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

Özlen Güzel-Akdemir,a Atilla Akdemir,*b Nilgün Karal,a and Claudiu T. Supuran*c

A series of 2/3/4-[(2-oxo-1,2-dihydro-3H-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) isosforms IX and XII – recently validated antitumor drug targets, being much less effective as inhibitors of the off-target cytosolic isoforms CA I and II.

Results and discussion

Chemistry

Sulfonamides (RSO₂NH₂) constitute the most important and investigated class of CAIIs.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 isosforms of human (h) origin.¹⁷,¹⁸ As isosforms hCA I and II are widespread and play important physiological functions,¹³–¹⁵ it is of great interest to design inhibitors targeting the tumor-associated isosforms hCA IX and XII, which, at the same time, show weak affinity for the off-target isosforms hCA I and II. Some Schiff bases incorporating sulfonamide moieties were among the first types of CAIs showing selective inhibition of some CA isosforms 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-methyl-isatins substituted with methyl, halogens, nitro or trifluoromethoxy 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 isosforms (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 Kᵢs ranging...
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 $10^1$–$728$ 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: $\sim 15$-fold; hCA XII: $\sim 19$-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$_2$NH$^-$ 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

<table>
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<tr>
<th>Compounds</th>
<th>$K_I$ (nM)</th>
<th>Selectivity ratio</th>
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additional interactions except for compound 4f, which has a NO2 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 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. 1H-NMR, D2O-exch., HSQC and HMBC spectra were obtained on Varian INOVA 500 and Bruker Avance DPX 400 spectrophotometers using DMSO-d$_6$.

**Synthesis of** 2/3/4-{[(2-oxo-1,2-dihydro-3H-indol-3-ylidene)amino]benzenesulfonamides (3a–e, 4a–f, 5a–l). Equimolar quantities of 1H-indole-2,3-diones (1) [0.01 mol] and 2-aminobenzenesulfonamide/3-aminobenzenesulfonamide/4-aminobenzenesulfonamide (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.

2-{(2-Oxo-1,2-dihydro-3H-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), 1327, 1151 (S=O); $^1$H-NMR (DMSO-d$_6$, 400 MHz) δ (ppm): 6.68–8.25 (10H, m, Ar–H, SO$_2$NH$_2$), 10.58, 10.96 (1H, 2s, indole NH). Anal. Calcd for C$_{14}$H$_{11}$N$_3$O$_3$S: 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-{[3-Methyl-2-oxo-1,2-dihydro-3H-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$_2$NH$_2$), 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...
C_{12}H_{11}N_{2}O_{5}S (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-Fluro-2-oxo-1,2-dihydrop-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); ^{1}H-NMR (DMSO-d_{6}, 500 MHz) δ (ppm): 6.81 (1H, d, J = 7.32 Hz, phenyl C₁-H), 6.83 (1H, t, J = 7.81 Hz, phenyl C₃-H), 6.86 (1H, dd, J = 8.29, 4.39 Hz, indole C₁-H), 7.15 (1H, d, J = 9.26, 2.93 Hz, indole C₉-H), 7.32–7.36 (2H, m, indole C₅-H, phenyl C₁-H), 7.51 (1H, dd, J = 7.81, 0.98 Hz, phenyl C₁-H), 7.64, 8.36 (2H, 2s, SO₃NH₂, D₂O exh.), 10.64 (1H, s, indole NH, D₂O exh.). HSQC (DMSO-d₆) δ (ppm): 111.73 (δ = 7.66 Hz, indole C₂), 114.36 (δ = 25.87 Hz, indole C₁), 117.07 (phenyl C₂), 117.53 (δ = 23.48 Hz, indole C₁), 118.29 (phenyl C₁), 123.42 (phenyl C₉), 123.95 (phenyl C₇), 130.98 (δ = 8.15 Hz, indole C₃a), 133.81 (phenyl C₄), 138.10 (indole C₂a), 138.11 (indole C₁), 143.81 (phenyl C₂), 158.50 (δ = 237.71, indole C₁), 174.00 (indole C₉). Anal. Calcld for C_{14}H_{10}F_{3}N_{3}O_{5}S (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-Fluoro-2-oxo-1,2-dihydrop-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); ^{1}H-NMR (DMSO-d₆, 500 MHz) δ (ppm): 6.82 (1H, t, J = 7.81 Hz, phenyl C₁-H), 6.89 (1H, d, J = 8.29 Hz, indole C₉-H), 7.37 (1H, dd, J = 7.81, 1.46 Hz, phenyl C₁-H), 7.36 (1H, dd, J = 8.29, 2.44 Hz, indole C₂-H), 6.86, 8.41 (2H, 2s, SO₃NH₂, D₂O exh.), 7.52 (1H, dd, J = 7.81, 2.44 Hz, phenyl C₃-H), 7.54 (1H, d, J = 2.44 Hz, phenyl C₉-H), 7.64 (1H, s, indole NH, D₂O exh.). Anal. Calcld for C_{15}H_{13}F_{4}N_{3}O_{5}S (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.

2-[5-Fluoro-2-oxo-1,2-dihydrop-3H-indol-3-ylidene]amino]benzenesulfonamide (4a). Yellow powder, yield 30%; m.p. 261–263 °C; IR (KBr) (ν, cm⁻¹): 3289, 3187 (NH), 1733 (C=O), 1339, 1163 (S=O); ^{1}H-NMR (DMSO-d₆, 500 MHz) δ (ppm): 6.82 (1H, d, J = 8.29 Hz, indole C₉-H), 6.86 (1H, d, J = 7.81 Hz, phenyl C₁-H), 6.95 (1H, d, J = 8.29 Hz, indole C₂-H), 7.32 (1H, dd, J = 8.29, 1.95 Hz, indole C₉-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 C₁₆-H), 7.66, 8.46 (2H, 2s, SO₃NH₂), 10.81 (1H, s, indole NH). HMBC (DMSO-d₆) δ (ppm): 111.86 (indole C₇), 117.10 (phenyl C₂), 118.41 (indole C₄), 119.49 (OCF₃), 120.57 (indole C₁₀), 123.37 (phenyl C₁), 124.07 (phenyl C₉), 124.46 (phenyl C₇), 130.99 (indole C₃), 133.86 (phenyl C₄), 141.09 (indole C₂), 143.73 (phenyl C₉), 143.85 (indole C₄), 174.05 (indole C₁). Anal. Calcld for C_{15}H_{16}F₂N₂O₅S (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.

2-[5-Fluoro-2-oxo-1,2-dihydrop-3H-indol-3-ylidene]amino]benzenesulfonamide (4b). Yellow powder, yield 32%; m.p. 243–245 °C; IR (KBr) (ν, cm⁻¹): 3269, 3175 (NH), 1731 (C=O), 1326, 1154 (S=O); ^{1}H-NMR (DMSO-d₆, 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, SO₃NH₂), 10.77, 10.88 (1H, 2s, indole NH). Anal. Calcld for C_{14}H_{13}F₄N₂O₅S (313.54): 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.

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4-[1-Methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene]amino]-
252–253 °C; IR (KBr) (v, cm−1): 3328, 3213 (NH), 1725 (C=O),
1321, 1154 (S=O); 1H-NMR (DMSO-d6, 500 MHz) δ (ppm): 
3.07, 3.19 (3H, 2s, 1-CH3), 6.36, 6.74 (1H, 2d, J = 7.81 Hz, 
inode C6-H), 6.80–7.16 (4H, m, indole C3,4-H, phenyl C3,5-H), 7.30, 7.36 (2H, 2s, SO2NH2), 7.46, 7.56 (1H, 2t, J = 7.81 Hz, 
inode C5-H), 7.74, 7.90 (2H, 2d, J = 8.29 Hz, phenyl C2,6-H).
N, 10.66; S, 8.50. Found: C, 46.66; H, 2.85; N, 10.78; S, 8.50.

4-[5-Chloro-1-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene]-
amino]benzenesulfonamide (5h). Orange crystals, yield 40%;
m.p. 221–233 °C; IR (KBr) (v, cm−1): 3341, 3235 (NH), 1678, 1727 
(C=O), 1332, 1157 (S=O); 1H-NMR (DMSO-d6, 500 MHz) 
δ (ppm): 3.07, 3.20 (3H, 2s, 1-CH3), 6.30–7.62 (5H, m, indole 
C4,5,6-H, phenyl C2,6-H), 7.30, 7.41 (2H, 2s, SO2NH2), 7.75, 7.92 
(2H, 2dd, J = 8.78, 1.95 Hz, phenyl C2,6-H). Anal. Calcd for 
C15H12ClN3O3S (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-[3-Chloro-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) (v, cm−1): 3336, 3234 (NH), 1676, 1729 
(C=O), 1331, 1156 (S=O); 1H-NMR (DMSO-d6, 500 MHz) 
δ (ppm): 3.19 (3H, s, 1-CH3), 7.11 (1H, 2dd, J = 8.78, 3.42 Hz, 
inode C6-H), 7.17 (1H, d, J = 8.78 Hz, inode C5-H), 7.30 (1H, 
inode C4-H), 7.41 (2H, s, SO2NH2), 7.75 (2H, d, J = 8.30 Hz, 
phenyl C2,6-H), 7.92 (2H, 2d, J = 8.30 Hz, phenyl C2,6-H). Anal.
Calcd for C15H12BrN3O3S (394.32): 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) (v, cm−1): 3370, 3289, 3212 (NH), 1736 
(C=O), 1331, 1158 (S=O); 1H-NMR (DMSO-d6, 400 MHz) 
δ (ppm): 7.19 (2H, s, SO2NH2), 7.65 (4H, s, Ar-H), 6.55–7.89 
(3H, m, indole C4,5,6-H), 10.24 (1H, s, indole NH). Anal. Calcd for 
C15H12F2N3O3S (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 5%; m.p.
257–259 °C; IR (KBr) (v, cm−1): 1339, 3263 (NH), 1752 
(C=O), 1335, 1153 (S=O); 1H-NMR (DMSO-d6, 500 MHz) 
δ (ppm): 3.16, 3.20 (3H, 2s, 1-CH3), 7.17–8.47 (9H, m, Ar-H), 
11.59, 11.70 (1H, 2s, indole NH). Anal. Calcd for C15H12N2O3S 
(346.32): C, 48.55, H, 2.91; N, 16.18; S, 9.26. Found: C, 48.56;
H, 2.38; 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) (v, cm−1): 3312, 3241 (NH), 1678, 1741 
(C=O), 1339, 1157 (S=O); 1H-NMR (DMSO-d6, 500 MHz) 
δ (ppm): 3.16, 3.20 (3H, 2s, 1-CH3), 7.17–8.47 (9H, m, SO2NH2
and Ar-H). Anal. Calcd for C15H12N2SO3 (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 CO2 hydration
activity.26 Inhibitor and enzyme were preincubated for 15 min
for allowing the complete formation of the enzyme-inhibitor
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}([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 thiazole 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 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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Notes and references


