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Pyrazolylbenzo[*d*]imidazoles as new potent and selective inhibitors of carbonic anhydrase isoforms hCA IX and XII

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Abstract

Novel pyrazolylbenzo[*d*]imidazole derivatives (**2a-2f**) were designed, synthesized and evaluated against four human carbonic anhydrase isoforms belonging to α family comprising of two cytosolic isoforms hCA I and II as well as two transmembrane tumor associated isoforms hCA IX and XII. Starting from these derivatives that showed high potency but low selectivity in favor of tumor associated isoforms hCA IX and XII, we investigated the impact of removing the sulfonamide group. Thus, analogs **3a-3f** without sulfonamide moiety were synthesized and biological assay revealed a good activity as well as an excellent selectivity as inhibitors for tumor associated hCA IX and hCA XII and the same was analyzed by molecular docking studies.

Key words: Carbonic anhydrase isoforms, pyrazolylbenzo[*d*]imidazoles, acetazolamide, inhibition constant (K_i)

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) constitute ubiquitous family of metalloenzymes containing Zn^{2+} at their active sites and are widely distributed in living organisms. CAs are particularly responsible for catalyzing the interconversion of carbon dioxide and water to produce bicarbonate and protons playing a crucial role in various physiological processes such as respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH and CO_2 homeostasis, electrolyte secretion, biosynthetic reactions such as gluconeogenesis, lipogenesis and ureagenesis, bone resorption, calcification, tumorigenicity and many other physiologic or pathologic processes.[1-9]

Out of 15 different isoforms of α-class of human associated CAs, hCA I and II are the cytosolic forms distributed in eye, kidney, central nervous system and inner ear and have been

targeted for clinically used diuretics, antiglaucoma and anticonvulsant drugs.[10-12] hCA IX is a multidomain protein existing in dimeric form being stabilized by a disulfide bond and is induced by hypoxic environment in tumor cells being mediated by the hypoxia- inducible transcription factor-1 (HIF-1). hCA XII is also induced by hypoxia but its induction by HIF-1 is not yet well established.Since hCA IX and XII have been established to contribute to pH regulation of tumor cells, cell proliferation, cell adhesion and malignant cell invasion, they have been considered as valuable markers for cancer and are being targeted for designing anticancer drugs.[13-16] Furthermore, specifically targeting hCA IX and XII over the physiologically relevant isoforms hCA I and II can be a promising strategy in developing anticancer drugs with minimum side effects.

Primary sulfonamides containing aromatic/heterocyclic compounds are the most widely investigated category of carbonic anhydrase inhibitors (CAIs) that led to the development of new classes of pharmaceutical agents including clinically used CAIs like acetazolamide(AZA), methazolamide(MZA), ethoxzolamide(EZA)etc (Figure 1).Over the years, azoles also constitute immensely important members of the biologically active aromatic heterocycles due to their presence in various CA inhibitors.[17] Recently, benzimidazole pharmacophore has been extensively studied as an important scaffold for the development of novel anticancer agents.[18,19] Omeprazole, pantoprazole, lansoprazole, rabeprazole drugs bearing benzimidazole nucleus in their structure have been reported to exhibit excellent anticancer activities besides having antiulcer properties (Figure 1).

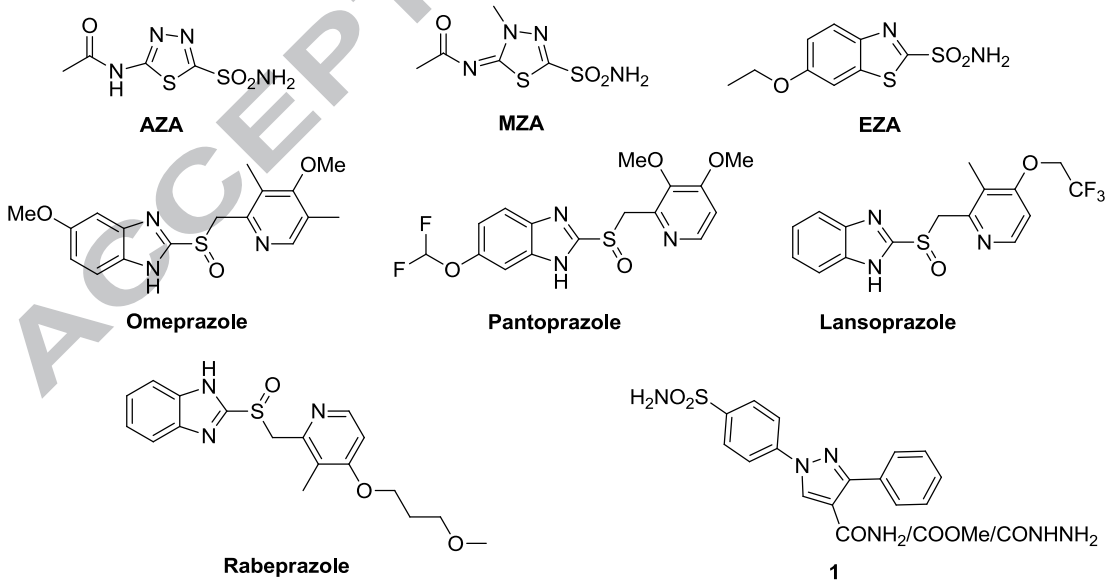


Figure 1. Some clinically used CA drugs and potential lead compounds

In addition to benzimidazoles, pyrazoles are also known to be more potent and selective for tumor targeting CA isoforms. Recently, our research group has reported the 4-functionalized pyrazoles **1** which were found to be selective CA inhibitors against tumor isoforms hCA IX and XII.[20-23]Motivated by the these findings coupled with our ongoing interest in heterocyclic scaffolds of medicinal interest,[24-30] we envisioned to synthesize pyrazolylbenzo[*d*]imidazoles **2a-2f** bearing a benzenesulfonamide moiety as novel scaffold for CA inhibition profile.Excellent inhibition but poor selectivity profile of **2a-2f** prompted us to study the effect of removing the sulfonamide group (**3a-3f**) on CA inhibition potency as well as selectivity which was further analyzed by docking studies.

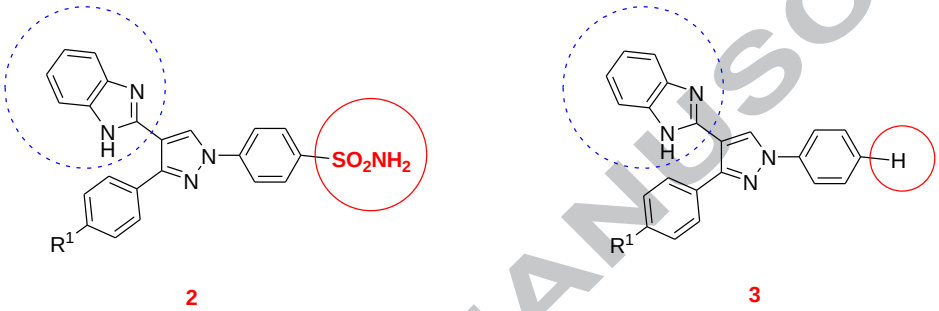


Figure 2. Pyrazolylbenzo[*d*]imidazoles (**2a-2f** and **3a-3f**) as CA inhibitors

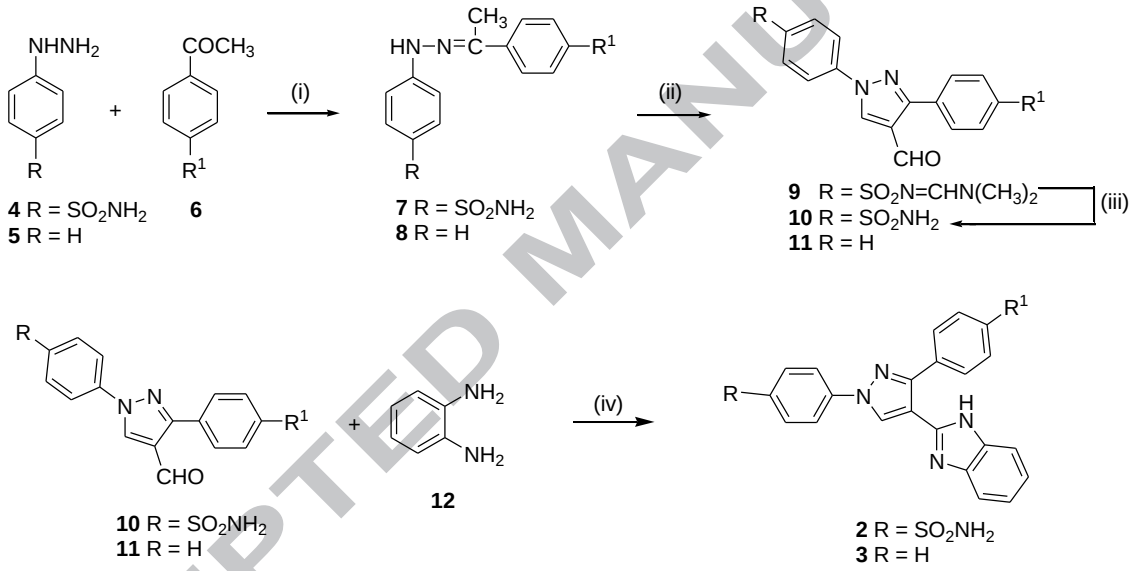
The molecular docking of compound **2a** having free SO_2NH_2 moiety in the active site of hCA XII was performed in order to find out the possible binding interactions with target enzymes. The molecular docking of compound **3a**(without sulfonamide moiety) against hCA I, II, IX and XII was also performed in order to find out the possible binding interactions of the selected compound in the target enzymes which throw some light into the high selectivity of compounds **3** for tumor associated isozymes hCA IX and XII when compared to compounds **2**.

2. Results and discussion

2.1. Chemistry

Synthesis of desired pyrazolylbenzo[*d*]imidazoles (**2**)has been accomplished by the condensation of appropriate 4-formylpyrazoles **10** with *ortho*-phenylenediamine (**12**) in refluxing DMF/H_2O using oxone as an oxidant (Scheme 1).Formylpyrazoles **10** in turn were prepared starting from appropriate acetophenones and phenylhydrazines following our earlier reported procedure.[28] In order to study the effect of free sulfonamide moiety on activity and selectivity pattern, analogs **3** (without SO_2NH_2 moiety) were also designed and synthesized following a similar protocol as depicted in Scheme 1. It is worthwhile to mention here that synthesis and characterization of all

these pyrazolylbenzo[*d*]imidazoles (**2** and **3**) completed in our labs in 2013. While CA inhibition studies and molecular docking studies of these compounds were underway,Reddy et al[31] reported the synthesis of a few pyrazolylbenzo[*d*]imidazoles (**3a**, **3c**, **3d**, **3e**) although via a different route using sodium metabisulphite. 4-Aminosulfonyl group present at *para* position of aromatic ring attached to N-1 of pyrazole ring in case of hydrazones **7** also got protected under Vilsmeier Haack conditions to afford intermediate **9** which was deprotected under basic conditions in THF-MeOH to yield 4-formylpyrazoles **10** having free 4-aminosulfonyl group.[28] The spectral data of target compounds **2** and **3** were in full agreement with their proposed structures. IR spectra of these compounds exhibits characteristic absorption band at ~ 3148 cm^{-1}, 1597 cm^{-1} and ~ 1512 cm^{-1}corresponding to N-H stretch, C=N stretch and N-H bending respectively.



Substitution pattern of pyrazolylbenzo[*d*]imidazoles (**2** and **3**) and intermediates

Compound	**R¹**					
	H	CH_3	OCH_3	F	Cl	Br
4 or **2**,**7**,**10** (R = SO_2NH_2), **3**,**8**,**11**(R = H) **9** (R = $SO_2N{=}CHN(CH_3)_2$)	**a**	**b**	**c**	**d**	**e**	**f**

Scheme 1. Synthesis of pyrazolylbenzo[*d*]imidazoles (**2** and **3**)**:** (i) Ethanol-water, CH_3COOH (catalytic), reflux, 30 min; (ii) $POCl_3$/DMF, 50-60 °C, 5-6h; (iii) NaOH/MeOH/THF overnight; (iv) oxone/DMF/H_2O, reflux 2-3h.

Further, in case of compounds **2** a set of characteristic SO_2 stretching bands appeared at ~ 1335 cm^{-1} and 1157 cm^{-1}. C_5-H proton of the pyrazole ring resonates in the range δ 9.05-9.30 as a singlet while a set of doublet of doublets corresponding to benzimidazole ring protons appeared at ~ δ 7.60 and ~ δ 7.25 in ^{1}H NMR spectra of these compounds.^{13}C NMR and mass spectral data further supported and confirmed the structures of the target compounds **2** and **3**.

2.2. Biological evaluation

2.2.1. In vitro carbonic anhydrase inhibition assay

All the newly synthesized compounds **2a-2f** and **3a-3f** were screened for their CA activity by stopped-flow, CO_2 hydrase assay against hCA I, II, IX and XII isoforms. CA inhibitory profile of all newly synthesized compounds, including **2a-2f** possessing sulfonamide group and, **3a-3f** without sulfonamide moiety against four human carbonic anhydrase isoforms hCA I, II, IX and XII was assayed by using stopped flow assay method[32]and results obtained regarding CA inhibition profile were presented in Table 1. Acetazolamide (AZA) was chosen as reference for the CA assay. Biological assay data of the newly synthesized compounds **2a-2f** and **3a-3f** provides interesting results.

The pyrazolylbenzo[*d*]imidazole molecules possessing the sulfonamide zinc binding group (**2a-2f**) showed a nanomolar activity against all the tested CAs. All the compounds showed a very similar activity against hCA I (K_i values between 32.4 and 87.6 nM) and hCA II (K_i values between 1.5 and 4.2 nM). For hCA IX compounds **2c** and **2f** showed a marked decrease of activity (K_i values of 554.0 and 641.5 nM, respectively) whereas the other four compounds showed K_i values from 24.9 to 74.7. Finally, with regards to hCA XII, compounds **2a** and **2e** showed a high potency, about ten-fold higher than that of compound **2b**. In terms of selectivity these compounds did not possess a good profile, as shown in Table 1, the most promising compounds seemed to be **2a** and **2e**, that showed only a good level of hCA XII selectivity against hCA I.

The removal of the sulfonamide zinc binding group determined an unexpected change of the biological results. Very interestingly, all the compounds showed a general inactivity against hCA I and hCA II, whereas they were able to maintain inhibition activity in the nanomolar range against both hCA IX and hCA XII. Independently on the R^1 substituent all the compounds were highly selective and as shown in Table 1 all six compounds were active hCA IX and hCA XII inhibitors with a selectivity ratio greater than 400 fold with respect to the cytosolic isoform hCA I and hCA II. Overall, the analysis of the biological activity data (with respect to potency and selectivity) allowed us to highlight that the removal of the sulfonamide zinc binding group for these pyrazolylbenzo[*d*]imidazole molecules produced astonishing results in terms of selectivity for the tumor associated transmembrane isoforms hCA IX and hCA XII. A possible explanation of this behavior was investigated via *in silico* docking experiments using currently available crystal structures of several CA isoforms.

Table 1. Carbonic anhydrase activity of pyrazolylbenzo[*d*]imidazoles **2** and **3** using stopped-flow, CO_2 hydrase assay.

Compounds	K_i (nM)*				Selectivity ratio			
	hCA **I**	hCA **II**	hCA **IX**	hCA **XII**	**I/IX**	**I/XII**	**II/IX**	**II/XII**
2a	33.9	2.7	24.9	0.9	1.36	37.66	0.10	3.0
2b	37.6	2.3	49.3	9.1	0.76	4.13	0.046	0.25
2c	32.4	4.2	554.0	5.5	0.058	5.89	0.0075	0.76
2d	35.0	1.5	74.7	3.7	0.46	9.45	0.20	0.40
2e	87.6	2.5	70.2	1.0	1.24	87.6	0.035	2.5
2f	33.4	1.9	641.5	7.6	0.052	4.39	0.0029	0.25
3a	>40000	>40000	55.9	7.3	>716	>5479	>716	>5479
3b	>40000	>40000	23.3	7.2	>1717	>5556	>1717	>5556
3c	>40000	>40000	48.7	9.4	>821	>4255	>821	>4255
3d	>40000	>40000	65.0	7.8	>615	>5128	>5128	>5128
3e	>40000	>40000	7.5	17.7	>5333	>2260	>5333	>2260
3f	33550	>40000	55.1	72.9	608.8	460.2	>726	>549
AZA	250	12.1	25	5.7	10.0	43.9	0.5	2.2

AZA = acetazolamide, reference compound, a standard sulfonamide CAI, is also provided for comparison.
* Mean from 3 different assays, errors were in the range of ± 5-10 % of the reported value.

2.3. Docking studies

The presence of the sulfonamide group in position 4 of the phenyl ring determines a nanomolar inhibitory activity for all the four carbonic anhydrase subtypes. As shown in Figure 3, the binding disposition of **2a** into hCA XII is driven by the sulfonamide portion of the molecule that acted as a zinc binding group, also showing two additional H-bond interactions with Thr227. The phenylpyrazole scaffold of the compound showed lipophilic interactions with V147 and L225, the benzimidazole system showed a H-bond interaction with Q117 whereas the phenyl ring was directed towards the solvent exposed region of the enzyme without highlighting important interactions.

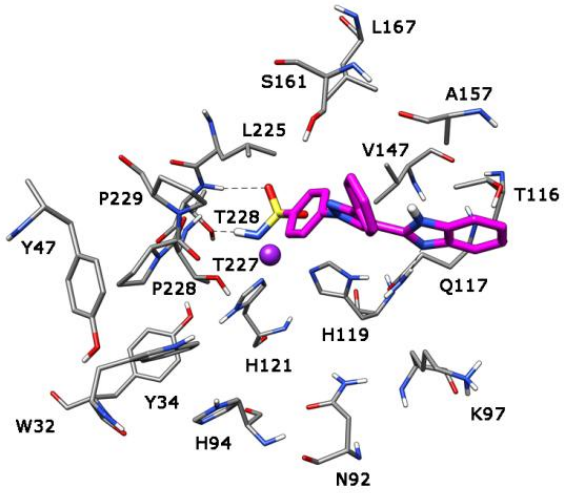


Figure 3. Docking analysis of compound **2a** into hCA XII

Compound **3a**, which correspond to one of the most promising ligands reported in this series, was docked using AUTODOCK 4.0 into a minimized structure of hCA I, II,IX and XII. Figure 4 shows the docking of the compound into the four enzymes. In the hCA XII binding site, the 3-phenyl ring of **3a** showed a π-π stacking interaction with H119, the 1-phenyl ring was inserted into a lipophilic cleft mainly delimited by T116, V147, A157, S161, L167 and L225, whereas the benzimidazole system was directed towards the solvent and showed an H-bond with K97 (Figure 4A). As shown in Figure 4B, the compound showed into the hCA IX a binding disposition very similar to that observed for hCA XII, with the π-π stacking interaction between H226 and the 3-phenyl ring, the lipophilic interactions of the 1-phenyl ring into the lipophilic cleft mainly delimited by L223,V253, V262, L266, L272 and L331 and the H-bond between the benzimidazole system of **3a** and Q203 that corresponded to the K97 in hCA XII. In the hCA II binding site the lipophilic cavity in which the 1-phenyl ring interacted in hCA IX and hCA XII was partially occluded by the non-conserved F130 and for this reason the compound showed a completely different binding mode with the lack of the above described interactions and both the 3-phenyl and benzimidazole rings that were located near the solvent exposed entrance of the binding site. A very similar analysis could also be carried out for the interaction of **3a** into hCA I; the lipophilic cavity in which the 1-phenyl ring interacted in hCA IX and hCA XII was partially occluded by the non-conserved F92 determining the impossibility for the compound to interact in this region. Furthermore, the presence of the non conserved H201 that replaced a threonine residue (T227 in hCA XII)prevented the π-π stacking interaction between H95 of the 3-phenyl ring. As a result, the compound was located near the entrance of the binding site with the two phenyl rings that were exposed to the solvent showing only lipophilic interactions with W6 and P203.

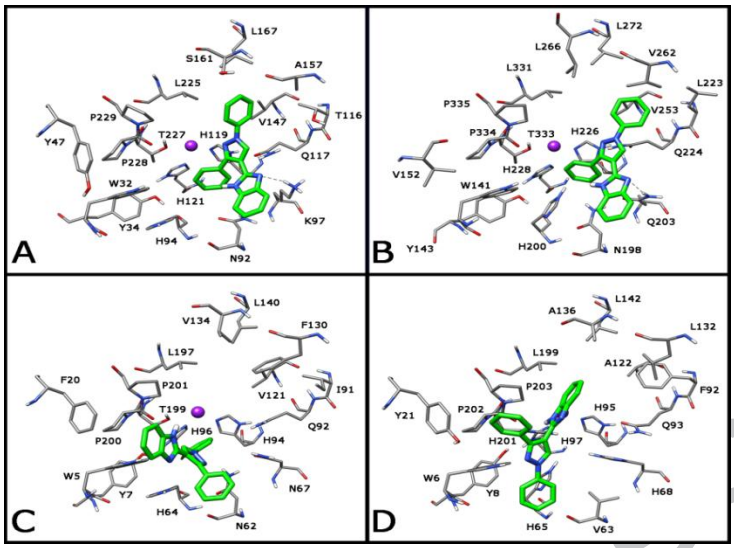


Figure 4. Docking analysis of compound **3a** into hCA XII (A), hCA IX (B), hCA II (C) and hCA I (D).

3. Conclusions

In the present study we have designed and synthesized two series of compounds bearing benzimidazole as well as pyrazole ring in the same molecular frame and *in vitro* evaluated them against four human carbonic anhydrase isoforms representing two cytosolic isoforms (hCA I and II) and two transmembrane tumor associated (hCA IX and XII) isoforms. In general, compounds **2a-2f** bearing a sulfonamide group exhibited excellent to good CA inhibition against all the four isoforms used in the present investigation. Furthermore, removing the sulfonamide group from these compounds generated analogs **3a-3f** that were found to be ineffective against cytosolic isoforms hCA I and II as indicated from their high inhibition constant values ($K_i \geq 33550$)and still active against hCA IX and XII, thus rendering these compounds highly selective inhibitors of hCA IX and XII over hCA I and II. The molecular docking study of compound **3a** into the four CA isoforms suggested that the removal of the sulfonamide zinc binding group allowed a high level of freedom in terms of ligand disposition inside the binding site. In the hCA IX and hCA XII binding sites the interaction of this compound seemed to be guided by π-π stacking and lipophilic interactions inside binding site clefts. Due to the presence of non-conserved residues, the same binding disposition could not be reproduced in the hCA I and II, thus suggesting a possible explanation of the selectivity showed by this class of compounds. Finally, the results of this study could also suggest that the removal of the sulfonamide zinc binding group could also be useful for other classes of compounds.

4. Experimental protocols

Melting points were determined in open glass capillaries in an electrical melting point apparatus and are uncorrected. The infrared (IR) spectra were recorded on ABB MB 3000 DTGS IR instrument using the KBr pellet technique. ^{1}H NMR and ^{13}C NMR spectra were recorded either in pure DMSO-d_6 or in $CDCl_3$ on Bruker NMR spectrometers at 300 MHz and 75.5 MHz respectively using tetramethylsilane (TMS) as internal standard. Chemical shifts are expressed in δ, ppm. ^{1}H NMR data are reported in order: multiplicity (br, broad; s, singlet; d, doublet; t, triplet; m, multiplet; ex, exchangeable by D_2O), approximate coupling constant in Hertz,number of protons and type of protons.The purity of the compounds was checked by ^{1}H NMR and thin layer chromatography (TLC) on silica gel plates using a mixture of petroleum ether and ethyl acetate as eluent. Iodine or UV lamp was used as a visualizing agent. Mass spectra were recorded on JEOL-AccuTOF JMS-T100LC Mass spectrometer having DART (*Direct Analysis in Real Time*) source in ES^+ mode.

4.1 General procedure for synthesis of 2-(1,3-diaryl-1*H*-pyrazol-4-yl)-1*H*-benzo[*d*]imidazoles (**2** and **3**)

To a solution of 4-formylpyrazole **10**[28],**11**[33,34] (1 mmol) and *ortho*-phenylenediamine **12**(1.2 mmol) in 10 ml of dimethylformamide was added oxone (1 mmol) and 1 mL of water. The reaction mixture was refluxed for 1-2 h when colour of reaction changed from greenish to reddish brown. The course of reaction was monitored by thin layer chromatography. Reaction mixture was poured in water whereupon a brown solid precipitated out. The solid so precipitated out was filtered, washed with hot water and dried to afford the target compound in good yield.

2-{1-(4-aminosulfonylphenyl)-3-phenyl-1*H*-pyrazol-4-yl}-1*H*-benzo[*d*]imidazole (2a)

m.p. 130-132 °C, yield 86%; IR (cm^{-1}): 1597 (s, C=N stretch), 1504 (N-H bend), 1335 & 1157 (s, SO_2 stretch); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.21 (s, 1H, C_5-H pyrazol), 8.19 (d, *J* = 8.4 Hz, 2H, Ar-H) , 8.04 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.90-7.88 (m, 2H, Ar-H), 7.60 (dd, *J* = 6.0, 3.3 Hz, 2H, Benzim-H), 7.49 (s, ex, 2H, SO_2NH_2), 7.45-7.43 (m, 3H, Ar-H), 7.24 (dd, *J* = 6.0, 3.3 Hz, 2H, Benzim-H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 151.7, 145.6, 142.5, 141.5, 138.8, 132.2, 131.4, 129.2, 128.8, 128.6, 128.0, 122.7, 119.1, 115.3, 113.5; DART MS: *m/z* 416 ($[M+H]^+$), $C_{22}H_{17}N_5O_2SH^+$Calcd. 416.

2-{1-(4-aminosulfonylphenyl)-3-(4-methylphenyl)-1*H*-pyrazol-4-yl}-1*H*-benzo[*d*]imidazole (2b)

m.p. 192-194 °C, yield 87%; IR (cm^{-1}): 1597 (s, C=N stretch), 1512 (N-H bend), 1335 & 1157 (s, SO_2 stretch); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.20 (s, 1H, C_5-H pyrazol), 8.17 (d, *J* = 8.7 Hz, 2H, Ar-H), 8.02 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.76 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.60 (dd, *J* = 6.0, 3.3 Hz, 2H,

Benzim-H), 7.49 (s, ex, 2H, SO_2NH_2), 7.27-7.23 (m, 4H, Ar-H, Benzim-H), 2.35 (s, 3H, CH_3); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 151.7, 145.6, 142.5, 141.5, 138.6, 131.4, 129.4, 128.5, 127.9, 127.2, 122.7, 119.0, 115.2, 113.3, 21.3 (CH_3); DART MS: *m/z* 430 ($[M+H]^+$), $C_{23}H_{19}N_5O_2SH^+$ Calcd.430.

2-{1-(4-aminosulfonylphenyl)-3-(4-methoxyphenyl)-1*H*-pyrazol-4-yl}-1*H*-benzo[*d*]imidazole (2c)

m.p. 172-174 °C, yield 83%; IR (cm^{-1}): 1597 (s, C=N stretch), 1512 (s, N-H bend), 1366 & 1157 (s, SO_2 stretch); ^1H NMR (300 MHz, DMSO-d_6): δ 9.18 (s, 1H, C_5-H pyrazol), 8.16 (d, *J* = 8.7 Hz, 2H, Ar-H), 8.02 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.85 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.60 (dd, *J* = 6.0, 3.3 Hz, 2H, Benzim-H), 7.48 (s, ex, 2H, SO_2NH_2), 7.24 (dd, *J* = 6.0, 3.0 Hz, 2H, Benzim-H), 7.01 (d, *J* = 8.7 Hz, 2H, Ar-H), 3.79 (s, 3H, OCH_3); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 160.2, 151.4, 145.7, 142.4, 141.5, 131.4, 130.0, 127.9, 124.6, 122.7, 118.9, 115.2, 114.2, 113.1, 55.6 (OCH_3); DART MS: *m/z* 446 ($[M+H]^+$), $C_{23}H_{19}N_5O_3SH^+$Calcd. 446.

2-{1-(4-aminosulfonylphenyl)-3-(4-flourophenyl)-1*H*-pyrazol-4-yl}-1*H*-benzo[*d*]imidazole (2d)

m.p. 202-204 °C, yield 90%; IR (cm^{-1}): 1597 (s, C=N stretch), 1512 (N-H bend), 1336 & 1157 (s, SO_2 stretch); ^1H NMR (300 MHz, DMSO-d_6): δ 9.22 (s, 1H, C_5-H pyrazol), 8.17 (d, *J* = 8.1 Hz, 2H, Ar-H), 8.04 (m, 4H, Ar-H), 7.59 (m 2H, Ar-H), 7.49 (s, ex, 2H, SO_2NH_2), 7.32-7.24 (m, 4H, Ar-H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 162.9 (d, $^1J_{CF}$ = 246.9 Hz), 150.7, 145.5, 142.6, 141.4, 131.4, 130.9 (d, $^3J_{CF}$ = 8.3 Hz), 128.8, 128.0, 122.6, 119.0, 115.6 (d, $^2J_{CF}$ = 21.9 Hz), 113.6; DART MS: *m/z* 434 ($[M+H]^+$), $C_{22}H_{16}N_5O_2SFH^+$Calcd. 434.

2-{1-(4-aminosulfonylphenyl)-3-(4-chlorophenyl)-1*H*-pyrazol-4-yl}-1*H*-benzo[*d*]imidazole(2e)

m.p. 188-190 °C, yield 84%; IR (cm^{-1}): 1597 (C=N stretch), 1512 (N-H bend), 1335 & 1157 (s, SO_2 stretch); ^1H NMR (300 MHz, DMSO-d_6): δ 9.22 (s, 1H, C_5-H pyrazol), 8.17 (d, *J* = 9.0 Hz, 2H, Ar-H), 8.03 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.95 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.60 (dd, *J* = 6.0, 3.3 Hz, 2H, Ar-H), 7.53 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.50 (s, ex, 2H, SO_2NH_2), 7.24 (dd, *J* = 6.0, 3.0 Hz, 2H, Ar-H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 150.4, 145.4, 142.7, 141.4, 133.9, 131.5, 131.2, 130.5, 128.8, 128.0, 122.7, 119.1, 115.2, 113.7; DART MS: *m/z* 450/452 ($[M+H/M+2+H]^+$), $C_{22}H_{16}N_5O_2SClH^+$Calcd. 450/452.

2-{1-(4-aminosulfonylphenyl)-3-(4-bromophenyl)-1*H*-pyrazol-4-yl}-1*H*-benzo[*d*]imidazole(2f)

m.p. 178-180 °C, yield 79%; IR (cm^{-1}): 1597 (s, C=N stretch), 1504 (N-H bend), 1335 & 1157 (s, SO_2 stretch); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.22 (s, 1H, C_5-H pyrazol), 8.17 (d, J = 8.4 Hz, 2H, Ar-H), 8.03 (d, J = 8.7 Hz, 2H, Ar-H), 7.89 (d, J = 8.4 Hz, 2H, Ar-H), 7.66 (d, J = 8.4 Hz, 2H, Ar-H), 7.59 (dd, J = 5.7, 3.0 Hz, 2H, Ar-H), 7.50 (s, ex, 2H, SO_2NH_2), 7.23 (dd, J = 5.7, 3.0 Hz, 2H, Ar-H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 150.5, 145.4, 142.7, 141.4, 131.7, 131.4, 130.8, 128.1, 128.0, 122.7, 119.1, 115.4, 113.2; DART MS: *m/z* 494/496 ($[M+H/M+2+H]^+$), $C_{22}H_{16}N_5O_2SBrH^+$Calcd. 494/496.

2-(1,3-phenyl)-1*H*-pyrazol-4-yl)-1*H*-benzo[*d*]imidazole (3a)

m.p. 188-190 °C, yield 84%; IR (cm^{-1}): 1597 (s, C=N stretch), 1504 (m, N-H bend); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.11 (s, 1H, C_5-H pyrazol), 7.98 (d, J = 7.8 Hz, 2H, Ar-H), 7.89 (d, J = 6.9 Hz, 2H, Ar-H), 7.61-7.59 (m, 4H, Ar-H, Benzim-H), 7.44-7.42 (m, 4H, Ar-H), 7.24 (dd, J = 5.7, 3.3 Hz, 2H, Benzim-H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 150.7, 145.6, 142.4, 141.2, 138.5, 132.6, 131.1, 129.7, 128.7, 128.6, 128.1, 122.7, 119.1, 115.3, 113.5; DART MS: *m/z* 337 ($[M+H]^+$), $C_{22}H_{16}N_4H^+$Calcd. 337.

2-(1-phenyl-3-(4-methylphenyl)-1*H*-pyrazol-4-yl)-1*H*-benzo[*d*]imidazole (3b)

m.p. 136-138 °C, yield 88%; IR (cm^{-1}): 3148 (m, N-H stretch), 2978 (m, C-H stretch) 1597 (s, C=N stretch), 1504 (m, N-H bend); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.19 (s, 1H, C_5-H pyrazol), 7.96 (d, J = 7.8 Hz, 2H, Ar-H), 7.71 (d, J = 8.1 Hz, 2H, Ar-H), 7.66 (dd, J = 6.1, 3.3 Hz, 2H, Benzim-H), 7.58 (d, J = 7.5 Hz, 2H, Ar-H), 7.44 (d, J = 7 2 Hz, 2H, Ar-H), 7.22 (dd, J = 6.0, 3.3 Hz, 2H, Benzim-H), 7.25 (d, J = 7.8 Hz, 2H, Ar-H), 2.34 (s, 3H, Ar-CH_3): ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 151.2, 145.3, 139.3, 138.6, 131.8, 130.2, 129.5, 129.3, 128.4, 127.7, 123.8, 119.2, 115.0, 21.3 (CH_3); DART MS: *m/z* 351 ($[M+H]^+$), $C_{23}H_{18}N_4H^+$Calcd. 351.

2-(1-phenyl-3-(4-methoxyphenyl)-1*H*-pyrazol-4-yl)-1*H*-benzo[*d*]imidazole (3c)

m.p. 142-144 °C, yield 85%; IR (cm^{-1}): 1597 (s, C=N stretch), 1512 (N-H bend); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.05 (s, 1H, C_5-H pyrazol), 7.96 (d, J = 8.1 Hz, 2H, Ar-H), 7.86 (d, J = 8.7 Hz, 2H, Ar-H), 7.61-7.57 (m, 4H, Ar-H, Benzim-H), 7.44-7.33 (m, 2H, Ar-H), 7.22 (dd, J = 6.0, 3.3 Hz, 2H, Benzim-H), 7.00 (d, J = 8.7 Hz, 2H, Ar-H), 3.80 (s, 3H, OCH_3): ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 159.9, 150.8, 149.3, 146.1, 139.3, 130.2, 129.8, 129.3, 127.5, 122.6, 119.2, 114.2, 112.5, 111.9, 55.5 (OCH_3); DART MS: *m/z* 367 ($[M+H]^+$), $C_{23}H_{18}N_4OH^+$Calcd. 367.

2-(1-phenyl-3-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-1*H*-benzo[*d*]imidazole (3d)

m.p. 260-262 °C, yield 82%; IR (cm^{-1}): 3148 (m, N-H stretch), 1597 (s, C=N stretch), 1512 (N-H bend); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.30 (s, 1H, C_5-H pyrazol), 7.96 (d, J = 7.5 Hz, 2H, Ar-H), 7.91-7.86 (m, 2H, Ar-H), 7.70 (dd, 6.0, 3.0 Hz, 2H, Benzim-H), 7.61 (t, J = 7.5 Hz, 2H, Ar-H),

7.47-7.44 (m, 1H, Ar-H), 7.22 (dd, *J* = 6.0, 3.0 Hz, 2H, Benzim-H), 7.30 (t, *J* = 8.7 Hz, 2H, Ar-H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 162.9 (d, $^{1}J_{CF}$ = 246.9 Hz), 150.4, 146.1, 141.9, 139.1, 135.3, 132.2, 130.8 (d, $^{3}J_{CF}$ = 8.3 Hz), 130.3, 128.0, 124.6, 119.4, 116.1 (d, $^{2}J_{CF}$ = 21.1 Hz), 114.8, 112.2; DART MS: *m/z* 355 ([M+H]$^+$), $C_{22}H_{15}N_4FH^+$Calcd. 355.

2-(1-phenyl-3-(4-chlorophenyl)-1*H*-pyrazol-4-yl)-1*H*-benzo[*d*]imidazole (3e)

m.p. 168-170 °C, yield 86%; IR (cm^{-1}): 3101 (m, N-H stretch), 1597 (C=N stretch), 1504 (N-H bend); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.12 (s, 1H, C_5-H pyrazole), 7.97 (d, *J* = 8.1 Hz, 4H, Ar-H), 7.61-7.57 (m, 4H, Ar-H, SO_2NH_2), 7.51 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.44-7.33 (m, 2H, Ar-H), 7.22 (dd, *J* = 6.0, 3.3 Hz, 2H, Benzim-H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 150.5, 145.3, 142.6, 141.3, 133.9, 131.5, 131.1, 130.5, 128.8, 128.0, 122.8, 119.1, 115.4, 113.6; DART MS: *m/z* 371/373 ([M+H/M+2+H]$^+$), $C_{22}H_{15}N_4ClH^+$Calcd. 371/373.

2-(1-phenyl-3-(4-bromophenyl)-1*H*-pyrazol-4-yl)-1*H*-benzo[*d*]imidazole (3f)

m.p. 184-188 °C, yield 80%; IR (cm^{-1}): 3140 (N-H, stretch), 1597 (C=N stretch), 1512 (N-H bend); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.13 (s, 1H, C_5-H pyrazol), 7.98 (m, 4H, Ar-H), 7.61-7.57 (m, 4H, Ar-H), 7.51 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.46-7.39 (m, 1H, Ar-H), 7.25 (dd, *J* = 6.0, 3.3 Hz, 2H, Benzim-H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 149.8, 145.6, 139.3, 133.7, 131.4, 131.0, 130.4, 130.2, 128.7, 127.7, 122.8, 119.2, 118.9, 115.2, 112.5; DART MS: *m/z* 415/417 ([M+H/M+2+H]$^+$), $C_{22}H_{15}N_4BrH^+$Calcd. 415/417

4.2 CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The unanalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min–72 h at room temperature (15 min) or 4 °C (all other incubation times) prior to assay, in order to allow for the formation of the E–I complex. Data from Table 1 were obtained after 15 min incubation as there was no difference of inhibitory power when the enzyme and inhibitors were kept for longer periods in incubation.[35]The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as

reported earlier,[36] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[37-40]

4.3 Docking methodologies.

The crystal structures of hCA I (pdb code 1AZM[41]), hCA II (pdb code 3HSA[42]), hCA IX (pdb code 3IAI[43]), and hCA XII (pdb code 1JD0[44]) were taken from the Protein Data Bank.[45] After adding hydrogen atoms and removing complex ligands, the four proteins were minimized using Amber 11[46] software and parm03 force field at 300 K. The four proteins were placed in a rectangular parallelepiped water box, an explicit solvent model for water, TIP3P, was used and the complexes were solvated with a 10 Å water cap. Sodium ions were added as counter ions to neutralize the system. Two steps of minimization were then carried out; in the first stage, we kept the protein fixed with a position restraint of 500 kcal/mol•Å^2 and we solely minimized the positions of the water molecules. In the second stage, we minimized the entire system through 5000 steps of steepest descent followed by conjugate gradient (CG) until a convergence of 0.05 kcal/Å•mol. Automated docking was carried out by means of the AUTODOCK 4.0 program;[47] Autodock Tools[48] was used in order to identify the torsion angles in the ligand, add the solvent model and assign the Kollman atomic charges to the protein. The ligand charge was calculated using the Gasteiger method.A grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant were used for the energetic map calculations. Using the Lamarckian Genetic Algorithm, the docked compound was subjected to 100 runs of the Autodock search, using 500000 steps of energy evaluation and the default values of the other parameters. Cluster analysis was performed on the results using an RMS tolerance of 2.0 Å and the best docked conformation was taken into account.

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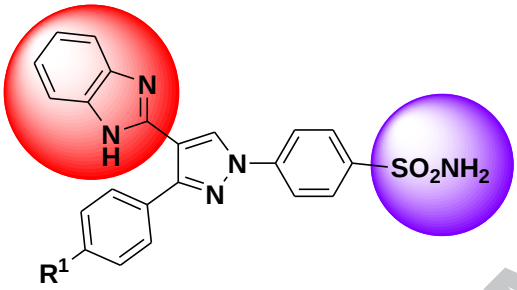
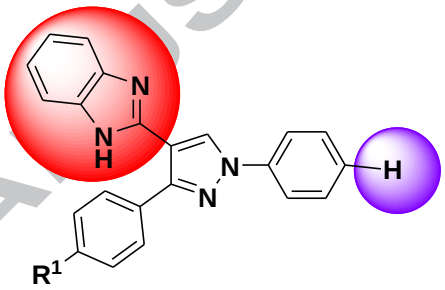
Graphical Abstract



Excellent inhibition against all isoforms hCA I, II, IX and XII
poor selectivity

2



Good inhibition against isoforms hCA IX and XII
high selectivity

3

Research Highlights

- 12 novel pyrazolylbenzo[*d*]imidazoles were designed and synthesized.
- Their inhibition potential was evaluated against hCA I, II, IX and XII.
- Compounds having sulfonamide moiety were better inhibitors with poor selectivity.
- Selectivity against tumor isoforms hCA IX abd XII enhanced on removing sulfonamide.
- Inhibition results were promising as compared to antitumor drug acetazolamide.