Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry 14 (2006) 2418–2427
Bioorganic & Medicinal Chemistry

Carbonic anhydrase inhibitors: Transepithelial transport of thioureido sulfonamide inhibitors of the cancer-associated isozyme IX is dependent on efflux transporters

Daniela Vullo,[a,b] Bente Steffansen,[a] Birger Brodin,[a] Claudiu T. Supuran,[b,*] Andrea Scozzafava[b] and Carsten Uhd Nielsen[a,*]

[a]*Molecular Biopharmaceutics, Department of Pharmaceutics and Analytical Chemistry, The Danish University of Pharmaceutical Sciences, 2-Universitetsparken, DK-2100 Copenhagen, Denmark*
[b]*Universita degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 1-50019 Sesto Fiorentino, Firenze, Italy*

Received 3 June 2005; revised 7 November 2005; accepted 11 November 2005
Available online 29 November 2005

Abstract—Sulfonamides and their derivatives inhibit the catalytic activity of carbonic anhydrases (CA, EC 4.2.1.1). Isozyme IX (CA IX) is a transmembrane isoform with the active site oriented toward the extracellular space. CA IX was recently shown to be a drug target, and it is highly overexpressed in hypoxic tumors with limited distribution in normal tissues. The present report deals with the drug design, synthesis, and biological investigation of a group of thioureido sulfonamides, which have been obtained by reaction of isothiocyanate-substituted aromatic sulfonamides with amines. These compounds have potent inhibitory properties against CA IX with K_I values in the range of 10–37 nM and P_{app}values $> 0.34 \times 10^{-6}$ cm/s for the absorptive transepithelial transport in Caco-2 cells. In Caco-2 cells, one of these compounds (**A6**) was shown to be a substrate for efflux transporters such as P-glycoprotein (P-gp). P-gp activity is not likely to be rate-limiting for intestinal absorption, but might be useful when targeting hypoxic tumors expressing both P-gp and CA IX.

1. Introduction

Sulfonamides inhibiting the ubiquitous metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1)[1,2] to various degrees also inhibit growth of tumor cells in vitro and in vivo.[1,3] The precise isozyme(s) involved in such processes were only recently discovered. The discovery of two isozymes, that is, CA IX[4] and then CA XII,[5,6] as the predominant CAs in tumors, offered a starting point for more detailed studies in the field. It has thereafter been shown that the level of CA IX dramatically increases in response to hypoxia via a direct transcriptional activation of the *CA9* gene by the hypoxia-inducible factor HIF-1,[7–10] and that the expression of CA IX in tumors is a sign of poor prognosis.[7–10] Thus, strong connections exist between an essential characteristic of tumor biology—hypoxia—and the activity of at least two CA isozymes, CA IX and XII.

Recently, we have shown that the acidic tumor pH_e, characteristic of many tumors is a consequence of the enzymatic activity of CA IX, which catalyzes the hydration of CO_2 to bicarbonate and H^+ ions.[1] This isozyme has a transmembrane localization, with the active site oriented toward the extracellular space.[1–4] It possesses very high catalytic activity, similar to that of the cytosolic isozyme CA II, which is ubiquitous in higher vertebrates and which is also involved in a variety of critical physiological processes.[11–13] The acidic pH of tumors can influence uptake of anticancer drugs and modulate the response of tumor cells to conventional chemo- and radiotherapy.[1,14] Acidification of the tumor microenvironment has generally been assigned to accumulation of lactic acid, that is excessively produced by glycolysis and poorly removed by inadequate tumor vasculature.[1,14] The high rates of glycolysis are important in hypoxic cells which, to a large degree, are dependent on anaerobic metabolism to generate their energy.[1,14]

Keywords: Carbonic anhydrase inhibitors; CAIX; Efflux transporters; Caco-2 cells.
* Corresponding authors. Tel.: +39 055 457 3005; fax: +39 055 457 3385 (C.T.S.); tel.: +45 3530 6472; fax: +45 3530 6030 (C.U.N.); e-mail addresses: claudiu.supuran@unifi.it; cun@dfuni.dk

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.11.019

However, experiments with glycolysis-deficient cells indicate that production of lactic acid is not the only process leading to tumor acidification; comparison of the metabolic profiles of the glycolysis-impaired and parental cells revealed that CO_2, in addition to lactic acid, is a significant source to increase acidity in tumors.[1,14] Thus, it has been demonstrated that CA IX has all the necessary requisites to act in tumor pH control under hypoxic conditions.[1] Furthermore, Svastova et al. have described how the tumor acidification effect may be reversed by inhibition of the CA IX with sulfonamides possessing potent inhibitory properties.[1] This opens up the possibility to develop novel approaches for the management (treatment and diagnostic tools) of hypoxic tumors, which are generally nonresponsive to classical chemo- and radiotherapy.[1,14] Ultimately, we have developed several classes of potent inhibitors targeting the tumor-associated isozyme IX.[15–18] However, only a few studies have looked at the membrane permeating properties of such anticancer sulfonamide CA inhibitors, although strategies to design membrane-impermeable compounds have been tried (considering the transmembrane localization of the target isozyme).[19,20] Recently, we showed that attaching the dipeptide L-carnosine to a sulfonamide CA inhibitor yielded a dipeptide analog with an affinity for the intestinal peptide transporter, hPEPT1, but at the same time this analog had a low absorptive flux across Caco-2 cells, which partly was due to its interaction with efflux mechanisms.[21] For further development of oral active anti-cancer compounds targeting CA IX, knowledge about transport across epithelial barriers is crucial. In the present study, we report the design of potent CA IX inhibitors with incorporated thioureido-moieties, which were investigated for their membrane permeating properties across Caco-2 cell monolayers. These investigations provide new insight into both the drug design of inhibitors as well as for their drug delivery properties.

2. Results and discussion

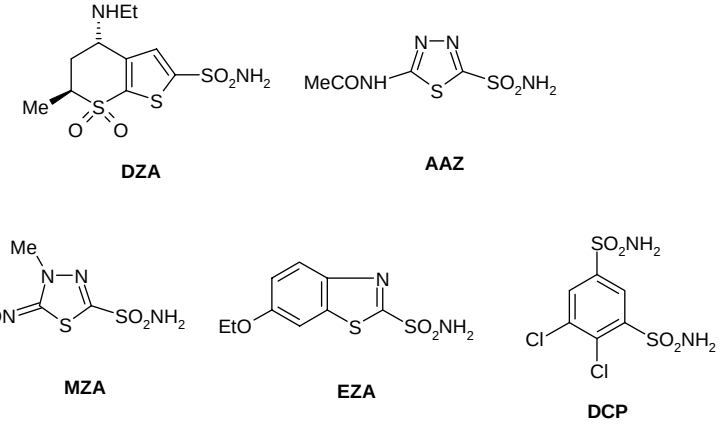
2.1. Chemistry

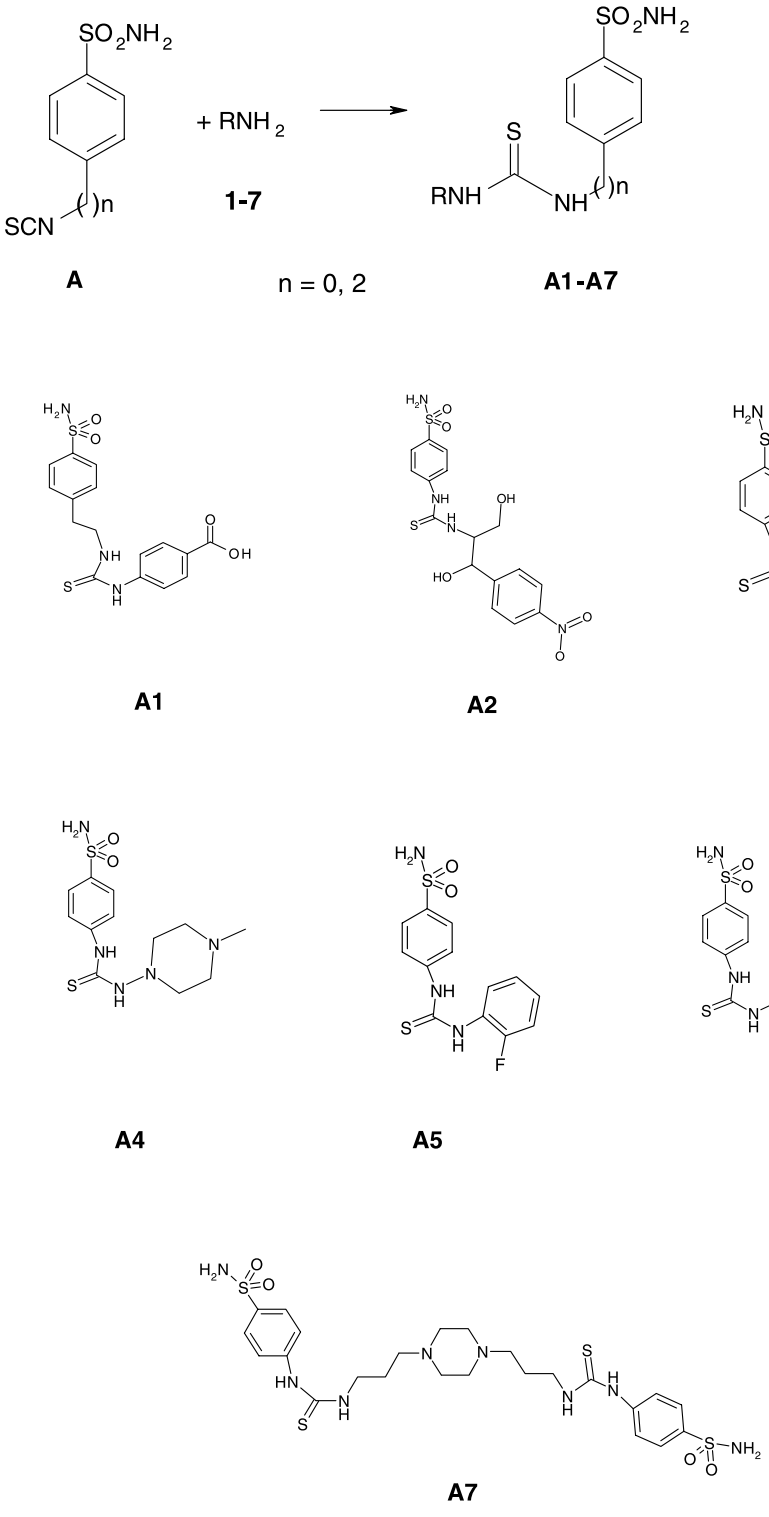
The sulfonamides investigated here were prepared by reacting 4-isothiocyanato- or 4-isothiocyanatoethyl-benzenesulfonamide **A** ($n = 0$ and 2, respectively) with amines **1**–**7**, leading to thioureas **A1**–**A7** by the procedure previously reported by this group (Scheme 1).[22–24]

Apart from designing compounds which fit well within the active site,[25] it is also important to design soluble compounds that are deliverable to the target. In previous reports, we have mainly investigated the preparation of derivatives incorporating amino acid moieties,[23,24] whereas for the drug design of the compounds reported here, we concentrated on different moieties aimed at increasing hydrosolubility.[26] Thus, the reaction of isothiocyanates **A** ($n = 0$ and 2) was performed with amines bearing moieties that can lead to an enhanced hydrosolubility, such as 4-amino-benzoic acid **1**, the amino diol **2**, the fluorine-containing amines **3** and **5**,[27–29] as well as the polyamines **4**, **6** or **7** (in the last case, the reaction involves two equivalents of isothiocyanate **A**, leading to the bis-sulfonamide **A7**). These moieties were also chosen because they presumably can adopt a conformation within the CA active site similar to that of the perfluorophenylhydrazine moiety of **A3**. The X-ray crystal structure of this perfluorophenylhydrazine moiety in complex with hCA II has recently been shown to make favorable interactions with critical amino acid residues in the enzyme, which are involved in the binding of inhibitors.[25] Similar X-ray structures were previously reported by Christianson's group, for simpler derivatives, but the tail of the inhibitor could not always be seen in the electronic density maps.[30,31] In addition, these moieties present in our compounds may also lead to an enhanced hydrosolubility of such sulfonamides. This is another important factor for the in vivo studies of the tumor-associated isozyme CA IX[1] and obviously for the bioavailability of a potential drug belonging to this class. Indeed, **A1** can form sodium salts that have enhanced water solubility as compared to the free acid (data not shown), whereas the protonated form of **A4**, **A6**, and **A7** can lead to hydrochloride or similar salts, which would also possess enhanced water solubility. We already proved earlier that many of the fluorine-containing inhibitors show a very good hydrosolubility.[27,28]

2.2. CA inhibition

Inhibition data for the compounds reported here as well as for standard CA inhibitors were generated for three physiologically relevant CA isozymes, that is, the





Scheme 1.

cytosolic, red blood human isozymes hCA I and II, and the transmembrane, tumor-associated isozyme, hCA IX (Table 1).

These inhibition data for the CA inhibitors **A1**–**A7**, and standard, clinically used drugs (dorzolamide **DZA**, acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, and dichlorophenamide **DCP**) lead to the following observations.

2.2.1. Isozyme I. Against the slow red blood cell isozyme, the derivatives **A1**–**A7** showed inhibition constants in the range of 24–450 nM (Table 1). Thus, the 2-fluoroaniline derivative **A5** was the best inhibitor, being

Table 1. Inhibition data of isozyme hCA I, II, and IX with compounds **A1**–**A7** and standard, clinically used CA inhibitors

Compound	K_I (nM)[a]		
	hCA I	hCA II	hCA IX[b]
A1	450	380	10
A2	135	27	32
A3	79	19	15
A4	80	75	37
A5	24	7	12
A6	100	33	20
A7	250	150	15
DZA	50,000	9	52
AAZ	250	12	25
MZA	50	14	27
EZA	25	8	34
DCP	1200	38	50

[a] Standard errors are in the range of ±10% of the reported value obtained from three different determinations.
[b] Catalytic domain of the human, cloned isozyme.

together with ethoxzolamide EZA, one of the most effective inhibitors against this isozyme reported. Derivatives **A3** and **A4** were also good inhibitors with inhibition constants of 79–80 nM, which is close to the magnitude observed for the clinically used sulfonamide, methazolamide. **A2**, **A6**, and **A7** showed K_I values in the range of 100–250 nM, being anyhow better inhibitors than acetazolamide, the CA inhibitor par excellence. Finally, the *p*-aminobenzoic acid derivative **A1** was the poorest hCA I inhibitor, with a K_I value of 450 nM.

2.2.2. Isozyme II. hCAII is generally considered the main therapeutic target of sulfonamide CA inhibitors. Thus, affinity of newly designed inhibitors for this isozyme is of critical relevance. The compounds **A1** and **A7** reported here showed modest hCA II inhibitory properties, with K_I values in the range of 150–380 nM. Thus, the bulky bis-sulfonamide **A7** as well as the carboxy-substituted thiourea **A1** may lead to unfavorable interactions with the active site of hCA II. This may explain their decreased efficacy as compared to those of other derivatives belonging to this series, which were much better inhibitors. The better inhibitors have K_I values in the range of 7–75 nM, which is close to the magnitude of the clinically used sulfonamides (K_I values in the range of 8–38 nM, see Table 1). The fluorine-containing derivatives **A3** and **A5** were particularly strong inhibitors. In fact, the X-ray crystal structure of the adduct of hCA II with **A3** may well explain these findings. **A6** also showed interesting inhibitory properties, although it does not have a cyclic moiety attached to the terminal thioureido functionality, in contrast to **A2**–**A5**.

2.2.3. Isozyme IX. Isozyme IX is a transmembrane isozyme with an extracellular CA domain possessing a catalytic activity similar to that of hCA II. The inhibition profile of this isozyme (catalytic domain) with the sulfonamides investigated here, as well as with the clinically used compounds **DZA**–**DCP**, is quite different from those of the other isozymes (CA I and II). All the sulfonamides investigated here were good hCA IX inhibitors with K_I values in the range of 10–37 nM, similar to the classical, clinically used sulfonamides (K_I values in the range of 27–52 nM). In particular, the compounds **A1**, **A3**, **A5**, and **A7** show very efficient inhibition against the tumor-associated isozyme hCA IX over the cytosolic isozymes I and II, with selectivity ratios between 38 and 45 for **A1** (against hCA II, and I, respectively). As a whole, this entire class of derivatives showed a very efficient inhibition profile of the tumor-associated isozyme hCA IX. As the X-ray crystal structure of hCA IX has not yet been reported, it is difficult to rationalize the SAR for this small number of sulfonamides.

2.3. Caco-2 cell transport studies

2.3.1. Transepithelial transport of hCA IX inhibitors A1–A7 across Caco-2 cell monolayers. Transport studies performed using Caco-2 cell monolayers are generally employed at discovery/development stages in order to estimate the oral in vivo bioavailability of drug candidates.[32] Often a pH gradient of 6.0/7.4 is applied in the in vitro experiments in order to simulate the real intestinal environment. Furthermore, bidirectional transport studies have been used to identify drug interactions with efflux transporter such as most importantly P-glycoprotein (P-gp).[33] The transepithelial transport across Caco-2 cell monolayers was measured for **A1**–**A7** at a donor concentration of 1 mM (Table 2). The

Table 2. The transepithelial flux (A) to basolateral (B) and B–A flux data for **A1**–**A7** across Caco-2 cell monolayers

Compound	A–B		B–A		J_{BA}/J_{AB}
	J (nmol min^{-1} cm^{-2})	P_{app} (10^{-6} cm s^{-1})	J (nmol min^{-1} cm^{-2})	P_{app} (10^{-6} cm s^{-1})	R
A1	0.1 ± 0.03	1.6 ± 0.6	0.64 ± 0.08	10.6 ± 1	6.7[a]
A2	0.23 ± 0.01	3.9 ± 0.2	1.08 ± 0.06	18.0 ± 1	4.6[b]
A3	0.7 ± 0.08	13 ± 1	1.3 ± 0.4	22.1 ± 1	1.9
A4	0.02 ± 0.007	0.34 ± 0.1	0.12 ± 0.02	2.04 ± 0.4	6.1[a]
A5	1.2 ± 0.4	21 ± 6	1.3 ± 0.3	21.6 ± 5	1.1
A6	0.09 ± 0.02	1.5 ± 0.3	0.85 ± 0.2	14.2 ± 4	9.4[a]
A7	1.8 ± 0.1	29 ± 2	2.4 ± 0.1	40 ± 2	1.4[b]

In all the experiments, the concentration of **A1**–**A7** was 1 mM on the donor (*cis*) side. The apical medium was pH 6.0 and basolateral medium was pH 7.4. Samples were taken from the receiver (*trans*) side and analyzed by HLPC–UV, as described in the method section. Transepithelial apical flux of the compounds **A1**–**A7** across Caco-2 cell monolayers. Experiments were performed in duplicate in three different Caco-2 cell passages. Values are given as average ± SE.
[a] $p < 0.05$.
[b] $p < 0.01$.

apical to basolateral flux, J_{AB}, ranged from 0.02 to 1.75 nmol min^{-1} cm^{-2} for **A4** and **A7**, respectively. The basolateral to apical flux, J_{BA}, ranged from 0.1 to 2.4 nmol min^{-1} cm^{-2} for **A4** and **A7**, respectively. The efflux ratio (J_{BA}/J_{AB}) was calculated for **A1**–**A7**, and the values are given in Table 2. For **A3**, **A5**, and **A7**, the efflux ratio was between 1 and 2, and for **A3** and **A5**, no significant difference in the directional transport was observed. For **A7**, the J_{BA} was significantly larger than J_{AB} due to low variation in the data series. For **A2** and **A6**, the efflux ratio was 4.6 and 9.4, respectively, and the J_{BA} was significantly larger than J_{AB}. For **A1** and **A4**, the efflux ratio was 6 and 7, respectively, and the J_{BA} was significantly larger than J_{AB}. For several of the compounds investigated, the efflux ratio ($R > 1$) indicates the involvement of efflux transporters in the transepithelial transport of these CA IX inhibitors. For other well-known P-gp substrates such as digoxin and paclitaxel, efflux ratios in Caco-2 cells are 4–6.5,[34] which are in the same range as the values obtained in this study. Several studies have addressed the question of correlation between oral drug absorption in humans and the apparent permeability in Caco-2 cell monolayers.[35–37] These publications suggest that compounds with a $P_{app} > 1–5 \times 10^{-6}$ cm/s would have a high oral absorption in humans.[32,35,38] Since **A3**, **A5**, and **A7** have P_{app} values $> 10 \times 10^{-6}$ cm/s, these compounds are expected to have oral bioavailabilities of >50%. The compounds **A1**, **A4**, and **A6** have P_{app} values of around 1×10^{-6} cm/s, and **A2** has a P_{app} value of 4×10^{-6} cm/s, and the likely oral absorption is thus difficult to predict since these compounds have both relatively low membrane permeability and are substrates for transporters which may lower the absorption of the compounds.

2.3.2. Transepithelial transport of A6 across Caco-2 cell monolayers in the presence of P-gp inhibitors. The compound **A6** was a fairly good inhibitor of CA IX and showed a high efflux ratio in the Caco-2 cell transport studies. **A6** was therefore selected for further investigation of the involvement of P-gp in the membrane transport. The transport of **A6** across Caco-2 cell monolayers was measured in the presence of the P-gp inhibitors verapamil and digoxin. It has previously been shown that verapamil is a substrate for P-gp,[39–42] or perhaps an inhibitor.[43] Likewise, digoxin is a substrate for P-gp,[44–46] perhaps even a specific inhibitor.[47] Since **A6**, in contrast to the other compounds, contains a tertiary amine, we determined the pK_a values for **A6** at 25 °C. **A6** has three measurable pK_a values. The determination of the pK_a values of **A6** is based on the assumption that pK_{a1} is due to one of the –NH groups of the thioureido part, that pK_{a2} is due to the tertiary amine, and that pK_{a3} is due to the sulfonamide. Accordingly, the values for pK_{a1}, pK_{a2}, and pK_{a3} were 8.20 ± 0.03, 9.32 ± 0.05, and 10.46 ± 0.03, respectively. The pK_a values for 1 and 3 are caused by N–H acidic protons, and therefore **A6** is predominantly (>99%) on its positive form (net charge +1) at pH 6.0, with a small fraction (<1%) being on the neutral form. At pH 7.4, **A6** is predominantly (~84%) on the cationic form, with a smaller fraction (~16%) being on the neutral form. In one series of Caco-2 cell experiments, we mimicked the intestinal pH gradient by having the apical pH of 6.0 and the basolateral pH of 7.4 (Table 3). In another series, both the apical and basolateral pH values were 7.4 (Table 3). This was done in order to avoid potential differences in directional transport due to changes in the effective concentration of the neutral species of **A6**. Under experimental conditions where a pH gradient has been applied, it has been shown that basic amino-containing compounds such as atenolol and metoprolol may show a polarized efflux which is not caused by P-gp.[48] This observation is a result of changed protonation of the permeating species in the basolateral chamber, thereby resulting in a false classification of the compounds as P-gp substrates.[48] In experiments with a pH gradient, the $P_{app/BA}$ of **A6** was significantly larger than $P_{app/AB}$, giving an efflux ratio of 5.5 (Table 3). In experiments without a pH gradient, the $P_{app/BA}$ of **A6** was still significantly higher than the $P_{app/AB}$, the efflux ratio being 3.3. The $P_{app/AB}$ was unaffected by both verapamil and digoxin, whereas the $P_{app/BA}$ in the presence of verapamil or digoxin was decreased to a level similar to the $P_{app/AB}$ of **A6** with an efflux ratio of 1.1 in the presence of verapamil. Taken together, these results indicate that the transport of **A6** observed in Caco-2 cells without a pH gradient depends on efflux transporters presumably P-gp, since the efflux ratio is higher than 1, and since both verapamil and digoxin inhibit the transport. Furthermore, the results indicate that a fraction of the transport of **A6** observed in Caco-2 cells with a pH gradient may partly be explained by the pH-partitioning theory, however, differences in the binding of the different species of **A6** to the efflux transporters may also be a part of the explanation and make a quantitative expla-

Table 3. The transepithelial flux of **A6** across Caco-2 cell monolayers

	P_{app} (10^{-6} cm s^{-1})				J_{BA}/J_{AB}	
	A–B		B–A		*R*	
	6.0/7.4	7.4/7.4	6.0/7.4	7.4/7.4	6.0/7.4	7.4/7.4
A6	1.6 ± 0.2	3.8 ± 0.7	8.3 ± 0.7	12 ± 0.5	5.5[a]	3.3[a]
A6 (verapamil)	1.4 ± 0.2	3.1 ± 0.6	7.3 ± 0.3	4.1 ± 0.5	5.5[a]	1.1
A6 (digoxin)	1.9 ± 0.6	2.6 ± 0.9	8.5 ± 0.2	4.8 ± 0.7	4.2[a]	1.8

P_{app} values and efflux ratios for **A6** (1 mM) and in the presence of verapamil (50 μM) or digoxin (50 μM). In all the experiments, the concentration of **A6** was 1 mM on the donor (*cis*) side. The apical pH was 6.0 or 7.4, and the basolateral pH was 7.4 Samples were taken from the receiver (*trans*) side and analyzed by HLPC–UV, as described in the methods section. Experiments were performed in duplicate in three different Caco-2 cell passages. Values are given as average ± SE.

[a] $p < 0.001$.

nation difficult. In cancer cells, efflux transporters are known to reduce uptake and cellular accumulation of classical anti-cancer compounds and collectively a partial cause for the phenomenon known as multi-drug resistance (MDR). Over-expression of P-gp, encoded by the MDR1 gene, confers resistance to a variety of structurally and functionally unrelated anti-tumor drugs. As mentioned earlier it has been shown that the level of CA IX dramatically increases in response to hypoxia via a direct transcriptional activation of the *CA9* gene by the hypoxia-inducible factor HIF-1.[7–10] Similarly, it has been shown that HIF-1 has a binding site on the MDR1 gene, and that inhibition of HIF-1 expression results in an inhibition of hypoxia-inducible MDR1 expression which causes a nearly complete loss of basal MDR1 expression.[49] Thus, strong connections exist between an essential characteristic of tumor biology—hypoxia—and the activity of CA IX and the expression of MDR1.

3. Conclusions

We here report, the drug design, synthesis, and biological investigation of a group of thioureido sulfonamides. The compounds **A1**–**A7** have been obtained by reaction of isothiocyanato-substituted aromatic sulfonamides with amines. **A1**–**A7** have potent inhibitory properties against CA IX with K_I values in the range of 10–37 nM. Some of the hCA IX inhibitors investigated in this study are substrates for P-gp, which potentially could be a disadvantage in terms of oral administration of the compounds (although a number of P-gp substrates display excellent oral availability). However, the findings open up theoretical perspectives on the influence of efflux transporters on the therapeutic use of hCA IX inhibitors. As mentioned earlier, CA IX and P-gp dramatically increase in response to hypoxia via a direct transcriptional activation of the *CA9* gene and MDR1 gene by the hypoxia-inducible factor HIF-1. Therefore, the use of CA IX inhibitors, which are P-gp substrates, may be an attractive anticancer therapy since the binding domain of this enzyme is extracellular. The pharmacological target is thus located outside the cytosol, and in theory the action of efflux transporters may keep high concentrations of the CA IX inhibitor in the vicinity of the enzyme. Furthermore, CA IX inhibitors, which are also P-gp substrates, may be restricted entry in tissues such as the brain, which may reduce undesired side effects associated with cancer treatment. Future studies will focus on exploiting this for increased therapeutic efficacy.

4. Experimental

4.1. Chemistry

Melting points were recorded with a heating plate microscope and are not corrected. ^{1}H NMR spectra were recorded in DMSO-d_6 as solvent, with a Bruker CPX200 or Varian 300 instrument. Chemical shifts are reported as δ values, relative to Me_4Si as internal standard. Elemental analyses were done by combustion for C, H, and N with an automated Carlo Erba analyzer and were ±0.4% of the theoretical values. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Sulfanilamide, 4-aminoethylbenzenesulfonamide, thiophosgene, and nucleophiles (amines, and their derivatives **1**–**7**) used in the synthesis were of highest purity, commercially available compounds (from Sigma–Aldrich, Fluka, E. Merck or Acros). Acetonitrile, acetone (E. Merck) or other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them under anhydrous conditions.

4.1.1. General procedure for the preparation of compounds (A1–A7). An amount of 2.5 mmol of 4-isothiocyanatobenzenesulfonamide **A**[24] and the stoichiometric amount of nucleophile **1**–**7** were suspended in 50–100 mL of dry acetone or acetonitrile and heated at reflux for 2–8 h (TLC control). The solvent was evaporated, and the crude product was recrystallized from ethanol or ethanol–water when the title derivatives were obtained in excellent yields (85–95%). For **A7**, the molar ratio of **A**:**7** used in the synthesis was of 2:1.

4.1.2. 4-[3-[2-(4-Sulfamoylphenyl)-ethyl]-thioureido]-benzoic acid (A1). White crystals, mp 212–213 °C (dec), ^{1}H NMR (DMSO-d_6), δ, ppm (*J*, Hz): 2.91 (t, 2H, 7.1), 3.49 (q, 2H, 6.7), 7.32 (s, 2H, SO_2NH_2), 7.43 (d, 2H, AA′BB′, 8.2), 7.46 (d, 2H, AA′BB′, 8.1), 7.76 (d, 2H, AA′BB′, 8.2), 7.79 (d, 2H, AA′BB′, 8.1), 8.49 (t, 1H, NHCS, 5.6); 8.63 (s, 1H, NHCS); Anal. Calcd for $C_{16}H_{17}N_3O_4S_2$: C, 50.65; H, 4.52; N, 11.07. Found: C, 50.49; H, 4.60; N, 11.00

4.1.3. 4-[3-[2-Hydroxy-1-hydroxymethyl-2-(4-nitrophenyl)-ethyl]thioureido]-benzenesulfonamide (A2). Tan crystals, mp 175–177 °C (dec), ^{1}H NMR (DMSO-d_6), δ, ppm (*J*, Hz): 2.72 (q, C*H*-NH, 5.9); 3.19 (dd, C*H* from CH_2OH, 6.2, 10.5); 3.36 (dd, C*H* from CH_2OH, 5.8, 10.5); 4.69 (d, C*H* from CHOH, 4.5); 7.23 (s, 2H, SO_2NH_2), 7.59(d, ArH from $O_2NC_6H_4$, 8.8); 7.78 (d, 2H, AA′BB′, 8.9), 7.91 (d, 2H, AA′BB′, 8.9), 8.18(d, ArH from $O_2NC_6H_4$, 8.8); 8.48 (m, 1H, NHCS); 8.60 (s, 1H, NHCS); Anal. Calcd for $C_{16}H_{18}N_4O_6S_2$: C, 45.06; H, 4.25; N, 13.14. Found: C, 44.83; H, 4.13; N, 13.07.

4.1.4. 4-Pentafluorophenyl-1-(4-sulfamoylphenyl)-thiosemicarbazide (A3). White crystals, mp 239–242; ^{1}H NMR (DMSO-d_6), δ, ppm (*J*, Hz): 7.13 (s, 2H, SO_2NH_2), 7.75 (d, 2H, AA′BB′, 8.9), 7.96 (d, 2H, AA′BB′, 8.9), 8.52 (m, 2H, NHCS); 8.65 (s, 1H, NHCS); Anal. Calcd for $C_{13}H_9F_5N_4O_2S_2$: C, 37.87; H, 2.20; N, 13.59. Found: C, 37.95; H, 1.96; N, 13.34.

4.1.5. 4-[3-(4-Methylpiperazin-1-yl)thioureido]-benzenesulfonamide (A4). White crystals, mp 256–258 °C; ^{1}H NMR (DMSO-d_6), δ, ppm (*J*, Hz): 2.31 (s, 3H, Me); 2.86 (t, 8H, 4 CH_2); 7.20 (s, 2H, SO_2NH_2), 7.74 (d, 2H, AA′BB′, 8.9), 7.93 (d, 2H, AA′BB′, 8.9), 8.49 (s, 1H, NHCS);

8.60 (s, 1H, NHCS); Anal. Calcd for $C_{12}H_{19}N_5O_2S_2$: C, 43.75; H, 5.81; N, 21.26. Found: C, 44.02; H, 5.69; N, 21.14.

4.1.6. 4-[3-(2-Fluorophenyl)thioureido]-benzenesulfonamide (A5). White crystals, mp 138–139 (dec); 7.18 (s, 2H, SO_2NH_2), 7.36–7.59 (m, 4H, C_6H_4F); 7.77 (d, 2H, AA′BB′, 8.9), 7.94 (d, 2H, AA′BB′, 8.9), 8.50 (s, 1H, NHCS); 8.66 (s, 1H, NHCS); Anal. Calcd for $C_{13}H_{12}FN_3O_2S_2$: C, 47.99; H, 3.72; N, 12.91. Found: C, 47.95; H, 3.90; N, 12.64.

4.1.7. 4-[3-(2-Dimethylaminoethyl)thioureido]-benzenesulfonamide (A6). White crystals, mp 250–251 °C (dec); 2.38 (s, 6H, 2Me); 2.80 (t, 2H, CH_2); 2.94 (q, 2H, CH_2); 7.15 (s, 2H, SO_2NH_2), 7.70 (d, 2H, AA′BB′, 8.9), 7.88 (d, 2H, AA′BB′, 8.9), 8.49 (t, 1H, NHCS); 8.57 (s, 1H, NHCS); Anal. Calcd for $C_{11}H_{18}N_4O_2S_2$: C, 43.69; H, 6.00; N, 18.53. Found: C, 43.35; H, 5.97; N, 18.25.

4.1.8. *N*,*N*′-Bis[3-(4-sulfamoylphenylthioureido)propyl]-piperazine (A7). White crystals, mp 125–127 °C; ^{1}H NMR (DMSO-d_6), δ, ppm (J, Hz): 2.16–2.54 (m, 12H, 6 CH_2 from propylene); 2.85 (t, 8H, 4 CH_2 from piperazine); 7.21 (br s, 4H, 2 SO_2NH_2), 7.76 (d, 4H, 2 AA′BB′, 8.9), 7.97 (d, 4H, 2 AA′BB′, 8.9), 8.49 (t, 2H, 2NHCS); 8.60 (s, 2H, 2 NHCS); Anal. Calcd for $C_{24}H_{36}N_8O_4S_4$: C, 45.84; H, 5.77; N, 17.82. Found: C, 45.90; H, 5.60; N, 17.62.

4.2. Cell assays

4.2.1. Materials. Caco-2 cells were obtained from the American Type Culture Collection (Manassas, Virginia). Cell culture media and Hank's balanced salt solution (HBSS) were obtained from Life Technologies (Høje Taastrup, Denmark). SDS was from BIO-RAD Laboratories (CA, USA). MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide), 2-(*N*-morpholino) ethanesulfonic acid (MES), and *N*-[2-hydroxyethyl]piperazine*N*′-[2-ethanesulfonate] (HEPES), verapamil, digoxin, and bovine serum albumin (BSA) were from Sigma (Saint Louis, Missouri). [^{3}H]mannitol with a specific activity of 50 mCi/mmol was from PerkinElmer Life Science, Inc. (Boston, MA, USA), Ultima Gold was from Packard (Groningen, The Netherlands).

4.2.2. Apparatus. Transepithelial electrical resistance (TEER) was measured in a tissue resistance measurement chambers (Endohm) with a voltohmmeter (EVOM), both from World Precision Instruments (Sarasota, Florida). The shaking plate used for cell culture experiments was a KS 10 DIGI shaker from Edmund Bühler. For assaying the hCA IX CO_2 hydration activity an SX.18MV-R Applied Photophysics stopped flow instrument was used.

4.2.3. Caco-2 cell culture. Caco-2 cells were cultured as previously described by Nielsen et al.[50] Briefly, cells were seeded in culture flasks and passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U ml^{-1} and 100 μg ml^{-1}, respectively), 1% L-glutamine, and 1% nonessential amino acids. Cells were seeded onto tissue culture treated Transwells (1.0 cm^2, 0.4 μm pore size) at a density of 10^5 cells cm^{-2}. TEER at room temperature was measured before the experiment. All TEER values were 310–500 Ω cm^2 depending on passage number. Transport experiments were performed on days 24–28 after seeding.

4.2.4. Purification of CA isozymes and CA inhibition. Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II. Enzymes were purified by affinity chromatography. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM^{-1} cm^{-1} for CA I and 54 mM^{-1} cm^{-1} for CA II, respectively, based on M_r = 28.85 kDa for CA I and 29.3 kDa for CA II, respectively. The cDNA of the catalytic domain of hCA IX 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 *E. coli* BL21 Codon Plus bacterial strain (from Stratagene). The bacterial cells were sonicated and then suspended in 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 (≈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, 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 obtained CA IX was further purified by sulfonamide affinity chromatography, the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate. An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM HEPES (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. Stock solutions of inhibitor (1 mM) were prepared in distilled de-ionized 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 de-ionized water. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature prior to assay, in order 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 means of such results.

4.2.5. Cytotoxicity study of sulfonamide-derivatives using the MTT assay. The MTT assay may be used to determine the toxic concentration of a substance to cells.

The principle behind the test is that the MTT is cleaved to a dark blue product by mitochondrial dehydrogenases in living but not in dead cells. Caco-2 cells were seeded onto a 96-well culture plate at a seeding density of 4×10^4 cells/well in DMEM culture medium. The cells were cultured for 3 days. Subsequently, the cells were incubated with metazolamide, acetazolamide, sulfanilamide or **A6** in a concentration range of 100 μM, 50 μM, 10 μM, 1 μM, 100 nM, 10 nM, and 1 nM, for 48 h. SDS was used as positive control in a concentration range of 5, 2, 1, 0.75, 0.25, and 0.1 mM, and HBSS was used as negative control (100% cells alive). The optical density was measured with an ELISA reader Labsystem Multiscan MS, using a test wavelength of 570 nm. None of the investigated compounds were toxic to the Caco-2 cells under the conditions employed here.

4.2.6. Transport experiments of hCA IX Inhibitors across Caco-2 cells. 4.2.6.1. General. The buffers used for cell experiments were HBSS supplemented with 0.05% BSA and 10 mM MES (pH 6) (MES-buffer) or 10 mM HEPES (pH 7.4) (HEPES-buffer). Apical (A) to basolateral (B) and B to A fluxes of the compounds were measured in MES- and HEPES-buffers. The concentration of the compounds on the donor (*cis*) side was 1 mM in all the experiments. 160 microliter samples were taken from the receiver and replaced with fresh buffer at $t = 0$, 30, 60, 90, 120, and 150 min. Samples were transferred to HPLC vials and analyzed by HPLC-UV as described below. After the experiment, the integrity of the Caco-2 cell monolayers was evaluated by [^{3}H]mannitol transport studies. Samples were taken from the donor chamber (20 μl) at 0, 20, 40, and 60 min, and from the receiver chamber (100 μl) at 0, 20, 40, and 60 min. Samples were transferred to scintillation vials, respectively, where 2 ml of scintillation fluid was added and the radioactivity was counted in a liquid scintillation analyzer. The mannitol permeability was calculated using Eqs. 1 and 2. The transport of compound **A6** was also measured in the presence of 50 μM verapamil or 50 μM digoxin on both donor and receiver sides, respectively. The experiments were carried out in a similar way as that described above. Mannitol permeabilities (P_{app}) obtained were 1.8 (±0.5) 10^{-6} cm/s (average ± SD, $n = 90$). This indicates that none of the compounds employed in the studies affected the barrier properties of the Caco-2 cell monolayers.

4.2.6.2. HPLC–UV analysis and analysis of pK_a values. *Apparatus*: High performance liquid chromatography (HPLC) was performed with a Waters Spherisorb S5ODS2 reversed-phase column (5 μm, 250 × 4.6 mm) in a Merck/Hitachi system consisting of an L-7100 pump, an L-7400 UV-detector, operated in a wavelength range between 223 and 275 nm depending on the maximum adsorption determined for the individual compounds, and an L-7200 autosampler connected with a D-7500 integrator. pK_a values were determined using a Sirius GlpK$_a$-meter from Sirius Analytical Instruments Ltd. (East Sussex UK) equipped with Sirius pK$_a$LOGPTH, version 5.2a software from Sirius Analytical Instruments Ltd. (East Sussex, United Kingdom).

General: All HPLC solvents were of analytical grade and chemicals used in buffer preparations were of laboratory grade. The sulfonamide derivatives were analyzed using a mobile phase system consisting of 80% 0.02 M phosphate buffer, 15% methanol, 5% acetonitrile, and 0.1% triethylamine (TEA) adjusted to pH 6 for **A4** and **A6**, and 97.5% 0.02 M phosphate buffer, 2.5% acetonitrile, and 0.1% TEA adjusted to pH 6 for **A1**, **A2**, **A3**, **A5**, and **A7** giving retention times of approximately 10 min for every compound, no other peaks being observed in the chromatograms. The flow rate was 1 ml/min. The detection and quantification limits (DL and QL, respectively) for the compounds on the L-7400 UV-detector were calculated from the standard curves using: QL: 10*(intercept/slope) and DL: 3.3*(intercept/slope). The QL was 40 μM for **A1**, 52 μM for **A2**, 85 μM for **A3**, 1.2 μM for **A4**, 42 μM for **A5**, 0.7μM for **A6**, 110 μM for **A7**, and the DL for **A1** was 13 μM, 17 μM for **A2**, 28 μM for **A3**, 0.4 μM for **A4**, 14 μM for **5**, 0.2 μM for **A6**, and 36 μM for **A7**.

The pK_a values for **A6** were determined by dissolving 2–4 mg solid compound in 10 ml ionic strength adjusted water (ISA-water) adjusted to 0.16 M with respect to KCl. The solution was titrated from pH 1.8–12.2. The same procedure was repeated in the pH range 2.5 → 10.5. The determination was performed at 25 °C in triplicate.

4.2.7. Data analysis of the transport experiments. Flux of the sulfonamides was calculated using Eq. 1:

$$J = (\mathrm{d}Q/\mathrm{d}t)/A, \tag{1}$$

where Q is the amount of compound transported over time t of the steady state part of the transport experiment and A is the surface area of the monolayers (cm^2).

Eq. 2 was used to determine the apparent permeability, P_{app}, from the flux:

$$P_{app} = (\mathrm{d}Q/\mathrm{d}t)/(A \cdot C_0) = J/(C_0), \tag{2}$$

where J is then the flux (nmol cm^{-2} min^{-1}), C_0 is the donor concentration of the test compound added to the donor compartment, and subsequently P_{app} (cm/s) is the apparent permeability coefficient.

4.2.8. Statistical analysis. Experiments with Caco-2 cell monolayers were performed in duplicate or triplicate in each cell passage, using several passages, where n is the number of cell passages used ($n = 3$–6). Values are given as means ± SE unless otherwise stated. The statistical significance of the results was determined using two-tailed Student's t test or an alternate t test when appropriate.

Acknowledgments

This work was supported by grants from EU (EUROXY project to C.T.S. and A.S.), being also supported by a Marie Curie Fellowship of the EU Program 'Improving the Human Research Potential and the

Socio-Economic Knowledge Base' under contract number HPMT-CT-2001-00403 (D.V.). Furthermore, a grant from the Hørslev foundation (to CUN) is also acknowledged. We are grateful to Susanne N. Sørensen for doing the cell culture work.

References and notes

1. Svastova, E.; Hulikova, A.; Rafajova, M.; Zat'ovicova, M.; Gibadulinova, A.; Casini, A.; Cecchi, A.; Scozzafava, A.; Supuran, C. T.; Pastorek, J.; Pastorekova, S. *FEBS Lett.* **2004**, *577*, 439–445.
2. Supuran, C. T; Scozzafava, A.; Conway, J. *Carbonic Anhydrase—Its Inhibitors and Activators*; CRC: Boca Raton, London, New York, 2004, pp 1–363.
3. Parkkila, S.; Rajaniemi, H.; Parkkila, A. K.; Kivela, J.; Waheed, A.; Pastorekova, S.; Pastorek, J.; Sly, W. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2220–2224.
4. Pastorek, J.; Pastorekova, S.; Callebaut, I.; Mornon, J. P.; Zelnik, V.; Opavsky, R.; Zat'ovicova, M.; Liao, S.; Portetelle, D.; Stanbridge, E. J., et al. *Oncogene* **1994**, *9*, 2877–2888.
5. Ivanov, S. V.; Kuzmin, I.; Wei, M. H.; Pack, S.; Geil, L.; Johnson, B. E.; Stanbridge, E. J.; Lerman, M. I. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12596–12601.
6. Tureci, O.; Sahin, U.; Vollmar, E.; Siemer, S.; Gottert, E.; Seitz, G.; Parkkila, A. K.; Shah, G. N.; Grubb, J. H.; Pfreundschuh, M.; Sly, W. S. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 7608–7613.
7. Loncaster, J. A.; Harris, A. L.; Davidson, S. E.; Logue, J. P.; Hunter, R. D.; Wycoff, C. C.; Pastorek, J.; Ratcliffe, P. J.; Stratford, I. J.; West, C. M. *Cancer Res.* **2001**, *61*, 6394–6399.
8. Turner, K. J.; Crew, J. P.; Wykoff, C. C.; Watson, P. H.; Poulsom, R.; Pastorek, J.; Ratcliffe, P. J.; Cranston, D.; Harris, A. L. *Br. J. Cancer* **2002**, *86*, 1276–1282.
9. Chia, S. K.; Wykoff, C. C.; Watson, P. H.; Han, C.; Leek, R. D.; Pastorek, J.; Gatter, K. C.; Ratcliffe, P.; Harris, A. L. *J. Clin. Oncol.* **2001**, *19*, 3660–3668.
10. Ivanov, S.; Liao, S. Y.; Ivanova, A.; Danilkovitch-Miagkova, A.; Tarasova, N.; Weirich, G.; Merrill, M. J.; Proescholdt, M. A.; Oldfield, E. H.; Lee, J.; Zavada, J.; Waheed, A.; Sly, W.; Lerman, M. I.; Stanbridge, E. J. *Am. J. Pathol.* **2001**, *158*, 905–919.
11. Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199–229.
12. Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Pat.* **2004**, *14*, 667–702.
13. Supuran, C. T. Carbonic Anhydrases: Catalytic and Inhibition Mechanisms, Distribution and Physiological Roles. In *Carbonic anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton, 2004; pp 1–23.
14. Pastorekova, S.; Pastorek, J. Cancer-related Carbonic Anhydrase Isozymes and their Inhibition. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton, 2004; pp 255–281.
15. Abbate, F.; Casini, A.; Owa, T.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 217–223.
16. Casey, J. R.; Morgan, P. E.; Vullo, D.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *J. Med. Chem.* **2004**, *47*, 2337–2347.
17. Vullo, D.; Franchi, M.; Gallori, E.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1005–1009.
18. Vullo, D.; Scozzafava, A.; Pastorekova, S.; Pastorek, J.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2351–2356.
19. Scozzafava, A.; Briganti, F.; Ilies, M. A.; Supuran, C. T. *J. Med. Chem.* **2000**, *43*, 292–300.
20. Supuran, C. T.; Scozzafava, A.; Ilies, M. A.; Briganti, F. *J. Enzyme Inhib.* **2000**, *15*, 381–401.
21. Nielsen, C. U.; Supuran, C. T.; Scozzafava, A.; Frokjaer, S.; Steffansen, B.; Brodin, B. *Pharm. Res.* **2002**, *19*, 1337–1344.
22. Cecchi, A.; Winum, J. Y.; Innocenti, A.; Vullo, D.; Montero, J. L.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5775–5780.
23. Innocenti, A.; Casini, A.; Alcaro, M. C.; Papini, A. M.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2004**, *47*, 5224–5229.
24. Casini, A.; Scozzafava, A.; Mincione, F.; Menabuoni, L.; Ilies, M. A.; Supuran, C. T. *J. Med. Chem.* **2000**, *43*, 4884–4892.
25. Di Fiore, A.; De Simone, G.; Menchise, V.; Pedone, C.; Casini, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1937–1942.
26. Mincione, F.; Starnotti, M.; Masini, E.; Bacciottini, L.; Scrivanti, C.; Casini, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3821–3827.
27. Abbate, F.; Casini, A.; Scozzafava, A.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 303–308.
28. de Leval, X.; Ilies, M.; Casini, A.; Dogne, J. M.; Scozzafava, A.; Masini, E.; Mincione, F.; Starnotti, M.; Supuran, C. T. *J. Med. Chem.* **2004**, *47*, 2796–2804.
29. Scozzafava, A.; Menabuoni, L.; Mincione, F.; Briganti, F.; Mincione, G.; Supuran, C. T. *J. Med. Chem.* **2000**, *43*, 4542–4551.
30. Boriack, P. A.; Christianson, D. W.; Kingery-Wood, J.; Whitesides, G. M. *J. Med. Chem.* **1995**, *38*, 2286–2291.
31. Nair, S. K.; Krebs, J. F.; Christianson, D. W.; Fierke, C. A. *Biochemistry* **1995**, *34*, 3981–3989.
32. Stewart, B. H.; Chan, O. H.; Lu, R. H.; Reyner, E. L.; Schmid, H. L.; Hamilton, H. W.; Steinbaugh, B. A.; Taylor, M. D. *Pharm. Res.* **1995**, *12*, 693–699.
33. Chan, L. M.; Lowes, S.; Hirst, B. H. *Eur. J. Pharm. Sci.* **2004**, *21*, 25–51.
34. Collett, A.; Tanianis-Hughes, J.; Hallifax, D.; Warhurst, G. *Pharm. Res.* **2004**, *21*, 819–826.
35. Artursson, P.; Karlsson, J. *Biochem. Biophys. Res. Commun.* **1991**, *175*, 880–885.
36. Gres, M. C.; Julian, B.; Bourrie, M.; Meunier, V.; Roques, C.; Berger, M.; Boulenc, X.; Berger, Y.; Fabre, G. *Pharm. Res.* **1998**, *15*, 726–733.
37. Yamashita, S.; Furubayashi, T.; Kataoka, M.; Sakane, T.; Sezaki, H.; Tokuda, H. *Eur. J. Pharm. Sci.* **2000**, *10*, 195–204.
38. Ren, S.; Lien, E. J. *Prog. Drug Res.* **2000**, *54*, 1–3.
39. Saitoh, H.; Aungst, B. J. *Pharm. Res.* **1995**, *12*, 1304–1310.
40. Sandstrom, R.; Karlsson, A.; Knutson, L.; Lennernas, H. *Pharm. Res.* **1998**, *15*, 856–862.
41. Adachi, Y.; Suzuki, H.; Sugiyama, Y. *Pharm. Res.* **2001**, *18*, 1660–1668.
42. Engman, H.; Tannergren, C.; Artursson, P.; Lennernas, H. *Eur. J. Pharm. Sci.* **2003**, *19*, 57–65.
43. Pauli-Magnus, C.; von Richter, O.; Burk, O.; Ziegler, A.; Mettang, T.; Eichelbaum, M.; Fromm, M. F. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 376–382.
44. Cavet, M. E.; West, M.; Simmons, N. L. *Br. J. Pharmacol.* **1996**, *118*, 1389–1396.

45. de Lannoy, I. A.; Silverman, M. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 551–557.
46. Drescher, S.; Glaeser, H.; Murdter, T.; Hitzl, M.; Eichelbaum, M.; Fromm, M. F. *Clin. Pharmacol. Ther.* **2003**, *73*, 223–231.
47. Taipalensuu, J.; Tavelin, S.; Lazorova, L.; Svensson, A.-C.; Artursson, P. *Eur. J. Pharm. Sci.* **2004**, *21*, 69–75.
48. Neuhoff, S.; Ungell, A. L.; Zamora, I.; Artursson, P. *Pharm. Res.* **2003**, *20*, 1141–1148.
49. Comerford, K. M.; Wallace, T. J.; Karhausen, J.; Louis, N. A.; Montalto, M. C.; Colgan, S. P. *Cancer Res.* **2002**, *62*, 3387–3394.
50. Nielsen, C. U.; Amstrup, J.; Steffansen, B.; Frokjaer, S.; Brodin, B. *Am. J. Physiol.* **2001**, *281*, G191–G199.