Carbonic Anhydrase Inhibitors: Synthesis and Inhibition of Cytosolic/ Membrane-Associated Carbonic Anhydrase Isozymes I, II, and IX with Sulfonamides Incorporating Hydrazino Moieties

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Targeting proteins overexpressed in hypoxic tumors is as an important means of controlling cancer disease. One such protein is the carbonic anhydrase (CA) isoenzyme IX, which in some types of tumors is overexpressed 150−200-fold. We report here a series of sulfonamide derivatives, prepared from 2-carbohydrazido- and 4-carbohydrazido-benzenesulfonamides, which were further derivatized by reaction with aryl isocyanates or arylsulfonyl isocyanates. Several low nanomolar CA IX inhibitors were detected in this way. SAR is discussed for the diverse types of inhibitors and their affinity for different isozymes, with the aim of obtaining isozyme-specific CA IX inhibitors, with putative applications as antitumor drugs.

Introduction

It has only recently been discovered that invasive growth and metastatic spread of many tumors types are closely associated with hypoxia.[1] Tumor hypoxia is the result of the abnormal process of neoplastic growth and crucially depends on oxygen/nutrients supply from the host.[1] Thus, changes in tumor metabolism and microenvironment connected with adaptation of cells to hypoxia are important components of tumor progression.[1,2] Hypoxic conditions elicit cellular responses designed to improve cell oxygenation and survival by means of several mechanisms such as neoangiogenesis, improved glycolysis, and enhanced energy production, as well as upregulation of molecules related to cell survival/apoptosis.[1] The most important molecule regulating the mammalian response to hypoxia is the heterodimeric protein hypoxia-inducible factor 1 (HIF-1), which in turn up-regulates genes involved in adaptation responses to hypoxic conditions.[1] Two such genes encode for the transmembrane carbonic anhydrase (CA, EC 4.2.1.1) isozymes CA IX and CA XII, containing extracellular enzyme active sites. These CAs appear to participate in tumorigenetic processes via their ability to catalyze hydration of CO_2 to bicarbonate and protons, regulating in this way the intratumoral pH.[2] In addition, CA IX, possessing a unique N-terminal domain, has a capacity to perturb E-cadherin-mediated cell−cell adhesion via interaction with β-catenin and may potentially contribute to tumor invasion.[2] CA IX shows restricted expression in normal tissues but is tightly associated with different types of tumors, mostly due to its strong induction by tumor hypoxia that involves HIF-1 binding to a hypoxia response element in the *CA9* gene promoter.[1,2] CA IX was proposed to serve as a marker of tumor hypoxia, and its predictive and prognostic potential has been demonstrated in numerous clinical studies (reviewed in ref 2) CA XII is present in many normal tissues and overexpressed in some tumors.[2] It is also induced by hypoxia, but the underlying molecular mechanism remains undetermined. Both CA IX and CA XII are negatively regulated by von Hippel Lindau tumor suppressor protein, and their expression in renal cell carcinomas is related to inactivating mutation of *VHL* gene.[2] The high catalytic activity of these two CA isoforms supports their role in acidification of tumor microenvironment that facilitates acquisition of metastatic phenotypes.[2−4] Therefore, modulation of extracellular tumor pH via inhibition of CA activity represents a promising approach to anticancer therapy.[2−4] Sulfonamide CA inhibitors (CAIs) were shown to compromise tumor cell proliferation and invasion in vitro and improve the effect of conventional chemotherapy in vivo.[2−5] However, their precise targets are not known in detail at this moment, but it is presumed that these two tumor-associated CA isozymes, i.e., CA IX and XII, may represent important molecules for targeting cancer cells, by an unconventional therapeutic approach.[2−5]

In previous work from this laboratory, we showed that CA IX is a target for which drugs can be developed.[6−9] In such papers we have explored the design of potent and preferably selective sulfamate/sulfonamide CA IX inhibitors belonging to various chemical classes.[6−9] It was thus observed among others that unlike for other CA isozymes (such as for example CA I, II, or V among others),[10−13] aromatic sulfonamides are generally better CA IX inhibitors, as compared to the heterocyclic derivatives. Thus, it appeared of interest to explore other chemical scaffolds incorporating aromatic (benzene) sulfonamide derivatives that led to the best CAIs

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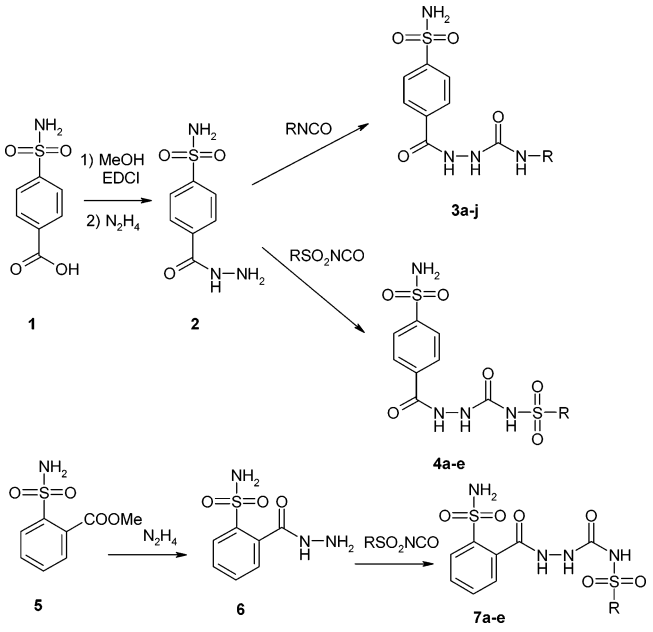
targeting CA IX reported up to now.[13] In this work we consider several simple approaches to obtain such compounds. A common denominator of all derivatives reported in this paper is the presence of a hydrazine moiety in their molecule. This was considered to be beneficial for CA IX targeting compounds, for several reasons: (i) the facile synthesis and subsequent derivatization of such compounds, leading to a chemical diversity necessary when such new targets are considered;[14] (ii) the hydrazine moiety is susceptible to redox chemistry which may be advantageous in the hypoxic environment present in some tumors;[1,2] and (iii) the presence of this moiety in some recently approved drugs, such as for example the HIV protease inhibitor atazanavir, which has been shown to be devoid of major toxicity and induced excellent bioavailability to this drug, which is the first protease inhibitor for once-a-day oral dosing.[15]

Chemistry. Benzenesulfonamide derivatives show well-known CA inhibitory properties, and a wide range of such compounds have been used in the design of inhibitors with various medicinal chemistry applications.[16−19] A drawback of some of these simple derivatives (such as orthanilamide, the halogenated sulfanilamides, the 4-amino-1,3-benzene-disulfonamides, etc.) is constituted sometimes by the rather low reactivity of amino groups grafted on the aromatic ring, which are deactivated for nucleophilic substitution reactions by the presence of the sulfamoyl moiety/moieties.[20] Thus, we decided to investigate whether the substitution of the amino moiety of some of these derivatives with the hydrazine one may lead to an enhanced reactivity, to further derivatize them for obtaining CAIs with various applications.

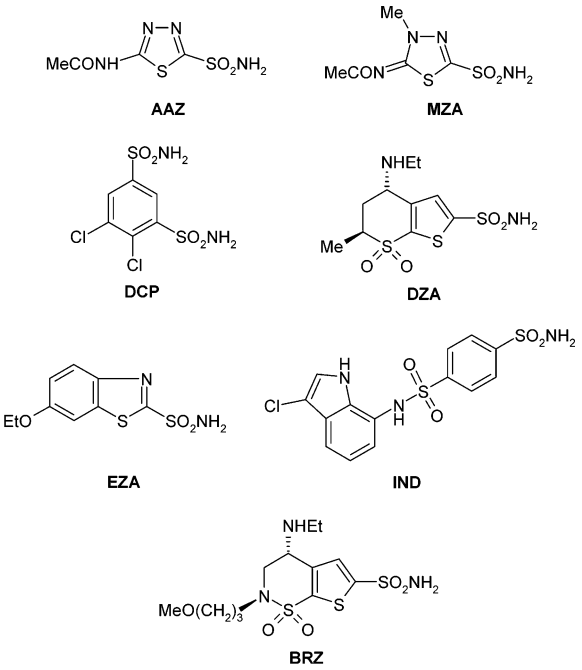
Two approaches have been considered here for achieving this goal: (i) starting from commercially available simple derivatives, such as 4-carboxybenzenesulfonamide **1** or 2-carboxymethylbenzenesulfonamide **5**, the corresponding hydrazides **2** and **6**, respectively, have been obtained,[16,17] by reaction of the corresponding methyl ester with hydrazine hydrate. The two hydrazides **2** and **6** were then further derivatized, by reaction with aryl isocyanates, leading to ureas **3a**−**j**,[21] or by reaction with arylsulfonylisocyanates,[22] leading to sulfonyl ureas **4a**−**e** and **7a**−**e**, respectively (Scheme 1). Indeed, in previous work from this laboratory it has been demonstrated that both ureido- as well as arylsulfonylureido-substituted aromatic/heterocyclic sulfonamides lead to potent CAIs targeting isozymes I, II, and IV,[21,22] and this has thereafter been rationalized after the report of the X-ray crystal structure of some of these derivatives (or their structurally related congeners) with hCA II. In such adducts it has been observed that the ureido/carboxamido part of the inhibitor participates in several favorable interactions with amino acid residues at the entrance of the active site, ensuring in this way a stabilized E−I complex, and as a consequence potent CA inhibitory properties to these compounds;[23] (ii) the conversion of the amino to the hydrazine moiety, in simple compounds such as sulfanilamide, halogenosulfanilamides, or orthanilamide (derivatives **8**−**10** and **17**) by means of the diazonium salts, which have been prepared (but not isolated) by reaction of the amine with in situ generated nitrous

Scheme 1



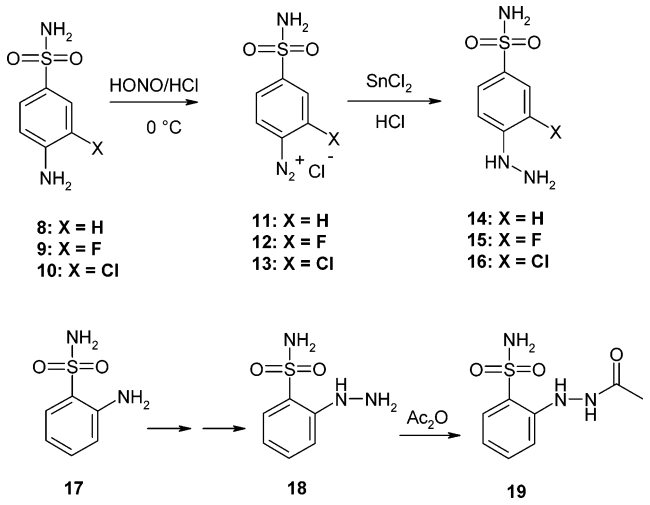
acid. The diazonium salts **11**−**13** were then reduced to hydrazines **14**−**16** and **18** with tin(II) chloride or sodium sulfite in acidic medium,[18] and the last compound has also been acetylated with acetic anhydride at room temperature, leading to compound **19** (Scheme 2).

CA Inhibition. Data of Table 1 show CA I, II, and IX inhibition with the new compounds reported here, as well as clinically used CAIs, such as acetazolamide (AAZ), methazolamide (MZA), ethoxzolamide (EZA), dichorophenamide (DCP), dorzolamide (DZA), and brinzolamide (BRZ). Indisulam (E7070) (IND), an antitumor



sulfonamide in phase II clinical trials for which we recently demonstrated potent CA inhibitory properties, has also been included for comparison in this study.[5d,10,24] Furthermore, the X-ray crystal structure of IND in adduct with isozyme hCA II has recently been reported by our group.[10]

Scheme 2



The following SAR should be noted from data of Table 1: (i) the derivatives **1**−**19** investigated here showed inhibitory activity against all three investigated CA isozymes, but affinity was in the micromolar range for the cytosolic isozyme hCA I, and generally in the (low) nanomolar range for the other cytosolic isozyme, hCA II, as well as the transmembrane, tumor-associated isozyme hCA IX. Thus, against hCA I, these compounds showed K_Is in the range of 0.55−48.3 μM, against hCA II in the range of 5.1−420 nM, and against hCA IX in the range of 3.2−680 nM; (ii) the best hCA I inhibitors were some of the compounds designed starting from 4-sulfamoylbenzenecarbohydrazide **2**, such as the ureas **3d** and **3e**, as well as the sulfonylureas **4b**−**e**, which showed K_Is in the range of 0.55−0.865 μM, whereas other substitution patterns, or derivatives designed starting from **6** as well as the simple hydrazine derivatives **14**−**16** and **18**, **19**, were much less effective inhibitors; (iii) against hCA II, the best inhibitors were the ureas **3a**−**h**, and the sulfonylureas **4c**−**e**, which showed K_Is in the range of 5.1−15 nM. It may be observed that again these compounds are derived from 4-sulfamoylbenzenecarbohydrazide **2**, and that the nature of the group present in the original isocyanate/sulfonylisocyanate (R), is the most important factor influencing CA II inhibitory properties. Thus, such 4-substituted-phenyl-, 3,4-dichlorophenyl-, or biphenyl-moieties seem to be quite beneficial for the hCA II inhibitory properties, whereas bulkier moieties (adamantly, fluorenyl, etc.), such as in **3g**, **3i**, and **3j**, lead to a decreased activity. The simple derivatives **1, 2**, and **14**−**18** were less effective hCA II inhibitors as compared to the above-mentioned compounds. Some of the best hCA II inhibitors among the new derivatives reported here showed the same level of hCA II inhibition as some of the clinically used compounds (acetazolamide, methazolamide, dorzolamide, indisulam, etc.), whereas dichlorophenamide, a clinically used systemic antiglaucoma drug,[4] is slightly less effective as hCA II inhibitor, Table 1. It should also be mentioned that the sulfonylureas isomeric to **4a**−**e**, of types **7a**−**e**, obtained from the *o*-carbohydrazidobenzenesulfonamide **6**, were much less effective hCA II inhibitors as compared to the para-substituted corresponding compounds. This may be due to the sterical impairment of the ortho-substituent for the binding of such compounds to the Zn(II) ion within the enzyme active site. This was also the reason why a smaller number of such derivatives has been synthesized, and our main interest has been concentrated on the para-substituted compounds; (iv) against hCA IX, the best inhibitors in the investigated series were again the ureas **3a**−**j** and the sulfonylureas **4c**−**e**, which showed K_Is in the range of 3.2−24 nM, being more effective than the clinically used sulfonamides (acetazolamide−brinzolamide), and better or equally potent hCA IX inhibitors as indisulam IND, the sulfonamide with the most impressive in vivo antitumor properties reported up to now.[5d,10,24] Thus, the nitrophenyl- (**3d**) and biphenyl-substituted ureas (**3f**) as well as the chlorophenylsuylfonylureido derivative **4e**, showed inhibition constants in the range of 3.2−5.4 nM, being among the most potent hCA IX inhibitors detected so far, and their potency compared to that of indisulam is 5−7-fold increased. As a whole, almost all substitution patterns of ureas **3** reported here were beneficial for the hCA IX inhibitory properties, and generally all these compounds were better hCA IX than hCA II inhibitors (except **3e**), a situation which is reversed as compared to the clinically used derivatives (including indisulam) which are all better hCA II than hCA IX inhibitors (Table 1). The simple derivatives **1** and **2**, as well as

Table 1. Inhibition Data for Derivatives **1**−**19** Investigated in the Present Paper and Standard Sulfonamide CAIs, against Isozymes I, II, and IX

		K_I[a] (nM)		
compd	R	hCA I[b]	hCA II[b]	hCA IX[c]
AAZ	-	250	12	25
MZA	-	50	14	27
EZA	-	25	8	34
DCP	-	1200	38	50
DZA	-	50000	9	52
BRZ	-	nt[e]	3	37
IND	-	31	15	24
1	-	3400	258	346
2	-	2950	124	175
3a	3,4-$Cl_2C_6H_3$	1150	13	7.9
3b	4-Ac-C_6H_4	1450	15	8.3
3c	4-EtOOC-C_6H_4	1200	9.0	8.5
3d	4-O_2N-C_6H_4	760	5.1	3.2
3e	4-Br-C_6H_4	865	7.3	8.6
3f	4-Ph-C_6H_4	1160	11	5.4
3g	4-PhO-C_6H_4	1450	18	7.9
3h	4-$PhCH_2$-C_6H_4	1300	15	7.0
3i	4-Ad-C_6H_4[d]	2400	33	24
3j	9-fluorenyl	1360	25	13
4a	Ph	1250	67	35
4b	2-Me-C_6H_4	800	54	32
4c	4-Me-C_6H_4	700	9.8	5.8
4d	4-F-C_6H_4	600	9.5	6.2
4e	4-Cl-C_6H_4	550	9.1	5.4
5	-	35800	420	680
6	-	24500	338	547
7a	Ph	4800	107	76
7b	2-Me-C_6H_4	4700	92	83
7c	4-Me-C_6H_4	3400	85	70
7d	4-F-C_6H_4	4900	97	64
7e	4-Cl-C_6H_4	3600	94	72
14	-	3900	83	265
15	-	3300	51	136
16	-	2800	76	149
17	-	45400	295	33
18	-	36000	78	48
19	-	48300	264	175

[a] Errors in the range of 5−10% of the reported value (from three different assays). [b] Human cloned isozyme, by the CO_2 hydration method. [c] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method. [d] Ad = 1-adamantyl. [e] nt = not tested.

the ortho-substituted derivatives **7a**−**e**, or the hydrazines **15**−**19**, were weaker hCA IX inhibitors, generally with inhibition constants in the range of 33−346 nM.

Experimental Section

To a stirring 0.1 M solution of methyl 2-(aminosulfonyl)-benzoate (commercially available, Sigma-Aldrich, Milan, Italy) or methyl 4-(aminosulfonyl)benzoate (prepared by esterification of the corresponding carboxylic acid with 1 equiv of EDCI in a mixture methylene chloride−methanol 1:1) in methanol was added 10 equiv of hydrazine hydrate. The solution was allowed to stir at room-temperature overnight then concentrated in vaccuo. The residue was coevaporated several times with toluene until obtaining the desired compound as a white powder, which was recrystallized in ethanol. Characterization of all the compounds is available as Supporting Information to this article.

General Procedure for the Coupling of Sulfamoylbenzoic Acid Hydrazides 2 and 6 with Arylsulfonyl Isocyanate and Aryl Isocyanate. To a stirred solution of sulfamoylbenzoic acid hydrazide **2** or **6** (1 equiv) in acetone was added at room temperature the sulfonyl isocyanate or aryl isocyanate (1 equiv). The reaction was monitored by TLC until starting material was consumed. Then the mixture was concentrated under vacuum, and the residue was triturated with ethylic ether. After filtration and washing with methylene chloride, the resulting powder can be purified by column chromatography to yield the expected product in 70% yield.

Synthesis of Hydrazinobenzenesulfonamides 14−16 and 18. Concentrated (36%) hydrochloric acid (6 mL) and 10 g of ice were added to 1 g of 4-amino-3-chloro/fluorobenzenesulfonamide (**9** or **10**). The suspension was cooled on ice and stirred. An amount of 1 g of sodium nitrite was dissolved into a minimal amount of water (2 mL), and this solution was added dropwise to the benzenesulfonamide solution (temperature < 5 °C). At the end of this step, 3 g of sodium sulfite was poured in the medium, and the obtained suspension was stirred for 12 h. The suspension was then dried by solvent evaporation under depression. The residue was suspended in acetone, and the insoluble part was harvested by filtration. This precipitate was dissolved in a minimal amount of water, and the pH was adjusted to 7 with sodium hydroxide. The solution was then extracted three times with diethyl ether, and the organic phases were collected, dried on magnesium sulfate, and evaporated under depression. The dried residue was dissolved in acetone, and drops of hydrochloric acid were added in order to precipitate the hydrazine hydrochlorides **15** and **16** which were filtered and washed with acetone. The purity of the final compound was verified by TLC (MeOH/$CHCl_3$: 3/7).

CA Inhibition. Human CA I, CA II, and CA IX cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) as previously described.[20,25] A variant of the previously published[6,7] CA IX purification protocol has been used for obtaining high amounts of hCA IX needed in these experiments. The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[25]) was amplified by using PCR and specific primers for the glutathione S-transferase (GST)-Gene Fusion Vector pGEX-3X. The obtained fusion construct was inserted in the pGEX-3X vector and then expressed in *Escherichia coli* BL21 Codon Plus bacterial strain (from Stratagene). The bacterial cells were sonicated and then suspended in the lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8, 150 mM NaCl and 0.2% Triton X-100). After incubation with lysozyme (approximately 0.01 g/L), the protease inhibitors Complete were added to a final concentration of 0.2 mM. The obtained supernatant was then applied to a prepacked Glutathione Sepharose 4B column and extensively washed with buffer, and the fusion (GST-CA IX) protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin. The advantage of this method over the previous one,[6,7] is that CA IX is not precipitated in inclusion bodies from which it has to be isolated by denaturing−renaturing in the presence of high concentrations of urea, when the yields in active protein were rather low, and the procedure much longer. The obtained CA IX was further purified by sulfonamide affinity chromatography,[20] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[6,7] The specific activity of the obtained enzyme was the same as the one previously reported,[6,7] but the yields in active protein were 5−6 times higher per liter of culture medium). An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA CO_2 hydration activity assays.[32] 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.[32] 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 15 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.

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Supporting Information Available: Complete characterization of the compounds described in this paper. This material is available free of charge via the Internet at http://pubs.acs.org.

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