# Carbonic Anhydrase Inhibitors. Inhibition of Cytosolic Isozymes I and II and Transmembrane, Tumor-Associated Isozyme IX with Sulfamates Including EMATE Also Acting as Steroid Sulfatase Inhibitors

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A series of sulfamates or bis-sulfamates incorporating aliphatic, aromatic, polycyclic (steroidal), and sugar 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 andII, and the transmembrane, tumor-associated isozymes CA IX. Some of these compounds were previously reported to act as inhibitors of steroid sulfatases, among which estrone sulfatase (ES) and dehydroepiandrosterone sulfatase (DHEAS) are the key therapeutic targets for estrogen-dependent tumors. Very potent (nanomolar) inhibitors were detected against the three investigated CA isozymes. Best CA I inhibitors were phenylsulfamate and some of its 4-halogeno derivatives, as well as the aliphatic compound *n*-octyl sulfamate. Against CA II, low nanomolar inhibitors (1.1−5 nM) were phenylsulfamate and some of its 4-halogeno/nitro derivatives, *n*-octyl sulfamate, and estradiol 3,17$\beta$-disulfamate among others. All the investigated sulfamates showed efficient CA IX inhibitory properties, with inhibition constants in the range of 18−63 nM. The best CA IX inhibitor detected so far was 4-chlorophenylsulfamate. These data are critical for the design of novel antitumor properties, mainly for hypoxic tumors that overexpress CA IX, which are nonresponsive to radiation or chemotherapy. The antitumor properties of the ES/DHEAS inhibitors in clinical trials may on the other hand also be due to their potent inhibitory properties of CA isozymes involved in tumorigenicity, such as CA II and CA IX.

## Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are involved in crucial physiological processes connected with respiration and transport of $CO_2$/bicarbonate between metabolizing tissues and the lungs, pH and $CO_2$ homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes.[1−3] Thus, it is not surprising that many of their isozymes (14 are presently known in higher vertebrates) have emerged as interesting targets for the design of inhibitors with clinical applications.[2,3] Examples of potent, clinically used inhibitors of CAs are represented by acetazolamide (AAZ), methazolamide (MZA), and ethoxzolamide (EZA), which all contain a terminal sulfonamide as anchoring group to coordinate the catalytic zinc ion of the enzyme, and possess the general formula $RSO_2NH_2$ (where R is generally an aromatic/heterocyclic moiety).[1−3] These sulfonamides are used clinically as antiglaucoma agents, but also for the therapy of other diseases, e.g., increased intracranial pressure, various neurological/neuromuscular pathologies, such as epilepsy, genetic hemiplegic migraine and ataxia, tardive diskinesia, hypokalemic periodic paralysis, essential tremor and Parkinson's disease, and mountain sickness. Accordingly, drugs belonging to this pharmacological class are under constant development.[2,3]

Recently, the X-ray crystallographic structure of sulfamic acid ($H_2NSO_3H$) bound to the physiologically relevant isozyme hCA II has been reported by this group.[4] It was shown that sulfamic acid binds to the zinc ion of the enzyme as a dianion, via its $(NH)SO_3^{2-}$ sulfamate bianionic species (Zn−N distance of 2.07 Å), whereas the NH moiety of the inhibitor also participates in a hydrogen bond with Thr1990$\gamma$. An additional hydrogen bond is formed from this NH to an adjacent water molecule at 2.75 Å distance. The second nearest contact of the ligand to Zn, an oxygen atom, leading to extra-coordination, is at a distance of about 3.07 Å from the metal ion, whereas the remaining two oxygens of the $SO_3$ moiety are involved in two other hydrogen bonds, one with the backbone NH group of Thr 199 at a distance of 2.99 Å and the other to a water molecule at a distance of 2.87 Å. This extended extra-coordination results in a distorted tetrahedral arrangement around the metal ion, the remaining three ligands of zinc being His 94, His 96, and His 119 (as in the uninhibited enzyme).[4] In summary, this very simple inhibitor shows a large number of favorable contacts in the binding pocket of CA and may be used as a lead molecule, since

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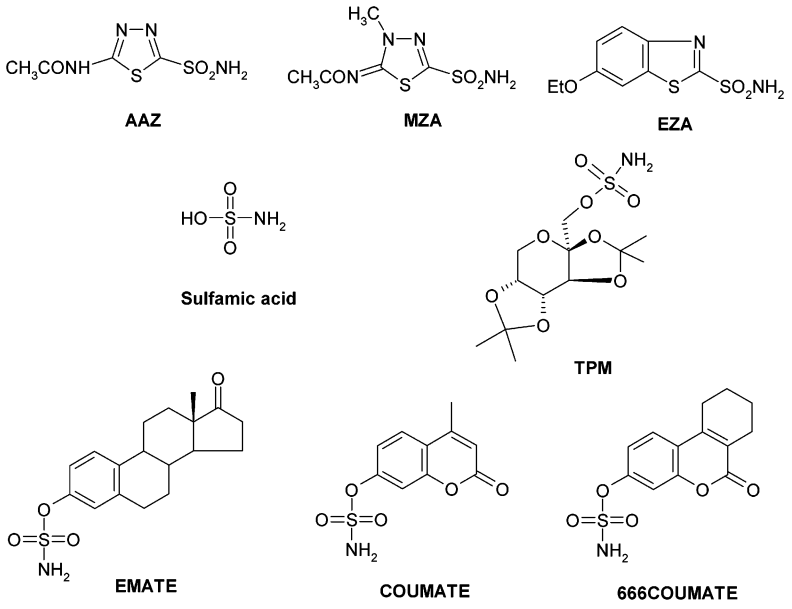
**Chart 1**



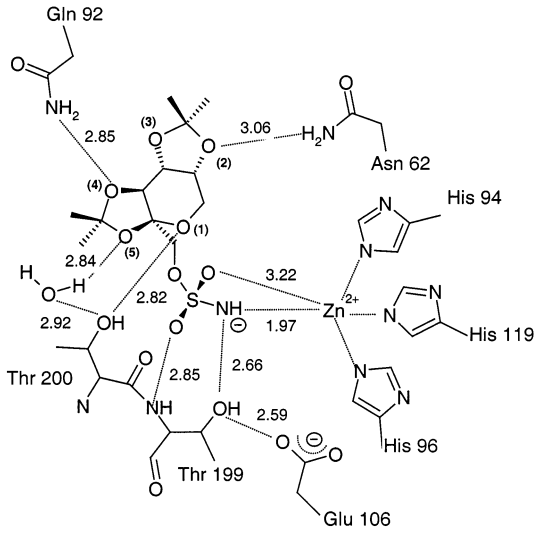
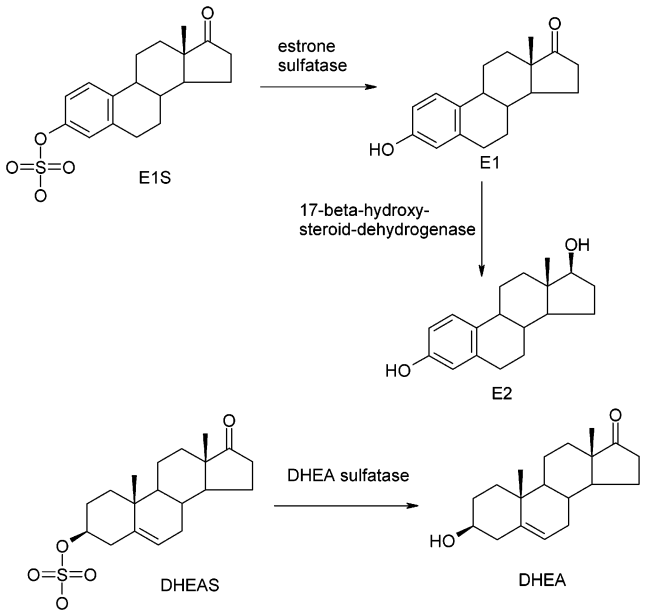




**Figure 1.** Binding of topiramate (TPM) to the hCA II active site: the seven hydrogen bonds with critical amino acid residues of the active site and the zinc coordination are emphasized, with the corresponding distances (Å).

**Scheme 1**

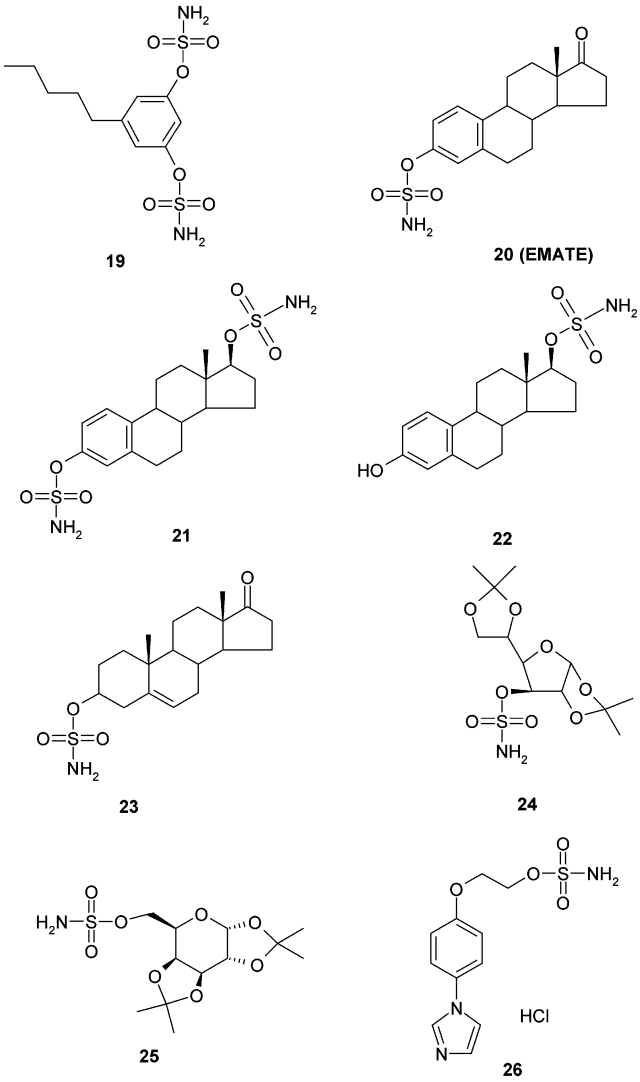



sulfamic acid itself is a weak inhibitor ($K_I$ of 21 $\mu$M against hCA I, and of 97 $\mu$M against hCA II, for the esterase activity of these enzymes).[5] Indeed, we have recently reported the strong CA II inhibitory properties of a series of sugar sulfamates, among which topiramate (TPM), which acts as a low nanomolar inhibitor.[6] Its X-ray structure in the complex with hCA II revealed a very tight association of the inhibitor to the enzyme, with a network of seven hydrogen bonds (to four amino acid residues and one water molecule) fixing topiramate within the active site, in addition to the Zn(II) coordination through the ionized sulfamate moiety (Figure 1).[6]

Sulfamates on the other hand also act as potent inhibitors of steroid sulfatases, among which estrone sulfatase (ES) and dehydroepiandrosterone sulfatase (DHEAS) are the key therapeutic targets for estrogen dependent tumors.[7−11] ES/DHEAS catalyze the hydrolysis of estrone sulfate (E1S) and DHEA-sulfates (DHEAS), respectively, releasing the corresponding unconjugated steroids (Scheme 1).[7,12] Inhibition of these enzyme by sulfamates such as EMATE, COUMATE, as well as many other such agents all possessing the general formula R-$OSO_2NH_2$ (where R is generally an aromatic moiety/polycyclic ring system)[7−11] is of critical interest for designing novel therapies against breast cancer,[7−11] androgen-dependent skin diseases,[13] in cognitive dysfunctions in which DHEAS inhibition was shown to enhance learning and spatial memory,[14] or for the design of immune modulators, as it was shown that ES/DHEAS inhibition has a role in regulating T-helper cell function.[15]

Considering the similarity between the two classes of inhibitors of the two types of enzymes mentioned here, i.e., sulfonamides/sulfamates as CA inhibitors, and sulfamates as ES/DHEAS inhibitors, as well as the fact that some CA isozymes, such as CA IX and CA XII are predominantly found in cancer cells, lacking from their normal counterparts,[16] it appeared of interest to inves-

**Chart 2**




tigate in detail the interaction of sulfamates with some CA isozymes. Here we report the finding that many sulfamates known to act as potent ES/DHEAS inhibitors also show very high (nanomolar) affinity for three CA isozymes, the cytosolic CA I and CA II, and the transmembrane, tumor-associated isozyme CA IX.

## Results

**Chemistry.** The sulfamates **1**−**26** investigated here as CAIs are shown in Table 1. They were prepared as shown in Scheme 2,[17−27] by reaction of the corresponding alcohols/phenols with sulfamoyl chloride.

**Carbonic Anhydrase Inhibitory Activity.** Inhibition data against three CA isozymes, the cytosolic hCA I and hCA II,[28−33] as well as the transmembrane, tumor-associated isozyme hCA IX, are shown in Table 1.[34−36] Standard, clinically used sulfonamide CAIs, such as acetazolamide, methazolamide, and ethoxzolamide, were also included in these assays, for comparison.

## Discussion

**Chemistry.** We attempted to synthesize sulfamates **1**−**25** by an original procedure using a new sulfamoylating reagent previously described by Winum et al.[17] followed by removal of the protecting group. (*N*-(*tert*-Butoxycarbonyl)-*N*-[4-(dimethylazaniumylidene)-1,4-dihydropyridin-1- ylsulfonyl]azanide) was allowed to react with the requisite alcohol or phenol, but unfortunately, no reaction was observed even after heating or base addition. This reagent is in fact effective only for sulfamoylation of amines. It appears that alcohol and phenol functions are not enough nucleophilic to react efficiently with this new sulfamoylating agent. We then conducted experiments using the unstable *N*-(*tert*-butoxycarbonyl)sulfamoyl chloride (obtained in situ from chlorosulfonyl isocyanate and *tert*-butyl alcohol). Under these conditions, N-Boc-sulfamate derivatives were obtained but some of them were not stable during

**Table 1.** Inhibition Data for Derivatives **1**−**26** Investigated in the Present Paper and Standard Sulfonamide CA Inhibitors, against Isozymes I, II, and IX

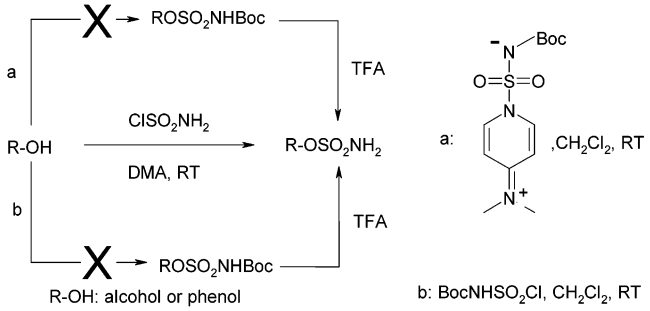
$RO\text{-}SO_2NH_2$

**1-18**

| inhibitor | | $K_I$[a] (nM) | | |
|---|---|---|---|---|
| no. | R | hCA I[b] | hCA II[a] | hCA IX[c] |
| acetazolamide | - | 900 | 12 | 25 |
| methazolamide | - | 780 | 14 | 27 |
| ethoxzolamide | - | 25 | 8 | 34 |
| sulfamic acid ($H_2NSO_3H$) | | 21 000 | 97 000 | nt |
| topiramate | - | 250 | 5 | nt |
| **1** | Ph | 2.1 | 1.3 | 63 |
| **2** | 4-Me-$C_6H_4$ | 3.8 | 1.9 | 59 |
| **3** | 4-Ph-$C_6H_4$ | 113 | 95 | 50 |
| **4** | 4-Cl-$C_6H_4$ | 4.6 | 1.1 | 18 |
| **5** | 4-Br-$C_6H_4$ | 7.3 | 1.5 | 19 |
| **6** | 4-I-$C_6H_4$ | 9.5 | 3.8 | 23 |
| **7** | 4-MeO-$C_6H_4$ | 33 | 1.6 | 34 |
| **8** | 4-PhO-$C_6H_4$ | 115 | 98 | 51 |
| **9** | 4-AcNH-$C_6H_4$ | 37 | 18 | 45 |
| **10** | 4-$O_2N$-$C_6H_4$ | 40 | 1.5 | 36 |
| **11** | 4-NC-$C_6H_4$ | 480 | 149 | 41 |
| **12** | 4-*t*-Bu-$C_6H_4$ | 43 | 2.9 | 33 |
| **13** | 4-$CF_3$-$C_6H_4$ | 369 | 138 | 54 |
| **14** | $C_6F_5$ | 415 | 113 | 47 |
| **15** | $C_6Cl_5$ | 432 | 125 | 39 |
| **16** | 2,4,6-$Cl_3C_6H_2$ | 454 | 138 | 37 |
| **17** | 2-naphthyl | 103 | 63 | 40 |
| **18** | *n*-$C_8H_{17}$ | 3.5 | 2.7 | 25 |
| **19** | A | 105 | 76 | 43 |
| **20** | A (EMATE) | 37 | 10 | 30 |
| **21** | A | 6 | 5 | 58 |
| **22** | A | 15 | 13 | 32 |
| **23** | A | 31 | 27 | 44 |
| **24** | A | 278 | 15 | 39 |
| **25** | A | 315 | 29 | 32 |
| **26** | A | 28 | 23 | 26 |

[a] Errors in the range of 5−10% of the reported value (from three different assays). [b]Human (cloned) isozymes, by the esterase method. [c]Catalytic domain of human, cloned isozyme, by the $CO_2$ hydration method. A: See structure in the text. nt = not tested.

**Scheme 2**



purification or Boc removal especially those with electron-withdrawing groups on the phenyl ring, the Boc-NH-$SO_2$ acting as a good leaving group. So, we chose to prepare sulfamates **1**−**25** by the procedure described by Okada et al.,[18] employing the reaction of phenols/alcohols with sulfamoyl chloride in *N*,*N*-dimethylacetamide as solvent. This procedure proved to be very efficient, and the different derivatives were obtained in high yields after purification either by crystallization or silica gel chromatography (Scheme 2). Sulfamoyl chloride was prepared from chlorosulfonyl isocyanate and formic acid as described by Appel et al.[19] All compounds were characterized by $^1$H and $^{13}$C NMR, IR, and mass spectroscopy. Some of these compounds were previously reported in the literature,[20−27] but they have not been tested as CA inhibitors (CAIs) in earlier studies. A rather large number of substitution patterns (R moieties) have been used in compounds **1**−**26** (aromatic, substituted-aromatic, aliphatic, steroidal backbones, etc.) together with some disulfamates which were also prepared, to detect best substitution pattern(s) for efficient CA inhibition.

**Carbonic Anhydrase Inhibitory Activity. Isozyme I.** As seen from data of Table 1, sulfamates **1**−**26** investigated here are much more effective CA I inhibitors as compared to the lead molecule from which they were obtained, i.e., sulfamic acid (inhibition constant of 21 $\mu$M). A large range of activities was observed for the different substitution patterns of the investigated sulfamates, as follows: (i) several derivatives, such as **1**, **2**, **4**−**6**, **18**, **21**, and **22**, acted as very potent CA I inhibitors, with inhibition constants in the range of 2.1−15 nM. It is interesting to note that this potent inhibitory activity is seen for simple aromatic derivatives (such as the phenyl, 4-tolyl or 4- halogenophenyl-substituted sulfamates), the aliphatic derivative **18**, or the condensated polycyclic derivatives **21** and **22**. It should be noted that these compounds behave as much more potent CA I inhibitors as compared to the clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, or topiramate (Table 1); (ii) derivatives **7, 9, 10, 12, 20, 23**, and **26** also showed potent CA I inhibitory activity, with inhibition constants in the range of 28−43 nM, being slightly less effective than the derivatives discussed above. Again SAR is difficult to envisage, since simple aromatic (**7**, **9**, **10**, **12**), polycyclic (**20** and **23**), or imidazolyl-phenyl-aliphatic (**26**) derivatives showed comparable activity. Comparing all the derivatives discussed up to now, it is clear that phenylsulfamate **1** is the best CA I inhibitor observed in this series, and that any substitution at its aromatic ring is detrimental to activity. Still, moieties that lead only to a slight decrease of activity were 4-methyl-, 4-halogeno-, and to a higher degree, 4-methoxy-, 4-acetamido-, 4-nitro-, and 4-*tert*-butyl; (iii) the other investigated derivatives showed inhibition constants in the range of 103−480 nM against hCA I, being better inhibitors than acetazolamide and methazolamide and showing a potency comparable to that of topiramate. Among these derivatives, it is obvious that other substitution patterns of the phenylsulfamate lead **1** were highly detrimental to inhibitory activity, such as 4-phenyl-, 4-phenoxy-, 4-cyano-, 4-trifluoromethyl- and perfluorophenyl-/perchlorophenyl-. The bis-sulfamate **19** was also weakly active, as well as the two sugar sulfamates **24** and **25** (with an activity comparable again with that of topiramate).

**Isozyme II**. This is considered the most important isozyme as target for obtaining inhibitors with pharmacological applications. All the sulfamates investigated here generally showed good CA II inhibitory properties, considering especially the very weak inhibition produced by the lead molecule, sulfamic acid (inhibition constant of 97 $\mu$M). Again a rather wide range of activities were evidenced, as follows: (i) very potent CA II inhibitors, with inhibition constants in the range of 1.1 − 15 nM proved to be compounds **1**, **2**, **4**−**7**, **10**, **12**, **18**, **20**−**22**, and **24**, which are all more effective or at the same levels of inhibition as the clinically used compounds acetazolamide, methazolamide, ethoxzolamide, or topiramate (Table 1). The best CA II inhibitor was the simple 4-chlorophenylsulfamate **4**, but very similar inhibition was produced by the structurally related halogenophenyl sulfamates **5** and **6**, the unsubstituted derivative **1**, the 4-methyl/methoxy- or 4-nitro-substituted compounds **2**, **7**, and **10**, as well as the *tert*-butyl derivative **12**. The very strong inhibitory activity of the simple, aliphatic sulfamate **18** is noteworthy (in fact this is the unique such derivative investigated up to now), which is among the best inhibitors detected here and belongs to a class of compounds which deserves much more attention. The steroidal sulfamates **20**, **21**, and **22** also showed excellent CA II inhibitory activity, with the best inhibitor being the bis-sulfamate **21**, followed by EMATE **20** and the monosulfamate **22**. It is interesting to note that the EMATE corresponding nonaromatic sulfamate **23** was 2.7 times less effective as a CA II inhibitor as compared to EMATE; (ii) less potent inhibitory activity was shown by compounds **9**, **17**, **19**, **23**, **25**, and **26**, with inhibition constants in the range of 18−76 nM. These compounds belong either to the aromatic type of sulfamates (**9, 17**, and **19**) but possess bulkier substituents/rings as compared to **1** (or in the case of **19**, two sulfamate moieties) or to the polycyclic/sugar type of derivatives (**23** and **25**). Compound **26** also showed this type of behavior; (iii) the most ineffective CA II inhibitors (compounds **3**, **8**, **11**, **13**−**16**) showed inhibition constants in the range of 95−149 nM and belong to the phenylsulfamate **1** type of derivative, substituted with moieties which led to an enhanced loss of affinity for the active site, such as 4-phenyl-/phenoxy-; 4-trifluoromethyl-; 4-cyano-; perfluorophenyl-/perchlorophenyl- or 2,4,6-trichlorophenyl. SAR is difficult to draw from all these data, but one may speculate that for substituted-phenyl sulfamates derived from **1**, best activity against CA II is observed for the halogeno-substituted compounds (together with the unsubstituted lead) and that generally bulky groups in position 4 (phenyl/phenoxy − but not *tert*-butyl!) are detrimental to activity. One must also mention that all sulfamates **1**−**26** showed higher affinity for CA II as compared to CA I, a behavior also observed for sulfonamide CAIs.[1−3]

**Isozyme IX**. The inhibition profile of this isozyme with the sulfamates **1**−**26** is very interesting, differing substantially from the inhibition profiles of the cytosolic isozymes I and II discussed earlier. Thus, as a first observation, all the investigated sulfamates as well as the three clinically used inhibitors (acetazolamide, methazolamide, and ethoxzolamide) showed inhibition

constants in the range of 18−63 nM. The range of activity is thus much more limited as compared to that of the other two isozymes CA I and II investigated here (Table 1). Several compounds, such as acetazolamide, methazolamide, and the sulfamates **4**−**6**, **18**, **20**, and **26** showed very strong CA IX inhibitory properties ($K_I$s in the range of 18−30 nM). Again it is rather difficult to draw SAR conclusions, since these compounds belong to different structural subtypes, with both halogenophenyl (**4**−**6**), aliphatic (**18** and **26**), and steroidal sulfamates showing strong inhibitory properties. It is noteworthy that **1**, which was one of the best CA I and CA II inhibitors among the investigated compounds, is in fact the worst CA IX inhibitor, although it appreciably inhibits this isozyme, too. In this case, all substitutions of **1** led to more effective inhibitors, culminating in the three halogenophenyl derivatives **4**−**6** which were the best compounds in the series. Still, other substitution patterns were equally effective, with the 4-methoxy-, 4-nitro-, 4-*tert*-butyl-, and the 2,4,6-trichloro- derivatives leading to some of the best inhibitors ($K_I$s in the range of 33−37 nM, comparable with that of ethoxzolamide). A very interesting behavior against CA IX was shown by the steroidal sulfamates **20**−**23**. Thus, EMATE **20** and the monosulfamate **22** are quite potent inhibitors ($K_I$s in the range of 30−32 nM), whereas **21** (which showed the best CA II and CA I inhibitory properties) was the worst CA IX inhibitor in this subseries ($K_I$ of 58 nM), being less effective than **23** ($K_I$ of 44 nM). The two sugar sulfamates **24** and **25** also showed good CA IX inhibitory properties, with inhibition constants in the range of 32−39 nM.

Data presented here are particularly important for the design of compounds able to interfere with the development of cancer cells. Thus, it has recently been demonstrated that the tumor-associated isozyme CA IX may be of considerable value as markers of tumor progression.[37] This is mostly due to its induction by hypoxia, a clinically important factor of tumor biology that significantly affects treatment outcome and disease progression.[37] Strong association between CA IX expression and intratumoral hypoxia has been demonstrated in the cervical, breast, head and neck, bladder, and nonsmall cell lung carcinomas (NSCLC).[38−41] Furthermore, such hypoxic tumors overexpressing CA IX do not respond to radiation or chemotherapy. It would be thus critically important to inhibit this isozyme in order to test whether such malignancies become again responsive to the anticancer therapy. It must also be stressed that some sulfonamide CAIs show themselves antitumor properties in vitro and in vivo, although the mechanism by which they exert this property is rather unclear for the moment.[42−45] Another point raised by our work is whether the antitumor properties of the steroid sulfatase inhibitors are due only to the inhibition of ES/DHEAS. Our data strongly suggest that this may be in part due to inhibition of some CA isozymes important for the tumor growth, such as CA IX and/or CA II (CA I), and that the mechanims of action of the inhibitors in clinical trials may be much more complicated as compared to those originally thought.

**Conclusions.** A series of sulfamates or bis-sulfamates incorporating aliphatic, aromatic, polycyclic (steroidal), and sugar 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. Some of these compounds were previously reported to act as inhibitors of steroid sulfatases, among which ES and DHEAS are the key therapeutic targets for estrogen-dependent tumors. Very potent (nanomolar) inhibitors were detected against the three investigated CA isozymes. The best CA I inhibitors were phenylsulfamate and some of its 4-halogeno derivatives, as well as the aliphatic compound *n*-octyl sulfamate. Against CA II, low nanomolar inhibitors (1.1−5 nM) were phenylsulfamate and some of its 4-halogeno-/nitro-derivatives, *n*-octyl sulfamate, and estradiol 3,17$\beta$-disulfamate among others. All the investigated sulfamates showed efficient CA IX inhibitory properties, with inhibition constants in the range of 18−63 nM. The best CA IX inhibitor detected so far was 4-chlorophenylsulfamate. The inhibition profile of the three investigated isozymes with this type of compounds 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. The antitumor properties of the ES/DHEAS inhibitors in clinical trials may on the other hand also be due to their potent inhibitory properties of CA isozymes involved in tumorigenicity, such as CA II and CA IX.

## 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 point 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-200 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 $\delta$ (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 were prepared by reacting the corresponding alcohol or phenol (1 equiv) with sulfamoyl chloride (3 equiv) in *N*,*N*-dimethylacetamide.[18] (Sulfamoyl chloride was prepared from chlorosulfonyl isocyanate and formic acid as described in ref 19). 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.

Phenylsulfamate **1**: mp 77−79 °C (lit. mp 77.6−81.2 °C);[9c] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8 (s, 2H), 7.55−7.2 (m, 5H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 151, 130.6, 127.4, 123; IR (KBr) 3418, 3305, 3071, 1550, 1486, 1370, 1182 cm$^{-1}$; MS *m*/*z* 196 (M + Na)$^+$.

*p*-Methylphenylsulfamate **2**: mp 75−76 °C (lit. mp 80 °C);[20] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 7.9 (s, 2H), 7.25 (d, $J$ = 7.2 Hz, 2H), 7.15 (d, $J$ = 7.2 Hz, 2H), 3.12 (s, 3H, Me); $^{13}$C NMR (DMSO-$d_6$, 400 MHz). 148.9, 136.7, 130.9, 122.8, 20.7; IR (KBr) 3381, 3275, 3056, 2920, 1598, 1535, 1355, 1178 cm$^{-1}$; MS *m*/*z* 210 (M + Na)$^+$.

*p*-Biphenylsulfamate **3**: mp 160−162 °C (lit. mp 165 °C);[20] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.1 (s, 2H), 7.7 (m, 4H), 7.4 (m, 5H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 150.5, 140, 139.4, 129.8, 128.9, 128.5, 127.6, 123.5; IR (KBr) 3418, 3290, 3071, 1542, 1482, 1377, 1175 cm$^{-1}$; MS *m*/*z* 272 (M + Na)$^+$.

*p*-Chlorophenylsulfamate **4**: mp 99−100 °C (lit. mp 104 °C);[20] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.1 (s, 2H), 7.55 (d, $J$ = 8.5 Hz, 2H), 7.3 (d, $J$ = 8.7 Hz, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 149.7, 130.5, 124.9, 117.7; IR (KBr) 3396, 3275, 3094, 1486, 1362, 1175, 1085 cm$^{-1}$; MS *m*/*z* 230 (M + Na)$^+$.

*p*-Bromophenylsulfamate **5**: mp 111−113 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.1 (s, 2H), 7.65 (d, $J$ = 8.5 Hz, 2H), 7.25 (d, $J$ = 8.5 Hz, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 150.2, 133.5, 125.3, 119.9; IR (KBr) 3381, 3275, 3094, 1535, 1482, 1355, 1171, 1066 cm$^{-1}$; MS *m*/*z* 276 (M + Na)$^+$.

*p*-Iodophenylsulfamate **6**: mp 134−136 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.1 (s, 2H), 7.8 (d, $J$ = 8.3 Hz, 2H), 7.15 (d, $J$ = 8.3 Hz, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 150.8, 139.4, 125.5, 92.5; IR (KBr) 3373, 3275, 3086, 1535, 1475, 1355, 1178; MS *m*/*z* 322 (M + Na)$^+$.

*p*-Methoxyphenylsulfamate **7**: mp 62−64 °C (lit. mp 65 °C);[21] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 7.9 (s, 2H), 7.2 (d, $J$ = 8.9 Hz, 2H), 7.0 (d, $J$ = 8.9 Hz, 2H), 3.5 (MeO); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 158.4, 144.4, 124.2, 115.4, 37.8; IR (KBr) 3381, 3267, 3094, 2966, 1598, 1542, 1362, 1246, 1156 cm$^{-1}$; MS *m*/*z* 226 (M + Na)$^+$.

*p*-Phenoxyphenylsulfamate **8**: mp 109−111 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8 (s, 2H), 7.35 (m, 2H), 7.1 (m, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 157.4, 155.7, 130.9, 124.8, 124.5, 120.4, 119.4; IR (KBr) 3381, 3298, 3071, 1587, 1490, 1355, 1250, 1175 cm$^{-1}$; MS *m*/*z* 288 (M + Na)$^+$.

*p*-Acetamidophenylsulfamate **9**: mp 175−177 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 10 (s, 1H), 8 (s, 2H), 7.6 (d, $J$ = 7.2 Hz, 2H), 7.2 (d, $J$ = 7.2 Hz, 2H), 2 (s, 3H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 169.2, 146.1, 138.5, 123.4, 120.9, 24.7; IR (KBr) 3373, 3184, 3090, 2996, 1666, 1606, 1546, 1370, 1156 cm$^{-1}$; MS *m*/*z* 253 (M + Na)$^+$.

*p*-Nitrophenylsulfamate **10**: mp 100−102 °C (lit. mp 94−96 °C);[22] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.4 (s, 2H), 8.35 (d, $J$ = 8.7 Hz, 2H), 7.55 (d, $J$ = 8.7 Hz, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 164.7, 127, 123.7, 116.6; IR (KBr) 3418, 3260, 3109, 1613, 1527, 1385, 1325, 1197 cm$^{-1}$; MS *m*/*z* 241 (M + Na)$^+$.

*p*-Cyanophenylsulfamate **11**: mp 152−153 °C (lit. mp 155 °C);[21] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.3 (s, 2H), 7.95 (d, $J$ = 8.7 Hz, 2H), 7.45 (d, $J$ = 8.7 Hz, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 162.4, 135.2, 123.9, 117.2, 101.8; IR (KBr): 3373, 3252, 2241, 1602, 1501, 1373, 1197 cm$^{-1}$; MS *m*/*z* 221 (M + Na)$^+$.

*p*-*tert*-Butylphenylsulfamate **12**: mp 95−97 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8 (s, 2H), 7.5 (d, $J$ = 8.7 Hz, 2H), 7.2 (d, $J$ = 8.7 Hz, 2H), 1.25 (s, 9H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 149.8, 148.7, 127.3, 122.5, 35.1, 32; IR (KBr) 3381, 3290, 3079, 2958, 1550, 1381, 1178 cm$^{-1}$; MS *m*/*z* 252 (M + Na)$^+$.

*p*-Trifluoromethylphenylsulfamate **13**: mp 100−102 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.2 (s, 2H), 7.85 (d, $J$ = 8 Hz, 2H), 7.5 (d, $J$ = 8 Hz, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 161.5, 153.8, 127.8, 123.8, 116.4; IR (KBr) 3381, 3275, 3079, 1613, 1538, 1366, 1163, 1062 cm$^{-1}$; MS *m*/*z* 240 (M − H)$^-$.

Pentafluorophenylsulfamate **14**: mp 104−106 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) 9.2 (s, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 138.3, 136, 133.6, 131.6; IR (KBr) 3381, 3305, 3094, 1527, 1370, 1223, 1141 cm$^{-1}$; MS *m*/*z* 262 (M − H)$^-$.

Pentachlorophenylsulfamate **15**: mp 210−213 °C (lit. mp 215 °C)[21]; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.8 (s, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 151.1, 131, 122.9, 122; IR (KBr) 3381, 3283, 1535, 1381, 1190 cm$^{-1}$; MS *m*/*z* 344 (M − H)$^-$.

2,4,6-Trichlorophenylsulfamate **16**: mp 143−144 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.5 (s, 2H), 7.8 (s, 2H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 149.3, 129.9, 128.9, 124.1; IR (KBr) 3373, 3275, 3071, 1565, 1445, 1377, 1235, 1193 cm$^{-1}$; MS *m*/*z* 298 (M + Na)$^+$.

2-Naphthylsulfamate **17**: mp 111−112 °C (lit. mp 112 °C);[23] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.2 (s, 2H), 8 (m, 4H), 7.55 (m, 3H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 148.6, 134.1, 132.1, 130.6, 128.6, 128.5, 127.7, 127, 122.5, 120; IR (KBr): 3411, 3267, 3094, 1550, 1362, 1167 cm$^{-1}$; MS *m*/*z* 246 (M + Na)$^+$.

*n*-Octylsulfamate **18**: mp 53−57 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 7.2 (s, 2H), 4 (t, $J$ = 6.4 Hz, 2H), 1.6 (m, 2H), 1.3 (m, 10H), 0.85 (t, $J$ = 6.6 Hz, 3H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 69.8, 32, 29.4, 29.3, 29.1, 26.1, 22.9, 14.7; IR (KBr) 3373, 3283, 2920, 1546, 1351, 1182 cm$^{-1}$; MS *m*/*z* 232 (M + Na)$^+$.

5-Pentylresorcinylsulfamate **19**: mp 77−79 °C; $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 8.1 (s, 4H), 7.1 (s, 3H), 2.6 (t, $J$ = 7.8 Hz, 2H), 1.6 (m, 2H), 1.3 (m, 4H), 0.85 (t, $J$ = 6.4 Hz, 3H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 151.1, 146.4, 12, 35.5, 31.6, 31, 22.7, 14.7; IR (KBr) 3403, 3358, 3275, 3086, 2943, 1598, 1531, 1370, 1186, 1103 cm$^{-1}$; MS *m*/*z* 361 (M + Na)$^+$.

Estrone sulfamate **20**: mp 197−200 °C (lit. mp 199−202 °C);[24] $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 7.9 (s, 2H), 7.35 (d, $J$ = 8.5 Hz, 1H), 7.1 (d, $J$ = 8.5 Hz, 1H), 7 (s, 1H), 2.85 (d, $J$ = 4.6 Hz, 2H), 2.45 (m, 2H), 2.25 (m, 1H), 2 (m, 3H), 1.8 (d, $J$ = 7.4 Hz, 1H), 1.5 (m, 6H), 0.85 (s, 3H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 148.9, 138.9, 138.8, 127.4, 122.7, 120.1, 50.3, 48.1, 44.4, 38.3, 36.2, 32.1, 29.8, 26.6, 26.2, 21.9, 14.3; IR (KBr) 3411, 3252, 2943, 1711, 1490, 1377, 1178 cm$^{-1}$; MS *m*/*z* 372 (M + Na)$^+$.

Estradiol 3,17$\beta$-disulfamate **21**: mp 186−190 °C (lit. mp 189−194 °C);[25] $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 7.9 (s, 2H), 7.4 (s, 2H), 7.35 (d, $J$ = 8.5 Hz, 1H), 7.05 (d, $J$ = 8.4 Hz, 1H), 6.95 (s, 1H), 4.35 (t, $J$ = 8.1 Hz, 1H), 2.8 (m, 2H), 2.3 (m, 1H), 2.2 (m, 2H), 2 (m, 1H), 1.85 (m, 1H), 1.7 (m, 2H), 1.3 (m, 6H), 0.75 (s, 3H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 148.8, 138.9, 138.8, 127.4, 122.7, 120.1, 88.2, 49.4, 44.2, 44.1, 38.6, 36.7, 29.8, 28.3, 27.2, 26.3, 23.4, 12.4; IR (KBr) 3381, 3320, 3094, 2928, 1546, 1377, 1190 cm$^{-1}$; MS *m*/*z* 453 (M + Na)$^+$.

Estradiol 17$\beta$-sulfamate **22**: mp 157−159 °C (lit. mp 158−161 °C);[25] $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 8.95 (s, 1H), 7.35 (s, 2H), 7.05 (d, $J$ = 8.4 Hz, 1H), 6.45 (dd, $J$ = 2.3 Hz, 8.3 Hz, 1H), 6.4 (d, $J$ = 2.2 Hz, 1H), 4.3 (t, $J$ = 8.5 Hz, 1H), 2.7 (**min,** 2H), 2.15 (m, 3H), 1.95 (m, 1H), 1.7 (m, 3H), 1.25 (m, 6H), 0.75 (s, 3H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 160.5, 142.6, 135.6, 131.7, 120.5, 118.3, 93, 54.2, 48.8, 48.2, 43.8, 41.5, 34.6, 33, 32.3, 31.3, 28.2, 17.2; IR (KBr) 3404, 3281, 3094, 2930, 1503, 1348, 1180 cm4; MS *m*/*z* 374 (M + Na)$^+$.

Dehydroepiandrosterone sulfamate **23**: mp 160−162 °C; $^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 7.4 (s, 2H), 5.4 (t, $J$ = 3.7 Hz, 1H), 4.25 (m, 1H), 2.45 (m, 4H), 2 (m, 5H), 1.7 (m, 4H), 1.45 (m, 2H), 1.3 (m, 2H), 1 (m, 5H), 0.8 (s, 3H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz): $\delta$ 140.3, 122.9, 80.2, 51.6, 50.4, 47.6, 39.3, 37.3, 36.9, 36.1, 31.9, 31.7, 31.1, 29, 22.2, 20.7, 19.7, 14; IR (KBr): 3373, 3275, 2943, 1730, 1568, 1381, 1182 cm$^{-1}$; MS *m*/*z* 390 (M + Na)$^+$.

1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose 3-sulfamate **24**: mp 136−138 °C (lit. mp 140 °C);[26] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 7.8 (s, 2H), 6 (d, $J$ = 3.7 Hz, 1H), 4.8 (m, 2H), 4.25 (m, 2H), 4 (m, 1H), 3.85 (m, 1H), 1.45 (s, 3H), 1.35 (s, 3H), 1.25 (s, 6H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 112.2, 108.8, 105.2, 83.2, 81.5, 79.6, 73.2, 65.6, 27.2, 26.8, 25.9; IR (KBr): 3350, 3237, 2988, 1568, 1381, 1216, 1085 cm$^{-1}$; MS *m*/*z* 362 (M + Na)$^+$.

1,2:3,4-Di-*O*-isopropylidene-α-D-galactopyranose 6-sulfamate **25**. Oil colorless (lit. syrup);[26] $^1$H NMR (DMSO-$d_6$, 250 MHz) $\delta$ 7.5 (s, 2H), 5.5 (d, $J$ = 4.9 Hz, 1H), 4.6 (dd, $J$ = 2.4 Hz, 1H), 4.4 (dd, $J$ = 2.4 Hz, 1H), 3.9−4.3 (m, 4H), 1.45 (s, 3H), 1.35 (s, 3H), 1.3 (s, 6H); $^{13}$C NMR (DMSO-$d_6$, 400 MHz) $\delta$ 109.5, 108.9, 96.4, 71, 70.8, 70.4, 68.6, 66.6, 26.7, 26.6, 25.6, 25; IR ($CH_2Cl_2$) 3404, 3338, 2970, 1604, 1384, 1186, 1069 cm$^{-1}$; MS *m*/*z* 362 (M + Na)$^+$.

Sulfamate **26**[27] was a gift from Prof. T. H. Maren (University of Florida at Gainesville).

**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.[28] Cell growth conditions were those described in

ref 29, and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[30] 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.[31,32]

The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[33]) 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 *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100, as described by Wingo et al.[34] 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, 1 mM oxidized glutathione. Active hCA IX was extensively dialyzed into a solution of 10 mM Hepes (pH 7.5), 10 mM Tris HC1, 100 mM $Na_2SO_4$, and l mM $ZnCl_2$. The amount of protein was determined by spectrophometric measurements and its activity by stopped-flow measurements, with $CO_2$ as substrate.

Initial rates of 4-nitrophenylacetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM-compatible PC.[35] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between $2 \times 10^{-2}$ and $1 \times 10^{-6}$ M, working at 25 °C. A molar absorption coefficient $c$ of 18 400 $M^{-1}$ $cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature.[35] Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. 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. The inhibition constant $K_I$ was determined as described by Pocker and Stone, for isozymes I and II.[35] Enzyme concentrations were 3.2 $\mu$M for CA II, 10.1 $\mu$M for CA I.

An SX.1 8MV−R Applied Photophysics stopped-flow instrument has been used for assaying the CA IX $CO_2$ hydration activity.[36] 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.[36] Stock solutions of inhibitors were prepared at a concentration of 1−3 mM (in DMSO−water 1:1, v/v) and dilutions up to 0.1 nM done with the assay buffer mentioned above. Enzyme concentration was 0.1 $\mu$M, and inhibition constants were calculated as described in ref 36.

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