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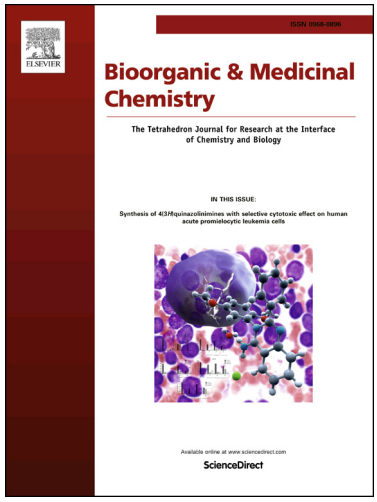

Novel sulfonamide-containing 2-indolinones that selectively inhibit tumor-associated alpha carbonic anhydrases

Nilgün Karalı, Atilla Akdemir, Füsun Göktaş, Pınar Eraslan Elma, Andrea Angeli, Merih Kızılırmak, Claudiu T. Supuran

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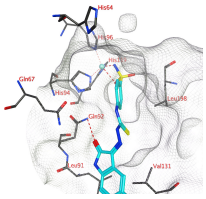

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Novel sulfonamide-containing 2-indolinones that selectively inhibit tumor-associated alpha carbonic anhydrases

Nilgün Karalı[a], Atilla Akdemir[b*], Füsun Göktaş[a], Pınar Eraslan Elma[a], Andrea Angeli[c], Merih Kızılırmak[a], and Claudiu T. Supuran[c*]

[a] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Istanbul University, Istanbul, 34116, Turkey*
[b] *Computer-Aided Drug Discovery Laboratory, Department of Pharmacology, Faculty of Pharmacy, Bezmialem Vakif University, Istanbul, 34093, Turkey*
[c] *Department of NEUROFARBA, Sezione di Scienze Farmaceutiche Universita degli Studi di Firenze, Sesto Fiorentino (Florence), 50019, Italy*
** Corresponding authors: AA: Email: aakdemir@bezmialem.edu.tr, Tel: +90-212-5232288/3142; CTS: Email: claudiu.supuran@unifi.it, Tel: +39-055-4573729/3005*

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ABSTRACT

Human carbonic anhydrases IX and XII are upregulated in many tumors and form a novel target for new generation anticancer drugs. Here we report the synthesis of novel 2-indolinone derivatives with the sulfonamide group as a zinc binding moiety. Enzyme inhibition assays confirmed that the compounds showed selectivity against hCA IX and XII over the widely distributed off-targets hCA I and II. Molecular modelling studies were performed to suggest modes of binding for these compounds.

1. Introduction

Carbonic anhydrases are enzymes that are widely distributed throughout almost all forms of live. They contain a zinc ion in their active site and they catalyze the hydration of carbon dioxide to bicarbonate, which is both a simple and at the same time a physiological important biochemical reaction.[1-6] It not only influences the acid/base balance in tissues and cells, but also is involved in carbon fixation and control of the bicarbonate levels. The human carbonic anhydrase isozymes IX and XII (hCA IX and XII) are upregulated in many tumors and may form a novel target for new generation anticancer drugs. hCA I and II are widely distributed throughout the human body and interference with these enzymes may cause side effects. As such, carbonic anhydrase inhibitors (CAIs) which show selectivity for hCA IX and/or hCA XII over hCA I and II are awaited. Here we focus on a novel class of 2-indolinone containing sulfonamides as putative CAIs of hCA IX/XII.

1*H*-Indole-2,3-dione and its derivatives have a broad spectrum of biological properties including anticancer, antiviral and antimicrobial activities. There are several reports on anticancer activities of 1*H*-indole-2,3-dione 3-thiosemicarbazone derivatives.[7-10] On the other hand, 2-indolinone derivatives carrying benzenesulfonamide moiety have been reported as selective carbonic anhydrase inhibitors.[4,11-14] Pharmacophore analysis of active compounds revealed that 1*H*-indole-2,3-dione 3-thiosemicarbazone moiety, aromatic/ hydrophobic features at the N4 position of the thiosemicarbazone and introduction of electron-withdrawing groups to 5 and/or 7 positions of 1*H*-indole-2,3-dione were essential for anticancer activity.[15-17] Additional studies showed that N^4-phenyl substituted thiosemicarbazone derivatives were significantly more active than N^4-alkyl and N^4-cycloalkyl thiosemicarbazone derivatives.[8,9]

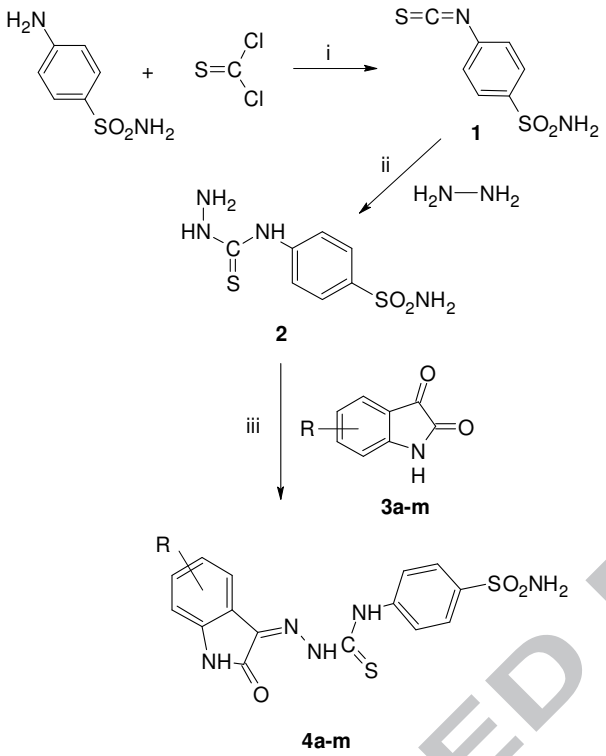
In the current study, we have synthesized novel 1*H*-indole-2,3-dione 3-(N-phenylthiosemicarbazones) derivatives that all contained a sulfonamide group as zinc binding moiety. These compounds were tested in enzyme inhibition assays of the intended target enzymes hCA IX and XII and the off-targets hCA I and II. The compounds showed selectivity for hCA IX/XII over hCA I/II. Molecular modelling studies were performed to gain more insight into the possible binding interactions of these novel class of CAIs.

2. Results and discussion

2.1. Chemistry

In this study, 4-isothiocyanatobenzenesulfonamide **1** was prepared by reacting of 4-aminobenzenesulfonamide with

thiophosgene in water containing concentrated hydrochloric acid.[18] Hydrazine hydrate was reacted with **1** in ethanol to give N-(4-sulfamoylphenyl)thiosemicarbazide **2**. A series of 1*H*-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] **4a-m** were synthesized by reacting **2** with 1*H*-indole-2,3-dione **3a-m** in ethanol containing a catalytic amount of sulfuric acid.[7,19,20] The structures of **4a-m** were confirmed by analytical and spectral (IR, ^{1}H NMR, ^{13}C-DEPT, HSQC-2D, HMBC-2D and LCMS-ESI) data (Scheme 1).



Scheme 1. Synthesis of **4a-m**. Reagents and conditions: i) H_2O, HCl, stirred ii) EtOH, stirred, cooled ii) EtOH, H_2SO_4, reflux

IR spectra of **4a-m** showed absorption bands in the 3394-3115 cm^{-1} region resulting from the NH stretching of the lactam, thioamide and sulfonamide functions. The lactam C=O and thioamide C=S stretching were observed in the 1712-1674 and 1224-1204 cm^{-1} regions, respectively. The sulfonamide S=O stretching showed two separate absorptions at 1343-1311 and 1161-1138 cm^{-1} regions.[21-23] ^{1}H NMR spectra of **4a-m** displayed the sulfonamide NH protons (δ 7.36-7.60 ppm), the NH protons of the thiosemicarbazone moiety (δ 10.93-11.22 and 12.65-12.92 ppm) and the indole NH proton (δ 11.15-11.86 ppm) as four separate singlets. HSQC-2D spectra of **4a** and HMBC-2D spectra of **4d** showed the C=O (δ 163.41 and 163.49 ppm) and the C=S (δ 177.04 and 177.09 ppm) peaks which verified the thiosemicarbazone structure.[22,24-26] In the mass spectra of **4a**, **4d** and **4m** $(M-H)^-$ peaks were observed which confirmed their molecular weights.

2.2. Carbonic anhydrase enzyme inhibition assays

The 13 ligands have been tested against the two tumor-associated membrane-bound hCA IX and XII and the widely distributed cytosolic off-targets hCA I and II (Table 1). The compounds show K_I values in the lower nanomolar range for hCA XII (1.2 – 87.3 nM) and hCA IX (13.3 – 66.3 nM).

Table 1. CA enzyme inhibition data (K_I, nM) of compounds **4** and acetazolamide (**AAZ**).

Compound	R	hCA I	hCA II	hCA IX	hCA XII
4a	H	1190	936	66.3	6.9
4b	5-CH_3	744	735	62.3	5.7
4c	5-OCF_3	481	420	45.7	6.0
4d	5-F	558	551	38.8	4.5
4e	5-Cl	650	616	54.2	5.8
4f	5-Br	882	851	64.8	6.1
4g	5-I	624.2	399.4	16.8	5.9
4h	5-SO_3Na	68.8	32.0	19.5	4.1
4i	5-NO_2	901	897	34.4	1.2
4j	7-F	57.6	26.9	20.8	45.5
4k	7-Cl	76.1	425.6	13.3	87.3
4l	5,7-diCl	554.4	782.1	21.3	82.3
4m	5,7-diBr	698	687	58.1	6.3
AAZ	---	250	12.1	25.0	5.7

The measured K_I values for hCA IX are higher compared to hCA XII (still in lower nanomolar region), but there is only approximately 5-fold difference between the highest and lowest measured K_I values, resulting in a flat SAR (Table 1).

The measured inhibition constant for hCA XII are lower for most compounds, except for **4j**, **4k** and **4l**. These three compounds have K_I values higher than 45 nM, while all other compounds have K_I values lower than 7 nM. The lowest K_I value has been measured for **4i** (1.2 nM). With the exception of compounds **4j**, **4k** and **4l** only approximately 6-fold difference in K_I values has been observed, which makes the structure-activity relationships (SAR) data rather flat and difficult to interpret. Compounds **4j**, **4k** and **4l** have either a fluorine or chlorine substituent at position 7 (in addition also 5-position for compound **4l**). However, compound **4m** has bromine substituents at the 5- and 7-positions, which is well tolerated. In addition, it seems that negatively charged substituents at the 5-position (such as **4i** and **4h**) show the lowest K_I values.

2.3. Molecular modelling studies

All K_I values of the tested compounds are in the lower nanomolar range (< 90 nM) for hCA IX and XII, with the lowest measured K_I value for compound **4i** (hCA XII; Table 1). The docked pose for this compound suggests that the nitro (at 5-position) and carbonyl groups chains of His64 and Lys67 of hCA XII, respectively (Figure 1). It should be noted that the nitro group is positioned close to the cationic nitrogen atom of Lys170 (distance < 3.0 Å) and that electrostatic interactions are possible. The sulfonamide tail interacts with the active site Zn^{2+} with its nitrogen and oxygen atoms to form distorted trigonal bipyramidal geometry. Compound **4h** has an identical binding pose and the

sulfonate group forms hydrogen bonds with both His64 and Lys170.

All docked compounds can adopt this docked pose in the hCA XII active site. The additional hydrogen bonds and electrostatic interactions formed by the nitro and sulfonate substituents at the 5-position are expected to be the cause for the lower K_I values of **4i** and **4h**.

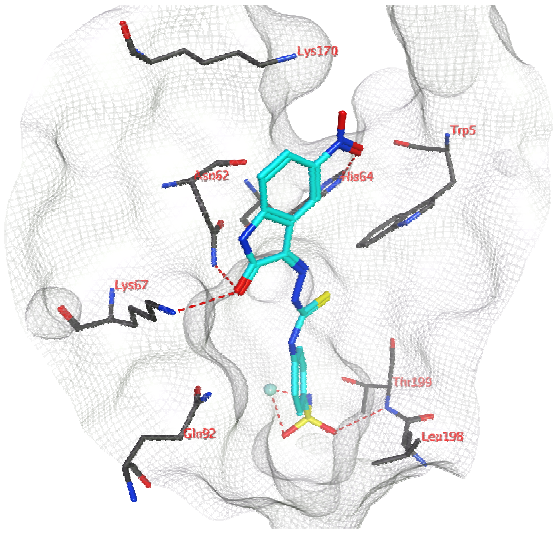


Figure 1. The docked pose of compound **4i** in the active site of hCA XII.

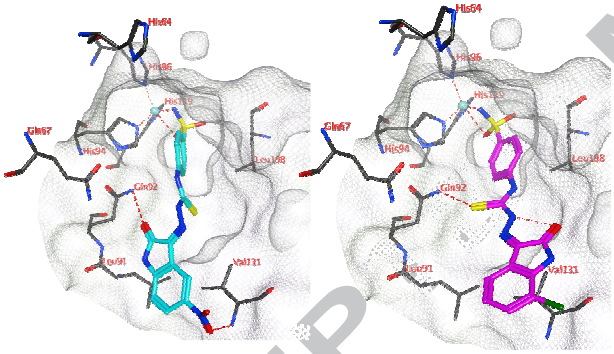


Figure 2. The docked poses of compounds **4i** (left panel; turquoise) and **4k** (right panel; magenta) in the active site of hCA IX.

The hCA IX active site shows some minor but important differences compared to the hCA XII site. Gln67 (hCA IX) is present in the active site instead of Lys67 (hCA XII) resulting in a loss of the hydrogen bond with the carbonyl of the 2-indolinone ring. Additionally, a negatively charged Glu170 is present in hCA IX instead of the cationic Lys170 in hCA XII. This may interfere with the negatively charged nitro or sulfonate substituents at the 5-position of the 2-indolinone ring. As such, the compounds adopt two different docked poses in the hCA IX active site (Figure 2). Both poses show hydrophobic interactions between their phenyl groups and the side chain of Leu198 and hydrogen bonds between either their carbonyl group or sulfur atoms and the side chain of Gln92. The nitro substituent at the 5-position of compound **4i** forms an additional hydrogen bond with the backbone of Val131. The chlorine substituent of the 7-position of compound **4k** interact with the side chain of Asp132. The other compounds can adopt similar poses as shown in Figure 2.

3. Experimental protocols

3.1. Chemistry

All reagents and solvents were purchased from Sigma-Aldrich (Darmstadt, Germany) or Meck (Germany) used as received. Compound **3c** was obtained from (Sigma-Aldrich, 390674). 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, HSQC-2D and HMBC-2D spectra were obtained on Varian UNITY INOVA 500 spectrophotometer using DMSO-d_6. Mass spectra were determined on an AGILENT 1100 MSD instrument.

*3.1.1. The synthesis of 4-isothiocyanatobenzenesulfonamide (**1**)*

4-Aminobenzenesulfonamide (3 mmol) was dissolved in 50 mL of water containing 12 mL of concentrated hydrochloric acid. To the solution, thiophosgene (3 mmol) was added in one portion. Stirring was begun immediately and continued until all of the red color of thiophosgene had disappeared and the product appeared as a white crystalline precipitate. The precipitate was filtered, washed thoroughly with water, and recrystallized from acetone-water.

*3.1.2. The synthesis of N-(4-sulfamoylphenyl) thiosemicarbazide (**2**)*

To a solution of hydrazine hydrate (5 mmol) in ethanol (10 mL), a suspension of 4-isothiocyanatobenzenesulfonamide **1** (5 mmol) in ethanol (10 mL) was added dropwise with vigorous stirring and cooling in an ice bath. The mixture was allowed to stand overnight. The crystals formed were recrystallized from ethanol.

*3.1.3. The synthesis of 1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4a-m**)*

N-(4-sulfamoylphenyl)hydrazinecarbothioamide (**2**) (3.5 mmol) was added to a solution of 1*H*-indole-2,3-diones **3a-m** (3.5 mmol) in ethanol (20 mL). After addition of a drop of concentrated sulfuric acid, the mixture was refluxed on a water bath for 4 h. The product formed after cooling was filtered and washed with ethanol or recrystallized from ethanol.

*3.1.4. 1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4a**)*[27]

Yellow powder (90%): mp 268°C; IR (KBr): υ 3374, 3274, 3182 (NH), 1674 (C=O), 1337, 1154 (S=O), 1207 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 6.94 (1H, d, J: 7.80 Hz, ind. C7-H), 7.10 (1H, dt, J: 7.80, 7.32 Hz, ind. C5-H), 7.36 (2H, s, SO_2NH_2, D_2O exch.), 7.37 (1H, t, J: 7.80 Hz, ind. C6-H), 7.76 (1H, d, J: 7.32 Hz, ind. C4-H), 7.85 (4H, s, phen.), 10.94 (1H, s, N4-H, D_2O exch.), 11.25 (1H, s, ind. NH, D_2O exch.), 12.89 (1H, s, N2-H, D_2O exch.). HSQC-2D (DMSO-d_6/125 MHz): 111.87 (ind. C7), 120.49 (ind. C3a), 122.17 (ind. C4), 123.11 (ind. C6), 125.98 (phen. C2,6), 126.67 (phen. C3,5), 132.34 (ind. C5), 133.55 (ind. C3), 141.79 (ind. C7a), 142.14 (phen. C4), 143.34 (phen. C1), 163.41 (ind. C2), 177.09 (C=S). LCMS-ESI (-): m/z (%) 374 (M-H-, 100). Anal. Calcd for $C_{15}H_{13}N_5O_3S_2$: C, 47.99; H, 3.49; N, 18.65 Found: C, 47.68; H, 3.56; N, 18.17.

*3.1.5. 5-Methyl-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4b**)*

Yellow powder (93%): mp 258-260°C; IR (KBr): υ 3296, 3269, 3173 (NH), 1685 (C=O), 1329, 1153 (S=O), 1212 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 2.30 (3H, s, CH_3), 6.83 (1H, d, J: 8.33 Hz, ind. C7-H), 7.18 (1H, d, J: 8.33 Hz, ind. C6-H), 7.36 (2H, s, SO_2NH_2), 7.59 (1H, s, ind. C4-H), 7.87 (4H, s, phen.), 10.93 (1H, s, N4-H), 11.15 (1H, s, ind. NH), 12.88 (1H, s, N2-H). Anal. Calcd for $C_{16}H_{15}N_5O_3S_2$: C, 49.34; H, 3.88; N, 17.98 Found: C, 49.12; H, 3.89; N, 17.85.

*3.1.6. 5-Trifluoromethoxy-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4c**)*

Light yellow powder (90%): mp 246-248°C; IR (KBr): υ 3339, 3237, 3115 (NH), 1693 (C=O), 1326, 1153 (S=O), 1204 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 7.03 (1H, d, J: 8.32 Hz, ind. C7-H), 7.35 (1H, dd, J: 8.32, 1.60 Hz, ind. C6-H), 7.37 (2H, s, SO_2NH_2, D_2O exch.), 7.78 (1H, d, J: 1.60, Hz, ind. C4-H), 7.84 (2H, s, phen. C2-H, C6-H), 7.85 (2H, s, phen. C3-H, C5-H), 11.01 (1H, s, N4-H, D_2O exch.), 11.41 (1H, s, ind. NH, D_2O exch.), 12.75 (1H, s, N2-H, D_2O exch.). Anal. Calcd for $C_{16}H_{12}F_3N_5O_4S_2$: C, 41.83; H, 2.63; N, 15.24 Found: C, 41.49; H, 2.52; N, 15.16.

*3.1.7. 5-Fluoro-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4d**)*

Red powder (92%): mp 260-262°C; IR (KBr): υ 3338, 3236, 3115 (NH), 1693 (C=O), 1326, 1154 (S=O), 1205 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 6.94 (1H, dd, J: 8.30, 3.91 Hz, ind. C7-H), 7.20 (1H, dd, J: 8.78, 2.44 Hz, ind. C6-H), 7.36 (2H, s, SO_2NH_2), 7.62 (1H, dd, J: 8.30, 2.44 Hz, ind. C4-H), 7.85 (4H, s, phen.), 10.96 (1H, s, N4-H), 11.25 (1H, s, ind. NH), 12.77 (1H, s, N2-H); HMBC-2D (DMSO-d_6/ 125 MHz): 109.06 (d, J: 25.88 Hz, ind. C4), 112.96 (d, J: 8.14 Hz, ind. C7), 118.56 (d, J: 24.44 Hz, ind. C6), 121.92 (d, J: 9.58 Hz, ind. C3a), 125.98 (phen. C2,6), 126.73 (phen. C3,5), 132.91 (d, J: 2.87 Hz, ind. C3), 139.60 (d, J: 1.91 Hz, ind. C7a), 141.94 (phen. C4), 141.95 (phen. C1), 158.98 (d, J: 237.71, ind. C5), 163.49 (ind. C2), 177.04 (C=S). LCMS-ESI (-): m/z (%) 392 (M-H-, 100). Anal. Calcd for $C_{15}H_{12}FN_5O_3S_2$: C, 45.79; H, 3.07; N, 17.80 Found: C, 45.46; H, 3.26; N, 17.33.

*3.1.8. 5-Chloro-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4e**)*

Orange powder (93%): mp 266-268°C; IR (KBr): υ 3323, 3291, 3165 (NH), 1691 (C=O), 1311, 1161 (S=O), 1204 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 6.95 (1H, d, J: 8.30 Hz, ind. C7-H), 7.40 (1H, dd, J: 8.30, 2.44 Hz, ind. C6-H), 7.84 (1H, d, J: 2.44 Hz, C4-H), 7.36 (2H, s, SO_2NH_2), 7.85 (4H, s, phen.), 10.99 (1H, s, N4-H), 11.34 (1H, s, ind. NH), 12.71 (1H, s, N2-H). Anal. Calcd for $C_{15}H_{12}ClN_5O_3S_2$: C, 43.96; H, 2.95 N, 17.09 Found: C, 43.64; H, 2.86; N, 17.09.

*3.1.9. 5-Bromo-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4f**)*

Dark yellow powder (96%): mp 254-255°C; IR (KBr): υ 3306, 3245, 3172 (NH), 1687 (C=O), 1326, 1151 (S=O), 1204 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 6.90 (1H, d, J: 8.33, Hz, ind. C7-H), 7.58 (1H, dd, J: 8.33, 2.25 Hz, ind. C6-H), 7.36 (2H, s, SO_2NH_2), 7.99 (1H, d, J: 2.25, Hz, ind. C4-H), 7.85 (4H, s, phen.), 11.00 (1H, s, N4-H), 11.35 (1H, s, ind. NH.), 12.70 (1H, s, N2-H). Anal. Calcd for $C_{15}H_{12}BrN_5O_3S_2$: C, 39.65; H, 2.66; N, 15.41 Found: C, 39.72; H, 2.66; N, 15.09.

*3.1.10. 5-Iodo-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4g**)*[28,29]

Yellow powder (73%): mp 286-288°C; IR (KBr): υ 3307, 3247, 3176 (NH), 1683 (C=O), 1327, 1151 (S=O), 1205 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 6.99 (1H, d, J: 8.30 Hz, ind. C7-H), 7.60 (2H, s, SO_2NH_2), 7.88 (1H, dd, J: 8.30, 1.20 Hz, ind. C6-H), 8.34 (1H, d, J: 1.20 Hz, ind. C4-H), 8.05 (4H, s, phen.), 11.22 (1H, s, N4-H), 11.56 (1H, s, ind. NH.), 12.92 (1H, s, N2-H); Anal. Calcd for $C_{15}H_{12}IN_5O_3S_2$: C, 35.94; H, 2.41; N, 13.97 Found: C, 35.81; H, 2.62; N, 13.71.

*3.1.11. 5-Nitro-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4h**)*

Dark yellow powder (85%): mp 265-267°C; IR (KBr): υ 3546, 3329, 3256 (NH), 1692 (C=O), 1343, 1155 (S=O), 1213 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 1.04 (3H, t, J: 6.83 Hz, CH_3CH_2OH), 3.43 (2H, q, J: 6.83 Hz, CH_3CH_2OH), 4.31 (1H, br.s, CH_3CH_2OH), 7.13 (1H, d, J: 8.78 Hz, ind. C7-H), 7.37 (2H, s, SO_2NH_2), 8.28 (1H, dd, J: 8.78, 2.44 Hz, ind. C6-H), 8.67 (1H, d, J: 2.44 Hz, ind. C4-H), 7.84 (2H, s, phen. C2-H, C6-H) , 7.86 (2H, s, phen. C3-H, C5-H), 11.19 (1H, s, N4-H), 11.86 (1H, s, ind. NH.), 12.65 (1H, s, N2-H); Anal. Calcd for $C_{15}H_{12}N_6O_5S_2.{}^1/_2C_2H_5OH$: C, 43.20; H, 3.37; N, 18.91 Found: C, 42.96; H, 3.38; N, 18.92.

*3.1.12. Sodium 1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] - 5-sulfonate (**4i**)*[28,29]

Dark yellow powder (66%): mp >300°C; IR (KBr): υ 3271, 3234 (NH), 1712 (C=O), 1313, 1153 (S=O), 1220 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 6.89 (1H, d, J: 8.40 Hz, ind. C7-H), 7.38 (2H, s, SO_2NH_2), 7.65 (1H, dd, J: 8.00, 1.60 Hz, ind. C6-H), 7.84 (4H, s, phen.), 8.16 (1H, d, J: 1.60 Hz, ind. C4-H), 11.20 (1H, s, N4-H), 11.33 (1H, s, ind. NH.), 12.86 (1H, s, N2-H); Anal. Calcd for $C_{15}H_{12}N_5NaO_6S_3.4H_2O$: C, 32.78; H, 3.85; N, 12.74 Found: C, 32.86; H, 3.69; N, 12.78.

*3.1.13. 7-Fluoro-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4j**)*[28,29]

Yellow powder (86%): mp 271-273°C; IR (KBr): υ 3394, 3277, 3136 (NH), 1681 (C=O), 1338, 1149 (S=O), 1220 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 7.14 (1H, dd (t), J: 7.80, 4.88 Hz, ind. C5-H), 7.31 (1H, dd, J: 8.29, 1.95 Hz, ind. C6-H), 7.39 (2H, s, SO_2NH_2, D_2O exch.), 7.63 (1H, d, J: 7.32 Hz, ind. C4-H), 7.85 (4H, s, phen.), 11.04 (1H, s, N4-H, D_2O exch.), 11.81 (1H, s, ind. NH, D_2O exch.), 12.86 (1H, s, N2-H, D_2O exch.); Anal. Calcd for $C_{15}H_{12}FN_5O_3S_2$: C, 45.79; H, 3.07; N, 17.80 Found: C, 45.64; H, 2.89; N, 17.52.

*3.1.14. 7-Chloro-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4k**)*[28,29]

Yellow powder (87%): mp 288-289°C; IR (KBr): υ 3331, 3271, 3130 (NH), 1693 (C=O), 1334, 1138 (S=O), 1224 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 7.15 (1H, t, J: 7.80 Hz, ind. C5-H), 7.46 (1H, d, J: 7.80 Hz, ind. C6-H), 7.39 (2H, s, SO_2NH_2, D_2O exch.), 7.75 (1H, d, J: 7.32 Hz, ind. C4-H), 7.85

(4H, s, phen.), 11.04 (1H, s, N4-H, D_2O exch.), 11.79 (1H, s, ind. NH, D_2O exch.), 12.83 (1H, s, N2-H, D_2O exch.); Anal. Calcd for $C_{15}H_{12}ClN_5O_3S_2$: C, 43.96; H, 2.95; N, 17.09 Found: C, 44.08; H, 3.34; N, 16.93.

*3.1.15. 5,7-Dichloro-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4l**)* [28,29]

Yellow powder (77%): mp 275-277°C; IR (KBr): υ 3321, 3251, 3136 (NH), 1693 (C=O), 1330, 1153 (S=O), 1222 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 7.37 (2H, s, SO_2NH_2), 7.59 (1H, d, J: 1.97 Hz, ind C6-H), 7.85 (3H, br.s, phen. C2-H, C6-H, ind. C4-H), 7.86 (2H, s, phen. C3-H, C5-H) , 11.04 (1H, s, N4-H), 11.82 (1H, s, ind. NH), 12.65 (1H, s, N2-H); Anal. Calcd for $C_{15}H_{11}Cl_2N_5O_3S_2$: C, 40.55; H, 2.50; N, 15.76 Found: C, 40.38; H, 2.80; N, 15.73.

*3.1.16. 5,7-Dibromo-1H-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] (**4m**)*

Yellow powder (95%): mp 283°C; IR (KBr): υ 3328, 3255, 3215 (NH), 1694 (C=O), 1329, 1151 (S=O), 1205 (C=S). ^{1}H NMR (DMSO-d_6/ 500 MHz) ppm: 7.37 (2H, s, SO_2NH_2), 7.80 (1H, d, J: 1.92 Hz, ind C6-H), 7.84 (2H, s, phen. C2-H, C6-H), 7.86 (2H, s, phen. C3-H, C5-H) , 8.01 (1H, d, J:1.92 Hz, ind. C4-H), 11.04 (1H, s, N4-H), 11.69 (s, 1H, ind. NH.), 12.65 (s, 1H, N2-H). ^{13}C-NMR (DEPT) (DMSO-d_6/125MHz) ppm: 124.00 (ind. C4), 126.17 (phen. C2, C6), 126.75 (phen. C3, C5), 138.00 (ind. C6). LCMS-ESI (-): m/z (%) 530, 532, 534 (M-H-, 43, 100, 52). Anal. Calcd for $C_{15}H_{11}Br_2N_5O_3S_2$: C, 33.79; H, 2.08; N, 13.13 Found: C, 33.85; H, 2.65; N, 13.20.

3.2. Carbonic anhydrase enzyme inhibition studies

A stopped-flow instrument (SX.18MV-R Applied Photophysics model) was used for assaying the CA-catalyzed CO_2 hydration activity.[30] 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 test compound (from 0.1 nM to 50 μM), by fitting the curves using PRISM (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 substrate at which the enzyme activity is at half maximal. All enzymes used were recombinant, produced in E. coli as reported earlier.[31] The concentrations of the 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.

3.3. Molecular modelling studies

3.3.1. Preparation of ligand structures

The three-dimensional structures of all ligands were prepared (MOE software package, v2016.08, Chemical Computing Group, Inc, Montreal, Canada). The sulfonamide nitrogen atoms were assigned a negative charge (R-SO_2NH^-) and the ligands were energy minimized using a steepest-descent protocol (MMFF94x force field). The ligand structures were saved as mol2 files.

3.3.2. Preparation of protein structures

All protein structures were obtained from the RCSB protein databank: 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). 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 (AMBER12:EHT 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).

3.3.3. Docking protocols

The GOLD Suite software package (v5.5, 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.

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