Bioorganic & Medicinal Chemistry Letters 19 (2009) 3170–3173

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Carbonic anhydrase inhibitors. Phenacetyl-, pyridylacetyl- and thienylacetyl-substituted aromatic sulfonamides act as potent and selective isoform VII inhibitors

Özlen Güzel [a,b], Alessio Innocenti [b], Andrea Scozzafava [b], Aydın Salman [a], Claudiu T. Supuran [b,*]

[a] Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 34116 Beyazıt, Istanbul, Turkey
[b] Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy

ARTICLE INFO

Article history:
Received 23 February 2009
Revised 22 April 2009
Accepted 25 April 2009
Available online 3 May 2009

Keywords:
Carbonic anhydrase
Cytosolic isoform
Isozyme VII
Epileptogenesis
Sulfonamide
Tail approach

ABSTRACT

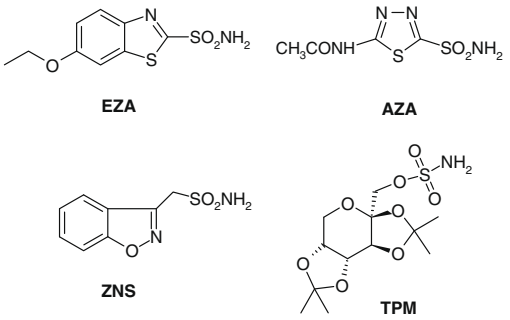
A series of aromatic/heterocyclic sulfonamides incorporating phenyl(alkyl), halogenosubstituted-phenyl- or 1,3,4-thiadiazole-sulfonamide moieties and thienylacetamido; phenacetamido- and pyridinylacetamido tails were prepared and assayed as inhibitors of cytosolic human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms hCA I, II and VII. The new compounds showed moderate inhibition of the two ubiquitous isoforms I and II (K_Is of 50–390 nM) and excellent inhibitory activity against the brain associated hCA VII (K_Is in the range of 4.7–8.5 nM). Isoform VII highly selective inhibitors are being detected for the first time, with selectivity ratios for inhibiting CA VII over CA II of 11–75, and for inhibiting CA VII over CA I of 10–49, which may be useful for understanding the role of CA VII in epileptogenesis and other physiologic processes.

There are 16 α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms expressed in mammals, CA I–CA XV, five of which, CA I–III, VII and XIII, being cytosolic ones.[1–4] These isozymes show a very different distribution in various tissues and organs as well as quite diverse catalytic properties for the physiologic reaction, that is, hydration of carbon dioxide to bicarbonate and a proton.[1–4] Indeed, CA II and VII possess very high efficiency as catalysts for hydration of carbon dioxide, CA I and XIII are 10–50 times less active as compared to CA II and VII, whereas CA III is a very poor catalyst for this reaction, showing around 1% of the catalytic activity of CA II, the most active mammalian CA (together with CA IX) and one of the best catalysts known in nature.[4–6] On the other hand, CA I and II are widely distributed in many tissues/cell types in humans,[7] CA III is present only in muscles and liver,[8] whereas CA VII and XIII show a rather limited distribution only in some organs/tissues, such as the brain for CA VII,[9] and the reproductive tract for CA XIII.[10] The precise physiologic role of some of these isozymes[7–10] is not clearly understood at this moment, with the least investigated and understood one being just CA VII. This isoform was shown to be highly expressed in the cortex, hippocampus and thalamus regions within the mammalian brain.[11,12] Furthermore, its expression is highly increased intrapyramidally at around postnatal day 12 (in mice, but presumably in other mammals too), being hypothesized that this developmental expression promotes excitatory response evoked by intense GABAergic activity, which is related to the epileptiform activity.[12,13] The ionic mechanism of the GABAergic excitation is dependent on bicarbonate, which is the only physiological ion in addition to chloride able to mediate a current through channels coupled to $GABA_A$ receptors.[12,13] CA VII, through its presence mainly in the brain, is currently considered to be involved in the mechanism of GABAergic excitation.[12,13] For example, ethoxzolamide **EZA**, a lipophilic, membrane-permeant CA inhibitor (CAI), acting at low nanomolar level against CA VII,[14] was shown to prevent GABAergic excitations, while membrane-impermeant CAIs had no such effects.[13] Such results clearly showed the involvement of the cytosolic isoform CA VII in the neuronal excitation and its inhibition as a possible antiepileptic mechanism.[15,16] Furthermore, some clinically used drugs, such as acetazolamide **AZA**, zonisamide **ZNS** and topiramate **TPM**, which act as broad-spectrum CAIs,[1] also show anticonvulsant activity and are used as antiepileptic drugs, although their mechanism of action is rather complex (at least for the last two agents).[15,16]

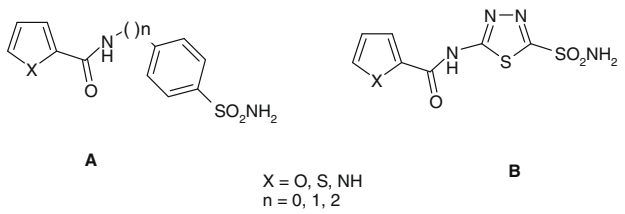
There are several reports[14,17] in which the inhibition of CA VII with various classes of sulfonamides and sulfamates has been evaluated. Although several low nanomolar or subnanomolar CAIs were detected (some of them also showing in vivo anticonvulsant

* Corresponding author. Tel.: +39 055 4573005; fax: +39 055 4573385.
E-mail address: claudiu.supuran@unifi.it (C.T. Supuran).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.04.123



activity[17a]) the main draw-back of such compounds is related to their low selectivity for inhibiting isoforms CA VII over other cytosolic (CA I, II, XIII) or membrane-associated isoforms (CA IV, IX, XII, XIV). In fact, the mammalian brain expresses in addition to CA VII, several other CA isoforms, such as CA I, II, III, VB, XII and XIV,[15,16] and the inhibitors investigated up to now[14,17] generally showed a very high activity towards most of them. Thus, a net discrimination between the effects of such drugs on the various isoforms and their relationship to the anticonvulsant activity is difficult if not impossible to achieve with broad spectrum CAIs which interact with many of these CAs.[18] It is thus of great importance to design compounds showing selective inhibition of different physiologically relevant CAs, and in particular, CA VII-selective inhibitors. Here, we report such a study which led to the discovery of the most CA VII-selective compounds reported to date.

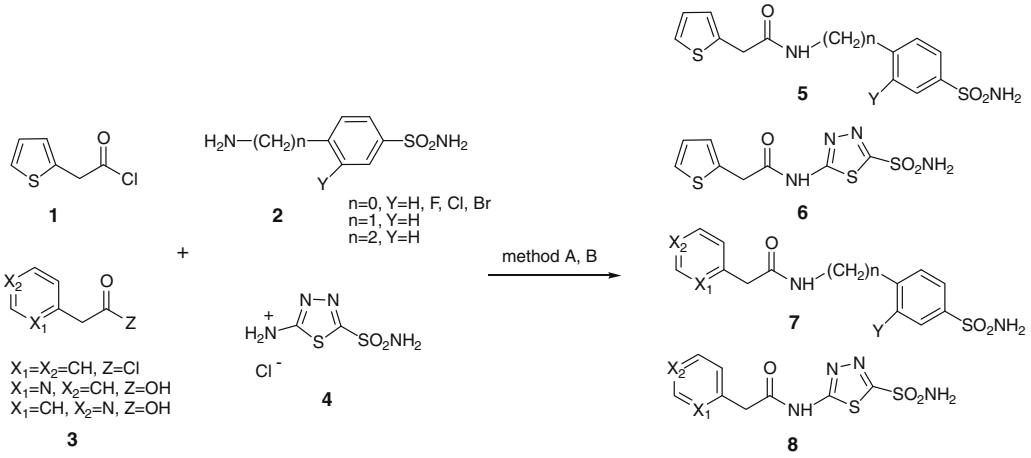


We used sulfonamides **A** and **B** reported earlier by our group[19] as lead molecules for the design of the new sulfonamides reported here. Indeed, sulfonamides of the type **A** and **B**, incorporating phenyl(alkyl), halogeno-phenyl- or 1,3,4-thiadiazole moieties as well as furan-2-ylcarboxamido; thien-2-ylcarboxamido or pyrrol-2-ylcarboxamido tails, were easily prepared from the corresponding amino sulfonamides by reactions with heterocyclic acyl halides or carboxylic acids in the presence of carbodiimides, by the tail approach.[19–21] 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.[19] The most active compounds were the thienyl derivatives, and here we extend the earlier work,[19] focusing on this substitution pattern. However, 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 terminal 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 posess longer tails as compared to the derivatives investigated earlier.[19] 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.[22,23] In that region of the active site are present 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 works showed that both favorable interactions as well as clashes with particular amino acid residues present only in some CA isozymes[24] are critical for the inhibition profile and isozyme selectivity issues of the sulfonamides and their bioisosteres such as the sulfamates and the sulfamides. 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 non-exceptional procedure (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 included in the study.[25]

The following structure–activity relationship (SAR) can be evidenced by considering data of Table 1:[26]

(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 clinically used compounds (**AZA, EZA, ZNS** and **TPM**) or the orphan drug benzolamide **BZA** show much more potent hCA II inhibitory activity, with K_Is in the range of 8–35 nM (Table 1). It should be also noted that the compounds investigated earlier,[19] 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).[19] These 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



Scheme 1. Synthesis of the new sulfonamides **5**–**8**. Method A: Z = Cl, NEt_3/MeCN; Method B: Z = OH, *N*-(3-dimethylpropyl)-*N*′-ethylcarbodiimide, DMAP/dioxane.

Table 1
Inhibition data of isoforms hCA I, II and VII with sulfonamides **5**–**8** and clinically used sulfonamides/sulfamates **EZA**–**TPM**, by a stopped flow CO_2 hydrase assay[26]

No.	n,Y	X_1	X_2	K_I* (nM)			Selectivity ratio	
				hCA I[a]	hCA II[a]	hCA VII[a]	I/VII	II/VII
5a	0, H	—	—	61	50	6.2	9.83	8.06
5b	0, F	—	—	161	390	7.0	23.00	55.71
5c	0, Cl	—	—	77	50	6.9	11.15	7.24
5d	0, Br	—	—	84	52	7.2	11.66	7.22
5e	1, H	—	—	60	52	7.1	8.45	7.32
5f	2, H	—	—	68	53	7.6	8.94	6.97
6	—		—	72	51	6.9	10.43	7.39
7[a]	0, H	CH	CH	108	107	4.7	22.97	22.76
7b	1, H	CH	CH	75	54	6.8	11.02	7.94
7c	2, H	CH	CH	60	67	5.5	10.90	12.18
7d	0, F	CH	CH	67	61	5.4	12.40	11.29
7e	0, Cl	CH	CH	61	58	6.9	8.84	8.40
7f	0, Br	CH	CH	263	395	5.3	49.62	74.52
7g	0, H	N	CH	75	69	7.7	9.74	8.96
7h	0, H	CH	N	71	74	7.9	8.98	9.36
7i	2, H	CH	N	73	97	8.5	8.58	11.41
8	—	CH	CH	63	51	6.1	10.32	8.36
EZA	—	—	—	25	8	0.8	31.25	10.00
AZA	—	—	—	250	12	2.5	100	4.80
ZNS	—	—	—	56	35	117	0.47	0.29
TPM	—	—	—	250	10	0.9	277.7	11.11

* Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[26]
[a] Human, recombinant isozymes.

case) may have drastic consequences for the enzyme inhibitory activity and selectivity profile against various isozymes of such derivatives.

(iii) The brain-associated isoform hCA VII was highly inhibited by all sulfonamides **5–8** investigated here, with K_Is in the range of 4.7–8.5 nM (Table 1). All these new compounds were highly active, irrespective of the aryl/hetaryl sulfonamide head and the heterocyclic/aromatic-acetamido tails present in their molecules. SAR is thus very simple and flat, and also difficult to rationalize as the X-ray crystal structure of CA VII is not known for the moment. However, this is a remarkable finding, considering that few compounds with such a strong inhibitory activity against this isoform have been reported up until now.

(iv) In addition of being highly potent CA VII inhibitors, compounds **5–8** reported here are also isoform VII selective over the ubiquitous cytosolic isozymes I and II (Table 1). Indeed, it may be observed that the selectivity ratios for inhibiting CA I over CA VII are in the range of 8.45–49.62, whereas that form inhibiting CA II over CA VII in the range of 7.22–74.52. This means that all these compounds have much higher affinity for CA VII than for CA II and I, which are ubiquitous, cytosolic isoforms.

In conclusion, we report here series of aromatic/heterocyclic sulfonamides incorporating phenyl(alkyl), halogenosubstituted-phenyl- or 1,3,4-thiadiazole-sulfonamide moieties and thienylacetamido; phenacetamido- and pyridinylacetamido tails, which were prepared and assayed as inhibitors of cytosolic human isoforms hCA I, II and VII. The new compounds showed moderate inhibition of the two ubiquitous isoforms I and II (K_Is of 50–390 nM) and excellent inhibitory activity against the brain associated hCA VII (K_Is in the range of 4.7–8.5 nM). Isoform VII highly selective inhibitors are being detected thus for the first time, with selectivity ratios for inhibiting CA VII over CA II of 11–75, and for inhibiting CA VII over CA I of 10–49, which may be useful for understanding the role of CA VII in epileptogenesis and other physiologic processes.

Acknowledgments

This research was financed in part by a grant of the 6th Framework Programme of the EU (DeZnIT project), by a 7th Framework Programme EU project (METOXIA) and by an Italian FIRB project (MIUR/FIRB RBNE03PX83_001).

References and notes

1. (a) Supuran, C. T. *Nat. Rev. Drug Disc.* **2008**, *7*, 168; (b) Supuran, C. T.; Scozzafava, A. *Bioorg. Med. Chem.* **2007**, *15*, 4336.
2. *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton (FL), USA, 2004; pp 1–376. and references cited therein.
3. (a) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146; (b) Winum, J. Y.; Rami, M.; Scozzafava, A.; Montero, J. L.; Supuran, C. *Med. Res. Rev.* **2008**, *28*, 445.
4. Di Fiore, A.; Monti, S. M.; Hilvo, M.; Parkkila, S.; Romano, V.; Scaloni, A.; Pedone, C.; Scozzafava, A.; Supuran, C. T.; De Simone, G. *Proteins* **2009**, *74*, 164.
5. (a) Nishimori, I.; Minakuchi, T.; Onishi, S.; Vullo, D.; Cecchi, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2007**, *15*, 7229; (b) Elder, I.; Fisher, Z.; Laipis, P. J.; Tu, C.; McKenna, R.; Silverman, D. N. *Proteins* **2007**, *68*, 337.
6. Hilvo, M.; Baranauskiene, L.; Salzano, A. M.; Scaloni, A.; Matulis, D.; Innocenti, A.; Scozzafava, A.; Monti, S. M.; Di Fiore, A.; De Simone, G.; Lindfors, M.; Janis, J.; Valjakka, J.; Pastorekova, S.; Pastorek, J.; Kulomaa, M. S.; Nordlund, H. R.; Supuran, C. T.; Parkkila, S. *J. Biol. Chem.* **2008**, *283*, 27799.
7. Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199.
8. Wetzel, P.; Papadopoulos, S.; Gros, G. *Pflugers. Arch.* **2002**, *443*, 762.

9. Rivera, C.; Voipio, J.; Payne, J. A.; Ruusuvuori, E.; Lahtinen, H.; Lamsa, K.; Pirvola, U.; Saarma, M.; Kaila, K. *Nature* **1999**, *397*, 251.
10. Lehtonen, J.; Shen, B.; Vihinen, M.; Casini, A.; Scozzafava, A.; Supuran, C. T.; Parkkila, A.-K.; Saarnio, J.; Kivelä, A.; Waheed, A.; Sly, W. S.; Parkkila, S. *J. Biol. Chem.* **2004**, *279*, 2719.
11. Halmi, P.; Parkkila, S.; Honkaniemi, J. *Neurochem. Int.* **2006**, *48*, 24.
12. Ruusuvuori, E.; Li, H.; Huttu, K.; Palva, J. M.; Smirnov, S.; Rivera, C.; Kaila, K.; Voipio, J. *J. Neurosci.* **2004**, *24*, 2699.
13. Rivera, C.; Voipio, J.; Kaila, K. *J. Physiol.* **2005**, *562*, 27.
14. Vullo, D.; Voipio, J.; Innocenti, A.; Rivera, C.; Ranki, H.; Scozzafava, A.; Kaila, K.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 971.
15. Thiry, A.; Dogné, J. M.; Supuran, C. T.; Masereel, B. *Curr. Top. Med. Chem.* **2007**, *7*, 855.
16. Thiry, A.; Dogné, J. M.; Supuran, C. T.; Masereel, B. *Curr. Pharm. Des.* **2008**, *14*, 661.
17. (a) Winum, J. Y.; Thiry, A.; El Cheikh, K.; Dogné, J. M.; Montero, J. L.; Vullo, D.; Scozzafava, A.; Masereel, B.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2685; (b) Thiry, A.; Masereel, B.; Dogné, J. M.; Supuran, C. T.; Wouters, J.; Michaux, C. *ChemMedChem* **2007**, *2*, 1273; (c) Thiry, A.; Rolin, S.; Vullo, D.; Frankart, A.; Scozzafava, A.; Dogné, J. M.; Wouters, J.; Supuran, C. T.; Masereel, B. *Eur. J. Med. Chem.* **2008**, *43*, 2853.
18. Supuran, C. T. *Epilepsy Res.* **2008**, *82*, 111.
19. Ilies, M.; Supuran, C. T.; Scozzafava, A.; Casini, A.; Mincione, F.; Menabuoni, L.; Caproiu, M. T.; Maganu, M.; Banciu, M. D. *Bioorg. Med. Chem.* **2000**, *8*, 2145.
20. (a) Scozzafava, A.; Menabuoni, L.; Mincione, F.; Briganti, F.; Mincione, G.; Supuran, C. T. *J. Med. Chem.* **1999**, *42*, 2641; (b) Supuran, C. T.; Vullo, D.; Manole, G.; Casini, A.; Scozzafava, A. *Curr. Med. Chem. Cardiovasc. Hematol. Agents* **2004**, *2*, 49.
21. (a) Alterio, V.; Vitale, R. M.; Monti, S. M.; Pedone, C.; Scozzafava, A.; Cecchi, A.; De Simone, G.; Supuran, C. T. *J. Am. Chem. Soc.* **2006**, *128*, 8329; (b) Stiti, M.; Cecchi, A.; Rami, M.; Abdaoui, M.; Barragan-Montero, V.; Scozzafava, A.; Guari, Y.; Winum, J. Y.; Supuran, C. T. *J. Am. Chem. Soc.* **2008**, *130*, 16130.
22. (a) De Simone, G.; Di Fiore, A.; Menchise, V.; Pedone, C.; Antel, J.; Casini, A.; Scozzafava, A.; Wurl, M.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2315; (b) Boriack, P. A.; Christianson, D. W.; Kingery-Wood, J.; Whitesides, G. M. *J. Med. Chem.* **1995**, *38*, 2286; (c) Casini, A.; Antel, J.; Abbate, F.; Scozzafava, A.; David, S.; Waldeck, H.; Schafer, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 841.
23. (a) Güzel, Ö.; Temperini, C.; Innocenti, A.; Scozzafava, A.; Salman, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 152; (b) Di Fiore, A.; Pedone, C.; D'Ambrosio, K.; Scozzafava, A.; De Simone, G.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 437; (c) Casini, A.; Abbate, F.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2759; (d) Di Fiore, A.; De Simone, G.; Menchise, V.; Pedone, C.; Casini, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1937.
24. Winum, J. Y.; Temperini, C.; El Cheikh, K.; Innocenti, A.; Vullo, D.; Ciattini, S.; Montero, J. L.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2006**, *49*, 7024.
25. Preparation of sulfonamides **5**–**8**: *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.*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-[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).
26. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes. 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),[19] 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,[19] 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.[19–21]