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Graphical abstract

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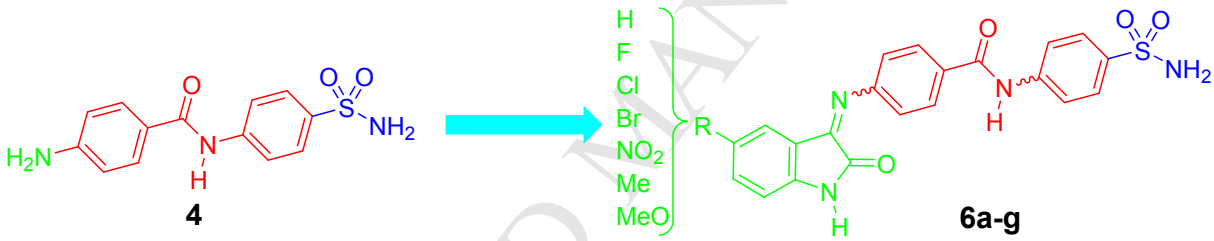
Two series of amido and ureido-sulfonamides conjugated with isatins were synthesized and evaluated for their inhibitory activity against a panel of hCAs; I, II (cytosolic) and IX, XII (transmembrane)



K_I **hCA XII = 2.7** nM

0.47 nM < K_I **hCA XII** < **1.76** nM

Amido/ureidosubstituted benzenesulfonamides-isatin conjugates as low nanomolar/subnanomolar inhibitors of the tumor-associated carbonic anhydrase isoform XII

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Abstract. By using a molecular hybridization approach, two series of amido/ureidosubstituted benzenesulfonamides incorporating substituted-isatin moieties were synthesized. The prepared derivatives were *in vitro* evaluated for their inhibitory activity against human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms I, II (cytosolic) and IX, XII (transmembrane, tumor-associated) isoforms. All these isoforms were inhibited in variable degrees by the sulfonamides reported here. hCA I was inhibited with K_Is in the range of 7.9–894 nM, hCA II in the range of 7.5–1645 nM (with one compound having a K_I > 10 μM); hCA IX in the range of 5.0–240 nM, whereas hCA XII in the range of 0.47–2.83 nM. As all these isoforms are involved in various pathologies, in which their inhibition can be exploited therapeutically, the derivatives reported here may represent interesting extensions to the field of CA inhibitors of the sulfonamide type.

Keywords: Carbonic anhydrase; tumor-associated isoforms; amido/ureido sulfonamides; isatin.

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1-Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in all life kingdoms [1,2]. They catalyze the efficient interconversion between CO_2 and bicarbonate, being involved in crucial physiologic processes. Most mammals, including humans, possess two blood isoforms denominated with a total concentration of these proteins as high as 0.2 mM [1]. The catalytic activity of the human (h) isoform hCA I (slow blood enzyme) is much lower compared to that of hCA II and in addition hCA I is inhibited by the chloride and bicarbonate present in the plasma, leaving a lot of questions regarding the physiologic function of this isoform [1,2]. The high activity isoform hCA II (rapid blood enzyme) which is one of the best catalysts known in nature, is involved in the secretion of electrolytes in a multitude of tissues, such as the bicarbonate rich aqueous humor in the anterior chamber of the eyes, the cerebrospinal fluid and in pH and CO_2 homeostasis all over the body [1,2]. Dysregulation of the activity of these two isoforms in one or more tissues has important pathologic consequences, such as glaucoma and oedema [1,2]. There are several types of cancer, in which CA II was observed to be overexpressed - alone or together with other isoforms such as CA IX and XII [1,2]. However most solid tumors overexpress two transmembrane isoforms, hCA IX and XII, which are involved in tumor progression and metastases formation [1,2]. Thus, many of the hCA isoforms are validated drug targets for various applications, such as diuretics, anticonvulsants, antiglaucoma agents, and antitumor/antimetastatic drugs [1,2].

Benzenesulfonamides incorporating an amido or ureido moiety represent interesting classes of CA inhibitors (CAIs) [1-5]. Akdemir *et al*. [3] developed a new class of substituted-phenylacetamido benzenesulfonamides as potent inhibitors of the four physiologically relevant hCA isoforms, (hCA I, II, IX and XII). Interestingly, the K_I values were in the nanomolar range for the tumor-associated hCA IX/XII, in particular, compound **I** has excellent inhibitory action against hCA XI (0.32 nM) and hCA XII (0.64 nM), Figure 1.

Figure 1

Moreover, incorporation of urea functionality in the aromatic/heterocyclic sulfonamides represents the most recent and promising trend in the design of CAIs with selectivity profile for inhibiting the transmembrane, tumor associated isoforms (CAs IX and XII) over the off-target cytosolic CAs I and II [6-15]. SLC-0111 **II**, Figure 1, an ureido benzenesulfonamide derivative discovered by one of our groups, is currently in clinical evaluations. SLC-0111 **II** is characterized by

its selectivity towards inhibition of the transmembrane isoforms hCA IX/XII (over the cytosolic isoforms hCA I/II), which attributable to the presence of the ureido functionality as linker between the benzene sulfonamide fragment (which coordinates in deprotonated form at the sulfamoyl group to the zinc ion from the CA active site) and the tail of the inhibitor. This ureido linker allows a great flexibility to the tail of the molecule which imparts the possibility for the inhibitor to adopt a variety of orientations when bound within the enzyme active site. These orientations allow the specific interactions between the inhibitor tail and amino acid residues at the entrance of the active site cavity, which is the most variable region in the various a-CA isoforms [16-22]. SLC-0111 **II** was also able to block human breast cancer invasion, delay tumor growth and diminish the cancer stem cell population in vivo. Furthermore, combination of SLC-0111 **II** with paclitaxel significantly inhibits tumor growth and attenuates spontaneous lung metastasis in this aggressive orthotopic breast cancer model [23]. Besides, recent study [24] has reported 2,4-dichloro-5-(3-(4-sulfamoylphenyl) ureido)benzenesulfonamide (**III)** as a potent CA IX and XII inhibitor with K_I values of 15.8 and 3.6 nM, respectively, Figure 1.

Isatin is a privileged heterocyclic scaffold, as its derivatives possesses interesting biological activity profiles and are well-tolerated in humans [25]. In 2015, a series of 2/3/4-[(2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)amino]benzenesulfonamides was synthesized, showing low nanomolar inhibitory activity against the tumor associated CA isoforms IX and XII. Compound **IV** was one of the potent analogs within this series [26]. In addition, the isatin-based 1,3,4-thiadiazole-2-thione derivative **V** was found to inhibit the tumor-associated hCA IX with K_I value of 1.25 μM [27], Figure 1.

VEGFR inhibitors restrain tumor growth by suppressing tumor angiogenesis and blocking the developing vasculature of tumors. Regrettably, resistance is common and individual responses are variable; some patients exhibiting resistance from the outset (intrinsic resistance) and others going on to develop resistance following a short time of disease control (acquired resistance) [28-31]. Recent studies have highlighted the possibility that anti-angiogenic therapy induces an increasingly hypoxic tumor microenvironment, thence, increased expression of hypoxia-regulated genes and proteins, including CA IX and XII, in response to treatment was observed. For example, CA-IX expression correlates with poor prognosis in most tumor types and with worse outcome in bevacizumab-treated metastatic colorectal cancer patients, malignant astrocytoma and recurrent malignant glioma [32-34]. Continuing our interest in developing compounds which inhibit enzymes/proteins overexpressed in hypoxic tumors, here we report two series of benzenesulfonamides-isatin conjugates **6a-g** and **10a-g** incorporating amido or ureido linkers, which

have been assayed for the inhibition of cytosolic and transmembrane CA isoforms involved in various pathologies.

2-Results and Discussion

2.1. Chemistry

The synthetic pathways employed to prepare the targeted sulfonamides are depicted in schemes 1 and 2. Firstly, the reaction of 4-nitrobenzoyl chloride (**1**) with 4-aminobenzenesulfonamide (**2**) in THF at ambient temperature in the presence of TEA afforded the corresponding 4-nitro-*N*-(4-sulfamoylphenyl)benzamide (**3**) (Scheme 1). Reduction of the 4-nitrobenzamide derivative **3** using Pd/C resulted in the formation of 4-amino-*N*-(4-sulfamoylphenyl)benzamide (**4**) in 85% yield. 4-((2-Oxoindolin-3-ylidene)amino)-*N*-(4-sulfamoylphenyl)benzamides **6a-g** were prepared *via* refluxing 4-amino-*N*-(4-sulfamoylphenyl)benzamide **4** with isatins **5a-g**, respectively, in glacial acetic acid (Scheme 1).

Scheme 1

Next, 1-isocyanato-4-nitrobenzene (**7**) was treated with 4-aminobenzenesulfonamide (**2**) in acetonitrile under reflux to give 4-(3-(4-nitrophenyl)ureido)benzenesulfonamide (**8**). Reduction of the latter over Pd/C produced 4-(3-(4-aminophenyl)ureido)benzenesulfonamide (**9**) in 81% yield. Finally, the reaction of compound **9** with isatins **5a-g** afforded 4-(3-(4-((2-oxoindolin-3-ylidene)amino)phenyl)ureido)benzenesulfonamides **10a-g**, respectively (Scheme 2).

Scheme 2

The structures of amides **6a-g** and ureas **10a-g** were confirmed under the basis of their spectral data and elemental analyses. It is worthy to mention that ^{1}H NMR spectra of these compounds revealed their presence as *cis/trans* conformers and *E/Z* geometrical isomers due to the amide group and exocyclic -C=N- double bond, respectively (Figure 2) [35-38]. Moreover, it was reported that the compounds containing imine bond are present in higher percentage as *E* isomer in dimethyl-d_6 sulfoxide solution. According to the literature, the ratio of the isomers is solvent-dependent [37, 38]. However, the spectral data of **6a-g** and **10a-g**, in our hands, is similar to that reported for isatin imine *E/Z* isomers [26] and isatin amide *cis/trans* conformers [39]. The latter facts noticed for some compounds of amides **6a-g**

and urea derivatives **10a-g** through the appearance of additional NMR signals due to their isomerization. We could identify some proton signals of isomers especially for NHs in ^{1}H NMR spectra whereas the overlapping of symmetrical carbon signals of isomers appeared in several cases in ^{13}CNMR spectra. For example, ^{1}H and ^{13}C NMR spectra of amide **6d** revealed the signals of protons and carbons of its possible isomers whereas ^{1}H NMR spectra of amides **6f** and **6g** appeared their isomerization through the signals of their 2NH protons. ^{1}H NMR spectra of urea derivatives **10d** and **10g** showed their isomerization through the integration and position of their three NHs (isatin NH and urea 2NHs), respectively. For example, ^{1}H NMR spectrum of **10d** showed two sets of signals for its three D_2O exchangeable NHs with the same ratio of isomerization at 8.89 and 8.95 (s, 1H, NH urea), 9.08 (s, 1H, NH urea), 10.96 and 11.71 (s, 1H, NH isatin), respectively.

Figure 2

2.2. Carbonic anhydrase inhibition

Sulfonamides **4**, **9**, **6a-g** and **10a-g** were screened for their ability to inhibit four physiologically relevant hCA isoforms, hCA I, II (cytosolic) as well as hCA IX and XII (transmembrane, tumor-associated isoforms). Table 1 shows inhibition data of the prepared conjugates and the sulfonamide acetazolamide **AAZ** (as a standard inhibitor) against the four isoforms [42]. The following structure-activity relationship (SAR) should be noted regarding the inhibition data of Table 1:

(i) Isoform hCA I was inhibited by the prepared conjugates reported here with K_Is ranging between 7.9 and 894 nM. Concerning activity of the amido analogs **4** and **6a-g**, all of them showed better activity (K_Is: 7.9–95.6 nM) than that of the standard drug **AAZ** (K_I of 250 nM against this isoform). Among them, compounds **6b-6f** emerged as efficient hCA I inhibitors with K_Is values of 9, 9.2, 7.9, 10.2 and 8.5 nM, respectively, with more than 25-fold increased activity than **AAZ**. As the precise physiologic function of hCA I is largely unknown, these inhibitors may be valuable tools for investigating this enzyme in more details. The effective inhibitors discovered here incorporated the amide linker and the following substituents at the isatin ring: 5-halogens (F, Cl, Br), 5-nitro and 5-methyl. All these derivatives were around one order of magnitude better hCA I inhibitors compared to **6a** which has hydrogen at the isatin ring, proving that substitution of isatin at 5-position led to dramatic differences of biological activity. The compounds incorporating the ureido linker **9** and

10a-g were on the other hand less effective as hCA I inhibitors, as reported in the original series of derivatives to which SLC-0111 belongs [17, 18]. In fact these derivatives showed inhibition constants in the range of 45.2–894 nM (Table 1).

Table 1

(ii) Isoform hCA II was effectively inhibited by most sulfonamides reported here, except **10d** (K_I > 10 μM) and **10b**, **10c**, **10e** and **10g** (K_Is in the range of 585–1645 nM) (Table 1). It may be thus observed that among the ureido derivatives reported here only **9** and **10f** were potent hCA II inhibitors (K_I of 15.7 and 33 nM, respectively) whereas all other derivatives were weak or ineffective inhibitors of this isoform, similar to the uerido-benzenesyulfonamides described earlier by one of our groups [17,18]. This behavior has also been explained from the structural viewpoint, since in the X-ray crystal structures of five such inhibitors bound to hCA II, a very high flexibility of the arylureido tail has been evidenced, which led to very diverse orientations of the inhibitor within the active site. As a consequence of this flexibility, the inhibitors belonging to this chemotype may participate in a multitude of favorable and unfavorable interactions with amino acid residues from the active site, leading thus to a versatile inhibitory behavior [17,18]. The compounds incorporating the amide linker, **4** and **6a-6g** showed a better hCA II inhibitory action. Incorporation of unsubstituted isatin moiety led to compound **6a** with moderate activity against hCA II (K_I of 116 nM), but its congeners incorporating halogens, nitro, methyl and methoxy substituents at the 5-position of isatin ring were much more effective (K_Is in the range of 7.5–14.5 nM, Table 1).

(iii) The transmembrane isoform hCA IX was inhibited by all sulfonamides investigated here, with K_Is in the range of 5.0–240 nM. The best inhibitory activity against this isoform was observed for counterparts **6b, 10e, 10f** and **10g** (K_Is = 5.0, 14.8, 16.7 and 8.5 nM, respectively) whereas the remaining compounds were medium potency inhibitors (K_Is in the range of 159–240 nM). Notably, grafting of nitro, methyl or methoxy groups at the 5-position of isatin moiety in the ureido series **10a-g**, greatly enhanced the activity against hCA IX. Thence, the order of activities of the isatin-substituted members in this series, was decreased in the order of OMe > NO_2 > Me >>> halogens. Differently, the SAR for the amido series **6a-g** is difficult to interpret as compounds in this series

showed effective activity, although rather similar derivatives (e.g., **6b** and **6c**) had a rather different activity profile.

(iv) The second transmembrane isoform investigated here, hCA XII, was highly inhibited by the sulfonamides reported here, many of which were subnanomolar inhibitors. Superiorly, sulfonamides **9**, **6b-6g**, **10c** and **10e** showed K_Is in the subnanomolar range of 0.47–0.71, nM whereas the remaining derivatives were also highly effective single-digit nanomolar hCA XII inhibitors (K_Is in the range of 1.17–2.83 nM). Thus, all compounds investigated here act as highly potent hCA XII inhibitors, indiscriminately of the linker or substitution pattern at the isatin ring.

Finally, we can deduce that grafting of different substituents (as halogens, nitro, methyl and methoxy) at the 5-position of isatin moiety is advantageous for the activity of the amido series **6a-g** against hCA I, II and XII, regardless of the electronic nature of substituent. Also, substitution of isatin moiety in the ureido series **10a-g** with an electron withdrawing group as 5-nitro or electron donating group like 5-methyl or 5-methoxy is indispensable for the activity against hCA IX.

3. Conclusion

In conclusion, we report here the synthesis of two series of amido/ureido-substituted benzenesulfonamides incorporating substituted-isatin moieties, utilizing a molecular hybridization approach. The prepared derivatives were *in vitro* evaluated for their inhibitory activity against a panel of hCA I, II (cytosolic) and IX, XII (transmembrane, tumor-associated) isoforms. All the tested isoforms were inhibited in variable degrees by the prepared sulfonamides reported here. Best activity was observed against the tumor-associated isoform hCA XII. Sulfonamides **9**, **6b-6g**, **10c** and **10e** displayed outstanding potency with K_Is in the subnanomolar range of 0.47–0.71, nM. Moreover, the remaining derivatives emerged as single-digit nanomolar hCA XII inhibitors (K_Is in the range of 1.17–2.83 nM). The preliminary SAR study revealed the importance of various substituents at the 5-position of isatin for activity of the amido series **6a-g** against hCA I, II and XII, besides, the significance of grafting 5-nitro, 5-methyl and 5-methoxy groups to the isatin ring for activity of the ureido series **10a-g** against hCA IX. Meanwhile, the electronic nature of such substituents has no effect on the enzyme inhibitory activity of the compounds.

4. Experimental

4.1. Chemistry

4.1.1. General

Melting points were determined with a Stuart apparatus and are uncorrected. IR spectra were recorded on Schimadzu FT-IR 8400S spectrophotometer and expressed in wave number (cm^{-1}). The NMR spectra were recorded on Varian Gemini-300BB at 300 MHz (Varian Inc., Palo Alto, CA) or Bruker spectrophotometer at 400 MHz. Chemical shifts (δ_H) are reported relative to TMS as internal standard. All coupling constant (*J*) values are given in hertz. The abbreviations used are as follows: s, singlet; d, doublet; m, multiplet. Microanalyses were carried out using Perkin Elmer PE 2400 CHN Elemental Analyzer. Reaction courses and product mixtures were routinely monitored by thin layer chromatography (TLC) on silica gel precoated F_{254} Merck plates. All the starting materials and reagents used were purchased from Sigma-Aldrich. Unless otherwise noted, all solvents and reagents were used without further purification.

Compounds **3** [40], **6** [41], **10a-c** [41] are previously reported.

4.1.2. 4-Amino-*N*-(4-sulfamoylphenyl)benzamide (**4**)

The nitro derivative **3** (1.61 g, 5 mmol) was suspended in MeOH (25 mL) and 10% palladium on carbon (0.5 g) was added. The reaction mixture was stirred at room temperature under a hydrogen atmosphere for 3 h. Then, DMF (10 mL) was added to dissolve the product, the catalyst was filtered off, and the filtrate was concentrated in vacuum. The residual solid was collected and washed with AcOEt–hexane to give **4** (1.23 g, 85%) as crystals: m.p. > 280 °C; IR (KBr, *v* cm^{-1}): 3344, 3302, 3238 (NH, NH_2), 1648 (C=O) 1314, 1171, (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 5.85 (s, 2H, D_2O exchangable, NH_2), 6.61 (d, 2H, *J*= 8.7 Hz, Ar-H), 7.23 (s, 2H, D_2O exchangable, SO_2NH_2), 7.73 (d, 2H, *J*= 6.8 Hz, Ar-H), 7.75 (d, 2H, *J*= 6.8 Hz, Ar-H), 7.91 (d, 2H, *J*= 8.7 Hz, Ar-H), 10.06 (s, 1H, D_2O exchangable, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 113.07, 119.86, 126.87, 128.00, 130.05, 138.32, 143.31, 152.89, 166.0; Anal. calcd. For $C_{13}H_{13}N_3O_3S$ (291.07): C, 53.60; H, 4.50; N, 14.42. Found C, 53.72; H, 4.54; N, 14.45.

4.1.3. General procedure for preparation of target 4-((2-oxoindolin-3-ylidene)amino)-N-(4-sulfamoylphenyl)benzamides **6a-g**.

A mixture of equimolar quantities of isatins **5a-g** (1 mmol) and 4-Amino-*N*-(4-sulfamoylphenyl)benzamide **4** (1 mmol) was refluxed in glacial acetic acid (10 mL) for 5 h. The precipitate formed was collected by filtration while hot, washed with hot ethanol, dried and crystallized from ethanol/DMF to afford compounds **6a-g** with 41-65% yield.

4.1.3.1. 4-(2-Oxoindolin-3-ylideneamino)-*N*-(4-sulfamoylphenyl)benzamide (**6a**): Yellow powder (yield 59%); m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3340, 3289, 3238 (NH, NH_2), 1718, 1655 (2C=O), 1342, 1156 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 6.39-7.20 (m, 4H, Ar-H), 7.26 (s, 2H, D_2O exchangable, SO_2NH_2), 7.35-7.97 (m, 6H, Ar-H), 8.10 (d, *J*= 7.8 Hz, 2H, Ar-H), 10.04, 10.45 and 10.56 (s, 1H, D_2O exchangable, NH), 10.95, 11.01 and 11.13 (s, 1H, D_2O exchangable, NH); Anal. calcd. For $C_{21}H_{16}N_4O_4S$ (420.44): C, 59.99; H, 3.84; N, 13.33; Found C, 60.05; H, 3.81; N, 13.39.

4.1.3.2. 4-(5-Fluoro-2-oxoindolin-3-ylideneamino)-*N*-(4-sulfamoylphenyl)benzamide (**6b**): Orange powder (yield 61%); m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3350, 3304, 3247 (NH, NH_2), 1733, 1653 (2C=O), 1310, 1153 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 6.07 (d, *J*= 7.8 Hz, 1H, Ar-H), 6.60-7.25 (m, 2H, Ar-H), 7.27 (s, 2H, D_2O exchangable, SO_2NH_2), 7.71-7.80 (m, 6H, Ar-H), 8.11 (d, *J*= 8.4 Hz, 2H, Ar-H), 10.21, 10.38 and 10.59 (s, 1H, D_2O exchangable, NH), 10.48, 11.03 and 11.15 (s, 1H, D_2O exchangable, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 117.08, 118.13, 119.79, 126.51, 128.43, 129.66, 130.09, 130.98, 138.09, 138.65, 142.15, 142.54, 143.61, 153.03, 163.23, 165.17, 168.77; Anal. calcd. For $C_{21}H_{15}FN_4O_4S$ (438.43): C, 57.53; H, 3.45; N, 12.78; Found C, 57.71; H, 3.49; N, 12.63.

4.1.3.3. 4-(5-Chloro-2-oxoindolin-3-ylideneamino)-*N*-(4-sulfamoylphenyl)benzamide (**6c**): Orange powder (yield 60%); m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3359, 3307, 3246 (NH, NH_2), 1733, 1654 (2C=O), 1306, 1155 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 6.32 (s, 1H, Ar-H), 6.60-7.20 (m, 2H, Ar-H), 7.27 (s, 2H, D_2O exchangable, SO_2NH_2), 7.43-8.00 (m, 6H, Ar-H), 8.12 (d, *J*= 8.4 Hz, 2H, Ar-H), 10.23, 10.49 and 10.60 (s, 1H, D_2O exchangable, NH), 10.40, 11.04 and 11.17 (s, 1H, D_2O exchangable, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 116.77, 117.06, 119.80, 122.56, 124.73, 125.33, 126.52, 129.64, 130.90, 134.21, 138.51, 142.13, 145.08, 153.09, 154.08, 162.92, 165.10; Anal. calcd. For $C_{21}H_{15}ClN_4O_4S$ (454.88): C, 55.45; H, 3.32; N, 12.32; Found C, 55.33; H, 3.34; N, 12.38.

4.1.3.4. 4-(5-Bromo-2-oxoindolin-3-ylideneamino)-*N*-(4-sulfamoylphenyl)benzamide (**6d**): Orange powder (yield 65%); m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3351, 3298, 3249 (NH, NH_2), 1731, 1678 (2C=O)

1303, 1154 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 6.45-7.28 (m, 3H, Ar-H), 7.29 (s, 2H, D_2O exchangable, SO_2NH_2), 7.55-8.15 (m, 8H, Ar-H), 10.24, 10.51 and 10.62 (s, 1H, D_2O exchangable, NH), 10.41, 11.05 and 11.18 (s, 1H, D_2O exchangable, NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 113.46, 114.24, 114.53, 117.67, 117.78, 118.60, 118.99, 120.21, 120.25, 120.33, 123.45, 125.90, 127.01, 128.07, 128.89, 128.94, 129.26, 130.14, 130.63, 131.37, 137.22, 137.41, 138.97, 139.01, 139.16, 142.65, 142.76, 142.79, 143.05, 145.60, 146.82, 152.90, 153.03, 153.63, 154.50, 158.58, 163.29, 165.60, 165.75, 165.94, 169.31; Anal. calcd. For $C_{21}H_{15}BrN_4O_4S$ (499.34): C, 50.51; H, 3.03; N, 11.22; Found C, 50.26; H, 3.09; N, 11.31.

4.1.3.5. 4-(5-Nitro-2-oxoindolin-3-ylideneamino)-*N*-(4-sulfamoylphenyl)benzamide (**6e**): Yellow powder (yield 55%); m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3318, 3176 (NH, NH_2), 1747, 1659 (2C=O) 1325, 1150 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 7.10 (d, 1H, *J*= 8.4 Hz, Ar-H); 7.22 (d, 2H, J= 8.1 Hz, Ar-H); 7.30 (s, 2H, D_2O exchangable, SO_2NH_2), 7.81 (d, 2H, *J*= 7.8 Hz, Ar-H), 7.96 (d, 2H, *J*= 8.1 Hz, Ar-H), 8.01 (s, 1H, Ar-H), 8.15 (d, 2H, *J*= 7.8 Hz, Ar-H), 8.28 (d, 1H, *J*= 8.4 Hz, Ar-H), 10.54 and 10.64 (s, 1H, D_2O exchangable, NH), 11.62 and 11.75 (s, 1H, D_2O exchangable, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 115.55, 117.22, 119.86, 126.23, 127.60, 128.43, 129.68, 130.49, 131.10, 138.70, 141.56, 142.09, 152.51, 153.49, 158.68, 163.51, 164.96; Anal. calcd. For $C_{21}H_{15}N_5O_6S$ (465.44): C, 54.19; H, 3.25; N, 15.05; Found C, 54.31; H, 3.31; N, 14.92.

4.1.3.6. 4-(5-Methyl-2-oxoindolin-3-ylideneamino)-*N*-(4-sulfamoylphenyl)benzamide (**6f**): Beige powder (yield 41%); m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3315, 3247, 3213 (NH, NH_2), 1742, 1655 (2C=O) 1315, 1153 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 1.98 and 2.09 (s, 3H, CH_3), 6.24 (s, 1H, Ar-H), 6.77-7.26 (m, 2H, Ar-H), 7.26 (s, 2H, D_2O exchangable, SO_2NH_2), 7.71-8.01 (m, 6H, Ar-H), 8.11 (d, *J*= 8.1 Hz, 2H, Ar-H), 10.05, 10.23 and 10.40 (s, 1H, D_2O exchangable, NH), 10.49, 10.57 and 10.91 (s, 1H, D_2O exchangable, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 20.4, 24.09, 115.59, 117.11, 117.29, 118.44, 125.76, 126.46, 128.43, 128.72, 129.24, 129.35, 129.58, 130.51, 130.59, 135.33, 142.18, 142.54, 145.01, 153.61, 155.03, 163.27, 165.22, 168.78; Anal. calcd. For $C_{22}H_{18}N_4O_4S$ (434.47): C, 60.82; H, 4.18; N, 12.90; Found C, 60.98; H, 4.12; N, 13.01.

4.1.3.7. 4-(5-Methoxy-2-oxoindolin-3-ylideneamino)-*N*-(4-sulfamoylphenyl)benzamide (**6g**): Red powder (yield 49%); m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3313, 3237 (NH, NH_2), 1748, 1662 (2C=O) 1310, 1150 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 3.46 and 3.78 (s, 3H, OCH_3), 5.90 (s, 1H, Ar-H), 6.81-7.21 (m, 2H, Ar-H), 7.26 (s, 2H, D_2O exchangable, SO_2NH_2), 7.71-8.00 (m, 6H, Ar-H), 8.10 (d,

J= 8.1 Hz, 2H, Ar-H), 10.22, 10.57 and 10.71 (s, 1H, D_2O exchangable, NH), 10.40, 10.49 and 10.83 (s, 1H, D_2O exchangable, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 55.00, 112.19, 116.04, 117.08, 117.26, 119.77, 126.50, 128.43, 128.75, 129.58, 129.81, 130.69, 138.47, 138.64, 140.60, 142.16, 153.56, 153.99, 155.22, 155.45, 163.27, 165.17; Anal. calcd. For $C_{22}H_{18}N_4O_5S$ (450.47): C, 58.66; H, 4.03; N, 12.44; Found C, 58.86; H, 4.06; N, 12.37.

4.1.4. 4-(3-(4-Aminophenyl)ureido)benzenesulfonamide (**9**)

The compound **9** was prepared from **8** in a manner similar to that described for **4** to yield a white solid (81%): m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3411, 3384, 3293 (NH, NH_2), 1630 (C=O) 1310, 1146 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 4.80 (s, 2H, D_2O exchangable, NH_2), 6.51 (d, 2H, *J*= 8.7 Hz, Ar-H); 7.07 (d, 2H, *J*= 8.7 Hz, Ar-H); 7.13 (s, 2H, D_2O exchangable, SO_2NH_2),7.55 (d, 2H, *J*= 8.7 Hz, Ar-H); 7.68 (d, 2H, *J*= 8.7 Hz, Ar-H); 8.23 (s, 1H, D_2O exchangable, NH), 8.84 (s, 1H, D_2O exchangable, NH); Anal. calcd. For $C_{13}H_{14}N_4O_3S$ (306.08): C, 50.97; H, 4.61; N, 18.29. Found C, 51.08; H, 4.74; N, 18.45.

4.1.5. General procedure for preparation of target 4-(3-(4-((2-oxoindolin-3-ylidene)amino)phenyl)ureido)benzenesulfonamides **10a-g**.

A mixture of 4-(3-(4-aminophenyl)ureido)benzenesulfonamide **9** (1 mmol) and the appropriate isatin **5a-g** (1 mmol) in glacial acetic acid (10 mL) was heated under reflux for 2 h, filtered while hot and the precipitate was washed with ethanol. The solid product was collected and crystallized from an ethanol/DMF to furnish compounds **10a-g** with 57-73% yield.

4.1.5.1. 4-(3-(4-(5-Bromo-2-oxoindolin-3-ylideneamino)phenyl)ureido)benzenesulfonamide (**10d**): Orange powder (yield 73%); m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3123-3400 broad band (NH, NH_2), 1724, 1612 (2C=O),1307, 1150 (SO_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 6.56-7.24 (m, 6H, 4H Ar-H and 2H SO_2NH_2), 7.44-7.76 (m, 7H, Ar-H), 8.91 and 8.98 (s, 1H, D_2O exchangeable, NH urea), 9.10, 9.12 (s, 1H, D_2O exchangeable, NH urea), 10.98 and 11.10 (s, 1H, D_2O exchangeable, NH isatin); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 112.84, 113.41, 117.41, 118.76, 119.12, 124.13, 126.77, 136.20, 136.86, 137.30, 137.77, 142.75, 143.95, 145.90, 152.14, 153.34, 163.15; Anal. calcd. For $C_{21}H_{16}BrN_5O_4S$ (514.35): C, 49.04; H, 3.14; N, 13.62; Found C, 48.98; H, 3.11; N, 13.69.

4.1.5.2. 4-(3-(4-(5-Nitro-2-oxoindolin-3-ylideneamino)phenyl)ureido)benzenesulfonamide (**10e**): Yellow powder (yield 69%); m.p. > 280 °C; IR (KBr, ν cm^{-1}): 3200-3404 broad band (NH, NH_2), 1728, 1615 (2C=O), 1304, 1147 (SO_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.05-7.12 (m, 2H, Ar-H),

7.20 (s, 2H, D_2O exchangeable, SO_2NH_2), 7.34 (d, *J* =8.1 Hz, 1H, Ar-H), 7.48 (d, *J* =8.4 Hz, 1H, Ar-H), 7.62-7.65 (m, 4H, Ar-H), 7.73 (d, *J* = 8.7 Hz, 2H, Ar-H), 8.27 (d, *J* = 8.7 Hz, 1H, Ar-H), 8.96 and 9.03 (s, 1H, D_2O exchangeable, NH urea), 9.12 (s, 1H, D_2O exchangeable, NH urea), 11.53 and 11.65 (s, 1H, D_2O exchangeable, NH isatin); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 115.65, 117.12, 119.06, 120.10, 126.77, 128.98, 129.94, 136.90, 137.82, 138.48, 141.50, 142.71, 143.33, 150.09, 152.13, 163.91, 189.22; Anal. calcd. For $C_{21}H_{16}N_6O_6S$ (480.45): C, 52.50; H, 3.36; N, 17.49; Found C, 52.76; H, 3.41; N, 17.41.

4.1.5.3. 4-(3-(4-(5-Methyl-2-oxoindolin-3-ylideneamino)phenyl)ureido)benzenesulfonamide (**10f**): Biege powder (yield 57%); m.p. > 280 °C; IR (KBr, *v* cm^{-1}): 3192-3415 broad band (NH, NH_2), 1685, 1614 (2C=O), 1310, 1146 (SO_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.01 and 2.29 (s, 3H, CH_3), 6.54-7.14 (m, 5H, Ar-H), 7.24 (s, 2H, D_2O exchangeable, SO_2NH_2), 7.58-7.75 (m, 6H, Ar-H), 8.87 and 8.93 (s, 1H, D_2O exchangeable, NH urea), 9.08 and 9.12 (s, 1H, D_2O exchangeable, NH urea), 10.74 and 10.85 (s, 1H, D_2O exchangeable, NH isatin); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 21.03, 111.75, 115.28, 116.29, 117.60, 119.34, 121.32, 122.10, 125.83, 127.25, 131.72, 135.14, 137.28, 143.19, 144.92, 152.57, 154.99, 164.20 ;Anal. calcd. For $C_{22}H_{19}N_5O_4S$ (449.48): C, 58.79; H, 4.26; N, 15.58; Found C, 58.71; H, 4.30; N, 15.66.

4.1.5.4. 4-(3-(4-(5-Methoxy-2-oxoindolin-3-ylideneamino)phenyl)ureido)benzenesulfonamide (**10g**): Red powder (yield 65%); m.p. > 280 °C; IR (KBr, *v* cm^{-1}): 3270-3489 broad band (NH, NH_2), 1721, 1688 (2C=O), 1302, 1149 (SO_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 3.49 and 3.77 (s, 3H, O$\underline{CH_3}$), 6.18-7.17 (m, 5H, Ar-H), 7.21 (s, 2H, D_2O exchangeable, SO_2NH_2), 7.42-7.76 (m, 6H, Ar-H), 8.86 and 8.92 (s, 1H, D_2O exchangeable, NH urea), 9.09, 9.10 (s, 1H, D_2O exchangeable, NH urea), 10.65 and 10.77 (s, 1H, D_2O exchangeable, NH isatin); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 55.74, 56.11, 107.63, 111.97, 112.10, 112.41, 114.65, 116.69, 117.57, 117.92, 117.97, 118.63, 119.15, 119.42, 119.93, 120.49, 121.43, 122.15, 123.14, 127.25, 127.30, 136.77, 137.33, 137.40, 137.64, 139.34, 140.78, 143.05, 143.30, 143.77, 144.98, 152.70, 152.80, 153.01, 154.42, 155.53, 155.62, 159.34, 164.20; Anal. calcd. For $C_{22}H_{19}N_5O_5S$ (465.48): C, 56.77; H, 4.11; N, 15.05; Found C, 56.86; H, 4.09; N, 14.93.

4.2 CA inhibitory assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity [42]. The enzymes are recombinant proteins prepared in our lab. 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 uncatalyzed 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 at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as reported earlier [43-36], and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier [43-46].

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Captions page

Figure 1. Structures of the reported carbonic anhydrase inhibitors **I-V** and the target conjugates **6a-g** and **10a-g.**

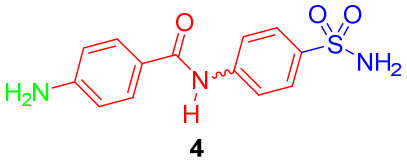
Figure 2. *Cis/Trans* conformers around amide function and *E/Z* geometrical isomers around -C=N- of compounds **6a-g**.

Scheme 1. Reagents and conditions: **(i)** THF / TEA / r.t. 5h; **(ii)** H_2 / Pd/C / MeOH / r.t. 3h; **(iii)** Glacial acetic acid / reflux 5h.

Scheme 2. Reagents and conditions: **(i)** CH_3CN, reflux 2h; **(ii)** H_2 / Pd/C / MeOH / r.t. 3h; **(iii)** Glacial acetic acid / reflux 2h.

Table 1. Inhibition data of human CA isoforms hCA I, II, IX and XII with sulfonamides **4**, **9**, **6a-g** and **10a-g** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO_2 hydrase assay.

Table 1: Inhibition data of human CA isoforms hCA I, II, IX and XII with sulfonamides **4**, **9**, **6a-g** and **10a-g** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO_2 hydrase assay.



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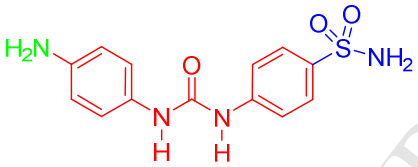

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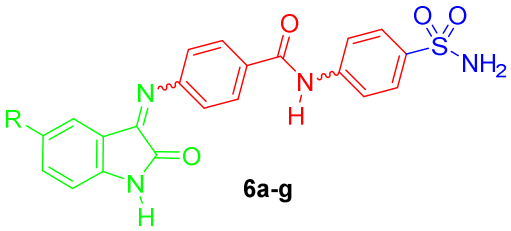
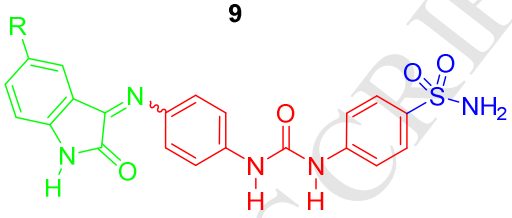


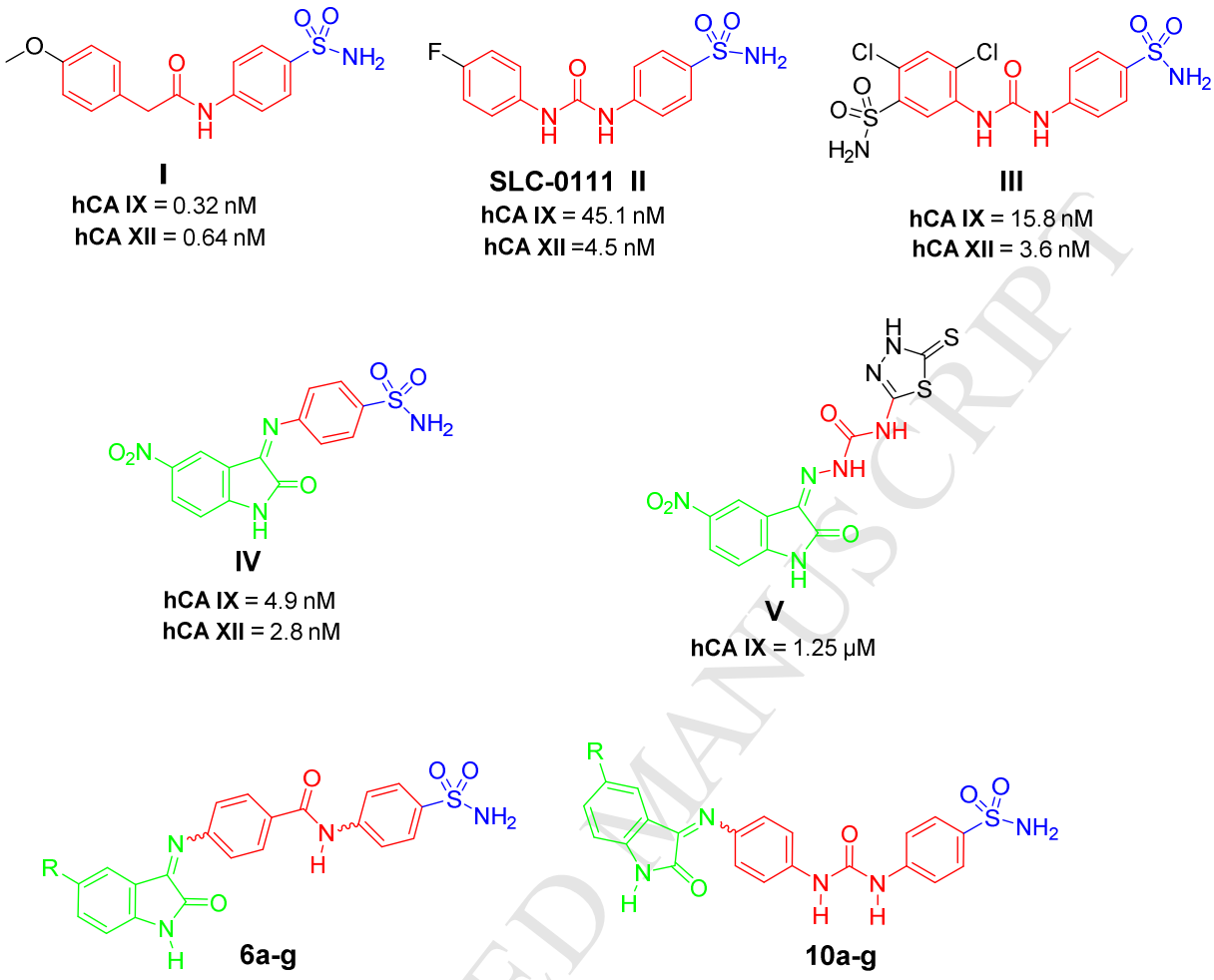
6a-g



10a-g

Compound	R	K_I (nM)			
		hCAI	**hCA II**	**hCA IX**	**hCA XII**
4	-	78.4	28.1	159	2.7
9	-	94.9	15.7	171	0.71
6a	H	95.6	116	240	1.76
6b	F	9.0	7.7	5.0	0.69
6c	Cl	9.2	8.4	194	0.54
6d	Br	7.9	7.5	208	0.58
6e	NO_2	10.2	9.9	237	0.69
6f	Me	8.5	9.1	201	0.63
6g	MeO	29.3	14.5	175	0.47
10a	H	679	157	217	2.32
10b	F	894	585	235	2.83
10c	Cl	85.9	1645	239	0.67
10d	Br	462	>10000	192	2.31
10e	NO_2	62.8	877	14.8	0.64
10f	Me	45.2	33.0	16.7	1.17
10g	MeO	387	690	8.5	1.62
AAZ	-	250	12	25	5.7

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10 % of the reported values).



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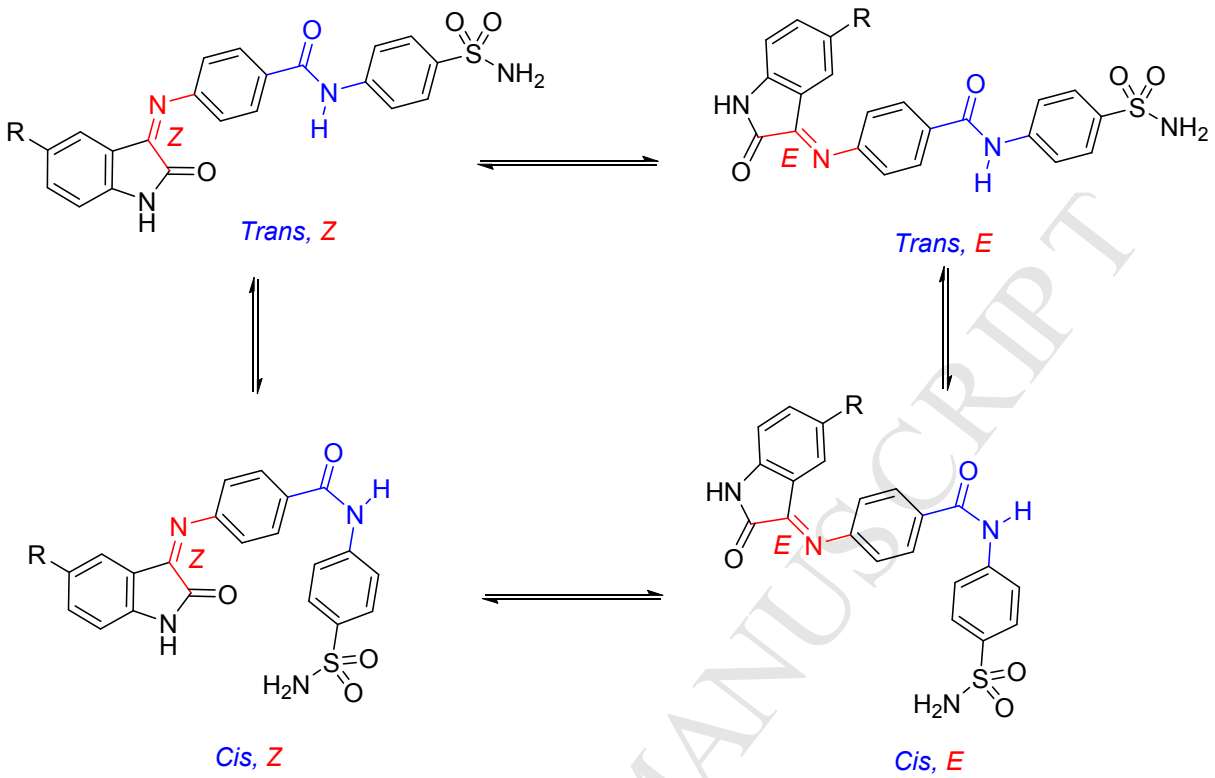

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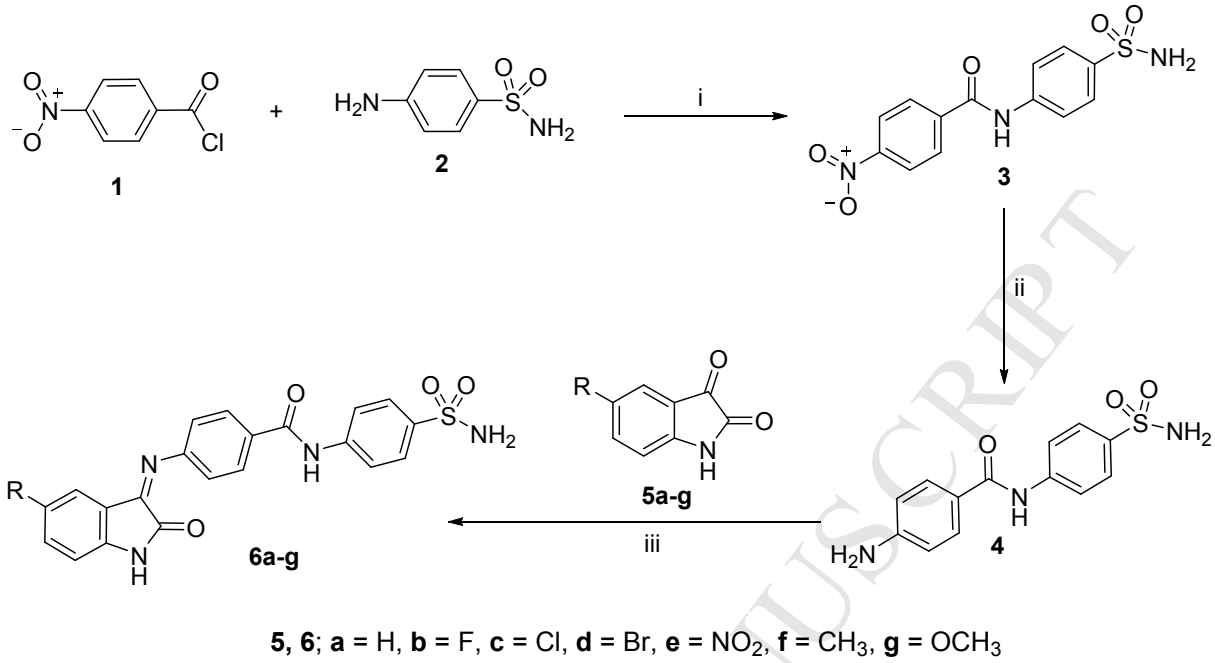

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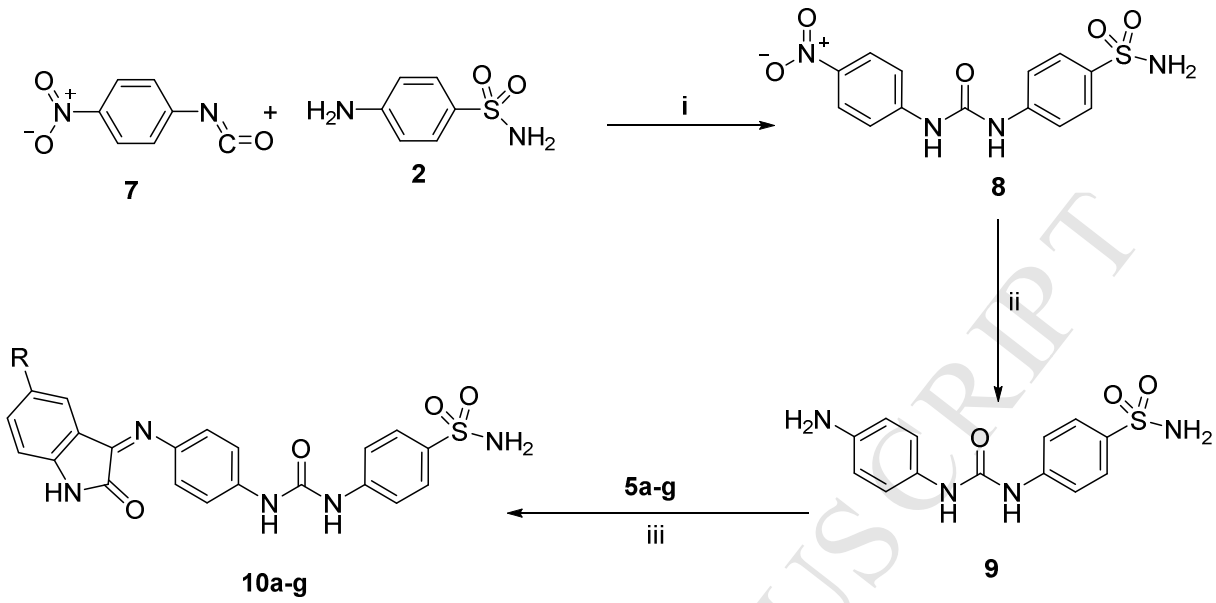

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Highlights

- Different sulfonamides incorporating amido or ureido linkers were synthesized.
- Inhibitory activity of these sulfonamides was evaluated toward hCA I, II, IX and XII.
- **9**, **6b-g**, **10c** and **10e** displayed outstanding potency against CA XII (K_I: 0.47–0.71 nM).
- Other derivatives emerged as single-digit nanomolar CA XII inhibitors (1.17–2.83 nM).