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Carbonic anhydrase inhibitors: synthesis and inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, IX, and XII with *N*-hydroxysulfamides—a new zinc-binding function in the design of inhibitors

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Abstract—A small library of *N*-hydroxysulfamides was synthesized by an original approach in order to investigate whether this zinc-binding function is efficient for the design of inhibitors targeting the cytosolic (hCA I and II) and transmembrane, tumor-associated (hCA IX and XII) isozymes of carbonic anhydrase (CA, EC 4.2.1.1). The parent derivative, *N*-hydroxysulfamide was a more potent inhibitor as compared to sulfamide or sulfamic acid against all isozymes, with inhibition constants in the range of 473 nM–4.05 μM. Its substituted *n*-decyl-, *n*-dodecyl-, benzyl-, and biphenylmethyl-derivatives were less inhibitory against hCA I (K_Is in the range of 5.8–8.2 μM) but more inhibitory against hCA II (K_Is in the range of 50.5–473 nM). The same situation was true for the tumor-associated isozymes, with K_Is in the range of 353–790 nM against hCA IX and 372–874 nM against hCA XII. Some sulfamides/sulfamates possessing similar substitution patterns have also been investigated for the inhibition of these isozymes, being shown that in some particular cases sulfamides are more efficient inhibitors as compared to the corresponding sulfamates. Potent CA inhibitors targeting the cytosolic or tumor-associated CA isozymes can thus be designed from various classes of sulfonamides, sulfamides, or sulfamates and their derivatives, considering the extensive interactions in which the inhibitor and the enzyme active site are engaged, based on recent X-ray crystallographic data.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are wide-spread enzymes, present in mammals in at least 15 different isoforms. Some of these isozymes are cytosolic (CA I, CA II, CA III, CA VII, CA XIII), others are membrane-bound (CA IV, CA IX, CA XII, and CA XIV), CA VA, and CA VB are present in mitochondria throughout the body, whereas CA VI is secreted in the saliva and milk.[1–4] Three cytosolic acatalytic forms are also known (CARP VIII, CARP X, and CARP XI). The catalytically active isoforms, which play important physiological and patho-physiological functions, are strongly inhibited by aromatic and heterocyclic sulfonamides, sulfamates, sulfamides, and some of their derivatives.[1–4] The catalytic and inhibition mechanisms of these enzymes are understood in great detail, and this was helpful for the design of potent inhibitors, some of which possess clinical applications for the treatment or prevention of a multitude of diseases.[1]

Recently, the involvement of some CAs and their sulfonamide inhibitors in cancer has been investigated: many potent CA inhibitors (CAIs) were shown to inhibit the growth of several tumor cell lines in vitro and in vivo, constituting thus interesting leads for developing novel antitumor therapies.[5,6] Indeed, Svastova et al.[7] showed that the acidic extracellular pH, which is a typical attribute of the tumor microenvironment, is generated by the activity of one of the tumor-associated CA isozymes,

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that is, CA IX, and that this acidification can be perturbed by deletion of the enzyme active site and inhibited by CA IX-selective inhibitors of the sulfonamide type, which bind only to hypoxic cells containing CA IX (the involvement of the other tumor-associated isozyme, that is, CA XII, in such processes has not been investigated for the moment). Thus, it appears of critical importance to pursue the development of CAIs targeting the tumor-associated CA isozymes CA IX and XII, eventually belonging to novel classes of compounds, less investigated up to now.

Among the various classes of CAIs reported by this group in the last years, there were also some compounds incorporating modified sulfonamide/sulfamate moieties, such as *N*-hydroxysulfonamides,[8] phosphorylated sulfonamides,[9,10] or phosphorylated sulfamates.[11] The classical, clinically used CAIs belong to the unsubstituted aromatic/heterocyclic sulfonamide types possessing the very simple general formula RSO_2NH_2,[1–3] but it has been shown mostly by this group that many other zinc-binding functions (ZBFs) except the sulfonamide one can lead to potent CAIs.[8–12] Some of the most investigated such moieties are shown in Figure 1, and they include the sulfamate one, the phosphorylated sulfamate, the sulfamide, the sulfonylated sulfamide/sulfamate, the sulfamide Schiff's base, the *N*-hydroxy-, *N*-amino-, *N*-cyano-, or *N*-phosphorylated sulfonamide, the urea, hydroxyurea, or hydroxamate ones.[1,13,14] The *N*-hydroxysulfamides have not been investigated up to now for their interaction with CAs, although this ZBF is included in Figure 1.

Recently, it has been claimed[15] that sulfamides are ineffective as ZBFs for the design of CAIs, based on two examples of sugar sulfamates and the corresponding sulfamides (topiramate, RWJ-37947 and their corresponding sulfamide derivatives), and two bicyclic compounds possessing the general formula $RCH_2–XSO_2NH_2$ where X = O or NH, and R = benzofuran and benzodioxolan. This claim is as a matter-of-fact not true, since it has been demonstrated earlier by us[4] that low nanomolar hCA I and hCA II inhibitors belonging to the sulfamide class can be obtained. Indeed, in a series of aromatic sulfamides possessing the general formula $X–C_6H_4–NHSO_2NH_2$, compounds with inhibition constants in the range of 8–39 nM against hCA I and 7–36 nM against hCA II have been detected.[4] Furthermore, some of the corresponding sulfamates have also been reported subsequently,[16] being shown that in some cases the sulfamides are more potent hCA I, hCA II, or hCA IX inhibitors as compared to them (e.g., in the case of the 4-trifluoromethyl-phenyl, pentafluorophenyl-, or 2-naphthyl-sulfamides, among others). Thus, the statement of Maryannof et al.[15] that sulfamides cannot lead to the design of potent CAIs can be definitively rebutted. Continuing our work in finding novel ZBFs for the design of CAIs, we report here that *N*-hydroxysulfamides constitute interesting such candidates. A small library of such derivatives has been synthesized and tested for the inhibition of four physiologically relevant isozymes, the cytosolic, ubiquitous hCA I and II, as well as the transmembrane, tumor-associated isozymes hCA IX and XII (h means isozyme of human origin), comparing the activity of these new CAIs with that of the sulfamates and sulfamides investigated in more detail in recent years.

2. Chemistry

The *N*-alkyl-*N*′-hydroxysulfamide derivatives **4a**–**f** were prepared using the reaction sequence summarized in Scheme 1. The commercially available *O*-(*tert*-butyldimethylsilyl) hydroxylamine **1** was sulfamoylated with *N*-Boc-protected sulfamoyl chloride, prepared ab initio by reacting chlorosulfonyl isocyanate with *tert*-butanol.[17] The key intermediate **2** was allowed to react with different aliphatic alcohols R–OH, with R = octyl, decyl, dodecyl, benzyl, biphenylmethyl, under standard Mitsunobu conditions to afford compounds **3** in very good yield (>90% for each compound). Compounds **3** were then fully deprotected using trifluoroacetic acid (TFA) with 5% of water to yield the *N*-alkyl-*N*′-hydroxysulfamides **4b**–**f**. *N*-Hydroxysulfamide **4a**, previously

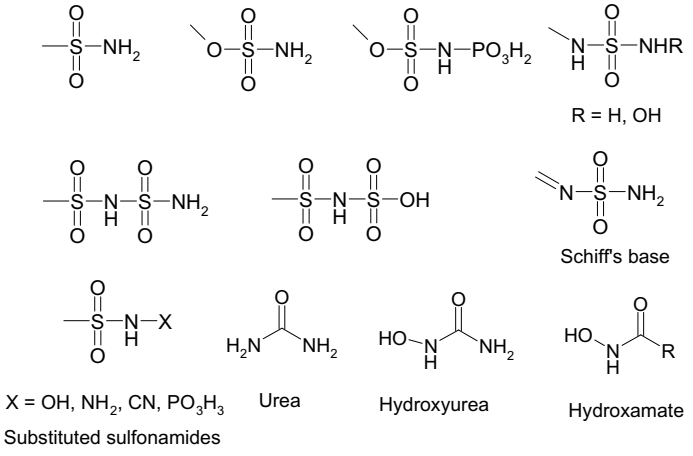
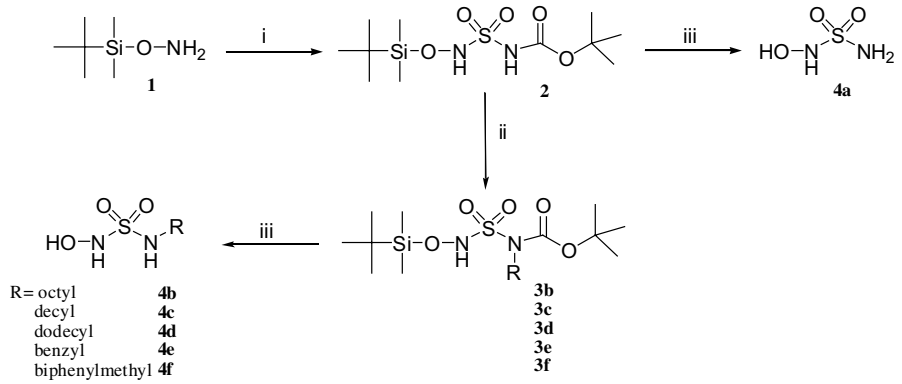


Figure 1. Zinc-binding functions (ZBFs) for the design of potent CAIs: sulfonamides, sulfamates, sulfamides, substituted sulfonamides/sulfamates/sulfamides, Schiff's bases, urea, hydroxyurea, and hydroxamates. All these groups bind in deprotonated form, as anions to the Zn(II) ion within the enzyme active site, presumably in monodentate fashion.



Scheme 1. Synthesis of *N*-alkyl-*N*′-hydroxysulfamides. Reagents and conditions: (i) chlorosulfonyl isocyanate, *t*-BuOH, CH_2Cl_2; (ii) R–OH, PPh_3, DIAD, THF, Mitsunobu conditions; (iii) TFA–H_2O 5%.

reported (in the salt form) by Montero's group and prepared with a modified procedure,[18] was obtained efficiently in high yield upon treatment of **2** with a mixture of TFA–H_2O 5%. All these compounds were fully characterized by NMR spectroscopy and mass spectrometry.[19]

3. CA inhibition data

Inhibition data against isozymes hCA I, II, IX, and XII with *N*-hydroxysulfamides **4** as well as sulfamide and sulfamic acid or organic sulfamides/sulfamates **5** and **6** as standard inhibitors are presented in Table 1.[20]

The following SAR is evidenced from the data of Table 1: (i) against isozyme hCA I both sulfamide and sulfamic acid (as anions)[12] act as very weak, millimolar inhibitors, whereas *N*-hydroxysulfamide **4a** showed an enhanced affinity, with an inhibition constant of 4.05 μM. The substituted alkyl/aryl–alkyl derivatives **4b–e** on the other hand are less efficient hCA I inhibitors as compared to the parent hydroxysulfamide **4a**, showing K_Is in the range of 5.8–8.2 μM; (ii) against the physiologically most relevant isozyme, hCA II, the unsubstituted parent compound **4a** again behaves as a more potent inhibitor as compared to sulfamide or sulfamic acid (millimolar inhibitors, with K_Is in the range of 0.39–1.13 mM), with an inhibition constant of 566 nM. For this isozyme, the substituted derivatives **4b–e** lead to enhanced inhibition, with K_Is in the range of 50.5–473 nM. Thus, the *n*-decyl- and the benzyl derivatives **4b** and **4d** are medium potency inhibitors (K_Is in the range of 313–473 nM), whereas the slight increase

Table 1. Inhibition of isozymes hCA I, II, IX, and XII with sulfamide, sulfamic acid, *N*-hydroxysulfamides **4**, sulfamides **5**, and the corresponding sulfamates **6**

$RNHSO_2NHOH$ **4** $RNHSO_2NH_2$ **5** $ROSO_2NH_2$ **6**

Inhibitor	R	K_I[a] (nM)			
		hCA I[b]	hCA II[b]	hCA IX[c]	hCA XII[c]
$H_2NSO_2NH_2$	—	0.31×10^6	1.13×10^6	9.6×10^3	13.2×10^3
H_2NSO_3H[d]	—	0.21×10^5	0.39×10^6	9.2×10^3	10.7×10^3
4a	H	4050	566	865	1340
4b	*n*-$C_{10}H_{23}$	5800	473	506	874
4c	*n*-$C_{12}H_{25}$	6000	89.6	485	539
4d	$PhCH_2$	8200	313	790	633
4e	4-$PhC_6H_4CH_2$	8100	50.5	353	372
5a	4-$CF_3C_6H_4$	8	7	26	48
6a	4-$CF_3C_6H_4$	369	138	54	103
5b	4-CNC_6H_4	20	16	30	45
6b	4-CNC_6H_4	480	149	41	76
5c	C_6F_5	34	32	40	19
6c	C_6F_5	415	113	47	34
5d	2-Naphthyl	39	36	38	30
6d	2-Naphthyl	103	63	40	62

Data for compounds **4** against all isozymes, as well as those regarding hCA IX inhibition with sulfamides **5**, and hCA XII inhibition with all these (**4–6**) compounds are new. Sulfamide/sulfamic acid hCA I, II, and IX data were previously published,[21] as well as the inhibition data of sulfamides **5**,[4] and sulfamates **6**[22] against two isozymes (hCA I and II).

[a] Errors in the range of 5–10% of the shown data, from three different assays.

[b] Human recombinant isozymes.

[c] Catalytic domain of the human recombinant isozyme, CO_2 hydrase assay method.[20]

[d] As sodium salt.

in size to *n*-dodecyl- or a supplementary phenyl moiety in **4d**, leading to the biphenyl-methyl derivative **4e**, has as consequence a dramatic increase in inhibitory power, the later two compounds being efficient hCA II inhibitors (K_Is in the range of 50.5–89.6 nM); (iii) sulfamide and sulfamic acid are much more potent hCA IX inhibitors as compared to their affinity for the cytosolic isozymes hCA I and II,[21] with K_Is in the range of 9.2–9.6 μM. *N*-Hydroxysulfamide **4a** is also a more potent hCA IX inhibitor as compared to sulfamide or sulfamic acid, with an inhibition constant of 865 nM, whereas its substituted derivatives **4b–e** showed an even increased affinity for this tumor-associated isozyme, with K_Is in the range of 353–790 nM (Table 1). Again the best inhibitors were the *n*-dodecyl- and biphenyl-methyl derivatives (**4c** and **4e**) whereas the *n*-decyl- and benzyl-substituted compounds showed a less efficient inhibition profile, similarly with the situation observed for hCA II; (iv) similarly to hCA IX, hCA XII shows higher affinity for sulfamide and sulfamic acid, as compared to the cytosolic isozymes I and II, with K_Is in the range of 10.7–13.2 μM. Sulfamic acid is slightly more inhibitory than sulfamide (which is in fact true against all isozymes investigated up to now). *N*-Hydroxysulfamide **4a** is more potent an inhibitor as compared to the above-mentioned compounds, with a K_I of 1340 nM, whereas its derivatives **4b–e** showed better hCA XII inhibitory properties, with K_Is in the range of 372–874 nM. In this small library of derivatives the best substitution patterns for obtaining potent CA II, IX or XII inhibitors were identical, that is, the *n*-dodecyl- and biphenyl-methyl-substituted compounds were the most effective as CAIs.

At this point it is important to re-examine various ZBFs for the design of CAIs, since our results[4] that sulfamides may lead to effective such inhibitors were recently contested by Maryannof et al.[15] In Table 1 we also show some examples of sulfamides and the corresponding sulfamates, which have been tested up to now only as hCA I and II inhibitors (the sulfamides **5**)[4] or as hCA I, II, and IX inhibitors (the sulfamates **6**).[22] Although the general trend is that sulfamates are slightly more efficient hCA I and II inhibitors as compared to the corresponding sulfamides possessing the same substitution pattern,[4,22] this finding cannot be generalized since we have observed many cases in which the reverse is true, that is, sulfamides being more inhibitory than the corresponding sulfamates. In Table 1 several such examples are provided in which pairs of sulfamides/sulfamates **5** and **6**, respectively, have been tested as inhibitors of isozymes I, II, IX, and XII. Thus, it may be observed that for the 4-trifluoromethylphenyl-, 4-cyano-phenyl-, pentafluorophenyl-, or 2-naphthyl-substituted derivatives among others, sulfamides **5a–d** are much more effective CAIs as compared to the corresponding sulfamates **6a–d** against all investigated isozymes, and of particular importance are the hCA IX and hCA XII such data, since sulfamides and sulfamates have not been investigated up to now for the inhibition of these tumor-associated isozymes. It may be observed that sulfamides **5** behave as effective hCA IX inhibitors, with K_Is in the range of 26–40 nM, whereas sulfamates **6** are slightly less effective (K_Is in the range of 40–54 nM). The same is true against hCA XII, with sulfamides **5** showing K_Is in the range of 19–48 nM, and sulfamates **6** in the range of 34–103 nM. The first partial conclusion is that potent hCA I, II, IX, and XII inhibitors can be designed both with sulfamide or sulfamate (or sulfonamide) ZBFs. Indeed, considering detailed X-ray crystallography work from this laboratory, mainly on isozymes I and II in adducts with many structural types of sulfonamide and sulfamate inhibitors,[23–30] it appears that a multitude of factors influence the binding of such a compound within the enzyme active site (Fig. 2). Thus, the ZBF of the inhibitor (irrespective which one of those shown in Fig. 1 is considered) is deprotonated in order to be able to coordinate monodentately to the Zn(II) ion from the active site, and also participates in a network of hydrogen bonds with the neighboring amino acid residues

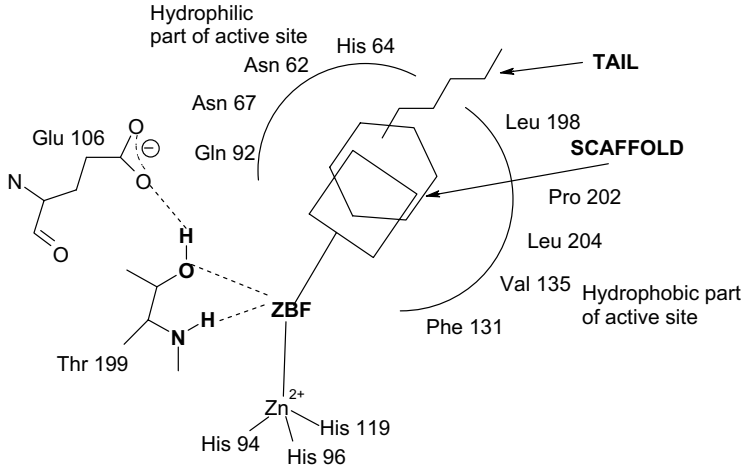


Figure 2. The general structure of a CAI complexed to the enzyme (α-CA) active site: ZBF = zinc-binding function; the organic scaffold may be present or absent; the tail too. These structural elements interact both with the hydrophobic as well as the hydrophylic halves of the active site (representative amino acid residues involved in binding are shown), whereas ZBF interacts with the Zn(II) ion as well as the neighboring residues Thr 199 and Glu 106.

(conserved in all α-CAs)[1] Thr 199 and Glu 106. These fundamental interactions have already been observed in the X-ray crystal structure of the adducts of hCA II with the simplest compounds incorporating a sulfonamide moiety, that is, sulfamide and sulfamic acid, reported by us.[12] However, these compounds are weak CAIs (in the milli- to micromolar range), since other interactions with the enzyme active site are precluded due to their intrinsic simplicity, but these X-ray structures provided strong evidence that such compounds may be used as lead molecules for designing CAIs with enhanced affinity for various isozymes, which we confirmed subsequently for derivatives possessing also an organic scaffold and sulfamide/sulfamate ZBFs.[4,22,25] Indeed, a potent CAI should possess such an organic scaffold (Fig. 2) possibly substituted with various tails that should enable its interaction with both the hydrophobic as well as hydrophilic halves of the enzyme active site.[23–30] It has been observed that the amino acid residues belonging to the two above-mentioned active site regions (i.e., His 64, Asn 62, Asn 67, and Gln 92 for the hydrophilic half, and Phe 131, Val 135, Leu 202, Leu 204, and Leu 198, respectively, for the hydrophobic half) are generally conserved in all CA isozymes.[1–3] With such a multitude of interactions between the inhibitor and the active site, low nanomolar inhibitors may be obtained belonging to different classes of compounds, in addition to the classical sulfonamide CAIs.

4. Conclusions

A small library of *N*-hydroxysulfamides was synthesized by an original approach in order to investigate whether this ZBF is efficient for the design of inhibitors targeting the cytosolic (hCA I and II) and transmembrane, tumor-associated (hCA IX and XII) isozymes. The parent derivative, *N*-hydroxysulfamide was a more potent inhibitor as compared to sulfamide or sulfamic acid against all isozymes, with inhibition constants in the range of 473 nM–4.05 μM. Its substituted *n*-decyl-, *n*-dodecyl-, benzyl-, and biphenylmethyl-derivatives were less inhibitory against hCA I (K_Is in the range of 5.8–8.2 μM) but more inhibitory against hCA II (K_Is in the range of 50.5–473 nM). The same situation was true for the tumor-associated isozymes, with K_Is in the range of 353–790 nM against hCA IX and 372–874 nM against hCA XII. Some sulfamides/sulfamates possessing the same substitution pattern have also been investigated for the inhibition of these isozymes, being shown that for these particular cases sulfamides are more efficient inhibitors as compared to the corresponding sulfamates. Potent CA inhibitors targeting the cytosolic or tumor-associated CA isozymes can thus be designed from various classes of sulfonamides, sulfamides, or sulfamates and their derivatives.

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References and notes

1. Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199–229.
2. Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146–189.
3. Scozzafava, A.; Owa, T.; Mastrolorenzo, A.; Supuran, C. T. *Curr. Med. Chem.* **2003**, *10*, 925–953.
4. Casini, A.; Winum, J. Y.; Montero, J. L.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 837–840.
5. Supuran, C. T.; Scozzafava, A. *Eur. J. Med. Chem.* **2000**, *35*, 867–874.
6. Supuran, C. T.; Scozzafava, A. *J. Enzyme Inhib.* **2000**, *15*, 597–610.
7. Svastova, E.; Hulikova, A.; Rafajova, M.; Zat'ovicova, M.; Gibadulinova, A.; Casini, A.; Cecchi, A.; Scozzafava, A.; Supuran, C. T.; Pastorek, J.; Pastorekova, S. *FEBS Lett.* **2004**, *577*, 439–445.
8. Mincione, F.; Menabuoni, L.; Briganti, F.; Mincione, G.; Scozzafava, A.; Supuran, C. T. *J. Enzyme Inhib.* **1998**, *13*, 267–284.
9. Fenesan, I.; Popescu, R.; Scozzafava, A.; Crucin, V.; Mateiciuc, E.; Bauer, R.; Ilies, M. A.; Supuran, C. T. *J. Enzyme Inhib.* **2000**, *15*, 297–310.
10. Fenesan, I.; Popescu, R.; Supuran, C. T.; Nicoara, S.; Culea, M.; Palibroda, N.; Moldovan, Z.; Cozar, O. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 721–729.
11. Bonnac, L.; Innocenti, A.; Winum, J. Y.; Casini, A.; Montero, J. L.; Scozzafava, A.; Barragan, V.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 275–278.
12. Abbate, F.; Supuran, C. T.; Scozzafava, A.; Orioli, P.; Stubbs, M. T.; Klebe, G. *J. Med. Chem.* **2002**, *45*, 3583–3587.
13. Briganti, F.; Mangani, S.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T. *J. Biol. Inorg. Chem.* **1999**, *4*, 528–536.
14. Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2003**, *11*, 2241–2246.
15. Maryanoff, B. E.; McComsey, D. F.; Costanzo, M. J.; Hochman, C.; Smith-Swintowsky, V.; Shank, R. P. *J. Med. Chem.* **2005**, *48*, in press.
16. Winum, J. Y.; Vullo, D.; Casini, A.; Montero, J. L.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2003**, *46*, 2197–2204.
17. Winum, J.-Y.; Toupet, L.; Barragan, V.; Dewynter, G.; Montero, J.-L. *Org. Lett.* **2001**, *3*, 2241–2243.
18. Hajri, A.-H. D. G.; Criton, M.; Dilda, P.; Montero, J.-L. *Heteroatom. Chem.* **2001**, *12*, 1–5.
19. Experimental procedure for the synthesis of compound **2**: To a solution of *tert*-butanol (1.2 equiv) in 8 mL of methylene chloride was added at 0 °C chlorosulfonyl isocyanate (1.2 equiv). The resulting solution was then added to a solution of *O*-(*tert*-butyldimethylsilyl)hydroxylamine (1 equiv) and triethylamine (3 equiv) in methylene chloride (30 mL). The reaction mixture was stirred at room temperature for 3 h, then poured in ethyl acetate, and washed several times with water. The organic phase was dried on anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel chromatography to afford the expected compound in 61% yield. ^{1}H NMR (DMSO-d_6, 250 MHz) δ 10.55 (s, 1H), 8.75 (s, 1H), 0.65 (s, 9H), 0.4 (s, 9H), 0.05 (s, 6H). MS ESI$^+$ *m/z* 349 $(M+Na)^+$. ESI$^-$ *m/z* 325 $(M-H)^-$.
General procedure for the synthesis of compounds **3**: To a solution of compound **2** (1 equiv), alcohol R–OH (1 equiv) and triphenylphosphine (1.1 equiv) in 5 mL of THF was added di-isopropylazodicarboxylate DIAD (1.1 equiv). The mixture was stirred at room temperature for 2 h, then concentrated in vacuo. The residue was purified by

silica gel chromatography to yield compound **3** as a yellow oil. Compound **3b**: ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.8 (s, 1H), 3.5 (t, 2H, J = 5.1 Hz), 1.3 (s, 9H), 1.1–1.2 (m, 12H), 0.8 (s, 9H), 0.7 (t, 3H, J = 3.3 Hz), 0.1 (s, 6H); MS ESI$^+$ m/z 461 (M+Na)$^+$. ESI$^-$ m/z 437 (M−H)$^-$. Compound **3c**: ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.7 (s, 1H), 3.6 (t, 2H, J = 2.5 Hz), 1.4 (s, 9H), 1.1–1.3 (m, 16H), 0.8 (s, 9H), 0.7 (t, 3H, J = 3.3 Hz), 0.1 (s, 6H); MS ESI$^+$ m/z 489 (M+Na)$^+$. ESI$^-$ m/z 465 (M−H)$^-$. Compound **3d**: ^{1}H NMR (DMSO-d_6, 250 MHz) δ 9.6 (s, 1H), 3.5 (t, 2H, J = 2.6 Hz), 1.4 (s, 9H), 1.2 (m, 20H), 0.8 (s, 9H), 0.7 (t, 3H, J = 7.4 Hz), 0.1 (s, 6H); MS ESI$^+$ m/z 517 (M+Na)$^+$. ESI$^-$ m/z 493 (M−H)$^-$. Compound **3e**: ^{1}H NMR (DMSO-d_6, 250 MHz) δ 9.9 (s, 1H), 7.4 (m, 5H), 4.75 (s, 2H), 1.4 (s, 9H), 0.9 (s, 9H), 0.15 (s, 6H); MS ESI$^+$ m/z 439 (M+Na)$^+$. ESI$^-$ m/z 415 (M−H)$^-$. Compound **3f**: ^{1}H NMR (DMSO-d_6, 250 MHz) δ 9.7 (s, 1H), 7.1–7.8 (m, 9H), 4.9 (s, 2H), 1.6 (s, 9H), 0.9 (s, 9H), 0.5 (s, 6H); MS ESI$^+$ m/z 515 (M+Na)$^+$. ESI$^-$ m/z 491(M−H)$^-$.

General procedure for the synthesis of compounds **4**: Compound **3** was dissolved and stirred at 0 °C in a mixture of TFA–H_2O 5%. The reaction was monitored by TLC until the complete disappearance of starting material. The mixture was then diluted with ethyl acetate, washed with a saturated aqueous solution of $NaHCO_3$, and then washed several times with water. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel chromatography to give compound **4** in moderate to good yield. For the synthesis of compound **4a**, the same procedure was used starting from compound **2**. Compound **4a**: mp: 75–80 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9 (s, 1H), 8.4 (s, 1H), 6.7 (s, 2H); MS ESI$^-$ m/z 110 (M−H)$^-$. Compound **4b**: mp: 75–77 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.8 (s, 1H), 7.2 (t, 1H, J = 5.7 Hz), 2.85 (q, 2H J = 13.3 Hz, J = 6.6 Hz), 1.4 (s, 2H), 1.2–1.3 (m, 10H), 0.9 (t, 3H, J = 6.4 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 43.2, 32.1, 30.0, 29.5, 26.4, 27.1, 22.9, 14.8; MS ESI$^+$ m/z 247 (M+Na)$^+$. Compound **4c**: mp: 89–92 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.1 (d, 1H, J = 3.3 Hz), 8.75 (d, 1H, J = 3.3 Hz), 7.3 (t, 1H, J = 5.8 Hz), 2.9 (q, 2H,J = 13.2 Hz, J = 6.4 Hz), 1.1–1.5 (m, 16H), 0.9 (t, 3H, J = 6.6 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) 42.8, 31.8, 29.6, 29.5, 29.4, 29.2, 29.1, 26.7, 22.6, 14.4; MS ESI$^+$ m/z 275 (M+Na)$^+$. Compound **4d**: mp: 95–97 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 9.1 (d, 1H, J = 3.2 Hz), 8.75 (d, 1H, J = 3.3 Hz), 7.35 (t, 1H, J = 5.8 Hz), 2.9 (q, 2H, J = 13.4 Hz, J = 6.9 Hz), 1.1–1.5 (m, 20H), 0.8 (t, 3H, J = 6.3 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 42.8, 31.8, 29.6, 29.5, 29.4, 29.2, 29.1, 26.6, 26.2, 25.9, 22.6, 14.4; MS ESI$^+$ m/z 275 (M+Na)$^+$. Compound **4e**: mp: 88–93 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.1 (s, 1H), 8.9 (s, 1H), 7.8 (t, 1H, J = 6.3 Hz), 7.1–7.4 (m, 5H), 4 (d, 2H, J = 6.3 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 139.1, 129.3, 129.1, 128.6, 127.9, 127.4, 46.3; MS ESI$^+$ m/z 225 (M+Na)$^+$. Compound **4f**: mp:149–151 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.4 (d, 1H, J = 3.1 Hz), 9.2 (d, 1H, J = 3.1 Hz), 8.1 (t, 1H, J = 6.3 Hz), 8–7.5 (m, 9H), 4.3 (d, 2H, J = 6.3 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 140.4, 139.5, 138.3, 129.4, 128.6, 127.8, 127.1, 127.0, 126.9, 45.9; MS ESI$^+$ m/z 301 (M+Na)$^+$.

20. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573. An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for measuring the initial velocities by following the change in absorbance of a pH indicator. 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 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total obseverd rates. The kinetic constants k_{cat} and k_{cat}/K_m were obtained by nonlinear least-squares methods using SigmaPlot. Stock solutions of inhibitors were prepared at a concentration of 1–3 mM (in DMSO–water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. K_Is of the inhibitors were determined by using Lineweaver–Burk plots, as reported earlier.[4–7]
21. Innocenti, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 567–571.
22. Winum, J.-Y.; Vullo, D.; Casini, A.; Montero, J.-L.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2003**, *46*, 2197–2204.
23. Casini, A.; Antel, J.; Abbate, F.; Scozzafava, A.; David, S.; Waldeck, H.; Schäfer, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 841–845.
24. Abbate, F.; Casini, A.; Owa, T.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 217–223.
25. Abbate, F.; Winum, J.-Y.; Potter, B. V. L.; Casini, A.; Montero, J.-L.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 231–234.
26. Weber, A.; Casini, A.; Heine, A.; Kuhn, D.; Supuran, C. T.; Scozzafava, A.; Klebe, G. *J. Med. Chem.* **2004**, *47*, 550–557.
27. Abbate, F.; Casini, A.; Scozzafava, A.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 303–308.
28. Casini, A.; Abbate, F.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett* **2003**, *13*, 2763–2769.
29. Abbate, F.; Coetzee, A.; Casini, A.; Ciattini, S.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 337–341.
30. Abbate, F.; Casini, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2357–2361.