PAPER

Discovery of novel isatin-based sulfonamides with potent and selective inhibition of the tumor-associated carbonic anhydrase isoforms IX and XII

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A series of 2/3/4-[(2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)amino]benzenesulfonamides, obtained from substituted isatins and 2-, 3- or 4-aminobenzenesulfonamide, showed low nanomolar inhibitory activity against the tumor associated carbonic anhydrase (CA, EC 4.2.1.1) isoforms IX and XII – recently validated antitumor drug targets, being much less effective as inhibitors of the off-target cytosolic isoforms CA I and II.

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes with catalytic versatility, having as substrates CO_2, COS, CS_2, cyanamide, carboxylic, phosphoric and thiocarboxylic esters.[1–4] Although their main physiological functions are related to the inter-conversion between CO_2, bicarbonate and protons, they play crucial functions in pH regulation, electrolyte secretion, biosynthetic reactions, and carcinogenesis, among others.[5–8] Of the 15 isoforms described in humans (hCA I–XIV), many are drug targets for pharmacological agents such as diuretics,[9] antiglaucoma drugs,[10] antiepileptics,[11] anti-obesity[12] and ultimately, antitumor drugs/cancer diagnostic agents.[13] The transmembrane isoforms CA IX and XII are overexpressed in hypoxic tumors as a consequence of the HIF-1α (hypoxia inducible factor-1α) activation pathway, and their inhibition by small molecules/antibodies was recently shown to lead to significant antitumor action.[13,14] Furthermore, as these enzymes are present in normal tissues in a very small concentration compared to the tumors, they can also be used for imaging hypoxic tumors.[15] Recently, a sulfonamide CA inhibitor (CAI) targeting CA IX and XII entered phase I clinical trials for the treatment of advanced metastatic solid tumors.[16]

Results and discussion

Chemistry

Sulfonamides (RSO_2NH_2) constitute the most important and investigated class of CAIs.[17]

A large number of structurally diverse sulfonamides were investigated for their CA inhibitory properties.[17,18] However the main problem with sulfonamides is their promiscuous behavior as strong inhibitors of many of the 15 CA isoforms of human (h) origin.[17,18] As isoforms hCA I and II are widespread and play important physiological functions,[1–3] it is of great interest to design inhibitors targeting the tumor-associated isoforms hCA IX and XII, which, at the same time, show weak affinity for the off-target isoforms hCA I and II. Some Schiff bases incorporating sulfonamide moieties were among the first types of CAIs showing selective inhibition of some CA isoforms of interest for medicinal chemistry applications,[19,20] and this is the reason why we explore here these types of compounds which incorporate substituted isatin moieties (Scheme 1). Reaction of isatins with aromatic sulfonamides was in fact investigated earlier by our and other groups,[21–25] and a limited number of such compounds have been reported. Here we extend the previous studies, reporting a series of 23 such derivatives which incorporate orthanilamide, metanilamide or sulfanilamide moieties, as well as isatin or *N*-methylisatins substituted with methyl, halogens, nitro or trifluoromethyloxy moieties at the heterocyclic ring. We have chosen these substitution patterns at the isatin fragment of the molecule in order to investigate the structure–activity relationship (SAR) for the inhibition of four CA isoforms (hCA I, II, IX and XII) with this class of derivatives.[26]
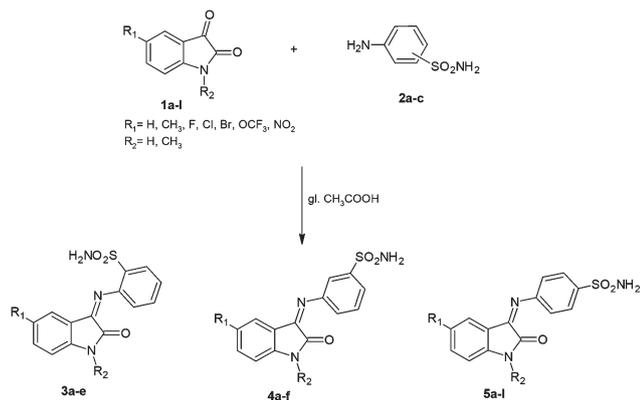
Enzyme inhibition data

hCA I was inhibited moderately by the reported compounds irrespective of the substitution pattern, with K_Is ranging

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Scheme 1 Preparation of 2/3/4-[(2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]benzenesulfonamides.

between 146 and 816 nM. The same was true for the cytosolic dominant isoform hCA II; for which the inhibition constants were in the range of 101–728 nM (Table 1). All K_I values were in the lower nanomolar region, in a narrow range, for both tumor-associated hCA isozymes (hCA IX: 1.0–15.6 nM; hCA XII: 2.8–53.8 nM; Table 1). The K_I values for the widespread hCA I/II were significantly larger (hCA I: 146–816 nM; hCA II: 101–728 nM) and thus, the new compounds showed a discrete selectivity for the tumor-associated isozymes (Table 1). The difference in K_I values is relatively small for the tumor-associated isozymes (hCA IX: ∼15-fold; hCA XII: ∼19-fold) and a conclusive SAR analysis is difficult to perform. Compound **4b** drew our attention since it shows very low K_I values for the tumor-associated isozymes (hCA IX: 1.1 nM; hCA XII: 3.3 nM) and it shows the highest selectivity for the tumor-associated isozymes compared to the widely distributed hCA I and II (Table 1).
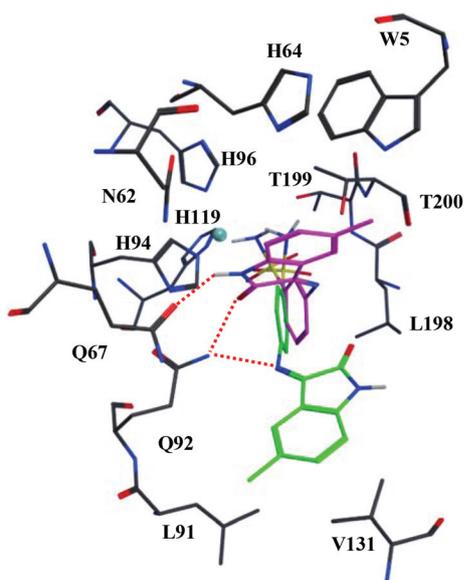
Molecular modelling studies

Compound **4b** has one of the lowest measured K_I values for hCA IX and shows the highest selectivity towards hCA IX compared to the other isozymes (Table 1). Molecular modelling studies were applied to suggest a rationale for this selectivity. Available crystal structures of hCA isozymes with sulfonamide-containing ligands such as acetazolamide bound to their active site indicate that the sulfonamide moiety is oriented in a very similar way to the Zn^{2+}-ion of the hCA active sites. The nitrogen atom of the SO_2NH^- group is coordinated to the Zn^{2+}-ion and forms a hydrogen bond with the side-chain of Thr199, whereas one of the sulfonamide oxygen atoms forms a hydrogen bond with the backbone NH of the same residue. A similar orientation and binding-interactions were enforced upon the ligands in our docking studies.

Docking studies on hCA IX. The docked pose of compound **4b** in the active site of hCA IX reveals that the vicinal nitrogen and carbonyl group of the indole ring form hydrogen bonds with the side-chains of Gln67 and Gln92, respectively (Fig. 1). The other analogs with sulfonamide groups on the *meta* position of the phenyl ring (compound series **4**) adopted similar docked poses and the range of K_I values was 1.1–9.8 nM. The various substituents on the isatin ring did not form any

Table 1 hCA I, II, IX and XII inhibition data of compounds 3, 4 and 5 by a stopped-flow CO_2 hydrase assay[26]

Compounds			K_I (nM)				Selectivity ratio			
#	R1	R2	I	II	IX	XII	I/IX	II/IX	I/XII	II/XII
3a	H	H	816	728	9.3	53.8	88	78	15	14
3b	CH₃	H	600	711	4.2	49.7	143	169	12	14
3c	F	H	652	428	1.2	41.3	543	357	16	10
3d	Cl	H	778	652	5.7	52.5	136	114	15	12
3e	OCF₃	H	742	683	10.3	53.0	72	66	14	13
4a	H	H	579	618	8.4	43.9	69	74	13	14
4b	CH₃	H	510	565	1.1	3.3	464	514	155	171
4c	F	H	426	264	1.3	30.8	328	203	14	9
4d	Cl	H	490	547	4.8	41.1	102	114	12	13
4e	OCF₃	H	539	484	9.8	36.5	55	49	15	13
4f	NO₂	H	378	250	5.7	3.1	66	44	122	81
5a	H	H	422	523	6.5	29.3	65	80	14	18
5b	H	CH₃	586	549	5.9	6.7	99	93	87	82
5c	CH₃	H	414	454	1.0	18.5	414	454	22	25
5d	CH₃	CH₃	536	491	4.1	39.7	131	120	14	12
5e	F	H	375	468	4.0	32.5	94	117	12	14
5f	F	CH₃	259	432	1.6	36.4	162	270	7	12
5g	Cl	H	249	309	3.3	25.2	75	94	10	12
5h	Cl	CH₃	368	462	4.7	44.6	78	98	8	10
5i	Br	CH₃	457	514	15.6	37.8	29	33	12	14
5j	OCF₃	H	295	236	3.9	3.8	76	61	78	62
5k	NO₂	H	229	164	4.9	2.8	47	33	82	59
5l	NO₂	CH₃	146	101	4.2	4.3	35	24	34	23
AZ	—	—	250	12	25.0	5.7	10	0	44	2

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Fig. 1 The docked poses of compounds **4b** (magenta) and **5c** (green) in the active site of hCA IX. Interactions of the sulfonamide group with the protein are not shown for clarity. Hydrogen bonds are indicated with red dashed lines. The Zn^{2+}-ion is indicated with a turquoise sphere.

additional interactions except for compound **4f**, which has a NO_2 group capable of forming a hydrogen bond with Trp5.

Compound **5c** is very similar to **4b**, except for the fact that the sulfonamide is substituted on the *para* position instead of the *meta* position. This reorients the isatin fragment to form hydrogen bonds with Gln92 *via* the imine group between the 2-indolinone and the phenyl ring (Fig. 1). In addition, hydrophobic interactions were observed between the isatin moiety and the sidechain of Val131. The analogs with a sulfonamide in the *para* position (compound series **5**) showed a similar docked pose. Their range of K_I values is 1.0–15.6 nM and the varying substituents do not form additional interactions with the active site, as they point towards the solvent.

Differences in active sites between hCA IX and hCA XII. Gln67 and Gln92 are involved in hydrogen bonding to compound **4b** and are believed to be responsible for the low K_I value observed for this compound. Gln67 is not conserved amongst the other hCA isozymes (Table 2). hCA XII has a Lys67 instead of the Gln67 of hCA IX and the ligand cannot form the same interactions as observed in Fig. 1. Gln92 is conserved in both structures and the backbone is located at a similar position, but the sidechain conformation is slightly different. As such, no hydrogen bond is observed in the docking, but it should be possible after sidechain reorientation.

Differences in active sites between hCA IX and hCA I. The bulky His67 is present in hCA I instead of the Gln67 of hCA IX (Table 2). In addition, Val131 and Thr200 of hCA IX are replaced by the larger Leu131 and His200 in hCA I (Table 2). The presence of His200 forces a reorientation of Trp5, which

Table 2 The differences and similarities in the active site lining of the investigated hCA isozymes

hCA IX	hCA XII	hCA I	hCA II
Trp5[a]	Trp5	Trp5	Trp5
Gln67	Lys67	His67	Asn67
Gln92[a]	Gln92	Gln92	Gln92
Val131	Ala131	Leu131	Phe131
Thr199	Thr199	His200	Thr199

[a] This residue is conserved but has a different orientation in at least one crystal structure.

enters the active site more deeply and sterically interferes with the docked ligands. These changes in the binding site do not allow for the adoption of similar poses as observed in Fig. 1.

Differences in active sites between hCA IX and hCA II. Asn67 and Phe131 are present in hCA II (Table 2). Asn67 is shorter than its Gln67 counterpart observed in hCA IX, while Phe131 points into the active site to a larger degree compared to Val131.

Experimental

Synthetic procedures

Melting points were estimated with a Buchi 540 melting point apparatus in open capillaries and are uncorrected. Elemental analyses were performed on a Thermo Finnigan Flash EA 1112 elemental analyzer. IR spectra were recorded on KBr discs, using a Perkin-Elmer Model 1600 FT-IR spectrometer. ^{1}H-NMR, D_2O-exch., HSQC and HMBC spectra were obtained on Varian[UNITY] INOVA 500 and Bruker Avance DPX 400 spectrophotometers using DMSO-d_6.

Synthesis of 2/3/4-[(2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)-amino]benzenesulfonamides (3a–e, 4a–f, 5a–l). Equimolar quantities of 1*H*-indole-2,3-diones (**1**) (0.01 mol) and 2-amino-benzenesulfonamide/3-aminobenzenesulfonamide/4-amino-benzenesulfonamide (**2**) were refluxed in glacial acetic acid (10 ml) for 6 h. The reaction mixture was allowed to stand for 24 h at room temperature. The product was filtered and recrystallized from ethanol.[21]

2-[(2-Oxo-1,2-dihydro-3*H*-indol-3-ylidene)amino]benzenesulfonamide (3a). Yellow powder, yield 66%; m.p. 249–250 °C; IR (KBr) (ν, cm^{-1}): 3291, 3180 (NH), 1735 (C=O), 1337, 1151 (S=O); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 6.68–8.25 (10H, m, Ar–H, SO_2NH_2), 10.58, 10.96 (1H, 2s, indole NH). Anal. Calcd for $C_{14}H_{11}N_3O_3S$ (301.32): C, 55.80, H, 3.68; N, 13.95; S, 10.64. Found: C, 55.51; H, 3.94; N, 13.84; S, 10.50.

2-[(5-Methyl-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)amino]-benzenesulfonamide (3b). Orange powder, yield 24%; m.p. 235–236 °C; IR (KBr) (ν, cm^{-1}): 3274, 3192 (NH), 1732 (C=O), 1327, 1160 (S=O); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 1.94, 2.29 (3H, 2s, 5-CH_3), 6.09–7.65 (6H, m, Ar–H), 6.94, 7.03 (2H, 2s, SO_2NH_2), 7.80, 7.95 (1H, 2d, J = 7.81 Hz, phenyl C_6-H), 10.68, 10.86 (1H, 2s, indole NH). Anal. Calcd for

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C₁₅H₁₃N₃O₃S (315.34): C, 57.13, H, 4.16; N, 13.33; S, 10.17. Found: C, 57.55; H, 4.44; N, 13.14; S, 9.90.

2-[(5-Fluoro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (3c). Orange powder, yield 85%; m.p. 269–271 °C; IR (KBr) (ν, cm⁻¹): 3290, 3187 (NH), 1731 (C=O), 1338, 1168 (S=O); ¹H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 6.81 (1H, d, J = 7.32 Hz, phenyl C₃–H), 6.83 (1H, t, J = 7.81 Hz, phenyl C₅–H), 6.86 (1H, dd, J = 8.29, 4.39 Hz, indole C₇–H), 7.15 (1H, dt, J = 9.26, 2.93 Hz, indole C₆–H), 7.32–7.36 (2H, m, indole C₄–H, phenyl C₄–H), 7.51 (1H, dd, J = 7.81, 0.98 Hz, phenyl C₆–H), 7.64, 8.36 (2H, 2s, SO₂NH₂, D₂O exch.), 10.64 (1H, s, indole NH, D₂O exch.). HSQC (DMSO-d_6) δ (ppm): 111.73 (d, J = 7.66 Hz, indole C₇), 114.36 (d, J = 25.87 Hz, indole C₄), 117.07 (phenyl C₅), 117.53 (d, J = 23.48 Hz, indole C₆), 118.29 (phenyl C₃), 123.42 (phenyl C₆), 123.95 (phenyl C₁), 130.98 (d, J = 8.15 Hz, indole C₃ₐ), 133.81 (phenyl C₄), 138.10 (indole C₇ₐ), 138.11 (indole C₃), 143.81 (phenyl C₂), 158.50 (d, J = 237.71, indole C₅), 174.00 (indole C₂). Anal. Calcd for C₁₄H₁₀FN₃O₃S (319.31): C, 52.66, H, 3.16; N, 13.16; S, 10.04. Found: C, 52.52; H, 3.13; N, 13.12; S, 10.11.

2-[(5-Chloro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (3d). Orange powder, yield 40%; m.p. 248–250 °C; IR (KBr) (ν, cm⁻¹): 3266, 3197 (NH), 1731 (C=O), 1328, 1156 (S=O); ¹H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 6.82 (1H, t, J = 7.81 Hz, phenyl C₅-H), 6.89 (1H, d, J = 8.29 Hz, indole C₇–H), 7.35 (1H, dd, J = 7.81, 1.46 Hz, phenyl C₃–H), 7.36 (1H, dd, J = 8.29, 2.44 Hz, indole C₆–H), 6.86, 8.41 (2H, 2s, SO₂NH₂, D₂O exch.), 7.52 (1H, dd, J = 7.81, 2.44 Hz, phenyl C₄–H), 7.54 (1H, d, J = 2.44 Hz, phenyl C₆–H), 7.64 (1H, s, indole C₄–H), 10.75 (1H, s, indole NH, D₂O exch.). Anal. Calcd for C₁₄H₁₀ClN₃O₃S (335.76): C, 50.08, H, 3.00; N, 12.51; S, 9.55. Found: C, 49.77; H, 3.12; N, 12.35; S, 9.48.

2-[(2-Oxo-5-(trifluoromethoxy)-1,2-dihydro-3H-indol-3-ylidene)-amino]benzenesulfonamide (3e). Orange powder, yield 30%; m.p. 261–263 °C; IR (KBr) (ν, cm⁻¹): 3289, 3187 (NH), 1733 (C=O), 1339, 1163 (S=O); ¹H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 6.82 (1H, d, J = 8.29 Hz, indole C₇–H), 6.86 (1H, d, J = 7.81 Hz, phenyl C₃–H), 6.95 (1H, d, J = 8.29 Hz, indole C₆–H), 7.32 (1H, dd, J = 8.29, 1.95 Hz, indole C₄–H), 7.36 (1H, dd, J = 7.81, 1.46 Hz, phenyl C₅–H), 7.52 (2H, dd, J = 7.81, 1.46 Hz, phenyl C₄,₆–H), 7.66, 8.46 (2H, 2s, SO₂NH₂), 10.81 (1H, s, indole NH). HMBC (DMSO-d_6) δ (ppm): 111.86 (indole C₇), 117.10 (phenyl C₅), 118.41 (indole C₄), 119.49 (OCF₃), 120.57 (indole C₆), 123.37 (phenyl C₃), 124.07 (phenyl C₆), 124.46 (phenyl C₁), 130.99 (indole C₃ₐ), 132.69 (indole C₅), 133.86 (phenyl C₄), 141.09 (indole C₃), 143.73 (phenyl C₂), 143.85 (indole C₇ₐ), 174.05 (indole C₂). Anal. Calcd for C₁₅H₁₀F₃N₃O₄S (385.32): C, 46.76, H, 2.62; N, 10.91; S, 8.32. Found: C, 46.44; H, 2.84; N, 10.56; S, 8.32.

3-[(2-Oxo-1,2-dihydro-3H-indol-3-ylidene)amino]benzenesul-fonamide (4a). Yellow powder, yield 24%; m.p. 263–264 °C; IR (KBr) (ν, cm⁻¹): 3361, 3259 (NH), 1725, 1746 (C=O), 1330, 1147 (S=O); ¹H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 6.32–7.69 (8H, m, Ar–H), 7.33, 7.41 (2H, 2s, SO₂NH₂), 10.88, 10.98 (1H, 2s, indole NH). Anal. Calcd for C₁₄H₁₁N₃O₃S (301.32): C, 55.80, H,

3.68; N, 13.95; S, 10.64. Found: C, 55.89; H, 4.06; N, 13.64; S, 10.65.

3-[(5-Methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (4b). Orange powder, yield 32%; m.p. 243–245 °C; IR (KBr) (ν, cm⁻¹): 3269, 3175 (NH), 1731 (C=O), 1326, 1154 (S=O); ¹H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 1.95, 2.24 (3H, 2s, 5-CH₃), 6.16–7.70 (7H, m, Ar–H), 7.33, 7.43 (2H, 2s, SO₂NH₂), 10.77, 10.88 (1H, 2s, indole NH). Anal. Calcd for C₁₅H₁₃N₃O₃S (315.34): C, 57.13, H, 4.16; N, 13.33; S, 10.17. Found: C, 56.83; H, 4.14; N, 12.82; S, 10.01.

3-[(5-Fluoro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (4c). Orange crystals, yield 43%; m.p. 247–250 °C; IR (KBr) (ν, cm⁻¹): 3274, 3180 (NH), 1731 (C=O), 1329, 1156 (S=O); ¹H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 5.97–7.33 (4H, m, indole C₄,₆–H, phenyl C₂,₄–H), 6.90, 6.92 (1H, 2dd, J = 8.30, 4.39 Hz, indole C₇–H), 7.34, 7.44 (2H, 2s, SO₂NH₂), 7.48, 7.66 (1H, 2t, J = 7.80 Hz, phenyl C₅–H), 7.56, 7.71 (1H, 2d, J = 7.80 Hz, phenyl C₆–H), 10.90, 11.02 (1H, 2s, indole NH). Anal. Calcd for C₁₄H₁₀FN₃O₃S (319.31): C, 52.66, H, 3.16; N, 13.16; S, 10.04. Found: C, 52.29; H, 3.58; N, 13.18; S, 10.38.

3-[(5-Chloro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (4d). Orange powder, yield 43%; m.p. 252–254 °C; IR (KBr) (ν, cm⁻¹): 3284, 3173 (NH), 1733 (C=O), 1325, 1148 (S=O); ¹H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 6.87–7.73 (7H, m, Ar–H), 7.35, 7.46 (2H, 2s, SO₂NH₂), 11.10, 11.12 (1H, 2s, indole NH). Anal. Calcd for C₁₄H₁₀ClN₃O₃S (335.76): C, 50.08, H, 3.00; N, 12.51; S, 9.55. Found: C, 49.96; H, 3.01; N, 12.32; S, 9.79.

3-[(2-Oxo-5-(trifluoromethoxy)-1,2-dihydro-3H-indol-3-ylidene)-amino]benzenesulfonamide (4e). Orange powder, yield 25%; m.p. 205–207 °C; IR (KBr) (ν, cm⁻¹): 3338, 3282 (NH), 1667, 1733 (C=O), 1327, 1150 (S=O); ¹H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.31 (2H, s, SO₂NH₂), 7.42–7.71 (6H, m, Ar–H), 8.13 (1H, s, indole C₄–H), 10.19 (1H, s, indole NH). Anal. Calcd for C₁₅H₁₀F₃N₃O₄S (385.32): C, 46.76, H, 2.62; N, 10.91; S, 8.32. Found: C, 46.97; H, 2.32; N, 10.76; S, 8.27.

3-[(5-Nitro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (4f). Orange powder, yield 30%; m.p. 175–178 °C; IR (KBr) (ν, cm⁻¹): 3311, 3262 (NH), 1748 (C=O), 1307, 1154 (S=O). ¹H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 7.31, 7.42 (2H, 2s, SO₂NH₂), 6.97–8.65 (7H, m, Ar–H), 10.19, 11.26 (1H, 2s, indole NH). Anal. Calcd for C₁₄H₁₀N₄O₅S (346.32): C, 48.55, H, 2.91; N, 16.18; S, 9.26. Found: C, 48.74; H, 3.04; N, 16.18; S, 9.46.

4-[(2-Oxo-1,2-dihydro-3H-indol-3-ylidene)amino]benzenesulfo-namide (5a).[22] Yellow powder, yield 68%; m.p. 278–279 °C; IR (KBr) (ν, cm⁻¹): 3324, 3233 (NH), 1723, 1750 (C=O), 1329, 1144 (S=O); ¹H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 6.33, 7.60 (1H, 2d, J = 7.81 Hz, indole C₇–H), 6.73, 7.06 (1H, 2t, J = 7.81 Hz, indole C₆–H), 6.86, 6.89 (1H, 2d, J = 7.81 Hz, indole C₄–H), 7.08, 7.15 (2H, 2d, J = 8.30 Hz, phenyl C₃,₅–H), 7.28, 7.35 (2H, 2s, SO₂NH₂), 7.35, 7.46 (1H, 2t, J = 7.81 Hz, indole C₅–H), 7.73, 7.89 (2H, 2d, J = 8.30 Hz, phenyl C₂,₆–H), 10.89, 11.00 (1H, 2s, indole NH). Anal. Calcd for C₁₄H₁₁N₃O₃S (301.32): C, 55.80; H, 3.68; N, 13.95; S, 10.64. Found: C, 56.05; H, 4.05; N, 13.86; S, 10.53.

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4-[(1-Methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (5b).[23] Orange powder, yield 62%; m.p. 252–253 °C; IR (KBr) (ν, cm^{-1}) 3328, 3213 (NH), 1725 (C=O), 1321, 1154 (S=O); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 3.07, 3.19 (3H, 2s, 1–CH$_3$), 6.36, 7.64 (1H, 2d, J = 7.81 Hz, indole C$_7$–H), 6.80–7.16 (4H, m, indole C$_{4,6}$–H, phenyl C$_{3,5}$–H), 7.30, 7.36 (2H, 2s, SO$_2$NH$_2$), 7.46, 7.56 (1H, 2t, J = 7.81 Hz, indole C$_5$–H), 7.74, 7.90 (2H, 2d, J = 8.29 Hz, phenyl C$_{2,6}$–H). Anal. Calcd for C$_{15}$H$_{13}$N$_3$O$_3$S (315.34): C, 57.13, H, 4.16; N, 13.33; S, 10.17. Found: C, 57.37; H, 4.57; N, 13.20; S, 10.34.

4-[(5-Methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (5c).[24] Orange powder, yield 58%; m.p. 265–266 °C; IR (KBr) (ν, cm^{-1}) 3323, 3232 (NH), 1723, 1751 (C=O), 1320, 1147 (S=O); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.24, 2.27 (3H, 2s, 5–CH$_3$), 5.77, 6.11 (1H, 2s, indole C$_4$–H), 6.57, 6.79 (2H, 2d, J = 8.79 Hz, phenyl C$_{3,5}$–H), 6.85, 7.36 (2H, 2s, SO$_2$NH$_2$, D$_2$O exch.), 7.07, 7.29 (1H, 2d, J = 8.29 Hz, indole C$_6$–H), 7.14, 7.28 (1H, 2d, J = 8.29 Hz, indole C$_7$–H), 7.38, 7.43 (2H, 2d, J = 8.79 Hz, phenyl C$_{2,6}$–H), 10.78, 10.89 (1H, 2s, indole NH, D$_2$O exch.). Anal. Calcd for C$_{15}$H$_{13}$N$_3$O$_3$S (315.34): C, 57.13, H, 4.16; N, 13.33; S, 10.17. Found: C, 56.89; H, 4.14; N, 12.95; S, 10.53.

4-[(1,5-Dimethyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (5d). Orange powder, yield 20%; m.p. 223–225 °C; IR (KBr) (ν, cm^{-1}) 3325, 3235 (NH), 1723, 1753 (C=O), 1328, 1147 (S=O); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 1.99, 2.31 (3H, 2s, 5–CH$_3$), 3.04, 3.17 (3H, 2s, 1–CH$_3$), 6.14, 7.47 (1H, 2s, indole C$_4$–H), 6.98, 7.07 (1H, 2d, J = 8.30 Hz, indole C$_6$–H), 7.01, 7.14 (2H, 2d, J = 8.30 Hz, phenyl C$_{3,5}$–H), 7.18, 7.28 (1H, 2d, J = 8.30 Hz, indole C$_7$–H), 7.29, 7.37 (2H, 2s, SO$_2$NH$_2$), 7.73, 7.89 (2H, 2d, J = 8.30 Hz, phenyl C$_{2,6}$–H). Anal. Calcd for C$_{16}$H$_{15}$N$_3$O$_3$S (329.37): C, 58.34, H, 4.59; N, 12.76; S, 9.74. Found: C, 58.01; H, 4.90; N, 12.34; S, 9.65.

4-[(5-Fluoro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (5e). Orange powder, yield 20%; m.p. 275–277 °C; IR (KBr) (ν, cm^{-1}): (C=O); 1H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 6.08, 7.52 (1H, 2dd, J = 7.81, 4.32 Hz, indole C$_7$–H), 6.95, 6.99 (1H, 2dd, J = 8.78, 2.44 Hz, indole C$_6$–H), 7.18, 7.25 (2H, 2d, J = 8.30 Hz, phenyl C$_{3,5}$–H), 7.34, 7.40 (1H, 2dd, J = 8.78, 2.44 Hz, indole C$_4$–H), 7.37, 7.46 (2H, 2s, SO$_2$NH$_2$), 7.81, 7.98 (2H, 2d, J = 8.30 Hz, phenyl C$_{2,6}$–H), 10.97, 11.11 (1H, 2s, indole NH). Anal. Calcd for C$_{14}$H$_{10}$FN$_3$O$_3$S (319.31): C, 52.66, H, 3.16; N, 13.16; S, 10.04. Found: C, 52.99; H, 3.17; N, 12.90; S, 9.85.

4-[(5-Fluoro-1-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene)-amino]benzenesulfonamide (5f). Orange powder, yield 44%; m.p. 247–249 °C; IR (KBr) (ν, cm^{-1}): 3327, 3225 (NH), 1734 (C=O), 1322, 1155 (S=O); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 3.06, 3.19 (3H, 2s, 1–CH$_3$), 6.03–7.52 (5H, m, indole C$_{4,6,7}$–H, phenyl C$_{3,5}$), 7.31, 7.40 (2H, 2s, SO$_2$NH$_2$), 7.75, 7.92 (2H, 2d, J = 8.29 Hz, phenyl C$_{2,6}$–H). Anal. Calcd for C$_{15}$H$_{12}$FN$_3$O$_3$S (333.33): C, 54.05, H, 3.63; N, 12.61; S, 9.62. Found: C, 54.06; H, 3.71; N, 12.46; S, 9.70.

4-[(5-Chloro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (5g).[25] Yellow powder, yield 61%; m.p. 271–272 °C; IR (KBr) (ν, cm^{-1}): 3330, 3259 (NH), 1723, 1745 (C=O), 1328, 1151 (S=O); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 6.28–7.50 (3H, m, indole C$_{4,6,7}$–H), 7.11, 7.17 (2H, 2dd, J = 6.83, 1.95 Hz, phenyl C$_{3,5}$–H), 7.29, 7.40 (2H, 2s, SO$_2$NH$_2$), 7.74, 7.91 (2H, 2dd, J = 6.83, 1.95 Hz, phenyl C$_{2,6}$–H), 11.03, 11.14 (1H, 2s, indole NH). Anal. Calcd for C$_{14}$H$_{10}$ClN$_3$O$_3$S (335.76): C, 50.08, H, 3.00; N, 12.51; S, 9.55. Found: C, 49.96; H, 3.24; N, 12.48; S, 9.20.

4-[(5-Chloro-1-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene)-amino]benzenesulfonamide (5h). Orange crystals, yield 40%; m.p. 221–223 °C; IR (KBr) (ν, cm^{-1}): 3341, 3235 (NH), 1678, 1727 (C=O), 1332, 1157 (S=O); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 3.07, 3.20 (3H, 2s, 1-CH$_3$), 6.30–7.62 (5H, m, indole C$_{4,6,7}$–H, phenyl C$_{3,5}$–H), 7.30, 7.41 (2H, 2s, SO$_2$NH$_2$), 7.75, 7.92 (2H, 2dd, J = 8.78, 1.95 Hz, phenyl C$_{2,6}$–H). Anal. Calcd for C$_{15}$H$_{12}$ClN$_3$O$_3$S (349.79): C, 51.51, H, 3.46; N, 12.01; S, 9.17. Found: C, 49.05; H, 3.93; N, 11.56; S, 9.53.

4-[(5-Bromo-1-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene)-amino]benzenesulfonamide (5i). Orange crystals, yield 16%; m.p. 235–237 °C; IR (KBr) (ν, cm^{-1}): 3336, 3234 (NH), 1676, 1729 (C=O), 1331, 1156 (S=O); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 3.19 (3H, s, 1-CH$_3$), 7.11 (1H, 2dd, J = 8.78, 3.42 Hz, indole C$_6$–H), 7.17 (1H, d, J = 8.78 Hz, indole C$_7$–H), 7.30 (1H, s, indole C$_4$–H), 7.41 (2H, s, SO$_2$NH$_2$), 7.75 (2H, d, J = 8.30 Hz, phenyl C$_{3,5}$–H), 7.92 (2H, d, J = 8.30 Hz, phenyl C$_{2,6}$–H). Anal. Calcd for C$_{15}$H$_{12}$BrN$_3$O$_3$S (394.24): C, 45.70, H, 3.07; N, 10.66; S, 8.13. Found: C, 45.72; H, 3.24; N, 10.96; S, 8.17.

4-[(2-Oxo-5-(trifluoromethoxy)-1,2-dihydro-3H-indol-3-ylidene)-amino]benzenesulfonamide (5j). Yellow powder, yield 20%; m.p. 193–195 °C; IR (KBr) (ν, cm^{-1}): 3370, 3289, 3212 (NH), 1736 (C=O), 1331, 1158 (S=O); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 7.19 (2H, s, SO$_2$NH$_2$), 7.65 (4H, s, Ar–H), 6.55–7.89 (3H, m, indole C$_{4,6,7}$–H), 10.24 (1H, s, indole NH). Anal. Calcd for C$_{15}$H$_{10}$F$_3$N$_3$O$_4$S (385.32): C, 46.76, H, 2.62; N, 10.91; S, 8.32. Found: C, 46.66; H, 2.85; N, 10.78; S, 8.50.

4-[(5-Nitro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]-benzenesulfonamide (5k).[25] Orange powder, yield 15%; m.p. 257–259 °C; IR (KBr) (ν, cm^{-1}): 3339, 3263 (NH), 1752 (C=O), 1335, 1153 (S=O); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 7.32, 7.43 (2H, 2s, SO$_2$NH$_2$), 7.05–8.37 (7H, m, Ar–H), 11.59, 11.70 (1H, 2s, indole NH). Anal. Calcd for C$_{14}$H$_{10}$N$_4$O$_5$S (346.32): C, 48.55, H, 2.91; N, 16.18; S, 9.26. Found: C, 48.56; H, 3.28; N, 16.27; S, 9.56.

4-[(1-Methyl-5-nitro-2-oxo-1,2-dihydro-3H-indol-3-ylidene)-amino]benzenesulfonamide (5l). Yellow powder, yield 27%; m.p. 230–232 °C; IR (KBr) (ν, cm^{-1}): 3312, 3241 (NH), 1678, 1741 (C=O), 1339, 1157 (S=O); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 3.16, 3.20 (3H, 2s, 1-CH$_3$), 7.17–8.47 (9H, m, SO$_2$NH$_2$ and Ar–H). Anal. Calcd for C$_{15}$H$_{10}$N$_4$O$_5$S (360.34): C, 50.00, H, 3.36; N, 15.55; S, 8.90. Found: C, 50.48; H, 3.84; N, 15.53; S, 9.03.

Enzyme inhibition assay

A stopped-flow instrument (SX.18MV-R Applied Photophysics model) was used for assaying the CA-catalyzed CO$_2$ hydration activity.[26] Inhibitor and enzyme were preincubated for 15 min for allowing the complete formation of the enzyme–inhibitor

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adduct. IC_{50} values were obtained from dose response curves working at seven different concentrations of the test compound (from 0.1 nM to 50 μM), by fitting the curves using PRISM (http://www.graphpad.com) and non-linear least squares methods, the obtained values representing the mean of at least three different determinations. The inhibition constants (K_I) were derived from the IC_{50} values by using the Cheng–Prusoff equation, as follows: $K_I = IC_{50}/(1 + [S]/K_m)$ where [S] represents the CO_2 concentration at which the measurement was carried out, and K_m the concentration of the substrate at which the enzyme activity is at half maximal. All enzymes used were recombinant, produced in *E. coli* as reported earlier.[27–30] The concentrations of enzymes used in the assay were: hCA I, 12.4 nM; hCA II, 8.7 nM; hCA IX, 9.2 nM and hCA XII, 10.8 nM.

Molecular modelling studies

Preparation of ligand structures. The isatin structures **3**, **4** and **5** were prepared in 3D with the MOE software package (v2013.08.02, Chemical Computing Group Inc., Montreal, Canada) and the ligands were energy minimized using a steepest-descent protocol (MMFF94x force field).

Preparation of hCA crystal structures for docking studies. The structures of hCA I (PDB: 3LXE, 1.90 Å), hCA II (PDB: 4E3D, 1.60 Å), hCA IX (PDB: 3IAI; 2.20 Å) and hCA XII (PDB: 1JD0; 1.50 Å) were obtained from the protein databank. The protein atoms and the active site zinc ions were retained and all other atoms were omitted. The remaining structure was protonated using the MOE software package and subsequently the obtained structure was energy-minimized (AMBER99 force field). Finally, the obtained protein models were superposed on the hCA I structure using the backbone Cα-atoms and all Zn^{2+}-ions, zinc-binding histidines and the overall backbone atoms superposed well (RMSD value: 1.281 Å).

Docking of the compounds into the hCA structures. The GOLD Suite software package (v5.2, CCDC, Cambridge, UK) and the ChemScore scoring function were used to dock the compounds into the hCA structures (50 dockings per ligand). The binding pocket was defined as all residues within 13 Å of a centroid (*x*: −17.071, *y*: 35.081, 43.681; corresponding approximately to the position of the thiadiazole ring of acetazolamide in the 1JD0 structure). Position restraints were applied to the sulfur and nitrogen atoms of the acetazolamide sulfonamide tail of hCA XII (default settings) and were also applied to the other three hCA structures due to the low RMSD value of the superpositions.

Conclusions

We report here a panel of 23 new sulfonamides incorporating Schiff base moieties. They were obtained by reactions of variously substituted isatins with 2-, 3- and 4-amino-benzenesulfonamides. These new derivatives were tested as inhibitors of four physiologically relevant CA isoforms, involved in crucial physiological and pathological processes: the house-keeping cytosolic hCA I and II, as well as the transmembrane, tumor-associated hCA IX and XII, validated drug targets for theranostics for the management of hypoxic tumors. The new sulfonamides were moderate–weak hCA I/II inhibitors and highly potent, low nanomolar hCA IX/XII inhibitors. By using docking studies we also explained the differential inhibition of the four CA isoforms and the structural reasons connected with the selective inhibition of the transmembrane over the cytosolic isoforms. As a sulfonamide CA IX/XII inhibitor recently entered Phase I clinical trials for the management of metastatic solid tumors, compounds of the type reported here may be useful for designing different derivatives with such properties.

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