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Letter to the editor

Carbonic anhydrase inhibitors - Part 94. 1,3,4-Thiadiazole-2-sulfonamide derivatives as antitumor agents?[#]

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Abstract – Potent sulfonamide inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), derivatives of 1,3,4-thiadiazole-2-sulfonamide, possessing inhibition constants in the range of 10^{-8}–10^{-9} M against isozymes II and IV, were shown to act as efficient in vitro tumour cell growth inhibitors with GI_{50} (molarity of inhibitor producing a 50% inhibition of tumour cell growth) values typically in the range of 0.1–30 μM against several leukaemia, non-small cell lung cancer, ovarian, melanoma, colon, CNS, renal, prostate and breast cancer cell lines. The mechanism of antitumour action with the new sulfonamides reported here is unknown, but it might involve either inhibition of several CA isozymes (such as CA IX, CA XII, CA XIV) present predominantly in tumour cell membranes, acidification of the intracellular environment as a consequence of CA inhibition, uncoupling of mitochondria and/or strong CA V inhibition, or a combination of several such mechanisms. Such derivatives might lead to the development of effective novel types of anticancer agents/therapies. © 2000 Éditions scientifiques et médicales Elsevier SAS

sulfonamide / carbonic anhydrase / 1,3,4-thiadiazole-2-sulfonamide / tumour growth inhibition

1. Introduction

A number of 14 different carbonic anhydrase (CA, EC 4.2.1.1) isozymes were described up to now in higher vertebrates, including humans. These abundant zinc enzymes are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH homeostasis, electrolyte secretion in a variety of tissues/organs, or biosynthetic reactions, such as lipogenesis, gluconeogenesis and ureagenesis among others [2]. Some of these isozymes are cytosolic (such as CA I, CA II, CA III, CA VII), others are membrane-bound (CA IV, CA IX, CA XII and CA XIV), CA V is present only in mitochondria, CA VI is secreted in saliva, whereas several acatalytic forms have also been isolated in recent years (CA VIII, CA X and CA XI) [2–7].

Recently, three novel membrane-bound CA isozymes, CA IX [2], XII [6] and XIV [7], in addition to the 'classical' one, CA IV, previously reported [2] have been isolated and characterized. Some of them were identified predominantly in tumour cells, and little is known for the moment regarding the physiological consequences of their inhibition/activation [6, 7]. Although inhibition of CAs by aromatic/heterocyclic sulfonamides has been exploited clinically for more than 45 years in the treatment of a variety of diseases such as glaucoma, epilepsy congestive heart failure, mountain sickness, gastric and duodenal ulcers or as diuretic agents [8], their potential use as antitumour drugs has been little explored up to now. Several classical clinical agents from this class include acetazolamide **1**, methazolamide **2** or ethoxzolamide **3** [8] (*figure 1*). In an important work, Teicher et al. [9] reported that one of these derivatives, acetazolamide **1**, which is a well-studied, potent inhibitor of several CA isozymes (CA II, CA IV, CA V and CA VII among others) [8] might also function as a modulator of anticancer therapies in combination with different cytotoxic agents, such as alkylating agents; nucleoside analogues; platinum derivatives, etc. These authors hypothesized that the anticancer effects of acetazolamide (alone or in combination with other drugs) might be due to the acidification of the intra-tumoural environment ensued after CA inhi-

[#] See reference [1]

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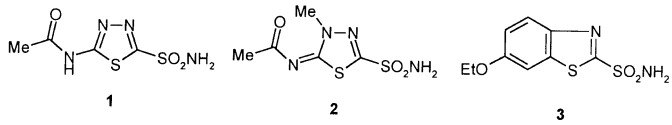


Figure 1. Structures of acetazolamide **1**, methazolamide **2** and ethoxzolamide **3**.

bition, although other mechanisms of action of this drug were not excluded. Our and Puscas' groups [10] also showed earlier that by modulating the CA activity (by means of inhibitors or activators of this enzyme) the pH of the tumour environment can be changed, which may favourably influence the anticancer effect of the drug per se (i.e. the sulfonamide CA inhibitor) or that of another anticancer agent used concomitantly with the CA inhibitor/activator. Chegwidden and Spencer [11] then showed that two other potent, clinically used sulfonamide CA inhibitors, methazolamide **2** and ethoxzolamide **3** (in concentrations of 0.4–1 mM for methazolamide and around 10 μM for **3**) inhibited in vitro in cell cultures, the growth of human lymphoma cells, showing that this is probably due to a reduced provision of bicarbonate for nucleotide synthesis (HCO_3^- is the substrate of carbamoyl phosphate synthetase II) as a consequence of CA inhibition [11]. Since different isozymes such as CA I, II and IV were recently shown to be present and probably involved in other types of proliferative conditions, such as von Hippel-Lindau tumours [8], progressive polycystic kidney disease, acinar-ductal carcinomas of the pancreas or autoimmune/idiopathic chronic pancreatitis [8], it appeared of great interest to further explore the connections between CAs and tumours. The development of specific inhibitors for some of the isozymes presumably involved in these processes would be highly beneficial for both obtaining novel types of drugs/therapies, as well as for a better understanding of the physiology of the CAs.

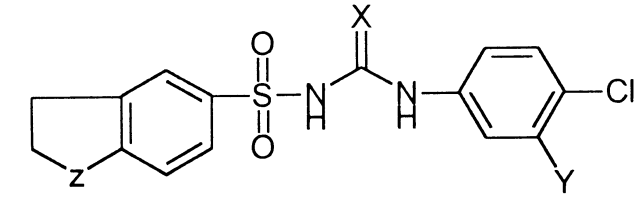
Here we report such a study, showing that several 1,3,4-thiadiazole-2-sulfonamide derivatives possessing potent CA inhibitory properties (typically in the low nanomolar range) also act as effective in vitro tumour cell growth inhibitors of different leukaemia, non-small cell lung cancer, melanoma, ovarian, renal, prostate and breast cancer cell lines. As far as we know, this is one of the first systematic studies reporting sulfonamide CA inhibitors with such a high potency as in vitro tumour cell growth inhibitors, against such a variety of tumour cell types/lines.

2. Results and discussion

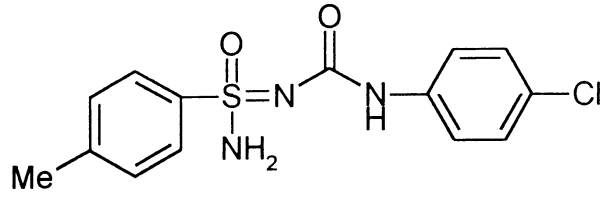
Although a very large number of aromatic/heterocyclic sulfonamide CA inhibitors were synthesized in the last 45 years in the search for different types of drugs [12–14], derivatives with potential use as antitumour agents from this class of pharmacological agents were not reported up to now. Still, several non-CA inhibitor sulfonamide derivatives were recently investigated for their anticancer properties. Thus, some arylsulfonyl-ureas/hydroxyguanidines or sulfonimideamides of types **4**–**8** have been reported by researchers from Eli Lilly [15] and by Chern et al. [16], whereas Medina et al. [17] reported N-substituted polyhalogeno-benzenesulfonamides of type **9** (*figure 2*), which strongly inhibited the growth of multidrug-resistant MCF-7/ADR cancer cells in vitro. We stress again that sulfonamides **4**–**9** do not act as CA inhibitors, being substituted at the sulfonamide moiety by bulky groups, which impair their binding to the Zn(II) ion of this enzyme, and thus its inhibition [8]. It is assumed that the cytotoxicity of derivatives **4**–**8** might be a consequence of the uncoupling of mitochondria [18], where high concentrations of a CA V are also present [8]. Still, up to now no studies have been performed in order to investigate whether such anticancer compounds interfere with CA activity, although in a previous work from this laboratory [19], we hypothesized as very probable a strong in vivo CA V inhibition (due to the hydrolysis of the cytotoxic agent, leading to the formation of unsubstituted sulfonamides among others). The polyhalogeno-benzenesulfonamides **9** on the other hand, exert their cytotoxic effects due to the irreversible binding to an amino acid residue (Cys 239) of β-tubulin, resulting thus in the disruption of cellular microtubules [17].

In this paper we report the observation that several potent CA sulfonamide inhibitors, incorporating 1,3,4-thiadiazole-2-sulfonamide moieties in their molecule, of the type **10–16** (*figure 3*), also show strong in vitro antitumour properties, against a large variety of tumour types and cell lines.

Inhibition data against three CA isozymes, hCA I, hCA II and bCA IV with the derivatives **10–16** (*table I*) prove that they all act as very powerful CA II and CA IV inhibitors, whereas their efficiency towards CA I is somehow reduced, in agreement with literature data for similar sulfonamide derivatives [8]. All these compounds practically inhibit, in the nanomolar range, the physiologically relevant isozymes CA II and CA IV, and they act as slightly worse CA I inhibitors. A notable exception from this behaviour is constituted by the urea/thiobiguanide derivatives **14–16**, which act as very powerful CA I inhibitors, with affinities of the same order of



4: X = O, Y = H, Z = CH_2 - sulofenur
5: X = O, Y = Cl, Z = O - LY295501
6: X = S, Y = H, Z = CH_2



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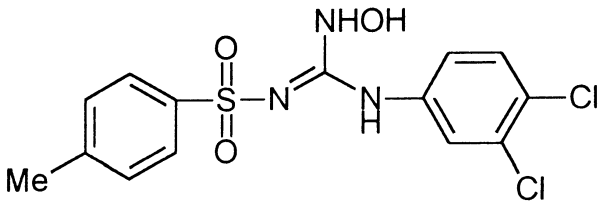

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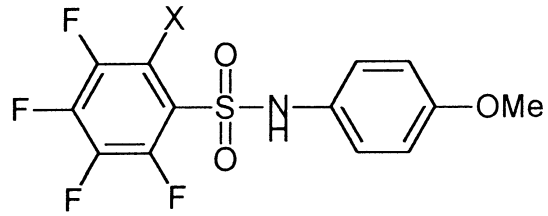
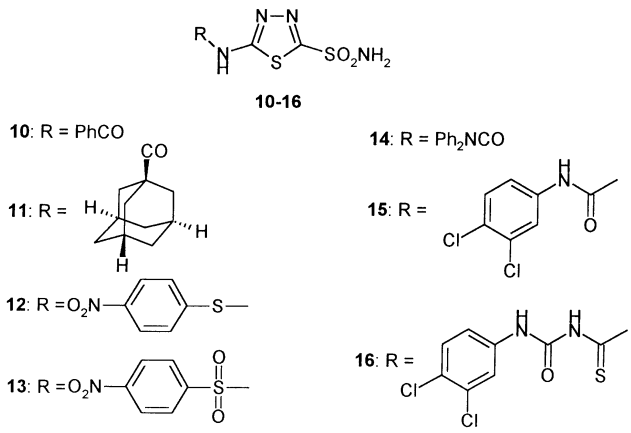


9: X = H, F, Br

Figure 2. Structure of the anticancer, non-CA inhibitory sulfonamides **4–9**.

magnitude (in the nanomolar range) for CA I and CA II, and (slightly lower for) CA IV. A typical sulfonamide CA inhibitor (such as acetazolamide **1** or methazolamide **2**) generally shows a 50–75 times lower affinity for CA I as compared to CA II (ethoxzolamide **3** is an exception from this rule), and many aromatic/heterocyclic derivatives



10-16

10: R = PhCO
11: R =
12: R = O_2N—S—
13: R = O_2N—S(=O)(=O)—
14: R = Ph_2NCO
15: R =
16: R =

Figure 3. Structure of the 1,3,4-thiadiazole-2-sulfonamide derivatives **10–16** investigated in the present study for their antitumour properties.

show the same type of activity [8, 12–14, 19–23]. In fact, in a previous communication [23] we showed that this type of derivative might lead to the development of isozyme I-specific CA inhibitors, a difficult goal considering the high avidity for sulfonamides of isozyme CA II. Interestingly, the unique new derivative reported here, **14**, which is again a urea derivative, showed the same behaviour mentioned above.

The antitumour activity of the sulfonamide CA inhibitors **10–16**, against a variety of cancer cell lines has been assayed at the NIH National Cancer Institute, Bethesda, MD, USA, with the samples furnished from this laboratory. A large variety of cancer/cell line types were included in these assays, such as leukaemia; non-small cell lung cancer; colon cancer; CNS cancer; melanoma; ovarian cancer; renal cancer; prostate and breast cancer among others (*table II*).

The following should be noted regarding the tumour cell growth inhibition data with the test compounds **10–16**: i) different cancer cell lines, of the same tumour type, possessed a very variable response to inhibition of growth in the presence of the new derivatives. For example, the OVCAR-4 ovarian cancer cells were very susceptible to inhibition by **10** and **11** (GI_{50} in the range of 100–500 nM), whereas other ovarian cancer cell lines (such as IGROV-1; OVCAR-5, OVCAR-8) showed in-

Table I. CA inhibition data with standard inhibitors **1–3**, and the sulfonamides **10–16** investigated in the present study, against isozymes I, II and IV.

Inhibitor	K_I* (nM)		
	hCA I[a]	hCA II[a]	bCA IV[b]
Acetazolamide, **1**	900	12	220
Methazolamide, **2**	780	14	245
Ethoxzolamide, **3**	25	8	13
10	900	15	100
11	850	10	65
12	9	0.5	10
13	2	0.1	3
14	7	8	15
15	3	6	8
16	3	6	28

* Standard error for the determination of K_Is was of 10–15% (from three different assays); [a]human (cloned) isozyme; [b]isolated from bovine lung microsomes.

significant levels of inhibition at concentrations as high as 100 μM of inhibitor; ii) all the investigated cancer lines were generally inhibited by one or the other sulfonamide tested, typically with GI_{50} values in the range of 12–30 μM, but some of the tumours investigated here responded very well to inhibition with these new sulfonamides. These included: OVCAR-4 with **10** and **11**; NCI-H522 non-small cell lung cancer with **15** and **16**; COLO-205 colon cancer with **15**; LOX IMVI melanoma with **14**–**16**, etc. (*table II*); iii) important differences of activity between the investigated sulfonamides were detected. Thus, sulfonamides **10–13** were generally less active than derivatives **14–16**, with the important exception of the OVCAR-4 tumour cells, for which the best inhibitors were just two compounds of the first group, i.e. **10** and **11**. The inhibitors of the second group, i.e. **14–16**, showed a much more homogenous behaviour as compared to those of the first group (**10–13**), in the sense that generally they inhibited all the investigated tumours with GI_{50} values in the range of 12–70 μM, whereas the first derivatives showed good activity only against a very limited number of cell lines. For example **10** was active only against two tumour types/cell lines, whereas **16** inhibited all the 52 different cell lines investigated here. Among the 'active' derivatives of the second subseries, i.e. **14–16**, generally best inhibition was exhibited by **15**, followed by **16** and then **14**, but many exceptions from this rule were observed; iv) the inhibition of growth of tumour 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 note that from the chemical point of view, the tumour cell growth inhibitors of the second subtype (**14–16**) are all urea derivatives, a structural feature which is absent in the derivatives of the first subgroup (**10–13**), although all these compounds are obviously 1,3,4-thiadiazole-2-sulfonamide derivatives.

One must also note that the sulfonamides investigated here are much more potent tumour cell growth inhibitors as compared to methazolamide **2** or ethoxzolamide **3**, previously investigated on a single cell line [11]. Some preliminary data with several of these new derivatives in a hollow fibre assay [24] of transplanted tumours showed these sulfonamides to be active in vivo against various tumour types (data not shown and unpublished data).

The mechanism of tumour growth inhibition with these sulfonamides is unknown for the moment, but several hypotheses may be made regarding this. Thus, as suggested by Chegwidden and Spencer [11], these compounds, similarly to the classical inhibitors methazolamide **2** or ethoxzolamide **3**, might reduce the provision of bicarbonate needed for the nucleotide synthesis mediated by carbamoyl phosphate synthetase II. An alternative, more probable mechanism, might contemplate the acidification of the intracellular environment as a consequence of CA inhibition by these potent CA inhibitors, as shown in the seminal work of Teicher et al. [9] 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 present preponderantly in tumour cells, such as CA IX, XII and XIV, but this hypothesis is not easily verifiable at the present time, since clones of these new isozymes are not easily available. Finally, a mechanism based on uncoupling of mitochondria [15] as for the relatively similar diarylsulfonylureas mentioned above should not be excluded, especially considering the fact

Table II. In vitro tumour cell growth inhibition data with the sulfonamide CA inhibitors **10**–**16**, against different tumours/cell lines.

Tumour	cell line	GI_{50} (μM)*						
		10	**11**	**12**	**13**	**14**	**15**	**16**
Leukaemia	K–562	–	–	–	33	33	31	30
	MOLT–4	> 100	> 100	40	26	21	32	29
	RPMI–8226	> 100	89	49	21	17	17.5	26
	SR	> 100	> 100	59	32	–	22	–
	CCRF–CEM	–	–	–	–	20	14	28
	HL–60(TB)	–	–	–	–	16	29	27
Non-small cell lung cancer	A549/ATCC	> 100	> 100	> 100	> 100	26	31	32
	HOP–92	65	> 100	> 100	> 100	19	16	13.5
	NCI–H460	> 100	60	> 100	> 100	–	19	–
	NCI–H322M	> 100	16	> 100	> 100	23	20	22
	NCI–H522	> 100	68	23	29	16	12	15
	EKVX	–	–	–	–	25	29	26
	HOP–62	–	–	–	–	27	18	22
	NCI–H226	–	–	–	–	41	23	24
	NCI–H23	–	–	–	–	19	18	18
Colon cancer	COLO–205	> 100	> 100	> 100	> 100	38	14	19.5
	HCT–15	> 100	> 100	> 100	> 100	22	21	19
	HT29	> 100	> 100	> 100	> 100	27	17	25.5
	SW–620	> 100	> 100	> 100	> 100	30	28	22.5
	HCT–116	–	–	–	–	29	19.5	17
	KM12	–	–	–	–	21	27	18.5
CNS cancer	SF–268	> 100	55	> 100	> 100	30	25	27.5
	SNB–19	> 100	> 100	> 100	> 100	24	21	28
	SNB–75	> 100	> 100	> 100	> 100	18	–	17
	U251	> 100	> 100	> 100	> 100	43	29	36
	SF–295	–	–	–	–	34	31	21
	SF–539	–	–	–	–	17	19	24
Melanoma	LOX IMVI	> 100	> 100	> 100	30	16	18	17
	MALME–3M	> 100	> 100	> 100	> 100	21	17.5	23
	M14	> 100	> 100	> 100	> 100	15	17	18
	SK–MEL–2	> 100	> 100	> 100	> 100	31	25	32
	SK–MEL–28	> 100	> 100	> 100	> 100	39	17	37
	SK–MEL–5	> 100	95	> 100	69	22	16	21
	UACC–62	–	–	–	–	22	31	17.5
Ovarian cancer	IGROV1	> 100	–	–	> 100	17	–	17
	OVCAR–4	0.5	0.1	48	> 100	28	30	19.5
	OVCAR–5	> 100	> 100	> 100	> 100	25	24.5	25
	OVCAR–8	> 100	> 100	> 100	> 100	20	20	22
	OVCAR–3	–	–	–	–	19	25	21
	SK–OV–3	–	–	–	–	59	20.5	20.5
Renal cancer	768–0	> 100	> 100	> 100	37	23	24	28
	ACHN	> 100	> 100	> 100	> 100	–	34	–
	CAKI–1	> 100	> 100	92	> 100	28	26	23.5
	RXF 393	> 100	61	> 100	> 100	18	16	19
	UO–31	> 100	> 100	> 100	> 100	24	27	20
	SN12C	–	–	–	–	16	19	27
Prostate cancer	PC–3	> 100	–	> 100	> 100	33	–	27
	DU–145	> 100	> 100	> 100	> 100	14	29	21
Breast cancer	MCF7	> 100	> 100	> 100	> 100	38	27	38
	MDA–MB–435	> 100	> 100	> 100	> 100	70	20	19
	MDA–N	> 100	> 100	> 100	> 100	25	20.5	21
	BT–549	> 100	82	> 100	> 100	16	20	21
	T–47D	> 100	90	> 100	35	–	19	–
	NCI/ADR–RES	–	–	–	–	18	21	25
	HS 578T	–	–	–	–	25	29	24
	MDA–MB–231/ATCC	–	–	–	–	–	26	30

* Molarity of inhibitor producing a 50% inhibition of growth of the tumour 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).

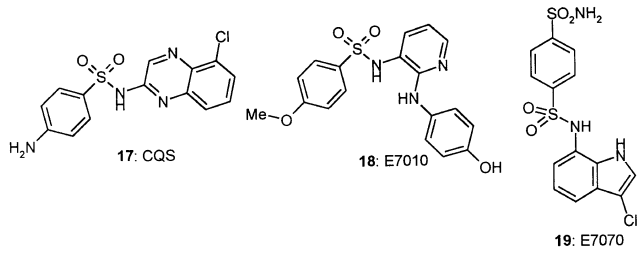


Figure 4. Structure of the antitumour sulfonamide derivatives **17–19**.

that three of our derivatives (**14–16**) possess di/tri-substituted urea moieties in their molecule. A combination of several of the mechanisms proposed above is also plausible.

One of the reviewers of this paper comented to us that three other antitumour sulfonamides have been investigated: CQS, 5-chloroquinoxaline-2-sulfanilamide **17** [25–27], E7010 **18** [28] and E7070 **19** [29, 30] (*figure 4*). The first two derivatives possess substituted sulfonamide moieties, and probably do not act as CA inhibitors. The mechanism of antitumour action of **18** has been studied in some detail [28], showing that this compound is a tubulin polymerisation inhibitor, binding at the colchicine site. On the other hand, the molecular targets of CQS and E7070 are still unclear. One must mention that the latter compound, possessing a free sulfonamide moiety probably acts as a strong CA inhibitor, thus being remarkably similar to the antitumour sulfonamides reported here. Work is in progress in our laboratory for assessing the mechanism of antitumour action, CA inhibitory and antitumour properties of some other structurally-related sulfonamides of the type reported in the present paper. This might provide a new insight into the research and development of a new class of antitumour drugs.

3. Experimental Protocols

3.1. Chemistry

Melting points: heating plate microscope (not corrected); IR spectra: KBr pellets, 400–4 000 cm^{-1} Perkin-Elmer 16PC FTIR spectrometer; ^{1}H-NMR spectra: Varian 300CXP apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard); Elemental analysis: Carlo Erba Instrument CHNS Elemental Analyzer, Model 1106. Reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm pre-coated silica gel plates (E. Merck). Sulfonamides **10–16** were previously reported by our group (except for one derivative which is new, and will be reported here for the first time) as follows: **10** in ref. [20]; **11** in ref. [21]; **12** in ref. [21]; **13** in ref. [22]; **15** and **16** in ref. [23]; **14** is new, and was prepared by previously reported procedures [13], as described below. Reagents used in the synthesis were commercially available (from Sigma-Aldrich, Fluka or E. Merck) and were used without further purification.

3.1.1. 5-(N,N-Diphenylcarbamoylamido)-1,3,4-thiadiazole-2-sulfonamide **14**

An amount of 180 mg (1 mmol) of 5-amino-1,3,4-thiadiazole-2-sulfonamide [20] was suspended in 50 mL of dry acetonitrile and the stoichiometric amounts of N,N-diphenyl carbamoyl chloride (232 mg) and triethylamine (108 μL) dissolved in the same solvent were added to the reaction mixture, which was stirred at 4 °C for 4–5 h (TLC control). The solvent was evaporated in vacuo, the residue taken up in 50 mL of cold water, brought to pH 5 with 5% citric acid, and the precipitated sulfonamide filtered and recrystallized from ethanol (59% yield). White crystals, m.p. 243–244 °C, IR (KBr), cm^{-1}: 1 162 (SO_2); 1 375 (SO_2); 1 610 (C=N); 1 670 (C=O), 3 060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.08–7.75 (m, 10H, ArH); 7.81 (s, 1H, CONH); 8.15 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 118.8; 119.9; 128.1; 130.3; 159.5 (C-2 of thiadiazole); 171.3 (C-5 of thiadiazole); 176.2 (CONH). Anal. found: C, 48.23; H, 3.36; N, 18.54%; $C_{15}H_{13}N_5O_3S_2$ requires: C, 47.99; H, 3.49; N, 18.65%.

3.2. Pharmacology

3.2.1. CA inhibition assay

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 previously [13] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described previously [13], and enzymes were purified by affinity chromatography [13]. 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 29.30 kDa for CA II, respectively [13]. CA IV was isolated from bovine lung microsomes as described previously [13].

Initial rates of 4-nitrophenyl acetate hydrolysis catalysed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC [13]. Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and $1 \times$

10^{-6} M, working at 25 °C. A molar absorption coefficient ε of 18 400 $M^{-1}.cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature [13]. 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 [15]). Inhibitor and enzyme solutions were pre-incubated 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 previously [13]. Enzyme concentrations were 3.5 nM for hCA II, 9.8 nM for hCA I and 25 nM for bCA IV (this isozyme has a decreased esterase activity [13] and higher concentrations had to be used for the measurements).

3.2.2. Inhibition of tumour cell growth assay

Stock solutions of inhibitor (1 mM) were prepared in DMSO, and dilutions up to 10 nM done with distilled deionized water. The percentage growth (PG) of the cells 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) [24]:

$$PG = 100 \times (\text{mean } OD_{test} - \text{mean} OD_0)/ \quad (1)$$

$$(\text{mean } OD_0), \text{ when } (\text{mean } OD_{ctrl} - \text{mean } OD_0) \geq 0,$$

$$PG = 100 \times (\text{mean } OD_{test} - \text{mean } OD_0)/ \quad (2)$$

$$\text{mean } OD_0, \text{ when } (\text{mean } OD_{test} - \text{mean } OD_0) < 0,$$

where:mean OD_0 = the average optical density measurements of sulforhodamine B (SRB)-derived colour just before exposure of cells to the test compounds; mean OD_{test} = the average optical density measurements of SRB-derived colour after 48 h exposure of cells to the test compounds; mean OD_{ctrl} = the average optical density measurements of SRB-derived colour 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 tumour 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} represents the molarity of inhibitor at which PG = 50% [24]. The standard sulforhodamine B (SRB) protein assay has been used to estimate cell viability or growth [24].

Note added in proof

Since we have submitted this paper for publication, Sly's group [31] reported that acetazolamide **1** reduced in vitro the invasiveness of some renal cancer cell lines of the type investigated by us here with compounds **10–16**, presumably due to the inhibition of isozymes CA II and CA XII which have been evidenced in three of the four cell lines investigated. 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.

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