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Carbonic anhydrase inhibitors: Inhibition of cytosolic/tumor-associated isoforms I, II, and IX with iminodiacetic carboxylates/hydroxamates also incorporating benzenesulfonamide moieties

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Abstract—The synthesis of a new class of sulfonamide carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs), also possessing carboxylate/hydroxamate moieties in their molecule, is reported. These compounds may act on dual antitumor targets, the tumor-associated CA isozymes (CA IX) and some matrix metalloproteinases (MMPs). The compounds were prepared by an original method starting from iminodiacetic acid, and assayed as inhibitors of three isozymes, hCA I, II (cytosolic), and IX (transmembrane). The new derivatives showed weak inhibitory activity against isozyme I (K_Is in the range of 95–8300 nM), were excellent to moderate CA II inhibitors (K_Is in the range of 8.4–65 nM), and very good and selective CA IX inhibitors (K_Is in the range of 3.8–26 nM). The primary sulfonamide moiety is a better zinc-binding group in the design of CAIs as compared to the carboxylate/hydroxamate one, but the presence of hydroxamate functionalities in the molecule of CAIs leads to selectivity for the tumor-associated isozyme IX over the ubiquitous, cytosolic isoform II.

Inhibitors of zinc metalloenzymes need precise structural requirements in order to tightly bind to the metal ion(s) present in their active sites.[1] For example, primary sulfonamides (RSO_2NH_2), sulfamates ($ROSO_2NH_2$), and sulfamides ($RNHSO_2NH_2$) show high affinity for binding the Zn(II) ion present in α-carbonic anhydrases (CAs, EC 4.2.1.1), acting as potent inhibitors with clinical applications as antiglaucoma, diuretic, antiobesity or antitumor drugs.[2–6] Various CA isoforms are responsible for specific physiological functions, and drugs with such a diversity of actions target in fact quite different isozymes of the 15 presently known in humans.[2–6] In all of them, the sulfonamide/sulfamate/sulfamide drug binds in deprotonated form to the catalytically critical Zn(II) ion, also participating in extensive hydrogen bond and van der Waals interactions with amino acid residues both in the hydrophobic and hydrophilic halves of the enzyme active site, as shown by X-ray crystallographic studies of enzyme–inhibitor complexes.[7–9] Although the Zn(II) coordination at the catalytic domain of CAs is identical to that of the matrix metalloproteinases (MMPs) see Figure 1a,[1] a family of zinc endopeptidases degrading extracellular matrix (and many other substrates),[1] being constituted of three histidine ligands and a water molecule/hydroxide ion acting as nucleophile in the hydrolytic processes, sulfonamides, sulfamates or sulfamides do not bind to MMPs with high affinity.[1,10] Instead, the main classes of MMP inhibitors (MMPIs) are constituted by compounds incorporating hydroxamate or carboxylate zinc-binding functions.[1,10–14] Indeed, X-ray crystallographic studies showed both the carboxylate as well as the hydroxamate deprotonated moieties to be bidentately coordinated to the catalytic Zn(II) ions present in many of the more than 25 MMPs presently known.[1,10–14] On the other hand, hydroxamates do show CA inhibitory properties,[10] whereas some structurally related zinc-binding groups, such as the hydroxyurea one (a derivatized hydroxamate), seem to be appropriate for designing

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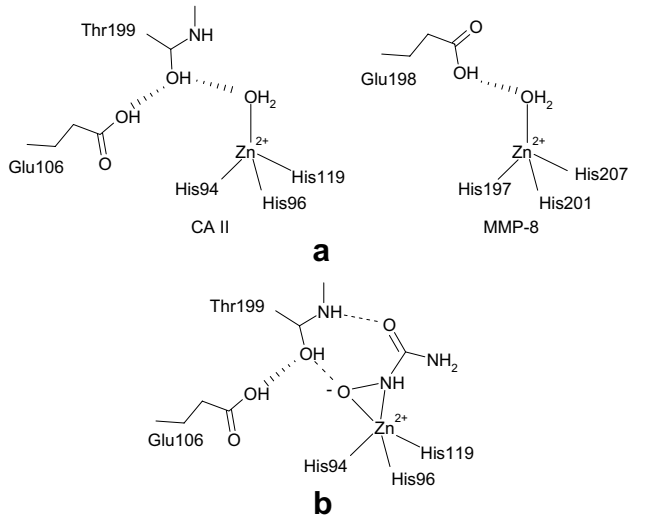
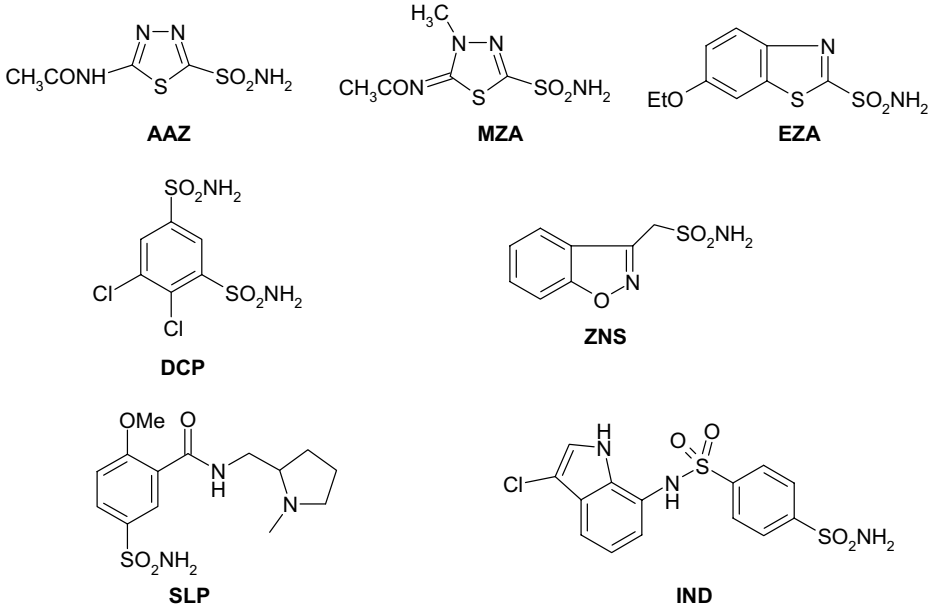


Figure 1. (a) Zn(II) coordination sphere in CAs and MMPs. The Zn(II) ion in both active sites is coordinated by three histidine residues and a water molecule. The main difference is that the water molecule interacts with the OH moiety of a Thr residue (hydrogen-bonded to a carboxylate of a glutamic acid) in CAs, whereas it is hydrogen-bonded directly to the carboxylate of a glutamic acid residue in MMPs.[1–4] (b) Binding of *N*-hydroxyurea, a hydroxamate-like derivative, to CA II as shown by X-ray crystallography.[15b]

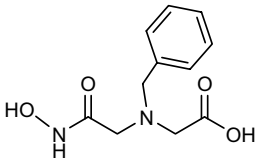
both CA[15] as well as MMP[13] inhibitors. The X-ray crystal structure of *N*-hydroxyurea with CA II has recently been reported by this group,[15b] being shown that unexpectedly, the ligand bidentately coordinates to the Zn(II) ion within the CA active site through the NH and OH groups present in its molecule (Fig. 1b). Since some MMPs as well as several CA isozymes are both involved in tumorigenesis,[1,10,16–20] it appeared of interest to investigate compounds that may potentially inhibit both these enzymes, continuing our previous work[10] in searching ''cross-reactivities'' between the two types of unrelated zinc enzymes (i.e., the CAs and the MMPs).

Here we report an inhibition study directed against the ubiquitous cytosolic isozymes CA I and II,[2–4] as well as the transmembrane, tumor-associated isoform CA IX[16,18] (all of human origin, i.e., hCA I, hCA II, and hCA IX), with a series of hydroxamates/carboxylates known to possess MMP inhibitory activity,[1,22] which have been derivatized in such a way as to incorporate in their molecules primary sulfamoyl moieties, the zinc-binding function *par excellence*, present in CA inhibitors (CAIs) in clinical use such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, zonisamide **ZNS**, sulpiride **SLP**, and indisulam **IND**.

The compounds reported here are similar to the new class of non-peptidic hydroxamate/carboxylate-based MMP inhibitors, i.e., the *N*-aryl derivatives of iminodiacetic acid (IDA),[21–23] which were designed in such a way as to mimic a truncated variant of the potent peptidic succinyl-hydroxamate MMPIs, such as marimastat or batimastat.[12a] Some of these compounds were previously reported as MMPIs showing interesting activity.[21–23] They were obtained starting from the simple iminodiacetic acid (IDA) scaffold which by reaction with arylsulfonyl halides led to the key intermediates N-substituted derivatives **1** (with the aim of improving the efficacy and specificity of their interaction within the S1' pocket of the MMPs' active site region).[12,19] Starting from this type of derivatives **1**, we report here the effect of replacing one of its carboxylic acid moieties by an amide chain incorporating 4-aminoethylbenzenesulfonamide functionalities. Thus, we introduced the aromatic sulfonamide 'head' typical of strong CA inhibitors in the molecules of the compounds investigated earlier as MMPIs, in order to prepare derivatives with potentially dual action, as MMPIs and CAIs. We evaluated the CA inhibitory activity of these new compounds against three physiologically relevant human isoforms, i.e., hCA I, II, and IX.



The preparation of the new compounds **2**–**11** reported in this paper is presented in Schemes 1 and 2.[21] Coupling of IDA with arylsulfonyl halides led to the key intermediate dicarboxylic acid **1** as previously reported.[21–23] Derivatives **1** were first amidated at one of their carboxylic groups (which has been pre-activated with ethylchloroformate (ECF) in the presence of *N*-methylmorpholine (NMM)) with 4-aminoethylbenzenesulfonamide (AEBS), thus leading to derivatives **2**–**4** (Scheme 1). The second step involved the formation of the hydroxamic acid functionality by condensation of the carboxylic acids **2-4** with hydroxylamine, using the standard activation of the carboxylic acid group with ECF/NMM, thus leading to hydroxamates **5**–**7**. By reacting **1** with AEBS in the same conditions as above, but working at 1:2 molar ratios between the reagents, the bis-amide incorporating two primary sulfamoyl moieties **8** has been obtained (Scheme 1). Alternatively, AEBS was reacted with *N*-benzyliminodiacetic anhydride **9**[11] leading to derivative **10**, which has been thereafter transformed to the hydroxamate **11** by the same procedure used for the preparation of compounds **5**–**7** (Scheme 2). The non-sulfonamide derivatives **12** and **13** have also been included in the series of compounds investigated as CAIs here, in order to better understand the contribution of the primary sulfamoyl/carboxylate/hydroxamate moieties to the binding of this series of compounds to the target enzymes.



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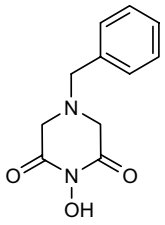

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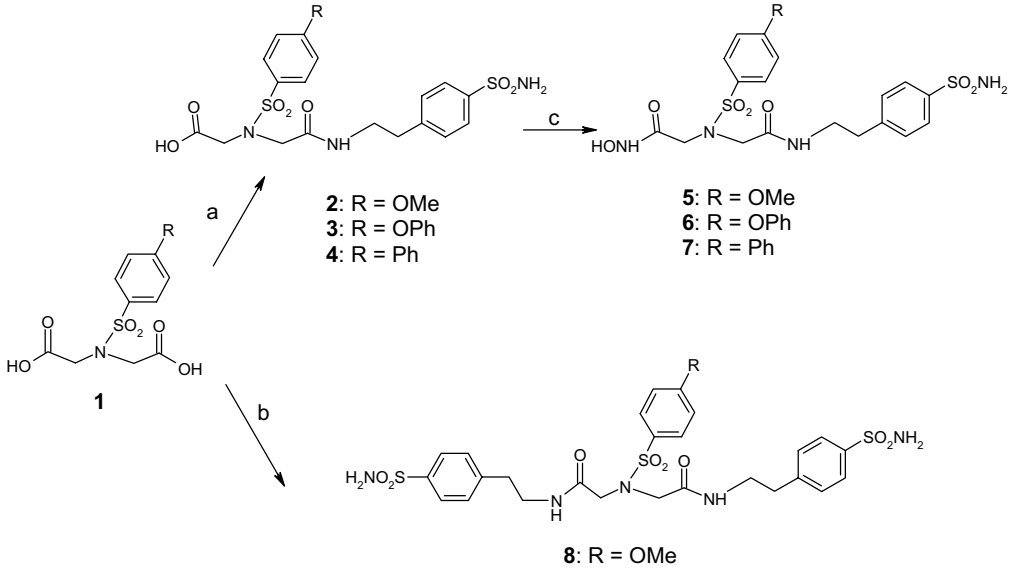
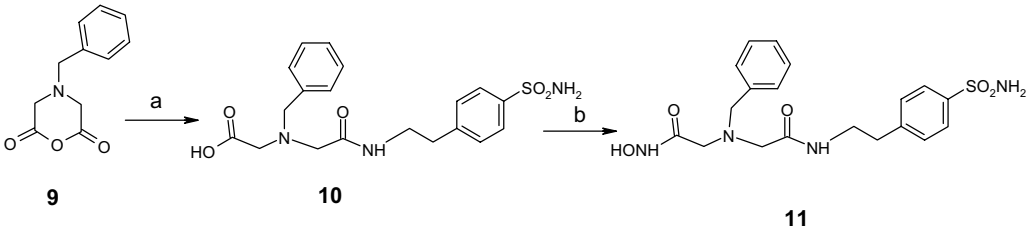
Data of Table 1 show the inhibition of three physiologically relevant CA isoforms, i.e., CA I and II (ubiquitous, cytosolic isoforms)[2–4] and CA IX (predominantly tumor-associated isozyme)[2,3,24] with compounds **2**–**13** described here and standard CAIs of the sulfonamide type, such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, zonisamide **ZNS**, sulpiride **SLP**, and indisulam **IND** (for comparison).[25]

All compounds investigated here, of types **2**–**13**, showed inhibitory activity against the three CA isozymes known to be important medicinal chemistry targets, i.e., CA I,



Scheme 1. Synthesis of compounds **2**–**8**. Reagents and conditions: (a) (ECF, NMM)/THF, 1 equiv AEBS/THF, 0 °C; (b) (ECF, NMM)/THF, 2 equiv AEBS/THF, 0 °C; (c) (ECF, NMM)/THF, NH_2OH/CH_3OH, 0 °C.



Scheme 2. Synthesis of compounds **10** and **11**. Reagents and conditions: (a) AEBS, CH_3CN, 0 °C; (b) (ECF, NMM)/THF, NH_2OH/CH_3OH, 0 °C.

Table 1. Human (h) CA I, II, and IX inhibition data with compounds **2**–**13**, *N*-hydroxyurea,[15a] and standard sulfonamide CA inhibitors (**AAZ**–**IND**), for the CA catalyzed CO_2 hydration reaction, measured by a stopped-flow assay[25]

Compound	K_I (nM)[c]		Selectivity ratio	
	hCA I[a]	hCA II[a]	hCA IX[b]	$K_{I(CA\ II)}/K_{I(CA\ IX)}$
2	7230	12	8.5	1.41
3	3430	9.3	6.4	1.45
4	116	8.4	3.8	2.21
5	1765	57	13	4.38
6	95	65	12	5.41
7	2487	35	13	2.69
8	4290	47	3.9	12.05
10	7450	18	17	1.05
11	2339	63	26	2.42
12	8307	380	9412	0.04
13	2835	821	6907	0.11
N-hydroxyurea[15a]	nt	28,000	23,000	1.21
AAZ	250	12	25	0.48
MZA	50	14	27	0.51
EZA	25	8	34	0.25
DCP	1200	38	50	0.76
ZNS	56	35	5	7.00
SLP	1200	40	31	1.29
IND	31	15	24	0.62

[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method.[25]

[b] Catalytic domain of the human, recombinant enzyme, stopped flow CO_2 hydrase assay method.[25]

[c] Errors were in the range of 5–10% of the reported values, from three different assays; nt, not tested.

II, and IX. The following SAR can be observed from data of Table 1: (i) the derivatives investigated here, possessing either primary sulfonamide or hydroxamate zinc-binding functions (ZBFs), showed inhibitory activity against all three investigated CA isoforms, but with different inhibition profiles as compared to those of the clinically used sulfonamide drugs **AAZ**–**IND**. Thus, the compounds possessing only hydroxamate moieties as ZBF (such as **12**, **13**, and *N*-hydroxyurea) were generally much weaker inhibitors of all CA isozymes as compared to the derivatives incorporating SO_2NH_2 moieties as ZBF (derivatives **2**–**11** and **AAZ**–**IND**); (ii) against the cytosolic isozyme hCA I, all the new derivatives investigated here except **4** and **6** behaved as weak inhibitors, with inhibition constants in the range of 1.765–8.307 μM. This is a general feature of isozyme I, which has less affinity for sulfonamides as compared to hCA II or IX, due to the fact that its active site is much more restricted as compared to those of isozymes II and IX.[2–4] This phenomenon is in turn due to the presence of the bulky His200 (which is Thr in most other CA isozymes including CA II and IX) in the neighborhood of the catalytic zinc ion, and His67 (which is Asn in CA II and Gln in CA IX) at the entrance of the cavity.[15,26] Thus, derivatives investigated by us here, possessing generally a rather bulky tail extending from the 4-aminoethylcarboxamido moiety substituting the benzenesulfonamide head, are no exception from this rule, being obviously too bulky for allowing a tighter binding within this smaller active site cavity of hCA I. However, two of these new compounds, i.e., **4** and **6**, showed medium potency against this isozyme (K_Is in the range of 95–116 nM), being however much less effective hCA I inhibitors as compared to methazolamide, ethoxzolamide, zonisamide or indisulam, which showed K_Is in the range of 25–56 nM (Table 1); (iii) against the ubiquitous isozyme hCA II, the hydroxamates **12**, **13**, and hydroxyurea behaved as weak inhibitors (K_Is in the range of 380–28,000 nM), whereas a number of new derivatives, such as **5**–**8** and **11**, showed efficient inhibitory activity, with K_Is of 35–65 nM. However, compounds **2**–**4** and **10** showed excellent hCA II inhibitory action, with inhibition constants in the range of 8.4–18 nM, of the same order of magnitude as those of many clinically used sulfonamide CAIs (**AAZ**, *MZA*, *IND*, etc.). It may be observed that the carboxylates are always more active than the corresponding hydroxamates (compare **2** with **5**, **3** with **6**, **4** with **7**, **10** with **11**), and that monosulfonamides (less bulky) are better inhibitors than the corresponding bis-sulfonamides (too bulky—compare **2** and **8**). The substitution pattern of the arylsulfonyl moiety linked to the central nitrogen atom of the IDA scaffold also influences activity, with the biphenylsulfonamide-derivatives being the best inhibitors both for carboxylates **2**–**4** as well as hydroxamates **5**–**7** (Table 1). Thus, it is obvious from the data above that when two possible ZBFs are present in the same molecule of a CAI, the hydroxamate/carboxylate one and the primary sulfonamide one, it is always the last one to achieve the anchoring of the inhibitor to the Zn(II) ion from the enzyme active site; (iv) against the tumor-associated isoform hCA IX, the hydroxamates **12**, **13**, and hydroxyurea showed quite weak inhibitory activity (K_Is of 6.9–23 μM), but all other compounds reported for the first time here, i.e., derivatives **2**–**11**, acted as quite potent inhibitors, with K_Is in the range of 3.8–26 nM. Thus, these compounds are generally much more effective CA IX inhibitors than the sulfonamides in clinical use (except for **ZNS**, which with a K_I of 5 nM is one of the best hCA IX inhibitors reported until now),[27] making them quite interesting candidates for further anti-tumor studies. In fact, it is well established[1–4,20,24] that many tumors overexpress both CA IX and some MMPs, and compounds with a dual inhibitory action against these enzymes may be of interest. SAR for these derivatives is rather similar to what is observed for CA II inhibition: carboxylates **2**–**4** were more effective CA IX inhibitors as compared to the corresponding hydroxamates **5**–**7**. However, apparently the active site cavity of CA IX is larger than that of CA II, and this may explain why these quite bulky derivatives showed such a good inhibitory action against the tumor-associated isozyme. Although the X-ray crystal structure of CA IX was not reported yet, in earlier studies we already observed this effect, i.e., compounds incorporating bulky moieties, which showed moderate CA II inhibitory activity, were excellent CA IX inhibitors (in the low nanomolar range);[18,28] (v) most of the new sulfonamides reported here were rather selective CA IX over CA II inhibitors (Table 1). Thus, for the clinically used sulfonamides (except for **ZNS**), the inhibition ratio between the two isozymes was always <1, meaning that such sulfonamides were better CA II than CA IX inhibitors. This is obviously not desired for a

compound that should target primarily the tumor-associated isozyme IX and not the ubiquitous, house-keeping enzyme CA II.[2–4] However, it may be observed that similarly to **ZNS** (which shows a selectivity ratio of 7), many of the new compounds reported in the present study possess selectivity ratios >1, some of them in the range of 4.38–12.05. The most CA IX selective compounds were the hydroxamates **5**–**7** and the bis-sulfonamide **8**. On the other hand, the most CA II selective compounds were the hydroxamates **12** and **13**, which were 9- to 25-fold better CA II than CA IX inhibitors.

In conclusion, we report the synthesis of a new class of sulfonamide CAIs, also possessing carboxylate/hydroxamate moieties in their molecule. As a consequence, these compounds may act on dual antitumor targets, such as the tumor-associated CA isozymes (e.g., CA IX) and some MMPs. The new compounds were prepared by an original method starting from iminodiacetic acid, and assayed as inhibitors of three CA isozymes, hCA I, II, and IX. The new derivatives showed weak inhibitory activity against isozyme I (K_Is in the range of 95–8300 nM), were excellent to moderate CA II inhibitors (K_Is in the range of 8.4–65 nM), and very good and selective CA IX inhibitors (K_Is in the range of 3.8–26 nM). The sulfonamide is a better zinc-binding group in the design of CAIs as compared to the carboxylate/hydroxamate one, but the presence of hydroxamate functionalities in the molecule of such CAIs leads to selectivity for the tumor-associated isozyme IX over the ubiquitous, cytosolic isoform CA II.

Acknowledgments

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21. *N-(4-phenoxybenzenesulfonyl)-N-((2-(4-sulfamoylphenyl) ethylcarbamoyl)methyl)-aminoacetohydroxamic acid (**6**)*: m.p 151–153 °C; ^{1}H NMR (D_2O, pD *ca* 9): δ 7.78 (d, J = 9.0 Hz, 2H, Ar***H***), 7.74 (d, J = 8.1 Hz, 2H, Ar***H***), 7.48 (t, J = 8.1 Hz, 2H, Ar***H***), 7.36 (d, J = 8.4 Hz, 2H, Ar***H***), 7.32 (t, 1H, J = 7.2 Hz, Ar***H***), 7.17–7.12 (2d, 4H, Ar***H***),

3.85 (s, 2H, C***H***$_2$CONHCH$_2$), 3.79 (s, 2H, C***H***$_2$CONHOH), 3.41 (t, J = 6.8 Hz, 2H, NHC***H***$_2$CH$_2$PhSO$_2$NH$_2$), 2.84 (t, J = 6.8 Hz, 2H, NHCH$_2$C***H***$_2$PhSO$_2$NH$_2$); *m/z* (HRMS) calcd for $(MNa)^+$: 585.10843. Found 585.10743; calcd for $(MH)^+$: 563.12648, found 563.12580. Elem. Analysis: Calcd ($C_{24}H_{26}N_4O_8S_2$ 0.4CH_2Cl_2): C, 49.00; H, 4.52; N, 9.36; S, 10.71%. Found: C, 49.07; H, 4.88; N, 9.63; S, 10.39%.

*N,N-di((2-(4-sulfamoylphenyl)ethylcarbamoyl)methyl)-4-methoxybenzenesulfonamide (**8**)*: M.p. 118–120 °C; ^{1}H NMR (CD_3OD): δ 7.74 (d, J = 8.1 Hz, 4H, Ar***H***), 7.68 (d, J = 9.3 Hz, 2H, Ar***H***), 7.35 (d, J = 7.8 Hz, 4H, Ar***H***), 7.01 (d, J = 8.4 Hz, 2H, Ar***H***), 3.79 (s, 3H, OC***H***$_3$), 3.66 (s, 4H, C***H***$_2$CONH), 3.36 (t, J = 6.7 Hz, 4H, NHC***H***$_2$CH$_2$PhSO$_2$NH$_2$), 2.82 (t, J = 6.5 Hz, 4H, NHCH$_2$C***H***$_2$PhSO$_2$NH$_2$); *m/z* (HRMS) calcd for $C_{27}H_{34}N_5O_9S_3$: 668.15187; found 668.15165.

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25. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An applied photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. 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. The CO_2 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 for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. 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, from Lineweaver–Burk plots, as reported earlier, and represent the mean from at least three different determinations.[7–10] Recombinant isozymes CA I, II and IX were prepared as described earlier.[15,18]
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