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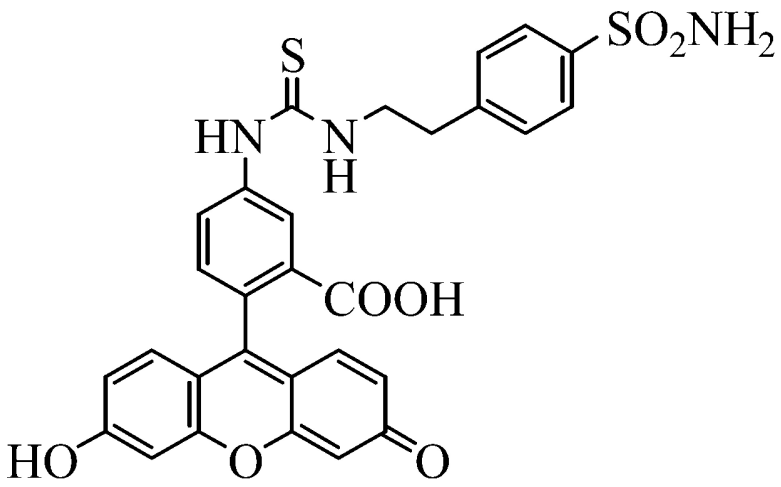

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Carbonic Anhydrase Inhibitors. Design of Fluorescent Sulfonamides as Probes of Tumor-Associated Carbonic Anhydrase IX That Inhibit Isozyme IX-Mediated Acidification of Hypoxic Tumors†

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Sulfonamides inhibit the catalytic activity of carbonic anhydrases (CAs, EC 4.2.1.1), enzymes participating in the regulation of acid−base balance and ion transport in many tissues. Carbonic anhydrase IX (CA IX), a transmembrane isoform with predominant association with tumors and limited distribution in normal tissues, is strongly overexpressed by hypoxia. Hypoxia increases the catalytic performance of CA IX contributing to microenvironmental acidosis, which influences cancer progression and treatment outcome. CA IX represents a target for detection and therapy of hypoxic tumors. Sulfonamide CA IX selective inhibitors accumulate only in hypoxic cells containing CA IX, reversing acidification mediated by this enzyme. The design of fluorescent sulfonamides that preferentially inhibit the activity of CA IX, showing reduced penetration through the plasma membranes and binding to hypoxic cells expressing CA IX, is reported here. These inhibitors represent promising candidates for developing anticancer therapies based on tumor-associated CA isozyme inhibition and offer interesting tools for imaging and further investigation of hypoxic tumors.

Introduction

It was known for several years that many sulfonamides possessing carbonic anhydrase (CA, EC 4.2.1.1)[1,2] inhibitory properties also inhibit in various degrees the growth of tumor cells in vitro and in vivo.[1−5] The precise isozyme(s) involved in such processes, among the 15 presently characterized human CAs, was not known until recently, but the discovery of CA IX [6] and then of CA XII [7] (as isozymes overexpressed in tumors) offered a starting point for more detailed studies in the field. Another issue little understood in the first years of "CA-tumors connection" research was why various tumor cell lines belonging to the same tumor type (for example, leukemia, non-small-cell lung cancer, ovarian, melanoma, colon, central nervous system (CNS), renal, prostate, and breast cancer) showed very different sensitivity to inhibition by sulfonamides, with GI_{50} (molarity of inhibitor producing a 50% inhibition of tumor cell growth) values typically in the range of 30 μM to 10 nM.[4,5] It was discovered only later that CA IX and CA XII are not present in all tumor types[2] and furthermore that the levels of isozyme IX, the best studied one at this moment, dramatically increase in response to hypoxia via a direct transcriptional activation of the CA9 gene by the hypoxia inducible factor HIF-1.[8] It has also been proven thereafter that the expression of CA IX in tumors is a sign of poor prognosis.[9]

Recent functional studies have revealed that CA IX is not just a surrogate marker of tumor hypoxia but could also contribute to tumorigenesis via its participation in the control of cell adhesion and in pH regulation.[10,11] Using a transfected cell model with the constitutive level of CA IX, we demonstrated that hypoxia activates the capacity of CA IX to acidify extracellular pH.[11] Hypoxic induction of both transcription and catalytic activity of CA IX indicates a protective role for CA IX in the cells exposed to hypoxia. This may have important implications for tumor biology because hypoxia causes extensive changes in the gene expression profile, selects for cells that can adapt to low oxygen stress, and thus increases the tumor aggressiveness.[12] Acidic extracellular pH (pHe), which is associated with hypoxia, supports tumor progression by different molecular mechanisms.[13−16] It can influence the uptake of anticancer drugs and modulate the response of tumor cells to conventional chemo- and radiotherapy.[2,13a] Noteworthy is that CA IX mediated acidification was reversed by selective sulfonamides not only in the transfected cells but also in the tumor cell lines that naturally express the CA IX protein. This effect was observed only under hypoxia and not in the normoxic cell cultures. In addition, a fluorescein-labeled sulfonamide became bound only to the hypoxic cell that expressed CA IX and not to their normoxic counterparts or to the control cells lacking CA IX.[11] Here, we show that this sulfonamide accumulates only in the cells containing CA IX with intact catalytic domain and

† This paper is dedicated to the memory of Mircea D. Banciu (1942−2005).

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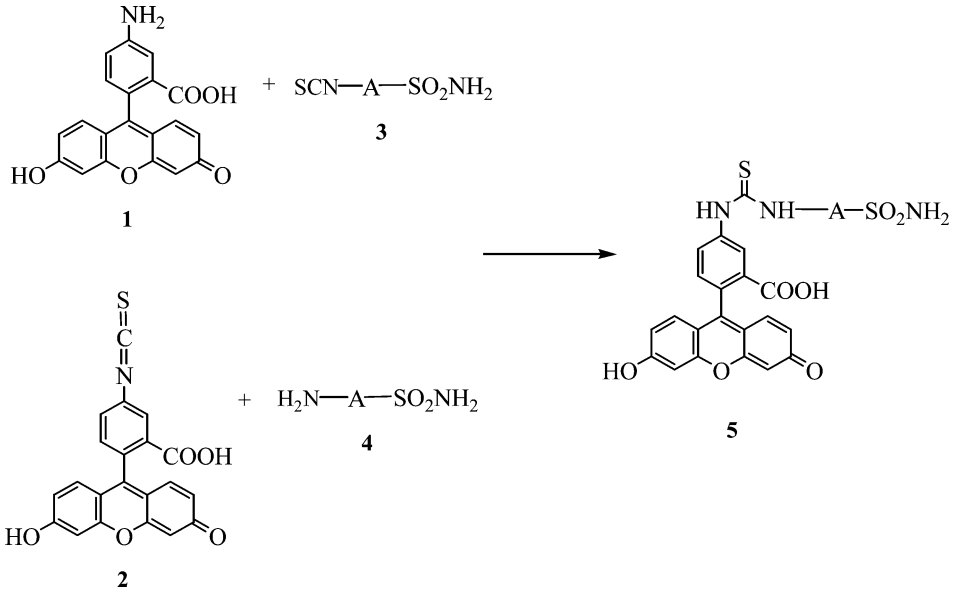
Scheme 1



report the design of a new class of strong CA IX inhibitors bearing fluorescent tails, which may become useful tools for imaging hypoxic tumor cells via binding to this isozyme or for inhibiting it with reduction of tumor acidosis.

Results

Chemistry. The fluorescent sulfonamides reported here have been prepared by two approaches, as shown in Scheme 1: (i) reaction of the aminofluorescein derivative **1** with isothiocyanato-aromatic/heterocyclic sulfonamides **3**[17] or (ii) reaction of fluorescein isothiocyanate (FITC) **2** with amino-substituted aromatic/heterocyclic sulfonamides **4**, as previously reported for structurally related thioureas[18] (Scheme 1). The new sulfonamides **5a**−**5j** (Chart 1) reported here were fully characterized, and their structures have been confirmed (see Experimental Section for details).

CA Inhibition. Inhibition data against isozymes I, II, and IX with **5** reported here are shown in Table 1.[19] The data of some standard inhibitors as well as compounds previously reported by our group are also shown for comparison.

Ex Vivo Penetration through Red Blood Cell Membranes. Levels of sulfonamides in red blood cells after incubation of human erythrocytes with millimolar solutions of inhibitor for various periods of time (starting with 30−60 min till 48 h) are shown in Table 2.[20−22]

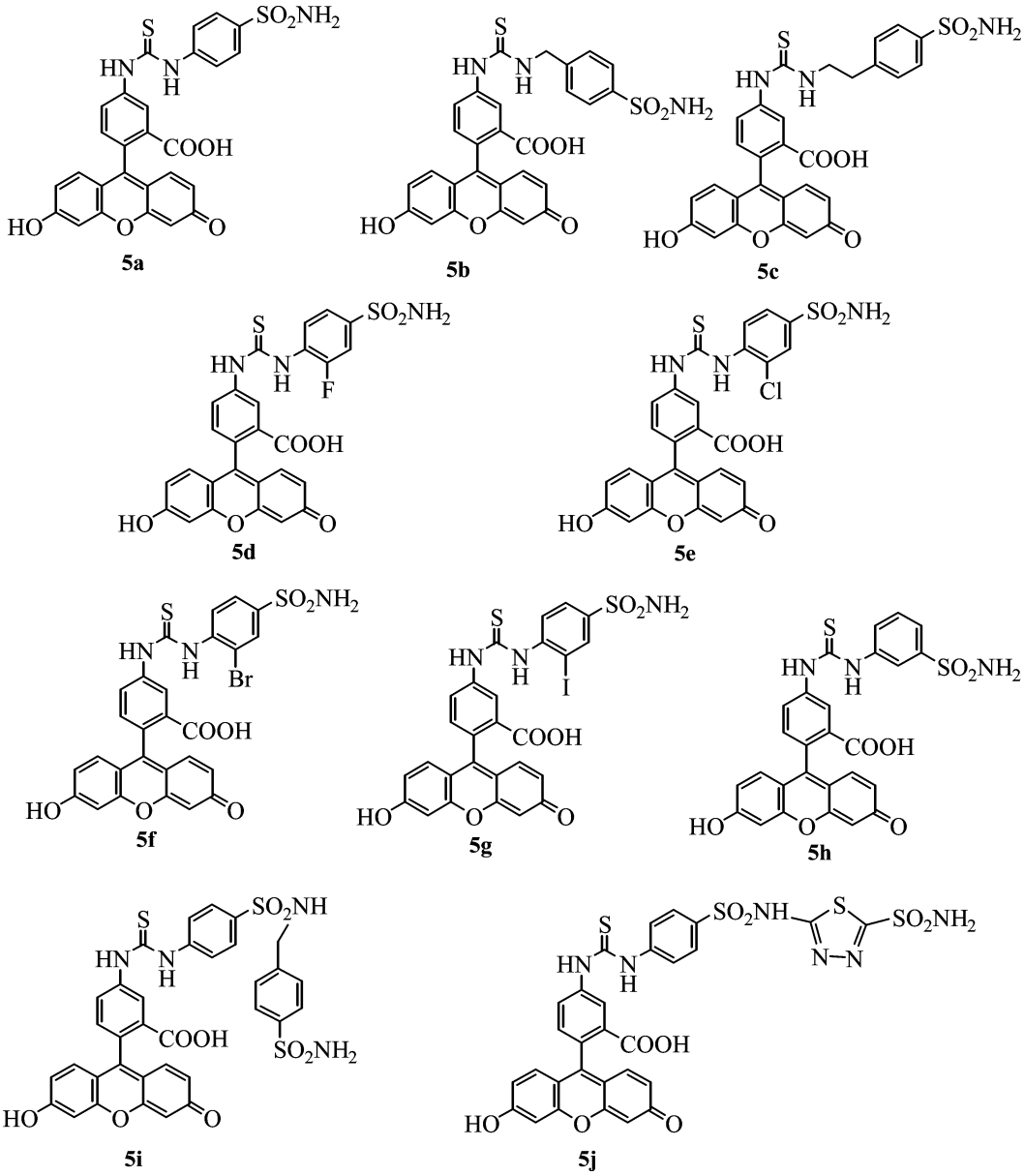
Inhibition of CA IX Mediated Acidification of the Extracellular pH by Sulfonamides. The CA IX transfected MDCK cells and mock-transfected controls used for determining the pHe values in hypoxia/normoxia were treated with three different sulfonamide CAIs including the fluorescent derivative **5c**. The sulfonamides binding to hypoxic MDCK-CA IX cells and their effect on the pHe are shown in Figure 1. The sulfonamides were added to MDCK-CA IX cells just before their transfer to hypoxia, and the pHe was measured 48 h later.

Sulfonamide Binding to Cells Expressing CA IX Deletion Variants. To prove that the accumulation of sulfonamide in hypoxic cells containing CA IX is mediated by the CAI interaction with the catalytic domain of CA IX, we examined the binding of the fluorescent sulfonamide **5c** to MDCK cells transfected with two CA IX deletion variants (Figure 2a). The variant designated ΔPG was lacking an N-terminal proteoglycan-like region (aa 42−130) but contained an intact catalytic domain. In contrast, a large part of the catalytic domain (aa 167−405) was missing from the ΔCA variant, which was shown earlier as being enzyme-dead and unable to acidify pHe.[11] In accord with the selective inhibition of CA IX catalytic activity, the fluorescent sulfonamide **5c** accumulated in the hypoxic MDCK cells expressing ΔPG variant with the complete CA domain but not in the cells containing inactive ΔCA variant (Figure 2b).

Discussion

Chemistry. A series of fluorescent sulfonamides of type **5** have been designed for studying the binding of inhibitors to the tumor-associated isozyme CA IX in normoxia or hypoxia conditions. Two approaches have been used for obtaining the new sulfonamides **5**: (i) reaction of aminofluorescein **1** with isothiocyanato derivatives of aromatic/heterocyclic sulfonamides **3** and (ii) reaction of fluorescein isothiocyanate (FITC) **2** with aminosulfonamides **4**. In both cases the thioureas **5** have been obtained with good yields, and the desired fluorescent compound has been purified by flash chromatography. During the synthesis of **5f**, an unexpected side reaction occurred, leading to the formation of large amounts of the bis-fluorescein-thiourea **6** (Scheme 2), which could be separated chromatographically from the desired sulfonamide **5f**. A similar side reaction has also been noticed earlier for the preparation of ureidosulfonamides by our group,[18] but it is the first time we observe this during the synthesis of thioureido derivatives. It is probable that **5f** formed according to Scheme 1 reacts thereafter with another equivalent of amine **1**, with displacement of the bromosulfanilyl moiety and formation of the bis-fluorescein-thiourea **6**, as depicted in Scheme 2. It is also interesting to note that this side reaction has not been encountered for the structurally

Chart 1



related halogenosulfanilamides used for the preparation of other **5** derivatives.

CA Inhibition. The data of Table 1 show the inhibition properties against the cytosolic isozymes hCA I and II, the transmembrane and tumor-associated isozyme hCA IX of the new sulfonamides **5** reported here, and standard, clinically used inhibitors (acetazolamide AAZ, methazolamide MZA, ethoxzolamide EZA, dichlorophenamide DCP, and indisulam IND) or other sulfonamides previously investigated by us for targeting the tumor-associated CAs (such as **7** and **8**) (Chart 2).[23−25]

The following should be noted regarding the data of Table 1: (i) The fluorescent sulfonamides **5** reported here behave as moderate to weak inhibitors against the slow cytosolic isozyme hCA I, with inhibition constants in the range of 480−1500 nM. It is in fact well-known[1,2] that this isozyme has a lower affinity for sulfonamides, compared to hCA II or hCA IX. Thus, these fluorescent sulfonamides show similar affinities for this isozyme as the clinically used compounds AAZ, MZA, or DCP, whereas ethoxzolamide EZA and indisulam IND are much more potent CA I inhibitors (K_I values in the range of 25−31 nM). (ii) Compounds **7** and **8** also show modest hCA I inhibitory properties (Table 1) against the major cytosolic isozyme hCA II, and the fluorescent sulfonamides **5** show a very compact behavior as efficient inhibitors, with K_I values in the range of 27−52 nM. This behavior is probably due to the fact that the bulky fluorescein tail is probably the main contributor to activity in addition to the binding of the ionized sulfonamide moiety of the inhibitor to the Zn(II) ion within the enzyme active site.[26,27] In fact, several recent X-ray crystallographic studies on adducts of hCA II with sulfonamides showed that the tails attached to the aromatic/heterocyclic sulfonamide scaffold make extensive contacts with amino acid residues both in the middle and at the entrance of the active site, leading thus to nanomolar affinity for the enzyme.[26,27] Thus, the best hCA II inhibitors in this series of sulfonamides were the aminobenzolamide derivative **5j** and the sulfanilylhomosulfanilamide **5i**, but the other compounds (as mentioned above) were only slightly less inhibitory

Table 1. Inhibition Data of Fluorescent Sulfonamides **5** Reported in the Present Paper and Standard CA Inhibitors and Data of the 4-Aminoethylbenzenesulfonamide **7**[22] and the 2,4,6-Trimethylpyridinium Derivative of Homosulfanilamide **8**[23,24] Used in the ex Vivo Studies versus Isozymes I, II, and IX

	K_I [a] (nM)		
inhibitor	hCA I[b]	hCA II[b]	hCA IX[c]
AAZ	900	12	25
MZA	780	14	27
EZA	25	8	34
DCP	1200	38	50
IND	31	15	24
5a	1500	41	29
5b	1450	44	26
5c	1300	45	24
5d	980	47	30
5e	950	52	32
5f	1100	43	35
5g	1070	40	31
5h	1400	52	34
5i	630	34	20
5j	480	27	16
7	2100	160	33
8	7000	50	38

[a] Errors in the range of 5−10% of the reported value (from 3 different assays). [b] Human (cloned) isozymes, by the CO_2 hydration method. [c] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.

than **5i,j**. These compounds are less efficient CA II inhibitors compared to the clinically used derivatives, which typically showed K_I values in the range of 8−15 nM (DCP is the less effective such inhibitor, with a K_I of 38 nM). (iii) The simple derivatives **7** and **8** are also less effective CA II inhibitors (K_I values in the range of 50−160 nM) against the tumor-associated isozyme CA IX, and the fluorescent sulfonamides **5** showed very good inhibitory properties, with K_I values in the range of 16−35 nM. Similar to the situation observed for CA II, there are no important variations of activity for the diverse structures included in the study, and the explanation may be the one mentioned above. But it is important to note that all these compounds act as better hCA IX inhibitors than as hCA II inhibitors, which constitutes a remarkable finding, since possible drugs based on CA IX inhibitors should bind as much as possible to the target cancer-associated isozymes (i.e., CA IX and XII) but not to the other ubiquitous CA isozymes, such as CA II, IV, and V. This is probably due to the fact that

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 (3 mM Sulfonamide in 5 mM Tris Buffer, pH 7.4)[a]

	[sulfonamide], μM[b]					
	t = 30 min		t = 60 min		t = 48 h	
inhibitor	HPLC	ES	HPLC	ES	HPLC	ES
AAZ	136	139	160	167	163	168
MZA	170	169	168	168	167	169
7	132	138	162	165	167	168
8	0.3	0.5	0.4	0.5	0.3	0.5
5b	0.5	0.8	0.8	0.8	10.1	12.5
5c	0.4	0.9	0.6	1.2	10.4	13.0

[a] The concentrations of sulfonamide have been determined by two methods: HPLC and electronic spectroscopy (ES) (see Experimental Section for details). [b] Standard error (from three determinations) is less than 5% by the HPLC method[20] and by the rhe electronic spectroscopic method.[21]

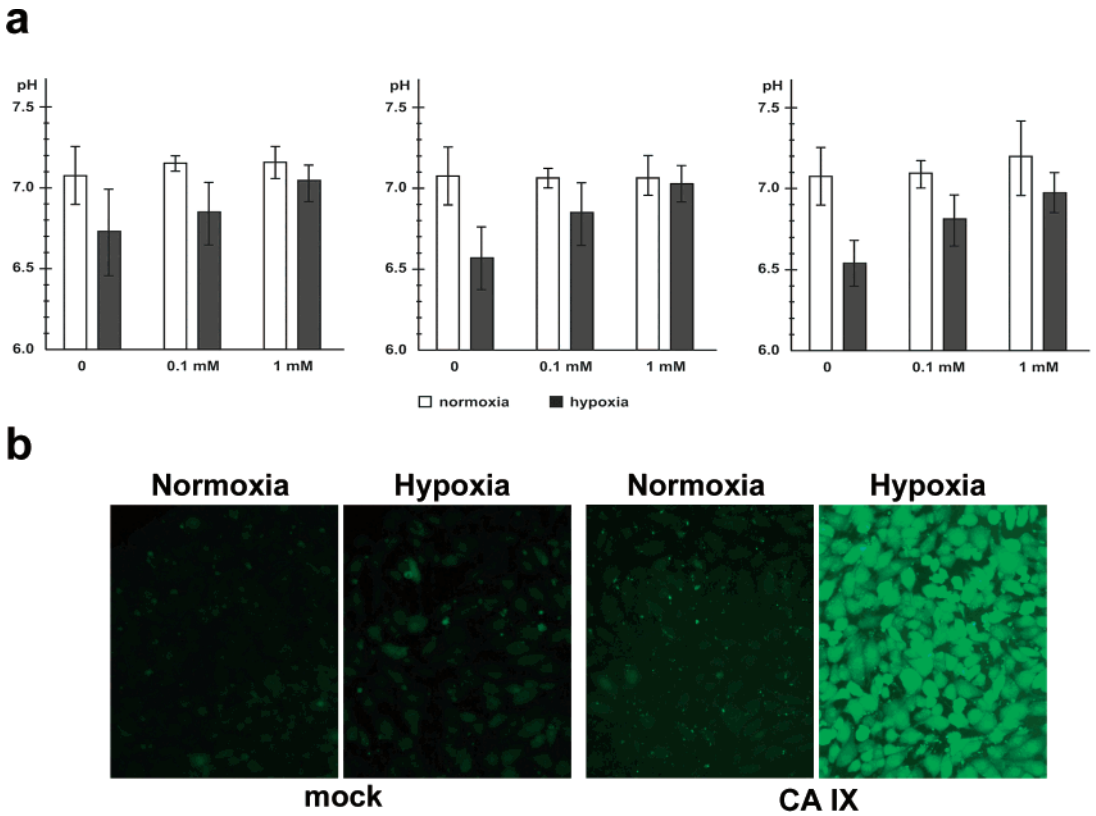


Figure 1. Sulfonamide inhibition and binding to hypoxic MDCK−CA IX cells. (a) The sulfonamides **8**, **7**, and **5c** (at 0.1 and 1 mM) respectively were added to MDCK-CA IX cells just before their transfer to hypoxia, and pHe was measured 48 h later. At least three independent experiments with three parallel dishes per sample were performed for each inhibitor. (b) Fluorescence analysis of the transfected MDCK cells plated on the glass coverslips. The cells were treated with the FITC-labeled compound **5c** throughout the 48 h of incubation in normoxia and hypoxia, respectively.

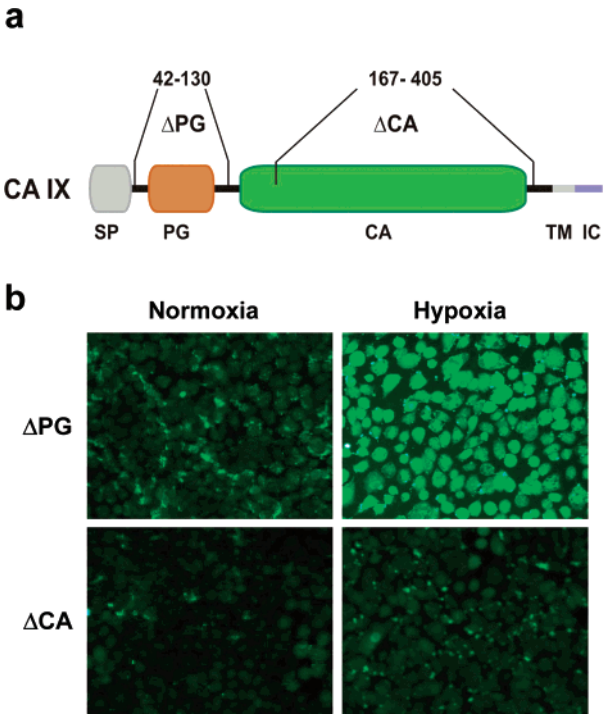


Figure 2. Sulfonamide binding to MDCK cells expressing deletion variants of CA IX. (a) Scheme of the domain composition of CA IX protein with indicated amino acid positions of the regions removed in the deletion variants: SP, signal peptide, PG, proteoglycan-like region; CA, carbonic anhydrase domain; TM, transmembrane region; IC, cytoplasmic tail. (b) Fluorescence analysis of the transfected MDCK cells plated on the glass coverslips. The cells were treated with the FITC-labeled compound **5c** throughout the 48 h of incubation in normoxia and hypoxia, respectively.

the hCA IX active site is larger than that of the cytosolic isozyme hCA II, as already reported earlier by us.[25] It must also be noted that the CA IX inhibitory properties of these new sulfonamides **5** are in the same range as those of the clinically used sulfonamides, including indisulam, an antitumor sulfonamide in clinical trials.[26a]

Ex Vivo Penetration through Red Blood Cell Membranes. Levels of sulfonamides **5b**,**c**, **7**, **8**, AAZ, and MZA in 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)[22] after incubation periods of 30 min, 60 min, or 48 h were determined in order to investigate the penetrability of these compounds through biological membranes. Since hCA IX is a transmembrane protein with the active site exposed out of the cell, membrane-impermeant derivatives (or derivatives with decreased permeability) may lead to the selective inhibition of hCA IX and not of the cytosolic CA isozymes CA I or II. This is considered a very desirable property of a future drug belonging to this class of compounds. We have already shown previously that the positively charged pyridinium-substituted sulfonamides, of which **8** is a representative, are indeed membrane-impermeable, in contrast to classical sulfonamides that cross membranes easily because of the fact they are nonpolar and uncharged (although in equilibrium with the ionized sulfonamide, which is the species binding to the enzyme active site).[24,25,28]

Indeed, it may be observed (Table 2) that the uncharged sulfonamides AAZ, MZA, and aminoethylbenzenesulfonamide **7** easily penetrate biological membranes, practically saturating red blood cells (RBCs) after 1 h. After 48 h, identical levels (within the limits of experimental errors) of these three sulfonamides in RBCs were observed. In contrast, the pyridinium, charged compound **8**, has been detected only in very small amounts within the RBCs, proving that it is unable to penetrate the membranes obviously because of its cationic nature. Even after incubation times as long as 48 h, only traces of the cationic sulfonamide were present inside the RBCs, as proved by the two assay methods used for their identification in the cell lysate, which were in good agreement with each other (the very small amount of sulfonamide detected may be due to contamination of the lysates with minute amounts of membranes) (Table 2). The fluorescein sulfonamide derivatives **5b** and **5c** investigated here showed decreased membrane permeability at exposure times of 30−60 min but were more permeant after 48 h of exposure. These findings may be explained by the fact that because of the presence of the carboxylic acid moiety in these compounds and under the conditions of our experiments (pH 7.4), most of the fluorescent sulfonamides are in the anionic carboxylate form, which leads to a decreased penetration through membranes, similar to the cationic sulfonamide **8**. Still, these carboxylates are in chemical equilibrium with the corre-

Scheme 2

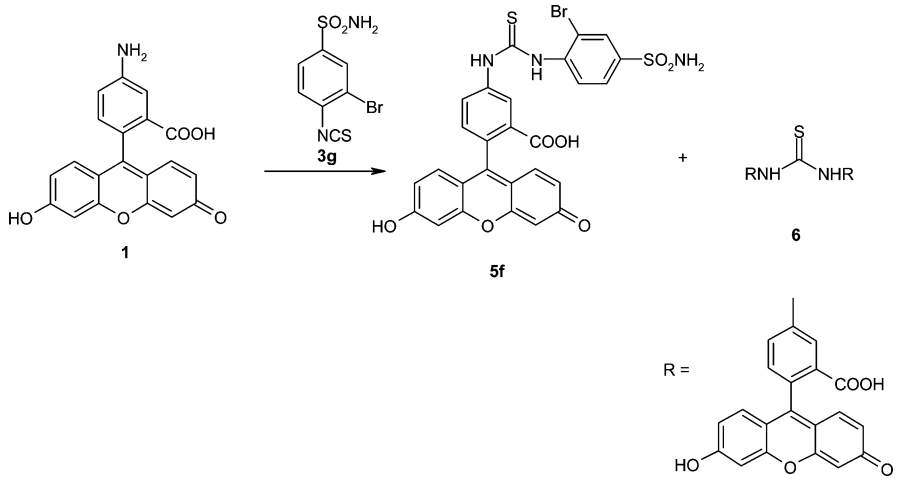
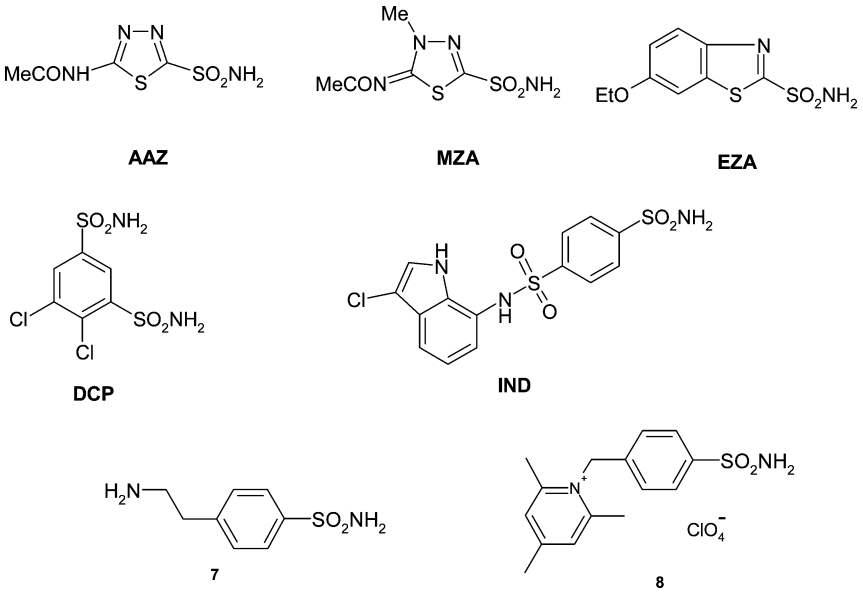


Chart 2



sponding acids (neutral molecules), which are membrane-permeant, and this may explain why after 48 h of incubation some sulfonamides crossed the membranes (on the other hand **8** is not in equilibrium with any neutral molecule and this is the reason that the compound cannot cross membranes even after 48 h of incubation with RBCs). Still, these levels are quite small and considering the fact that **5** showed a better affinity for hCA IX than for hCA II, in vivo, we hypothesize that the cancer-associated transmembrane isozyme IX is predominantly inhibited by these compounds.

Inhibition of CA IX-Mediated Acidification of the Extracellular pH by Sulfonamides. In a previous study we showed that hypoxic incubation of MDCK cells with the constitutive ectopic expression of CA IX leads to an enhanced acidification of the extracellular pH.[11] Together with additional evidence, this finding led us to conclude that hypoxia induces both high levels and high enzyme activity of CA IX present in tumors. This was the first evidence that lactic acid is not the only cause of tumor acidosis and that CA IX significantly contributes to this phenomenon. Because microenvironmental acidosis supports development of the malignant tumor phenotype,[13a] inhibition of hypoxia-induced CA IX activity by sulfonamides may represent a new approach toward reduction of tumor growth and/or spread. Despite a principally nonspecific action of sulfonamides against different CA isoenzymes, their structure and properties can be modified to introduce a preference for certain isoforms. We tested three CA IX potent and rather selective CAIs for their effects on pHe: the fluorescent compound **5c** designed in this study, the membrane-permeable sulfonamide **7**, and the membrane-impermeant sulfonamide **8**, all of which possess high affinity for CA IX (K_I values in the range of 24−38 nM; see Table 1). We found that all three sulfonamides were able to reduce the extracellular acidification of MDCK-CA IX cells in hypoxia and their effect on the normoxic pHe was negligible (Figure 1). Moreover, the fluorescent sulfonamide **5c** was used for the treatment and fluorescence analysis of both CA IX positive and CA IX negative cells incubated either in normoxia or in hypoxia for 48 h. In accord with the previous data, the fluorescence signal produced by **5c** was detected only in the hypoxic MDCK-CA IX cells but was absent from their normoxic counterparts and from both hypoxic and normoxic mock-transfected controls (Figure 1). This observation indicates that the newly designed fluorescent sulfonamide **5c** did not interact with other CA isoforms and that it binds only to hypoxia-activated CA IX. Altogether, these results offer reliable proof that CA IX activity is essential for the medium acidification in hypoxic MDCK-CA IX cells and that this acidification is reversed by inhibiting CA IX with sulfonamides. It may be observed that similar effects were obtained both with membrane-permeable and with membrane-impermeant CAIs (compounds **5c**, **7**, and **8**), proving that this is a general feature of these pharmacological agents, thus allowing some optimism regarding the possible design of novel anticancer therapies based on CA IX inhibitors.

Sulfonamide Binding to Cells Expressing CA IX Deletion Variants. The results presented above were further supported by the capability of the sulfonamide **5c** to bind only to hypoxic cells that express the CA IX deletion variant ΔPG with the intact catalytic domain and not to cells containing the ΔCA variant, from which most of the CA domain was removed. This finding is in agreement with our previous data that showed the full capacity of the ΔPG variant to acidify extracellular pH in the transfected MDCK cells maintained under hypoxia and, on the other hand, the inability of ΔCA variant to do so.[11] Furthermore, this result indicates that the intact catalytic domain exposed on the surface of the hypoxic cells is the sole requirement for the binding of CA IX selective sulfonamides analyzed in this study. In addition to their potential application in anticancer therapy, the exclusive binding of the fluorescent inhibitor **5c** to hypoxic cells with activated CA IX offers an attractive possibility for the use of similar sulfonamide-based compounds for imaging of the hypoxic tumors. Clearly, this assumption warrants further studies.

Conclusions

A novel class of fluorescent, potent CA IX inhibitors was designed. Representative compounds belonging to it selectively bound to the enzyme only under hypoxic conditions. Since it was previously shown that many sulfonamides possess appreciable tumor cell growth inhibitory properties in vitro and in vivo, these findings constitute the proof-of-concept that anticancer therapies based on tumor-associated CA isozyme inhibition can be developed and can also offer interesting tools for investigating hypoxic tumors and for their imaging.

Experimental Section

General. ^{1}H spectra were recorded on a Bruker DRX-400 a spectrometer using DMSO-d_6 as the solvent and tetramethylsilane as the internal standard. Chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (J) are expressed in hertz. Electron ionization mass spectra (30 eV) were recorded in positive or negative mode on a Water MicroMass ZQ spectrometer.

General Procedure for the Preparation of Compound 5. Method A. Fluorescein isothiocyanate (1 mmol) and the aminosulfonamide derivative (1 mmol) were dissolved in 5 mL of dimethylformamide. Then triethylamine (1 mmol) was added and the mixture was stirred at room temperature until completion of the reaction (TLC monitoring). The reaction mixture was then dissolved in water and was extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. The resulting product was then purified by flash chromatography.

Method B. Fluorescein amine (1 mmol) and the isothiocyanate sulfonamide (1 mmol) were dissolved in 5 mL of *N*,*N*-dimethylacetamide, and then the mixture was stirred at room temperature until completion of the reaction (TLC monitoring). The reaction mixture was then dissolved in water and was extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. The resulting product was then purified by flash chromatography.

(4-Sulfamoylphenyl)thioureidofluorescein (5a): ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.45 (s, 1H), 10.35 (s, 1H), 10.15 (s, 2H), 8.2 (d, 1H, J = 1.85 Hz), 7.85 (dd, 1H, J = 8.2 Hz, J = 2 Hz), 7.8 (d, 2H, J = 8.7 Hz), 7.7 (d, 2H, J = 8.7 Hz), 7.35 (s, 2H), 7.25 (d, 1H, J = 8.2 Hz), 6.7 (d, 2H, J = 2 Hz), 6.6 (m, 4H); MS ESI$^+$ m/z 562 $(M + H)^+$; ESI$^-$ m/z 560 $(M - H)^-$.

Detailed spectroscopic and analytic data for the other compounds described in the paper are provided in the Supporting Information.

Penetrability through Red Cell Membranes. An amount of 10 mL of freshly isolated human red 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 3 mM solution of sulfonamide inhibitor. Incubation has been done at 37 °C with gentle stirring, for periods of 30−120 min. After the incubation times of 30 min, 60 min, and 48 h, the red 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. 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 CAs), and sulfonamides possibly present have been assayed in each sample by two methods: an HPLC method[20] and spectrophotometrically.[21]

HPLC. A variant of the methods of Gomaa[20] has been 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, and with 0.3 mg/mL sulfadiazine (Sigma) as the 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, 8.76 min for **7**, 4.12 min for **8**, 6.50 min for **5b**, and 6.27 min for **5c**. The eluent was monitored continuously for absorbance (at 254 nm for acetazolamide and with wavelength in the range of 270−310 nm in the case of the other sulfonamides).

Spectrophotometry. A variant of the pH-induced spectrophotometric assay of Abdine et al.[21] has been used, working for instance at 260 and 292 nm for acetazolamide, at 225 and 265 nm for sulfanilamide, etc. Standardized solutions of each inhibitor have been prepared in the same buffer as the one used for the membrane penetrability experiments.

Cell Cultures. MDCK cells and their transfected derivatives were grown in DMEM with 10% FCS (BioWhittaker, Verviers, Belgium) buffered with 22.4 mM bicarbonate and containing supplements as described before.[11] To maintain standard experimental conditions, the cells were always plated in 3 mL of culture medium at a density of $(0.8-8) \times 10^6$ per 6 cm dish 24 h before the transfer to hypoxia (2% O_2 and 5% CO_2 balanced with N_2) generated in a Napco 7000 incubator, where they were grown for an additional 48 h (if not stated otherwise). Parallel normoxic dishes were incubated in air with 5% CO_2. At the end of each experiment, the pH of the culture medium was immediately measured using a portable ARGUS pH meter with IFSET Hot-Line CupFET pH sensor (Sentron, Roden, The Netherlands). The lactic acid content in the medium was determined with a standard assay kit (Sigma, St. Louis, MO), and the cells were counted to ensure that the resulting cultures are comparable. Parallel dishes were processed for immunofluorescence.

Sulfonamide Treatment of Cells. The sulfonamides were dissolved in PBS with 20% DMSO at 100 mM and diluted in a culture medium to the required final concentration just before their addition to the cells. Immediately after beginning of the treatment with sulfonamides, the cells were transferred to hypoxia and incubated for 48 h. Parallel cultures were maintained for the same time period in normoxia. At the end of the experiment, the pH of the culture medium was measured as described above, and also measured was the binding of the fluorescent sulfonamide **5c** to living cells, which were washed three times with PBS. The fluorescence was viewed by a Nikon E400 epifluorescence microscope equipped with PlanFluor 20× objectives and photographed. Images were acquired by Nikon Coolpix 990.

Cloning of CA IX Mutants and Transfection. Cloning of the deletion mutants of CA IX that lack either the N-terminal PG domain or the central CA domain was performed as described.[11] MDCK and HeLa cell lines constitutively expressing CA IX protein or its mutated forms were obtained by cotransfection of individual recombinant plasmids pSG5C-CA IX, pSG5C-ΔCA, and pSG5C-ΔPG with pSV2neo plasmid in a 10:1 ratio using a GenePorter II transfection kit from Gene Therapy Systems (San Diego, CA). The transfected cells were subjected to selection in the presence of 500−1000 μg/mL G418 (Life Technologies, Gaithersburg, MD), cloned, tested for expression of CA IX, and expanded. At least three clonal cell lines expressing each CA IX form were analyzed to eliminate the effect of clonal variations. The cells cotransfected with empty pSG5C and pSV2 neo and subjected to the same selection and cloning procedures were used as negative controls.

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Supporting Information Available: Spectroscopic data and results of elemental analysis for the described compounds in the paper. This material is available free of charge via the Internet at http://pubs.acs.org.

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