Carbonic Anhydrase Inhibitors. Inhibition of Tumor-Associated Isozyme IX by Halogenosulfanilamide and Halogenophenylaminobenzolamide Derivatives†

Marc A. Ilies,[‡,§] Daniela Vullo,[||] Jaromir Pastorek,[⊥] Andrea Scozzafava,[||] Monica Ilies,[‡,||] Miron T. Caproiu,[⊗] Silvia Pastorekova,[⊥] and Claudiu T. Supuran*[,||]

University of Agricultural Sciences and Veterinary Medicine, Faculty of Biotechnologies, Department of Chemistry, B-dul Marasti nr. 59, 71331-Bucharest, Roumania, Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy, Institute of Virology, Slovak Academy of Sciences, Dubravska cesta 9, 842 45 Bratislava, Slovakia, and "C.D. Nenitzescu" Institute of Organic Chemistry, Splaiul Independentei 202B, Bucharest, Roumania

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Two series of halogenated sulfonamides have been prepared. The first consists of mono/dihalogenated sulfanilamides, whereas the second one consists of the mono/dihalogenated aminobenzolamides, incorporating equal or different halogens (F, Cl, Br, and I). These sulfonamides have been synthesized from the corresponding anilines by acetylation (protection of the amino group), chlorosulfonylation, followed either by amidation, or reaction with 5-amino-1,3,4-thiadiazole-2-sulfonamide (and eventually deacetylation). All these compounds, together with the six clinically used sulfonamide inhibitors (acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, and brinzolamide) were investigated as inhibitors of the transmembrane, tumor-associated isozyme carbonic anhydrase (CA) IX. Inhibition data against the classical, physiologically relevant isozymes I, II, and IV were also obtained. CA IX shows an inhibition profile which is generally completely different from those of isozymes I, II, and IV, with potent inhibitors (inhibition constants in the range of 12−40 nM) among both simple aromatic (such as 3-fluoro-5-chloro-4-aminobenzenesulfonamide) as well as heterocyclic compounds (such as acetazolamide, methazolamide, 5-amino-1,3,4-thiadiazole-2-sulfonamide, aminobenzolamide, and dihalogenated aminobenzolamides). This first detailed CA IX inhibition study revealed many interesting leads, suggesting the possibility to design even more potent and eventually CA IX-selective inhibitors, with putative applications as antitumor agents.

Introduction

Among the zinc enzymes extensively studied in the last period, the carbonic anhydrases (CAs, EC 4.2.1.1) occupy a special place for several reasons: (i) these enzymes are ubiquitous in all kingdoms, starting with *Archaea*, bacteria, algae, and green plants, and ending with superior animals, including vertebrates;[1−4] (ii) their physiological function is essential for these organisms, as CAs catalyze a very simple physiological reaction, the interconversion between carbon dioxide and bicarbonate.[1−4] This reaction is critical for respiration and transport of CO_2 between metabolizing tissues and excretion sites, secretion of electrolytes in a variety of tissues and organs, pH regulation and homeostasis, CO_2 fixation (for algae and green plants), several metabolic biosynthetic pathways (in vertebrates), etc.;[1−4] (iii) inhibition (but also activation) of these enzymes may be exploited clinically in the treatment or prevention of a variety of disorders.[1−3] In consequence, CA inhibitors (CAIs) and to a less extent up to now, CA activators possess a variety of applications in therapy.[1,2] Four such pharmacological agents, acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, and dichlorophenamide **DCP**, have been used for more than 40 years as systemic CAIs, whereas additional two drugs dorzolamide **DZA** (clinically launched in 1995) and the structurally related brinzolamide **BRZ** (used since 1999) are topically acting antiglaucoma CAIs.[1,2]

CAs are encoded by three distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, bacteria, algae, and cytoplasm of green plants), the β-CAs (predominantly in bacteria, algae and chloroplasts of both mono- as well as dicotyledons) and the γ-CAs (mainly in *Archaea* and some bacteria), respectively.[1,2,5] In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) were described, with very different subcellular localization and tissue distribution.[1,2,5] Basically, there are several cytosolic forms (CA I−III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII, and CA XIV), and one mitochondrial form (CA V) as well as a secreted CA isozyme, CA VI.[1,2,5] Not much is known about the cellular localization of other isozymes.

Some of the isozymes mentioned above, such as CA IX and CA XII, are predominantly found in cancer cells.[6] The first tumor-associated CA isozyme discovered was CA IX, a transmembrane protein with a suggested function in maintaining the acid−base balance and intercellular communication. It consists of an N-terminal proteoglycan-like domain that is unique among the

* Correspondence author. Tel: +39-055-457 3005; fax: +39-055-4573385; e-mail: claudiu.supuran@unifi.it.
† Dedicated to the memory of Professor Ovidiu Maior.
‡ University of Agricultural Sciences and Veterinary Medicine.
§ Present address: Texas A & M University at Galveston, Department of Marine Sciences, Galveston, TX.
|| Università degli Studi di Firenze.
⊥ Institute of Virology, Slovak Academy of Sciences.
⊗ "C.D. Nenitzescu" Institute of Organic Chemistry.

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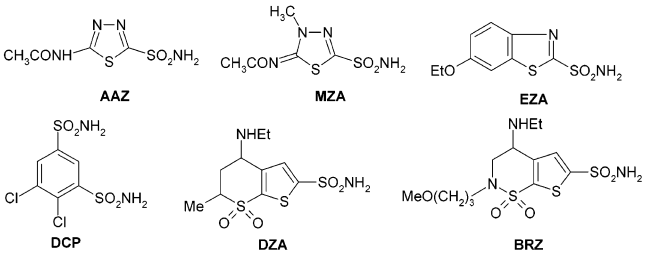
Chart 1



CAs, a highly active CA catalytic domain, a single transmembrane region, and a short intracytoplasmic tail.[7] CA IX is particularly interesting for its ectopic expression in a multitude of carcinomas derived from cervix uteri, kidney, lung, esophagus, breast, colon, etc., contrasting with its restricted expression in normal tissues, namely in the epithelia of the gastrointestinal tract.[7−14]

It has recently been demonstrated that such tumor-associated CAs (mainly CA IX) may be of considerable value as markers of tumor progression. This is mostly due to their induction by hypoxia, a clinically important factor of tumor biology that significantly affects treatment outcome and disease progression.[9] Strong association between CA IX expression and intratumoral hypoxia (either measured by microelectrodes, or detected by incorporation of a hypoxic marker Pimonidazole or by evaluation of extent of necrosis), has been demonstrated in the cervical, breast, head and neck, bladder, and nonsmall cell lung carcinomas (NSCLC).[10−13] Moreover, in NSCLC and breast carcinomas, correlation between CA IX and a constellation of proteins involved in angiogenesis, apoptosis inhibition, and cell−cell adhesion disruption has been observed, possibly contributing to strong relationship of this enzyme to a poor clinical outcome.[13] Hypoxia is linked with acidification of extracellular milieu that facilitates tumor invasion, and CA IX is believed to play a role in this process via its catalytic activity.[14] Thus, inhibition of this enzyme may constitute a novel approach to the treatment of cancers in which CA IX is expressed.

Acetazolamide, one of the best-studied, classical CAI used clinically, was on the other hand shown to function as a modulator in anticancer therapies, in combination with different cytotoxic agents (such as alkylating agents, nucleoside analogues, platinum derivatives, etc.) and to reduce the invasive capacity of several renal carcinoma cell lines (Caki-1, Caki-2, ACHN, and A-498).[15,16] Such valuable studies constitute a proof-of-concept demonstration that CAIs may be used in the management of tumors that overexpress one or more CA isozymes. It should also be mentioned that our group reported the design and in vitro antitumor activity of several classes of sulfonamide CA inhibitors, shown to act as nanomolar inhibitors against the classical isozymes known to possess critical physiological roles, such as CA I, CA II, and CA IV.[17−20] No data regarding CA IX inhibition with different types of sulfonamides are available up to now, although inhibition of this isozyme may be clinically exploited for designing novel anticancer therapies. However, a key issue for a possible cancer therapy approach by means of CA inhibition would be the selectivity of such compounds for different CA isozymes, to avoid or limit the side effects associated with this kind of therapy. In fact, this is also a major aim for improving the present treatment of CA-related diseases based on systemic or topical delivery of carbonic anhydrase inhibitors.[1,2]

On these premises, we report here the first CA IX inhibition study with a series of aromatic and heterocyclic sulfonamides derived from sulfanilamide and aminobenzolamide, as well as their inhibitory activity against the classical isozymes CA I, II and IV.

Results

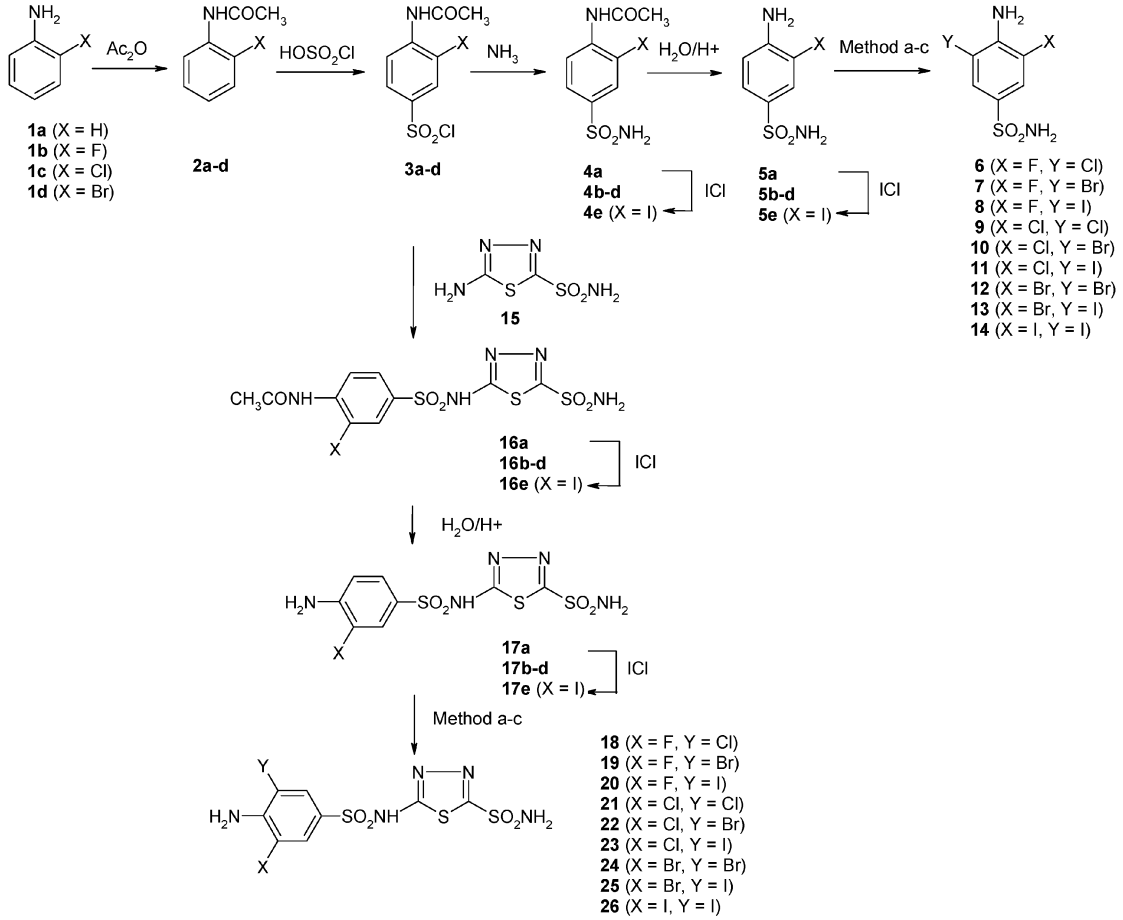
Chemistry. Synthesis of derivatives **4**−**26** investigated in detail here is shown in Scheme 1. These compounds were prepared by using classical procedures for the synthesis of sulfanilamide[21,22] or aminobenzolamide[23] derivatives, starting from the same key intermediates **3a**−**d**. All these compounds were characterized by standard analytical and spectroscopic methods, that confirmed the proposed structures.

CA Inhibition Data. Inhibition data against four CA isozymes, CA I, II, IV, and IX, with compounds **4**−**26** and standard sulfonamide CAIs are shown in Table 1.

Discussion

Chemistry. Two series of halogenosulfonamides were prepared, one derived from sulfanilamide (compounds **4**−**14**), the other one based on aminobenzolamide[23] as parent structure (compounds **16**−**26**) (Scheme 1). In the case of aromatic compounds **4**−**14** several methods of synthesis are available. In fact compounds **4**, **5**, **9**, **12**, and **14** were previously reported, being usually obtained by the direct halogenation of sulfanilamide, but their CA inhibition data were not reported up to now.[24−27] Reinvestigating these old synthetic methods, we found that they are reliable only for the preparation of the dihalogenated compounds **9**, **12**, and **14**. The monohalogenated products obtained via the old literature procedures[24−27] were always impurified with the dihalogenated derivatives. To obtain the pure monohalogenated sulfanilamides **5a**−**e** in large quantities and to avoid expensive purification steps, we opted for an alternative procedure, i.e., the classical synthesis of this type of derivatives, by chlorosulfonation of the corresponding acetanilides.[21,22,28] Pure chlorosulfonic acid was used as electrophile, due to the deactivation of the aromatic nucleus by the halogens present in intermediates **2a**−**d** (prepared from the corresponding anilines **1a**−**d** and acetic anhydride, Scheme 1). This procedure allowed the generation of the desired aromatic compounds **4**−**14**, but also the quick access to the halogenated aminobenzolamide derivatives via the key sulfonyl chlorides **3a**−**d**. In the case of the iodine-substituted derivatives, the reported literature method[27] was used in our syntheses too (Scheme 1). The original halogenation methods (involving the use of HCl/H_2O_2,[24,25] HBr/H_2O_2,[25] ICl/AcOH[27] as source of halogens) were used to further obtain the mixed halogenated derivatives **6**−**8**, **10**, **11**, **13**, **18**−**20**, **22**, **23**, and **25**, which are all new compounds (Scheme 1). It is noteworthy that the previously reported compounds were for the first time extensively characterized here by modern spectroscopic techniques. The heterocyclic compounds **16** were obtained by reaction of sulfonyl chlorides **3a**−**d** with 2-amino-1,3,4-thiadiazole-5-sulfonamide **15**,[29,30] in Schot-

Scheme 1. Synthesis of Halogenosulfonamides **4**−**26**[a]



[a] Method a: HCl/H_2O_2. Method b: HBr/H_2O_2. Method c: ICl/AcOH.

ten−Baumann conditions (Scheme 1).[31] After deprotection, the obtained derivatives **17** were halogenated with the standard methods described above, to yield the mixed halogenated aminobenzolamides **18**−**26**. Iodine derivatives **16e**, **17e**, and **26** were obtained each time by direct iodination of the nonhalogenated, parent compound (Scheme 1).

CA Inhibition. Isozyme I. As seen from data of Table 1, a very net difference of affinity between the aromatic derivatives **4**−**14** on one hand, and the heterocyclic ones **16**−**26** on the other one, is observed for this isozyme. Thus, the halogenosulfanilamides **4**−**14** show inhibition constants in the range of 3300−28000 nM, whereas the aminobenzolamides **16**−**26** are several orders of magnitude stronger inhibitors, with inhibition constants in the range of 1.0−10.2 nM. The 5-amino-1,3,4-thiadiazole-2-sulfonamide key intermediate **15** shows rather weak inhibitory properties against this isozyme, with a K_I of 8600 nM, resembling thus more the aromatic than the heterocyclic compounds mentioned above. For the aromatic compounds, the acetylated derivatives **4b**−**d** were more active than the corresponding deacetylated sulfonamides **5b**−**d**, whereas for the heterocylic subseries just the opposite is true, with the amines **17a**−**e** more active than the corresponding acetamido derivatives **16a**−**e**. The mixed halogen aminobenzolamides **18**−**26** were all very potent hCA I inhibitors—with inhibition constants in the range of 1.0−2.1 nM (as compared for example with the clinically used compounds **AAZ**, **MZA**, etc., showing constants in the range of 25−50000 nM), being in fact the most potent isozyme I inhibitors ever observed up to now. This may be rather relevant from the pharmacological point of view, although the physiological function of CA I is still unknown.[1,2]

Isozyme II. hCA II is generally considered the main therapeutic target of sulfonamide CAIs.[1,2] Thus, affinity of newly designed inhibitors for this isozyme is of critical relevance. As for isozyme I discussed above, again a net distinction is observed for the aromatic sulfonamides **4**−**14** on one hand, and the heterocyclic derivatives **16**−**26** on the other one, regarding their affinity for isozyme II. The aromatic derivatives **4**−**14** investigated here showed inhibition constants in the range of 25−300 nM, whereas aminobenzolamides **16**−**26** in the range of 0.2−3.1 nM (again **15** had a behavior similar to that of the sulfanilamides **4**−**14**, with a K_I of 60 nM). The same pattern of inhibition as for isozyme I has been observed for the acetylated/deacetylated compounds: thus, for the aromatic subseries the acetylated derivatives **4** were more inhibitory than the corresponding deacetylated compounds **5**, whereas for the heterocyclic subseries the opposite behavior was observed. The presence of the acetyl moieties or the different number of halogen atoms (as well as their nature) influenced only minimally hCA II inhibition with the aminobenzolamide derivatives **16**−**26**, as all these compounds behave as extremely potent inhibitors, much more effective than the clinically used compounds shown in Table 1. It should also be mentioned that whereas a large difference of affinity

Table 1. Inhibition Data with Compounds **4**−**26** and Standard, Clinically Used Inhibitors against Isozymes CA I, II, IV, and IX.

	K_I[a] (nM)			
inhibitor	hAC I[b]	hCA II[b]	bCA IV[c]	hCA IX[d]
AAZ	250	12	70	25
MZA	50	14	36	27
EZA	25	8	13	34
DCP	1200	38	380	50
DZA	50000	9	43	52
BRZ	nt	3	45	37
4b	4200	29	105	213
4c	6250	74	245	196
4d	3400	25	58	203
4e	3300	47	115	209
5a	28000	300	3000	294
5b	8300	60	180	245
5c	9800	110	320	264
5d	6500	40	66	269
5e	6000	70	125	285
6	3800	32	95	12
7	3900	33	113	69
8	3700	29	125	34
9	7000	50	165	27
10	7600	54	210	33
11	7800	61	200	63
12	9650	73	175	68
13	12500	107	250	86
14	14000	124	280	85
15	8600	60	540	41
16a	10.2	3.1	8.2	75
16b	5.7	1.2	4.0	79
16c	5.4	1.3	4.2	71
16d	5.0	1.5	4.6	78
16e	5.2	1.7	4.5	nt
17a	6.4	2.1	5.0	38
17b	3.1	0.8	2.5	46
17c	2.5	0.6	2.4	46
17d	1.9	0.7	2.4	47
17e	2.1	0.6	2.5	nt
18	1.4	0.3	2.0	40
19	1.0	0.2	1.9	27
20	1.7	0.3	2.1	21
21	1.1	0.5	1.9	24
22	1.3	0.6	2.0	23
23	1.6	0.8	2.7	nt
24	1.0	0.5	1.7	39
25	1.9	0.6	2.3	nt
26	2.1	0.9	2.3	41

[a] Errors in the range of 5−10% of the reported value (from three different assays). [b]Human cloned isozyme, by the esterase method. [c]Bovine isozyme, isolated from lung microsomes, by the esterase method. [d]Catalytic domain of human, cloned isozyme, by the CO_2 hydration method. nt = not tested.

for isozyme I versus isozyme II was observed for the aromatic derivatives **4**−**14** (which are generally 2 orders of magnitude more potent isozyme II inhibitors as compared to hCA I inhibitors), for the heterocyclic compounds **16**−**26** the affinities for the two isozymes are rather similar, with hCA II being only slightly more avid for the sulfonamides as compared to hCA I.

Isozyme IV. The membrane-bound isozyme CA IV showed a behavior rather similar to that of the cytosolic isozyme CA II in its interaction with the sulfonamides reported here. Thus, the sulfanilamides **4**−**14** showed inhibition constants in the range of 58−3000 nM, whereas the aminobenzolamides **16**−**26** in the range of 1.7−8.2 nM. It is clear from these data that these sulfonamides show an affinity for CA IV intermediate between that for CA I and CA II, respectively. For the aromatic subseries a more efficient inhibition was observed with the acetylated compounds **4** as compared to the corresponding deacetylated derivatives **5**, whereas in the heterocyclic subseries the opposite behavior was evidenced (the amino-derivatives **17** were better inhibitors than the corresponding acetamido derivatives **16**). For the monohalogenated sulfanilamides **4**, **5**, the nature of the halogen atom quite irregularly influenced activity (not only against CA IV, but against the previously mentioned isozymes too), generally with the bromo-derivatives more active than the fluoro-derivatives, which in turn were more active than the iodo-derivatives, which were more active than the chloro-derivatives, whereas the unhalogenated compounds showed the worst inhibitory properties. When two different halogens were present in these sulfanilamides, the behavior was quite complicated, but generally fluorine-containing compounds (such as **6**−**8**) showed best activity. The halogeno-aminobenzolamides **16**−**26** on the other hand showed very potent inhibitory properties against this isozyme, too, irrespective of the substitution pattern (nature and number of halogens), being much more effective bCA IV inhibitors as compared to the clinically used sulfonamides showed in Table 1.

Isozyme IX. It is a transmembrane isozyme with extracellular CA domain, similarly to CA IV, but possessing a catalytic activity more akin to that of CA II. This is the first detailed CA IX inhibition study with a series of sulfonamides. The inhibition profile of this isozyme with the aromatic/heterocyclic sulfonamides investigated here, as well as with the six clinically used compounds, is very different from that of the other isozymes (CA I, II, and IV). Thus, very potent inhibitors (K_Is less or around 40 nM) were detected among the aromatic compounds (such as **6**, **8**, **9**, and **10**) as well as among the heterocyclic derivatives (such as the clinically used compounds acetazolamide, methazolamide, ethoxzolamide and brinzolamide, as well as the investigated sulfonamides **15**, **17a**, **18**−**24**, and **26**). The best CA IX inhibitor detected up to now was to our greatest surprise the very simple derivative **6** (3-fluoro-5-chloro-4-aminobenzenesulfonamide, K_I of 12 nM) which is a two times more active compound as compared to the CAI par excellence, acetazolamide (K_I of 25 nM). The monohalogenated sulfanilamides (both acetylated and deacetylated) of types **4** and **5** generally behaved as moderate CA IX inhibitors, with inhibition constants in the range of 196−294 nM, whereas activity was greatly improved for the dihalogenated derivatives **6**−**14** (inhibition constants in the range of 12−86 nM). It is again quite surprising that **15** is a potent CA IX inhibitor (although it acts as a moderate CA II inhibitor, K_I of 60 nM), being practically as potent as aminobenzolamide **17a** (which is a very potent CA II inhibitor, K_I of 2 nM). The acetylated aminobenzolamides **16** were more ineffective CA IX inhibitors as compared to the corresponding deacetylated compounds **17**, whereas activity further increased for the dihalogenated aminobenzolamides **19**−**26** (which are all as potent as the most active clinically used inhibitors, **AAZ**, **MZA**, **EZA**, and **BRZ**). It is rather difficult at this point to understand how the nature and the number of the halogens substituting the aromatic ring in **16**−**26** influence CA IX inhibitory properties, but it seems that two halogens, of which at least one is fluorine or chlorine, produce the highest affinity of the

inhibitor for the active site. Since the CA IX activity is generally associated with tumors, the fact that the inhibition profile of CA IX is so different compared to that of the other isozymes, constitutes a positive feature for the possible design of CA IX-specific inhibitors to be used as a novel therapeutic approach against cancer.

Conclusion. A series of halogenated sulfanilamides and halogenated aminobenzolamides, incorporating one or two (equal or different) halogens has been obtained from the corresponding anilines. These compounds, together with the six clinically used sulfonamide CAIs (acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, and brinzolamide) were investigated as inhibitors of the transmembrane, tumor-associated isozyme CA IX. Inhibition data against the classical, physiologically relevant isozymes I, II, and IV were also obtained. CA IX shows an inhibition profile which is generally completely different from those of isozymes I, II and IV, with both simple aromatic (such as 3-fluoro-5-chloro-4-aminobenzenesulfonamide) and heterocyclic compounds (such as acetazolamide, methazolamide, 5-amino-1,3,4-thiadiazole-2-sulfonamide, aminobenzolamide, and dihalogenated aminobenzolamides) behaving as potent inhibitors (some of them showing inhibition constants in the range of 12−40 nM). Few compounds (such as **5a**, **8**, **11**, and **12**) show comparable inhibition constants for CA IX and CA II. This is the first detailed CA IX inhibition study, and many interesting leads were discovered so far, with the possibility to design even more potent and eventually CA IX-selective inhibitors, with putative applications as antitumor agents.

Experimental Section

General. Melting points: heating plate microscope (not corrected); IR spectra: KBr pellets, 400−4000 cm^{-1} Karl Zeiss Jena UR-20 spectrometer; NMR spectra: Varian Gemini 300BB apparatus, operating at 300 MHz for ^1H NMR and at 75 MHz for ^{13}C NMR (chemical shifts are expressed as δ values relative to Me_4Si as internal standard for proton spectra and to the solvent resonance for carbon-13 spectra). Attributions are given based on APT, COSY ($^1H-^1H$), and HETCOR ($^1H-^{13}C$) experiments. Elemental analysis (±0.4% of the theoretical values, calculated for the proposed structures): Carlo Erba Instruments CHNS Elemental Analyzer, Model 1106. All reactions were monitored by thin-layer chromatography (TLC), using 0.25 mm-thick precoated silica gel plates (E. Merck) eluted with MeOH:$CHCl_3$ 1:4 v/v unless specified otherwise. 2-Fluoroaniline, 2-chloroaniline, 2-bromoaniline, acetic anhydride, chlorosulfonic acid were from Merck or Aldrich Chemical Co. Acetonitrile, methanol, ethanol, chloroform, ethyl acetate (E. Merck, Darmstadt, Germany), or other solvents used in the synthesis were double distilled and kept on molecular sieves in order to maintain them in anhydrous conditions.

Chemistry. General Procedure for the Synthesis of Halogenosulfonamides 5a−d. Step 1. Synthesis of Acetanilides 2b−d. 2-Halogenoaniline **1** (0.1 mol) was treated under stirring and external cooling (ice bath) with 21 mL (0.2 mol) of acetic anhydride, and the mixture was then refluxed for 30 min. The excess of acetic anhydride and the resulted acetic acid were rotoevaporated under reduced pressure, affording the corresponding pure acetanilide **2b−d** (yield of 100%) as evidenced by TLC (acetone/petroleum ether: 3/7 v/v).

Step 2. Synthesis of Sulfonyl Chlorides 3b−d. The crude acetanilide **2b−d** (0.1 mol) obtained in the previous step was cooled to 0 °C (when crystallization occurred) and treated dropwise with chlorosulfonic acid (33.5 mL, 0.5 mol), under stirring (CAUTION: the solid dissolved and corrosive gases evolved!). When the addition was complete the cooling bath was replaced with an oil bath, and the resulted homogeneous mixture was heated under stirring at 70−85 °C for 3−4 h (70 °C/3 h for **2b**; 70−80 °C/3.5 h for **2c**; 80−85 °C/4 h for **2d**). The gas evolution ceased almost completely, and the mixture was cooled and poured on 200 g of crushed ice in a 400 mL beaker (WARNING: violent decomposition of the remained chlorosulfonic acid occurred; corrosive gases evolve!). The obtained sticky sulfonyl chloride was shaken vigorously in order to adhere to the walls of the beaker, and the aqueous phase was discarded. After a brief washing with ice-cold water and separation, the crude sulfonyl chloride was used directly in the next steps. The pure compound can be obtained by taking the sticky precipitate in chloroform, drying the chloroform solution over sodium sulfate, evaporation of the solvent, and recrystallization of the residue from benzene.

Step 3. Synthesis of 4-Acetamido-3-halogeno-benzenesulfonamides 4b−d. The crude sulfonyl chloride obtained in the previous step was carefully treated with 25 mL of NH_4OH (25%) when the sticky precipitate usually dissolved. The mixture was stirred for 30 min at 70 °C in the hood in order to remove excess ammonia (precipitation of the sulfonamide ammonium salt occurred). The desired sulfonamide was finally precipitated from the suspension with HCl 5%, filtered, washed with cold water, and air-dried. Overall yields (step 2 and step 3) ranged between 40 and 65%. The crude compounds were recrystallized from water or ethanol−water mixtures, with charcoal, to yield the pure title compounds.

Step 4. Deacetylation of 4-Acetamido-3-halogeno-benzenesulfonamides 4b−d. The acetyl halogenosulfanilamide **4b−d** was suspended in 50 mL of HCl (20%) and refluxed for 45 min. The resulted homogeneous solution was concentrated in a vacuum to one forth of the initial volume, cooled, and brought to pH = 4 when the corresponding halogenosulfanilamide **5b−d** precipitated. It was filtered, washed with cold water, and recrystallized from water or water−ethanol mixtures (with charcoal), affording the pure products in 70−90% yields.

General Procedures for Halogenation of Halogenosulfonamides. Method a. Chlorination of Halogenosulfonamides (adapted from ref 24). The appropriate halogenosulfonamide (10 mmol) was dissolved (suspended) in 30 mL of HCl (20%), heated at 50 °C, and treated with 30% hydrogen peroxide (1.1 mL, 10.5 mmol). The mixture was stirred 5 min at 50 °C, then heated slowly to 80 °C and kept at this temperature for 1 h. The desired product precipitated on cooling and was filtered, washed with cold water, and air-dried. In the case of fluorinated compounds, the yield can be increased by extracting the mother liquor with ethyl acetate, drying the organic layer over sodium sulfate and evaporation of the solvent to dryness. The crude products were recrystallized from ethanol 95% to generate the pure compounds in 30−67% yield.

Method b. Bromination of Halogenosulfonamides (adapted from ref 25). The appropriate halogenosulfonamide (10 mmol) was dissolved (suspended) in a solution obtained by dissolving 2 mL of HBr (62%) in 20 mL of water. The mixture was heated under stirring at 85 °C, then 30% hydrogen peroxide (1.1 mL, 10.5 mmol) was added and the suspension stirred for other 2 h at 85 °C. The suspension was filtered while hot, and the resulted compound was recrystallized from ethanol 95%. Yields ranged from 60 to 88%.

Method c. Iodination of Halogenosulfonamides (adapted from ref 27). Iodine monochloride (1.7 g, 10.5 mmol) was dissolved in 10 mL of glacial acetic acid, and the resulted solution was added dropwise to a solution/suspension of the appropriate halogenosulfonamide (10 mmol) in 25 mL of glacial acetic acid, preheated at 60 °C. The resulted solution was stirred for 2.5 h at 85−90 °C, then poured into boiling water (200 mL). The resulted precipitate was filtered immediately, washed with hot water, and recrystallized from ethanol 95%. The final product was washed with diethyl ether (5 mL) and dried. Yields ranged from 42 to 90%.

General Procedure for the Synthesis of Halogenoacetamidobenzolamides 16a−d.[31] 2-Amino-5-sulfonamido-

1,3,4-thiadiazole **15**[29,30] (5.4 g, 30 mmol) was dissolved in 16 mL of NaOH (2.5 N) and cooled to 10 °C. The appropriate sulfonyl cloride **3a**−**d** (6 mmol) was added to this solution, together with 3 mL of NaOH (5 N), and stirred at 10 °C until the entire sulfonyl chloride reacted; the procedure was repeated four times (30 mmol of sulfonyl chloride and 15 mL of NaOH (5 N) overall). After the last batch of sulfonyl chloride was dissolved, the solution was stirred for 2 h, brought to pH = 9 with concentrated aqueous ammonia, filtered, and acidified to pH = 2 with HCl 5%. The precipitated product was filtered, washed with cold water, and air-dried. Recrystallization from EtOH (50%) (with charcoal) afforded the desired pure compounds in 35−68% yield.

General Procedure for the Synthesis of Halogenoaminobenzolamides 17a−d.[31] The appropriate halogenoacetamidobenzolamide **16a**−**d** (10 mmol) was refluxed with 100 mL of HCl (20%) for 50 min. The homogeneous solution was evaporated to dryness, and the residue was taken in 30 mL of distilled water. The pH was adjusted to 9 (with 25% aqueous ammonia), and the resulted solution was filtered of any impurities and acidified to pH = 4 with glacial acetic acid. Cooling the solution overnight yielded the desired product, which was filtered, washed with cold water, and air-dried. Yields were in the range of 60−90%. Recrystallization from ethanol 95% (once or twice) generated the pure title compounds.

4-Acetamido-3-fluorobenzenesulfonamide **4b**: mp 203−205 °C (lit.[32] mp 203−204 °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 10.03 (s, 1H: CONH), 8.20 (dt, J = 8.3, 8.2, 0.8 Hz, 1H: H5), 7.64 (ddd, J = 10.8, 2.0, 0.8 Hz, 1H: H2), 7.61 (dd, J = 8.2, 2.0 Hz, 1H: H6), 7.41 (s, 2H: SO_2NH_2), 2.13 (s, 3H: CH_3); ^{13}C NMR (DMSO-d_6), δ, ppm: 169.27 (CO), 151.94 (d, $J_{Cipso-F}$ = 249.2 Hz, C3Ph), 139.63 (d, J_{Cm-F} = 5.8 Hz, C1Ph), 129.75 (d, J_{Co-F} = 11.3 Hz, C4Ph), 123.12 (C6Ph), 122.11 (d, J_{Cm-F} = 3.4 Hz, C5Ph), 113.11 (d, J_{Co-F} = 22.6 Hz, C2Ph), 23.73 (CH_3); IR (KBr), cm^{-1}: 1160, 1315, 1325, 1540, 1600, 1670, 3250, 3345; Anal. $C_8H_9FN_2O_3S$ (C, H, N).

4-Acetamido-3-chlorobenzenesulfonamide **4c**: mp 205−206 °C (lit.[26] mp 202 °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 9.74 (s, 1H: CONH), 8.02 (d, J = 8.5 Hz, 1H: H5), 7.87 (d, J = 2.1 Hz, 1H: H2), 7.73 (dd, J = 8.5, 2.1 Hz, 1H: H6), 7.43 (s, 2H: SO_2NH_2), 2.15 (s, 3H: CH_3); ^{13}C NMR (DMSO-d_6), δ, ppm: 169.09 (CO), 140.66 (C4Ph), 138.09 (C1Ph), 126.80 (C2Ph), 125.22 (C6Ph), 125.08 (C3Ph), 124.86 (C5Ph), 23.58 (CH_3); IR (KBr), cm^{-1}: 1165, 1310, 1350, 1520, 1675, 3280, 3570; Anal. $C_8H_9ClN_2O_3S$ (C, H, N).

4-Acetamido-3-bromobenzenesulfonamide **4d**: mp 206−208 °C (lit.[26] mp °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 9.65 (s, 1H: CONH), 8.04 (d, J = 2.1 Hz, 1H: H2), 7.88 (d, J = 8.5 Hz, 1H: H5), 7.78 (dd, J = 8.5, 2.1 Hz, 1H: H6), 7.46 (s, 2H: SO_2NH_2), 2.13 (s, 3H: CH_3); ^{13}C NMR (DMSO-d_6), δ, ppm: 168.98 (CO), 141.38 (C4Ph), 139.48 (C1Ph), 129.98 (C2Ph), 126.30 (C5Ph), 125.39 (C6Ph), 116.50 (C3Ph), 23.50 (CH_3); IR (KBr), cm^{-1}: 1170, 1300, 1360, 1520, 1680, 3290, 3580; Anal. $C_8H_9BrN_2O_3S$ (C, H, N).

4-Acetamido-3-iodobenzenesulfonamide **4e**: mp 216−217 °C (lit.[27] mp 216 °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 9.56 (s, 1H: CONH), 8.25 (d, J = 2.1 Hz, 1H: H2), 7.78 (dd, J = 8.5, 2.1 Hz, 1H: H6), 7.65 (d, J = 8.5 Hz, 1H: H5), 7.43 (s, 2H: SO_2NH_2), 2.10 (s, 3H: CH_3); ^{13}C NMR (DMSO-d_6), δ, ppm: 168.71 (CO), 142.74 (C4Ph), 141.92 (C1Ph), 136.12 (C2Ph), 126.59 (C5Ph), 125.94 (C6Ph), 95.18 (C3Ph), 23.39 (CH_3); IR (KBr), cm^{-1}: 1160, 1300, 1320, 1530, 1680, 3280, 3370; Anal. $C_8H_9IN_2O_3S$ (C, H, N).

4-Amino-3-fluorobenzenesulfonamide **5b**: mp 169 °C (lit.[32] mp 169−170.5 °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.37 (ddd, J = 11.6, 2.1, 1.1 Hz, 1H: H2), 7.33 (dd, J = 8.5, 2.1 Hz, 1H: H6), 7.07 (s, 2H: SO_2NH_2), 6.80 (dt, J = 8.5, 8.4, 1.1 Hz, 1H: H5), 5.69 (d, J = 4.0 Hz, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 148.73 (d, $J_{Cipso-F}$ = 240.2 Hz, C3Ph), 140.13 (d, J_{Co-F} = 13.0 Hz, C4Ph), 130.21 (d, J_{Cm-F} = 5.2 Hz, C1Ph), 123.05 (C6Ph), 114.72 (d, J_{Cm-F} = 4.7 Hz, C5Ph), 112.93 (d, J_{Co-F} = 20.8 Hz, C2Ph); IR (KBr), cm^{-1}: 1150, 1310, 1515, 1640, 3280, 3470; Anal. $C_6H_7FN_2O_2S$ (C, H, N).

4-Amino-3-chlorobenzenesulfonamide **5c**: mp 161−163 °C (lit.[26] mp 161−162 °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.60 (d, J = 2.1 Hz, 1H: H2), 7.44 (dd, J = 8.5, 2.1 Hz, 1H: H6), 7.08 (s, 2H: SO_2NH_2), 6.83 (d, J = 8.5 Hz, 1H: H5), 6.10 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 147.81 (C4Ph), 131.24 (C1Ph), 127.13 (C2Ph), 125.83 (C6Ph), 115.70 (C3Ph), 114.14 (C5Ph); IR (KBr), cm^{-1}: 1160, 1310, 1495, 1630, 3380, 3470; Anal. $C_6H_7ClN_2O_2S$ (C, H, N).

4-Amino-3-bromobenzenesulfonamide **5d**: mp 152−154 °C (lit.[26] mp 153 °C, lit.[33] mp 155 °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.76 (d, J = 2.1 Hz, 1H: H2), 7.48 (dd, J = 8.5, 2.1 Hz, 1H: H6), 7.09 (s, 2H: SO_2NH_2), 6.83 (d, J = 8.5 Hz, 1H: H5), 6.04 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 148.93 (C4Ph), 131.69 (C1Ph), 130.30 (C2Ph), 126.40 (C6Ph), 114.09 (C5Ph), 105.60 (C3Ph); IR (KBr), cm^{-1}: 1150, 1310, 1490, 1625, 3380, 3480; Anal. $C_6H_7BrN_2O_2S$ (C, H, N).

4-Amino-3-iodobenzenesulfonamide **5e**: mp 178−180 °C (lit.[27] mp 179−180 °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.95 (d, J = 2.1 Hz, 1H: H2), 7.49 (dd, J = 8.5, 2.2 Hz, 1H: H6), 7.05 (s, 2H: SO_2NH_2), 6.78 (d, J = 8.5 Hz, 1H: H5), 5.92 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 151.60 (C4Ph), 136.55 (C2Ph), 132.21 (C1Ph), 127.04 (C6Ph), 112.83 (C5Ph), 80.81 (C3Ph); IR (KBr), cm^{-1}: 1150, 1320, 1480, 1610, 3380, 3480; Anal. $C_6H_7IN_2O_2S$ (C, H, N).

4-Amino-5-chloro-3-fluorobenzenesulfonamide **6**: mp 173−174.5 °C (EtOH); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.62 (s, 1H: H6), 7.53 (d, J_{FH} = 10.6 Hz, 1H: H2), 7.34 (s, 2H: SO_2NH_2), 6.25 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 153.02 (d, $J_{Cipso-F}$ = 244.1 Hz, C3Ph), 141.34 (d, J_{Co-F} = 15.3 Hz, C4Ph), 134.16 (d, J_{Cm-F} = 6.8 Hz, C1Ph), 127.04 (C6Ph), 121.16 (d, J_{Cm-F} = 5.8 Hz, C5Ph), 115.71 (d, J_{Co-F} = 21.3 Hz, C2Ph); IR (KBr), cm^{-1}: 1145, 1315, 1620, 3340, 3425; Anal. $C_6H_6ClFN_2O_2S$ (C, H, N).

4-Amino-5-bromo-3-fluorobenzenesulfonamide **7**: mp 181−182.5 °C (EtOH); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.75 (d, J_{FH} = 1.3 Hz 1H: H5), 7.55 (d, J_{FH} = 10.8 Hz, 1H: H2), 7.34 (s, 2H: SO_2NH_2), 6.19 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 152.63 (d, $J_{Cipso-F}$ = 245.5 Hz, C3Ph), 142.45 (d, J_{Co-F} = 15.0 Hz, C4Ph), 134.81 (d, J_{Cm-F} = 6.2 Hz, C1Ph), 130.03 (C6Ph), 116.22 (d, J_{Co-F} = 21.4 Hz, C2Ph), 110.43 (d, J_{Cm-F} = 4.3 Hz, C5Ph); IR (KBr), cm^{-1}: 1140, 1315, 1620, 3330, 3420; Anal. $C_6H_6BrFN_2O_2S$ (C, H, N).

4-Amino-3-fluoro-5-iodobenzenesulfonamide **8**: mp 195−195.5 °C (EtOH); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.91 (br.s, 1H: H6), 7.52 (dd, J = 10.8, 1.8 Hz, 1H: H2), 7.30 (s, 2H: SO_2NH_2), 6.03 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 151.20 (d, $J_{Cipso-F}$ = 245.8 Hz, C3Ph), 144.71 (d, J_{Co-F} = 13.4 Hz, C4Ph), 135.24 (d, J_{Cm-F} = 6.3 Hz, C1Ph), 136.04 (C6Ph), 116.67 (d, J_{Co-F} = 21.5 Hz, C2Ph), 85.6 (C5Ph); IR (KBr), cm^{-1}: 1140, 1315, 1610, 3340, 3475; Anal. $C_6H_6FIN_2O_2S$ (C, H, N).

4-Amino-3,5-dichlorobenzenesulfonamide **9**: mp 207−209 °C (lit.[26] mp 203−205 °C, lit.[24] 205−205.5 °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.61 (s, 2H: H2,H6), 7.25 (s, 2H: SO_2NH_2), 6.31 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 144.20 (C4Ph), 131.30 (C1Ph), 125.71 (2C: C2,C6Ph), 116.96 (2C: C3,C5Ph); IR (KBr), cm^{-1}: 1165, 1310, 1610, 3340, 3430; Anal. $C_6H_6Cl_2N_2O_2S$ (C, H, N).

4-Amino-5-bromo-3-chlorobenzenesulfonamide **10**: mp 219−221 °C (EtOH); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.86 (d, J = 2.0 Hz, 1H: H6), 7.75 (d, J = 2.0 Hz, 1H: H2), 7.35 (s, 2H: SO_2NH_2), 6.28 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 149.11 (C4Ph), 136.04 (C1Ph), 132.95 (C6Ph), 130.32 (C2Ph), 120.74 (C3Ph), 110.5 (C5Ph); IR (KBr), cm^{-1}: 1150, 1310, 1610, 3330, 3410; Anal. $C_6H_6BrClN_2O_2S$ (C, H, N).

4-Amino-3-chloro-5-iodobenzenesulfonamide **11**: mp 216−218 °C (EtOH); ^{1}H NMR (DMSO-d_6), δ, ppm: 8.05 (d, J = 2.0 Hz, 1H: H6), 7.76 (d, J = 2.0 Hz, 1H: H2), 7.33 (s, 2H: SO_2NH_2), 6.06 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 151.34 (C4Ph), 139.32 (C6Ph), 137.14 (C1Ph), 131.01 (C2Ph), 119.18 (C3Ph), 87.54 (C5Ph); IR (KBr), cm^{-1}: 1150, 1310, 1610, 3335, 3430; Anal. $C_6H_6ClIN_2O_2S$ (C, H, N).

4-Amino-3,5-dibromobenzenesulfonamide **12**: mp 239−240 °C (lit.[25] mp 237 °C, lit.[26] mp 239−240 °C); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.79 (s, 2H: H2,H6), 7.25 (s, 2H: SO_2NH_2), 6.10

(s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 145.83 (C4Ph), 132.66 (C1Ph), 129.48 (2C: C2,C6Ph), 106.11 (2C: C3,C5Ph); IR (KBr), cm^{-1}: 1150, 1315, 1610, 3340, 3430; Anal. $C_6H_6Br_2N_2O_2S$ (C, H, N).

4-Amino-3-bromo-5-iodobenzenesulfonamide **13**: mp 233−235 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.08 (s, 1H: H6), 7.91 (s, 1H: H2), 7.33 (s, 2H: SO_2NH_2), 5.98 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 151.92 (C4Ph), 139.94 (C6Ph), 137.62 (C1Ph), 134.20 (C2Ph), 108.53 (C3Ph), 85.44 (C5Ph); IR (KBr), cm^{-1}: 1150, 1310, 1610, 3330, 3420; Anal. $C_6H_6BrIN_2O_2S$ (C, H, N).

4-Amino-3,5-diiodobenzenesulfonamide **14**: mp 274−276 °C (lit.[27] mp 265 °C); ^1H NMR (DMSO-d_6), δ, ppm: 7.99 (s, 2H: H2,H6), 7.20 (s, 2H: SO_2NH_2), 5.71 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 149.92 (C4Ph), 136.57 (2C: C2,C6Ph), 134.33 (C1Ph), 79.80 (2C: C3,C5Ph); IR (KBr), cm^{-1}: 1160, 1310, 1610, 3320, 3340, 3400; Anal. $C_6H_6I_2N_2O_2S$ (C, H, N).

5-(4-Acetamido-3-fluorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **16b**: mp 223−224 °C; ^1H NMR (DMSO-d_6), δ, ppm: 10.09 (s, 1H: CONH), 8.50 (s, 3H: SO_2NH_2 + SO_2NH), 8.28 (m, 1H: H5), 7.68 (dd, J = 10.6, 2.1 Hz, 1H: H2), 7.65 (dd, J = 8.1, 2.1 Hz, 1H: H6), 2.14 (s, 3H: CH_3); ^{13}C NMR (DMSO-d_6), δ, ppm: 169.33 (CO), 167.72 (C5 thiadiazole), 158.16 (C2 thiadiazole), 151.77 (d, $J_{Cipso-F}$ = 249.6 Hz, C3Ph), 136.09 (d, J_{Cm-F} = 6.0 Hz, C1Ph), 130.83 (d, J_{Co-F} = 11.1 Hz, C4Ph), 122.92 (C6Ph), 122.62 (d, J_{Cm-F} = 3.3 Hz, C5Ph), 113.36 (d, J_{Co-F} = 22.6 Hz, C2Ph), 23.76 (CH_3); IR (KBr), cm^{-1}: 1160, 1310, 1330, 1510, 1560, 1605, 1680, 3350; Anal. $C_{10}H_{10}FN_5O_5S_3$ (C, H, N).

5-(4-Acetamido-3-chlorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **16c**: mp 236−238 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 9.74 (s, 1H: CONH), 8.49 (s, 3H: SO_2NH_2+ SO_2NH), 8.08 (d, J = 8.6 Hz, 1H: H5), 7.86 (d, J = 2.1 Hz, 1H: H2), 7.77 (dd, J = 8.6, 2.1 Hz, 1H: H6), 2.15 (s, 3H: CH_3); ^{13}C NMR (DMSO-d_6), δ, ppm: 169.22 (CO), 167.90 (C5 thiadiazole), 158.25 (C2 thiadiazole), 139.08 (C4Ph), 137.44 (C1Ph), 126.94 (C2Ph), 125.32 (C6Ph), 125.14 (C3Ph), 125.03 (C5Ph), 23.69 (CH_3); IR (KBr), cm^{-1}: 1155, 1300, 1310, 1510, 1580, 1685, 3330; Anal. $C_{10}H_{10}ClN_5O_5S_3$ (C, H, N).

5-(4-Acetamido-3-bromobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **16d**: mp 242−244 °C (EtOH/H2O); ^1H NMR (DMSO-d_6), δ, ppm: 9.65 (s, 1H: CONH), 8.49 (s, 3H: SO_2NH_2+ SO_2NH), 8.00 (d, J = 2.0 Hz, 1H: H2), 7.94 (d, J = 8.6 Hz, 1H: H5), 7.81 (dd, J = 8.6, 2.1 Hz, 1H: H6), 2.13 (s, 3H: CH_3); ^{13}C NMR (DMSO-d_6), δ, ppm: 169.03 (CO), 167.92 (C5 thiadiazole), 158.26 (C2 thiadiazole), 140.43 (C4Ph), 138.13 (C1Ph), 130.03 (C2Ph), 126.08 (C5Ph), 125.80 (C6Ph), 116.25 (C3Ph), 23.58 (CH_3); IR (KBr), cm^{-1}: 1150, 1300, 1325, 1515, 1580, 1680, 3340, 3370; Anal. $C_{10}H_{10}BrN_5O_5S_3$ (C, H, N).

5-(4-Acetamido-3-iodobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **16e**: mp 249−250.5 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 9.57 (s, 1H: CONH), 8.48 (s, 3H: SO_2NH_2+ SO_2NH), 8.22 (d, J = 2.1 Hz, 1H: H2), 7.81 (dd, J = 8.6, 2.1 Hz, 1H: H6), 7.72 (d, J = 8.6 Hz, 1H: H5), 2.11 (s, 3H: CH_3); ^{13}C NMR (DMSO-d_6), δ, ppm: 168.81 (CO), 167.86 (C5 thiadiazole), 158.21 (C2 thiadiazole), 142.81 (C4Ph), 140.50 (C1Ph), 136.19 (C2Ph), 126.58 (C6Ph), 126.33 (C5Ph), 96.06 (C3Ph), 23.46 (CH_3); IR (KBr), cm^{-1}: 1160, 1300, 1320, 1510, 1575, 1680, 3370; Anal. $C_{10}H_{10}IN_5O_5S_3$ (C, H, N).

5-(4-Amino-3-fluorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **17b**: mp 231−233 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.48 (s, 3H: SO_2NH_2+ SO_2NH), 7.37 (dt, J = 11.0, 2.1, 1.7 Hz, 1H: H2), 7.25 (dd, J = 8.4, 2.1 Hz, 1H: H6), 6.83 (dt, J = 8.5, 8.4, 1.7 Hz, 1H: H5), 6.10 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 166.59 (C5 thiadiazole), 157.68 (C2 thiadiazole), 150.43 (d, $J_{Cipso-F}$ = 240.4 Hz, C3Ph), 141.30 (d, J_{Co-F} = 12.9 Hz, C4Ph), 126.13 (d, J_{Cm-F} = 5.3 Hz, C1Ph), 123.73 (C6Ph), 114.90 (d, J_{Cm-F} = 4.7 Hz, C5Ph), 113.08 (d, J_{Co-F} = 20.8 Hz, C2Ph); IR (KBr), cm^{-1}: 1150, 1315, 1510, 1580, 1620, 3390, 3480; Anal. $C_8H_8FN_5O_4S_3$ (C, H, N).

5-(4-Amino-3-chlorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **17c**: mp 245−245.5 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.47 (s, 3H: SO_2NH_2+ SO_2NH), 7.57 (d, J = 2.1 Hz, 1H: H2), 7.47 (dd, J = 8.6, 2.2 Hz, 1H: H6), 6.86 (d, J = 8.6 Hz, 1H: H5), 6.27 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 166.61 (C5 thiadiazole), 157.72 (C2 thiadiazole), 148.92 (C4Ph), 127.33 (C1Ph), 127.28 (C2Ph), 126.33 (C6Ph), 115.85 (C3Ph), 114.41 (C5Ph); IR (KBr), cm^{-1}: 1160, 1310, 1500, 1580, 1620, 3360, 3430; Anal. $C_8H_8ClN_5O_4S_3$ (C, H, N).

5-(4-Amino-3-bromobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **17d**: mp 253.5−255 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.46 (s, 3H: SO_2NH_2+ SO_2NH), 7.70 (d, J = 2.1 Hz, 1H: H2), 7.49 (dd, J = 8.6, 2.2 Hz, 1H: H6), 6.84 (d, J = 8.6 Hz, 1H: H5), 6.23 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 166.66 (C5 thiadiazole), 157.71 (C2 thiadiazole), 149.99 (C4Ph), 130.42 (C2Ph), 127.80 (C1Ph), 126.85 (C6Ph), 114.30 (C5Ph), 105.59 (C3Ph); IR (KBr), cm^{-1}: 1150, 1315, 1510, 1580, 1620, 3350, 3400; Anal. $C_8H_8BrN_5O_4S_3$ (C, H, N).

5-(4-Amino-3-iodobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **17e**: mp 260−261 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.45 (s, 3H: SO_2NH_2+ SO_2NH), 7.94 (d, J = 2.2 Hz, 1H: H2), 7.49 (dd, J = 8.6, 2.2 Hz, 1H: H6), 6.79 (d, J = 8.6 Hz, 1H: H5), 6.10 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 166.63 (C5 thiadiazole), 157.69 (C2 thiadiazole), 157.70 (C4Ph), 135.87 (C2Ph), 128.20 (C1Ph), 127.43 (C6Ph), 112.87 (C5Ph), 80.92 (C3Ph); IR (KBr), cm^{-1}: 1160, 1315, 1520, 1575, 1620, 3360, 3430; Anal. $C_8H_8IN_5O_4S_3$ (C, H, N).

5-(4-Amino-5-chloro-3-fluorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **18**: mp 230−232 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.43 (s, 3H: SO_2NH_2 + SO_2NH), 7.84 (br.s, 1H: H6), 7.31 (dd, J = 10.5, 1.7 Hz, 1H: H2), 4.96 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 169.19 (C5 thiadiazole), 157.58 (C2 thiadiazole), 148.81 (d, $J_{Cipso-F}$ = 239.0 Hz, C3Ph), 140.39 (d, J_{Co-F} = 12.8 Hz, C4Ph), 136.82 (d, J_{Cm-F} = 13.8 Hz, C5Ph), 128.50 (d, J_{Cm-F} = 4.8 Hz, C1Ph), 12123.48 (C6Ph), 113.14 (d, J_{Co-F} = 20.5 Hz, C2Ph); IR (KBr), cm^{-1}: 1150, 1315, 1520, 1580, 1620, 3350, 3460; Anal. $C_8H_7ClFN_5O_4S_3$ (C, H, N).

5-(4-Amino-5-bromo-3-fluorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **19**: mp 252−254 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.46 (s, 3H: SO_2NH_2+ SO_2NH), 7.61 (dd, J = 2.0, 1.2 Hz 1H: H6), 7.46 (d, J = 10.5, 2.0 Hz, 1H: H2), 6.27 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 167.24 (C5 thiadiazole), 157.94 (C2 thiadiazole), 148.58 (d, $J_{Cipso-F}$ = 244.7 Hz, C3Ph), 139.45 (d, J_{Co-F} = 14.6 Hz, C4Ph), 127.15 (d, J_{Cm-F} = 5.8 Hz, C1Ph), 126.24 (C6Ph), 112.32 (d, J_{Co-F} = 21.5 Hz, C2Ph), 106.37 (d, J_{Cm-F} = 4.8 Hz, C5Ph); IR (KBr), cm^{-1}: 1160, 1320, 1520, 1575, 1620, 3340, 3430; Anal. $C_8H_7BrFN_5O_4S_3$ (C, H, N).

5-(4-Amino-3-fluoro-5-iodobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **20**: mp 242−244 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.45 (s, 3H: SO_2NH_2+ SO_2NH), 7.76 (dd, J = 2.0, 1.2 Hz, 1H: H6), 7.44 (dd, J = 10.6, 2.0 Hz, 1H: H2), 6.08 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 167.23 (C5 thiadiazole), 157.88 (C2 thiadiazole), 147.20 (d, $J_{Cipso-F}$ = 245.2 Hz, C3Ph), 141.81 (d, J_{Co-F} = 13.1 Hz, C4Ph), 132.25 (d, $J_{Cpara-F}$ = 2.4 Hz, C6Ph), 128.45 (d, J_{Cm-F} = 6.0 Hz, C1Ph), 112.86 (d, J_{Co-F} = 21.5 Hz, C2Ph), 81.67 (d, J_{Cm-F} = 2.8 Hz, C5Ph); IR (KBr), cm^{-1}: 1150, 1315, 1520, 1570, 1620, 3340, 3460;Anal. $C_8H_7FIN_5O_4S_3$ (C, H, N).

5-(4-Amino-3,5-dichlorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **21**: mp 286−288 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.50 (s, 3H: SO_2NH_2 + SO_2NH), 7.62 (s, 2H: H2,H6), 6.50 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 167.25 (C5 thiadiazole), 157.97 (C2 thiadiazole), 145.20 (C4Ph), 127.78 (C1Ph), 125.94 (2C: C2,C6Ph), 117.20 (2C: C3,-C5Ph); IR (KBr), cm^{-1}: 1160, 1325, 1515, 1580, 1620, 3400, 3500; Anal. $C_8H_7Cl_2N_5O_4S_3$ (C, H, N).

5-(4-Amino-5-bromo-3-chlorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **22**: mp 263−265 °C (EtOH); ^1H NMR (DMSO-d_6), δ, ppm: 8.49 (s, 3H: SO_2NH_2+ SO_2NH), 7.73 (d, J = 2.0 Hz, 1H: H6), 7.64 (d, J = 2.0 Hz, 1H: H2), 6.39 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 167.28 (C5 thiadiazole), 157.97 (C2 thiadiazole), 146.05 (C4Ph), 129.10 (C6Ph), 128.54 (C1Ph), 126.48 (C2Ph), 116.90 (C3Ph), 106.60 (C5Ph);

IR (KBr), cm^{-1}: 1160, 1320, 1520, 1580, 1620, 3360, 3450; Anal. $C_8H_7BrClN_5O_4S_3$ (C, H, N).

5-(4-Amino-3-chloro-5-iodobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **23**: mp 269−271 °C (EtOH); ^{1}H NMR (DMSO-d_6), δ, ppm: 8.48 (s, 3H: SO_2NH_2+ SO_2NH), 7.88 (d, J = 2.0 Hz, 1H: H6), 7.65 (d, J = 2.0 Hz, 1H: H2), 6.28 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 167.27 (C5 thiadiazole), 157.90 (C2 thiadiazole), 148.31 (C4Ph), 135.21 (C6Ph), 129.41 (C1Ph), 126.93 (C2Ph), 115.50 (C3Ph), 84.82 (C5Ph); IR (KBr), cm^{-1}: 1160, 1315, 1520, 1575, 1620, 3340, 3460; Anal. $C_8H_7ClIN_5O_4S_3$ (C, H, N).

5-(4-Amino-3,5-dibromobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **24**: mp 283−284 °C (EtOH); ^{1}H NMR (DMSO-d_6), δ, ppm: 8.46 (s, 3H: SO_2NH_2 + SO_2NH), 7.77 (s, 2H: H2,H6), 6.25 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 167.32 (C5 thiadiazole), 157.95 (C2 thiadiazole), 146.77 (C4Ph), 129.63 (2C: C2,C6Ph), 129.28 (C1Ph), 106.27 (2C: C3,-C5Ph); IR (KBr), cm^{-1}: 1150, 1325, 1515, 1580, 1615, 3380, 3485; Anal. $C_8H_7Br_2N_5O_4S_3$ (C, H, N).

5-(4-Amino-3-bromo-5-iodobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **25**: mp 273−275 °C (EtOH); ^{1}H NMR (DMSO-d_6), δ, ppm: 8.48 (s, 3H: SO_2NH_2+ SO_2NH), 7.91 (s, 1H: H6), 7.80 (s, 1H: H2), 6.19 (v.br.s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 167.25 (C5 thiadiazole), 157.87 (C2 thiadiazole), 148.94 (C4Ph), 135.77 (C6Ph), 129.88 (C1Ph), 129.83 (C2Ph), 105.02 (C3Ph), 82.61 (C5Ph); IR (KBr), cm^{-1}: 1150, 1320, 1520, 1580, 1620, 3360, 3480; Anal. $C_8H_7BrIN_5O_4S_3$ (C, H, N).

5-(4-Amino-3,5-diiodobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **26**: mp 288−290 °C (EtOH); ^{1}H NMR (DMSO-d_6), δ, ppm: 8.47 (s, 3H: SO_2NH_2+ SO_2NH), 7.95 (v.br.s, 2H: H2,H6), 5.89 (s, 2H: NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm: 167.04 (C5 thiadiazole), 157.90 (C2 thiadiazole), 150.92 (C4Ph), 136.64 (2C: C2,C6Ph), 130.78 (C1Ph), 79.97 (2C: C3,C5Ph); IR (KBr), cm^{-1}: 1145, 1320, 1515, 1580, 1595, 3365, 3475; Anal. $C_8H_7I_2N_5O_4S_3$ (C, H, N).

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.[34] Cell growth conditions were those described in ref 35, and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[36] 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.[37,38] bCA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide.[39]

The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[7a]) 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.[40] The homogenate thus obtained was extensively centrifuged (11000*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 HCl, 100 mM Na_2SO_4, and 1 mM $ZnCl_2$. The amount of protein was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate.[43]

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.[41] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25 °C. A molar absorption coefficient ϵ of 18400 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.[41] 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, II, and IV.[41] Enzyme concentrations were 3.3 μM for CA II, 10.7 μM for CA I and 23 μM for CA IV (this isozyme has a decreased esterase activity [42] and higher concentrations had to be used for the measurements).

An SX.18MV−R Applied Photophysics stopped-flow instrument has been used for assaying the CA IX CO_2 hydration activity.[43] 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. 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 μM, and inhibition constants were calculated as described in ref 43.

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