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Carbonic Anhydrase Inhibitors: Inhibition of the Tumor-Associated Isozyme IX with Aromatic and Heterocyclic Sulfonamides

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Abstract—The inhibition of the tumor-associated transmembrane carbonic anhydrase IX (CA IX) isozyme has been investigated with a series of aromatic and heterocyclic sulfonamides, including the six clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) were also provided for comparison. A very interesting and unusual inhibition profile against CA IX with these sulfonamides has been observed. Several nanomolar (K_I-s in the range of 14–50 nM) CA IX inhibitors have been detected, both among the aromatic (such as orthanilamide, homosulfonilamide, 4-carboxy-benzenesulfonamide, 1-naphthalenesulfonamide and 1,3-benzenedisulfonamide derivatives) as well as the heterocylic (such as 1,3,4-thiadizole-2-sulfonamide, etc.) sulfonamides examined. Because CA IX is a highly active isozyme predominantly expressed in tumor tissues with poor prognosis of disease progression, this finding is very promising for the potential design of CA IX-specific inhibitors with applications as anti-tumor agents.
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Introduction

Among the zinc enzymes extensively studied in the last period, the carbonic anhydrases (CAs, EC 4.2.1.1) occupy a special place for several reasons: (i) these enzymes are ubiquitous in all kingdoms, starting with *Archaea*, *Bacteria*, algae and green plants, and ending with superior animals, including vertebrates;[1–4] (ii) their physiological function is essential for these organisms, as CAs catalyze a fundamental physiological reaction, the interconversion between carbon dioxide and bicarbonate.[1–4] This reaction is critical for respiration and transport of CO_2 between metabolizing tissues and excretion sites, secretion of electrolytes in a variety of tissues and organs, pH regulation and homeostasis, CO_2 fixation (for algae and green plants), several metabolic biosynthetic pathways (in vertebrates), and so on;[1–4] (iii) inhibition (but also activation) of these enzymes may be exploited clinically in the treatment or prevention of a variety of disorders.[1–3] In consequence, CA inhibitors (CAIs) and to a less extent up to now, CA activators possess a variety of applications in therapy.[1,2] Four such pharmacological agents, acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, and dichlorophenamide **DCP**, have been used for more than 40 years as systemic CAIs, whereas two additional drugs dorzolamide **DZA** (clinically launched in 1995) and the structurally-related brinzolamide **BRZ** (used since 1999) are topically acting antiglaucoma CAIs.[1,2]

CAs are encoded by three distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, *Bacteria*, algae and cytoplasm of green plants), the β-CAs (predominantly in *Bacteria*, algae and chloroplasts of both mono- as well as dicotyledons) and the γ-CAs (mainly in *Archaea* and some *Bacteria*), respectively.[1,2,5] In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) were described, with very different subcellular localization and tissue distribution.[1,2,5] Basically, there are several

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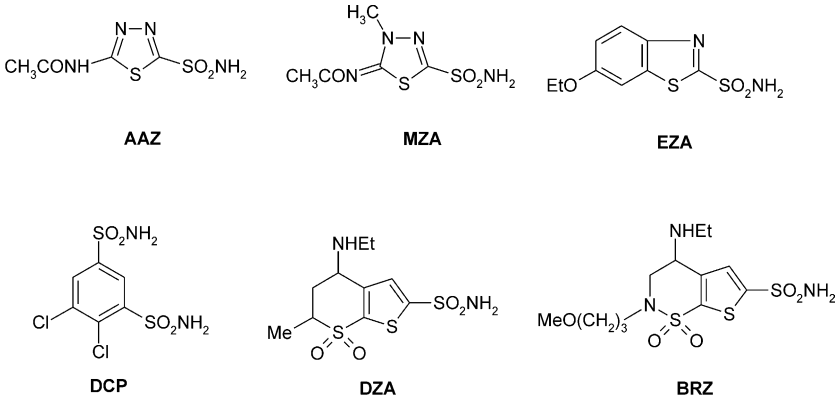


cytosolic forms (CA I–III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII and CA XIV), one mitochondrial form (CA V) as well as a secreted CA isozyme, CA VI.[1,2,5] Not much is known about the cellular localization of the other isozymes.

Some of the isozymes mentioned above, such as CA IX and CA XII, are overexpressed in cancer cells.[6] The first tumor-associated CA isozyme discovered was CA IX, a transmembrane protein with a suggested function both in maintaining acid-base balance and in intercellular communication. It consists of an N-terminal proteoglycan-like domain that is unique among the CAs, a highly active CA catalytic domain, a single transmembrane region and a short intracytoplasmic tail.[7] CA IX is particularly interesting for its ectopic expression in a multitude of carcinomas derived from cervix uteri, kidney, lung, esophagus, breast, colon and so on, contrasting with its restricted expression in normal tissues, namely in the epithelia of the gastrointestinal tract.[7–14]

It has recently been demonstrated that such tumor-associated CAs (mainly CA IX) may be of considerable value as markers of tumor progression. This is mostly due to their induction by hypoxia, a clinically important factor of tumor biology that significantly affects treatment outcome and disease progression.[9] Strong association between CA IX expression and intratumoral hypoxia (either measured by microelectrodes, or detected by incorporation of a hypoxic marker pimonidazole, or by evaluation of extent of necrosis) has been demonstrated in the cervical, breast, head and neck, bladder and non-small cell lung carcinomas (NSCLC).[10–13] Moreover, in NSCLC and breast carcinomas, correlation between CA IX and a constellation of proteins involved in angiogenesis, apoptosis inhibition and cell–cell adhesion disruption has been observed, possibly contributing to strong relationship of this enzyme to a poor clinical outcome.[13] Hypoxia is linked with acidification of extracellular milieu that facilitates tumor invasion and CA IX is believed to play a role in this process via its catalytic activity.[14] Thus, inhibition of this enzyme may constitute a novel approach to the treatment of cancers in which CA IX is expressed.

In fact, acetazolamide, one of the best-studied, classical CAI used clinically, was shown to function as a modulator in anticancer therapies, in combination with different cytotoxic agents (such as alkylating agents; nucleoside analogues; platinum derivatives, etc.), to suppress tumor metastasis and to reduce the invasive capacity of several renal carcinoma cell lines (Caki-1, Caki-2, ACHN, and A-498).[15,16] Such valuable studies constituted a proof-of-concept demonstration that CAIs may be used in the management of tumors that overexpress one or more CA isozymes. It should also be mentioned that our group reported the design and in vitro antitumor activity of several classes of sulfonamide CAIs, shown to act as nanomolar inhibitors against the classical isozymes known to possess critical physiological roles, such as CA I, CA II and CA IV. These compounds were also shown to exert potent inhibition of cell growth in several leukemia, non-small cell lung, ovarian, melanoma, colon, CNS, renal, prostate and breast cancer cell lines, with GI_{50} values of 10–75 nM in some cases.[17–20] On the other hand, no data regarding inhibition of tumor-associated CA IX with different types of sulfonamides are available up to now, although inhibition of this particular isozyme might be clinically exploited for designing novel anticancer therapies. In this paper we report the first CA IX inhibition study with a series of aromatic and heterocyclic sulfonamides, as well as with the six clinically used CAIs mentioned above.

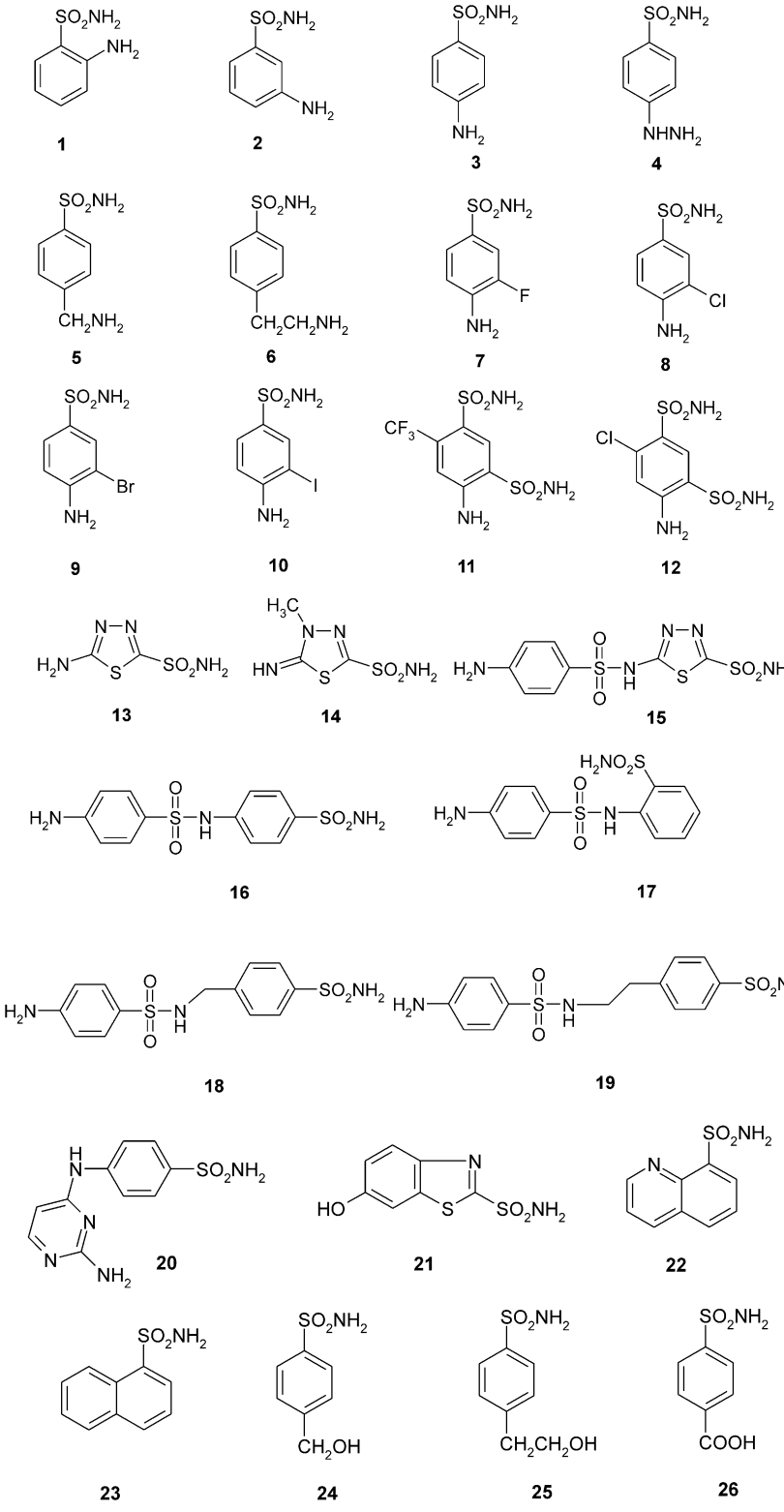
Chemistry

Sulfonamides investigated for the inhibition of the tumor-associated isozyme CA IX, of types **1–26** are shown below. Compounds **1–6**, **11–12**, **20** and **26** are commercially available, whereas **7–10**,[21] **13–19**[22] and **21–25**[23] were prepared as reported earlier. The six clinically used compounds were also assayed, since no such data are available in the literature.

CA Inhibition Data

Inhibition data against four CA isozymes, CA I, II, IV and IX,[24–26] with the above mentioned compounds **1–26** and the six clinically used inhibitors, are shown in Table 1.

These data are rather surprizing, since the inhibition profile of isozyme CA IX is very different from that of the classical isozymes CA I and II (cytosolic) as well as



CA IV (membrane-bound). The following particular features may be noted: (i) all the 32 sulfonamides investigated here act as CA IX inhibitors, with inhibition constants in the range of 14–285 nM (the corresponding affinities for the other three isozymes vary in a much wider range, as seen from data in Table 1). Based on these data, it can be noted that CA IX is a sulfonamide avid CA, similarly to CA II, the isozyme considered up to now to be responsible for the majority of pharmacological effect of sulfonamides.[1–3] Still, many other differences are observed between CA IX and other isozymes for which inhibitors were developed for clinical use; (ii) for CA I, II and IV, generally, aromatic sulfonamides behave as weaker inhibitors as compared to heterocyclic derivatives (compare **1**–**6**, or **DCP**), as aromatic compounds, with **15**, **21**, **AAZ**, **MZA**, **EZA**, **DZA**

Table 1. CA I, II, IV and IX inhibition data with sulfonamides **1–26** and clinically used inhibitors

Inhibitor	K_I* (nM)			
	hCA I[a]	hCA II[a]	bCA IV[b]	hCA IX[c]
1	45,400	295	1310	33
2	25,000	240	2200	238
3	28,000	300	3000	294
4	78,500	320	3215	305
5	25,000	170	2800	103
6	21,000	160	2450	33
7	8300	60	180	245
8	9800	110	320	264
9	6500	40	66	269
10	6000	70	125	285
11	5800	63	154	24
12	8400	75	160	39
13	8600	60	540	41
14	9300	19	355	30
15	6	2	5	38
16	164	46	129	34
17	185	50	144	20
18	109	33	72	31
19	95	30	72	24
20	690	12	154	16
21	55	8	17	14
22	21,000	125	415	32
23	23,000	133	438	30
24	24,000	125	560	21
25	18,000	110	450	22
26	135	40	86	26
AAZ	250	12	70	25
MZA	50	14	36	27
EZA	25	8	13	34
DCP	1200	38	380	50
DZA	50,000	9	43	52
BRZ	—	3	45	37

[a]Human cloned isozymes, esterase assay method.[26]
[b]Isolated from bovine lung microsomes, esterase assay method.[26]
[c]Human cloned isozyme, CO_2 hydrase assay method.[24,25]

or **BRZ** among others (as heterocyclic sulfonamides). In the case of CA IX, such a fine distinction is rather difficult to be made, since both aromatic (such as **1**, **6**, **11**, **12**, **17**, **18**, **22–26**) derivatives, as well as heterocyclic compounds (such as **14**, **15**, **21**, and the clinically used sulfonamides—except dichlorophenamide) possess rather similar inhibition constants, in the range of 14–50 nM; (iii) orthanilamide derivatives (such as **1**, **17** and **22**) behave as very potent CA IX inhibitors (K_i-s in the range of 20–33 nM), although they are weak or medium-weak inhibitors of CA I, II and IV; (iv) 1,3-benzenedisulfonamide derivatives (such as **11**, **12** and **DCP**) are again strong CA IX inhibitors, with K_i-s in the range of 24–50 nM, although their CA II, I and IV inhibition profile is not particularly strong; (v) metanilamide **2**, sulfanilamide **3**, and 4-hydrazino-benzenesulfonamide **4** show CA IX inhibition data quite similar with those against CA II, whereas homosulfanilamide **5** and 4-aminoethyl-benzensulfonamide **6** act as better CA IX inhibitors as compared to CA II inhibition; (vi) the halogenosulfanilamides **7–10** are much weaker inhibitors of CA IX than of CA II, a finding difficult to interpret at this moment; (vii) the strongest CA II inhibitor among the investigated compounds, 4-aminobenzolamide **15** (K_i of 2 nM) is not the strongest CA IX inhibitor (K_i of 38 nM). Instead, the best CA IX inhibitor detected so far is the ethoxzolamide phenol **21** (K_i of 14 nM). It is interesting to note that **21** and **EZA** have the same affinity for CA II, whereas their affinity for CA IX is rather different, with the phenol more active than the ethoxy-derivative; (viii) among the clinically used compounds, the best inhibitor is acetazolamide, followed by methazolamide, ethoxzolamide and brinzolamide. The most ineffective (but appreciably inhibiting the isozyme IX) are dichlorophenamide and dorzolamide; (ix) sulfonamides **20** and **22–26** behave as very good CA IX inhibitors, with K_i-s in the range of 16–32 nM, being slightly more effective than the clinically used CAIs mentioned above, and among the best CA IX inhibitors detected so far. It is thus envisageable that such compounds may be used as lead molecules for obtaining more potent and eventually specific CA IX inhibitors, with applications as antitumor agents.

Conclusion

We report here the first inhibition study of the tumor-associated, transmembrane isozyme CA IX with a series of aromatic and heterocyclic sulfonamides, including also the six clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) are also provided for comparison. Very interesting inhibition profile against CA IX with these sulfonamides has been detected, which is a promising discovery for the potential design of CA IX-specific inhibitors, with applications as antitumor agents. Several nanomolar CA IX inhibitors have been detected, both among the aromatic (such as orthanilamide, homosulfanilamide, 4-carboxy-benzenesulfonamide, 1-naphthalene- sulfonamide and 1,3-benzenedisulfonamide derivatives) as well as the heterocyclic (such as 1,3,4-thiadiazole-2-sulfonamide, benzothiazole-2-sulfonamide, etc.) sulfonamides investigated.

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24. The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[7a]) was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100, as described by: Wingo, T.; Tu, C.; Laipis, P. J.; Silverman D. N. *Biochem. Biophys. Res. Comm.* **2001**, *288*, 666. The homogenate thus obtained was extensively centrifuged in order to remove soluble and membrane associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione, 1 mM oxidized glutathione. Active hCA IX was extensively dialysed into a solution of 10 mM Hepes (pH 7.5), 10 mM Tris HCl, 100 mM Na_2SO_4 and 1 mM $ZnCl_2$. The amount of protein was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate.[25]
25. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561 An SX.18MV-R Applied Photophysics stopped-flow instrument has been used. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 1–3 mM (in DMSO–water 1:1, v/v) and dilutions up to 0.1 nM done with the assay buffer mentioned above.
26. A stopped flow variant of the Poker and Stone spectrophotometric method (Pocker, Y.; Stone, J. T. *Biochemistry* **1967**, *6*, 668) has been employed, using an SX.18MV-R Applied Photophysics stopped flow instrument, as described previously.[21]