Carbonic Anhydrase Inhibitors: Inhibition of Transmembrane, Tumor-Associated Isozyme IX, and Cytosolic Isozymes I and II with Aliphatic Sulfamates

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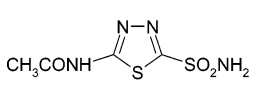
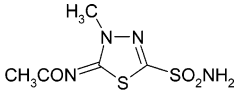
A series of aliphatic sulfamates and related derivatives incorporating cyclic/polycyclic (steroidal) moieties in their molecules has been synthesized and assayed as inhibitors of the zinc enzyme carbonic anhydrase (CA) and, more precisely, of the cytosolic isozymes CA I and II and the transmembrane, tumor-associated isozyme CA IX. The most potent CA I inhibitor was *n*-tetradecyl sulfamate and some (substituted)benzyl/phenethyl sulfamates (inhibition constants in the low micromolar range). Against CA II, low nanomolar inhibitors (0.7−3.4 nM) were *n*-decyl sulfamate and the (substituted)benzyl/phenethyl derivatives mentioned above. Effective CA II inhibition was also observed for the hydroxy/keto derivatives of dehydroepiandrosterone sulfamate. Efficient CA IX inhibitory properties, with inhibition constants in the range of 9−23 nM, were observed for the aliphatic sulfamates C_{10}−C_{16} (with the most potent inhibitor being the *n*-dodecyl derivative) and the (substituted)benzyl/phenethyl sulfamates. The inhibition profile of the three investigated isozymes with this type of compound was rather different, allowing us to hope that the preparation of CA IX-selective inhibitors is possible (selectivity ratios toward hCA IX versus hCA II in the range of 5−63 has been observed for some of these compounds, whereas for the clinically used sulfonamides this parameter is in the range of 0.23−0.51). These data are critical for the design of novel antitumor therapies, mainly for hypoxic tumors that overexpress CA IX, which are nonresponsive to radiation or chemotherapy.

Introduction

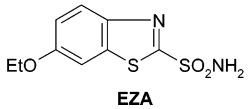
In a previous paper[1] we showed that sulfamates or bis-sulfamates incorporating aromatic, aliphatic, polycyclic (steroidal), and sugar moieties previously reported to act as inhibitors of steroid sulfatases, enzymes considered to be the key therapeutic targets for estrogen dependent tumors,[2] also act as very potent inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) and, more precisely, of the cytosolic isozymes CA I and II and the transmembrane, tumor-associated isozyme CA IX.[3−5] Indeed, CAs are critical in a multitude of physiological and physiopathological processes, including tumorigenesis.[3−8] Recently, at least two tumor-associated CA isozymes have been identified (CA IX and CA XII)[8] among the 14 isoforms isolated in humans.[3−5] Among them, CA IX shows restricted expression in normal tissues but is tightly associated with different types of tumors, mostly due to its strong induction by tumor hypoxia that involves hypoxia inducible factor type 1 (HIF-1) binding to a hypoxia response element in the *CA9* gene promoter.[7,8] CA XII is present in many normal tissues and overexpressed in some tumors, also being induced by hypoxia, but the underlying molecular mechanism remains undetermined. Both CA IX and CA XII are negatively regulated by von Hippel Lindau tumor suppressor protein, and their expression in renal cell carcinomas is related to inactivating mutation of the *VHL* gene.[7,8] The high catalytic activity of these two isozymes supports their role in the acidification of tumor microenvironment that facilitates acquisition of the metastatic phenotype.[7,8] Therefore, modulation of the extracellular tumor pH via inhibition of CA activity represents a promising approach to novel anticancer therapies.[6−8] Indeed, sulfonamide/sulfamate CA inhibitors (CAIs) with nanomolar activity against the tumor-associated isozyme IX were recently reported by this group, both among the classical sulfonamide drugs such as acetazolamide (AZA), methazolamide (MZA), and



AZA



MZA



EZA

ethoxzolamide (EZA), as well as for derivatives designed de novo for this target.[1,9] Among the sulfamates recently investigated as CAIs,[1] most were aromatic derivatives. The unique exception was constituted by *n*-octyl sulfamate (*n*-C_8H_{17}−OSO_2NH_2), which was one of the most

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Table 1. Inhibition Data for Derivatives **1**−**29** Investigated in the Present Paper and Standard Sulfonamide CA Inhibitors, against Isozymes I, II, and IX

$RO\text{-}SO_2NH_2$

1-29

compd	inhibitor R group	K_I [d] hCA I[a] (μM)	hCA II[a] (nM)	hCA IX[b] (nM)	selectivity ratio[c] K_I(hCA II)/ K_I(hCA IX)
AZA		0.90	12	25	0.48
MZA		0.78	14	27	0.51
EZA		0.025	8	34	0.23
1	Me	40	6000	>1000	
2	Et	38	5500	>1000	
3	*n*-Pr	3.70	750	>1000	
4	*i*-Pr	690	>10000	>1000	
5	*n*-Bu	3.10	70	>1000	
6	*n*-C_5H_{11}	0.71	58	126	0.46
7	*n*-$C_{10}H_{21}$	0.53	0.7	23	0.03
8	*n*-$C_{11}H_{23}$	0.43	4.7	17	0.27
9	*n*-$C_{12}H_{25}$	0.27	10	9	1.11
10	*n*-$C_{14}H_{29}$	0.15	87	15	5.80
11	*n*-$C_{16}H_{33}$	58	97	22	4.41
12	*n*-$C_{18}H_{37}$	65	129	120	1.07
13	CF_3CH_2	7.8	845	458	1.84
14	*n*-$C_6F_{13}CH_2CH_2$	400	8000	335	23.88
15	*n*-$C_8F_{17}CH_2CH_2$	>1000	9000	142	63.38
16	$(CF_3)_2CH$	3.54	1580	279	5.66
17	$CH_2{=}CHCH_2CH_2$	800	883	386	2.28
18	$CH{\equiv}CCH_2CH_2$	990	5900	633	9.32
19	$ClCH_2CH_2CH_2$	4.62	570	>1000	
20	c-C_5H_9	>1000	>10000	>1000	
21	c-C_6H_{11}	59	60	>1000	
22	$PhCH_2$	0.76	3.4	14	0.24
23	$PhCH_2CH_2$	0.41	1.1	12	0.09
24	*p*-Me-$C_6H_4CH_2$	0.10	2.7	13	0.20
25	*p*-Ph-$C_6H_4CH_2$	>1000	>10000	>1000	
26	fluorenylmethyl[e]	38	4500	>1000	
27	cholesteryl[e]	>1000	>10000	>1000	
28	*e*	0.40	13	65	0.20
29	*e*	0.41	23	76	0.30

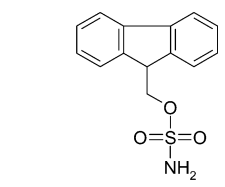
[a] Human (cloned) isozymes, by the CO_2 hydration method. [b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method. [c] Selectivity ratio toward hCA IX as compared to hCA II. [d] Errors in the range of 5−10% of the reported value (from three different assays). [e] See structure in the text.

potent inhibitors in the entire series of investigated compounds, with inhibition constants of 3.5 nM against isozyme hCA I, 2.7 nM against isozyme hCA II, and 25 nM against the tumor-associated isozyme hCA IX, respectively.[1] Considering this simple compound as lead molecule, we decided to investigate a larger series of aliphatic sulfamates and related derivatives, to detect potent CA I, II, and IX inhibitors belonging to this class. Such compounds may exert a dual antitumor mechanism of action, by inhibiting at least two enzymes involved in tumorigenesis, CAs and steroid sulfatases.[1,10]

Results

Chemistry. The sulfamates **1**−**29** investigated here as CAIs are shown in Table 1. They were prepared as described previously,[1] by reaction of the corresponding alcohols with sulfamoyl chloride. Some of these derivatives were previously reported in the literature,[11−16] but they were not investigated as CAIs until the present study.

Carbonic Anhydrase Inhibitory Activity. Inhibition data against three CA isozymes, the cytosolic hCA I and hCA II[17−22] as well as the transmembrane, tumor-associated isozyme hCA IX, are shown in Table 1.[23,24] Standard, clinically used sulfonamide CAIs, such as acetazolamide, methazolamide, and ethoxzolamide were also included in these assays, for comparison.



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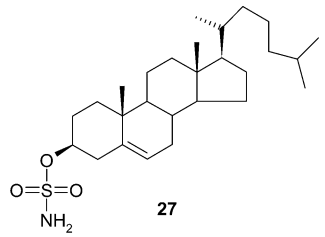

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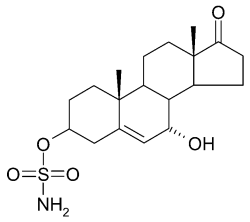

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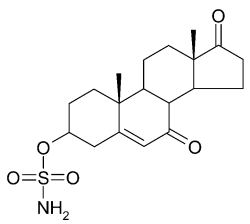

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Discussion

Chemistry. The sulfamates **1**−**29** investigated here were prepared by the classical procedure[1] involving the reaction of the appropriate alcohol with sulfamoyl chloride.[11−16] Some of these derivatives have been previously reported in the literature,[11−16] but no data regarding their interaction with CA isozymes are available. Compounds **1**−**29** have been thoroughly characterized by physicochemical methods (see Experimental Section for details) that confirmed the proposed structures.

Carbonic Anhydrase Inhibitory Activity. Isozyme I. As seen from the data of Table 1, sulfamates **1**−**29** investigated here show a varied degree of CA I inhibitory activity, as follows: (i) Derivatives **15**, **20**, **25**, and **27** showed almost no inhibitory activity against this isozyme, possessing K_I values in the millimolar range, whereas compounds **4**, **14**, **17**, and **18** were also very weak inhibitors, with K_I values in the range of 400−990 μM. It may be seen that all these compounds share some structural features: they either contain bulky aliphatic/steroidal moieties (such as isopropyl, cyclopentyl, and cholestryl), polyfluorinated long aliphatic chains (such as in compounds **14** and **15**), or unsaturated aliphatic moieties (such as in **17** and **18**). These data are rather surprising, especially when comparing the K_I values of the isopropyl and *n*-propyl derivatives (**4** and **3**, respectively) or of the cyclopentyl and cyclohexyl derivatives (**20** and **21**, respectively). In both cases, the first compound of the pair is much less active than the second one, although they are isomers (the case of **4** and **3**) or close analogues (the case of **20** and **21**). Such huge differences of activity between structurally very similar compounds have never been detected up to now either for sulfonamide or sulfamate CAIs,[1,3−5] and they are difficult to explain at this moment, with no X-ray structures available for adducts of such inhibitors with CA isozymes. The data of the polyfluorinated derivatives **14** and **15** are also surprising for at least two reasons: on one hand, the corresponding perhydro derivatives *n*-octyl sulfamate[1] and compound **7** are very potent CA I inhibitors, and on the other one, the presence of polyfluorinated tails in aliphatic,[25] aromatic,[26] and heterocyclic[26] sulfonamides was shown to

lead to very potent inhibitors, typically in the low nanomolar range. Thus, definitively, the presence of long aliphatic polyfluorinated groups is detrimental for the CA I inhibitory properties of sulfamate CA inhibitors. (ii) Several derivatives, such as **1**, **2**, **11**−**13**, **21**, and **26**, acted as moderate CA I inhibitors, with inhibition constants in the range of 7.8−65 μM. These derivatives incorporate either small aliphatic moieties (methyl, ethyl, or trifluoroethyl), very long aliphatic chains (C_{16} and C_{18}), or cyclic moieties (cyclohexyl and fluorenemethyl). (iii) Derivatives **3**, **5**, **16**, and **19** also showed good CA I inhibitory activity, with inhibition constants in the range of 3.10−4.62 μM, being more effective than the derivatives discussed above. These compounds incorporate medium aliphatic chains (*n*-propyl and *n*-butyl moieties) or halogenated groups such as 3-chloropropyl or hexafluoro-isopropyl. (iv) The most potent CA I inhibitors were compounds **6**−**10**, **22**−**24**, and **28**, **29**, with inhibition constants in the range of 0.10−0.76 μM, on the same order of magnitude as the clinically used compounds acetazolamide and methazolamide. These inhibitors incorporate long aliphatic chains (from C_5 to C_{14}), benzyl, or (substituted)phenethyl moieties. It is interesting to note how the CA I inhibitory properties augment with the elongation of the aliphatic chain from C_1 to C_{14} (*n*-alkyl chains), this last sulfamate being one of the best CA I inhibitors in the entire series. A further elongation to the C_{16} and C_{18} sulfamates leads to a drastic diminution of the CA I inhibitory properties, the same situation being also true for the polyfluorination of the alkyl chains, as mentioned above. Another optimal substitution pattern for obtaining potent CA I inhibitors of this type consists of the benzyl/phenethyl type of sulfamates (compounds **22**−**24**) or the dehydroepiandrosterone derivatives **28** and **29** (Table 1).

Isozyme II. This is considered the most important isozyme as target for obtaining inhibitors with pharmacological applications. The sulfamates **1**−**29** investigated here showed a very large variation of the CA II inhibitory properties, a feature rarely seen for a homologous series of CA inhibitors. As for isozyme I, again a rather wide range of activities were evidenced, as follows: (i) Weak inhibitors, with inhibition constants in the range from 1.5 to >10 μM, were compounds **1**, **2**, **4**, **14**−**16**, **18**, **20**, and **25**−**27**. As mentioned for isozyme I, these weak inhibitors belong to several subcategories: small aliphatic derivatives (methyl, ethyl, and isopropyl sulfamates); polyfluorinated aliphatic sulfamates (**14**−**16**); unsaturated derivatives; and the bulky compounds incorporating cyclopentyl, biphenylylmethyl, fluorenylmethyl, and cholesteryl moieties, respectively. The huge difference in activity between the cyclopentyl and cyclohexyl sulfamates **20** and **21** are again dramatic and difficult to explain. It may also be seen that the elongation of the aliphatic chain already for the first members of the series (methyl and ethyl) is beneficial for the CA II inhibitory effects. (ii) A group of four compounds, i.e., **3**, **13**, **17**, and **19**, showed moderate CA II inhibitory properties, with inhibition constants in the range of 570−883 nM. They incorporate C_2–C_4 aliphatic chains, possibly substituted with halogens or unsaturated. (iii) Potent inhibitors, with inhibition constants in the range of 13−129 nM, were derivatives **5**, **6**, **9**−**12**, **21**, **28**, and **29**. It may be observed that an elongation of alkyl chain in the aliphatic sulfamates C_4 and C_5 is further beneficial for the CA II inhibitory effects, these properties varying quite linearly from C_1 to C_{10}, which is the best (subnanomolar) inhibitor in the series (see later in the text). The further elongation starts then to be detrimental, with the C_{11}−C_{18} derivatives gradually acting as weaker inhibitors, but some of them (for example **9**) are of the same potency as the clinically used sulfonamides acetazolamide, methazolamide, or ethoxzolamide (see Table 1). The cyclohexyl sulfamate **21** and the dehydroepiandrosterone derivatives **28** and **29** also show good CA II inhibitory properties, especially the alcohol **28**, which is as good as acetazolamide as a hCA II inhibitor. (iv) Very potent CA II inhibitors were sulfamates **7**, **8**, and **22**−**24**, with inhibition constants in the range of 0.7−4.7 nM. These compounds incorporate the medium to long aliphatic chains C_{10} and C_{11} as well as the benzyl/phenethyl moieties which were also optimal for obtaining CA I inhibitors. It is important to note that for the aliphatic derivatives, the best CA I inhibitor was the sulfamate C_{14} (**10**), whereas the best CA II inhibitor was the sulfamate C_{10} (**7**). Finally, as it will be discussed shortly, the best CA IX inhibitor was the sulfamate C_{12} (**9**).

Isozyme IX. The inhibition profile of this isozyme with the sulfamates **1**−**29** is very interesting, differing substantially from the inhibition profiles of the cytosolic isozymes I and II discussed above (Table 1). Thus, (i) derivatives **1**−**5**, **19**−**21**, and **25**−**27** were ineffective CA IX inhibitors, with K_I values >1 μM. It may be observed that these compounds incorporate either the small aliphatic chains C_1−C_4, the cyclic moieties (cyclopentyl and cyclohexyl), or the bulky biphenylbenzyl, fluorenemethyl, or cholesteryl moieties, which also led to weak CA I and CA II inhibitors. (ii) Medium potency inhibitors were derivatives **6** and **12**−**18**, with inhibition constants in the range of 120−633 nM. These inhibitors incorporate medium aliphatic chains (C_5), long aliphatic chains (C_{18}), polyfluorinated alkyl, and unsaturated alkyl moieties. It is very interesting to note that although the polyfluorinated sulfamates **14** and **15** are quite inefficient CA I and CA II inhibitors, they show much higher affinity for CA IX, a finding impossible to explain at this point. Also the alkenyl/alkynyl derivatives **17** and **18** showed higher affinity for CA IX than for the cytosolic isozymes I and II, which may be considered an encouraging result for the design of CA IX-specific inhibitors, a goal of critical importance for the potential use of CAIs as antitumor agents.[6] (iii) Strong CA IX inhibitors were compounds **7**−**11**, **22**−**24**, **28**, and **29**, with inhibition constants in the range of 9−76 nM. The SAR is rather clear in this subseries, as best activity is observed again, as for CA II, for sulfamates with an elongated aliphatic chain (from C_{10} to C_{12}), whereas a further elongation of the molecule (C_{14}−C_{18}) leads to decreased activity. The best CA IX inhibitor is the C_{12} sulfamate (**9**, K_I of 9 nM) followed by the benzyl/phenethyl type of derivatives (**22**−**24**), which showed K_I values in the range of 12−14 nM. All these sulfamates are at least 2 times more effective CA IX inhibitors as compared to the clinically used sulfonamides acetazolamide, methazolamide, and ethoxzolamide, some of which were shown to inhibit tumor cell

growth in vitro and in vivo.[6−8] It should also be stressed that isozymes CA II and CA IX generally show quite different inhibition profiles with this type of inhibitors. For example, in addition to the compounds mentioned above, showing higher avidity for CA IX, other inhibitors (such as **22**−**24** and **28**, **29**) possess higher affinity for CA II than for CA IX. Thus, in a rather simple series of sulfamate derivatives, most of which are aliphatic, cyclic, or hydroxy/keto derivatives of dehydroepiandrosterone sulfamate, quite diverse and unexpected inhibition profiles were observed for the two major cytosolic isozymes CA I and CA II and the transmembrane, tumor-associated isozyme CA IX.

Selectivity toward hCA IX over hCA II. As seen from data reported here and also previous studies of our group,[1,9] both isozymes hCA II and hCA IX show high affinity for sulfonamide inhibitors, and hence, it may be rather difficult to detect compounds with specificity toward one of this isozyme. In this specific case, the target isozyme for designing novel antitumor therapies is obviously hCA IX, and the problem is the affinity of the potent hCA IX inhibitors also for hCA II. As seen from data of Table 1, most inhibitors reported here (compounds **1**−**7** and **19**−**29**) showed a selectivity ratio hCA II over hCA IX of less than 1, which means that they act as more potent inhibitors toward isozyme II than toward isozyme IX. This feature is also shared by the clinically used sulfonamides acetazolamide, methazolamide, and ethoxzolamide, which showed selectivity ratios in the range of 0.48−0.23. Four of the new inhibitors reported here, i.e., **9**, **12**, **13** and **17**, showed selectivity ratios in the range of 1.07−2.28, being thus slightly better hCA IX inhibitors than hCA II inhibitors. Finally, six other compounds, **10**, **11**, **14**−**16**, and **18**, showed selectivity ratios in the range of 5.66−63.38, and they may be considered as the most "CA IX-selective" inhibitors reported up to now. Among them, the last four compounds were medium-potency CA IX inhibitors, and thus they may be considered as leads for developing new low nanomolar CA IX-selective inhibitors, but compounds **10** and **11** are low nanomolar inhibitors per se, showing at least a 4−5 times higher affinity for CA IX over CA II.

Conclusions. A series of sulfamates incorporating aliphatic, cyclic, and steroidal moieties in their molecules has been synthesized and assayed as inhibitors of the cytosolic isozymes CA I and II and the transmembrane, tumor-associated isozymes CA IX. The most potent CA I inhibitor was *n*-tetradecyl sulfamate and some (substituted)benzyl/phenethyl sulfamates. Against CA II, low-nanomolar inhibitors (0.7−3.4 nM) were *n*-decyl sulfamate and the (substituted)benzyl/phenethyl derivatives mentioned above. Good CA II inhibition was also observed for the hydroxy/keto derivatives of dehydroepiandrosterone sulfamate. Efficient CA IX inhibitory properties, with inhibition constants in the range of 9−23 nM, were observed for the aliphatic sulfamates C_{10}−C_{16} (with the best inhibitor being the *n*-dodecyl derivative), and the (substituted)benzyl/phenethyl sulfamates mentioned above. The inhibition profile of the three investigated isozymes with this type of compound was rather different, allowing us to hope that the preparation of CA IX-selective inhibitors is possible. These data are critical for the design of novel antitumor therapies, mainly for hypoxic tumors that overexpress CA IX, which are nonresponsive to radiation or chemotherapy.

Experimental Section

General. All reagents and solvents were of commercial quality and used without further purification. All reactions were carried out under an inert atmosphere of nitrogen. TLC analyses were performed on silica gel 60 F_{254} plates (Merck Art.1.05554). Spots were visualized under 254 nm UV illumination or by ninhydrin solution spraying. Melting points were determined on a Büchi Melting Point 510 and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer FT-IR spectrometer S1000. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-250 and/or Bruker DRX-400 spectrometer using DMSO-d_6 as solvent and tetramethylsilane as internal standard. For ^{1}H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (J) are expressed in hertz. Electron ionization mass spectra (30 eV) were recorded in positive or negative mode on a Water MicroMass ZQ.

Preparation of Sulfamates. General Procedure. Sulfamates **1**−**29** were prepared by reacting the requisite alcohol (1 equiv) with sulfamoyl chloride (3 equiv) in *N*,*N*-dimethylacetamide[11] (sulfamoyl chloride was prepared from chlorosulfonyl isocyanate and formic acid as described in ref 12) 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.

Methyl sulfamate (1): mp 23−25 °C (lit.[13,14] mp 22−24 °C, 25−27 °C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.45 (s, 2H), 3.7 (s, 3H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 56.3; IR (CH_2Cl_2) 3448, 3338, 2970, 1542, 1380, 1183 cm^{-1}; MS ESI$^-$ m/z 110 (M − H)$^-$.

Ethyl sulfamate (2): mp 37−39 °C (lit.[14] mp 40−41°C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.40 (s, 2H), 4.07 (q, 2H, J = 7.4 Hz, J = 1.4 Hz), 1.26 (t, 3H, J = 7.4 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 66.0, 15.3; IR (KBr) 3375, 3286, 1556, 1355, 1172 cm^{-1}; MS ESI$^+$ m/z 148 (M + Na)$^+$, ESI$^-$ m/z 124 (M − H)$^-$.

***n*-Propyl sulfamate (3):** mp 21−22 °C (lit.[13] mp 22−23 °C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.38 (s, 2H), 3.97 (t, 2H, J = 6.4 Hz), 1.64 (m, 2H), 1.25 (t, 3H, J = 7.3 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 71.3, 22.6, 10.8; IR (CH_2Cl_2) 3441, 3338, 2963, 1545, 1373, 1179 cm^{-1}; MS ESI$^+$ m/z 162 (M + Na)$^+$, ESI$^-$ m/z 138 (M − H)$^-$.

Isopropyl sulfamate (4): mp <18 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.34 (s, 2H), 4.62 (m, 1H), 1.28 (d, 6H, J = 6.2 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 75.3, 23.2; IR (CH_2Cl_2) 3433, 3338, 2963, 1542, 1373, 1241, 1179 cm^{-1}; MS ESI$^+$ m/z 162 (M + Na)$^+$, ESI$^-$ m/z 138 (M − H)$^-$.

***n*-Butyl sulfamate (5):** mp 26−27 °C (lit.[14] mp 27−28 °C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.38 (s, 2H), 4 (t, 2H, J = 6.4 Hz), 1.6 (m, 2H), 1.36 (m, 2H), 0.9 (t, 3H, J = 7.2 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 69.5, 31.1, 19.1, 14.2; IR (CH_2Cl_2) 3441, 3330, 2977, 1545, 1377, 1179 cm^{-1}; MS ESI$^+$ m/z 176 (M + Na)$^+$, ESI$^-$ m/z 152 (M − H)$^-$.

***n*-Pentyl sulfamate (6):** mp 37−39 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.4 (s, 2H), 4 (t, 2H, J = 6.4 Hz), 1.64 (m, 2H), 1.3(m, 4H), 0.88 (t, 3H, J = 6.6 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 69.8, 28.8, 28.1, 22.4, 14.6; IR (KBr) 3375, 3279, 2933, 2860, 1545, 1465, 1347, 1175, 1040 cm^{-1}; MS ESI$^+$ m/z 190 (M + Na)$^+$, ESI$^-$ m/z 166 (M − H)$^-$.

***n*-Decyl sulfamate (7):** mp 69−71 °C (lit.[16] mp 81.2−83.6 °C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.4 (s, 2H), 4 (t, 2H, J = 6.4 Hz), 1.6 (m, 2H), 1.28 (s, 14H), 0.86 (t, 3H, J = 6 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 69.8, 32.1, 29.8, 29.6, 29.4, 29.2, 25.9, 22.9, 14.7; IR (KBr) 3367, 3279, 2919, 2845, 1542, 1468, 1344, 1175 cm^{-1}; MS ESI$^+$ m/z 260 (M + Na)$^+$, ESI$^-$ m/z 236 (M − H)$^-$.

***n*-Undecyl sulfamate (8):** mp 76−77 °C (lit.[16] mp 77.8−79.6 °C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.4 (s, 2H), 4.0 (t,

2H, J = 6.4 Hz), 1.65 (m, 2H), 1.25 (m, 14H), 0.85 (t, 3H, J = 5.7 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 69.7, 32.2, 29.9, 29.8, 29.6, 29.4, 29.2, 25.9, 22.9, 14.7; IR (KBr) 3375, 3286, 2926, 2845, 1545, 1468, 1351, 1179 cm^{-1}; MS ESI$^-$ m/z 250 (M − H)$^-$.

***n*-Dodecyl sulfamate (9):** mp 79−80 °C (lit.[16] mp 82−84 °C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.4 (s, 2H), 3.95 (t, 2H, J = 6.4 Hz), 1.6 (m, 2H), 1.3 (m, 16H), 0.85 (t, 3H, J = 6 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 69.7, 32.2, 29.95, 29.9, 29.85, 29.8, 29.6, 29.4, 29.1, 25.9, 22.9, 14.7; IR (KBr) 3375, 3286, 2919, 2845, 1542, 1468, 1351, 1179 cm^{-1}; MS ESI$^+$ m/z (M + Na)$^+$, ESI$^-$ m/z 264 (M − H)$^-$.

***n*-Tetradecyl sulfamate (10):** mp 84−85 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.4 (s, 2H), 3.9 (t, 2H, J = 6.4 Hz), 1.6 (m, 2H), 1.35 (m, 20H), 0.85 (t, 3H, J = 5.9 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 69.7, 32.2, 30, 29.95, 29.9, 29.8, 29.6, 29.4, 29.2, 25.9, 23, 14.7; IR (KBr) 3375, 3286, 2911, 2852, 1542, 1468, 1351, 1183 cm^{-1}; MS ESI$^-$ m/z 292 (M − H)$^-$.

***n*-Hexadecyl sulfamate (11):** mp 88−89 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.4 (s, 2H), 4 (t, 2H, J = 6.4 Hz), 1.6 (m, 2H), 1.3 (m, 26H), 0.85 (t, 3H, J = 6 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 69.7, 32.2, 30, 29.95, 29.9, 29.8, 29.6, 29.4, 29.2, 25.9, 22.9, 14.7; IR (KBr) 3367, 3286, 2919, 2845, 1538, 1468, 1347, 1183 cm^{-1}; MS ESI$^-$ m/z 320 (M − H)$^-$.

***n*-Octadecyl sulfamate (12):** mp 91−92 °C; ^{1}H NMR ($CDCl_3$-DMSO-d_6, 250 MHz) δ 7.15 (s, 2H), 3.95 (t, 2H, J = 6.5 Hz), 1.6 (m, 2H), 1.2 (m, 30H), 0.8 (t, 3H, J = 6.3 Hz); ^{13}C NMR ($CDCl_3$-DMSO-d_6, 400 MHz) δ 69.8, 32.2, 30, 29.95, 29.9, 29.8, 29.6, 29.4, 29.2, 25.9, 23, 14.6; IR (KBr) 3375, 3286, 2911, 2852, 1538, 1472, 1351, 1183 cm^{-1}; ESI$^-$ m/z 348 (M − H)$^-$.

1*H*,1*H*-Perfluoroethyl sulfamate (13): mp 51−53 °C (lit.[15] mp 50−51 °C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8 (s, 2H), 4.64 (q, 2H, J = 8.2 Hz, 17 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 119.6−127.8, 65.1−64; IR (KBr) 3397, 3294, 1531, 1366, 1311, 1271, 1179, 1047 cm^{-1}; MS ESI$^+$ m/z 202 (M + Na)$^+$, ESI$^-$ m/z 178 (M − H)$^-$.

1*H*,1*H*,2*H*,2*H*-Perfluorooctyl sulfamate (14): mp 59−61 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.66 (s, 2H), 4.3 (t, 2H, J = 5.6 Hz), 2.75 (m, 2H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 106−121, 61.6, 30.6; IR (KBr) 3397, 3286, 1560, 1369, 1238, 1186, 1139, 1076 cm^{-1}; MS ESI$^+$ m/z 465 (M + Na)$^+$, ESI$^-$ m/z 442 (M − H)$^-$.

1*H*,1*H*,2*H*,2*H*-Perfluorodecyl sulfamate (15): mp 119−121 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.6 (s, 2H), 4.28 (t, 2H, J = 5.8 Hz), 2.65 (m, 2H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 122−107, 61.5, 30.5; IR (KBr) 3404, 3301, 1556, 1369, 1208, 1146 cm^{-1}; MS ESI$^+$ m/z 565 (M + Na)$^+$, ESI$^-$ m/z 542 (M − H)$^-$.

1*H*-Perfluoroisopropyl sulfamate (16): mp 50−52 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.54 (s, 2H), 6.20 (m, 2H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 117.1−125.6, 70.8−72.1; IR (KBr) 3397, 3294, 2985, 1538, 1377, 1355, 1307, 1245, 1194, 1106, 1069 cm^{-1}; MS ESI$^-$ m/z 446 (M − H)$^-$.

But-3-enyl sulfamate (17): yellow oil; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.45 (s, 2H), 5.80 (m, 1H), 5.1 (m, 2H), 4.05 (m, 2H), 2.4 (m, 2H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 134.7, 118.3, 68.7, 33.5; IR (CH_2Cl_2) 3433, 3338, 3073, 2977, 1549, 1373, 1241, 1186 cm^{-1}; MS ESI$^+$ m/z 174 (M + Na)$^+$, ESI$^-$ m/z 150 (M − H)$^-$.

But-3-ynyl sulfamate (18): mp 51−53 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.55 (s, 2H), 4.05 (t, 2H, J = 6.2 Hz), 2.85 (s, 1H), 2.58 (t, 2H, J = 6 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 81.2, 73.5, 67.5, 19.5; IR (KBr) 3375, 3272, 2970, 1545, 1362, 1175 cm^{-1}; MS ESI$^+$ m/z 172 (M + Na)$^+$, ESI$^-$ m/z 148 (M − H)$^-$.

3-Chloropropyl sulfamate (19): yellow oil; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.45 (s, 2H), 4.15 (t, 2H, J = 6 Hz), 3.70 (t, 2H, J = 6.4 Hz), 2.10 (m, 2H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 66.6, 42.1, 32.2; IR (CH_2Cl_2) 3433, 3338, 2963, 1542, 1377, 1179 cm^{-1}; MS ESI$^+$ m/z 196 (M + Na)$^+$, ESI$^-$ m/z 172 (M − H)$^-$.

Cyclopentyl sulfamate (20): mp 58−60 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.4 (s, 2H), 4.9 (m, 1H), 1.9−1.4 (m, 8H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 83.7, 33.4, 23.6; IR (KBr) 3367, 3264, 3147, 2963, 2867, 1534, 1443, 1340, 1179, 1153, 1069 cm^{-1}; MS ESI$^+$ m/z 188 (M + Na)$^+$, ESI$^-$ m/z 164 (M − H)$^-$.

Cyclohexyl sulfamate (21): yellow oil; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.4 (s, 2H), 4.4 (m, 1H), 1.2 (m, 10H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 79.5, 32.6, 25.3, 23.9; IR (CH_2Cl_2) 3433, 3338, 2941, 1380, 1183 cm^{-1}; MS ESI$^+$ m/z 202 (M + Na)$^+$, ESI$^-$ m/z 178 (M − H)$^-$.

Benzyl sulfamate (22): mp 74−76 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.65 (s, 2H), 7.4 (s, 5H), 5.1 (s, 2H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 137.5, 129.4, 71.1; IR (KBr) 3360, 3279, 1542, 1457, 1340, 1183 cm^{-1}; MS ESI$^+$ m/z 210 (M + Na)$^+$, ESI$^-$ m/z 186 (M − H)$^-$.

2-Phenylethyl sulfamate (23): oil; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.5 (s, 2H), 7.3 (m, 5H), 4.25 (t, 2H, J = 6.8 Hz), 3 (t, 2H, J = 6.6 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 138.2, 129.7, 129.2, 127.3, 70, 35.3; IR (CH_2Cl_2) 3441, 3338, 2963, 1545, 1377, 1183 cm^{-1}; MS ESI$^+$ m/z 224 (M + Na)$^+$, ESI$^-$ m/z 200 (M − H)$^-$.

***p*-Methylbenzyl sulfamate (24):** mp 23−25 °C (lit.[13,14] mp 22−24 °C, 25−27 °C); ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.6 (s, 2H), 7.3 (d, 2H, J = 8.2 Hz), 7.22 (d, 2H, J = 8.3 Hz), 5.0 (s, 2H), 2.3 (s, 3H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 138.9, 132.4, 129.9, 129.4, 71.1, 21.6; IR (KBr) 3375, 3279, 3139, 1545, 1336, 1179, 1069 cm^{-1}; MS ESI$^+$ m/z 224 (M + Na)$^+$, ESI$^-$ m/z 200 (M − H)$^-$.

***p*-Biphenylylmethyl sulfamate (25):** mp >220 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.8−7.4 (m, 11H), 5.12 (s, 2H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 141.3, 140.4, 134.7, 129.9, 129.8, 128.5, 127.6, 127.5; IR (KBr) 3367, 3279, 1545, 1487, 1358, 1175, 1076, 1051 cm^{-1}; MS ESI$^+$ m/z 286 (M + Na)$^+$, ESI$^-$ m/z 262 (M − H)$^-$.

Fluorenylmethyl sulfamate (26): mp 150−152 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.9 (d, 2H, J = 6.4 Hz), 7.7 (d, 2H, J = 6.8 Hz), 7.6 (s, 1H), 7.5−7.3 (m, 4H), 4.42 (s, 2H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 143.9, 141.6, 128.7, 128.1, 125.9, 121.1, 70.6, 47.1; IR (KBr) 3375, 3264, 1534, 1450, 1344, 1164 cm^{-1}; MS ESI$^+$ m/z 298 (M + Na)$^+$.

Cholesteryl sulfamate (27): mp 167−168 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.4 (s, 2H), 5.35 (m, 1H), 3.3 (m, 1H), 1(s, 3H), 0.9 (s, 3H), 0.8 (d, 6H, J = 6.6 Hz), 0.6 (s, 3H), 0.6−1.8 (m, 28H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 140.1, 123.2, 80.2, 56.9, 56.5, 50.3, 42.6, 39.2, 37.4, 36.7, 36.6, 36.2, 32.2, 29, 28.6, 28.3, 24.2, 23.4, 23.1, 19.7, 19.3, 12.4; IR (KBr) 3375, 3272, 2948, 1465, 1347, 1197, 1161 cm^{-1}; MS ESI$^+$ m/z 488 (M + Na)$^+$, ESI$^-$ m/z 464 (M − H)$^-$.

7-α-Hydroxydehydroepiandrosterone sulfamate (28): mp 131−133 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.2 (s, 2H), 5.3 (d, 1H, J = 4.3 Hz), 4.1 (m, 1H), 3.55 (t, 1H, J = 3.9 Hz), 2.2 (m, 3H), 1.8 (m, 5H), 1.6 (m, 1H), 1.45−1.15 (m, 7H), 0.9 (m, 2H), 0.75 (s, 3H), 0.6 (s, 3H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 172.2, 142.6, 126.9, 80.1, 60.5, 47.2, 45.3, 42.5, 39.1, 37.7, 37.6, 36.9, 36.1, 31.8, 28.9, 22.1, 21.6, 20.4, 18.5, 13.8; IR (KBr) 3492, 3338, 3176, 3066, 2948, 1721, 1575, 1465, 1377, 1267, 1172 cm^{-1}; MS ESI$^+$ m/z 406 (M + Na)$^+$, ESI$^-$ m/z 382 (M − H)$^-$.

7-Oxodehydroepiandrosterone sulfamate (29): mp 180−182 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.45 (s, 2H), 5.7 (s, 1H), 4.3 (m, 1H), 2.65 (m, 3H), 2.5 (t, 1H, J = 11.2 Hz), 2.4 (dd, 1H, J = 19.2 Hz, J = 8.26 Hz), 2 (m, 3H), 1.5−1.8 (m, 7H), 1.2 (m, 5H), 0.8 (s, 3H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 219.8, 201.1, 165.7, 126.6, 78.8, 50.1, 48.1, 45.8, 44.6, 38.8, 38.7, 36.2, 35.8, 31.2, 28.6, 24.6, 20.9, 17.6, 14.3; IR (KBr) 3367, 3213, 3095, 2948, 1725, 1673, 1377, 1300, 1183 cm^{-1}; MS ESI$^+$ m/z 404 (M + Na)$^+$.

CA Inhibition. Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog's group.[17] Cell growth conditions were as described,[18] and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[19] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM^{-1}·cm^{-1} for CA I and 54 mM^{-1}·cm^{-1} for CA II, respectively, based on M_r = 28.85 kDa for CA I and

29.3 kDa for CA II, respectively.[20,21] The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[22]) was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene, Milan, Italy). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *E. coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenized in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100, as described by Wingo et al.[23] The homogenate thus obtained was extensively centrifuged (11 000*g*) in order to remove soluble and membrane-associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM l-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione, and 1 mM oxidized glutathione. Active hCA IX was extensively dialyzed into a solution of 10 mM Hepes (pH 7.5), 10 mM Tris HCl, 100 mM Na_2SO_4, and 1 mM $ZnCl_2$. The protein was further purified by sulfonamide affinity chromatography,[23] the amount of enzyme was determined by spectrophometric measurements, and its activity was determined by stopped-flow measurements, with CO_2 as substrate.[24] The activity of this preparation was identical to that reported by Wingo et al.,[23] with k_{cat}/K_M of 55 μM^{-1} s^{-1}. An SX.18MV-R Applied Photophysics stopped-flow instrument was used for assaying the CA CO_2 hydration activity assays.[24] Phenol red (at a concentration of 0.2 mM) was 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.[24] 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 10 min at room temperature prior to assay, 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. Enzyme concentrations were 1.0 μM for hCA I, 10 nM for hCA II, and 0.1 μM for hCA IX. Inhibition constants were calculated as described.[24]

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