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Carbonic anhydrase inhibitors: Inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, and IX with benzo[*b*]thiophene 1,1-dioxide sulfonamides

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Abstract—A series of selected benzo[*b*]thiophene-5- and 6-sulfonamide derivatives previously reported to show cytotoxic activity and some others newly synthesized has been tested for the interactions with several CA isozymes, some of which are known to be involved in tumorigenesis (hCA IX), whereas others are ubiquitously found in many normal tissues (the cytosolic isoforms hCA I and II). The unsubstituted sulfonamides inhibited hCA I with inhibition constants in the range of 63–138 nM, hCA II with inhibition constants in the range of 6.3–8.8 nM, and hCA IX with inhibition constants in the range of 2.8–15 nM, being thus more active than clinically used inhibitors such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide or indisulam (E 7070). Some of these derivatives also showed some selectivity for the inhibition of the tumor-associated (hCA IX) over the cytosolic isozyme hCA II. Although these derivatives may act on many targets other than the CAs (such as the NADH oxidase) or may induce apoptosis by accumulation of reactive oxygen species, it is quite important to try to decipher as many as possible of the potential mechanisms that lead to derivatives with potent antitumor activity in order to develop novel therapeutic strategies for the management of cancer.

It was known for several years that many sulfonamides possessing carbonic anhydrase (CA, EC 4.2.1.1)[1–3] inhibitory properties also inhibit in various degrees the growth of tumor cells in vitro and in vivo.[4–6] The precise isozyme(s) involved in such processes, among the 15 presently characterized human CAs, were not known until recently, but the discovery of CA IX[7,8] and then of CA XII[9] as isozymes predominantly present in tumors offered a starting point for more detailed studies in the field.[10] Another issue little understood in the first years of 'CA–tumors connection' research was why various tumor cell lines belonging to the same tumor type (for example, leukemia, non-small cell lung cancer, ovarian, melanoma, colon, CNS, renal, prostate or breast cancer) showed very different sensitivity to inhibition by sulfonamides, with GI_{50} (molarity of inhibitor producing a 50% inhibition of tumor cell growth) values typically in the range of 30 μM–10 nM.[4,5,11] It was discovered only later that CA IX/XII are not present in all tumor types,[1–3] and furthermore, that the levels of isozyme IX—the best studied one at this moment—dramatically increase in response to hypoxia via a direct transcriptional activation of the *CA9* gene by the hypoxia inducible factor HIF-1.[12–16] It was proven thereafter that the expression of CA IX in tumors is a sign of poor prognosis.[12–16]

Recently, the involvement of sulfonamide CA inhibitors in cancer has been investigated in more detail: Svastova et al.[17] showed that the acidic extracellular pH, which is a typical attribute of the tumor microenvironment, is generated by the activity of one of the tumor-associated CA isozymes, i.e., CA IX, and that this acidification can be perturbed by deletion of the enzyme active site and inhibited by potent CA IX inhibitors of the sulfonamide type, which bind only to hypoxic cells containing CA IX (the involvement of the other tumor-associated isozyme, i.e., CA XII, in such processes has not been investigated

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at the moment). Thus, it appears of critical importance to pursue the development of CA inhibitors targeting the tumor-associated CA isozymes CA IX and XII, eventually belonging to novel classes of compounds, less investigated up to now.

One of our groups reported[18] recently the synthesis and potent cytotoxic activity of some benzo[*b*]thiophene sulfonamide derivatives, which showed good activity against a wide range of tumor types, with GI_{50} values in the low nanomolar range. Some of these compounds possessed unsubstituted sulfamoyl or N-substituted such moieties with rather compact groups (ethyl, cyclopropyl, etc.), and it appeared thus of interest to investigate their interaction with several CA isozymes, some of which, as shown above, were involved in tumorigenesis. We report here an inhibition study of the cytosolic ubiquitous isozymes CA I and II, as well as of the tumor-associated isozyme CA IX (all of human origin) with a selected small library of benzo[*b*]thiophene sulfonamide derivatives reported earlier[18] to possess potent in vitro antitumor properties, and compared with other derivatives without cytotoxic activity against human tumoral cell lines.

The standard, clinically used CA inhibitors (acetazolamide **AZA**, methazolamide **MZA**, ethoxzolamide **EZA**, and dichlorophenamide **DCP**) were commercially available from Sigma–Aldrich, whereas indisulam (**IND**), a compound in phase II clinical trials as an antitumor agent,[10,19,20] was prepared as reported earlier. The benzo[*b*]thiophene sulfonamide derivatives **1a**–**e**, **2a**, and **3a**–**b** (Table 1) were obtained from the corresponding 6-aminobenzo[*b*]thiophene 1,1-dioxide derivative, its 2,3-dihydro derivative, and 5-aminobenzo[*b*]thiophene 1,1-dioxide, respectively, following the method described by us before.[18] The 6-amino-2,3-dihydrobenzo[*b*]thiophene 1,1-dioxide[21] was obtained by reduction of 6-amino-benzo[*b*]thiophene-1,1-dioxide using NaOH and an excess of Zn powder.[22] Compounds **2b** and **2c** from Table 1 were prepared from **1a** by reaction with the corresponding alcohol in diluted KOH.[23]

Inhibition data against isozymes hCA I, II, and IX with derivatives **1**–**3** as well as standard sulfonamide inhibitors are presented in Table 1.

The following SAR is evidenced from the data of Table 1: (i) Against isozyme hCA I the primary sulfonamides **1a**, **1b**, **2a**–**c**, and **3a** showed moderate inhibitory activity, with inhibition constants in the range of 63–138 nM, being thus less inhibitory than the very potent hCA I inhibitors ethoxzolamide and indisulam, but much better inhibitors than the clinically used drugs acetazolamide, methazolamide, and dichlorophenamide (K_I's in the range of 780–1200 nM). N-substitution of the sulfamoyl moiety of **1** with ethyl or cyclopropylmethyl moieties (**1c** and **1d**) led to a decrease of the hCA I inhibitory properties (K_I's in the range of 493–530 nM), but these derivatives were anyhow much better inhibitors than **AZA** and **MZA**. Thus, this is one of the first reports showing that some N-substituted sulfonamides may act as moderate hCA I inhibitors. The much bulkier benzyl (**1e**) or 3-methoxyphenyl (**3b**) substituents in such derivatives led, on the other hand, to a total loss of the CA inhibitory activities, not only against hCA I but also against the other two isozymes investigated here. Obviously, these bulky groups impede the binding of these sulfonamides to the Zn(II) ion within the enzyme active site. (ii) Against the ubiquitous, physiologically relevant isozyme hCA II, again the unsubstituted sulfonamides **1a**, **1b**, **2a**–**c**, and **3a** showed very good inhibition, with K_I's in the range of 6.3–8.8 nM. Thus, the nature of the ring (benzo[*b*]thiophene or the corresponding dihydro ring) or the position of the sulfamoyl moiety (5- or 6-substituted sulfonamides) attached to this ring does not show major influence on the excellent inhibitory properties of these derivatives against hCA II. The presence of the lipophilic R^1 moiety in compounds **2b** and **2c** was also beneficial for the CA inhibitory properties probably due to the interaction of these moieties with the hydrophobic half of the active site, as seen in many sulfonamide/sulfamate–hCA II adducts for which the X-ray crystal structure has been reported.[24] All these compounds showed an activity comparable to that of

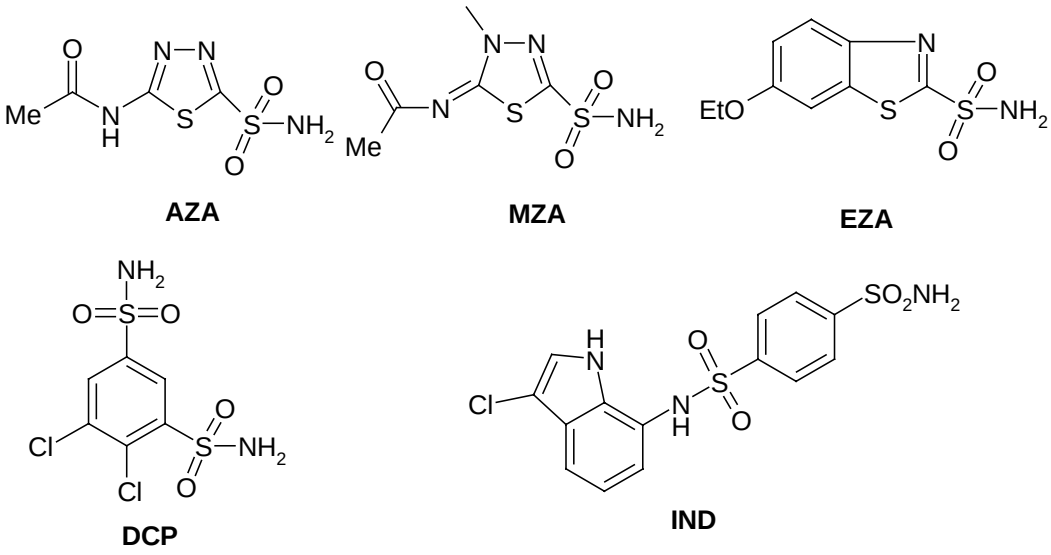
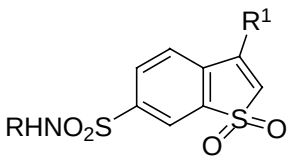
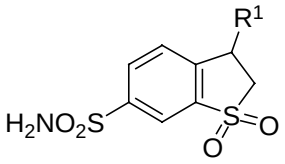


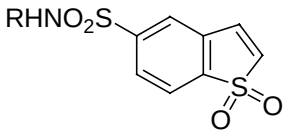
Table 1. Inhibition data for sulfonamides **1**–**3** investigated in the present paper and standard sulfonamide CA inhibitors, against isozymes hCA I, II, and IX[30]



1



2



3

Inhibitor	R	R[1]	K_I* (nM)			Selectivity ratio
			hCA I[a]	hCA II[a]	hCA IX[b]	K_I (hCA II)/K_I (hCA IX)
AZA	—		900	12	25	0.48
MZA	—		780	14	27	0.52
EZA	—		25	8	34	0.23
DCP	—		1200	38	50	0.76
IND	—		31	15	24	0.62
1a	H	H	63	7.5	15	0.50
1b	H	Me	85	8.3	13	0.63
1c	Et	H	530	436	362	1.20
1d	c-PrCH_2	H	493	475	76	6.25
1e	PhCH_2	H	>1000	>1000	>1000	—
2a	—	H	72	8.8	10	0.88
2b	—	O-*n*-C_4H_9	104	6.3	3.1	2.03
2c	—	OCH_2Ph	138	7.9	2.8	2.82
3a	H	—	75	7.4	13	0.57
3b	3-MeO-C_6H_4	—	>1000	>1000	>1000	—

[a] Human (cloned) isozymes, by the CO_2 hydration method.
[b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.[29]
* Errors in the range of 5–10% of the reported value (from three different assays).

ethoxzolamide, one of the best hCA II inhibitors known,[1–3] being more active than **AZA**, **MZA**, **DCP** or **IND**, all inhibitors in clinical use/clinical trials for the management of diverse CA-related disorders, tumors included.[1–3] As for hCA I, the substituted derivatives incorporating not very bulky moieties at the sulfonamide group (**1c** and **1d**) showed weak hCA II inhibitory activity (K_I's in the range of 436–475 nM), whereas the much bulkier derivatives (**1e** and **3b**) were devoid of activity (K_I's >1000 nM). (iii) The tumor associated isozyme hCA IX was also inhibited well by the unsubstituted sulfonamides **1a**, **1b**, **2a**–**c**, and **3a**, which showed K_I's in the range of 2.8–15 nM. It is obvious that SAR is very much similar to what was mentioned above for isozyme II, but these compounds are better inhibitors than all the clinically used derivatives, which showed inhibition constants in the range of 24–50 nM. It is also interesting to note the rather good hCA IX inhibitory properties of the cyclopropylmethyl-substituted compound **1d**, which with a K_I of 76 nM behaves as a moderate inhibitor. The *N*-ethyl-derivative **1c**, was on the other hand, a much weaker hCA IX inhibitor, with a K_I of 362 nM. The two bulky compounds **1e** and **3b** were devoid of hCA IX inhibitory effects. (iv) A critical issue in the design of CA inhibitors is represented by the specificity of the inhibitor for the target isozyme over the ubiquitous ones (hCA I and II, widely distributed throughout the body).[25,26] In the case of most of the compounds designed here (**1a**–**1c**, **2a**, and **3a**), as for the clinically used inhibitors, no selectivity of the active compounds for hCA IX over hCA II has been observed (Table 1). Indeed, the selectivity ratio of most of the compounds investigated here was in the range of 0.50–1.20. The compound with a good selectivity ratio for the inhibition of hCA IX was **1d** (selectivity ratio of 6.25), but this derivative is only a moderate hCA IX inhibitor. The highly active hCA IX inhibitors **2b** and **2c** showed an interesting selectivity for inhibiting this isozyme over hCA II, being 2.03–2.82 times better inhibitors of the tumor associated than the cytosolic isozyme (Table 1). (v) However, the cytotoxicity of benzo[*b*]thiophenesulfonamide 1,1-dioxides against HT-29, CCRF-CEM, K-562, HTB-54 and MEL-AC human tumoral cell lines or normal human lung fibroblasts (reported in the previous contributions)[18,21] seems to follow different patterns of action, probably due to the interaction of these compounds with other enzymes than the CAs. Thus, **1a**, **1c**, **1e**, **3a**, and **3b** were cytotoxic, whereas compounds **1b** and **2a** were inactive.[18,21] The substitution on position 3 of the benzo[*b*]thiophene nucleus or the reduction of its 2,3-double bond leads to non-cytotoxic derivatives. Furthermore, it has been demonstrated that benzo[*b*]thiophenesulfonamide 1,1-dioxides produce their antitumor effect through inhibition of a NADH oxidase activity found in the plasma membrane and conditioned culture medium of different kinds of tumour cells,[27,28] as well as through a process of apoptosis by accumulation reactive oxygen species (ROS).[29,30] In conclusion, many times the antitumor activity of a compound may include the interaction with more than one target, leading to complex pharmacological and biochemical processes that in the end lead to a

diminished growth of the tumor cell.[31] However, it is quite important to try to decipher as many as possible of these potential mechanisms that lead to derivatives with potent antitumor activity.

A series of benzo[*b*]thiophene-5- and 6-sulfonamide derivatives previously reported to show cytotoxic activity and some others newly synthesized has been tested for the interactions with several CA isozymes, some of which are known to be involved in tumorigenesis (hCA IX), whereas others are ubiquitously found in many normal tissues (the cytosolic isoforms hCA I and II). The unsubstituted sulfonamides inhibited hCA I with inhibition constants in the range of 63–138 nM, hCA II with inhibition constants in the range of 6.3–8.8 nM, and hCA IX with inhibition constants in the range of 2.8–15 nM, being thus more active than clinically used inhibitors such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide or indisulam (E 7070). Some of these derivatives also showed some selectivity for the inhibition of the tumor-associated (hCA IX) over the cytosolic isozyme hCA II. The 2,3-dihydrobenzo[*b*]thiophene derivatives could be considered as new lead compounds for the design of CAIs because of their absence of cytotoxicity, whereas substitutions on the sulfonamide group could model a certain degree of selectivity toward some CA isozymes (e.g., CA IX vs CA II). Although these derivatives may act on many targets other than the CAs (such as the NADH oxidase) or may induce apoptosis by accumulation reactive oxygen species, it is quite important to try to decipher as many as possible of the potential mechanisms that lead to derivatives with potent antitumor activity in order to develop novel therapeutic strategies for the management of cancer.

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23. Example of synthesis for **2b**. To a stirred solution of 0.66 g of **1a** (2.68 mmol) in 5 mL of 1-butanol, a solution of 0.35 g of KOH in 2 mL of 1-butanol was drop wise added, and the stirring was continued at room temperature for 1.5 h. Subsequently, butanol was removed in vacuum and the solid residue was dissolved in 10 mL of water. The aqueous layer was washed with ether (2×10 mL) and acidified with HCl to reach pH 2. The solid material was collected by filtration and recrystallised from water to give **2b** (0.32 g, 37% yield): Mp 159–160 °C; IR (KBr, cm^{-1}): 1147, 1345 (SO_2), 3319, 3247 (NH_2); ^1H NMR ($CDCl_3$ δ): 8.29 (s, 1H, H-7), 8.19 (d, $J = 8.06$ Hz, 1H), 7.80 (d, 1H), 5.27 (t, $J = 6.23$ Hz, 1H); 4.97 (s, 2H), 3.99–3.89 (dd, $J = 13.18$ Hz, 1H), 3.66 (t, $J = 6.23$ Hz, 2H), 3.56–3.44 (dd, 1H), 1.73–1.37 (m, 4H), 0.95 (t, $J = 7.33$ Hz, 3H).
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