Carbonic Anhydrase Inhibitors. Design of Selective, Membrane-Impermeant Inhibitors Targeting the Human Tumor-Associated Isozyme IX

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A series of positively charged sulfonamides were obtained by reaction of aminobenzolamide [5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide] with tri-/tetrasubstituted pyrilium salts possessing alkyl-, aryl- or combinations of alkyl and aryl groups at the pyridinium ring. The new compounds reported here were assayed for the inhibition of four physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isozymes: the cytosolic hCA I and II, the membrane-anchored bCA IV, and the membrane-bound, tumor-associated isozyme hCA IX. They showed potent inhibitory activity against all investigated isozymes, although with different profiles. For CA I the new derivatives showed inhibition constants in the range of 3−12 nM, for CA II in the range of 0.20−5.96 nM, against CA IV in the range of 2.0−10.3 nM, and against CA IX in the range of 3−45 nM, respectively. These new compounds are membrane-impermeant due to their salt-like character. Some of these derivatives were also tested for their inhibitory activity against the Cl^-/HCO_3^- anion exchanger AE1: two derivatives showed inhibitory activity in the low micromolar range, whereas one compound was inactive at these concentrations. The high affinity of these new derivatives for the tumor-associated isozyme CA IX and their membrane impermeability make this type of CA inhibitor interesting candidates for the selective inhibition of only the tumor-associated isozyme and not the cytosolic ones, for which they also show high potency. Furthermore, we prove here for the first time that the CA−AE metabolon can be inhibited by the same type of sulfonamide derivative.

Introduction

There are many connections between the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) and cancer; for example, some CA isozymes (CA IX and XII) are predominantly found in cancer cells but are lacking from their normal counterparts.[1−3] Teicher et al.[4] reported that acetazolamide **1**—the prototypical CA inhibitor (CAI)—functions as a modulator in anticancer therapies, in combination with different cytotoxic agents, such as alkylating agents, nucleoside analogues, and platinum derivatives. It was hypothesized that the anticancer effects of acetazolamide (alone or in combination with such drugs) might be due to the acidification of the intratumoral environment ensued after CA inhibition, although other mechanisms of action of this drug were not excluded.[3] Chegwidden et al.[3] hypothesized that the in vitro inhibition of growth in cell cultures of human lymphoma cells with two other potent, clinically used sulfonamide CAIs, methazolamide (**2**) and ethoxzolamide (**3**), is probably due to a reduced provision of bicarbonate for nucleotide synthesis (HCO_3^- is the substrate of carbamoyl phosphate synthetase II) as a consequence of CA inhibition.[3] All six classical CAIs **1**−**6** used in clinical medicine or as diagnostic tools show some tumor growth inhibitory properties,[5,6] whereas indisulam (E7070) **7**, a compound in phase II clinical trials as an antitumor drug, shows a complex mechanism of action also involving CA inhibition of several isozymes involved in tumorigenesis, such as CA II and CA IX, among others (Chart 1).[5−8] The development of CAIs possessing potent tumor cell growth inhibitory properties was then reported by this group, and such sulfonamides were initially tested only as inhibitors of isozymes I, II, and IV.[9]

Most of the presently available compounds are systemically acting CAIs, showing several undesired side effects due to inhibition of many of the different CA isozymes present in the target tissues/organs (14 isoforms are presently known in humans).[6,7] Therefore, many attempts to design and synthesize novel classes of CAIs were recently reported, to avoid such side effects.[6,7] At least four CA isozymes (CA IV, CA IX, CA XII, and CA XIV) are associated with cell membranes, with the enzyme active site generally oriented extracellularly.[6,7] Some of these isozymes were shown to play pivotal physiological roles (such as CA IV and XII in the eye, lungs, and kidneys and CA IX in the gastric mucosa and many tumor cells),[1−3] whereas the function of other such isozymes (CA XIV) is for the moment less well understood.[6] Due to the extracellular location of these isozymes, it would be possible to design membrane-impermeant CA inhibitors (CAIs), which in this way

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Chart 1

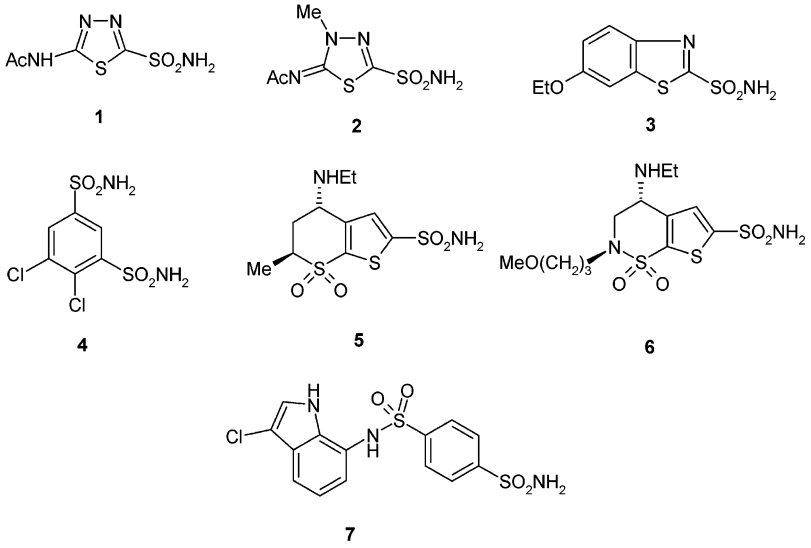
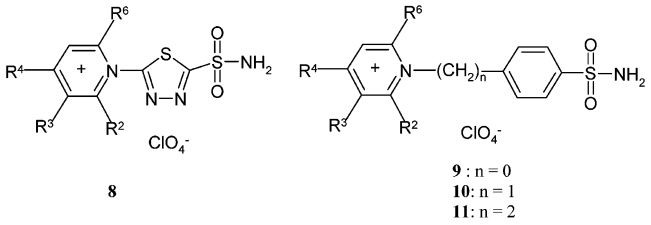


Chart 2



become specific inhibitors for the membrane-associated CAs. This possibility has been recently explored in this laboratory, by designing positively charged sulfonamides,[10,11] whereas an alternative approach consisted of designing polymeric (high molecular weight) inhibitors, but such compounds were not very useful in vivo due to the usual problems connected with polymers (i.e., allergic reactions and problems of bioavailability).[12]

A program of developing cationic sulfonamides has been initiated in our laboratory with compounds of types **8**−**11** (Chart 2) prepared by reaction of aromatic/heterocyclic sulfonamides containing free NH_2 groups with pyrylium salts, affording pyridinium derivatives. The inhibitors **8**−**11** obtained in this way showed nanomolar affinities both for CA II, as well as CA IV, and more importantly, they were unable to cross the plasma membranes in vivo.[10] In two model systems (human red blood cells and perfusion experiments in rats, respectively), this new class of potent, positively charged CAIs was able to discriminate between the membrane-bound versus the cytosolic isozymes, selectively inhibiting only CA IV (the only membrane-bound isozyme present in the investigated systems).[10] Such data constituted the proof-of-concept for the specific in vivo inhibition of membrane-associated CA isozymes but also for the eventual development of novel anticancer therapies, since—as mentioned above—it has been shown that some tumor cells predominantly express only some membrane-associated CA isozymes, such as CA IX and CA XII.[1,6,7] This type of selective CAI may also be of great relevance for different physiological studies. For example, Sterling et al.[13] investigated the functional and physical relationship between the downregulated in adenoma bicarbonate transporter and CA II, by using membrane-impermeant inhibitors of type **8** (in addition to the classical inhibitors such as acetazolamide), which could clearly discriminate between the contribution of the cytosolic and membrane-associated isozymes in these physiological processes.

Up to now no CA IX inhibition studies with membrane-impermeant CAIs have been reported. Thus, we decided to design novel pyridinium derivatives incorporating sulfamoyl groups in their molecule and to explore their interaction with the tumor-associated isozyme IX, recently cloned and purified by this group, as well as the cytosolic, physiologically relevant isozymes CA I II and the membrane-anchored isozyme CA IV.[14,15]

Results

Chemistry. Reaction of aminobenzolamide [5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide,[11] **12**] with 2,6-di-, 2,4,6-tri-, or 2,3,4,6-tetrasubstituted pyrylium salts **13** afforded the pyridinium salts **14** investigated here, by the general synthesis of such derivatives with nucleophiles (Scheme 1).[16]

CA Inhibition. Inhibition data against isozymes I, II, IV, and IX with compounds **14** reported here are shown in Table 1.[17−20]

Ex Vivo Penetration through Red Blood Cells. Levels of sulfonamides in red blood cells after incubation of human erythrocytes with millimolar solutions of inhibitor for 30−60 min (both classical as well as positively charged sulfonamides were used in such experiments) are shown in Table 2.[21−31]

Inhibition of AE1. The rate of the Cl^-/NO_3^- exchange measured for AE1-transfected cells, before and after treatment with several CAIs synthesized here or without subsequent CAI treatment (control), and the relative rate of transport measured in the presence of a range of CAI concentrations are shown in Table 3 and Figures 1 and 2.[32−35]

Discussion

Chemistry. A large number of positively charged sulfonamides, prepared by reaction of amino sulfonamides with pyrylium salts, of types **8**−**11**, were recently

Scheme 1

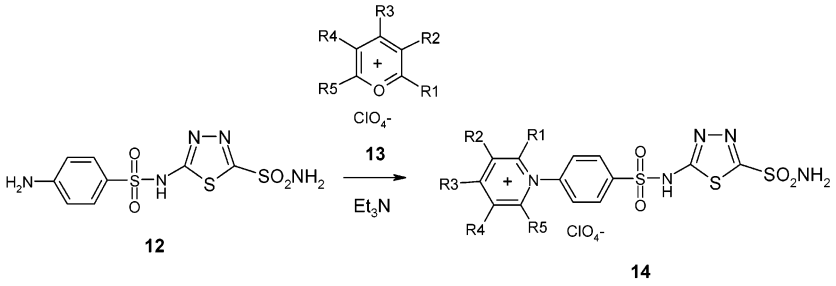
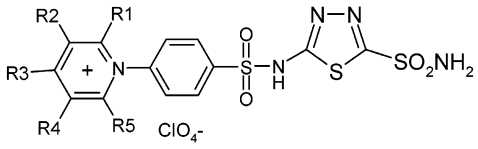


Table 1. Inhibition of Isozymes hCA I,[a] hCA II,[a] bCA IV,[b] and hCA IX[c] with the Pyridinium Salts **14**



14

						K_1[d] (nM)			
14	R^1	R^2	R^3	R^4	R^5	hCA I[a]	hCA II	bCA IV	hCA IX
a	Me	H	Me	H	Me	4	0.26	2.1	3
b	*i*-Pr	H	Me	H	Me	4	0.39	3.0	5
c	*i*-Pr	H	Me	H	*i*-Pr	7	1.54	4.7	16
d	*t*-Bu	H	Me	H	*t*-Bu	11	3.13	9.4	34
e	Me	H	Ph	H	Me	3	0.20	2.0	6
f	Et	H	Ph	H	Et	4	0.21	2.3	9
g	*n*-Pr	H	Ph	H	*n*-Pr	9	3.45	8.1	35
h	*n*-Bu	H	Ph	H	*n*-Bu	10	4.62	10.3	40
i	*i*-Pr	H	Ph	H	*i*-Pr	5	1.61	4.1	30
j	Me	H	Ph	H	Ph	4	1.21	3.0	24
k	Et	H	Ph	H	Ph	5	1.14	3.8	29
m	*n*-Pr	H	Ph	H	Ph	8	3.90	6.0	40
n	*i*-Pr	H	Ph	H	Ph	6	3.74	4.5	32
o	*n*-Bu	H	Ph	H	Ph	8	4.95	8.4	45
p	*t*-Bu	H	Ph	H	Ph	12	4.11	7.0	43
q	Ph	H	Me	H	Ph	6	4.78	5.8	12
r	Ph	H	Ph	H	Ph	5	5.96	5.6	12
s	Ph	H	H	H	Ph	5	4.93	5.4	16
t	Me	Me	Me	H	Me	3	0.30	2.4	5
u	Me	Me	Ph	H	Me	3	1.24	5.2	15
v	Me	R^3,R^5 = $-(CH_2)_9-$		H	Me	3	1.37	4.6	12
aminobenzolamide, **12**						6	2.04	5.1	38
acetazolamide, **1**						250	12	70	25
methazolamide, **2**						50	14	36	27
dichlorophenamide, **4**						1200	38	380	50
indisulam, **7**						30	15	65	24

[a] Human (cloned) isozymes, esterase (4-nitrophenyl acetate as substrate) assay method.[17] [b] From bovine lung microsomes, esterase (4-nitrophenyl acetate as substrate) assay method.[17] [c] Catalytic domain of the human, cloned isozyme, CO_2 hydrase assay method.[18] [d] Errors in the range of ±10% of the reported value, from three different determinations.

reported by this group and generally tested as inhibitors of the "classical" isozymes CA I, II, and IV.[10,11] On the basis of QSAR studies on several series of CA inhibitors, including some positively charged derivatives of type **8**,[19] it emerged that the enhancement of CA inhibitory activity is correlated with increased positive charges on the heterocyclic/aromatic ring incorporated in such molecules, as well as with "long" inhibitor molecules per se (i.e., molecules extending on the direction passing through the Zn(II) ion of the enzyme, the sulfonamide nitrogen atom, and the long axis of the inhibitor).[19] It appeared thus of interest to try to explore this result, designing positively charged, long, sulfonamide CAIs. Thus, we thought of attaching substituted-pyridinium moieties to an already potent and long CAI molecule suitable for reaction with pyrylium salts **13**, i.e., aminobenzolamide **12**.[11] Indeed, this compound acts as a very potent CAI against isozymes I, II, and IV (with inhibition constants in the low nanomolar range; see later in the text). The substitution pattern of the pyridinium ring was previously shown[10,11] to be critical for the biological activity of this type of sulfonamide CAIs. Thus, a large series of of 2,4,6-trialkylpyridinium, 2,6-dialkyl-4-phenylpyridinium, 2-alkyl-4,6-diphenylpyridinium, 2,4,6-triphenylpyridinium, various 2,6-disubstituted-pyridinium, and 2,3,5,6-tetrasubstituted-pyridinium aminobenzolamide derivatives of type **14** have been prepared by the reaction described in Scheme 1.

Although apparently simple, the reaction between a pyrylium salt and an amine, leading to pyridinium salts, is in reality a complicated process (Scheme 2), as established by detailed spectroscopic and kinetic data from Balaban's and Katritzky's groups.[16] Thus, the nucleophilic attack of a primary amine RNH_2 on pyrylium cations generally occurs in the α position, with the

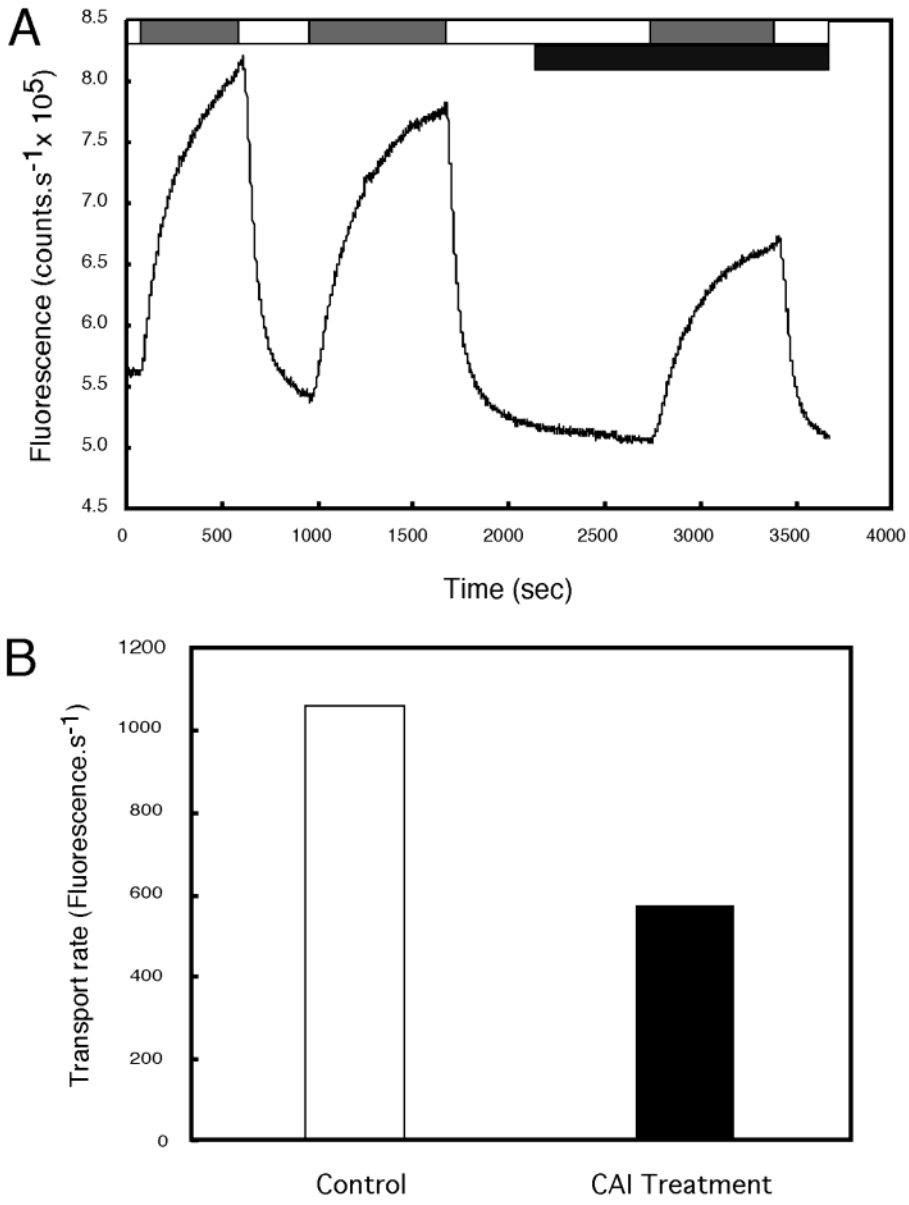


Figure 1. Anion exchange transport experiment after treatment with **14g**. HEK 293 cells grown on coverslips were transiently transfected with human AE1 cDNA and loaded with the Cl^- sensitive dye SPQ. Cells were perfused with Ringer's buffer containing either 140 mM KCl (open bar) or 140 mM KNO_3 (grey bar), as indicated in the top of the figure. Fluorescence was measured with an excitation wavelength of 350 nm and emission wavelength of 428 nm. (A) Cells were perfused with (black bar) CAI compound **14g**, starting with the Ringer's buffer containing 140 mM KCl 10 min before switching to the Cl^--free Ringer's buffer. (B) Bars show the Cl^- transport activity of the AE1, for the experiment above, after normalization and subtraction of the background transport. Rates were determined from the slope of the curve during the first 100 s of the Cl^- efflux. The white bar shows the first Cl^- efflux control pulse and the black bar shows the Cl^- efflux pulse treated with compound **14g** at 4 μM.

formation of intermediates of type **15**, which by deprotonation in the presence of bases leads to the 2-aminotetradehydropyran derivatives **16**. In many cases the deprotonation reaction is promoted by the amine itself, when this is basic enough (this being the reason in many cases one works at molar ratios pyrylium:amine of 1:2 when pyridinium salts are prepared by this method), or by external catalysts added to the reaction mixture, such as triethylamine.[16] The derivatives **16** are generally unstable, being tautomers with the ketodieneamines **17**, which are the key intermediates for the conversion of pyryliums into pyridiniums.[16] In acidic media, in the rate-determining step of the whole process, ketodieneamines **17** may be converted to the corresponding pyridinium salts **18**, although other products, such as vinylogous amides with diverse structures, have also been isolated in such reactions.[16] A supplementary complication appears when the moiety substituting the 2- and/or 6-position(s) of the pyrylium ring is methyl, cases in which a concurrent cyclization with formation of the anilines **19** in addition to the pyridinium salts **18** may take place too.[16] These concurrent reactions mentioned above are generally important when the amine to be converted into the pyridinium salt possesses weak nucleophilicity or basicity. This happens to be the case of aminobenzolamide **12**. In fact, reaction of **12** with several pyrylium salts, performed under a variety of conditions [different solvents, such as low molecular weight alcohols (MeOH, EtOH, *i*-PrOH), DMF, methylene chloride, acetonitrile; diverse molar ratios of the reagents; temperatures from 25 to 150 °C; reaction times between 15 min and 48 h] led only to the isolation

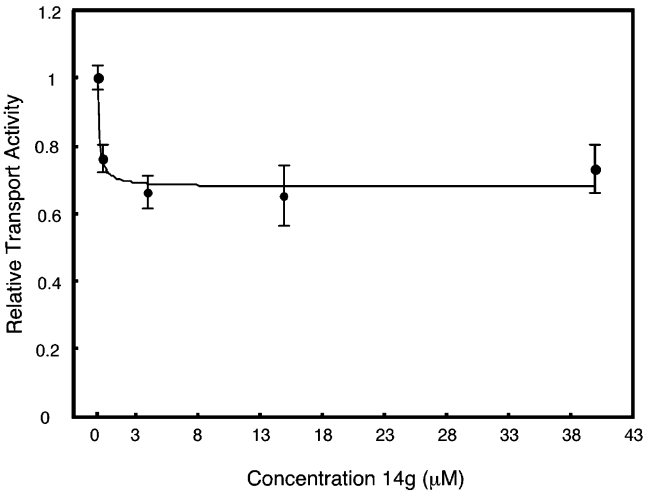


Figure 2. The rate of Cl^- transport measured for AE1 transfected cells, before and after treatment with **14g**. The relative rate of transport was calculated as the rate with **14g**/rate without inhibitor.

Table 2. Levels of Sulfonamide CA Inhibitors (μM) in Red Blood Cells at 30 and 60 min, after Exposure of 10 mL of Blood to Solutions of Sulfonamide (2 mM sulfonamide in 5 mM Tris buffer, pH 7.4

	[sulfonamide], μM[a]					
	t = 30 min			t = 60 min		
inhibitor	HPLC	ES	EI	HPLC	ES	EI
acetazolamide	136	139	140	160	167	163
methazolamide	170	169	165	168	168	167
benzolamide	110	108	112	148	146	149
12	125	127	122	154	156	158
14a	0.3	0.5	0.5	0.4	0.5	0.3
14f	1.0	1.1	1.0	1.1	1.2	1.1
14t	0.3	0.2	0.5	0.3	0.6	0.4
14v	0.4	0.3	0.5	0.3	0.6	0.5

[a] Standard error (from three determinations) < 5% by the HPLC method, the electronic spectroscopic (ES) method,[22] and the enzymatic (EI) method.[17]

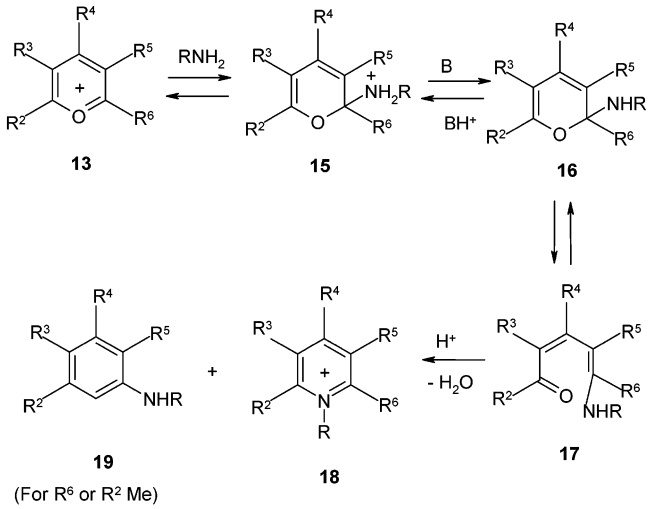
Table 3. Rate of the Cl^-/NO_3^- Exchange Measured for AE1 Transfected Cells, before and after Treatment with CAI or without Subsequent CAI Treatment (control)[a]

CAI compd	concn (mM)	relative transport activity	maximum inhibition (%)	IC_{50}[b] (mM)
control	-	1.00 ± 0.037	-	-
14v	4	1.00 ± 0.117	-	-
14g	4	0.651 ± 0.052[c]	32	0.126
14h	4	0.709 ± 0.052[c]	34	1.51

[a] The relative rate of transport was calculated as the rate with CAI/rate without CAI. The relative rate of transport was measured in the presence of a range of CAI concentrations. Shown is the relative rate of transport at 4 μM of each compound. [b] IC_{50} refers to the concentration required to observe the half-maximum inhibition. [c] CAIs that inhibited AE1 transport activity, with statistical significance (P < 0.01). Maximum inhibition is the maximum obtained by curve fitting.

of the unreacted raw materials. The only conditions that led to the formation of the pyridinium salts **14** were the following: anhydrous methanol in the presence of acetic anhydride as solvent and triethylamine as catalysts for the deprotonation of the intermediates **15**. Acetic anhydride had the role of reacting with the water formed in the condensation reaction. This water may in fact act as a competitive nucleophile with aminobenzolamide when reacting with the pyrylium cation, and as a consequence, the yields in pyridinium salts would dramatically be decreased. After the rapid formation of the ketodieneamine, catalyzed by triethylamine (and in the presence of the acetic anhydride as water scavenging agent), the cyclization to the pyridinium ring (the rate-determining step) has been achieved by refluxation in the presence of acetic acid (2−5 h, see Experimental Section for details). Still the yields were not always good, especially for the 2-methyl-containing derivatives.

Scheme 2



The new compounds reported in the present work were characterized by standard chemical and physical methods (elemental analysis, within ±0.4% of the theoretical values, and IR and NMR spectroscopy) that confirmed their structure (see Experimental Section for details) and were assayed for the inhibition of isozymes hCA I, hCA II, bCA IV, and hCA IX.

Carbonic Anhydrase Inhibitory Activity. Isozyme I. As seen from data of Table 1, all derivatives **14a**−**v** reported here act as very efficient CAIs against this isozyme, which is generally the most "resistant" to inhibitors of this type.[6,20] Indeed, aminobenzolamide **12** is already a highly potent CA I inhibitor (K_I of 6 nM), whereas inhibitors **14** show inhibition constants in the range of 3−12 nM, in contrast to the clinically used sulfonamide CAIs, which are much less effective inhibitors, with K_I values in the range of 30−1200 nM (Table 1). Thus, derivatives possessing several bulky groups (*i*-Pr, *t*-Bu, *n*-Pr, *n*-Bu, Ph, etc.) substituting the pyridinium moiety of **14**, such as **14c**,**d**,**g**,**h**,**m**,**o**,**p**, showed a decreased inhibitory activity as compared to aminobenzolamide, with K_I values in the range of 7−12 nM (aminobenzolamide has a K_I of 6 nM against hCA I): The rest of the compounds **14** were more efficient as compared to aminobenzolamide in inhibiting this isozyme, with K_I values in the range of 3−5 nM. The best CA I inhibitors were **14e**,**t**−**v** (K_I of 3 nM), all of which contain either only alkyl moieties or 4-Ph and other alkyl moieties substituting the pyridinium ring. These are probably the best CA I inhibitors ever reported up to now, since the clinically used CAIs show much higher inhibition constants against isozyme I (Table 1).

Isozyme II. Aminobenzolamide is already a very potent CA II inhibitor, with an inhibition constant around 2 nM. Several of the new inhibitors **14**, such as

14d,**g**,**h**,**m**−**s** act as weaker CA II inhibitors as compared to aminobenzolamide, with K_I values in the range of 3.13−5.96 nM (but all these compounds act as potent inhibitors, being much more effective than the clinically used CAIs acetazolamide, methazolamide, dichlorophenamide, or indisulam; see Table 1). Again, the substitution pattern at the pyridinium ring is the main discriminator of activity for these compounds: all the less active derivatives mentioned above incorporate at least two bulky/long aliphatic groups, mainly in positions 2- and 6- of the pyridinium ring (*n*-Pr, *t*-Bu, *n*-Bu, and Ph). The best CA II inhibitors among derivatives **14** were those incorporating more compact 2,6-substituents at the pyridinium ring (such as Me, Et), together with a 4-Me or 4-Ph moiety, or those incorporating only aliphatic such groups, such as **14a**−**c**,**e**,**f**,**i**−**k**,**t**−**v**, which showed K_I values in the range of 0.20−1.61 nM (thus, for the best inhibitors a factor of 10 increase in inhibitory power as compared to aminobenzolamide). It should be mentioned that isopropyl-substituted compounds (**14c**,**i**) are active as CA II inhibitors, although their activity against CA I was not so good.

Isozyme IV. Most sulfonamides show inhibitory activity against CA IV intermediate between those toward CA I (less susceptible) and CA II (very high affinity for sulfonamides). This is also the trend observed with the sulfonamides investigated here, derivatives of aminobenzolamide. Thus, the parent sulfonamide **12** is a potent CA IV inhibitor, with a K_I value around 5 nM. The new derivatives **14** incorporating bulky pyridinium-ring substituents (such as **14d**,**g**,**h**,**m**,**o**−**s**,**u**) were less effective than aminobenzolamide, showing K_I values in the range of 5.2−10.3 nM, whereas the compounds showing the other substitution pattern mentioned above were better CA IV inhibitors, showing K_I values in the range of 2.0−4.7 nM.

Isozyme IX. Aminobenzolamide **12** is less inhibitory against this isozyme (K_I of 38 nM) as compared to the other isozymes discussed above. This behavior is difficult to explain at this point, since no X-ray crystal structure of this isozyme has been reported. A very encouraging result obtained with the new derivatives **14** reported here was the observation that several of them show very high affinity for CA IX, with K_I values in the range of 3−9 nM (derivatives **14a**,**b**,**e**,**f**,**t**). It may be seen that all of them incorporate aliphatic moieties (Me, Et, and i-Pr) in positions 2 and 6 of the pyridinium ring and either 4-Me or 4-Ph moieties. Only one compound is tetrasubstituted (**14t**), again possessing only methyl moieties. The best CA IX inhibitor (and the best ever reported up to now) was **14a**, which is almost 13 times more effective than benzolamide in inhibiting this isozyme. Another group of new derivatives, such as **14c**,**d**,**g**,**i**,**j**,**k**,**n**,**q**−**s**,**u**,**v**, showed effective CA IX inhibition, with K_I values in the range of 12−35 nM, being thus more effective than aminobenzolamide. They incorporate slightly bulkier groups as compared to the previously discussed ones. Again, the less effective inhibitors (K_I values in the range of 40−43 nM) were those incorporating several bulky pyridinium substituents, such as **14h**,**o**,**p**, which contained either two *n*-Bu or one Ph and *n*-Bu/*t*-Bu in positions 2 and 6 of the pyridinium ring. Thus, the SAR is now rather clear for this type of CAI: the best CA IX inhibitors should contain either only small, compact aliphatic moieties substituting the pyridinium ring or a 4-Ph moiety, but the 2,6-substituents should again be small, compact aliphatic moieties. In this particular case, 2,4,6-trisubstituted-pyridinium derivatives were equally effective CA IX inhibitors as the tetrasubstituted derivatives (for example, **14t**, with a K_I of 5 nM).

Membrane Impermeability. As seen from data of Table 2, incubation of human red blood cells (which contain high concentrations of isozymes I and II, i.e., 150 μM hCA I and 20 μM hCA II, but not the membrane-bound CA IV or CA IX)[23] with millimolar concentrations of different sulfonamide inhibitors, such as acetazolamide or methazolamide, led to saturation of the two isozymes present in erythrocytes with inhibitor, after short periods of incubation (30 min), whereas for benzolamide or aminobenzolamide, a similar effect is achieved after somewhat longer periods (60 min) (Table 2). This is obviously due to the high diffusibility through membranes of the first three inhibitors, whereas benzolamide/aminobenzolamide with a pK_a of 3.2 for the second sulfonamido group,[24] being present mainly as an (di)anion at the pH at which the experiment has been done (7.4), is already less diffusible and penetrates membranes in a longer time. Different cationic sulfonamides synthesized by us here, such as **14a**,**f**,**t**,**v**, under the same conditions, were detected only in very small amounts within the red blood cells, proving that they were unable to penetrate through the membranes, obviously due to their cationic nature. Even after incubation times as long as 1 h (and longer, data not shown), only traces of such cationic sulfonamides were present inside the red blood cells, as proved by the three assay methods used for their identification in the cell lysate, which were in good agreement with each other (Table 2). This demonstrates that the proposed approach for achieving membrane impermeability works well for the designed positively charged sulfonamide CAIs **14**, since the very small amount of sulfonamide detected may be due to contamination of the lysates with a very small amount of membranes.

AE Inhibition. Some sulfonamide CAIs have been shown to inhibit transport mediated by Cl^-/HCO_3^- anion exchangers (AEs),[36] which is not surprising since the active sites of both CAs and AEs bind the same anion, HCO_3^-. CAIs indirectly inhibit Cl^-/HCO_3^- exchange activity by reducing availability of substrate HCO_3^-.[37] Therefore, it is not possible to use Cl^-/HCO_3^- assays to assess direct effects of CAIs on the AE1 transport rate. To assess the direct effects of CAIs on AE1 activity, we measured the rate of Cl^-/NO_3^- exchange mediated by AE1, which is independent of CA activity.[34] Figure 1 shows the change of fluorescence associated with SPQ-loaded, AE1-transfected cells, when perfusion solution was changed from Cl^--containing Ringer's buffer to Cl^- free (Cl^- replaced by NO_3^-) buffer. As Cl^- leaves the cell, the fluorescence of Cl^- sensitive SPQ is unquenched and fluorescence rises. Similarly, fluorescence fell upon switching to Cl^--containing Ringer's buffer. The inhibitory effect of compound **14g** used in this experiment is evident from the reduced slope in the third curve. The downward shift of the baseline fluorescence results from SPQ leakage from the cell and

was corrected, as described in the Experimental Section. In control experiments, three repeated pulses of switching to Cl^--containing, to Cl^--free, and back to Cl^--containing Ringer's buffer showed that there was no statistical difference in rates, when corrected for loss of SPQ from the cells. The effect of CAIs was assessed over a range of concentrations, as seen in Figure 2. There was a clear and potent inhibition of AE1 transport activity by **14g**. However, maximal inhibition approached a limit of approximately 30% of total transport activity. Table 3 summarizes the effects of CAIs on transport activity. Strikingly, while **14g** and **14h** potently inhibited AE1 transport activity, **14v** was without effect on AE1 activity, a result difficult to interpret at this moment.

Thus, among the three CA inhibitors studied, **14g** and **14h** potently inhibited AE1 transport activity, while **14v** did not. The ability to inhibit both CA and AE activities may be desirable in a drug candidate, since it could enhance the biological activity of the compound, which in this way interferes with the activity of both components of the CA−AE metabolon.[38] Indeed, CA and AE work together in protein complex denominated metabolons, to modulate cell pH by transport of the base, HCO_3^-.[38] However, the specificity of **14g** and **14h** is compromised, in comparison to **14v** (but this is a problem observed with many types of pharmacological agents; see a recent example for the COX-2 selective inhibitors of the sulfonamide type, which are nanomolar inhibitors of several CA isozymes).[39] Although, AE1 is expressed only in erythrocytes, kidney, and heart, AE1 is a member of a family of Cl^-/HCO_3^- exchangers that includes AE2 and AE3.[38] Since these proteins are closely related in their amino acid sequence and share sensitivity to the stilebene disulfonate family of inhibitors, it is likely that AE2 and AE3, like AE1, are sensitive to **14g** and **14h**. AE2 and AE3 are broadly expressed across tissues so that **14g** and **14h** will have effects on bicarbonate homeostasis that extend beyond tissues that express CA IX. The differential sensitivity of AE1 to CAIs underscores the difficulty in preparation of truly selective inhibitors.

Conclusions

We report here a general approach for the preparation of positively charged, membrane-impermeant sulfonamide CA inhibitors with high affinity for the cytosolic isozymes CA I and CA II, as well as for the membrane-bound ones CA IV and CA IX. They were obtained by attaching substituted-pyridinium moieties to aminobenzolamide, a very potent CA inhibitor itself. Ex vivo studies showed the new class of inhibitors reported here to discriminate for the membrane-bound versus the cytosolic isozymes. Correlated with the low nanomolar affinity of some of these compounds for the tumor-associated isozyme CA IX, this report constitutes the basis of selectively inhibiting only the target, tumor-associated CA IX in vivo, whereas the cytosolic isozymes would remain unaffected. Furthermore, we prove here for the first time that the CA−AE metabolon can be inhibited by the same type of sulfonamide derivative, a feature that we consider positive for a potential antitumor drug belonging to this class of pharmacological agents.

Experimental Section

General. Melting points were determined with a heating plate microscope (not corrected). IR spectra were recorded on KBr pellets, at 400−4000 cm^{-1}, with a Perkin-Elmer 16PC FTIR spectrometer. ^1H NMR spectra were recorded with a Varian 300CXP apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard). Elemental analysis were determined with a Carlo Erba Instrument CHNS elemental analyzer, model 1106. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Pyrylium salts were prepared by literature procedures, generally by olefin (or their precursors) bisacylation, as described in the literature,[16] whereas aminobenzolamide was prepared as described earlier.[11] Other sulfonamides used as standards were commercially available.

General Procedure for the Preparation of Compounds 14. An amount of 2.9 mM of aminobenzolamide **12**[11] and 2.9 mM of pyrylium salt **13** were suspended in 5 mL of anhydrous methanol and poured into a stirred mixture of 14.5 mM of triethylamine and 5.8 mM of acetic anhydride. After 5 min of stirring, another 10 mL of methanol was added to the reaction mixture, which was heated to reflux for 15 min. Then 14.5 mM of acetic acid was added and heating was continued for 2−5 h. The role of the acetic anhydride is to react with the water formed during the condensation reaction between the pyrylium salt and the aromatic amine, to shift the equilibrium toward the formation of the pyridinium salts of type **14**. In the case of aminobenzolamide, this procedure is the only one that gave acceptable yields in pyridinium salts, probably due to the deactivating effect of the sulfamoylaminothiadiazole moiety on the amine group, which becomes poorly nucleophilic and unreactive toward these reagents. The precipitated pyridinium salts obtained were purified by treatment with concentrated ammonia solution (which also converts the eventually unreacted pyrylium salt to the corresponding pyridine, which is soluble in acidic medium), reprecipitation with perchloric acid, and recrystallization from water with 2−5% $HClO_4$.

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-trimethylpyridinium perchlorate, 14a: white crystals, mp >300 °C. IR (KBr), cm^{-1} (bands in italics are due to the anion): 595, *625*, 664, 787, 803, 884, 915, *1100*, 1150, 1190, 1200, 1285, 1360, 1495, 1604, 3065. ^1H NMR (D_2O), δ, ppm: 3.08 (s, 6H, 2,6-Me_2), 3.11 (s, 3H, 4-Me), 7.30−8.06 (m, AA′BB′,4H, ArH from phenylene), 9.05 (s, 2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal. $C_{16}H_{18}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-isopropyl-4,6-dimethylpyridinium perchlorate, 14b: colorless crystals, mp 290−1 °C. IR (KBr), cm^{-1}: 625, 680, 720, 1100, 1165, 1330, 1640, 3020, 3235. ^1H NMR (TFA), δ, ppm: 1.50 (d, 6H, 2Me from *i*-Pr), 2.80 (s, 3H, 6-Me), 2.90 (s, 3H, 4-Me), 3.49 (heptet, 1H, CH from *i*-Pr), 7.25−8.43 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 7.98 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{18}H_{22}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-diisopropyl-4-methylpyridinium perchlorate, 14c: tan crystals, mp 278−9 °C. IR (KBr), cm^{-1}: 625, 685, 820, 1100, 1165, 1340, 1635, 3030, 3250. ^1H NMR (TFA), δ, ppm: 1.51 (d, 12H, 4Me from 2 *i*-Pr), 2.83 (s, 3H, 4-Me), 3.42 (heptet, 2H, 2CH from 2 *i*-Pr), 7.31−8.51 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 8.05 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{20}H_{26}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-dimethyl-4-phenylpyridinium perchlorate, 14e: white crystals, mp > 300 °C. IR (KBr), cm^{-1}: 625, 690, 770, 1100, 1170, 1330, 1635, 3030, 3260, 3330. ^1H NMR (TFA), δ, ppm: 2.62 (s, 6H, 2,6-$(Me)_2$), 8.10−9.12 (m, 11H, ArH from 1,4-phenylene, pyridinium, and 4-Ph). Anal. $C_{21}H_{20}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-diethyl-4-phenylpyridinium perchlorate, 14f: tan crystals, mp 267−8 °C. IR (KBr), cm^{-1}: 625, 695, 765,

1100, 1180, 1340, 1630, 3040, 3270, 3360. ^{1}H NMR (TFA), δ, ppm: 1.43 (t, 6H, 2 Me from ethyl), 2.82 (q, 4H, 2 CH_2 from Et), 7.68−8.87 (m, 11H, ArH from 1,4-phenylene, pyridinium, and 4-Ph). Anal. $C_{23}H_{24}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-di-*n*-propyl-4-phenylpyridinium perchlorate 14g: colorless crystals, mp 235−7 °C. IR (KBr), cm^{-1}: 625, 695, 770, 1100, 1180, 1340, 1630, 3050, 3220, 3315. ^{1}H NMR (TFA), δ, ppm: 1.06 (t, 6H, 2 Me from propyl), 1.73 (sextet, 4H, 2CH_2 (β) from *n*-Pr), 2.84 (t, 4H, 2 CH_2 (α) from *n*-Pr), 7.55−8.71 (m, 11H, ArH from 1,4-phenylene, pyridinium, and 4-Ph). Anal. $C_{25}H_{28}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-di-isopropyl-4-phenylpyridinium perchlorate, 14i: white crystals, mp 278−9 °C. IR (KBr), cm^{-1}: 625, 690, 765, 1100, 1180, 1340, 1625, 3040, 3270, 3315. ^{1}H NMR (TFA), δ, ppm: 1.45 (d, 12H, 4 Me from *i*-Pr), 2.95 (heptet, 2H, 2 CH from *i*-Pr), 7.92−8.97 (m, 11H, ArH from 1,4-phenylene, pyridinium, and 4-Ph). Anal. $C_{25}H_{28}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-methyl-4,6-diphenylpyridinium perchlorate, 14j: white crystals, mp 298−9 °C. IR (KBr), cm^{-1}: 625, 710, 770, 1100, 1170, 1345, 1625, 3040, 3245, 3350. ^{1}H NMR (TFA), δ, ppm: 2.75 (s, 3H, 2-Me), 7.53−8.70 (m, 16H, ArH from 1,4-phenylene, pyridinium, and 4,6-Ph_2). Anal. $C_{26}H_{22}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-ethyl-4,6-diphenylpyridinium perchlorate, 14k: white crystals, mp 254−5 °C. IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1180, 1340, 1620, 3040, 3250, 3350. ^{1}H NMR (TFA), δ, ppm: 1.52 (t, 3H, Me from ethyl), 2.97 (q, 2H, CH_2), 7.40−8.57 (m, 16H, ArH from 1,4-phenylene, pyridinium, and 4,6-Ph_2). Anal. $C_{27}H_{24}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-*n*-propyl-4,6-diphenylpyridinium perchlorate, 14m: white crystals, mp 214−5 °C. IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1180, 1340, 1620, 3030, 3270, 3350. ^{1}H NMR (TFA), δ, ppm: 1.03 (t, 3H, Me from propyl), 1.95 (sextet, 2H, β-CH_2 from *n*-Pr), 2.88 (t, 2H, α-CH_2 from *n*-Pr), 7.39−8.55 (m, 16H, ArH from 1,4-phenylene, pyridinium, and 4,6-Ph_2). Anal. $C_{28}H_{26}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-isopropyl-4,6-diphenylpyridinium perchlorate, 14n: white crystals, mp 186−8 °C. IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1170, 1340, 1620, 3040, 3250, 3360. ^{1}H NMR (TFA), δ, ppm: 1.51 (d, 6H, 2 Me from *i*-Pr), 2.50−3.27 (m, 1H, CH from *i*-Pr), 7.32−8.54 (m, 16H, ArH from 1,4-phenylene, pyridinium, and 4,6-Ph_2). Anal. $C_{28}H_{26}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-*n*-butyl-4,6-diphenylpyridinium perchlorate, 14o: white crystals, mp 241−3 °C. IR (KBr), cm^{-1}: 625, 710, 770, 1100, 1180, 1335, 1625, 3040, 3260, 3345. ^{1}H NMR (TFA), δ, ppm: 0.93 (t, 3H, Me from butyl), 1.12−2.14 (m, 4H, $CH_3CH_2CH_2CH_2$ from *n*-Bu), 2.96 (t, 2H, α-CH_2 from *n*-Bu), 7.21−8.50 (m, 16H, ArH from 1,4-phenylene, pyridinium, and 4,6-Ph_2). Anal. $C_{29}H_{28}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-*tert*-butyl-4,6-diphenylpyridinium perchlorate, 14p: white crystals, mp 203−5 °C. IR (KBr), cm^{-1}: 625, 705, 765, 1100, 1160, 1310, 1620, 3060, 3270. ^{1}H NMR (TFA), δ, ppm: 1.91 (s, 9H, *t*-Bu), 6.80−8.74 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2, and 3,5-H from pyridinium). Anal. $C_{29}H_{28}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-triphenylpyridinium perchlorate, 14r: pale yellow crystals, mp >300 °C. IR (KBr), cm^{-1} (bands in italics are due to the anion): *625*, 635, 703, 785, 896, *1100*, 1150, 1204, 1355, 1410, 1520, 1600, 3065. ^{1}H NMR (D_2O), δ, ppm: 7.50−8.60 (m, 19H, ArH, 3Ph + C_6H_4), 9.27 (s, 2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal. $C_{31}H_{24}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-diphenyl pyridinium perchlorate, 14s: yellow crystals, mp 218−20 °C. IR (KBr), cm^{-1}: 625, 705, 765, 1100, 1160, 1335, 1615, 3050, 3260. ^{1}H NMR (TFA), δ, ppm: 6.75−8.43 (m, 17H, ArH from 1,4-phenylene, 2,6-Ph_2, and 3,4,5-H from pyridinium). Anal. $C_{25}H_{20}N_5O_4S_3^+ClO_4^-$ (C, H, N).

1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,3,4,6-tetramethylpyridinium perchlorate, 14t: tan crystals, mp >300 °C. IR (KBr), cm^{-1}: 625, 800, 1100, 1165, 1330, 1630, 3030, 3305. ^{1}H NMR (TFA), δ, ppm: 2.62 (s, 3H, 4-Me), 2.74 (s, 3H, 3-Me), 2.88 (s, 6H, 2,6-$(Me)_2$), 7.21−8.50 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 7.93 (s, 1H, ArH, 5-H from pyridinium). Anal. $C_{17}H_{20}N_5O_4S_3^+ClO_4^-$ (C, H, N).

Penetrability through Red Blood Cell Membranes. An amount of 10 mL of freshly isolated human red blood cells, thoroughly washed several times with Tris buffer (pH 7.40, 5 mM) and centrifuged for 10 min, was treated with 25 mL of a 2 mM solution of sulfonamide inhibitor. Incubation was done at 37 °C with gentle stirring, for periods of 30−120 min. After the incubation times of 30, 60, and 120 min, respectively, the red blood cells were centrifuged again for 10 min, the supernatant was discarded, and the cells were washed three times with 10 mL of the above-mentioned buffer, to eliminate all unbound inhibitor.[10] The cells were then lysed in 25 mL of distilled water and centrifuged to eliminate membranes and other insoluble impurities. The obtained solution was heated at 100 °C for 5 min (in order to denature CA-s), and the sulfonamides possibly present were assayed in each sample by three methods: chromatographically via HPLC;[21] spectrophotometrically,[22] and enzymatically.[17] Mention should be made that the three methods presented below led to results in good agreement, within the limits of the experimental errors.

Chromatographically. A variant of the HPLC methods of Gomaa[21] was developed by us as follows: a commercially available 5 μm Bondapak C-18 column was used for the separation, with a mobile phase made of acetonitrile−methanol−phosphate buffer (pH 7.4) 10:2:88 (v/v/v), at a flow rate of 3 mL/min, with 0.3 mg/mL sulfadiazine (Sigma) as internal standard. The retention times were 12.69 min for acetazolamide, 4.55 min for sulfadiazine, 10.54 min for benzolamide, 12.32 min for aminobenzolamide, 3.15 min for **14a**, 4.41 min for **14f**, 3.54 min for **14t**, and 4.24 min for **14v**. The eluent was monitored continuously for absorbance (at 254 nm for acetazolamide and in the range of 270−310 nm in the case of the other sulfonamides).

Spectrophotometrically. A variant of the pH-induced spectrophotometric assay of Abdine et al.[22] was used, working for instance at 260 and 292 nm for acetazolamide and at 225 and 265 nm for sulfanilamide, etc. Standardized solutions of each inhibitor were prepared in the same buffer as the one used for the membrane penetrability experiments.

Enzymatically. The amount of sulfonamide present in the lysate was evaluated on the basis of hCA II inhibition measured with the esterase method, as described above.[17] Standard inhibition curves were obtained previously for each sulfonamide, using the pure compound, which were used thereafter for determining the amount of inhibitor present in the lysate.

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.[25] Cell growth conditions were those described in ref 26, and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[27] 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.[28,29] CA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration was determined by titration with ethoxzolamide.[30] The cDNA of the catalytic domain of

hCA IX (isolated as described by Pastorek et al.[1]) was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene, Milan, Italy). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *E. coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100, as described by Wingo et al.[31] The homogenate thus obtained was extensively centrifuged (11 000*g*) in order to remove soluble and membrane-associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione, and 1 mM oxidized glutathione. Active hCA IX was extensively dialyzed into a solution of 10 mM Hepes (pH 7.5), 10 mM Tris HCl, 100 mM Na_2SO_4, and 1 mM $ZnCl_2$. The amount of protein was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate. The protein was further purified by sulfonamide affinity chromatography,[18] and the amount of enzyme was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate.[18] An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA CO_2 hydration activity assays.[18] Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10−100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate.[18] Stock solutions of inhibitor (1 mM) were prepared in distilled−deionized water with 10−20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, to allow for the formation of the E−I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results.

Initial rates of 4-nitrophenylacetate hydrolysis catalyzed by different CA isozymes (i.e., CA I, II, and IV) were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM-compatible PC.[17] 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 $\epsilon = 18\ 400\ M^{-1}.cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, under the conditions of the experiments (pH 7.40), as reported in the literature.[17] Nonenzymatic 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 in refs 17 and 18.

Tissue Culture. Human AE1 protein was expressed by transient transfection of HEK 293 cells[32] using the calcium phosphate method.[33] Cells were grown at 37 °C in an air/CO_2 (19:1) environment in Dulbecco's modified Eagle media (DMEM) supplemented with 5% (v/v) fetal bovine serum and 5% (v/v) calf serum. Cells were used 2 days post-transfection.

Anion Exchange Activity Assay. Anion exchange activity was monitored using a fluorescence assay described previously.[34] Briefly, HEK 293 cells grown on poly-L-lysine-coated coverslips in 60 mm dishes were transiently transfected with pRBG4-based plasmid pJRC9[35] as described earlier. Approximately 24 h post-transfection cells were incubated overnight at 37 °C in 4 mL of serum-supplemented DMEM medium, containing 10 mM 6-methoxy-*N*-(3-sulfopropyl)quinilinium, inner salt (SPQ) from Molecular Probes. Two days post-transfection, coverslips were mounted in a fluorescence cuvette and perfused at 3.5 mL/min alternately with Ringer's buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 25 mM sodium gluconate, 10 mM HEPES, pH 7.4), containing either 140 mM KCl or 140 mM KNO_3. Fluorescence was monitored using a Photon Technologies International RCR fluorometer at an excitation wavelength of 350 nm and an emission wavelength 438 nm. To correct for changes of fluorescence that occurred due to dye leakage from cells and cell loss from the coverslip, total fluorescence was corrected. A curve was fit to the heights of the first two Cl^- efflux pulses of an experiment. The height of each pulse was obtained by subtracting the maximum fluorescence to the baseline. Extrapolation to the time of the third Cl efflux pulse showed that the fluorescence was reduced compared to the first peak. After normalization of the fluorescence range, transport activity was consistent among the three determinations for the control group. The activity of the transporter was measured as the rate of Cl^- efflux, which was determined from the slope of the curve during the first 100 s by linear regression fitting (Kaleidagraph software). In all cases the transport activity of sham transfected cells was subtracted from the total rate to ensure that these rates consist only of AE1 transport activity.

Statistical Analysis. Values are expressed ± the standard error of measurement. Statistical significance was determined using an unpaired *t*-test with $p < 0.05$ considered significant.

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