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Carbonic anhydrase inhibitors. Aromatic/heterocyclic sulfonamides incorporating phenacetyl, pyridylacetyl and thienylacetyl tails act as potent inhibitors of human mitochondrial isoforms VA and VB

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ABSTRACT

A series of aromatic/heterocyclic sulfonamides incorporating phenyl(alkyl), halogenosubstituted-phenyl- or 1,3,4-thiadiazole-sulfonamide moieties and thienylacetamido; phenacetamido and pyridinylacetamido tails were prepared and assayed as inhibitors of four physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the cytosolic human (h) hCA I and hCA II, and the mitochondrial hCA VA and hCA VB. The new compounds showed moderate inhibition of the two cytosolic isoforms (K_Is of 50–390 nM) and excellent inhibitory activity against the two mitochondrial enzymes, with many low nanomolar inhibitors detected (K_Is in the range of 5.9–10.2 nM). All substitution patterns explored here lead to effective hCA VA/VB inhibitors. Some hCA VA/VB selective inhibitors were also detected, with selectivity ratios for inhibiting the mitochondrial over the cytosolic isozymes of around 55.5–56.9. As hCA VA/VB are involved in several biosynthetic processes catalyzed by pyruvate carboxylase, acetyl CoA carboxylase, and carbamoyl phosphate synthetases I and II, providing the bicarbonate substrate to these carboxylating enzymes involved in fatty acid biosynthesis, their selective inhibition may lead to the development of antiobesity agents possessing a new mechanism of action.

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1. Introduction

There are 16 α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms expressed in mammals, CA I–CA XV, two of which, CA VA and CA VB, being present in mitochondria.[1–3] These two isozymes are involved in several biosynthetic processes, such as ureagenesis, gluconeogenesis, and lipogenesis.[1,4,5] The provision of enough bicarbonate as substrate for several biosynthetic processes catalyzed by pyruvate carboxylase (PC), acetyl CoA carboxylase (ACC), and carbamoyl phosphate synthetases I and II, is assured mainly by the catalysis involving these mitochondrial isozymes CA VA and CA VB, probably assisted by the high activity cytosolic isozyme CA II, as shown schematically in Figure 1.[1,2] CAs thus play a key role in fatty acid biosynthesis. Mitochondrial pyruvate carboxylase (PC) is needed for efflux of acetyl groups from the mitochondria to the cytosol where fatty acid biosynthesis takes place.[1] Pyruvate is carboxylated to oxaloacetate in the presence of bicarbonate and under the catalytic influence of the mitochondrial isozymes CA VA and/or CA VB. The mitochondrial membrane is impermeable to acetyl-CoA which reacts with oxaloacetate, leading to citrate, which is thereafter translocated to the cytoplasm by means of the tricarboxylic acid

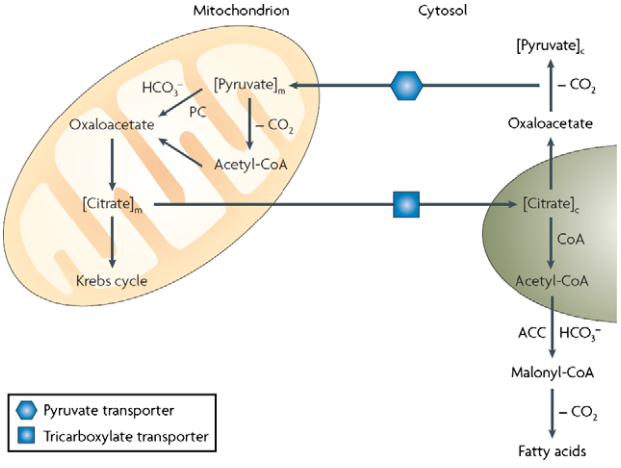

Fig. 1. The transfer of acetyl groups from the mitochondrion to the cytosol (as citrate) for the provision of substrate for de novo lipogenesis. All steps involving bicarbonate need the presence of at least two CA isozymes: CA VA/VB in the mitochondrion and CA II in the cytosol (see discussion in the text). Inhibition of the CA-mediated processes with sulfonamides lead to inhibition of lipogenesis.[1]

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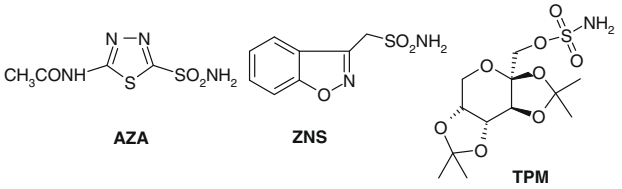
transporter. As oxaloacetate is unable to cross the mitochondrial membrane, its decarboxylation regenerates pyruvate which can be then transported into the mitochondria by means of the pyruvate transporter (Fig. 1). The acetyl-CoA thus generated in the cytosol is in fact used for the de novo lipogenesis, by carboxylation in the presence of ACC and bicarbonate, with formation of malonyl-CoA, the conversion between CO_2 and bicarbonate being assisted by CA II. Subsequent steps involving the sequential transfer of acetyl groups lead to longer chain fatty acids.[1–5] Therefore, several CA isozymes are critical to the entire process of fatty acid biosynthesis: CA VA and/or VB within the mitochondria (to provide enough substrate to PC), and CA II within the cytosol (for providing sufficient substrate to ACC). Inhibition of CAs by clinically used sulfonamides such as acetazolamide **AZA**, zonisamide **ZNS** or the sulfamate topiramate, **TPM**, can decrease lipogenesis in adipocytes in cell culture.[1–3] Furthermore, among some of the side effects of these drugs is also the weight loss,[6] which may in fact lead to novel antiobesity therapies.[7]

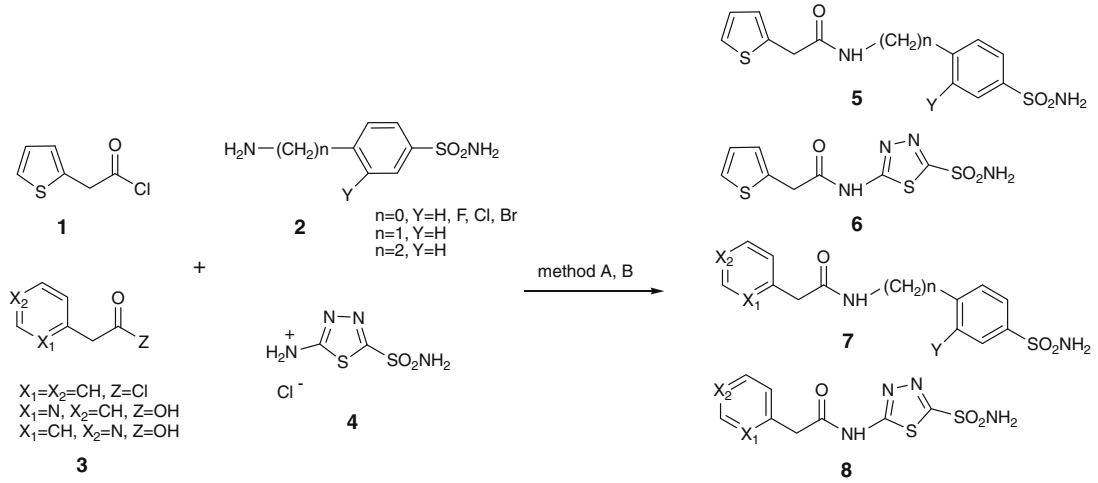


In earlier drug design studies of CA inhibitors (CAIs) from our group,[8] we have explored the preparation of aromatic/heterocyclic sulfonamides incorporating furan-, thiophene- and pyrrole-carboxamide moieties of the type RCONH-A-SO_2NH_2 (where A is a phenyl(alkyl), halogenosubstituted-phenyl- or 1,3,4-thiadiazole moiety and R = furan-2-yl; thien-2-yl or pyrrol-2-yl group). Such compounds have been tested as inhibitors of three physiologically relevant mammalian isoforms, that is, hCA I and II and bCA IV (h = human, b = bovine isoform), and were also active in vivo in an animal glaucoma model.[8] Some of the best such inhibitors were those incorporating thiophene-2-carboxamide moieties. Here we report a study dealing with the design of new sulfonamides possessing some structural similarity with the previously investigated derivatives,[8] prepared for targeting the mitochondrial isoforms hCA VA and Vb. Compounds of the type R-CH_2–CONH-A-SO_2NH_2 (R = thien-2-yl; Ph- and pyridinyl; A is a phenyl(alkyl), halogenosubstituted-phenyl- or 1,3,4-thiadiazole moiety) were obtained and assayed as inhibitors of four physiologically relevant isoforms, the cytosolic hCA I and II, as well as the mitochondrial hCA VA and hCA VB.

2. Results and discussion

2.1. Chemistry

Sulfonamides of the type RCONH-A-SO_2NH_2 (where A is a phenyl(alkyl), halogeno-phenyl- or 1,3,4-thiadiazole moiety and R = furan-2-yl; thien-2-yl or pyrrol-2-yl group) were previously reported by this group,[8] being easily prepared from the corresponding amino sulfonamides by their reactions with heterocyclic acyl halides or carboxylic acids in the presence of carbodiimides, by the tail approach.[8–11] The in vitro and in vivo biological activity of such compounds was interesting, with some of them showing low nanomolar inhibitory profiles against isoforms CA I, II and IV, as well as antiglaucoma activity in an animal model of this disease.[8] Thus, compounds of type RCONH-A-SO_2NH_2 have been used as lead molecules in the present work. As many of the thiophene-carboxamides reported earlier[8] were among the most potent CAIs identified in the study, we focused on this substitution pattern for the compounds reported here, but some structurally related derivatives incorporating phenylacetamido- and pyridyl-acetamido moieties were also prepared and assayed as CAIs, in order to understand the role that the termianl part of the tail plays in modulating the isoform selectivity profile of such compounds. The sulfonamides reported here (Scheme 1) differ of the previously investigated ones by the incorporation of the aryl-/hetarylacetamido moiety in their molecules instead of the hetarylcarboxamido one. Thus, they possess longer tails as compared to the derivatives investigated earlier.[8] The rationale for this modification resides in the fact that the tails present in CAIs interact with amino acid residues situated towards the exit of the CA active site or on its edge, as shown by extensive X-ray crystallographic work on such enzyme-inhibitor adducts.[12,13] On the other hand, these are the amino acid residues which are less conserved among the various mammalian CA isoforms,[1,2] and their interactions with the tails incorporated in the inhibitors explain why most of the novel generation inhibitors usually show a better (i.e., more isozyme-selective) inhibition profile as compared to the classical sulfonamides, of which acetazolamide **AZA** is the best studied representative.[1,2] X-Ray crystal structures and homology modeling are available for the interaction of many sulfonamides with isoforms hCA I, II, and XIII,[1–3] whereas fewer such reports for hCA IX[9] and hCA VA[14] were published. All these works showed that both favorable interactions as well as clashes with particular amino acid residues present only in some CA isozymes[15] are critical for the inhibition profile and isozyme selectivity issues of the sulfonamides and their bioisosteres such as the sulfamates and the sulfamides.



Scheme 1. Synthesis of the new sulfonamides **5**–**8**. Method A: Z = Cl, Et_3N/MeCN; Method B: Z = OH, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide, DMAP/dioxane.

Reaction of 2-thienylacetyl chloride **1** or phenyl-/pyridyl-acetyl halides/carboxylic acids **3** with aminosulfonamides **2** and **4**, led to the acylated sulfonamides **5–8** by the procedure reported earlier[8] (Scheme 1). In addition to sulfanilamide, homosulfanilamide, 4-aminoethyl-benzenesulfonamide and halogenated sulfanilamides **2**, the heterocyclic derivative 5-amino-1,3,4-thiadiazole-2-sulfonamide **4** was also included in the study. All these aminosulfonamides have been shown earlier to lead to effective CAIs by derivatization reactions employing the tail approach.[8–10] The new compounds **5–8** have been characterized by spectroscopic and analytic methods which confirmed their structures (see Experimental or details).

2.2. Carbonic anhydrase inhibition

Inhibition data against four catalytically active human (h) CA isoforms, that is, hCA I, hCA II (cytosolic) and hCA VA and hCA VB (mitochondrial enzymes) with the new sulfonamides **5–8** reported here, as well as the standard compounds **AZA, ZNS** and **TPM** are shown in Table 1.

The following structure–activity relationship (SAR) can be drawn by considering data of Table 1:[16]

(i) Isoform hCA I was moderately inhibited by sulfonamides **5–8** reported here. Thus, several derivatives, such as **5b, 7a** and **7f**, showed medium inhibitory activity, with inhibition constants in the range of 108–263 nM, in the same range as the clinically used compounds acetazolamide and topiramate (K_I of 250 nM for both compounds against this isoform, Table 1). The remaining new sulfonamides were more effective hCA I inhibitors as compared to derivatives discussed above, with K_Is in the range of 60–84 nM. The best hCA I inhibitor was the clinically used compound zonisamide **ZNS** (K_I of 56 nM). Obviously both the aromatic sulfonamide head as well as the aryl/hetaryl-acetyl moiety influence the biological activity of these hCA I inhibitors. It may be observed that efficient inhibitors incorporate both sulfanilamide, halogenated sulfanilamide, homosulfanilamide, 4-aminoethyl-benzenesulfonamide and 5-amino-1,3,4-thiadiazole-2-sulfonamide moieties, whereas the tail present in the acylating agent probably modulates and fine-tunes the binding. Except for the three less active compounds mentioned above (**5b, 7a** and **7f**) which incorporate a 2-thienylacetyl (**5b**) or a phenacetyl moiety (**7a** and **7f**), the remaining compounds showed a quite compact behavior of moderately-efficient hCA I inhibitors. Thus, the aryl/hetaryl-acetamido moieties present in these compounds lead to significant hCA I inhibition, but all these compounds possess K_Is > 50 nM, being thus only moderately active.

(ii) A rather similar situation was observed for the inhibition of hCA II (Table 1), a physiologically dominant and highly relevant isoform.[1] Indeed, again the same three compounds (**5b, 7a** and **7f**) showed weaker hCA II inhibitory activity, with K_Is in the range of 107–395 nM whereas all the remaining derivatives behave as moderate inhibitors (K_Is in the range of 50–97 nM). It should be noted that the three clinically used compounds (**AZA, ZNS** and **TPM**) show much more potent hCA II inhibitory activity, with K_Is in the range of 10–35 nM (Table 1). It should be also noted that the compounds investigated earlier,[8] having a CH_2 moiety less than the present ones, showed much better hCA II inhibitory activity, with K_Is in the range of 3–12 nM (but they were less effective as hCA I inhibitors, with K_Is in the range of 120–365 nM).[8] Theses finding clearly illustrate that a very small variation in the structure of a CAI (such as the presence of an additional CH_2 moiety, in this case) may have drastic consequences for the enzyme inhibitory activity and selectivity profile against various isozymes of such derivatives.

(iii) The first mitochondrial isoform, hCA VA, showed excellent inhibition with the new compounds **5–8** investigated here, as all of them possessed low nanomolar affinity for it (K_Is in the range of 6.7–10.2 nM, Table 1). Basically SAR is quite flat again, since all the substitution patterns present in the new compounds investigated here are beneficial for obtaining potent hCA VA inhibitors. This is a quite unexpected finding, but such a behavior was in fact observed earlier for some 1,3,4-thiadiazole-sulfamides,[17] which showed very good hCA VA/VB inhibitory activity and selectivity for the mitochondrial over cytosolic isoforms, although the structurally related 1,3,4-thiadiazole-sulfonamides are devoid of these interesting properties, being much better hCA II than hCA VA/VB inhibitors.[1–3] It should be also noted that the clinically used compounds (except **ZNS**, K_I of 20 nM) are much weaker hCA VA inhibitors compared to derivatives **5–8** reported here. Indeed, both **AZA** and **TPM** show K_Is of 63 nM for inhibiting hCA VA.

(iv) The second mitochondrial isoform, hCA VB, has an inhibition profile with derivatives **5–8** very similar to that of hCA VA. Thus, all these compounds are very good hCA VB inhibitors, with K_Is in the range of 5.9–9.3 nM, Table 1, being much better inhibitors compared to the clinically used drugs which show K_Is in the range of 30–6033 nM.

(v) A very interesting property of compounds **5–8** reported here, compared to most sulfonamides and sulfamates investigated earlier,[18,19] regards their selectivity for inhibiting the mitochondrial (hCA VA/VB) over cytosolic isoforms (hCA I/II). Indeed, all sulfonamides and sulfamates investigated up to now as CAIs generally showed a much higher affinity for hCA II (a ubiquitous and catalytically very efficient isoform)[1–3] compared to hCA VA and hCA VB.[18,19] This can be also observed for the three standard inhibitors present in Table 1, **AZA, ZNS** and **TPM**. On the contrary, many of the compounds **5–8** reported here show selectivity ratios for

Table 1
Inhibition data of human CA isozyms I, II (cytosolic), VA and VB (mitochondrial) with compounds **5–8** and standard inhibitors (acetazolamide **AZA**, zonisamide **ZNS** and topiramate **TPM**), by a stopped-flow, CO_2 hydration assay[16]

No.	n,Y	X_1	X_2	K_I* (nM)			
				hCA I[a]	hCA II[a]	hCA VA[b]	hCA VB[b]
5a	0, H	—	—	61	50	7.2	7.0
5b	0, F	—	—	161	390	6.9	7.9
5c	0, Cl	—	—	77	50	7.7	8.6
5d	0, Br	—	—	84	52	7.5	8.7
5e	1, H	—	—	60	52	9.1	7.2
5f	2, H	—	—	68	53	10.2	8.0
6	—	—	—	72	51	7.6	7.4
7a	0, H	CH	CH	108	107	6.7	7.9
7b	1, H	CH	CH	75	54	8.6	8.3
7c	2, H	CH	CH	60	67	8.1	8.2
7d	0, F	CH	CH	67	61	8.3	8.1
7e	0, Cl	CH	CH	61	58	8.2	8.1
7f	0, Br	CH	CH	263	395	7.1	5.9
7g	0, H	N	CH	75	69	6.8	6.8
7h	0, H	CH	N	71	74	7.3	9.0
7i	2, H	CH	N	73	97	7.6	9.3
8	—	CH	CH	63	51	8.4	6.1
AZA	—	—	—	250	12	63	54
ZNS	—	—	—	56	35	20	6033
TPM	—	—	—	250	10	63	30

* Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[16]
[a] Human, recombinant isozymes.
[b] Full length, recombinant human isoforms.[8–10]

inhibiting hCA VA over hCA II of 56.5 (compound **5b**), 55.6 (**7f**) and 15.9 (**7a**) whereas most of them have this parameter in the range of 5.2–6.9. On the other hand, the selectivity ratio for inhibiting hCA VA over hCA II with **AZA** is of 0.19, clearly showing that the standard drug has a much higher affinity for hCA II than for hCA VA. The same situation is true for the selectivity ratio of inhibiting hCA VB over hCA II. Indeed, the most selective inhibitors were again **5b, 7a** and **7f**, possessing selectivity ratios in the range of 13.5–66.9. Most of the other compounds **5–8** have this parameter of around 5.8–10.9, being anyhow much more isozyme V-selective as compared to the clinically used drugs (selectivity ratios in the range of 0.05–0.33 for **AZA, ZNS** and **TPM**). Thus, we have detected here a new class of isozyme VA/VB selective inhibitors, which may be useful for the investigation of the antiobesity activity of this class of derivatives.

3. Conclusions

A library of aromatic/heterocyclic sulfonamides incorporating phenyl(alkyl), halogenosubstituted-phenyl- or 1,3,4-thiadiazole-sulfonamide moieties and thienylacetamido; phenacetamido and pyridinylacetamido tails were prepared and assayed as inhibitors of four physiologically relevant CA isoforms, the cytosolic hCA I and hCA II, and the mitochondrial hCA VA and hCA VB. The new compounds show moderate inhibition of the two cytosolic isoforms and excellent inhibitory activity against the two mitochondrial enzymes, with many low nanomolar inhibitors detected (K_Is in the range of 5.9–10.2 nM). All substitution patterns explored here lead to effective hCA VA/VB inhibitors. Some hCA VA/VB selective compounds were also detected, with selectivity ratios for inhibiting the mitochondrial over the cytosolic isozymes of >50. As hCA VA/VB are involved in several biosynthetic processes catalyzed by pyruvate carboxylase, acetyl CoA carboxylase, and carbamoyl phosphate synthetases I and II, providing the bicarbonate substrate to these carboxylating enzymes involved in fatty acid biosynthesis, their selective inhibition may lead to the development of new antiobesity agents possessing a totally new mechanism of action.

4. Experimental

4.1. Chemistry

Buffers, sulfonamides **2, 4** and other chemicals (e.g., **1** and **3**) were from Sigma–Aldrich (Milan, Italy) of highest purity available, and were used without further purification. Halogenosulfanilamides were prepared as reported earlier.[20] All CA isozymes were recombinant ones produced and purified in our laboratory as described earlier.[9,12–15] All melting points were measured in open capillary tubes with a Buchi 540 instrument and are uncorrected. The compounds were checked for purity by TLC on silica gel HF_{254} (E.Merck, Darmstadt, Germany). IR (KBr) spectra were recorded using a Perkin–Elmer 1600 FTIR spectrophotometer and all values are expressed as ν_{max}, cm^{-1}. ^1H NMR spectra were recorded on a Varian 300 MHz spectrometer using DMSO-d_6 as solvent, and chemical shifts are given in ppm with TMS as standard.

4.1.1. Preparation of sulfonamides 5–8

4.1.1.1. Method A. An amount of 5 mmol aminosulfonamide **2** or **4** was suspended/dissolved in 20–30 mL anhydrous MeCN and 0.78 mL (0.56 g, 5.5 mmol) triethylamine was added under stirring. The mixture was cooled to 0–5 °C, then a solution of 5 mmol phenylacetyl chloride/2-thienylacetyl chloride **1** or **3** dissolved in 3 mL MeCN was added dropwise during 10 min. (immediately, precipitate appeared). The reaction was stirred overnight or until a reasonable conversion was reached (TLC control). The solvent was evaporated in vacuum and the resulted product was treated with 15–20 mL cold water. The crude solid product was filtered, washed with water and air dried. The obtained compounds were further purified by recrystallisation from ethanol.

4.1.1.2. Method B. Five millimoles of aminosulfonamide **2** or **4** dissolved in 30 mL anhydrous dioxane were treated, under stirring, with 0.95 g (5 mmol) *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride and 0.06 g (0.5 mmol) dimethylaminopyridine (DMAP); the reaction mixture was kept under nitrogen and 0.86 g (5 mmol) of pyridine-2-yl-acetic acid /pyridine-4-yl-acetic acid **3** (Z = OH) was added. The obtained homogeneous colorless solution turned orange-cream and in around 20 min a precipitate appeared. The stirring was continued overnight, then the precipitate was filtered, washed with ethanol and further purified by recrystallisation from ethanol.

4.1.1.3. 4-[2-(2-Thienyl)acetamido]benzenesulfonamide 5a. Yield 40%; mp 207–208 °C; IR(KBr) (ν, cm^{-1}), 1663 (C=O), 1160, 1309 (SO_2); ^1H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.94 (2H, s, CH_2CO), 7.01 (2H, s, thiophene $C_{3,4}$–H), 7.29 (2H, s, SO_2NH_2), 7.42 (1H, s, thiophene C_5–H), 7.78 (4H, s, phenyl $C_{2,3,5,6}$–H), 10.57 (1H, s, CONH). Elem. Anal. Calcd for $C_{12}H_{12}N_2O_3S_2$ (296.36): C, 48.63; H, 4.08; N, 9.45. Found: C, 48.51; H, 4.32; N, 9.18.

4.1.1.4. 4-[2-(2-Thienyl)acetamido]-3-fluorobenzenesulfonamide 5b. Yield 69%; mp 176–177 °C; IR(KBr) (ν, cm^{-1}), 1676 (C=O), 1151, 1320 (SO_2); ^1H NMR (DMSO-d_6, 300 MHz) δ (ppm): 4.04 (2H, s, CH_2CO), 7.01 (2H, s, thiophene $C_{3,4}$–H), 7.44 (3H, s, thiophene C_5–H and SO_2NH_2), 7.62–7.74 (2H, m, phenyl $C_{2,6}$–H), 8.20 (1H, d, J = 6.00 Hz, phenyl C_5–H), 10.31 (1H, s, CONH). Elem. Anal. Calcd for $C_{12}H_{11}FN_2O_3S_2$ (314.35): C, 45.85; H, 3.53; N, 8.91. Found: C, 45.68; H, 3.65; N, 8.82.

4.1.1.5. 4-[2-(2-Thienyl)acetamido]-3-chlorobenzenesulfonamide 5c. Yield 65%; mp 188–189 °C; IR(KBr) (ν, cm^{-1}), 1683 (C=O), 1166, 1334 (SO_2); ^1H NMR (DMSO-d_6, 300 MHz) δ (ppm): 4.03 (2H, s, CH_2CO), 6.99–7.05 (2H, m, thiophene $C_{3,4}$–H), 7.43 (1H, dd, J = 5.10, 1.20 Hz, thiophene C_5–H), 7.49 (2H, s, SO_2NH_2), 7.76 (1H, dd, J = 8.40, 2.10 Hz, phenyl C_6–H), 7.90 (1H, d, J = 2.10 Hz, phenyl C_2–H), 8.03 (1H, d, J = 8.40 Hz, phenyl C_5–H), 10.20 (1H, s, CONH). Elem. Anal. Calcd for $C_{12}H_{11}ClN_2O_3S_2$ (330.81): C, 43.57; H, 3.35; N, 8.47. Found: C, 43.86; H, 3.19; N, 8.50.

4.1.1.6. 4-[2-(2-Thienyl)acetamido]-3-bromobenzenesulfonamide 5d. Yield 30%; mp 191–192 °C; IR(KBr) (ν, cm^{-1}), 1670 (C=O), 1163, 1334 (SO_2); ^1H NMR (DMSO-d_6, 300 MHz) δ (ppm): 4.04 (2H, s, CH_2CO), 7.01–7.05 (2H, m, thiophene $C_{3,4}$–H), 7.42–7.44 (1H, m, thiophene C_5–H), 7.49 (2H, s, SO_2NH_2), 7.79 (1H, dd, J = 8.70, 2.10 Hz, phenyl C_6–H), 7.93 (1H, d, J = 8.70 Hz, phenyl C_5–H), 8.06 (1H, d, J = 2.10 Hz, phenyl C_2–H), 9.90 (1H, s, CONH). Elem. Anal. Calcd for $C_{12}H_{11}BrN_2O_3S_2$ (375.26): C, 38.41; H, 2.95; N, 7.47. Found: C, 38.73; H, 3.12; N, 7.21.

4.1.1.7. 4-[2-(2-Thienyl)acetamidomethyl]benzenesulfonamide 5e. Yield 43%; mp196–197 °C; IR(KBr) (ν, cm^{-1}), 1644 (C=O), 1162, 1335 (SO_2); ^1H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.75 (2H, s, CH_2CO), 4.36 (2H, s, NHCH_2), 6.97 (2H, s, thiophene $C_{3,4}$–H), 7.34 (2H, s, SO_2NH_2), 7.43 (3H, d, J = 6.60 Hz, thiophene C_5–H and phenyl $C_{3,5}$–H), 7.78 (2H, d, J = 6.90 Hz, phenyl $C_{2,6}$–H), 8.65 (1H, s, CONH). Elem. Anal. Calcd for $C_{13}H_{14}N_2O_3S_2$ (310.39): C, 50.30; H, 4.55; N, 9.03. Found: C, 50.39; H, 4.27; N, 8.84.

4.1.1.8. 4-[2-(2-Thienyl)acetamidoethyl]benzenesulfonamide 5f. Yield 31%; mp 174–175 °C; IR(KBr) (ν, cm^{-1}), 1631 (C=O), 1156,

1336 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.79 (2H, t, J = 7.20 Hz, $NHCH_2CH_2$), 3.38 (2H, s, CH_2CO), 3.60 (2H, d, J = 6.30 Hz, $NHCH_2$), 6.87 (1H, d, J = 3.60 Hz, thiophene C_3–H), 6.96 (1H, t, J = 3.60 Hz, thiophene C_4–H), 7.33 (2H, s, SO_2NH_2), 7.35–7.37 (3H, m, thiophene C_5–H and phenyl $C_{3,5}$–H), 7.72 (2H, d, J = 8.10 Hz, phenyl $C_{2,6}$–H), 8.22 (1H, s, CONH). Elem. Anal. Calcd for $C_{14}H_{16}N_2O_3S_2$ (324.41): C, 51.83; H, 4.97; N, 8.63. Found: C, 52.05; H, 4.80; N, 8.39.

4.1.1.9. 5-[2-(2-Thienyl)acetamido]-1,3,4-thiadiazole-2-sulfonamide 6. Yield 76%; mp 248–249 °C; IR(KBr) (ν, cm^{-1}), 1686 (C=O), 1169, 1371 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 4.10 (2H, s, CH_2CO), 6.99–7.03 (2H, m, thiophene $C_{3,4}$–H), 7.43–7.45 (1H, m, thiophene C_5–H), 8.34 (2H, br s, SO_2NH_2). Elem. Anal. Calcd for $C_8H_8N_4O_3S_3$ (304.36): C, 31.57; H, 2.65; N, 18.41. Found: C, 31.43; H, 2.90; N, 18.24.

4.1.1.10. 4-(2-Phenylacetamido)benzenesulfonamide 7a. Yield 35%; mp 205–206 °C; IR(KBr) (ν, cm^{-1}), 1691 (C=O), 1170, 1323 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.70 (2H, s, CH_2CO), 7.26 (2H, s, SO_2NH_2), 7.35 (5H, d, J = 4.50 Hz, Ar-H), 7.77 (4H, d, J = 3.60 Hz, Ar-H), 10.50 (1H, s, CONH). Elem. Anal. Calcd for $C_{14}H_{14}N_2O_3S$ (290.33): C, 57.92; H, 4.86; N, 9.65. Found: C, 58.09; H, 4.64; N, 9.61.

4.1.1.11. 4-(2-Phenylacetamidomethyl)benzenesulfonamide 7b. Yield 46%; mp 204–205 °C; IR(KBr) (ν, cm^{-1}), 1624 (C=O), 1151, 1324 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.48 (2H, s, CH_2CO), 4.31 (2H, d, J = 5.70 Hz, $NHCH_2$), 7.24 (2H, s, SO_2NH_2), 7.28 (5H, m, Ar-H), 7.37 (2H, d, J = 8.40 Hz, phenyl $C_{3,5}$–H), 7.73 (2H, d, J = 8.40 Hz, phenyl $C_{2,6}$–H), 8.60 (1H, s, CONH). Elem. Anal. Calcd for $C_{15}H_{16}N_2O_3S$ (304.36): C, 59.19; H, 5.30; N, 9.20. Found: C, 59.36; H, 4.98; N, 9.04.

4.1.1.12. 4-(2-Phenylacetamidoethyl)benzenesulfonamide 7c. Yield 77%; mp 184–185 °C; IR(KBr) (ν, cm^{-1}), 1626 (C=O), 1156, 1334 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.79 (2H, t, J = 6.90 Hz, $NHCH_2CH_2$), 3.29 (2H, s, CH_2CO), 3.40 (2H, d, J = 7.80 Hz, $NHCH_2$), 7.21–7.29 (5H, m, Ar-H), 7.32 (2H, s, SO_2NH_2), 7.36 (2H, d, J = 8.40 Hz, phenyl $C_{3,5}$–H), 7.74 (2H, d, J = 8.40 Hz, phenyl $C_{2,6}$–H), 8.14 (1H, s, CONH). Elem. Anal. Calcd for $C_{16}H_{18}N_2O_3S$ (318.39): C, 60.36; H, 5.70; N, 8.80. Found: C, 60.51; H, 5.49; N, 8.53.

4.1.1.13. 4-(2-Phenylacetamido)-3-fluorobenzenesulfonamide 7d. Yield 65%; mp 190–191 °C; IR(KBr) (ν, cm^{-1}), 1675 (C=O), 1150, 1338 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.82 (2H, s, CH_2CO), 7.29 (1H, d, J = 3.90 Hz, Ar-H), 7.35 (4H, d, J = 4.80 Hz, Ar-H), 7.44 (2H, s, SO_2NH_2), 7.62 (1H, d, J = 8.40 Hz, phenyl C_6–H), 7.68 (1H, d, J = 2.10 Hz, phenyl C_2–H), 8.18 (1H, t, J = 8.40 Hz, phenyl C_5–H), 10.24 (1H, s, CONH). Elem. Anal. Calcd for $C_{14}H_{13}FN_2O_3S$ (308.32): C, 54.54; H, 4.25; N, 9.09. Found: C, 54.40; H, 3.91; N, 8.83.

4.1.1.14. 4-(2-Phenylacetamido)-3-chlorobenzenesulfonamide 7e. Yield 36%; mp 174–175 °C; IR(KBr) (ν, cm^{-1}), 1672 (C=O), 1162, 1334 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.83 (2H, s, CH_2CO), 7.30–7.38 (5H, m, Ar-H), 7.47 (2H, s, SO_2NH_2), 7.76 (1H, dd, J = 8.70, 3.00 Hz, phenyl C_6–H), 7.91 (1H, d, J = 3.00 Hz, phenyl C_2–H), 8.00 (1H, d, J = 8.70 Hz, phenyl C_5–H), 9.92 (1H, s, CONH). Elem. Anal. Calcd for $C_{14}H_{13}ClN_2O_3S$ (324.78): C, 51.77; H, 4.03; N, 8.63. Found: C, 52.06; H, 3.94; N, 8.57.

4.1.1.15. 4-(2-Phenylacetamido)-3-bromobenzenesulfonamide 7f. Yield 30%; mp 180–181 °C; IR(KBr) (ν, cm^{-1}), 1667 (C=O), 1164, 1338 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.80 (2H, s, CH_2CO), 7.28–7.36 (5H, m, Ar-H), 7.47 (2H, s, SO_2NH_2), 7.79 (1H, dd, J = 8.40, 2.10 Hz, phenyl C_6–H), 7.89 (1H, d, J = 8.40 Hz, phenyl C_5–H), 8.05 (1H, d, J = 2.10 Hz, phenyl C_2–H), 9.80 (1H, s, CONH). Elem. Anal. Calcd for $C_{14}H_{13}BrN_2O_3S$ (369.23): C, 45.54; H, 3.55; N, 7.59. Found: C, 45.27; H, 3.68; N, 7.26.

4.1.1.16. 4-(2-Pyridin-2-ylacetamido)benzenesulfonamide 7g. Yield 30%; mp 219–220 °C; IR(KBr) (ν, cm^{-1}), 1666 (C=O), 1161, 1333 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 4.28 (2H, s, CH_2CO), 7.30 (2H, s, SO_2NH_2), 7.74–7.79 (5H, m, phenyl $C_{2,3,5,6}$–H and pyridine C_5–H), 7.88 (1H, d, J = 7.80 Hz, pyridine C_3–H), 8.34 (1H, t, J = 7.50 Hz, pyridine C_4–H), 8.81 (1H, d, J = 5.40 Hz, pyridine C_6–H), 10.99 (1H, s, CONH). Elem. Anal. Calcd for $C_{13}H_{13}N_3O_3S$ (291.32): C, 53.60; H, 4.50; N, 14.42. Found: C, 53.86; H, 4.33; N, 14.28.

4.1.1.17. 4-(2-Pyridin-4-ylacetamido)benzenesulfonamide 7h. Yield 33%; mp 144–145 °C; IR(KBr) (ν, cm^{-1}), 1707 (C=O), 1155, 1331 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 4.14 (2H, s, CH_2CO), 7.29 (2H, s, SO_2NH_2), 7.78 (4H, s, phenyl $C_{2,3,5,6}$–H), 8.00 (2H, d, J = 6.00 Hz, pyridine $C_{3,5}$–H), 8.86 (2H, d, J = 5.40 Hz, pyridine $C_{2,6}$–H), 11.00 (1H, s, CONH). Elem. Anal. Calcd for $C_{13}H_{13}N_3O_3S$ (291.32): C, 53.60; H, 4.50; N, 14.42. Found: C, 53.45; H, 4.74; N, 14.13.

4.1.1.18. 4-(2-Pyridin-4-ylacetamidoethyl)benzenesulfonamide 7i. Yield 32%; mp 133–134 °C; IR(KBr) (ν, cm^{-1}), 1651 (C=O), 1156, 1333 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.73 (2H, s, $NHCH_2CH_2$), 3.02 (2H, s, CH_2CO), 3.45 (2H, s, $NHCH_2$), 7.21 (2H, s, SO_2NH_2), 7.34 (2H, s, pyridine $C_{3,5}$–H), 7.38 (2H, d, J = 7.20 Hz, phenyl $C_{3,5}$–H), 7.74 (2H, d, J = 7.20 Hz, phenyl $C_{2,6}$–H), 8.31 (1H, s, CONH), 8.47 (2H, s, pyridine $C_{2,6}$–H). Elem. Anal. Calcd for $C_{15}H_{17}N_3O_3S$ (319.37): C, 56.41; H, 5.37; N, 13.16. Found: C, 56.62; H, 5.19; N, 13.04.

4.1.1.19. 5-(2-Phenylacetamido)-1,3,4-thiadiazole-2-sulfonamide 8. Yield 68%; mp 246–247 °C; IR(KBr) (ν, cm^{-1}), 1688 (C=O), 1170, 1372 (SO_2); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.90 (2H, s, CH_2CO), 7.29 (2H, d, J = 6.00 Hz, phenyl $C_{2,6}$–H), 7.35 (3H, s, phenyl $C_{3,4,5,}$–H), 8.35 (2H, s, SO_2NH_2). Elem. Anal. Calcd for $C_{10}H_{10}N_4O_3S_2$ (298.34): C, 40.26; H, 3.38; N, 18.78. Found: C, 39.90; H, 3.46; N, 18.54.

4.2. CA inhibition assay

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes as reported by Khalifah.[16] Phenol Red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration),[8] following the CA-catalyzed CO_2 hydration reaction for a period of 5–10 s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO–water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. At least seven different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[9] and represent the mean from at least three different determinations. All CA isozymes used here were recombinant proteins obtained as reported earlier by our group.[12–15]

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