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Carbonic anhydrase inhibitors: synthesis and inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, and IX with sulfonamides incorporating thioureido-sulfanilyl scaffolds

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Abstract—The tumor-associated transmembrane carbonic anhydrase (CA, EC 4.2.1.1) isozyme IX (CA IX) is overexpressed in hypoxic tumors and appears to be involved in acidification of the tumor microenvironment, a process correlated with cancer progression and bad prognosis. The acidification may be reduced by inhibiting the enzyme with potent sulfonamide/sulfamate CA inhibitors. A series of such aromatic sulfonamides incorporating thioureido-sulfanilyl moieties has been prepared and investigated for its interaction with the catalytic domain of the human isozyme hCA IX. The key intermediates in the synthesis were obtained by reacting sulfanilamide, homosulfanilamide, or 4-aminoethylbenzenesulfonamide with 4-acetamido-benzenesulfonyl chloride followed by deacetylation and reaction with thiophosgene. The obtained isothiocyanato sulfonamides were reacted with aliphatic or aromatic primary amines or hydrazines, leading to the corresponding thioureas. Some of these compounds showed excellent inhibitory properties against isozymes I, II, and IX, with several inhibitors also presenting selectivity for the inhibition of CA IX over that of the ubiquitous isozyme CA II. Such sulfonamides may constitute interesting candidates for the development of novel antitumor therapies based on the inhibition of the CA isozymes overexpressed in hypoxic tumors. Due to the highest expression of CA IX in clear renal cell carcinoma and its chemo/radioresistance, our efforts are first of all directed to generate effective therapeutic strategies for the cure of this malignancy.

1. Introduction

We have recently shown[1] that the tumor-associated carbonic anhydrase (CA, EC 4.2.1.1) isozyme IX, hCA IX, is involved in acidification of hypoxic tumors, and that this process may be diminished (and probably abolished) by inhibiting the enzyme with potent sulfonamide inhibitors. This constitutes the proof-of-concept demonstration that inhibiting the tumor-associated CAs (two such isozymes are presently known, CA IX and CA XII)[2–6] may be relevant for the design of novel antitumor therapies. Thus, much work is currently being done in this and other laboratories for discovering either small molecule, iRNA-s or immunologic-based agents targeting these proteins present in a multitude of hypoxic tumors.[7–10]

Sulfonamide CA inhibitors (CAIs), such as acetazolamide **AZA**, methazolamide **MZA**, ethoxzolamide **EZA**, or dichlorophenamide **DCP** among others, initially played a crucial role in the understanding of renal physiology and pharmacology, leading then to the development of widely used drugs such as the benzothiadiazine and high ceiling diuretics.[1–5] More recently, this class of drugs showed important applications for the development of antiglaucoma agents,[2] being also useful for the design of other types of pharmacological agents, as it will be shown here shortly. Thus, a recent and new

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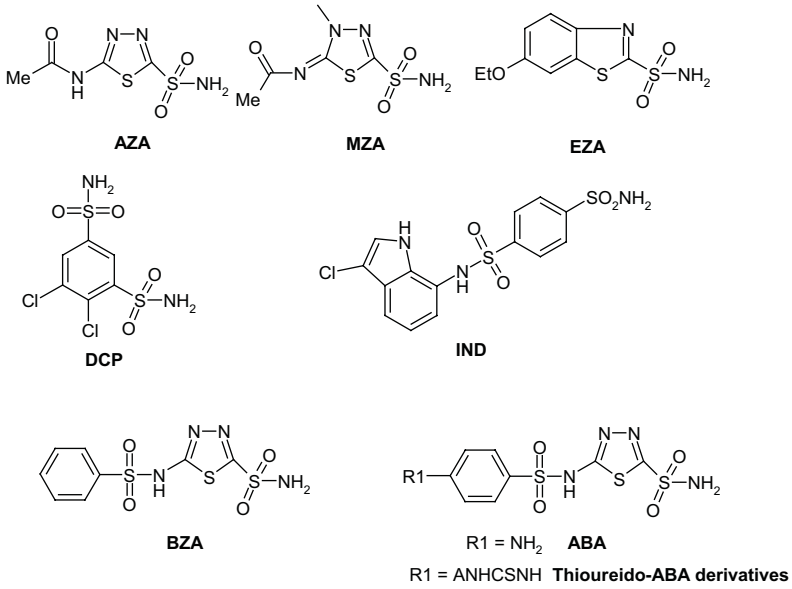


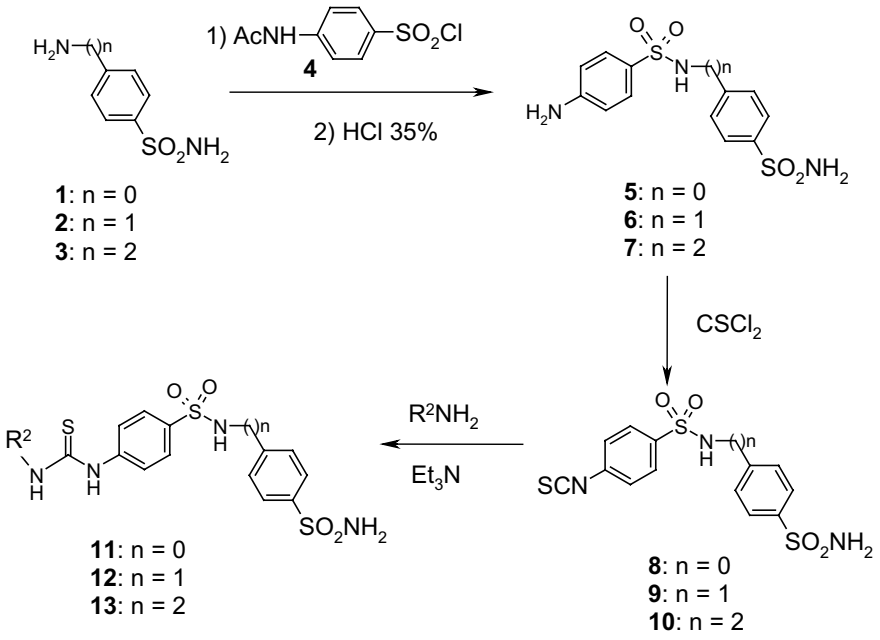
field in CAI research has been opened by the report of the potent antitumor properties of a rather large number of sulfonamide CAIs, as well as by the isolation, purification, and characterization of the isozymes predominantly present in tumor cells, such as CA IX and CA XII.[1–6] The mechanisms by which these isozymes participate in the tumor cell growth and differentiation, as well as those by which their inhibitors interfere with the tumor growth only now begin to be understood,[1] but important advances in these directions have recently been achieved.[1] Indeed, several laboratories are involved in the synthesis, evaluation, and in vitro/in vivo antitumor testing of novel types of CAIs with potential application as anticancer therapeutic agents.[6–12] Furthermore, a compound of this type—indisulam **IND**—has progressed to Phase II clinical trials for the treatment of solid tumors.[6,9,10]

CA IX gene regulation is tightly dependent on the HIF-1α gene expression, which is evoked by environmental hypoxic stimuli. Initially, the strategy to counteract hypoxic tumor growth was by targeting of the HIF-1α protein, but its expression is usually transient and its half-life is very short.[5–7] On the contrary, CA IX is a very stable protein. The activity of CA IX, whose half-life is of at least 96 h, is long lasting, even in the case of hypoxia abrogation. Finally, CA IX is anchored to the outermost layer of the cell and not placed in the inner nuclear compartment (as HIF-1α is), making this enzyme a druggable interesting target.[1,5–7]

In previous work from this laboratory,[13] we have developed thioureido-containing sulfonamide CAIs derived from simple aromatic sulfonamides, such as sulfanilamide **1** and homosulfanilamide **2**, which showed excellent CA I, II, and IV inhibitory properties and were effective topically acting antiglaucoma agents in an animal model of this disease (some of the above-mentioned isozymes are involved in aqueous humor secretion within the eye, being the targets of antiglaucoma sulfonamides).[1–4] Continuing this work and the same type of chemistry,[13] but using as lead molecule benzolamide **BZA** (an orphan drug belonging to the family of CAIs),[2] we then reported novel thioureido-containing benzolamide-like compounds, which were also shown to act as very potent inhibitors of the transmembrane, tumor-associated isozyme CA IX (in addition to the strong inhibition of the ubiquitous, cytosolic isozymes I and II).[14] Using these last compounds as lead molecules,[14] we report this time novel derivatives incorporating thioureido-sulfanilyl scaffolds. Thus, the thiadiazole scaffold of the above mentioned lead compounds has been changed to an 1,4-phenylene moiety, and the spacer between this structural element and the thioureido-sulfanilyl scaffold has been also varied, with 0, 1, and 2 CH_2 moieties present in the new compounds reported in this paper. Some of these compounds showed excellent inhibitory properties against isozymes CA I, II, and IX, as well as some specificity for inhibiting the last isozyme over the ubiquitous cytosolic isoform CA II.

2. Chemistry

The reaction of amino-substituted sulfonamides such as sulfanilamide **1**, homosulfanilamide **2**, and 4-aminoethylbenzenesulfonamide **3** with alkyl/arylsulfonyl halides has been investigated in detail by our group.[15] During the previous studies, we also reported the sulfanylated derivatives **5**–**7**,[15a] which were obtained by treating the parent amino sulfonamides **1**–**3** with 4-acetamidobenzenesulfonyl chloride **4**, followed by deacetylation with concentrated hydrochloric acid for removal of the protecting group. Derivatives **5**–**7** obtained as reported earlier[15a] were treated with thiophosgene in acidic medium, in order to prepare the key intermediates needed in the present study, the isothiocyanato derivatives **8**–**10** (Scheme 1), by the procedure already reported for preparing the isothiocyanato derivatives of the simpler sulfonamides **1** and **2**.[13,16]



Scheme 1.

The isothiocyanates **8**–**10** prepared as outlined above were then converted to thioureas **11**–**13** by reaction with primary amines/hydrazines in the presence of triethylamine as catalyst, by the procedure reported for the preparation of other thioureido-substituted sulfonamides.[13,14] The amines/hydrazines used for obtaining the novel derivatives reported here, of types **11**–**13**, were chosen in such a way as to incorporate moieties, which: (i) were previously shown to lead to potent CA IX inhibitors,[14] such as dimethylaminoethyl, morpholinyl, *N*-methylpiperazinyl, morpholinyl-*N*-ethyl, sulfamoylphenylethyl,[17,18] etc.; and (ii) may assure an acceptable water solubility to such compounds, eventually as salts with pharmaceutically acceptable acids, such as hydrochlorides, trifluoroacetates, triflates, etc.[19] Indeed, many of the moieties mentioned above (dimethylaminoethyl, morpholinyl, *N*-methylpiperazinyl, and morpholinyl-*N*-ethyl) are able to be protonated, leading to salts with enhanced water solubility as compared to the sulfonamides from which they are derived. A strong and possibly water-soluble CA IX inhibitor would constitute an excellent tool for investigating in more detail the role(s) of the tumor-associated CA isozymes in tumor growth and differentiation, as well as potential therapeutic options exploiting their specific inhibition.

3. Carbonic anhydrase inhibition

Inhibition data against three physiologically relevant isozymes, that is, the cytosolic isoforms hCA I and II and the membrane-bound, tumor-associated isozyme hCA IX (all of them of human origin) with the new compounds **11**–**13** as well as the standard, clinically used CA inhibitors acetazolamide **AZA**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND**, are shown in Table 1. Inhibition data for the parent sulfonamides from which the new derivatives were obtained (such as **1**–**3** and **5**–**7**) are also provided for comparison, as they were recently reported by our group.[8]

The following SAR should be noted from, data of Table 1: (i) the cytosolic slow isozyme hCA I is inhibited by all the new compounds **11**–**13** investigated here, with inhibition constants in the range of 3.1–355 nM. Thus, whereas the simple sulfonamides **1**–**3** act as weak CA I inhibitors (K_Is in the range of 21–28 μM), the corresponding sulfanylated derivatives **5**–**7** are much better inhibitors, with K_Is in the range of 95–109 nM (Table 1).[8] It may be seen that for all these compounds the inhibitory properties increase with the increase of the number of carbon atoms (from 0 to 2) between the benzene ring bearing the primary sulfamoyl group and the amino moiety. For the thioureas **11**–**13**, SAR is more complicated. Thus, a group of derivatives, such as **11a**, **11c**, **11d**, **12a**, **12c**, **12d**, **12f**, and **13a**, behave as weaker hCA I inhibitors as compared to the corresponding parent sulfonamides from which they were obtained (**5**–**7**), showing K_Is in the range of 105–355 nM. It is also noteworthy to observe that the most active derivative in such a subseries possessing the same substitution pattern (e.g., **11a**, **12a**, and **13a**) is the sulfanilamide compound ($n = 0$), which is more active than the homosulfanilamide derivative ($n = 1$), which in turn is more active than the compound with $n = 2$ (just the reverse situation as compared to the parent sulfonamides **5**–**7** discussed above). Another group of derivatives, including **11b**, **12b**, **12e**, and **12g** act as very potent hCA I inhibitors, with K_Is in the range of 3.1–9.5 nM. These compounds are stronger inhibitors than the parent sulfonamides from which they were derived (**5**–**7**) or than the clinically used sulfonamides, which showed inhibition constants in the range of 25 nM (ethoxzolamide)–1.2 μM

Table 1. Inhibition data for sulfonamides **1**–**13** investigated in the present paper and standard sulfonamide CA inhibitors, against isozymes I, II, and IX

Inhibitor	R^2	K_I[a] (nM)			Selectivity ratio K_I (hCA II)/K_I (hCA IX)
		hCA I[b]	hCA II[b]	hCA IX[c]	
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
1[d]	—	28,000	300	294	1.02
2[d]	—	25,000	170	103	1.65
3[d]	—	21,000	160	33	6.49
5[d]	—	164	46	34	1.35
6[d]	—	109	33	31	1.06
7[d]	—	95	30	24	1.25
11a	$Me_2NCH_2CH_2$	105	8.1	6.7	1.20
11b	$[O(CH_2CH_2)_2N]CH_2CH_2$	9.5	180	5.6	32.14
11c	Me–$[N(CH_2CH_2)_2N]$	175	7.2	4.8	1.50
11d	$[O(CH_2CH_2)_2N]$	233	290	6.7	43.28
12a	$Me_2NCH_2CH_2$	210	12	5.4	2.22
12b	$[O(CH_2CH_2)_2N]CH_2CH_2$	3.1	220	4.8	45.83
12c	Me–$[N(CH_2CH_2)_2N]$	246	10.3	5.0	2.06
12d	$[O(CH_2CH_2)_2N]$	268	278	6.4	43.43
12e	$PhCH_2CH_2$	8.6	640	5.0	128.00
12f	2-Pyridyl-CH_2	355	290	5.2	55.76
12g	4-$H_2NO_2SC_6H_4CH_2CH_2$	3.7	46	4.6	10.00
13a	$Me_2NCH_2CH_2$	308	10.1	6.1	1.65

[a] Errors in the range of 5–10% of the reported value (from three different assays).
[b] Human (cloned) isozymes, by the CO_2 hydration method.
[c] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.
[d] From Ref. 8.

(dichlorophenamide). Thus, it may be observed that the morpholinyl-*N*-ethyl as well as phenethyl moieties substituting the thiourea group of compounds **11**–**13** lead to the best hCA I inhibitors, irrespective of the spacer between the benzenesulfonamide and the substituted-thioureido moieties, whereas other substitution patterns investigated here lead to such less effective inhibitors; (ii) against the rapid cytosolic isozyme hCA II, the compounds reported here showed inhibition constants in the range of 8.1–640 nM. Again, as for isozyme I, the simple aromatic sulfonamides **1**–**3** show moderate inhibitory properties (K_Is in the range of 160–300 nM), which increase with the length of the $(CH_2)_nNH_2$ moiety for n from 0 to 2 (Table 1). The same is true for the sulfanylated derivatives **5**–**7**, which act as stronger hCA II inhibitors (K_Is in the range of 30–46 nM) than the sulfonamides **1**–**3** from which they are derived. For the thioureas **11**–**13**, several substitution patterns, such as those present in compounds **11b**, **11d**, **12b**, and **12d**–**f**, lead to a decrease of the inhibitory properties as compared to the parent sulfonamides **5**–**7**, as these thioureas showed K_Is in the range of 180–640 nM. On the contrary, derivatives **11a**, **11c**, **12a**, **12c**, **12g**, and **13a** showed excellent hCA II inhibitory properties (similar to those of the clinically used sulfonamides shown in Table 1), with K_Is in the range of 8.1–46 nM. It may be seen that the best substitution patterns for obtaining good hCA II inhibitors include the dimethylaminoethyl- and *N*-methyl-piperazine moieties as substituents of the thioureido functionality. It is also important to note that these are just the moieties that led to ineffective hCA I inhibitors. Thus, we are for the first time in the presence of a series of compounds in which we obtained good hCA I inhibition and ineffective hCA II inhibition for some representatives (such as **11b**, **12b**), whereas for other compounds we obtained just the inverse profile, with good hCA II inhibition and ineffective hCA I inhibition (e.g., **11a**, **11c**, **12a**, **12c**, and **13a**). It is difficult to explain these important differences of activity at this point, and work is in progress in this laboratory for resolving the X-ray crystal structures of some of these derivatives with the two isozymes discussed above; (iii) the compounds **11**–**13** reported here showed excellent hCA IX inhibitory properties, with K_Is in the range of 4.6–6.7 nM, being thus much more effective inhibitors as compared to the parent sulfonamides **5**–**7** (K_Is in the range of 24–34 nM), the simple sulfonamides **1**–**3** or the clinically used compounds shown in Table 1. It may be seen that the entire class of thioureas showed this compact behavior, similarly to the ABA-thioureido compounds reported earlier.[14] It may be thus stated that probably the thioureido scaffolds attached to sulfanylated-aromatic/hetetrocyclic sulfonamide scaffolds lead to effective and rather selective hCA IX inhibitors, with many optimal substitution patterns detected; (iv) some of the compounds reported here (such as **11a**, **11c**, **12a**, **12c**, and **13a**) showed selectivity ratios for inhibiting hCA II over hCA IX in the range of 1.20–2.06, being thus only slightly more effective hCA IX than hCA II inhibitors. Most of the new compounds, including **11b**, **11d**, **12b**, and **12d**–**g**, showed on the other hand much better selectivity ratios, in the range of 10.00–128.00, being thus much more effective inhibitors of the tumor-associated isozyme over the ubiquitous cytosolic

isozyme hCA II. Still, most of these compounds behave as potent hCA I inhibitors, but the real physiological role of this last isozyme is rather uncertain at this point.

4. Conclusions

A series of aromatic sulfonamides incorporating thioureido-sulfanilyl moieties have been prepared and investigated for its interaction with the catalytic domain of the human isozyme hCA IX as well as with the cytosolic isozymes I and II. The key intermediates in the synthesis were obtained by reacting sulfanilamide, homosulfanilamide, or 4-aminoethylbenzenesulfonamide with 4-acetamido-benzenesulfonyl chloride followed by deacetylation and reaction with thiophosgene. The obtained isothiocyanato sulfonamides were then reacted with aliphatic or aromatic primary amines or hydrazines, leading to the corresponding thioureas. Some of these compounds showed excellent inhibitory properties against isozymes I, II, and IX, with several inhibitors also presenting selectivity for the inhibition of CA IX over that of the ubiquitous isozyme CA II. Such sulfonamides may constitute interesting candidates for the development of novel antitumor therapies based on the inhibition of the CA isozymes overexpressed in hypoxic tumors. Clear renal cell carcinoma is the only human malignancy sharing both the highest CA IX expression and chemo/radiotherapy resistance.[20] To date, partial treatment response is achieved only with immunotherapy. Furthermore, CA IX is a tumor-associated antigen only in renal cell carcinoma. As a consequence, the present and previous results from our group attempt a new approach to be added to the mainstay of renal cell carcinoma therapy, based on inhibition of CA IX.

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16. Reaction of the isothiocyanates **8**–**10** with amines or hydrazines R^2NH_2 has been done in diethylether, water or *N*,*N*-dimethylacetamide as solvents, as previously described.[14]
1-[4-*N*-(2-(4-Sulfamoylphenyl)-phenylethyl)-sulfamoyl-phenyl]-3-(2-dimethylaminoethyl)-thiourea **13a**: mp 108–110 (EtOH–MeOH 1.1); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.30 (s, 6H, Me_2N), 2.57 (t, J = 5.8 Hz, 2H, CH_2NMe_2); 2.77 (t, J = 6.6 Hz, 2H, $C_6H_4CH_2$); 2.98 (t, J = 6.3 Hz, 2H, CH_2NHSO_2); 3.60 (m, 2H, $CSNHCH_2$); 7.33 (s, 2H, SO_2NH_2); 7.42–7.95 (m, 8H, ArH from $2C_6H_4$ moieties); 8.21 (s, 1H, SO_2NH). Elem. anal., found: C, 47.12; H,

5.33; N, 14.27; $C_{19}H_{27}N_5O_4S_3$ requires: C, 46.99; H, 5.60; N, 14.42.
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