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Ureido-substituted sulfamates show potent carbonic anhydrase IX inhibitory and antiproliferative activities against breast cancer cell lines

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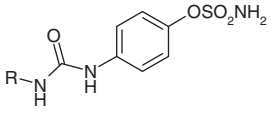
ABSTRACT

A series of 50 sulfamates were obtained by reacting 4-aminophenol with isocyanates followed by sulfamoylation. Most of the new compounds were nanomolar inhibitors of the tumor-associated carbonic anhydrase (CA, EC 4.2.1.1) isoforms IX and XII, whereas they inhibited less cytosolic offtarget isoforms CA I and II. Some of these sulfamates showed significant antiproliferative activity in several breast cancer cell lines, such as SKBR3, MCF10A, ZR75/1, MDA-MB-361 and MCF7, constituting interesting anticancer leads.

Recently we reported[1] a series of ureido-substituted benzenesulfonamides of type **A** which showed potent inhibitory activity against several isoforms of carbonic anhydrase (CA, EC 4.2.1.1) involved in tumorigenesis, such as CA IX and XII. Furthermore, in vivo, some of these compounds significantly inhibited the growth of both primary tumors and metastases in an animal model of breast cancer, making them interesting candidates for clinical development.[1,2] The CAs, are a superfamily of metalloenzymes which catalyze the interconversion between CO_2 and bicarbonate by using a metal hydroxide nucleophilic mechanism.[1–9] Five genetically distinct CA families are known to date, the α-, β-, -, δ- and ζ-class enzymes.[2–4] Inhibition and activation of these enzymes are well understood processes, with most classes of inhibitors binding to the metal center,[1–10] and activators binding at the entrance of the active site cavity. The sulfonamides and their isosteres (sulfamates, sulfamides) represent the main class of pharmacologically relevant CA inhibitors (CAIs), with many such derivatives in clinical use as antiglaucoma agents, anticonvulsants, diuretics or antiobesity drugs.[4–10]



3a – 3bb

It should be noted that recently, CA inhibitory mechanisms other than the binding to the metal center[11] were reported for α-CAs, which do not directly involve the metal ion from the enzyme active site.[12–15] For example polyamines bind to the enzyme by anchoring to the zinc-coordinated water/hydroxide ion,[13] whereas coumarins act as prodrugs and bind at the entrance of the active site cavity, quite distant from the metal ion, after being hydrolyzed by the esterase activity of the CAs to 2-hydroxy-cinnamic acids.[14,15]

CA inhibition has pharmacologic applications in the field of diuretics, antiglaucoma, anticonvulsant, and recently anticancer agents/diagnostic tools for imaging tumors.[1,4,5] Diverse isoforms of the 16 presently known in humans are involved in a variety of diseases.[1,2] The transmembrane isoforms CA IX and XII were

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shown to be involved in tumorigenesis,[2,4,5,16,17] being overexpressed in a broad range of tumor types.[16] Their expression negatively correlates with the prognosis of cancer patients and is regulated by hypoxia through the hypoxia-inducible factor (HIF) pathways.[2,4,5,16,17] Several groups reported recently that inhibition of CA IX/XII with sulfonamides or coumarins, in various types of tumors, has significant anticancer activity in several animal models of the disease (both alone, or in combination with radiation or cytotoxic drugs).[1,5,18]

Although several aliphatic/aromatic sulfamates have been investigated previously as CA IX/XII inhibitors,[19] no detailed such data are available for aromatic derivatives of this type. Considering the fact that sulfonamides and sulfamates have a similar mechanism of CA inhibition at the molecular level,[2,20] as both these classes of CAIs are zinc binders, but their pharmacologic properties are rather different, we report here a series of sulfamates which have been obtained by considering sulfonamides **A** as lead molecules.[1] Indeed, one of the most peculiar feature of the sulfonamides of type **A** was their selective inhibition of the tumor-associated isoforms CA IX and XII over the cytosolic offtarget isoforms CA I and II.[1] This has been subsequently explained by the report of the X-ray crystal structure of 5 of these compounds of type **A**,[21a] possessing various R moieties, which allowed the observation of a very variable orientation of the tail incorporating the RNHCONH moiety, in various sub-pockets of the CA active site.[21a] Such an orientation was considered possible due to the flexibility of the ureido linker, and has not been observed for example in structurally related compounds possessing the amide (CONH) linker instead of the ureido (NHCONH) one.[21]

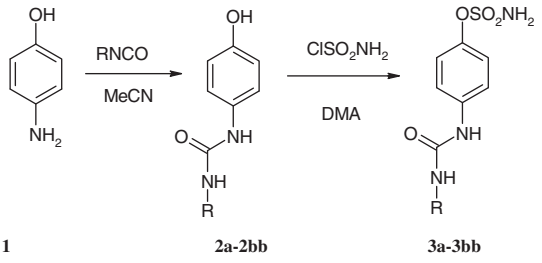
The lead compounds used in the present drug design study were the sulfonamides **A** reported earlier by our group.[1] The ureido part of those molecules incorporated a large number of moieties, of the aliphatic, aromatic and heterocyclic type, and we have chosen to maintain this chemical diversity also in the sulfamates reported here, as the nature of the R moiety was the main factor influencing in vitro and in vivo activity of sulfonamides A.[1] The sulfamates structurally related to sulfonamides **A** were prepared as depicted in Scheme 1. 4-Aminophenol **1** was reacted with a large number of commercially available isocyanates, leading to the ureido-phenols **2**. These intermediates were sulfamoylated with sulfamoyl chloride, leading to the series of 50 sulfamates **3** by the reported procedures[19] applied earlier to prepare aliphatic and heterocyclic sulfamates.[22]

Inhibition data against the offtarget[23] cytosolic CA isozymes h (h = human) hCA I and II, as well as the tumor-associated, target isoforms hCA IX and XII, with the new series of sulfamates **3a–3bb** are shown in Table 1. The following structure–activity relationship (SAR) can be demonstrated for this series of compounds:

(i) The cytosolic, widespread isoforms hCA I was poorly inhibited by sulfamates **3**, with inhibition constants in the range of 1.23–13.0 μM. The best inhibitor in the series was the 4-nitrophenyl-ureido compound **3m** (K_I of 1.23 μM), whereas most other substitution patterns led to compound with inhibition constants in the range of 2-6 μM (Table 1). The weak inhibition of this isoform may be considered a positive feature of this class of CAIs (and is shared also with the corresponding sulfonamides **A** reported earlier)[1] since hCA I, highly abundant in red blood cells, is undoubtedly an offtarget when considering antitumor CAIs.[23]

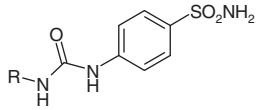
(ii) The physiologically dominant cytosolic isoform hCA II was moderately inhibited by sulfamates **3**, which showed K_Is in the range of 119–902 nM (Table 1). The simplest, unsubstituted member of this family of compounds, **3a**, was a medium potency hCA II inhibitor (K_I of 393 nM). Introducing various substituents, such as halogens, methoxy, cyano, phenyl, phenoxy, etc.) either lead to a slight increase in the inhibitory activity or maintain he inhibition at the same levels as for **3a** (e.g., compounds **3b–3l**). Interestingly, the 4-nitrosubstituted derivative **3m**, was a weaker hCA II inhibitor compared to the sulfamates discussed above. Other substitution patterns leading to a loss of activity compared to **3a** were those present in **3p** (3,5-dimethylphenyl), **3s** (2-bromo-4,6-difluorophenyl), **3u** (1-adamantyl), **3ab** (2-biphenyl), **3ac** (2-phenoxyphenyl), **3ad** (3-phenoxy-phenyl), **3ai** (2-ethoxyphenyl), **3am** (4-isopropylphenyl), **3ao** (9-fluorenyl), these compounds showing inhibition constants in he range of 430–900 nM (Table 1). The remaining derivatives, possessing a rather wide range of substitution patterns have an activity similar or slightly better than **3a**. Overall, these sulfamates show a moderate inhibitory action towards hCA II, which is the main offtarget isoform among the various mammalian CAs.[23]

(iii) The transmembrane isoforms hCA IX, and XII were on the other hand effectively inhibited by this new class of sulfamate CAIs, which showed K_Is in the range of 6–115 nM against hCA IX, and of 1–78 nM against hCA XII, respectively (Table 1). SAR is rather straightforward. For example, against hCA IX, the simplest member of the series, **3a**, was a potent inhibitor, with a K_I of 16 nM. Most substitution patterns to the second aromatic ring, such as halogens, cyano, methoxy, penta-/tetrafluoro, nitro, 1-naphthyl, etc., lead to compounds with smilar or slightly improved inhibitory activity (K_Is in the range of 6–17 nM). Few substitution patterns also led to a loss of the hCA IX inhibition, such as for example those present in sulfamates **3h** (4-biphenyl), **3i** (4-phenoxyphenyl); **3u** (1-adamantyl); **3ab** (2-biphenyl), **3ac** (2-phenoxyphenyl), **3ad** (3-phenoxy-phenyl); **3ag** (4-benzyloxyphenyl); **3ah** (4-methoxy-5-methylphenyl); **3ak** (diphenylmethyl); **3an** (2-isopropylphenyl); **3ao** (9-fluorenyl). These compounds incorporating rather bulky tails showed K_Is in the range of 24–115 nM, and with the exception of **3ad**, are in fact significantly strong hCA IX inhibitors. It should be noted that small structural changes in the nature of the moiety R present in these ureido sulfamates has drastic consequences for their inhibitory activity against most CA isoforms investigated so far, but these differences are particularly obvious for the inhibition of hCA IX (Table 1). The second tumor-associated isoform, hCA XII was even better inhibited by sulfamates **3** compared to hCA IX (Table 1). Thus starting form an excellent inhibitor which is **3a** (K_I of 10 nM), various substitution patterns led to an enhanced inhibitory power. Among such derivatives are compounds **3c** (4-chlorophenyl); **3g** (4-cyanophenyl); **3j** (pentafluorophenyl); **3k** (4-benzylphenyl); **3m** (4-nitrophenyl); **3n, 3o, 3p**, **3t** and **3y** (incorporating more halogens or methyl moieties, or the aminomethylphenyl one), **3ae** (4-acetylphenyl); **3ai** (2-ethoxyphenyl); 3ap



Scheme 1. Synthesis of sulfamates **3a–3bb** reported in the Letter.

Table 1
Inhibition of hCA isoforms I, II, IX and XII with sulfamates **3a–3bb**, by a stopped flow CO_2 hydrase assay, and selectivity ratio for the inhibition of the tumor-associated isoform CA IX over the cytosolic, offtarget isozyme hCA II[24]



A: lead compounds for the drug design
R = aromatic, aliphatic, heterocyclic moiety

No	R	K_I (nM)				Selectivity ratio
		hCA I	h CA II	hCAIX	hCA XII	hCA II/hCA IX
3a	Ph	3240	393	16	10	24.6
3b	4-F-C_6H_4	2800	287	13	9	22.1
3c	4-Cl-C_6H_4	2870	291	12	5	24.3
3d	4-Br-C_6H_4	3050	305	13	8	23.5
3e	4-I-C_6H_4	2100	186	10	10	18.6
3f	4-NC-C_6H_4	3280	279	9	6	31.0
3g	4-MeO-C_6H_4	2350	413	15	3	27.5
3h	4-Ph-C_6H_4	5400	284	24	12	11.8
3i	4-PhO-C_6H_4	4360	319	27	8	11.8
3j	C_6F_5	3180	145	6	1	24.2
3k	4-$PhCH_2$-C_6H_4	6500	286	16	5	17.9
3l	$PhCH_2CH_2$	5460	213	18	7	11.8
3m	4-O_2N-C_6H_4	1230	450	6	4	75.0
3n	4-Me_2N-C_6H_4	4370	348	9	2	42.7
3o	2,3,4-$F_3C_6H_2$	3500	286	5	3	57.2
3p	3,5-$Me_2C_6H_3$	5600	546	7	2	78.0
3q	4-EtO_2C-C_6H_4	2450	431	8	6	53.9
3r	1-naphthyl	8700	298	17	18	17.5
3s	2-Br-4,6-$F_2C_6H_2$	1390	641	9	6	71.2
3t	2,4,6-$Cl_3C_6H_2$	3240	338	11	5	30.7
3u	1-adamantyl	43000	467	21	46	22.2
3v	3,4-$Cl_2C_6H_3$	3300	285	10	4	28.5
3x	3-Cl-C_6H_4	4320	280	8	5	35.0
3y	2,4-$F_2C_6H_3$	2450	192	7	2	27.4
3z	2-Me-4-MeO-C_6H_3	2960	119	10	6	11.9
3ab	2-Ph-C_6H_4	8600	761	59	46	12.9
3ac	2-PhO-C_6H_4	9000	815	78	69	10.5
3ad	3-PhO-C_6H_4	10540	902	115	78	7.8
3ae	4-Ac-C_6H_4	4520	348	13	4	26.8
3af	3-Ac-C_6H_4	3480	413	18	9	22.9
3ag	4-$PhCH_2O$-C_6H_4	6640	285	28	13	10.2
3ah	2-MeO-5-Me-C_6H_3	3320	347	36	6	9.6
3ai	2-EtO-C_6H_4	4500	486	15	4	32.4
3aj	4-MeC_6H_4-CH_2	3600	310	10	10	31.0
3ak	Ph_2CH	8690	459	54	59	8.5
3am	4-iPr-C_6H_4	7510	613	30	18	20.4
3an	2-iPr-C_6H_4	6540	547	67	37	8.2
3ao	fluoren-9-yl	13000	750	75	26	10.0
3ap	3-MeS-C_6H_4	3480	344	9	4	38.2
3aq	2-naphthyl	12500	568	16	29	35.2
3ar	2-EtOOC-C_6H4	3100	435	8	8	54.4
3as	3-(2,3-Dihydrobenzofuran-5-yl)	2490	306	7	3	43.7
3at	3-EtOOC-C_6H_4	4530	448	12	5	37.3
3au	2-NC-C_6H_4	2470	541	18	11	30.1
3ax	1-naphthyl-CH_2CH_2	7650	317	15	25	21.1
3ay	thiophen-2-yl-CH_2CH_2	4300	236	9	3	26.2
3az	(2,3-dihydrobenzo[1,4]dioxin-6-yl)	3800	438	6	4	73
3aw	N-Boc-piperidin-4-yl	7640	257	7	2	36.7
3ba	pyridin-2-yl-CH_2	3010	421	12	5	35.0
3bb	pyridin-2-yl-CH_2 CH_2	3095	443	10	14	44.3

*Mean from 3 different assays. Errors were in the range of ± 10 of the reported values (data not shown).

(3-methylthiophenyl), and **3ay–3ba** (incorporating various heterocyclic moieties as substituents R). They all possess K_Is in the range of 1–5 nM. Thus, a quite large number of sulfamates **3** show excellent, low nanomolar (<5 nM) inhibitory activity against hCA XII.

(iv) The selectivity ratio for inhibiting the tumor-associated isoform hCA IX over the cytosolic offtarget one hCA II, was in the range of 7.8–78.0 for the new sulfamates **3** reported here. This is an excellent result, since most sulfonamide CAIs (acetazolamide, ethoxzolamide, dichlorophenamide, etc) show better hCA II than hCA IX inhibitory action.2 In the new series reported here, only a few compounds had a selectivity ratio in the range of 7.8–11.9 (compounds **3h**, **3i, 3l, 3z, 3ac, 3ad, 3ag, 3ah, 3ak, 3an, 3ao**), being thus relatively little CA IX-selective. The remaining ones had selectivity ratios in the range of 24–78.0, being thus highly selective CA IX/XII inhbitors (compared to the inhibition of CA II and I).

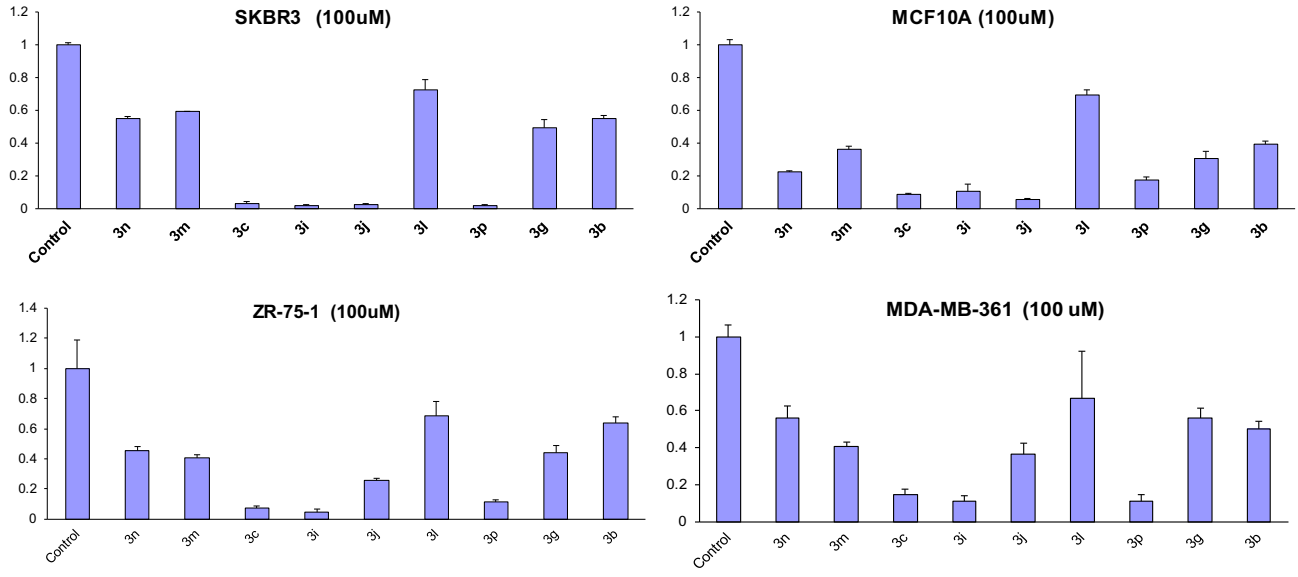


Figure 1. The effect of some of the novel compounds on the proliferation of a panel of breast cancer cell lines in normoxic conditions (21% O_2). SKBR3, MCF10A, ZR75/1 and MDA-MB-361 cells were plated out at a concentration of 1×10^3 cells per well of a 96-well plate and allowed to adhere for 48 h. Media was removed and the compounds were added at a concentration of 100 μM to assess efficacy in comparison with control cells (shown as 1.0). After 5 days of treatment, proliferation was assessed using SRB assays.[25]

In order to investigate whether this potent in vitro anti-CA IX effects are paralleled by effective antiproliferative action in vivo, we used 4 breast cancer cell lines (SKBR3, MCF10A, ZR75/1 and MDA-MB-361) which have been treated with 9 of the derivatives of type 3 described in this paper (see Fig. 1 and 2 for the derivatives used in the experiments) which possessed interesting in vitro potency as CA IX/XII inhibitors (Table 1).

We had tested initially these compounds at 100 μM under normoxic conditions and the obtained data is shown in Figure 1 for the four cell lines mentioned above. It was encouraging to observe that the potencies of the compounds **3** were of a similar order across these cell lines (Fig. 1). Indeed, data of Figure 1 show that compounds **3c, 3i, 3j** and **3p** were highly effective as antiproliferative agents (at the concentration of 100 μM used in this initial experiment) in all the breast cancer cell lines investigated, whereas derivatives **3n, 3m, 3l, 3g** and **3b** showed less effective such properties. It should be noted that although moist of these compounds are effective in vitro CA IX/XII inhibitors, there is not a straightforward correlation between the in vitro enzyme inhibitory activity and the in vivo antiproliferative results (but several of the highly effective inhibitors, for example **3p** and **3g** were also highly active in vivo).

Subsequently, under hypoxic conditions in the MCF7 cell line, when the expression of CA IX is highly induced by the shift from normoxia to hypoxia,[16] a dose-dependent and significant antiproliferative effect was observed with all the 9 compounds of type **3** (Fig. 2) which started at around 10 μM concentration of inhibitor and was increasing dramatically at 30 μM concentration of inhibitor.

In conclusion, we report here a series of 50 sulfamates, which were obtained by reacting 4-aminophenol with isocyanates followed by sulfamoylation. Most of the new derivatives were nanomolar inhibitors of the tumor-associated isoforms CA IX and XII, whereas they inhibited less the cytosolic, offtarget isoforms CA I and II. Some of these sulfamates showed significant antiproliferative activity in several breast cancer cell lines, such as SKBR3, MCF10A, ZR75/1, MDA-MB-361 and MCF7, constituting interesting anticancer leads.

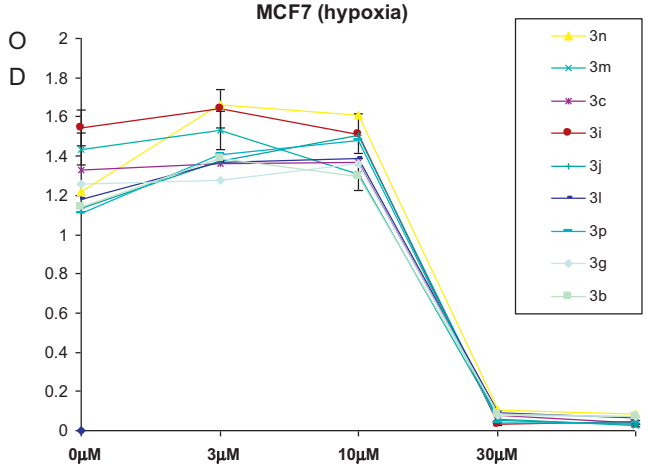


Figure 2. MCF7 breast cancer cells were plated on 96-well plates at a concentration of 3×10^3 per well, to allow for the inhibitory effect of hypoxia on cell proliferation. After 48 h adherence, plates were placed in a Don Whitley H35 hypoxystation set to 0.5% O_2 and left to equilibrate for 6 h, before the addition of compounds of type **3** made up in hypoxic media. Compounds were tested at a range of concentrations from 0.1–100 μM and dose responses assessed using SRB assays after 5 days of treatment.[25]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05. 083.

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22. 4-Aminophenol **1** (1 equiv.) was added to a solution of isocyanate (1 equiv.) in 15–20 ml of acetonitrile. The mixture was stirred at room temperature until the complete formation of the product (TLC monitoring). The resulting precipitate was then filtered and washed with ethyl acetate several times. Sulfamates were then prepared by reacting the requisite phenol (1 equiv.) with sulfamoyl chloride (3 equiv.) in *N*,*N*-dimethylacetamide (Okada, M.; Iwashita, S.; Koizumi, N. *Tetrahedron Lett.* **2000**, *41*, 7057). Sulfamoyl chloride was prepared from chlorosulfonyl isocyanate and formic acid as described previously: Appel, R.; Berger, G. *Chem. Ber.* **1958**, *91*, 1339). After completion of the reaction (TLC monitoring), the mixture was diluted with ethyl acetate and washed several times with water. The organic extract was dried ($MgSO_4$) and concentrated under vacuum. The residue was purified either by crystallization from ether/pentane or by chromatography on silica gel.
4-[(4-Fluorophenyl)ureido]phenyl sulfamate (3b): mp 180–182 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.8 (s, 1H), 8.75 (s, 1H), 7.9 (s, 2H), 7.5 (m, 4H), 7.2 (m, 4H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 159.4, 157, 153.4, 145.3, 138.9, 136.8, 123.5, 120.9, 120.2, 116.2, 116.1, 116, 115.9; MS ESI$^+$ m/z 348 (M+Na)$^+$. ESI$^-$ m/z 324 (M−H)$^-$.
*4-[(4-Chlorophenyl)ureido]phenyl sulfamate (**3c**)*: mp 200–201 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.8 (s, 2H), 7.9 (s, 2H), 7.5 (2d, 4H, J = 8.7 Hz), 7.3 (d, 2H, J = 8.7 Hz), 7.2 (d, 2H, J = 8.7 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 153.2, 145.4, 139.4, 138.8, 129.5, 129.4, 126.3, 123.5, 121.4, 120.6, 120.3, 120.1, 116; MS ESI$^+$ m/z 364 (M+Na)$^+$. ESI$^-$ m/z 340 (M−H)$^-$.
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24. Khalifah, R.J. *J. Biol. Chem.* **1971,** *246*, 2561. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed 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 20 mM Hepes (pH 7.5 for α-CAs) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. 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 (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[1] and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in house as reported earlier.[19–21]
25. Wells were fixed with 25% cold TCA for 1 h at 4 °C and the plates washed 10 times with distilled H_2O and allowed to dry. A 50 μl volume of SRB stain (Sulphorhodamine B (Sigma–Aldrich, UK) in 1% v/v acetic acid) was added to each well for 30 min at room temperature, and each plate washed 4 × with 1% v/v acetic acid to remove unbound dye. After drying the plates, bound SRB was solubilised by adding 150 μl of SRB assay buffer (10 mM Tris Base, pH 10.5) to each well for 1 h at room temperature before reading on a BioHit BP800 plate reader at 540 nM.