium *p*-toluenesulfonate 2k

White solid, 1.70 g (83%), mp: 179–180°C. Elemental analysis, calculated for $C_{22}H_{22}N_2O_4S$: C: 64.37, H: 5.40, N: 6.82, S: 7.81; found: C: 64.21, H: 5.51, N: 6.70, S: 7.94. LC-MS, calculated for cationic part, $C_{15}H_{15}N_2O$, *m/z*: 239.12; found: 239.10. FT-IR (υ_{max}, cm^{-1}): 3146 (C-H aromatic), 2615 (N^+-H), 1446 (NC). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ N^+-H signal was not detected, 9.90 (s, 1H, NC*H*N), 7.97–7.91 (m, 1H, $H_{Ar\text{-}benzimidazole}$), 7.84 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, *J* = 8.1 Hz), 7.52–7.42 (m, 3H, $H_{Ar\text{-}benzimidazole}$), 7.27 (d, 2H, $H_{Ar\text{-}Ph\text{-}4\text{-}}$

$_{OCH3}$, J = 8.7 Hz), 7.13 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, J = 8.1 Hz), 6.83 (d, 2H, $H_{Ar\text{-}Ph\text{-}4\text{-}OCH3}$, J = 8.7 Hz), 5.61 (s, 2H, $H_{CH2Ph\text{-}4\text{-}OCH3}$), 3.76 (s, 3H, $H_{CH2Ph\text{-}4\text{-}OCH3}$), 2.32 (s, 3H, $H_{CH3\text{-}PhSO3}$). ^{13}C NMR (75 MHz, $CDCl_3$, 298 K): δ 160.1, 142.4, 141.2 (NCHN), 140.1, 131.7, 130.9, 129.6, 128.9, 126.7, 126.4, 126.0, 124.8, 116.3, 114.7, 112.4, 55.3 (NCH_2Ph-4-OCH_3), 50.6 (NCH_2Ph-4-OCH_3), 21.3 (CH_3PhSO_3).

1-(3,4,5-Trimethoxybenzyl)-1*H*-benzimidazolium *p*-toluenesulfonate 2l

White solid, 2.10 g (89%), mp: 180–181°C. Elemental analysis, calculated for $C_{24}H_{26}N_2O_6S$: C: 61.26, H: 5.57, N: 5.95, S: 6.81; found: C: 61.18, H: 5.60, N: 5.84, S: 6.88. LC-MS, calculated for cationic part, $C_{17}H_{19}N_2O_3$, *m/z*: 299.14; found: 299.10. FT-IR (υ_{max}, cm^{-1}): 3149 (C-H aromatic), 2933 (C-H aliphatic), 2591 (N^+-H), 1460 (N═C). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ N^+-H signal was not detected, 10.06 (s, 1H, NC*H*N), 7.94–7.88 (m, 1H, $H_{Ar\text{-}benzimidazole}$), 7.82 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, J = 8.1 Hz), 7.56–7.43 (m, 3H, $H_{Ar\text{-}benzimidazole}$), 7.10 (d, 2H, $H_{Ar\text{-}CH3PhSO3}$, J = 8.0 Hz), 6.61 (s, 2H, $H_{Ar\text{-}Ph\text{-}3,4,5\text{-}(OCH3)3}$), 5.65 (s, 2H, $H_{NCH2Ph\text{-}3,4,5\text{-}(OCH3)3}$), 3.79 (s, 3H, $H_{Ph\text{-}3,4,5\text{-}(OCH3)3\text{-}p\ position}$), 3.73 (s, 3H, $H_{Ph\text{-}3,4,5\text{-}(OCH3)3\text{-}m\ position}$), 2.30 (s, 3H, $H_{CH3\text{-}PhSO3}$). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 153.7, 142.4, 141.6 (NCHN), 140.2, 138.3, 131.6, 130.9, 128.9, 128.7, 126.7, 126.4, 125.9, 116.2, 112.5, 105.4, 60.8 (Ph-3,4,5-$(OCH_3)_3$-*p* position), 56.3 (Ph-3,4,5-$(OCH_3)_3$-*m* position), 51.2 (NCH_2Ph-3,4,5-$(OCH_3)_3$), 21.3 (CH_3PhSO_3).

4.1.3 | General procedure for the synthesis of 1-substitue-1*H*-benzimidazolium iodides 3a,b

Compound **2d** or **2g** (0.6 mmol) and NaI (1 mmol) were stirred in 10 mL of methanol at room temperature during 24 h. After this period of time, the mixture was filtered through Celite® and diethyl ether (10 mL) was added to the mixture and colorless crystals were collected by filtration. Crystals were washed three times with diethyl ether (3 × 5 mL) and dried under reduced pressure.

1-(2-Methoxyethyl)-1*H*-benzimidazolium iodide 3a

White solid, 0.14 g (77%), mp: 213–214°C. Elemental analysis, calculated for $C_{10}H_{13}IN_2O$: C: 39.49, H: 4.31, N: 9.21; found: C: 39.63, H: 4.40, N: 9.02. FT-IR (υ_{max}, cm^{-1}): 2947 (C-H aliphatic), 2354 (N^+-H), 1441 (N═C). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 12.36 (br, 1H, N^+-H), 9.87 (s, 1H, NC*H*N), 8.12–8.06 (m, 1H, H_{Ar}), 7.81–7.75 (m, 1H, H_{Ar}), 7.62 (m, 2H, H_{Ar}), 4.84 (t, 2H, $H_{NCH2CH2OCH3}$, J = 4.7 Hz), 3.92 (t, 2H, $H_{NCH2CH2OCH3}$, J = 4.8 Hz), 3.35 (s, 3H, $H_{NCH2CH2OCH3}$).

1-Benzyl-1*H*-benzimidazolium iodide 3b

White solid, 0.16 g (79%), mp: 166–167°C. Elemental analysis, calculated for $C_{14}H_{13}IN_2$: C: 50.02, H: 3.90, N: 8.33; found: C: 50.21, H: 3.94, N: 8.17. FT-IR (υ_{max}, cm^{-1}): 3187 (C-H aromatic), 2996 (C-H aliphatic), 2610 (N^+-H), 1439 (N═C). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ N^+-H signal was not detected, 10.12 (s, 1H, NC*H*N), 8.01–7.97 (m, 1H, H_{Ar}), 7.53–7.25 (m, 8H, H_{Ar}), 5.80 (s, 2H, N_{CH2Ph}). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 139.7 (NCHN), 132.4, 131.0, 130.5, 129.5, 129.4, 128.3, 127.3, 126.9, 115.7, 112.9, 51.5 (NCH_2Ph).

4.2 | X-ray analysis

The single crystal X-ray diffraction data of **2d** were undertaken on a STOE-IPDS II diffractometer using graphite monochromated MoKα radiation in ω-scanning mode. Data collection and cell refinement were carried out using X-AREA[44] while data reduction was applied using X-RED32.[44] The structure was solved by a dual-space algorithm using SHELXT-2014[45] and refined with full-matrix least-squares calculations on F^2 using SHELXL-2018[46] implemented in WinGX[44] program suit. All hydrogen atoms were located in calculated positions as riding atoms with C–H = 0.93 (aromatic), 0.97 (methylene) and 0.96 Å (methyl), and with $U_{iso}(H) = 1.2U_{eq}$ ($1.5U_{eq}$ for CH_3). Crystal data, data collection, and structure refinement details are tabulated in Supporting Information Table S1. The molecular graphics were drawn by using ORTEP-3[47] and Mercury.[48]

4.3 | *In vitro* CA inhibition assay

4.3.1 | Preparation of hemolysate and purification from blood red cells

Blood samples (25 mL) were taken from healthy human volunteers. They were anticoagulated with acid-citrate-dextrose, centrifuged at 1000*g* for 20 min at 4°C and the supernatant was removed. The packed erythrocytes were washed three times with 0.9% NaCl and then hemolysed in cold water. The ghosts and any intact cells were removed by centrifugation at 3100*g* for 25 min at 4°C, and the pH of the hemolysate was adjusted to pH 8.5 with solid Tris-base. The 25 mL hemolysate was applied to an affinity column containing L-tyrosine-sulfonamide-Sepharose-4B[49] equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.5). The affinity gel was washed with 50 mL of 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.5). The human CA (hCA) isozymes were then eluted with 0.1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), which recovered hCA I and II, respectively. Fractions (3 mL) were collected and their absorbance was measured at 280 nm.

4.3.2 | Hydratase activity assay

Carbonic anhydrase activity was measured by the Wilbur and Anderson method, which is based on the determination of the time required for the pH to decrease from 10.0 to 7.4 due to CO_2 hydration.[50] The assay solution was 0.5 M Na_2CO_3/0.1 M $NaHCO_3$ (pH 10.0) and Phenol Red was added as the pH indicator. CO_2-hydratase activity [enzyme units (EU)] was calculated using the equation t_0–t_c/t_c, where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

4.3.3 | Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenyl-acetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (Biotek Power Wave XS) according to the method described in the literature.[51] The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M Tris-SO_4 buffer (pH 7.4), 1 mL of 3 mM 4-nitrophenylacetate, 0.5 mL H_2O, and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of the benzimidazolium salts were examined. All compounds were tested in triplicate at each concentration level. Different concentrations of the compounds were used.

4.3.4 | *In vitro* inhibition studies

For the inhibition studies of benzimidazolium salts, different concentrations of these compounds were added to the enzyme. Activity percentage values of CA for different concentrations of each salt were determined by regression analysis using Microsoft Office 2000 Excel (Microsoft, Redmond, WA). CA enzyme activity without a benzimidazolium salt solution was accepted to be 100% activity. Inhibitory effects of compounds **2a**–**l** and **3a**,**b** on enzyme activities were tested under *in vitro* conditions; K_i values given in Table 3 were calculated from the Lineweaver–Burk graphs by using five substrate concentrations range 0.5–1.4 mM as control and two different inhibitor concentrations indicated in the graphs.[52]

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

CCDC 1824660 contains the supplementary crystallographic data for the compound reported in this article. These data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Fax: +44 1223 336 033, e-mail: deposit@ccdc.cam.ac.uk, https://www.ccdc.cam.ac.uk/structures/].

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