Carbonic Anhydrase Inhibitors: Synthesis of Membrane-Impermeant Low Molecular Weight Sulfonamides Possessing in Vivo Selectivity for the Membrane-Bound versus Cytosolic Isozymes[1]

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Aromatic/heterocyclic sulfonamides act as strong inhibitors of the zinc enzyme carbonic anhydrase (CA; EC 4.2.1.1), but the presently available compounds do not generally discriminate between the 14 isozymes isolated in higher vertebrates. Thus, clinically used drugs from this class of pharmacological agents show many undesired side effects due to unselective inhibition of all CA isozymes present in a tissue/organ. Here we propose a new approach for the selective in vivo inhibition of membrane-bound versus cytosolic CA isozymes with a new class of positively charged, membrane-impermeant sulfonamides. This approach is based on the attachment of trisubstituted-pyridinium-methylcarboxy moieties (obtained from 2,4,6-trisubstituted-pyrylium salts and glycine) to the molecules of classical aromatic/heterocyclic sulfonamides possessing free amino, imino, hydrazino, or hydroxyl groups in their molecules. Efficient in vitro inhibition (in the nanomolar range) was observed with some of the new derivatives against three investigated CA isozymes: i.e., hCA I, hCA II (cytosolic forms), and bCA IV (membrane-bound isozyme) (h = human isozyme; b = bovine isozyme). Due to their salt-like character, the new type of inhibitors reported here, unlike the classical, clinically used compounds (such as acetazolamide, methazolamide, and ethoxzolamide), are unable to penetrate through biological membranes, as shown by ex vivo and in vivo perfusion experiments in rats. The level of bicarbonate excreted into the urine of the experimental animals perfused with solutions of the new and classical inhibitors undoubtedly proved that: (i) when using the new type of positively charged sulfonamides, only the membrane-bound enzyme (CA IV) was inhibited, whereas the cytosolic isozymes (CA I and II) were not affected; (ii) in the experiments in which the classical compounds (acetazolamide, benzolamide, etc.) were used, unselective inhibition of all CA isozymes (I, II, and IV) has been evidenced.

Introduction

At least 14 different carbonic anhydrase (CA; EC 4.2.1.1) isozymes or CA-related proteins (CA-RP) were described up to now in higher vertebrates, including humans.[2] These highly abundant proteins are involved in critical physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH homeostasis, electrolyte secretion in a variety of tissues/organs, and biosynthetic reactions, such as the gluconeogenesis and ureagenesis among others.[3−5] Inhibition of some of these enzymes by aromatic/heterocyclic sulfonamides has been exploited clinically for more than 45 years in the treatment of a variety of diseases such as glaucoma, epilepsy, congestive heart failure, mountain sickness, gastric and duodenal ulcers, etc.[3,4,6] It should be also noted that a sulfonamide CA inhibitor was the first nonmercurial diuretic in clinical use in the early 1950s and that it subsequently led to the development of the thiazide and high-ceiling diuretics, two classes of widely used pharmacological agents.[4,7] The main drawback of the presently available sulfonamide CA inhibitors is constituted by their total lack of specificity for the different CA isozymes. Thus, except for CA III, a muscle isozyme relatively resistant to the inhibition by sulfonamides,[8] other CA isozymes, such as the cytosolic CA I, II, and VII, the membrane-bound forms CA IV, IX, XII, and XIV, or the mitochondrial CA V, show very high and similar affinity (in the micromolar to nanomolar range) for this class of inhibitors.[2,9−14] Development of such isozyme-specific or at least organ-selective inhibitors would be highly beneficial for obtaining novel types of drugs, devoid of major side effects, as well as for many physiological studies in which specific/selective inhibitors would be valuable tools for understanding the physiology of these enzymes. Among the latest important developments in this field, one should note the recent isolation of three novel membrane-bound CA isozymes, CA IX,[10] XII,[13] and XIV,[14] in addition to the "classical" one, CA IV, purified several years before by Sly's group.[15] Some of these isozymes were identified only in tumor cells, and little is known at the moment regarding the physiological consequences of their inhibition/activation.[13−16]

In previous contributions from this laboratory[17−22] it was shown that by attaching different "tails" to the

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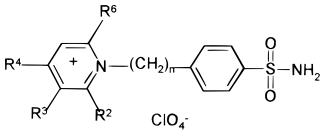
molecules of aromatic/heterocyclic sulfonamides of types **1**−**26** (Chart 1), possessing free amino, hydrazino, imino, or hydroxy groups, it is possible to obtain water-soluble, efficient inhibitors of several CA isozymes (such as CA I, II, and IV), some of which showed promising antiglaucoma activity via the topical route in experimental animals. It appeared thus of interest to explore a similar strategy with the tentative of obtaining membrane-impermeant sulfonamide inhibitors, which should inhibit only the membrane-bound isozymes but not the cytosolic isozymes.

In some preliminary communications[21,23] we reported that the reaction of sulfanilamide **3**, and its homologues **5** and **6**, or that of the heterocyclic derivative **14**, with substituted pyrylium salts affords new classes of tight-binding CA I, II, and IV inhibitors, of types **27**−**30**, which showed some selectivity in vivo for the inhibition of the membrane-bound isozyme bCA IV.



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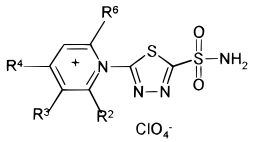

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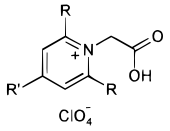
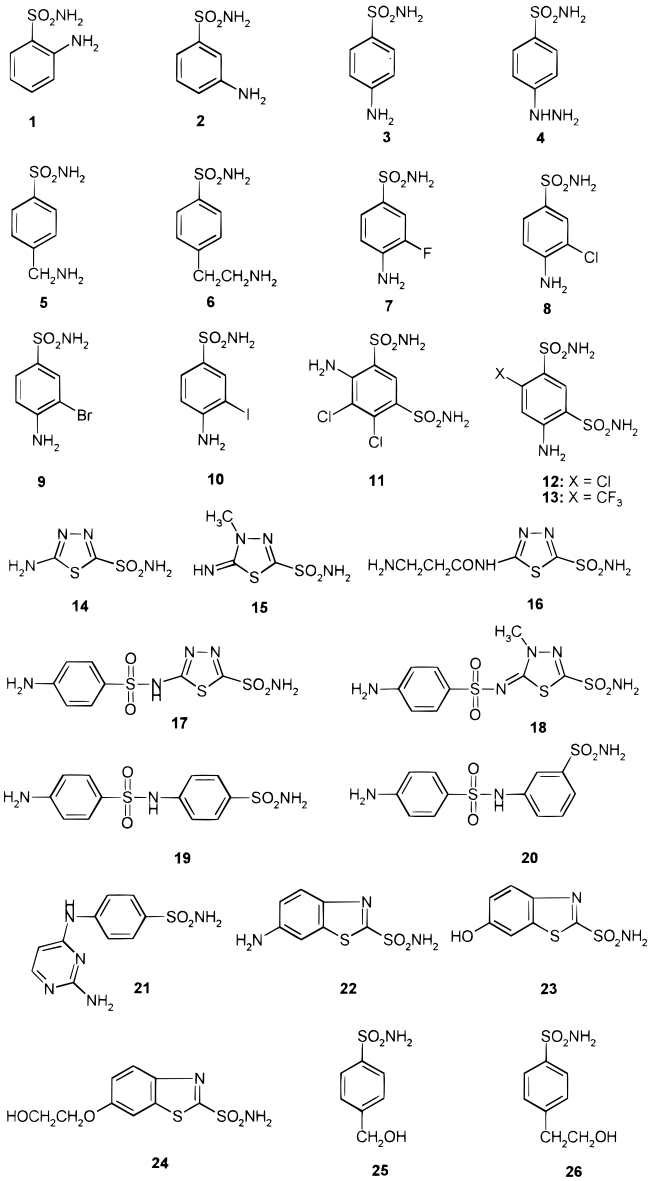
In this article we extend the above-mentioned studies[21,23] and report the reaction of 26 aromatic/heterocyclic sulfonamides containing a free amino, imino, hydrazino, or hydroxyl group, with 2,4,6-trisubstituted-pyridinium-methylcarboxylic acids of types **A**−**D** (obtained from glycine and pyrylium salts). The new



A: R = R' = Me
B: R = Me; R' = Ph
C: R = Et; R' = Ph
D: R = i-Pr; R' = Ph

compounds reported here were tested for the inhibition of three CA isozymes: hCA I, hCA II, and bCA IV (h = human isozyme; b = bovine isozyme). Affinities in the nanomolar range were detected for many compounds against isozymes II and IV. To show that the new inhibitors are membrane-impermeant, some ex vivo and in vivo perfusion experiments in rats have also been performed. Thus, incubation of red blood cells with positively charged as well as classical CA inhibitors showed that only in the second case did the erythrocytes become saturated with inhibitor, due to facilitated penetration of such compounds through the red blood cell membranes. The level of bicarbonate excreted into the urine of rats perfused with solutions of the new and classical inhibitors proved then that when using the positively charged sulfonamides, only the membrane-bound enzyme was inhibited, whereas the cytosolic isozymes were not affected, contrary to the experiments in which the classical compounds were used (such as acetazolamide, benzolamide, or methazolamide), which led to unselective inhibition of all CA isozymes (cytosolic and membrane-associated).

Chart 1

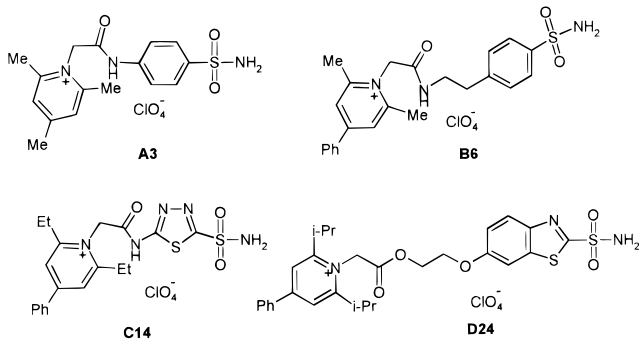


1 **2** **3** **4**
5 **6** **7** **8**
9 **10** **11** **12:** X = Cl **13:** X = CF_3
14 **15** **16**
17 **18**
19 **20**
21 **22** **23**
24 **25** **26**

Results

Synthesis. As a large number of derivatives are reported here, each compound will be designated by a letter identifying the positively charged carboxylic acid from which it is derived (**A**−**D**) and a figure identifying the sulfonamide of type **1**−**26** at which the substituted-pyridinium-methylcarboxy moiety has been attached. For instance, **A3** is *p*-(2,4,6-trimethylpyridinium-methylcarboxamido)benzenesulfonamide perchlorate; **B6** is *p*-(2,6-dimethyl-4-phenylpyridinium-methylcarboxamidoamidoethyl)benzenesulfonamide perchlorate; **C14** is 5-(2,6-diethyl-4-phenylpyridinium-methylcarboxamido)-1,3,4-thiadiazole-2-sulfonamide perchlorate; **D24** is 6-(2,6-diisopropyl-4-phenylpyridinium-methylcarboxyethoxy)-benzothiazole-2-sulfonamide, etc.

The new compounds **A(1−26)**−**D(1−26)** were prepared by reaction of the aromatic/heterocyclic sulfonamides **1**−**26** with the positively charged pyridinium derivatives **A**−**D**, obtained in turn from glycine and pyrylium salts.[23−25] Nonexceptional routine synthetic procedures were employed for the coupling reaction of



amines/imines/alcohols/phenols with carboxylic acids in the presence of carbodiimide derivatives, as reported previously by Whitesides[26,27] or this group[17−20] for structurally related sulfonamides.

CA Inhibitory Activity. Inhibition data against three CA isozymes, hCA I, hCA II, and bCA IV with compounds **A(1−26)−D(1−26)** as well as the original raw materials and standard sulfonamides are shown in Table 1. The esterase activity of CA isozymes against 4-nitrophenylacetate as substrate has been used in this assay (see Experimental Section for details).

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.

In Vivo Perfusion Experiments in Rats. Renal excretion of bicarbonate in rats after administration of sulfonamide CA inhibitors (acetazolamide, benzolamide, and some of the positively charged sulfonamides synthesized in the present study), as well as the level of sulfonamide present in the red blood cells of the experimental animals, 2 h after perfusion of the drug, are shown in Table 3.

Discussion

Chemistry. Although several thousand different aromatic/heterocyclic sulfonamide CA inhibitors were synthesized in the last 45 years in the search for diverse pharmacological agents,[3,4,17−29] the number of such membrane-impermeant derivatives is very low indeed. Thus, a first approach for inducing membrane impermeability to such compounds was that of attaching aromatic/heterocyclic sulfonamides to polymers, such as poly(ethylene glycol),[30] aminoethyldextran,[31] or dextran.[32] Compounds such as **31**−**33** (Chart 2), possessing molecular weights in the range of 3.5−99 kDa, showed membrane impermeability due to their high molecular weights and were shown to selectively inhibit (in vivo) only CA IV and not the cytosolic isozymes (primarily CA II), being used in several valuable renal and pulmonary physiologic studies.[30−32] Due to their macromolecular nature, such inhibitors could not be developed as drugs/diagnostic tools, since in vivo they should induce allergic reactions. A second approach for achieving membrane impermeability is that of using highly polar, salt-like compounds. Only one such sulfonamide has been used in physiological studies, QAS (quaternary ammonium sulfanilamide) **34** (Chart 2), which has been reported by Henry's group[33] to inhibit only extracellular CAs in a variety of arthropods (such as the crab *Callinectes sapidus*) and fish.[33] The main drawback of QAS is its relatively high toxicity in higher vertebrates.[4]

Table 1. CA Inhibition Data with Sulfonamides **1**−**26** and the Positively Charged Derivatives **A(1−26)**−**D(1−26)** Reported in the Present Study Against Isozymes I, II, and IV

	K_i* (nM)				K_i* (nM)		
inhib	hCA I[a]	hCA II[a]	bCA IV[b]	inhib	hCA I[a]	hCA II[a]	bCA IV[b]
1	45400	295	1310	**14**	8600	60	540
A1	32000	280	425	**A14**	300	10	50
B1	25300	215	290	**B14**	205	5	17
C1	27500	240	335	**C14**	300	7	25
D1	30500	275	350	**D14**	300	9	36
2	25000	240	2200	**15**	9300	19	355
A2	24000	250	410	**A15**	330	8	60
B2	19600	200	280	**B15**	200	6	16
C2	21800	205	310	**C15**	300	7	25
D2	23100	235	345	**D15**	300	8	35
3	28000	300	3000	**16**	455	3	125
A3	16000	145	190	**A16**	43	7	18
B3	13000	110	155	**B16**	33	4	15
C3	15000	125	170	**C16**	41	4	19
D3	15000	130	180	**D16**	41	6	16
4	78500	320	3215	**17**	6	2	5
A4	35000	320	450	**A17**	15	6	12
B4	26000	230	310	**B17**	10	2	9
C4	28000	290	340	**C17**	12	5	9
D4	34000	300	360	**D17**	14	5	10
5	25000	170	2800	**18**	1	0.6	0.8
A5	1500	43	80	**A18**	15	5	10
B5	1250	31	50	**B18**	11	2	8
C5	1400	39	67	**C18**	13	3	10
D5	1450	42	75	**D18**	15	4	10
6	21000	160	2450	**19**	42	6	50
A6	805	36	71	**A19**	55	16	78
B6	720	30	46	**B19**	41	8	54
C6	750	32	61	**C19**	46	11	63
D6	775	35	70	**D19**	50	15	73
7	8300	60	180	**20**	44	9	53
A7	750	19	44	**A20**	61	15	86
B7	700	12	36	**B20**	38	9	55
C7	750	16	39	**C20**	43	12	60
D7	750	18	39	**D20**	53	15	72
8	9800	110	320	**21**	690	12	154
A8	680	21	70	**A21**	52	19	69
B8	600	10	38	**B21**	35	11	50
C8	610	15	46	**C21**	45	15	58
D8	600	17	56	**D21**	47	16	62
9	6500	40	66	**22**	70	9	19
A9	800	45	77	**A22**	15	9	50
B9	600	13	35	**B22**	10	5	19
C9	615	21	55	**C22**	11	6	30
D9	645	36	70	**D22**	13	8	38
10	6000	70	125	**23**	55	8	17
A10	810	49	78	**A23**	13	8	29
B10	610	21	59	**B23**	11	5	17
C10	620	25	64	**C23**	11	7	27
D10	630	37	75	**D23**	12	8	39
11	6100	28	175	**24**	50	7	15
A11	550	18	67	**A24**	12	7	33
B11	380	12	31	**B24**	9	4	16
C11	400	13	35	**C24**	10	5	25
D11	470	15	49	**D24**	11	7	30
12	8400	75	160	**25**	24000	125	560
A12	360	19	64	**A25**	1800	79	140
B12	350	12	30	**B25**	1350	38	75
C12	350	15	36	**C25**	1700	51	97
D12	350	18	46	**D25**	1770	71	120
13	7500	62	140	**26**	18000	110	450
A13	320	19	58	**A26**	1700	78	130
B13	300	10	33	**B26**	1100	34	69
C13	310	14	33	**C26**	1500	46	84
D13	310	17	42	**D26**	1650	70	115

*Standard error for the determination of K_i's was 10−20% (from 2 different assays). [a] Human (cloned) isozyme. [b] Isolated from bovine lung microsomes.

Table 2. Levels of Sulfonamide CA Inhibitors (mM) 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] (mM)*					
	$t = 30$ min			$t = 60$ min		
inhibitor	HPLC[a]	ES[b]	EI[c]	HPLC[a]	ES[b]	EI[c]
sulfanilamide **3**	148	151	143	159	164	159
acetazolamide **35**	136	139	140	160	167	163
benzolamide **36**	110	108	112	148	146	149
methazolamide **37**	170	169	165	168	168	167
A17	0.4	0.6	0.5	0.6	0.5	0.5
A23	1.0	1.1	1.0	1.1	1.2	1.1
B7	0.7	0.9	0.9	0.9	1.0	1.0
B14	0.5	0.6	0.8	0.9	0.8	1.1
C13	1.4	1.5	1.7	1.5	1.5	1.9
C18	0.3	0.5	0.5	0.5	0.6	0.6
D15	0.4	0.7	0.6	0.5	0.9	0.9

*Standard error (from 2 determinations) was <5% by: [a]the HPLC method,[55] [b]the electronic spectroscopic (ES) method,[57] and [c]the enzymatic method (EI);[53] see Experimental Section for details.

Table 3. Renal Excretion of Bicarbonate in Rats after Administration of the Classical Sulfonamide CA Inhibitors Acetazolamide **35** and Benzolamide **36** and the Positively Charged Sulfonamides **B14**, **C18,** and **D15** and the Level of Sulfonamide Present in Red Blood Cells at 2 h after Administration of Drug

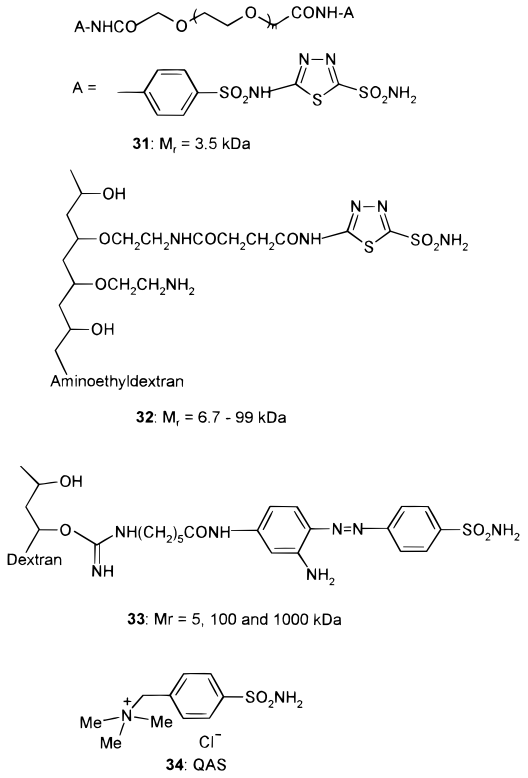
inhibitor	drug concn (mg/kg)	excreted HCO_3^- (mM)*	[sulfonamide][a] (mM)*
acetazolamide	10	110	164
benzolamide	5	115	155
B14	5	38	0.014
C18	4	40	0.010
D15	5	42	0.012

*Standard error (from 2 determinations) was <5%. [a] By the HPLC method.

A large number of positively charged sulfonamides, prepared by reaction of aminosulfonamides with pyrylium salts, of types **27**−**30** were recently reported by this group.[21,23] On the basis of QSAR studies on several series of CA inhibitors, including some positively charged derivatives of type **30**,[34] 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 in the direction passing through the Zn(II) ion of the enzyme, the sulfonamide nitrogen atom, and the long axis of the inhibitor).[34] It appeared thus of interest to try to explore this result, designing positively charged, "long" sulfonamide inhibitors. Thus, we thought of attaching substituted-pyridinium-methylcarboxy moieties to the amino, hydrazino, imino, or hydroxy groups of sulfonamides **1**−**26**. The substitution pattern of the pyridinium ring was previously shown[21,23,34] to be critical for the biological activity of this type of sulfonamide CA inhibitors. Best CA inhibitors were those incorporating 2,4,6-trimethylpyridinium or 4-phenyl-2,6-dialkylpyridinium moieties in their molecule.[21,23,34] These were just the types of moieties selected for the preparation of the new sulfonamides reported here (2,4,6-trimethylpyridinium, 4-phenyl-2,6-dimethylpyridinium, 4-phenyl-2,6-diethylpyridinium, and 4-phenyl-2,6-diisopropylpyridinium).

Reaction of sulfonamides **1**−**26** with the pyridinium derivatives **A**−**D** in the presence of EDCI (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide) or diisopropylcarbodiimide afforded the new derivatives **A(1**−**26)**−**D(1**−**26)**, by a procedure relatively similar to that reported by Whitesides'[26,27] and our[17−20] groups for the preparation of aminoacylcarboxamido sulfonamides.

Chart 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; IR; ^{1}H and ^{13}C NMR spectroscopy) that confirmed their structure (see Experimental Section for details) and were assayed for the inhibition of isozymes hCA I, hCA II, and bCA IV (Table 1).

In Vitro CA Inhibition. Inhibition data against three CA isozymes, hCA I, hCA II, and bCA IV, with the new derivatives (Table 1) prove that the substituted-pyridinium-methylcarboxy moieties attached to these sulfonamides generally led to an increase of the CA inhibitory properties for the obtained compound, as compared to the corresponding parent sulfonamides **1**−**26**. Particularly strong inhibitors were those derived from heterocyclic derivatives (1,3,4-thiadiazoles, 1,3,4-thiadiazolines, benzothiazoles). Slightly less active were the 1,3-benzenedisulfonamides **11**−**13** and the 3-fluoro/chlorosulfanilamide derivatives **7** and **8**, together with the pyrimidine-substituted sulfanilamides of type **21**, the sulfanilylsulfanilamides **19**, and the sulfanilylmetanilamides **20**. The simple aromatic derivatives were (as expected) less active than the previously mentioned heterocyclic sulfonamides. The efficiency of the obtained inhibitor generally varied in the following way, based on the parent sulfonamide from which it was prepared: the derivatives of *p*-hydrazinobenzenesulfonamide **4** < the orthanilamides **1** ≅ the metanilamides **2** < the sulfanilamides **3** < the homosulfanilamides **5** < the *p*-aminoethylbenzenesulfonamides **6** ≅ the halogeno-substituted sulfanilamides **7**−**10** ≅ the 1,3-benzenedi-

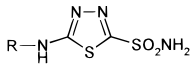
sulfonamides **11**−**13** ≅ the sulfanilylsulfanilamides **19** and the sulfanilylmetanilamides **20** < the 1,3,4-thiadiazole-2-sulfonamides **14**, **16,** and **17** ≅ 4-methyl-d_2-1,3,4-thiadiazoline-2-sulfonamides **15** and **18** ≅ the benzothiazole-2-sulfonamides **22**−**24**. On the basis of the carboxylic acid from which they were obtained, the trimethylpyridinium derivatives (**A** type) were less active than the 2,6-diisopropyl-4-phenylpyridinium derivatives (**D** type), which in turn were less active than the 2,6-diethyl-4-phenylpyridinium derivatives (**C** type). The best inhibitors in the whole series were those derived from 2,6-dimethyl-4-phenylpyridinium-methylcarboxylic acid (**B** type).

All three CA isozymes investigated here were susceptible to inhibition with this type of sulfonamides, with hCA II and bCA IV the most sensitive, whereas hCA I was generally less susceptible to inhibition as compared to the first two isozymes.

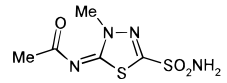
Ex Vivo Penetration through Red Blood Cells. Incubation of human red blood cells (which contain high concentrations of isozymes I and II, i.e., 150 mM hCA I and 20 mM hCA II, but not the membrane-bound CA IV)[35] with millimolar concentrations of different sulfonamide inhibitors, such as sulfanilamide **3**, acetazolamide **35**, or methazolamide **37**, led to saturation of the two isozymes present in erythrocytes with inhibitor, already after short periods of incubation (30 min), whereas for benzolamide **36** a similar effect is achieved after somehow longer incubation periods (60 min) (Table 2). This is obviously due to the high diffusibility through membranes of the first three inhibitors, whereas benzolamide, with a pK_a of 3.2 for the second sulfonamido group[36] being present mainly as a (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 **A17, A23, B7, B14, C13, C18, D15,** etc., in 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 CA inhibitors.

In Vivo Perfusion Experiments in Rats. To prove that in vivo, membrane-bound CA, but not cytosolic, isozymes are inhibited by the cationic sulfonamides reported here, additional experiments of intravenous (iv) perfusion in rats have been performed and the amount of bicarbonate excreted into the urine of the experimental animals has been analyzed, together with the level of sulfonamides present in their blood 2 h after administration of the drugs (Table 3).[30−33,37] It is well-established[30−33,37] that at least two CA isozymes, CA II and IV, are involved in bicarbonate reabsorption by the proximal and to a less extent also distal tubules in the kidneys of vertebrates (except those of *Reptilia*, in which other mechanisms were shown to be present[4b]). Administration of CA inhibitors (such as acetazolamide, benzolamide, ethoxzolamide, etc.) to vertebrates produces an alkaline urine, due to the renal excretion of bicarbonate, Na^+, K^+, and osmotically obligated water, as a consequence of CA inhibition.[4,30−33,37] Up to now, studies of selective inhibition of different CA isozymes present in the diverse compartments of renal tubular cells have been performed only with high molecular weight CA inhibitors of types **31**−**33**,[30−33] with molecular weights in the range of 3.5−99 kDa, which being membrane-impermeant due to their high molecular weight were shown to selectively inhibit only CA IV and not the cytosolic isozymes (primarily CA II). The particular nature of the compounds reported by us here allows for the first time to perform this type of study with low molecular weight inhibitors.

From data of Table 3 one can see that administration of classical low molecular weight inhibitors, such as acetazolamide **35** or benzolamide **36** (which inhibit both



35: R = CH_3CO
36: R = $C_6H_5SO_2$



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cytosolic as well as membrane-bound isozymes) leads to a peak of 105−110 mM bicarbonate excreted into urine in 12 h, as shown in the studies of Maren's group[4,30] and reconfirmed in the present work. Administration of positively charged sulfonamides such as **B14, C18,** or **D15**, in the same conditions as for the classical inhibitors mentioned above, produced bicarbonate elimination, but in a lower quantity due to the fact that only CA IV was inhibited by these membrane-impermeant compounds. On the other hand, after such experiments, only traces of positively charged sulfonamides have been detected in the red blood cells of the experimental animals, in contrast to the situation after the administration of acetazolamide or benzolamide, which produced a rapid saturation of the cytosolic isozymes present in erythrocytes (Table 3). Our data compare well with the recent data from Maren's laboratory[30] who reported an excretion of about 40 mM bicarbonate into urine, also in the rat, after 100 mg/kg of the 3.5 kDa inhibitor **31**. In their work it was concluded that both CA II and IV participate in the normal full renal reabsorption of bicarbonate, these data being in agreement with the data of Sly's laboratory[37] and obviously with the data reported by us here. Thus, corroborating the results of Tables 2 and 3, it is clear that ex vivo and in vivo, positively charged sulfonamides as those described here are able to discriminate between membrane-bound and cytosolic isozymes. This is not surprising after all, since many positively charged compounds, such as some reversible anticholinesterase agents (neostigmine, edrophonium, pyridostigmine, demecarium, etc.),[38] the quaternary ammonium antimuscarinic agents (such as methantheline),[39] or the neuromuscular blocking agents (tubocurarine, alcuronium, gallamine, atracurium, decamethonium, etc.),[40] all possessing quaternary ammonium or pyridinium moieties in their molecules, are known to be membrane-impermeant due to their cationic nature.

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 II, as well as for the membrane-bound CA IV. They were obtained by attaching pyridinium-methylcarboxylic acid moieties to the molecules of aromatic/heterocyclic sulfonamides incorporating free amino, imino, hydrazino, or hydroxyl groups in their molecule. Ring systems which have been derivatized by the above-mentioned procedures included: 2-, 3-, or 4-aminobenzenesulfonamides; 4-(ω-aminoalkyl)benzenesulfonamides; 3-halogeno-substituted-sulfanilamides; 1,3-benzenedisulfonamides; 1,3,4-thiadiazole-2-sulfonamides; and benzothiazole-2-sulfonamides, among others, and were chosen in such a way as to prove that the proposed approach is a general one. Ex vivo and in vivo studies, in two model systems (human red blood cells and perfusion experiments in rats, respectively), showed the new class of inhibitors reported here to discriminate for the membrane-bound versus cytosolic isozymes, selectively inhibiting only CA IV.

Experimental Section

General. Melting points, heating plate microscope (not corrected); IR spectra, KBr pellets, 400−4000 cm^{-1}, Perkin-Elmer 16PC FTIR spectrometer; ^1H NMR spectra, Varian 300CXP apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard); elemental analysis, 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). Analytical and preparative HPLC was performed on a reversed-phase C_{18} Bondapack column, with a Beckman EM-1760 instrument. Sulfonamides **1**−**26** used in synthesis were either commercially available compounds (from Sigma, Acros, or Aldrich) or prepared as described previously: 4-hydrazinobenzenesulfonamide **4** by diazotization of sulfanilamide, followed by reduction of the diazonium salt with tin(II) chloride;[41] halogenosulfanilamides **7**−**10** by halogenation of sulfanilamide as reported in the literature;[42] compound **15** from 5-amino-1,3,4-thiadiazole-2-sulfonamide (obtained from acetazolamide)[43] by acylation with the phthalimido derivative of β-alanine, followed by hydrazinolysis;[44] imine **14** by deprotection of methazolamide with concentrated hydrochloric acid;[45] aminobenzolamide **16** and the corresponding thiadiazoline **17** as reported in ref 30a; the sulfanilylsulfanilamide **18** and its *meta*-derivative **19** as reported in ref 29. The benzothiazole-2-sulfonamide derivatives **21**−**23** were prepared as described in ref 46, whereas the alcohols **24** and **25** were from the corresponding amines by diazotization followed by hydrolysis of the diazonium salts. The pyridinium-methylcarboxylic acids **A**−**D** were prepared as described in the literature[24,25] from glycine and the corresponding trisubstituted pyrylium salt. EDCI, diisopropyl carbodiimide, and triethylamine were from Sigma Chemical Co. Acetonitrile, acetone (E. Merck), or other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions.

General Procedure for the Preparation of Compounds A(1−26)−D(1−26). An amount of 3 mM sulfonamide **1**−**26** was dissolved/suspended in 50 mL of anhydrous acetonitrile and then treated with the stoichiometric amount (3 mM) of positively charged carboxylic acid **A**−**D**. An amount of 570 mg (3 mM) of EDCI. HCl was then added and the reaction mixture was magnetically stirred at room temperature for 15 min; then 90 μL (6 mM) of triethylamine was added and stirring was continued for 8−10 h at 4 °C (TLC control). The solvent was evaporated in vacuo and the residue taken up in ethyl acetate (5 mL), poured into a 5% solution of sodium bicarbonate (5 mL), and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate and filtered and the solvent was removed in vacuo. The pure compounds **A(1−26)−D(1−26)** were obtained either by means of preparative HPLC (C_{18} reversed-phase μ-Bondapack or Dynamax-60A (25 × 250 mm) columns; 80% acetonitrile/8% methanol/12% water, 30 mL/min) or by recrystallization from 5% aqueous perchloric acid.

4-(2,4,6-Trimethylpyridinium-*N*-methylcarboxamido)-benzenesulfonamide perchlorate, **A3**: white crystals, mp 305−7 °C dec; IR (KBr) cm^{-1} 625 (ClO_4^-), 1100 (ClO_4^-), 1143 (SO_2^{sym}), 1290 (amide III), 1352 (SO_2^{as}), 1560 (amide II), 1714 (amide I), 3365 (NH, NH_2); UV (MeOH) λ_{max} nm (log ϵ) 223 sh (4.58), 274 (3.94); ^1H NMR (TFA) δ 2.63 (s, 6H, 2,6-$(Me)_2$); 2.77 (s, 3H, 4-Me); 3.69 (s, 2H, CH_2CO); δ_A 7.18, δ_B 7.75 (AA′BB′system, 4H, J_{AB} = 7.9, ArH from 4-sulfamoylphenyl); 7.52 (br s, 2H, SO_2NH_2) (the CONH protons are in fast exchange with the solvent and are not seen in TFA); 8.10 (s, 2H, ArH, 3,5-H from pyridinium); ^{13}C NMR (TFA) δ 15.5 (s, Me); 17.9 (s, Me); 40.3 (s, CH_2CO); 128.7; 129.9; 132.1; 133.6; 135.5; 138.4; 142.5; 172.4 (CONH). Anal. ($C_{12}H_{19}N_2O_3S^+ClO_4^-$) C, H, N, S.

4-(2,6-Dimethyl-4-phenylpyridinium-methylcarboxamidoethyl)benzenesulfonamide perchlorate, B6: white crystals, mp 279−82 °C dec; IR (KBr) cm^{-1} 625 (ClO_4^-), 1100 (ClO_4^-), 1153 (SO_2^{sym}), 1295 (amide III), 1350 (SO_2^{as}), 1574 (amide II), 1715 (amide I), 3365 (NH, NH_2); UV (MeOH) λ_{max} nm (log ϵ) 223.5 sh (4.28), 294 (4.32); ^1H NMR (TFA) δ 3.00 (s, 6H, 2,6-$(Me)_2$); 3.07 (t, 2H, $^3J_{HH}$ = 6.7, αCH_2); 3.15 (s, 3H, N-Me); 3.74 (s, 2H, CH_2CO); 3.78 (t, 2H, $^3J_{HH}$ = 6.7, βCH_2); 7.10−7.77 (m, 9H, ArH from 4-sulfamoylphenyl + 4-Ph); 7.55 (br s, 2H, SO_2NH_2) (the CONH protons are in fast exchange with the solvent and are not seen in TFA); 8.04 (s, 2H, 3,5-H from pyridinium); ^{13}C NMR (TFA) δ 17.4 (s, Me); 30.3 (s, CH_2 of aminoethyl); 37.1 (s, CH_2 of aminoethyl); 41.3 (s, CH_2CO); 128.4; 130.7; 132.8; 133.6; 134.6; 145.5; 170.6 (CONH). Anal. ($C_{22}H_{23}N_2O_3S^+ClO_4^-$) C, H, N, S.

5-(2,6-Diethyl-4-phenylpyridinium-methylcarboxamido)-1,3,4-thiadiazole-2-sulfonamide perchlorate, C14: white crystals, mp > 310 °C; IR (KBr) cm^{-1} 625 (ClO_4^-), 1100 (ClO_4^-), 1176 (SO_2^{sym}), 1290 (amide III), 1369 (SO_2^{as}), 1572 (amide II), 1715 (amide I), 3060 (NH), 3365 (NH_2); UV (MeOH) λ_{max} nm (log ϵ) 221.7 sh (4.30), 294 (4.12); ^1H NMR (TFA) δ 1.55 (t, 6H, 2 Me from Et); 3.30 (q, 4H, 2 CH_2 from Et); 3.75 (s, 2H, CH_2CO); 7.08−8.63 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium); 7.25 (br s, 2H, SO_2NH_2); ^{13}C NMR (TFA) δ 13.8 (s, Me); 36.9 (CH_2 of Et); 41.5 (s, CH_2CO); 128.1; 130.5; 132.9; 133.4; 134.6; 145.8; 159.3 (C-2 of thiadiazole); 170.0 (C-5 of thiadiazole); 171.7 (CONH). Anal. ($C_{17}H_{19}N_5O_3S_2^+ClO_4^-$) C, H, N, S.

6-(2,6-Diisopropyl-4-phenylpyridinium-methylcarboxyethoxy)benzothiazole-2-sulfonamide perchlorate, **D24**: tan crystals, mp >310 °C; IR (KBr) cm^{-1} 625 (ClO_4^-), 1100 (ClO_4^-), 1149 (SO_2^{sym}), 1293 (amide III), 1365 (SO_2^{as}), 1578 (amide II), 1713 (amide I), 3060 (NH), 3365 (NH_2); UV (MeOH) λ_{max} nm (log ϵ) 225 (4.05), 296 (4.21); ^1H NMR (TFA) δ 1.55 (d, 12H, 4 Me from *i*-Pr); 3.53 (heptet, 2H, 2 CH from *i*-Pr); 7.28−8.65 (m, 14H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium+ 3H of benzothiazole); 8.25 (br s, 2H, SO_2NH_2); ^{13}C NMR (TFA) δ 10.5; 11.7; 30.3; 35.9 (s, CH_2CO); 40.8; 49.3; 128.6; 130.7; 132.0; 133.9; 137.5; 141.9; 149.8; 174.1 (CONH). Anal. ($C_{24}H_{25}N_3O_5S_2^+ClO_4^-$) C, H, N, S.

Enzyme Preparations. Human CA I and II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog et al.[47] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group,[48] and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[49] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1}\cdot cm^{-1}$ for CA I and 54 $mM^{-1}\cdot cm^{-1}$ for CA II, respectively, based on M_r = 28.85 kDa for CA I and 29.30 kDa for CA II, respectively.[50,51] CA IV was isolated from bovine lung mi-

crosomes as described by Maren et al., and its concentration was determined by titration with ethoxzolamide.[52]

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.[53] 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 14 800 $M^{-1} \cdot cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature.[53] Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor concentration, and the values reported throughout the article are the means 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.[53] Enzyme concentrations were 3.1 nM for hCA II, 8.5 nM for hCA I, and 31 nM for bCA IV (this isozyme has a decreased esterase activity[15] and higher concentrations had to be used for the measurements).

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.[54] The cells were then lysed in 25 mL of distilled water and centrifuged for eliminating membranes and other insoluble impurities. The obtained solution was heated at 100 °C for 5 min (in order to denature CAs) and sulfonamides possibly present were assayed in each sample by three methods: HPLC method,[55,56] spectrophotometrically,[57] and enzymatically.[53]

HPLC: A variant of the methods of Gomaa[55] and Iyer and Taft[56] 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, 4.12 min for sulfanilamide, 2.15 min for **A17**, 2.45 min for **B7**, 2.78 min for **A23**, 2.96 min for **B14**, 3.06 for **C13**, 2.87 for **C18**, and 3.24 min for **D15**. The eluent was monitored continuously for absorbance (at 254 nm for acetazolamide and wavelength 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.[57] was used, working for instance at 260 and 292 nm, respectively, for acetazolamide; at 225 and 265 nm, respectively, 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 based on hCA II inhibition measured with the esterase method, as described above.[53] 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. Mention should be made that the three methods presented above led to results in good agreement, within the limits of the experimental errors.

Intravenous Perfusion Experiments in Rats. Adult male Wistar rats were perfused intravenously with solutions of sulfonamide CA inhibitors in the concentration ranges of 3−15 mg/kg (equal volumes of inhibitor solutions were used in all experiments). Urine was collected for the next 12 h and the amount of bicarbonate present in it was determined enzymatically with a phosphoenol pyruvate carboxylase−malate dehydrogenase assay, with a kit from Gilford Systems (Oberlin, OH).[58] Three animals were used for each inhibitor, and the data reported in Table 3 are the means of such assays. Small amounts (0.5 mL) of blood were also prelevated from these animals, at 1 and 2 h after starting the perfusion experiment in order to determine the level of sulfonamides present in the erythrocytes. Red blood cells were treated as above (except that they were not incubated with any inhibitor) and sulfonamides present in the cell lysate were measured by the HPLC method mentioned above.

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