

Carbonic Anhydrase Inhibitors: Sulfonamides as Antitumor Agents?[☆]

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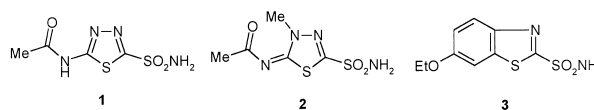
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Abstract—Novel sulfonamide inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) were prepared by reaction of aromatic or heterocyclic sulfonamides containing amino, imino, or hydrazino moieties with *N,N*-dialkyldithiocarbamates in the presence of oxidizing agents (sodium hypochlorite or iodine). The *N,N*-dialkylthiocarbamylsulfenamido-sulfonamides synthesized in this way behaved as strong inhibitors of human CA I and CA II (hCA I and hCA II) and bovine CA IV (bCA IV). For the most active compounds, inhibition constants ranged from 10^{−8} to 10^{−9} M (for isozymes II and IV). Three of the derivatives belonging to this new class of CA inhibitors were also tested as inhibitors of tumor cell growth in vitro. These sulfonamides showed potent inhibition of growth against several leukemia, non-small cell lung, ovarian, melanoma, colon, CNS, renal, prostate and breast cancer cell lines. With several cell lines, GI₅₀ values of 10–75 nM were observed. The mechanism of antitumor action with the new sulfonamides reported here remains obscure, but may involve inhibition of CA isozymes which predominate in tumor cell membranes (CA IX and CA XII), perhaps causing acidification of the intercellular milieu, or inhibition of intracellular isozymes which provide bicarbonate for the synthesis of nucleotides and other essential cell components (CA II and CA V). Optimization of these derivatives from the SAR point of view, might lead to the development of effective novel types of anticancer agents. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

Fourteen different carbonic anhydrase (CA, EC 4.2.1.1) isoforms have been identified in mammals (Table 1). Some of these are involved in crucial physiological processes connected with respiration and transport of CO₂/bicarbonate between metabolizing tissues and the lungs, pH homeostasis, electrolyte secretion in a variety of tissues/organs and biosynthetic reactions, such as the lipogenesis, gluconeogenesis and ureagenesis.^{2–6} It has been known for some time that several of these isozymes are cytosolic (CA I, CA II, CA III, CA VII), CA IV is membrane-bound, CA V is present only in mitochondria, and CA VI is secreted in saliva. Several acatalytic forms are also known (CA-RP VIII, CA-RP X and CA-RP XI),^{2–14} each of which lacks one or more of the histidine residues which are essential for the binding of the zinc ion in the active site.

More recently, three novel membrane-bound CA isozymes, CA IX,⁸ XII¹¹ and XIV,¹² have been discovered and partially characterized. Of these, CA IX shows close association with tumors and CA XII, although expressed in a wide variety of normal tissues, is significantly overexpressed in renal carcinoma of the clear cell type. Expression of both isozymes is down-regulated by the product of the von-Hippel-Lindau (VHL) tumor suppressor gene, strongly suggesting that they may play a role in VHL-mediated carcinogenesis.¹⁴ However, little is known currently regarding the physiological consequences of their activation or inhibition,^{11–14} although it has been suggested that CA IX may be involved in the pathogenesis of certain tumors.¹⁵ Recent evidence also implicates other CA isozymes in progressive polycystic kidney disease¹⁶ and in idiopathic chronic pancreatitis.¹⁷



[☆]This paper is part 88 of the series; for part 87, see Ref 1.

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Table 1. CA isozymes, their relative CO₂ hydrase activity and sub-cellular location

Isozyme	Catalytic activity (CO ₂ hydration)	Sub-cellular location
CA I	Low (10% of that of CA II)	Cytosol
CA II	High	Cytosol
CA III	Very low (1% of that of CA II)	Cytosol
CA IV	High	Membrane-bound
CA V	Moderate–high	Mitochondria
CA VI	Moderate	Secreted into saliva
CA VII	High	Cytosol
CA-RP VIII	Acatalytic	Probably cytosolic
CA IX ^a	High	Membrane-bound
CA-RP X	Acatalytic	Unknown
CA-RP XI	Acatalytic	Membrane-bound
CA XII ^a	Low	Membrane-bound
CA XIII ^b	Probably high	Unknown
CA XIV	Low	Membrane-bound

^aCA IX and CA XII are known to be tumor associated. CA II and CA IV have also been evidenced in some renal tumor cells.⁶²

^bCA XIII has not been isolated as a protein but has been identified from EST derived from a mouse mammary gland cDNA library.^{2a}

Although inhibition of CAs by aromatic and heterocyclic sulfonamides has been exploited clinically for more than 45 years in the treatment of a variety of diseases such as glaucoma,^{4,18} epilepsy,¹⁹ congestive heart failure,⁴ mountain sickness,²⁰ gastric and duodenal ulcers,²¹ or as diuretic agents,²² their potential use as antitumor drugs has been little explored up to now. Several classical clinical agents from this class include acetazolamide **1**, methazolamide **2** or ethoxzolamide **3**.⁴ Teicher et al.²³ reported that one of these derivatives, acetazolamide **1**, which is a strong inhibitor of several CA isozymes,^{3–5} is a potential modulator of anticancer therapies in combination with different cytotoxic agents (alkylating agents; nucleoside analogues; platinum derivatives, etc), probably due to the acidification of the intratumoral environment ensued after CA inhibition, although other mechanisms of action of this drug were not excluded.

The inhibition of growth of human cancer cells in culture by direct action of specific sulfonamide CA inhibitors was first reported by Chegwidan and Spencer,²⁴ who drew attention to their therapeutic potential in the treatment of cancer. They showed that potent, clinically used sulfonamide CA inhibitors, such as acetazolamide **1**, methazolamide **2**, or ethoxzolamide **3**, strongly inhibited the growth of human lymphoma cells, with GI₅₀ values ranging from 0.5 μM for ethoxzolamide to 0.25 mM for acetazolamide.^{5a,24} Since the inhibition was largely overcome by the addition of nucleotide precursors, they suggested that it may be attributed to a reduced provision of bicarbonate for nucleotide synthesis (HCO₃[–] is the substrate of carbamoyl phosphate synthetase II).^{5a,24}

It thus appeared of interest to further explore the connections between CAs and tumors, and the development of specific inhibitors for some of the isozymes presumably involved in such processes would be highly

beneficial for both obtaining novel types of drugs, as well as for a better understanding of the physiology of the CAs.

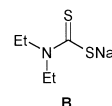
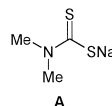
In previous contributions from this laboratory^{18,25–27} it was shown that by attaching different ‘tails’ to the molecules of aromatic and heterocyclic sulfonamides, it is possible to obtain potent CA inhibitors possessing the desired physico-chemical properties, such as enhanced water solubility (for their use as antiglaucoma drugs),^{18,25,26} membrane impermeability (important for obtaining isozyme-specific inhibitors),^{27a} and so forth, all requisites of interest for the drug design of novel types of pharmacological agents. Here we report a related approach, consisting in the incorporation of *N,N*-dialkylthiocarbamylsulfenamido moieties in the molecules of aromatic/heterocyclic sulfonamide CA inhibitors. This approach has been explored in a short communication of this group for the preparation of morpholine-thiocarbonylsulfenylamido sulfonamides possessing antitumor properties.^{27b} Here we extend the previous research,^{27b} and report some new compounds possessing high affinities for three physiologically relevant CA isozymes, hCA I, hCA II and bCA IV, with inhibition constants in the range of 10^{–8}–10^{–9} M for the most active derivatives. Furthermore, three of the new compounds proved to be effective in vitro tumor cell growth inhibitors of different leukemia, non-small cell lung cancer, melanoma, ovarian, renal, prostate and breast cancer cell lines, in some cases with GI₅₀ values as low as 10 nM.

So far as we know, this is the first systematic study reporting sulfonamide CA inhibitors with such a high potency as in vitro tumor cell growth inhibitors, against such a variety of tumor cell lines.

Results

Synthesis

As a relatively large number of derivatives is reported here, each compound will be designated by a letter identifying the *N,N*-dialkylthiocarbamate from which it was obtained, and a figure identifying the sulfonamide of type **4–28** at which the *N,N*-dialkylthiocarbamylsulfenamido moiety has been attached. For instance, **A4** is the *N,N*-dimethylthiocarbamylsulfenamido derivative of orthanilamide; **A9** is the *N,N*-dimethylthiocarbamylsulfenamido derivative of *p*-(2-aminoethyl)benzenesulfonamide; **B17** is 5-(*N,N*-diethylthiocarbamylsulfenamido)-1,3,4-thiadiazole-2-sulfonamide; **B28** is the *N,N*-diethylthiocarbamylsulfenamido derivative of dorzolamide **28**, and so forth (Chart 1).



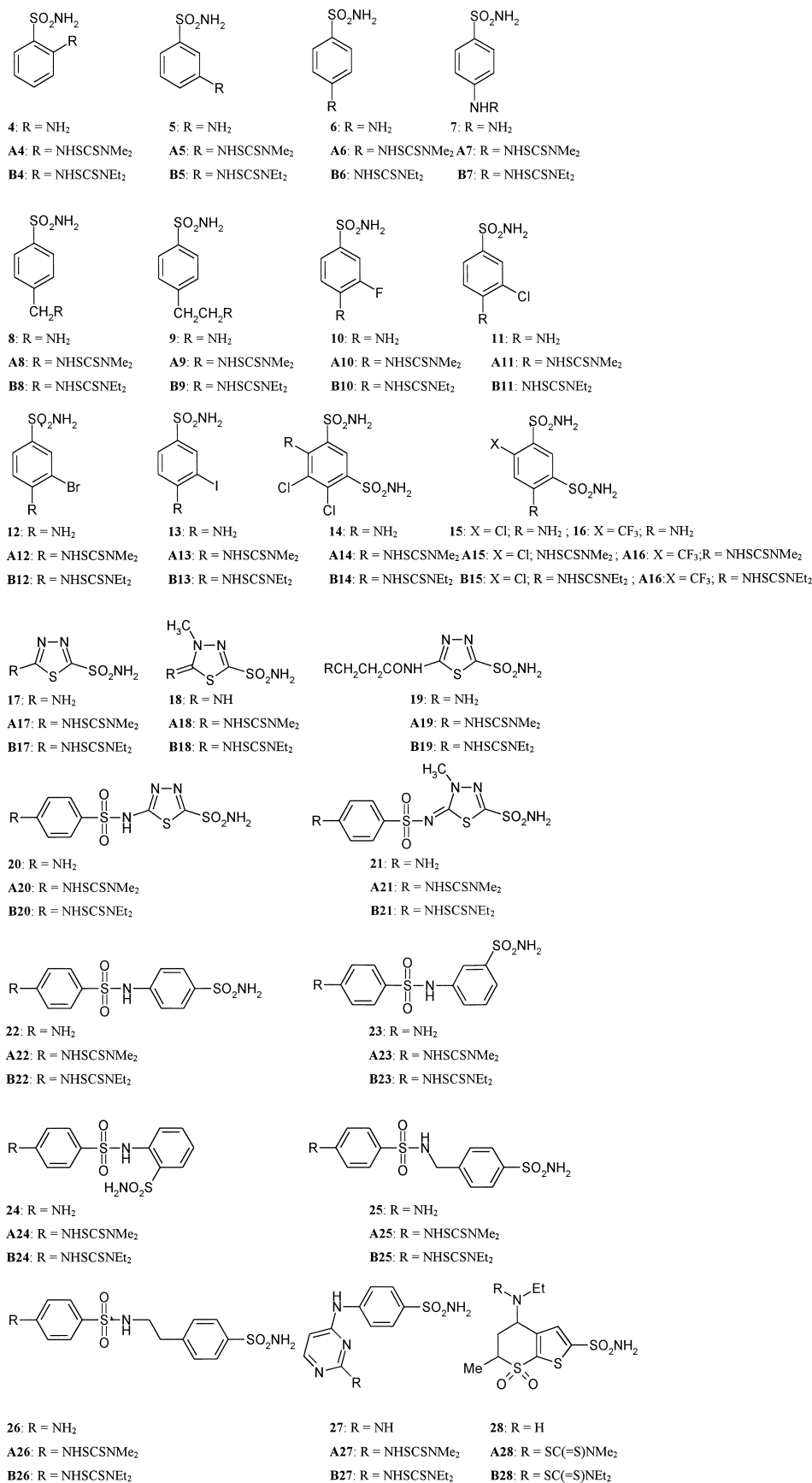
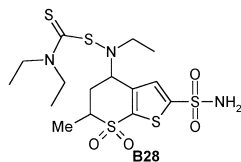
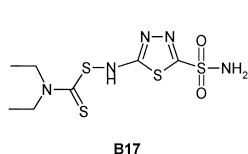
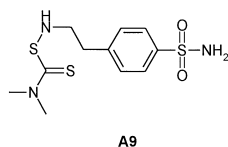
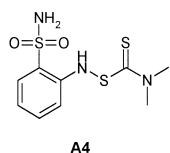


Chart 1.



The new compounds **A(4–28)**–**B(4–28)** were prepared by the oxidative thiocarbamylation reaction of the aromatic/heterocyclic sulfonamides **4–28** with sodium/potassium *N,N*-dialkyldithiocarbamates of types **A** and **B**, in the presence of oxidizing agents such as halogens or sodium hypohalogenites.^{27b,28–31}

Non-exceptional, new synthetic procedures were developed for the reaction of amines/imes/hydrazines with dialkyldithiocarbamates, as reported previously by this group for the synthesis of 1,3,4-thiadiazole-2,5-bissulfonamide^{28a} or sulfenamido-sulfonamides,^{27b,28b,c} or by other groups for the preparation of sulfenamides and thiocarbamylsulfenamides (with no CA inhibitory properties).^{29–31}

Carbonic anhydrase inhibitory activity

Inhibition data for three CA isozymes, hCA I, hCA II and bCA IV, with compounds **A(4–28)**–**B(4–28)** as well as the original raw materials and standard sulfonamides of type **1–28** are shown in Table 2. The esterase activity of CA isozymes with 4-nitrophenyl acetate as substrate has been used in this assay³² (see Experimental for details).

Table 2. CA inhibition data with standard inhibitors **1–3**, the parent sulfonamides **4–28** and the new derivatives **(A,B)4–28** reported in the present study, against isozymes I, II and IV

Inhibitor		K_i^a (nM)		
		hCA I ^b	hCA II ^b	bCA IV ^c
Acetazolamide,	1	200	7	120
Methazolamide,	2	100	9	145
Ethoxzolamide,	3	25	8	13
	4	45,400	295	1310
	5	25,000	240	2200
	6	28,000	300	3000
	7	78,500	320	3215
	8	25,000	170	2800
	9	21,000	160	2450
	10	8300	60	180
	11	9800	110	320
	12	6500	40	66
	13	6000	70	125
	14	6100	28	175
	15	8400	75	160
	16	7500	62	140
	17	8600	60	540
	18	9300	19	355
	19	455	3	125
	20	6	2	5
	21	1	0.6	0.8
	22	42	6	50
	23	44	9	53
	24	1800	54	109
	25	17	4	13
	26	9	4	7
	27	690	12	154
Dorzolamide,	28	50000	9	45
	A4	45000	290	1200
	A5	21000	230	1300
	A6	15500	75	190
	A7	55000	290	3000
	A8	1500	36	54
	A9	1100	30	51
	A10	950	13	38
	A11	2600	82	150
	A12	3100	76	144
	A13	3450	95	189
	A14	680	25	124
	A15	800	30	155
	A16	960	27	140
	A17	380	10	55
	A18	350	9	43
	A19	200	3	60
	A20	6	1.7	9
	A21	3	0.3	1
	A22	35	5	18
	A23	40	7	9
	A24	1500	48	93
	A25	13	3	10
	A26	10	4	5
	A27	355	11	75
	A28	> 50,000	9	39
	B4	29,500	1100	990
	B5	18,700	200	1150
	B6	15,000	48	140
	B7	54,000	240	2850
	B8	950	13	41
	B9	630	11	30
	B10	500	10	36
	B11	2500	77	134
	B12	3000	71	125
	B13	3350	87	170
	B14	590	17	115
	B15	650	20	150
	B16	800	16	120
	B17	300	7	33
	B18	290	6	40
	B19	200	2	29
	B20	5	1.5	6
	B21	2.7	0.8	1
	B22	31	5	12
	B23	28	8	9
	B24	1500	43	79
	B25	10	2.6	9
	B26	10	2.1	5
	B27	350	10	62
	B28	> 50,000	8	30

^aStandard error for the determination of K_i s was of 10–20% (from three different assays).

^bHuman (cloned) isozyme.

^cIsolated from bovine lung microsomes.

Inhibition of tumor cell growth

Data for the in vitro inhibition of tumor cell growth with three of the newly prepared sulfonamides, that is **B6**, **B8** and **B9**, as well as two acetazolamide-like inhibitors **36** and **37** (for comparison, since acetazolamide itself is too weak an inhibitor of tumor cell growth), against a variety of cancer cell lines are shown in Table 3. The reported measurements have been performed at the NIH National Cancer Institute, Bethesda, MD, USA, in the frame of a research program in which we have supplied diverse CA inhibitors for antitumor studies, by a standardized assay including a panel of around 60 different tumor cell lines.³³ The following cancer cell types were included in these assays: leukemia; non-small cell lung cancer; colon cancer; CNS cancer; melanoma; ovarian cancer; renal cancer; prostate and breast cancer.

The different cell lines used in these experiments are shown in detail in Table 3.

Discussion

Chemistry

Although a very large number of aromatic/heterocyclic sulfonamide CA inhibitors have been synthesized in the last 45 years in the search for different types of drugs,^{3,18,25–28,34,35} derivatives with potential use as antitumor agents from this class of pharmacological agents have been reported only recently among the well known, clinically used inhibitors acetazolamide **1**, methazolamide **2** and ethoxzolamide **3**.^{23,24} We have recently explored some morpholyl-sulfonylamido

Table 3. In vitro tumor growth inhibition data with some of the new CA inhibitors synthesized in the present work (**B6**, **B8** and **B9**) and the two acetazolamide-like sulfonamides **36** and **37**

Tumor	Cell line	GI ₅₀ (μM) ^a				
		B6	B8	B9	37^b	38^b
Leukemia	HL-60 (TB)	—	—	0.065	16	—
	MOLT-4	< 0.010	7.5	62	21	26
	RPMI-8226	22	—	18	17	21
	SR	25	0.17	< 0.010	—	32
Non-small cell lung cancer	A549/ATCC	75	—	12	26	> 100
	HOP-62	78	15	62	27	> 100
	NCI-H460	39	0.11	—	—	> 100
	NCI-H226	> 100	> 100	> 100	41	> 100
	NCI-H522	—	0.019	0.075	16	29
Colon cancer	COLO-205	68	—	29	38	> 100
	HCT-15	60	21	> 100	22	> 100
	HT29	16	9.7	9	27	> 100
	SW-620	19	31	13	30	> 100
CNS cancer	SF-268	58	16	90	30	> 100
	SF-295	44	5.4	> 100	34	> 100
	SNB-75	53	23	> 100	18	> 100
	U251	31	—	80	43	> 100
Melanoma	LOX IMVI	39	—	7	16	30
	MALME-3M	26	20	17	21	> 100
	M14	20	10	11	15	> 100
	SK-MEL-2	39	18	21	31	> 100
	SK-MEL-28	34	28	7	39	> 100
	SK-MEL-5	42	14	21	22	69
Ovarian cancer	IGROV1	73	—	0.06	17	> 100
	OVCAR-4	> 100	18	68	28	> 100
	OVCAR-5	21	13	37	25	> 100
	OVCAR-8	23	—	12	20	> 100
Renal cancer	768-0	19	15	21	23	37
	ACHN	33	17	18	—	> 100
	CAKI-1	30	17	12	28	> 100
	RXF 393	26	21	11	18	> 100
	UO-31	—	—	13	24	> 100
Prostate cancer	PC-3	37	12	14	33	> 100
	DU-145	86	16	13	14	> 100
Breast cancer	MCF7	0.038	—	—	38	> 100
	MDA-MB-435	33	17	12.5	70	> 100
	MDA-N	69	—	19	25	> 100
	BT-549	85	19	15	16	> 100
	T-47D	—	16	17	—	35

^aMolarity of inhibitor producing a 50% inhibition of growth of the tumor cells after 48 h exposure to variable concentrations (10^{−4}–10^{−8} M) of the test compound. Errors were in the range of ± 5–10% (from two determinations).

^bData from ref 48.

aromatic/heterocyclic sulfonamides possessing interesting CA inhibitory and antitumor properties.^{27b} In this paper we extend our previous research,^{27b} and report the synthesis of a new class of potent CA sulfonamide inhibitors, which also show strong in vitro antitumor properties.

The new derivatives **A(4–28)–B(4–28)** were prepared by the oxidative thiocarbamylation reaction of aromatic/heterocyclic sulfonamides possessing free amino, imino or hydrazino moieties, of type **4–28**, with sodium/potassium *N,N*-dimethyl-/diethyldithiocarbamate **A, B** in the presence of oxidizing agents such as iodine or sodium hypochlorite (Scheme 1).^{27b,28–31} This reaction has been thoroughly investigated for the preparation of heterocyclic sulfenamides,^{28,29b,30,31} as well as simple thiocarbamyl-sulfenamides.^{29a}

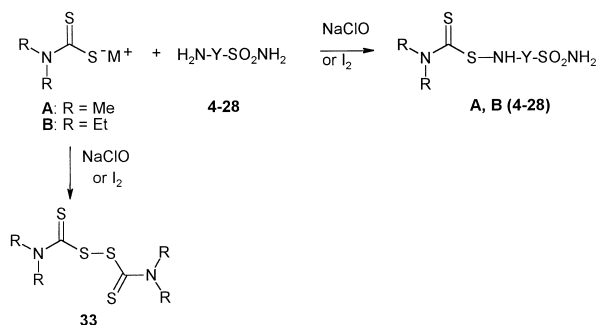
The thiocarbamylation of compounds **4–28** generally occurred with good yields for the aliphatic derivatives **8** and **9**, incorporating $\text{H}_2\text{N}-(\text{CH}_2)_n$ moieties, whereas yields were definitely lower for the aromatic/heterocyclic derivatives possessing H_2N -aryl- or H_2N -hetharyl moieties, for the secondary amine **28**, the imines **18, 21** and the hydrazine **7**. The main complication of this reaction consisted in the formation of tetra-alkylthiuram derivatives **36**. In order to avoid this problem, which would lead to impure compounds **A(4–28)–B(4–28)**,^{29a} the following synthetic strategies have been used: (i) the molar ratio amine **4–28**: dialkyldithiocarbamate **A,B**: oxidizing agent was generally of 1:1:1, but using an excess of 2–2.5 mol of amine (when possible, due to the limitation of costs and availability of these derivatives) led to higher yields and decreased formation of side products of type **33**; (ii) the temperature had to be maintained at 0–4 °C when iodine was used as oxidizing agent, and at 25–30 °C when sodium hypochlorite has been employed; (iii) the dialkyldithiocarbamates **A,B** (as water solutions) and the oxidizing agents were dropped slowly and *concomitantly* into the alkaline solution of amine **4–28**. This was in fact the most important factor leading to the formation of small amounts of tetraalkylthiurams **36**, and acceptable yields in thiocarbamylsulfenamides. Anyhow, the purification of the obtained derivatives **A(4–28)–B(4–28)** (even when variable amounts of **36** were concomitantly formed during the synthesis) was greatly facilitated by the fact that the obtained dialkyldithiocarbamylated sulfonamides **A(4–28)–B(4–28)** are soluble at cold (4 °C) in diluted (0.03–0.05 M)

alkaline solutions (NaOH or KOH), whereas the tetra-alkylthiurams **36** are not. The concentrations of the alkaline hydroxides used for such extraction should be precisely maintained in the limits mentioned above, since more concentrated bases (and higher temperatures) led to the dissolution of the tetra-alkylthiurams too, with regeneration of the dialkyldithiocarbamates **A, B**.²⁹

The new compounds reported in the present work were characterized by standard chemical and physical methods (elemental analysis, within $\pm 0.4\%$ of the theoretical values; IR; ^1H and ^{13}C NMR spectroscopy) that confirmed their structure (see Experimental for details), and were assayed for the inhibition of isozymes hCA I, hCA II and bCA IV (Table 2).

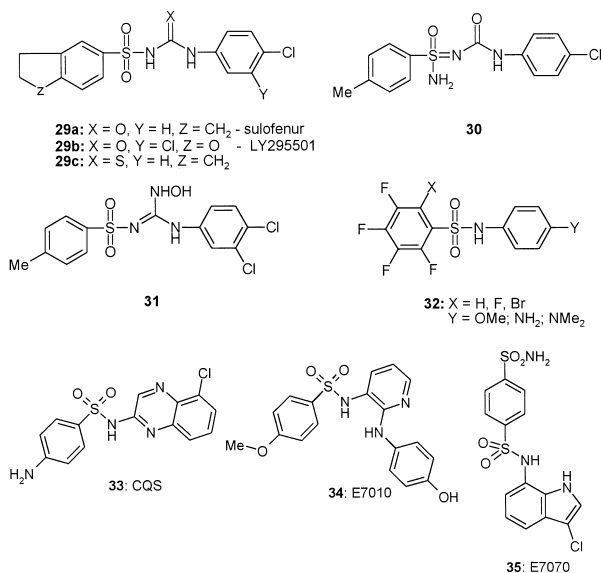
At this point one should mention that several non-CA inhibitor sulfonamide derivatives were recently investigated for their anticancer properties. Thus, some aryl-sulfonyl-ureas/hydroxyguanidines or sulfonimideamides of types **29–31** have been reported by researchers from Eli Lilly^{36a,b} and by Chern et al.,^{36c} whereas Medina's group at Tularik³⁷ prepared *N*-substituted polyhalogeno-benzenesulfonamides of type **32** (Chart 3), which strongly inhibited the growth of multidrug resistant MCF-7/ADR cancer cells in vitro. Furthermore, three other very interesting antitumor sulfonamides have been investigated in more detail: CQS, 5-chloro-quinoxaline-2-sulfanilamide **33** entered in phase II clinical trials in patients with stage III and IV non-small-cell lung cancer,³⁸ whereas E7010 **34**,³⁹ and E7070 **35** developed by Owa's group, reached advanced stages of clinical investigation and might be launched soon as novel antitumor drugs.^{40–42} The first two derivatives mentioned above, **33** and **34**, possess substituted sulfonamide moieties, and probably do not act as CA inhibitors. The mechanism of antitumor action of **34** has been studied in some detail,⁴⁰ being showed that this compound is a tubulin polymerisation inhibitor, binding at the colchicine site of this protein. On the other hand, the molecular targets of CQS and E7070 are still unclear.^{38,41–43} One must mention that the latter compound, **35**, possessing a free SO_2NH_2 moiety probably acts as a strong CA inhibitor, being thus remarkably similar with the antitumor sulfonamides reported here. It was shown that this compound interrupts cell cycle in the G1 phase, unlike E7010 which targets the M phase.^{39–43}

Sulfonamides **29–34** surely do not act as CA inhibitors, being substituted at the sulfonamido moiety with bulky groups, which impair their binding to the Zn(II) ion of the enzyme,⁴⁴ and thus its inhibition, but not the same may be said about **35**. It is a possibility that the cytotoxicity of derivatives **29–31** may be a consequence of the uncoupling of mitochondria.⁴⁵ Nonetheless, up to now, no studies have been reported regarding the possibility that such anticancer compounds (of type **29–31**) might interfere with the CA activity, although in a previous work from this laboratory,⁴⁶ we hypothesized as very probable a strong in vivo CA V inhibition (due to the hydrolysis of the cytotoxic agent, leading to the



Scheme 1.

formation of unsubstituted sulfonamides as principal products).^{36,47} On the other hand, it has been proven that the polyhalogeno-benzenesulfonamides **32** exert their cytotoxic activity due to the irreversible binding to an amino acid residue (Cys 239) of β -tubulin (by means of an aromatic nucleophilic substitution reaction involving the fluorine atom in position 4 as leaving group), resulting thus in the disruption of cellular microtubules.³⁷



In vitro CA inhibition

Inhibition data against three CA isozymes, hCA I, hCA II and bCA IV with the new derivatives (Table 2) prove that the dialkylthiocarbamyl moiety attached to these sulfonamides of types **A(4–28)–B(4–28)** generally led to an increase of the CA inhibitory properties for the obtained compound, as compared to the corresponding parent sulfonamide **4–28**. Particularly strong inhibitors were those derived from heterocyclic derivatives (1,3,4-thiadiazoles; 1,3,4-thiadiazolines; and thienothiopyran-sulfonamide) of types **A,B(17–21)** and **(A,B)28**. Slightly less active were the derivatives of 4-aminoalkyl-benzene-sulfonamides **8** and **9**, of 3-fluorosulfanilamide **10**, of the 1,3-benzene-disulfonamides **14–16**, together with those of the pyrimidine-substituted sulfanilamide **27**, and the sulfanilyl-sulfanilamide- and sulfanilyl-metanilamide derivatives of types **22, 23, 25** and **26**. The simple aromatic derivatives (obtained from sulfonamides such as **4–7; 11–13**) as well as the orthanilamide derivative **24** were less active than the previously mentioned derivatives. Based on the dialkyl-dithiocarbamate from which they were obtained the dimethyl (**A** type) derivatives were less active than corresponding diethyl derivatives (**B** type compounds).

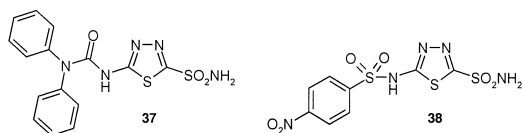
In vitro inhibition of growth of tumor cell lines

The antitumor activity of three of the newly reported CA inhibitors, **B6, B8** and **B9**, as well as two acetazolamide-like derivatives, **37** and **38** (for comparison), previously investigated by our group,⁴⁸ against a variety of

cancer cell lines has been assessed at the NIH National Cancer Institute. A large variety of cancer/cell line types were included in these assays, such as leukemia; non-small cell lung cancer; colon cancer; CNS cancer; melanoma; ovarian cancer; renal cancer; prostate and breast cancer among others (Table 3). The three new derivatives **B6, B8** and **B9** were chosen for detailed tumor cell growth inhibitory studies due to the fact that they act as relatively strong CA II inhibitors, and because they also possess a relatively balanced hydro- and liposolubility (data not shown) which might be important for their penetration through membranes, mitochondrial membranes, and so forth. This is the reason why **B20**, which is a very potent CA II inhibitor was not studied in depth, since this compound is very hydrophilic on one part, and poorly water soluble on the other one.

The following should be noted regarding the tumor cell growth inhibition data with the tested compounds **B6, B8** and **B9, 37** and **38**: (i) different cancer cell lines, of the same tumor type, possessed a very variable response to inhibition of growth in the presence of the new derivatives. For example, the MOLT-4 leukemia cells were very susceptible to inhibition by **B6** (GI₅₀ less than 10 nM), whereas other leukemia cell lines (such as RPMI-8226; SR, etc.) showed the same level of inhibition only at concentrations between 20 and 25 μ M of inhibitor. The same situation has been evidenced in the case of diverse non-small cell lung cancer cell lines, with **B9** acting as a very potent inhibitor (GI₅₀ = 75 nM) against the NCI-H522 line, whereas the related NCI-H226 line showed no measurable inhibition at concentrations as high as 100 μ M. Other cell lines of this tumor, such as HOP-62, had an intermediate behavior between the two extremes reported above (GI₅₀ = 62 μ M); (ii) all the investigated cancer lines were generally inhibited by one or the other sulfonamide tested, but some types of tumors, such as the leukemia, colon, renal or ovarian ones, were generally more susceptible to inhibition, whereas others, such as the CNS, melanoma, breast (except for the MCF7 line) or prostate cancer cell lines were slightly less susceptible; (iii) some of the tumors investigated here responded very well to inhibition with the new sulfonamides **B6, B8** and/or **B9**, with GI₅₀ values in the nanomolar range. These included: MOLT-4 leukemia with **B6**; HL-60 leukemia with **B9**; SR-leukemia with **B8** and especially **B9**; NCI-H522 non-small cell lung cancer with **B8** and **B9**; IGROV1 ovarian cancer with **B9**; as well as MCF7 breast cancer with **B6** (Table 3). The largest majority of susceptible tumors were generally inhibited by micromolar concentrations of the test compounds, with GI₅₀ values in the range of 7–40 μ M; (iv) no important differences of activity between the three investigated diethyldithiocarbamyl-sulfenyl-sulfonamides **B6, B8** and **B9**, were detected, in the sense that if a certain tumor type was susceptible to inhibition by one of the investigated sulfonamide, it generally responded similarly to its congeners too (for instance the strong inhibition of growth of NCI-H522 non-small cell lung cancers or SR-leukemia by both **B8** and **B9**). Generally, the most active compounds were **B8** and **B9**, followed by **B6**, but many

exceptions from this rule were observed (for example, in the case of SW-620; SK-MEL-28; SK-MEL-5; IGROV1; OVCAR-4 or BT-549 lines). On the contrary, this new family of sulfonamides (including **B6**, **B8** and **B9**) showed a much more potent antitumor action as compared to the acetazolamide-like derivatives **37** and **38** (or acetazolamide itself, which is inhibitory only in millimolar concentrations).⁴⁸ As seen from the data of Table 3, in contrast to the new sulfonamides reported in this paper, the acetazolamide-like derivatives are active only in the micromolar range, with GI_{50} values typically in the 15–70 μ M; (v) the inhibition of growth of tumor cells was dose-dependent of the concentration of sulfonamide inhibitor used in the experiments (data not shown), with growth inhibition increasing at increasing sulfonamide concentrations.



One should also note that some of the sulfonamides investigated here seem to be very much more potent tumor cell growth inhibitors than acetazolamide **1** methazolamide **2** or ethoxzolamide **3**, previously studied by Chegwiddden and Spencer,^{5a,b,24} (although the cell lines and methods used to assay the inhibition of growth of this and the above-cited²⁴ study are quite different) as well as than the previously mentioned acetazolamide-like compounds **37** and **38**. Some preliminary data with the new derivatives **B6**, **B8** and **B9**, with a hollow fiber assay⁴⁸ also showed them to be active against various tumor types in vivo (data not shown, and C. T. Supuran et al., in preparation).

The mechanism of tumor growth inhibition with these sulfonamides (**B6**, **B8** and **B9**) is not known at present, but several hypothesis may be proposed. Thus, as suggested by Chegwiddden and Spencer,^{5a,b,24} these compounds, similarly to the classical inhibitors acetazolamide **1**, methazolamide **2** or ethoxzolamide **3**, may reduce the provision of bicarbonate for the synthesis of nucleotides (mediated by carbamoyl phosphate synthetase II) and other cell components such as membrane lipids (mediated by pyruvate carboxylase). Such a mechanism would be likely to involve CA II and CA V. An alternative, or additional mechanism, may involve the acidification of intracellular milieu as a consequence of CA inhibition by these potent CA inhibitors, as shown by Teicher et al.²³ on the enhanced anticancer activity of different drugs in combination with acetazolamide **1**. It is also possible that the sulfonamides reported here interfere with the activity of the CA isozymes known to be present preponderantly in tumor cells, CA IX,⁸ XII.¹¹ Finally, a mechanism based on uncoupling of mitochondria,⁴⁵ as for the relatively similar diarylsulfonyleureas mentioned above, should not be excluded. (Although our new inhibitors have not yet been tested against the mitochondrial isozyme, CA V, potent inhibitors of CA II and IV generally possess high affinity for this isozyme.⁴⁹) A similar mechanism of action with E

7070 (**35**) is also possible for the compounds reported here,^{41–43} although the exact mechanism of tumor inhibition with this compound in advanced clinical studies is not clear at the moment, and it might be that E 7070 interacts with some CA isozymes present in tumor cells. A combination of several of the mechanisms proposed above is also possible. Since an entire range of structurally diverse, strong (aromatic/heterocyclic) sulfonamide CA inhibitors (including the starting amines from which the new inhibitors were prepared) tested in the same assays for tumor growth inhibitory properties, only gave modest results (data not shown, from our and NCI laboratories), we consider that the effective in vitro anticancer properties reported here constitute a feature of this new class of sulfonamides. Work is in progress in our laboratories to assess the CA inhibitory and antitumor properties of some other structurally-related sulfonamides of the type reported in the present paper, as well as to elucidate their mechanism of antitumor action.

Conclusions

We report here a general approach for the preparation of *N,N*-dialkylthio-carbamylsulfenamido-sulfonamides with high affinity for the cytosolic isozymes CA I and CA II, as well as for the membrane-bound CA IV. They were obtained by attaching *N,N*-dialkylthiocarbonyl-sulfenamido moieties to the molecules of aromatic/heterocyclic sulfonamides incorporating free amino, imino, or hydrazino groups in their molecule. Ring systems which have been derivatized by the above mentioned procedures included: 2-; 3- or 4-amino-benzene-sulfonamides and their derivatives; 4-(ω -aminoalkyl)-benzenesulfonamides; 3-halogeno-substituted-sulfanilamides; 1,3-benzene-disulfonamides; 1,3,4-thiadiazole-2-sulfonamides; and 4-methyl- δ^2 -1,3,4-thiadiazoline-2-sulfonamides among others, and were chosen in such a way as to prove that the proposed approach is a general one. In vitro inhibition of tumor cell growth experiments in a variety of cancer cell lines, showed some of the compounds reported here to act as powerful inhibitors of growth (in the 10–75 nM range in some cases) and make them attractive candidates for the development of novel anticancer agents. Some hypothesis regarding the mechanism of antitumor activity of the new derivatives are also presented, especially taking into account the structural resemblance between the compounds reported here, and an antitumor sulfonamide (E 7070) in advanced clinical trials in Europe.

Experimental

General

Melting points: heating plate microscope (not corrected); IR spectra: KBr pellets, 400–4000 cm^{-1} Perkin-Elmer 16PC FTIR spectrometer; ¹H NMR spectra: Varian 300CXP apparatus (chemical shifts are expressed as δ values relative to Me₄Si as standard); elemental analysis: Carlo Erba Instrument CHNS Elemental

Analyzer, Model 1106. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm pre-coated silica gel plates (E. Merck). Analytical HPLC was performed on a reversed-phase C₁₈ Bondapack column, with a Beckman EM-1760 instrument. Sulfonamides **4–28** used in synthesis were either commercially available compounds (from Sigma, Acros or Aldrich) or were prepared as described previously: 4-hydrazinobenzenesulfonamide **7** by diazotization of sulfanilamide followed by reduction of the diazonium salt with tin(II) chloride;⁵⁰ halogenosulfanilamides **10–13** by halogenation of sulfanilamide as reported in the literature;⁵¹ compound **19** from 5-amino-1,3,4-thiadiazole-2-sulfonamide (obtained from acetazolamide)⁵² by acylation with the phthalimido-derivative of β -alanine, followed by hydrazinolysis,⁵³ whereas imine **18** by deprotection of methazolamide with concentrated hydrochloric acid.⁵¹ Aminobenzolamide **20** and the corresponding thiadiazoline **21** as reported in ref 54a,b whereas the sulfanilyl-sulfanilamides/metanilamides/orthanilamides **22–26** by a method similar to that reported in ref 54c. Dorzolamide **28** was obtained as described in the literature.⁵⁵ Sodium/potassium *N,N*-dimethyl- or *N,N*-diethyldithiocarbamates were from E. Merck, whereas iodine and sodium hypochlorite were from Fluka, and were used without further purification. Solvents (E. Merck) used in these experiments were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions.

General procedure for the preparation of compounds (A,B)4-28

Method A. Sulfonamide (10 mmol) **4–28** was treated with 100 mL of a solution containing 0.8 g (20 mmol) sodium hydroxide (KOH could also be used). After dissolution of the sulfonamide, into the magnetically stirred reaction mixture were slowly (1–2 h) and concomitantly dropped the following two aqueous solutions: (i) 50 mL of a solution containing 10 mmol of sodium/potassium *N,N*-dialkyl-dithiocarbamate **A, B**; and (ii) 50 mL of NaClO solution, containing the stoichiometric amount (10 mmol) of oxidizing agent. The temperature was maintained in the range of 25–30 °C (generally cooling of the reaction mixture had to be done), with strong magnetic stirring, for 1–3 h (TLC control; the thiocarbamylsulfenamide and the thiurams **33** started to precipitate immediately after the addition of the oxidizing agent). A double-2.5-fold amount of initial sulfonamide could be used in the synthesis (for relatively inexpensive raw materials, such as **6–9**, for example), case in which the yields in thiocarbamylsulfenamides were increased. In order to purify the obtained thiocarbamyl-sulfenamides (**A,B**)4-28, the obtained raw precipitate was filtered and treated with an excess (100–150 mL) of a 0.03–0.05 M NaOH (or KOH) and magnetically stirred for 30 min at 4 °C. The insoluble thiuram was filtered and the sodium/potassium salts of the thiocarbamyl-sulfenamides acidified with a 10% HCl solution (till pH 5.5), when the pure (**A,B**)4-28 precipitated. They were then filtered, thoroughly washed with water and air dried. Yields were generally in the range of 25–40%, but for some derivatives they

were much higher (around 70% for **A6** and **B6**; around 75–80% for **A8, B8, A9** and **B9**), whereas for others definitely lower (6% for **A7**; around 15% for the derivatives obtained from **17, 18** and **27**, and around 10% for the dorzolamide derivatives **A,B28**).

Method B. Sulfonamide (10 mmol) **4–28** was treated with 100 mL of a solution containing 20 mmol of sodium/potassium hydroxide. After dissolution of the sulfonamide, into the magnetically stirred reaction mixture were slowly (1 h) and concomitantly dropped the following two aqueous solutions: (i) 50 mL of a solution containing 10 mmol of sodium/potassium *N,N*-dialkyl-dithiocarbamate **A, B**; and (ii) 100 mL of iodine-potassium iodide (KI₃) solution, containing the required amount (10 mmol) of oxidizing agent. The temperature was maintained at 0–4 °C by external cooling in an ice bath, with strong magnetic stirring, for 1–3 h (TLC control of the reaction product formation). The reaction mixture was then worked up as described above for method A. Yields were in this case higher (generally of around 30–55%, but some exceptions of the type mentioned above were also registered).

4-(*N,N*-Dimethylaminothiocarbamylsulfenamido)-benzenesulfonamide A6. Tan crystals, mp > 300 °C (dec.); IR (KBr), cm⁻¹: 1150 (SO₂^{sym}), 1310 (thioamide III), 1347 (SO₂^{as}), 1540 (thioamide II), 1740 (thioamide I), 3360 (NH, NH₂); ¹H NMR (DMSO-*d*₆), δ , ppm: 3.38 (s, 6H, NMe₂); 6.45 (s, 1H, SNH); δ_A 7.15, δ_B 7.73 (AA'/BB' system, 4H, *J*_{AB} = 7.9, ArH from 4-sulfamoylphenyl); 7.51 (br s, 2H, SO₂NH₂); ¹³C NMR (DMSO-*d*₆), δ , ppm: 34.9 (s, Me₂N); 129.9; 132.5; 135.9; 142.5; 176.5 (CSSNH). Anal. found: C, 37.21; H, 4.28; N, 14.07%; C₉H₁₃N₃O₂S₃ requires: C, 37.10; H, 4.50; N, 14.42%.

5-(*N,N*-Dimethylaminothiocarbamylsulfenamido)-1,3,4-thiadiazol-2-sulfonamide A17. Tan crystals, mp > 300 °C; IR (KBr), cm⁻¹: 1176 (SO₂^{sym}), 1315 (thioamide III), 1367 (SO₂^{as}), 1545 (thioamide II), 1750 (thioamide I), 3060 (NH), 3365 (NH₂); ¹H NMR (DMSO-*d*₆), δ , ppm: 3.41 (s, 6H, NMe₂); 6.89 (s, 1H, SNH); 7.22 (br s, 2H, SO₂NH₂); ¹³C NMR (DMSO-*d*₆), δ , ppm: 35.6 (s, Me₂N); 158.7 (C-2 of thiadiazole); 170.2 (C-5 of thiadiazole); 177.3 (CSSNH). Anal. found: C, 19.89; H, 3.01; N, 23.30%; C₅H₉N₅O₂S₄ requires: C, 20.06; H, 3.03; N, 23.39%.

4-(*N,N*-Diethylaminothiocarbamylsulfenamidoethyl)-benzenesulfonamide B8. Tan crystals, mp 279–81 °C; IR (KBr), cm⁻¹: 1154 (SO₂^{sym}), 1315 (thioamide III), 1357 (SO₂^{as}), 1543 (thioamide II), 1748 (thioamide I), 3360 (NH, NH₂); ¹H NMR (DMSO-*d*₆), δ , ppm: 2.72 (t, 6H, ³*J*_{HH} = 6.4, 2 Me from NEt₂); 3.03 (t, 2H, ³*J*_{HH} = 6.7, α CH₂ of aminoethylbenzenesulfonamide); 3.78 (t, 2H, ³*J*_{HH} = 6.7, β CH₂ of aminoethylbenzenesulfonamide); 3.84 (m, 4H, 2 CH₂ of Et₂N); 6.56 (s, 1H, SNH); δ_A 7.19, δ_B 7.77 (AA'/BB' system, 4H, *J*_{AB} = 8.1, ArH from 4-sulfamoylphenyl); 7.54 (br s, 2H, SO₂NH₂); ¹³C NMR (DMSO-*d*₆), δ , ppm: 25.6 (s, CH₃ of Et₂N); 30.5 (s, CH₂ of aminoethylbenzenesulfonamide); 37.4 (s, CH₂ of aminoethylbenzenesulfonamide); 43.1 (s, CH₂ of Et₂N);

130.4; 132.6; 134.0; 145.9; 175.8 (CSSNH). Anal. found: C, 44.54; H, 6.25; N, 11.83%; $C_{13}H_{21}N_3O_2S_3$ requires: C, 44.93; H, 6.09; N, 12.09%.

5,6-Dihydro-4-N-ethyl,N-(N,N-Diethylaminothiocarbamyl-sulfonyl)-6-methyl-4H-thieno-[2,3-b] thiopyran-2-sulfonamide 7,7-dioxide B28. White crystals, mp 296–297 °C (dec.); IR (KBr), cm^{-1} : 1138 (SO_2^{sym}), 1312 (thioamide III), 1347 (SO_2^{as}), 1552 (thioamide II), 1760 (thioamide I), 3360 (NH_2); 1H NMR (DMSO- d_6), δ , ppm: 1.30 (d, 3H, Me of dorzolamide); 1.36 (t, 3H, Me from the ethyl of dorzolamide); 2.55 (m, 1H, CH); 2.75 (t, 6H, $^3J_{HH}$ = 6.3, 2 Me from NEt_2); 2.81 (m, 1H, CH); 3.05–3.20 (m, 2H, CH_2 from N–Et of dorzolamide); 3.87 (m, 4H, 2 CH_2 of Et_2N); 4.37 (m, 2H, dorzolamide CH_2); 8.03 (s, 1H, CH, ArH from thienyl); 8.21 (br s, 2H, SO_2NH_2); ^{13}C NMR (DMSO- d_6), δ , ppm: 10.2; 11.8; 25.3 (s, CH_3 of Et_2N); 30.5; 40.6; 43.7 (s, CH_2 of Et_2N); 49.5; 51.6; 130.7; 137.4; 141.9; 149.6; 175.1 (CSSNH). Anal. found: C, 38.10; H, 5.52; N, 8.65%; $C_{15}H_{25}N_3O_4S_5$ requires: C, 38.19; H, 5.34; N, 8.91%.

Enzyme preparations

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog et al.⁵⁶ (the two plasmids were a gift from Professor Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group,⁵⁷ and enzymes were purified by affinity chromatography according to the method of Khalifah et al.⁵⁸ Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of $49\text{ mM}^{-1}\text{ cm}^{-1}$ for HCA I and $54\text{ mM}^{-1}\text{ cm}^{-1}$ for HCA II, respectively, based on M_r = 28.85 kDa for hCA I, and 29.30 kDa for hCA II, respectively.^{59,60} bCA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration was determined by titration with ethoxzolamide.⁶¹

Initial rates of 4-nitrophenyl acetate hydrolysis catalysed by different CA isozymes were monitored, at 400 nm, with a Cary 3 spectrophotometer interfaced with an IBM compatible PC.³² Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25 °C. A molar absorption coefficient ϵ of $18,400\text{ M}^{-1}\text{ cm}^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature.³² Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of inhibitor (1 mM) were prepared in DMSO and dilutions up to 0.01 nM were done thereafter with distilled-deionized water (DMSO is not inhibitory at these concentrations). Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constant K_I was determined as described by Pocker and Stone.³²

Enzyme concentrations were 3.6 nM for hCA II, 9.5 nM for hCA I and 30 nM for bCA IV.

Inhibition of tumor cell growth

Stock solutions of inhibitor (1 mM) were prepared in DMSO, and dilutions up to 10 nM prepared with distilled deionized water. The percentage growth (PG) of the cell lines in the presence of five concentrations (10^{-8} – 10^{-4} M) of inhibitor was calculated according to one of the following two expressions (1) or (2):⁴⁸

$$PG = 100 \times (\text{Mean OD}_{\text{test}} - \text{Mean OD}_0) / (\text{Mean OD}_{\text{ctrl}} - \text{Mean OD}_0),$$

when $(\text{Mean OD}_{\text{test}} - \text{Mean OD}_0) \geq 0$, (1)

$$PG = 100 \times (\text{Mean OD}_{\text{test}} - \text{Mean OD}_0) / \text{Mean OD}_0,$$

when $(\text{Mean OD}_{\text{test}} - \text{Mean OD}_0) < 0$, (2)

where: Mean OD_0 = the average optical density measurements of sulforhodamine B (SRB)-derived color just before exposure of cells to the test compounds; Mean OD_{test} = the average optical density measurements of SRB-derived color after 48 h exposure of cells to the test compounds; Mean OD_{ctrl} = the average optical density measurements of SRB-derived color after 48 h with no exposure of cells to the test compounds. GI_{50} represents the molarity of inhibitor producing a 50% inhibition of growth of the tumor cells after 48 h exposure to variable concentrations (10^{-4} – 10^{-8} M) of the test compound, measured as outlined before, and this parameter was obtained by interpolation. GI_{50} is in fact the molarity of inhibitor at which $PG = 50\%$.⁴⁸ The standard sulforhodamine B (SRB) protein assay has been used to estimate cell viability or growth.⁴⁸

Added note

Since this research has been terminated, Sly's group⁶² reported that acetazolamide **1**, at concentrations of 10 μM , strongly reduces in vitro the invasiveness of some renal cancer cell lines (such as CAKI-1, CAKI-2; ACHN and A-498), some of which have been also investigated by us here with compounds **B6–B9**. These authors stated that the observed effect is presumably due to the inhibition of isozymes CA II and CA XII which have been evidenced in three of the four tumor cell lines mentioned above. It is thus quite probable that the research line emphasized in this paper would lead to a better understanding of the relationship between sulfonamide CA inhibitors and cancer. The entire class of antitumor sulfonamides was recently reviewed by Owa and Nagasu.⁶³

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