



Synthesis 4-[2-(2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamides with subnanomolar carbonic anhydrase II and XII inhibitory properties



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ARTICLE INFO

Article history:

Received 1 June 2016

Revised 24 June 2016

Accepted 25 June 2016

Available online 27 June 2016

Keywords:

Carbonic anhydrase

Sulfonamide

Inhibitor

2-Mercapto-quinazolin-4-one

Tail approach

ABSTRACT

Condensation of substituted anthranilic acids with 4-isothiocyanatoethyl-benzenesulfonamide led to series of heterocyclic benzenesulfonamides incorporating 2-mercapto-quinazolin-4-one tails. These sulfonamides were investigated as inhibitors of the human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms hCA I and II (cytosolic isozymes), as well as hCA XII (a transmembrane, tumor-associated enzyme also involved in glaucoma-genesis). The new sulfonamides acted as medium potency inhibitors of hCA I (K_i s of 28.5–2954 nM), being highly effective as hCA II (K_i s in the range of 0.62–12.4 nM) and XII (K_i s of 0.54–7.11 nM) inhibitors. All substitution patterns present in these compounds (e.g., halogens, methyl and methoxy moieties, in positions 6, 7 and/or 8 of the 2-mercapto-quinazolin-4-one ring) led to highly effective hCA II/XII inhibitors. These compounds should thus be of interest as preclinical candidates in pathologies in which the activity of these enzymes should be inhibited, such as glaucoma (CA II and XII as targets) or some tumors in which the activity of isoforms CA II and XII is dysregulated.

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1. Introduction

In a previous work from our groups¹ we reported a series of benzenesulfonamides obtained from sulfanilamide, which was converted to the corresponding 4-isothiocyanato benzenesulfonamide, and reacted with anthranilic acid derivatives, leading thus to 2-mercapto-3H-quinazolin-4-ones. These sulfonamides possessed excellent inhibitory activity against the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). Several CA isoforms (of the 15 presently known in humans)^{2–4} were inhibited in the low nanomolar range with some of these compounds, among which the human (h) hCA II and XII (involved in glaucoma)^{2–4} as well as hCA IX and XII (transmembrane isoforms involved in tumorigenesis).^{5–7} Although no crystal structures for adducts of 2-mercapto-3H-quinazolin-4-one-benzenesulfonamides (such as **A–C**, Fig. 1) with any CA isozymes are available to date, we hypothesized that the presence of the substituted-mercaptoquinazolinone tails in their

scaffolds exerted a beneficial effect on the interaction with the enzyme active site, and thus decided to further investigate this type of tail for designing sulfonamide CA inhibitors (CAIs). Indeed, pharmacological agents belonging to this class are clinically used as diuretics,⁸ antiglaucoma,^{9,10} antiepileptic,^{11,12} antiobesity,¹³ and more recently antitumor agents,^{5–7} with one such derivative, SLC-0111 in Phase Ib clinical trials for the treatment of metastatic hypoxic cancers.^{14,15}

2. Results and discussion

2.1. Chemistry

The drug design strategy for obtaining novel sulfonamides used in this work is based on the tail approach developed by one of our groups earlier.¹⁶ Substituted anthranilic acids **1a–o** and 4-isothiocyanatoethyl-benzenesulfonamide **2** have been employed for preparing the new sulfonamides **3a–o** (Scheme 1) by a chemistry already reported in the previous work, in which the derivatives without the ethylene spacer, of types **A–C**, have been reported.^{1,17}

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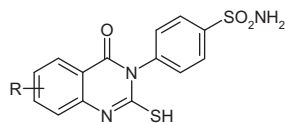


Figure 1. Sulfonamides **A–C** incorporating 2-mercapto-3H-quinazolin-4-one functionalities and their hCA II and XII inhibitory properties.¹ **A:** R = 8-Cl, K_i (hCA II) = 0.31 nM; K_i (hCA XII) = 5.5 nM; **B:** R = 6-Me, K_i (hCA II) = 0.25 nM; K_i (hCA XII) = 5.2 nM; **C:** R = 7,8-(MeO)₂, K_i (hCA II) = 0.61 nM; K_i (hCA XII) = 0.67 nM.

In fact we have prepared a large number of thioureas incorporating benzenesulfonamide functionalities, by reaction of aromatic sulfonamides incorporating isothiocyanato moieties with amines (aromatic, aliphatic or heterocyclic),^{18b} amino acids,^{18a} and oligopeptides.^{18c} Many of the derivatives obtained by such procedures (incorporating the NH-CS-NH-C₆H₄-SO₂NH₂ functionality) showed excellent CA inhibitory properties against isoforms such as hCA II, IV, IX and XII,¹⁸ involved in serious pathologies, such as glaucoma^{2–4} and cancer.^{5,6} Furthermore, some of these sulfonamides also showed good degrees of isoform selectivity inhibitory profiles.¹⁸ Thus, in this work we extend our previous investigations^{1,18} on the use of 4-isothiocyanatoethyl-benzenesulfonamide for obtaining CAIs incorporating novel heterocyclic scaffolds (Scheme 1).

The reaction of the substituted anthranilic acid **1a–o** with the isothiocyanate **2** initially led to an *ortho*-carboxyphenyl-thiourea, which has not been isolated, as in the reaction conditions we used it underwent cyclization with the formation of mercapto-quinazolinones **3a–o** (Scheme 1). The chemical diversity of the obtained series was achieved by using variously substituted-anthranilic acids which incorporated halogens (F, Cl, Br, I), methyl and methoxy moieties (one, two or three such moieties) in their molecules, similar with the sulfonamides reported in the previous work¹ (Table 1).

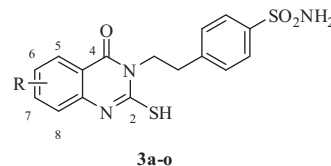
The structure of the new compounds was confirmed by means of NMR, MS and elemental analyses (see Section 4 for details). The ¹H NMR spectra showed D₂O exchangeable singlet peak corresponding to the SH group at around 13.0 ppm, D₂O exchangeable peaks due to the SO₂NH₂ protons at around 7.50 ppm. The aromatic protons were also detected at their expected respected regions. The ¹³C NMR spectra were also in agreement with the suggested structure of the target compounds (see Section 4 for details). The mass spectra (MS) showed the exact mass ion for each compound and the corresponding isotopes when halogens were present in the new compounds (see Section 4 for details).

2.2. Inhibition of CAs

We investigated the inhibition profile of sulfonamides **3a–3o** against the isoforms hCA I and II (cytosolic, off-target isoforms when considering the anti-cancer applications of CAIs, but target isoforms for glaucoma)¹⁹ and hCA XII (predominantly present in tumors but also overexpressed in glaucomatous eyes).⁹ For comparison reasons we also discuss the inhibition profiles of the standard, clinically used sulfonamide drug acetazolamide

Table 1

Inhibition data of human CA isoforms hCA I, II and XII with sulfonamides **3a–o** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO₂ hydrase assay¹⁹



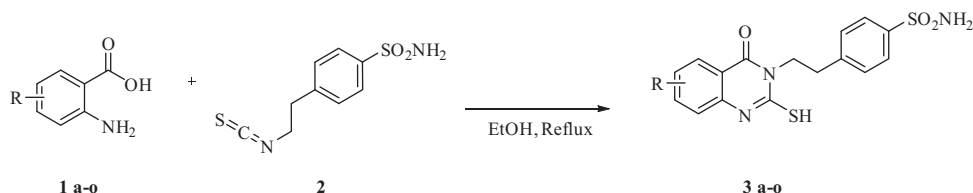
Compound	K_i (nM) [*]			
	R	hCA I	hCA II	hCA XII
3a	H	31.5	0.62	0.59
3b	6-F	212	12.4	0.62
3c	7-F	39.9	9.1	0.64
3d	6-Cl	153	6.5	0.54
3e	8-Cl	41.4	0.78	0.66
3f	7-Br	104	0.91	1.45
3g	6-I	34.3	0.71	0.76
3h	6-CH ₃	296	56.2	5.53
3i	8-CH ₃	2954	1.2	0.61
3j	6,8-CH ₃	1402	0.86	0.73
3k	6-OCH ₃	150	1.0	0.54
3l	7-OCH ₃	28.5	0.69	0.47
3m	8-OCH ₃	42.3	31.9	5.74
3n	6,7-OCH ₃	678	1.1	0.65
3o	6,7,8-OCH ₃	268	10.7	7.11
AAZ	—	250	12	5.7

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

(5-acetamido-1,3,4-thiadiazole-2-sulfonamide, **AAZ**) against the same isoforms (Table 1).

The following structure–activity relationship (SAR) can be observed from data of Table 1:

- hCA I, the slow, off-target cytosolic isoform was inhibited by all sulfonamides **3a–3o** with inhibition constants ranging between 28.5 and 2954 nM. Some derivatives showed good inhibitory properties, such as **3a**, **3c**, **3e**, **3g**, **3l** and **3m**, with K_i s ranging between 28.5 and 42.3 nM. They incorporated either H at the quinazolinone ring (**3a**) or 7-F, 8-Cl, 6-I, 7-MeO and 8-MeO substituents. Interestingly, small structural changes (such as the shift of the fluorine from the 7 to the 6 position of the heterocyclic ring, e.g., in **3b**), led to important differences of activity with **3b**, which resulted 5.3 fold weaker inhibitor of hCA I when compared to **3c** (Table 1). The same is true for the isomers **3d–3e**, **3k–3l**, etc. Some of these derivatives, such as **3b**, **3d**, **3f**, **3h**, **3k** and **3o** showed medium potency hCA I inhibition, in the same range as acetazolamide (K_i = 250 nM), with inhibition constants in the range of 104–268 nM. Three derivatives, **3i**, **3j** and **3n** were even weaker inhibitors, with K_i s ranging between 678 and 2954 nM. Thus, the main SAR conclusion is that



Scheme 1. General synthetic procedure for compounds **3a–o**.

the type and, more importantly, the position of the R substituent in the quinazolinone ring have great influence the hCA I inhibitory properties.

- (ii) SAR was even simpler for the inhibition of the physiologically dominant cytosolic isoform hCA II (Table 1). Thus, all the new sulfonamides **3a–3o** were highly effective inhibitors with two of them having K_i s in the range of 31.9–56.2 nM (compounds **3h** and **3m**) whereas the remaining ones acting as very strong inhibitors with K_i s in the range of 0.62–12.4 nM (as effective as **AAZ** or one order of magnitude better inhibitors compared to this clinically used compound). Thus, basically all substitution patterns present in these sulfonamides led to effective hCA II inhibitors except the 6-Me and 8-MeO groups which led to slightly weaker inhibitors.
- (iii) As hCA II, also the transmembrane isoform hCA XII was highly inhibited by all the new sulfonamides reported here with K_i s in the range of 0.54–7.11 nM. The SAR is thus very flat and all the substitution patterns from the new sulfonamides were highly beneficial for inducing high affinity for hCA II (Table 1).

3. Conclusions

Condensation of substituted anthranilic acids with 4-isothiocyanatoethyl-benzenesulfonamide led to series of heterocyclic benzenesulfonamides incorporating 2-mercapto-quinazolin-4-one tails. These sulfonamides were investigated as inhibitors of the following isoforms: hCA I and II (cytosolic isozymes), as well as hCA XII (a *trans*-membrane, tumor-associated enzyme also involved in glaucomagenesis). The new sulfonamides acted as medium potency inhibitors of hCA I (K_i s of 28.5–2954 nM), being highly effective as hCA II (K_i s in the range of 0.62–12.4 nM) and XII (K_i s of 0.54–7.11 nM) inhibitors. All substitution patterns present in these compounds (e.g., halogens, methyl and methoxy moieties, in positions 6, 7 and/or 8 of the 2-mercapto-quinazolin-4-one ring) led to highly effective hCA II/XII inhibitors. These compounds should thus be of interest as preclinical candidates in pathologies in which the activity of these enzymes should be inhibited, such as glaucoma (CA II and XII as targets) or some tumors in which the expression of isoforms CA II and XII is dysregulated.

4. Experimental protocols

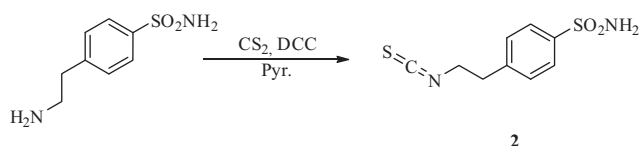
4.1. Chemistry

Anhydrous solvents and all reagents were purchased from Sigma–Aldrich, Alfa Aesar and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (^1H NMR, ^{13}C NMR) spectra were recorded using a Bruker Avance III 400 MHz spectrometer in DMSO- d_6 . Chemical shifts are reported in parts per million (ppm) and the coupling constants (J) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad singlet; dd, double of doublets. The assignment of exchangeable protons (OH and NH) was confirmed by the addition of D $_2$ O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck Silica gel 60 (230–400 mesh ASTM) as the stationary phase and ethyl acetate/*n*-hexane were used as eluents. Melting points (mp) were measured in open capillary tubes with a Gallenkamp MPD350. BM3.5 apparatus and are uncorrected.

4.1.1. General procedure for the synthesis of benzenesulfonamides **3a–o**¹

The commercially available 2-aminobenzoic acid derivatives **1a–o** (1.0 equiv) and isothiocyanato-benzenesulfonamide **2** (1.0 equiv) were suspended in EtOH, and the reaction was refluxed until starting materials were consumed (TLC monitoring). Then the mixture was cooled down to rt and the precipitate formed was collected by filtration to give a residue that was purified by silica gel column chromatography eluting with the appropriate ethyl acetate/*n*-hexane solution or by trituration from diethyl ether to afford the desired compounds **3a–o**.

4.1.2. Synthesis of 4-(2-isothiocyanato-ethyl)-benzenesulfonamide (**2**)^{2,3}

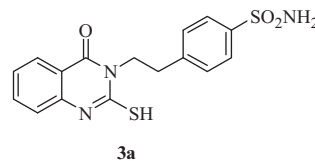


A suspension of ethylaminobenzene sulfonamide (1.0 g, 1.0 equiv) in dry pyridine (2.0 ml) was cooled to -10°C and then treated with CS $_2$ (5.0 g, 13.2 equiv) and DCC (1.0 g, 1.0 equiv). The reaction mixture was stirred at rt under a nitrogen atmosphere until consumption of the starting material (TLC monitoring). Then the solvent was removed under vacuo to give a pale yellow solid that was crystallized from acetone. The solution was recovered, concentrated under vacuo and the residue obtained was purified by silica gel column chromatography eluting with 5% MeOH/DCM to afford the titled compound as a pale yellow solid.

4-(2-Isothiocyanato-ethyl)-benzenesulfonamide **2**: 52% yield; silica gel TLC R_f 0.17 (MeOH/DCM 5% v/v); mp 142°C (lit.^{2,3} 146°C); δ_{H} (400 MHz, DMSO- d_6) 3.08 (2H, t, J 6.8, N-CH $_2$ -CH $_2$ -), 4.00 (2H, t, J 6.8, N-CH $_2$ -CH $_2$ -), 7.37 (2H, s, exchange with D $_2$ O, SO $_2$ NH $_2$), 7.52 (2H, d, J 8.4, Ar-H), 7.82 (1H, d, J 8.4, Ar-H); δ_{C} (100 MHz, DMSO- d_6) 35.8, 46.4, 126.7, 128.8 (–N=C=S), 130.3, 142.7, 143.6; Elemental analysis: calcd: C, 44.61; H, 4.16; N, 11.56; S, 26.47; found: C, 44.65; H, 4.15; N, 11.59; S, 26.44; m/z (ESI positive) 243.02 [M+H] $^+$.

4.1.3. Experimental data are in agreement with reported data^{2,3}

4.1.3.1. Synthesis of 4-[2-(2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (**3a**).

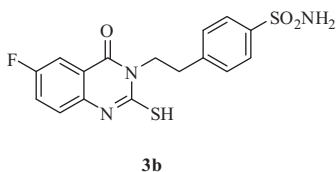


2-Aminobenzoic acid **1a** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.17 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux for 5 h according to the general procedure previously described. Then the solvent was removed under vacuo to give a residue that was trituated from diethyl ether to afford the title compound **3a** as a white solid.

4-[2-(2-Mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3a**: 74% yield; silica gel TLC R_f 0.21 (Ethyl acetate/*n*-hexane 40% v/v); mp 274°C sub.; δ_{H} (400 MHz, DMSO- d_6) 3.10

(2H, m, N-CH₂-CH₂-), 4.65 (2H, m, N-CH₂-CH₂-), 7.33 (2H, s, exchange with D₂O, SO₂NH₂), 7.39 (2H, m, Ar-H), 7.53 (2H, d, *J* 8.4, Ar-H), 7.78 (3H, m, Ar-H), 8.02 (1H, d, *J* 8.4, Ar-H), 13.05 (1H, s, exchange with D₂O, -SH); δ_{C} (100 MHz, DMSO-*d*₆) 33.0, 47.5, 116.4, 116.6, 125.5, 126.9, 128.2, 130.0, 136.4, 140.0, 143.3, 143.6, 160.1, 175.8; Elemental analysis: calcd: C, 53.17; H, 4.18; N, 11.63; S, 17.7; found: C, 53.20; H, 4.15; N, 11.62; S, 17.9; *m/z* (ESI positive) 361.06 [M+H]⁺.

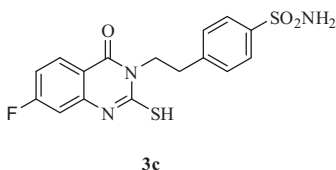
4.1.3.2. Synthesis of 4-[2-(6-fluoro-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3b).



2-Amino-5-fluorobenzoic acid **1b** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.16 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The precipitate formed was collected by filtration and triturated from diethyl ether to afford the title compound **3b** as a white solid.

4-[2-(6-Fluoro-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3b**: 80% yield; silica gel TLC *R_f* 0.19 (Ethyl acetate/*n*-hexane 50% v/v); mp > 310 °C; δ_{H} (400 MHz, DMSO-*d*₆) 3.10 (2H, t, *J* 5.6, N-CH₂-CH₂-), 4.64 (2H, t, *J* 5.6, N-CH₂-CH₂-), 7.35 (2H, s, exchange with D₂O, SO₂NH₂), 7.49 (1H, m, Ar-H), 7.52 (2H, d, *J* 8.4, Ar-H), 7.70 (1H, m, Ar-H), 7.72 (1H, d, *J* 8.1, Ar-H), 7.82 (2H, d, *J* 8.4, Ar-H), 13.13 (1H, s, exchange with D₂O, -SH); δ_{F} (376 MHz, DMSO-*d*₆) -116.43 (1F, s); δ_{C} (100 MHz, DMSO-*d*₆) 32.9, 47.6, 113.1 (d, ²*J*_{C-F} 24), 117.7 (d, ³*J*_{C-F} 8), 119.3 (d, ³*J*_{C-F} 8), 124.7 (d, ²*J*_{C-F} 24), 126.9, 130.0, 136.9, 143.3, 143.5, 159.3 (d, ¹*J*_{C-F} 241), 159.5 (d, ⁴*J*_{C-F} 3), 175.3; Elemental analysis: calcd: C, 50.65; H, 3.72; N, 11.07; S, 16.90; found: C, 50.66; H, 3.70; N, 11.10; S, 16.78; *m/z* (ESI positive) 380.06 [M+H]⁺.

4.1.3.3. Synthesis of 4-(2-(7-fluoro-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl)benzenesulfonamide (3c).

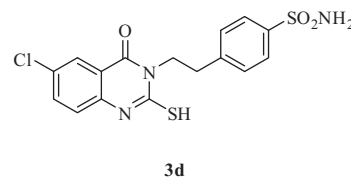


2-Amino-4-fluorobenzoic acid **1c** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.16 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The precipitate formed was collected by filtration and triturated from diethyl ether to afford the title compound **3c** as a white solid.

4-[2-(7-Fluoro-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3c**: 78% yield; silica gel TLC *R_f* 0.17 (Ethyl acetate/*n*-hexane 40% v/v); mp 246 °C; δ_{H} (400 MHz, DMSO-*d*₆) 3.09 (2H, t, *J* 5.6, N-CH₂-CH₂-), 4.62 (2H, t, *J* 5.6, N-CH₂-CH₂-), 7.14 (1H, dd, *J* 8.1, 2.2, Ar-H), 7.23 (1H, m, Ar-H), 7.35 (2H, s, exchange with D₂O, SO₂NH₂), 7.52 (2H, d, *J* 8.4, Ar-H), 7.83 (2H,

d, *J* 8.4, Ar-H), 8.08 (1H, m, Ar-H), 13.09 (1H, s, exchange with D₂O, -SH); δ_{F} (376 MHz, DMSO-*d*₆) -101.87 (1F, s); δ_{C} (100 MHz, DMSO-*d*₆) 33.0, 47.5, 102.7 (d, *J*_{C-F} 27), 113.7 (d, *J*_{C-F} 12), 113.9, 127.0, 130.1, 131.9, 141.8 (d, *J*_{C-F} 13), 143.5 (d, *J*_{C-F} 23), 159.5, 176.4; Elemental analysis: calcd: C, 50.65; H, 3.72; N, 11.07; S, 16.90; found: C, 50.68; H, 3.69; N, 11.11; S, 16.85; *m/z* (ESI positive) 380.06 [M+H]⁺.

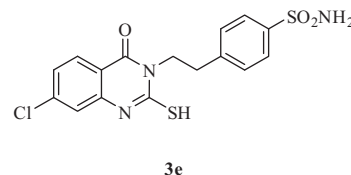
4.1.3.4. Synthesis of 4-[2-(6-chloro-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3d).



2-Amino-5-chlorobenzoic acid **1d** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.14 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The precipitate formed was collected by filtration and triturated from diethyl ether to afford the title compound **3d** as a light-brown solid.

4-[2-(6-Chloro-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3d**: 68% yield; silica gel TLC *R_f* 0.21 (Ethyl acetate/*n*-hexane 50% v/v); mp > 310 °C; δ_{H} (400 MHz, DMSO-*d*₆) 3.09 (2H, t, *J* 5.6, N-CH₂-CH₂-), 4.63 (2H, t, *J* 5.6, N-CH₂-CH₂-), 7.35 (2H, s, exchange with D₂O, SO₂NH₂), 7.45 (1H, d, *J* 8.1, Ar-H), 7.52 (2H, d, *J* 8.4, Ar-H), 7.83 (2H, d, *J* 8.4, Ar-H), 7.84 (1H, d, *J* 8.1, Ar-H), 7.95 (1H, s, Ar-H), 13.16 (1H, s, exchange with D₂O, -SH); δ_{C} (100 MHz, DMSO-*d*₆) 32.9, 47.7, 117.9, 119.0, 127.0, 127.2, 129.4, 130.1, 136.5, 138.9, 143.4, 143.6, 159.3, 175.8; Elemental analysis: calcd: C, 48.54; H, 3.56; N, 10.61; S, 16.20; found: C, 48.56; H, 3.58; N, 10.57; S, 16.17; *m/z* (ESI positive) 396.02 [M+H]⁺.

4.1.3.5. Synthesis of 4-[2-(7-chloro-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3e).

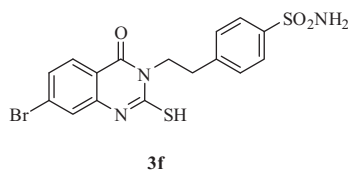


2-Amino-4-chlorobenzoic acid **1e** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.14 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The precipitate formed was collected by filtration and triturated from diethyl ether to afford the title compound **3e** as a white solid.

4-[2-(7-Chloro-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3e**: 65% yield; silica gel TLC *R_f* 0.25 (Ethyl acetate/*n*-hexane 50% v/v); mp > 310 °C; δ_{H} (400 MHz, DMSO-*d*₆) 3.09 (2H, t, *J* 5.6, N-CH₂-CH₂-), 4.63 (2H, t, *J* 5.6, N-CH₂-CH₂-), 7.35 (2H, s, exchange with D₂O, SO₂NH₂), 7.42 (2H, m, Ar-H), 7.52 (2H, d, *J* 8.4, Ar-H), 7.80 (2H, d, *J* 8.4, Ar-H), 8.00 (1H, s, Ar-H), 13.08 (1H, s, exchange with D₂O, -SH); δ_{C} (100 MHz, DMSO-*d*₆) 33.6, 48.2, 116.1, 116.6, 126.4, 127.6, 130.7, 131.2, 141.6,

141.6, 144.0, 144.3, 160.3, 177.0; Elemental analysis: calcd: C, 48.54; H, 3.56; N, 10.61; S, 16.20; found: C, 48.59; H, 3.60; N, 10.58; S, 16.16; m/z (ESI positive) 396.02 $[M+H]^+$.

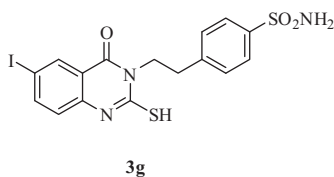
4.1.3.6. Synthesis of 4-[2-(7-Bromo-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3f).



2-Amino-4-bromobenzoic acid **1f** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.11 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The precipitate formed was collected by filtration and triturated from diethyl ether to afford the title compound **3f** as a white solid.

4-[2-(7-Bromo-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3f**: 55% yield; silica gel TLC R_f 0.40 (Ethyl acetate/*n*-hexane 50% v/v); mp 307 °C; δ_H (400 MHz, DMSO- d_6) 3.07 (2H, t, J 5.6, N-CH₂-CH₂-), 4.62 (2H, t, J 5.6, N-CH₂-CH₂-), 7.33 (2H, s, exchange with D₂O, SO₂NH₂), 7.54 (3H, m, Ar-H), 7.60 (1H, s, Ar-H), 7.79 (2H, d, J 8.4, Ar-H), 7.90 (1H, d, J 8.1, Ar-H), 13.07 (1H, s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 34.7, 49.5, 119.0, 125.0, 126.4, 128.0, 128.3, 130.0, 131.0, 141.0, 143.0, 149.1, 159.3, 177.1. Elemental analysis: calcd: C, 43.64; H, 3.20; N, 9.54; S, 14.56; found: C, 43.66; H, 3.25; N, 9.50; S, 14.52; m/z (ESI positive) 441.34 $[M+H]^+$.

4.1.3.7. Synthesis of 4-[2-(6-iodo-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3g).

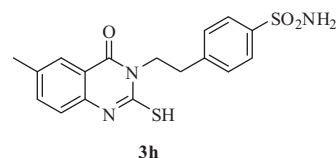


2-Amino-5-iodobenzoic acid **1g** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.09 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The precipitate formed was collected by filtration and triturated from diethyl ether to afford the title compound **3g** as a white solid.

4-[2-(6-Iodo-2-mercapto-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3g**: 68% yield; silica gel TLC R_f 0.12 (Ethyl acetate/*n*-hexane 40% v/v); mp 230 °C with dec; δ_H (400 MHz, DMSO- d_6) 3.10 (2H, t, J 5.6, N-CH₂-CH₂-), 4.63 (2H, t, J 5.6, N-CH₂-CH₂-), 7.40 (5H, m, SO₂NH₂, Ar-H), 7.79 (2H, d, J 8.4, Ar-H), 8.10 (1H, br s, Ar-H), 8.25 (1H, m, Ar-H), 13.11 (1H, s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 33.0, 47.5, 98.4, 120.0, 126.4, 127.1, 129.0, 130.2, 136.1, 137.2, 140.7, 143.6, 160.1, 177.0. Elemental analysis: calcd: C, 39.43; H, 2.90; N, 8.62; S,

13.16; found: C, 39.40; H, 2.87; N, 8.59; S, 13.12; m/z (ESI positive) 487.94 $[M+H]^+$.

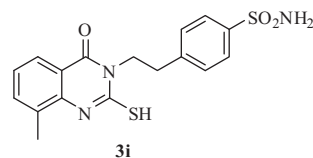
4.1.3.8. Synthesis of 4-[2-(2-mercapto-6-methyl-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3h).



2-Amino-5-methylbenzoic acid **1h** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.16 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux for 5 h according to the general procedure previously described. The precipitate formed was collected by filtration and was triturated from diethyl ether to afford the title compound **3h** as a light-brown solid.

4-[2-(2-Mercapto-6-methyl-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3h**: 32% yield; silica gel TLC R_f 0.25 (Ethyl acetate/*n*-hexane 40% v/v); mp > 310 °C; δ_H (400 MHz, DMSO- d_6) 2.41 (3H, s, CH₃), 3.10 (2H, t, J 5.6, N-CH₂-CH₂-), 4.66 (2H, t, J 5.6, N-CH₂-CH₂-), 7.35 (2H, s, exchange with D₂O, SO₂NH₂), 7.37 (1H, d, J 8.1, Ar-H), 7.52 (2H, d, J 8.4, Ar-H), 7.63 (1H, dd, J 8.1, 2.2, Ar-H), 7.82 (2H, d, J 8.4, Ar-H), 7.83 (1H, d, J 2.2, Ar-H), 13.12 (1H, s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 22.0, 33.4, 48.0, 121.0, 126.8, 127.1, 129.0, 130.2, 136.2, 137.0, 137.1, 140.7, 143.6, 160.1, 176.4. Elemental analysis: calcd: C, 54.38; H, 4.56; N, 11.19; S, 17.08; found: C, 54.40; H, 4.60; N, 11.17; S, 17.12; m/z (ESI positive) 376.09 $[M+H]^+$.

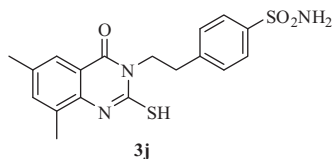
4.1.3.9. Synthesis of 4-[2-(2-mercapto-8-methyl-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3i).



2-Amino-3-methylbenzoic acid **1i** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.16 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux for 5 h according to the general procedure previously described. The precipitate formed was collected by filtration and was triturated from diethyl ether to afford the title compound **3i** as a light-brown solid.

4-[2-(2-Mercapto-8-methyl-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3i**: 20% yield; silica gel TLC R_f 0.22 (Ethyl acetate/*n*-hexane 40% v/v); mp 286 °C; δ_H (400 MHz, DMSO- d_6) 2.53 (3H, s, CH₃), 3.10 (2H, t, J 5.6, N-CH₂-CH₂-), 4.66 (2H, t, J 5.6, N-CH₂-CH₂-), 7.31 (1H, t, J 7.6, Ar-H), 7.35 (2H, s, exchange with D₂O, SO₂NH₂), 7.54 (2H, d, J 8.4, Ar-H), 7.63 (1H, dd, J 8.1, 2.2, Ar-H), 7.82 (2H, d, J 8.4, Ar-H), 7.89 (1H, d, J 7.6, Ar-H), 13.00 (1H, s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 18.2, 33.0, 47.8, 116.8, 125.5, 125.5, 126.2, 127.0, 130.1, 137.8, 138.5, 143.4, 143.7, 160.3, 176.2. Elemental analysis: calcd: C, 54.38; H, 4.56; N, 11.19; S, 17.08; found: C, 54.42; H, 4.58; N, 11.20; S, 17.15; m/z (ESI positive) 376.09 $[M+H]^+$.

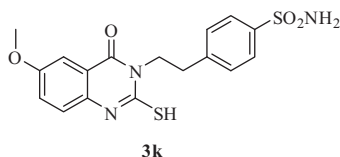
4.1.3.10. Synthesis of 4-[2-(2-mercapto-6,8-dimethyl-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3j).



2-Amino-3,5-dimethylbenzoic acid **1j** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.15 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux for 5 h according to the general procedure previously described. The precipitate formed was collected by filtration and was triturated from diethyl ether to afford the title compound **3j** as a light-brown solid.

4-[2-(2-Mercapto-6,8-dimethyl-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3j**: 22% yield; silica gel TLC R_f 0.30 (Ethyl acetate/*n*-hexane 40% v/v); mp 275 °C; δ_H (400 MHz, DMSO- d_6) 2.37 (3H, s, CH₃), 2.49 (3H, s, CH₃), 3.11 (2H, t, *J* 5.6, N-CH₂-CH₂-), 4.68 (2H, t, *J* 5.6, N-CH₂-CH₂-), 7.35 (2H, s, exchange with D₂O, SO₂NH₂), 7.47 (1H, s, Ar-H), 7.53 (2H, d, *J* 8.4, Ar-H), 7.70 (1H, s, Ar-H), 7.82 (2H, d, *J* 8.4, Ar-H), 11.80 (1H, s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 18.4, 20.0, 33.4, 48.1, 116.9, 124.4, 125.5, 126.0, 126.8, 130.2, 136.6, 138.5, 143.4, 143.6, 160.2, 176.2. Elemental analysis: calcd: C, 55.51; H, 4.92; N, 10.79; S, 16.47; found: C, 54.53; H, 4.89; N, 10.82; S, 16.50; *m/z* (ESI positive) 390.50 [M+H]⁺.

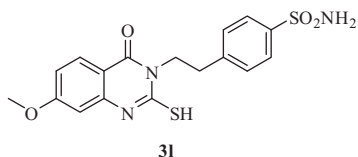
4.1.3.11. Synthesis of 4-[2-(2-mercapto-6-methoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3k).



2-Amino-5-methoxybenzoic acid **1k** (0.2 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.29 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The precipitate formed was collected by filtration and was triturated from diethyl ether to afford the title compound **3k** as a light-brown solid.

4-[2-(2-Mercapto-6-methoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3k**: 28% yield; silica gel TLC R_f 0.11 (MeOH/CDM 5% v/v); mp 273 °C; δ_H (400 MHz, DMSO- d_6) 3.09 (2H, t, *J* 5.6, N-CH₂-CH₂-), 3.87 (3H, s, OCH₃), 4.66 (2H, t, *J* 5.6, N-CH₂-CH₂-), 7.35 (2H, s, exchange with D₂O, SO₂NH₂), 7.42 (3H, m, Ar-H), 7.52 (2H, d, *J* 8.4, Ar-H), 7.82 (2H, d, *J* 8.4, Ar-H), 13.05 (1H, s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 33.6, 48.2, 57.3, 109.4, 117.8, 119.2, 126.4, 127.5, 130.8, 135.0, 143.5, 144.5, 157.8, 161.0, 174.9; Elemental analysis: calcd: C, 52.16; H, 4.38; N, 10.73; S, 16.38; found: C, 52.20; H, 4.35; N, 10.75; S, 16.40; *m/z* (ESI positive) 392.09 [M+H]⁺.

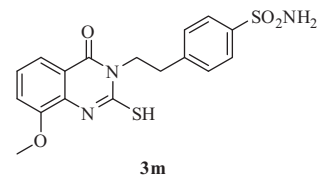
4.1.3.12. Synthesis of 4-[2-(2-mercapto-7-methoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3l).



2-Amino-4-methoxybenzoic acid **1l** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.14 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The solvent was removed under vacuo to give a residue that was triturated from diethyl ether and then purified by silica gel column chromatography eluting with 50% ethyl acetate/*n*-hexane to afford the title compound **3l** as a light-brown solid.

4-[2-(2-Mercapto-7-methoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3l**: 21% yield; silica gel TLC R_f 0.10 (MeOH/DCM 5% v/v); mp 235 °C; δ_H (400 MHz, DMSO- d_6) 3.07 (2H, t, *J* 5.6, N-CH₂-CH₂-), 3.90 (3H, s, OCH₃), 4.70 (2H, t, *J* 5.6, N-CH₂-CH₂-), 7.34 (2H, s, exchange with D₂O, SO₂NH₂), 7.48 (3H, m, Ar-H), 7.50 (2H, d, *J* 8.4, Ar-H), 7.90 (2H, d, *J* 8.4, Ar-H), 12.90 (1H, s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 34.0, 48.1, 56.1, 118.9, 119.4, 126.2, 128.0, 131.1, 135.4, 143.3, 144.0, 153.0, 156.1, 161.1, 177.6. Elemental analysis: calcd: C, 52.16; H, 4.38; N, 10.73; S, 16.38; found: C, 52.18; H, 4.36; N, 10.76; S, 16.35; *m/z* (ESI positive) 392.09 [M+H]⁺.

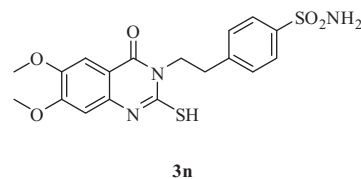
4.1.3.13. Synthesis of 4-[2-(2-mercapto-8-methoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3m).



2-Amino-3-methoxybenzoic acid **1m** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.14 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The solvent was removed under vacuo to give a residue that was triturated from diethyl ether to afford the title compound **3m** as a light-brown solid.

4-[2-(2-Mercapto-8-methoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3m**: 33% yield; silica gel TLC R_f 0.15 (MeOH/DCM 5% v/v); mp 295 °C; δ_H (400 MHz, DMSO- d_6) 3.10 (2H, t, *J* 5.6, N-CH₂-CH₂-), 4.00 (3H, s, OCH₃), 4.67 (2H, t, *J* 5.6, N-CH₂-CH₂-), 7.36 (3H, m, exchange with D₂O, SO₂NH₂, Ar-H), 7.44 (1H, m, Ar-H), 7.62 (2H, d, *J* 8.4, Ar-H), 7.68 (1H, m, Ar-H), 7.75 (2H, d, *J* 8.4, Ar-H), 11.80 (1H, br s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 33.6, 48.2, 56.0, 116.0, 118.8, 119.1, 126.0, 127.6, 130.7, 135.1, 143.5, 144.4, 152.9, 161.1, 176.9. Elemental analysis: calcd: C, 52.22; H, 4.39; N, 10.75; S, 16.40; found: C, 52.22; H, 4.39; N, 10.75; S, 16.40; *m/z* (ESI positive) 392.09 [M+H]⁺.

4.1.3.14. Synthesis of 4-[2-(2-mercapto-6,7-dimethoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (3n).

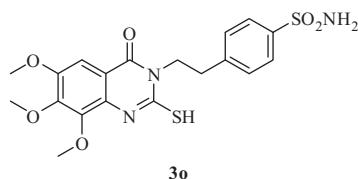


2-Amino-4,5-dimethoxybenzoic acid **1n** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.12 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according

to the general procedure previously described. The precipitate formed was collected by filtration and triturated from diethyl ether to afford the title compound **3n** as a white solid.

4-[2-(2-Mercapto-6,7-dimethoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3n**: 80% yield; silica gel TLC R_f 0.20 (MeOH/DCM 5% v/v); mp 302 °C; δ_H (400 MHz, DMSO- d_6) 3.09 (2H, t, J 5.6, N-CH₂-CH₂-), 3.88 (3H, s, OCH₃), 3.91 (3H, s, OCH₃), 4.66 (2H, t, J 5.6, N-CH₂-CH₂-), 6.98 (1H, s, Ar-H), 7.35 (3H, s, exchange with D₂O, SO₂NH₂, Ar-H), 7.52 (2H, d, J 8.4, Ar-H), 7.82 (2H, d, J 8.4, Ar-H), 12.86 (1H, br s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 33.2, 47.4, 56.8, 57.0, 107.8, 109.1, 126.9, 130.1, 130.2, 136.1, 143.3, 143.7, 147.8, 156.4, 159.7, 174.7; Elemental analysis: calcd: C, 51.29; H, 4.54; N, 9.97; S, 15.22; found: C, 51.30; H, 4.56; N, 10.01; S, 15.25; m/z (ESI positive) 422.09 [M+H]⁺.

4.1.3.15. Synthesis of 4-[2-(2-mercapto-6,7,8-trimethoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide (**3o**).



2-Amino-3,4,5-trimethoxybenzoic acid **1o** (0.1 g, 1.0 equiv) and 4-(2-isothiocyanato-ethyl)-benzenesulfonamide **2** (0.11 g, 1.0 equiv) were treated in EtOH (10 ml) at reflux O.N. according to the general procedure previously described. The precipitate formed was collected by filtration and triturated from diethyl ether to afford the title compound **3o** as a white solid.

4-[2-(2-Mercapto-6,7,8-trimethoxy-4-oxo-4H-quinazolin-3-yl)-ethyl]-benzenesulfonamide **3o**: 75% yield; silica gel TLC R_f 0.27 (Ethyl acetate/*n*-hexane 50% v/v); mp 260 °C; δ_H (400 MHz, DMSO- d_6) 3.09 (2H, t, J 5.6, N-CH₂-CH₂-), 3.92 (3H, s, OCH₃), 3.93 (3H, s, OCH₃), 3.94 (3H, s, OCH₃), 4.67 (2H, t, J 5.6, N-CH₂-CH₂-), 7.27 (1H, s, Ar-H), 7.35 (2H, s, exchange with D₂O, SO₂NH₂), 7.53 (2H, d, J 8.4, Ar-H), 7.82 (2H, d, J 8.4, Ar-H), 12.18 (1H, s, exchange with D₂O, -SH); δ_C (100 MHz, DMSO- d_6) 33.0, 48.2, 56.8, 56.9, 59.2, 117.2, 119.1, 127.0, 131.0, 131.2, 136.2, 143.0, 146.0, 148.1, 156.4, 160.4, 170.0. Elemental analysis: calcd: C, 50.54; H, 4.69; N, 9.31; S, 14.20; found: C, 50.57; H, 4.71; N, 9.27; S, 14.16; m/z (ESI positive) 453.00 [M+H]⁺.

4.2. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO₂ hydration activity.¹⁹ 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 buffer (pH 7.5) and 20 mM NaClO₄ for maintaining constant ionic strength, following the initial rates of the CA-catalyzed CO₂ hydration reaction for a period of 10–100 s, at 20 °C. The CO₂ 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 to determine the initial velocity. The uncatalyzed rates were measured in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water and dilutions down to 0.01 nM were made 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,^{20–22} and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in house as reported earlier,^{20–22} and their concentrations in the assay system were: 12.3 nM for hCA I, 8.5 nM for hCA II and 10.2 nM for hCA XII, respectively.

Acknowledgements

This project was supported by the Deanship of Scientific Research at Prince Sattam bin Abdulaziz University, Saudi Arabia under the research project number '2014/03/2048', and by two European Union FP7 projects, Metoxia and Dynano.

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