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Carbonic anhydrase inhibitors: Inhibition of the tumor-associated isozymes IX and XII with polyfluorinated aromatic/heterocyclic sulfonamides

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Abstract

The tumor-associated transmembrane carbonic anhydrase (CA, EC 4.2.1.1) isozymes IX (CA IX) and XII (CA XII) are involved in acidification of hypoxic tumors, a process correlated with poor prognosis and clinical outcome of patients harboring such tumors. This process may be reversed by inhibiting these enzymes with potent sulfonamide/sulfamate inhibitors. A series of such aromatic/heterocyclic sulfonamides incorporating 2,3,5,6-tetrafluorobenzoyl-, 2,3,5,6-tetrafluoro-phenylsulfonyl- and pentafluorophenylureido moieties has been investigated for its interaction with the catalytic domain of the human isozymes hCA IX and hCA XII. Some of these compounds showed excellent inhibitory properties against both isozymes IX and XII, with several subnanomolar inhibitors detected for the first time. These sulfonamides may constitute valuable candidates for the development of novel antitumor therapies based on the inhibition of such tumor-associated CA isozymes.

Keywords: *Carbonic anhydrase, isozymes IX and XII, antitumor drug, sulfonamide, polyfluorinated derivative, inhibitors*

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 reverted 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 two proteins present in a multitude of hypoxic tumors [7–10].

CA inhibitors (CAIs) are clinically used mostly as antiglaucoma agents, as they are highly effective in reducing elevated intraocular pressure, after systemic administration of drugs such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** or dichlorophenamide **DCP,** or after topically administered sulfonamides, such as dorzolamide **DZA,** or brinzolamide **BRZ** [2–4]. They are also useful for the treatment or prevention of other diseases, since different CA isozymes are widely distributed in higher vertebrates [2–4]. In these organisms, including humans, the physiological functions of CAs have thoroughly been

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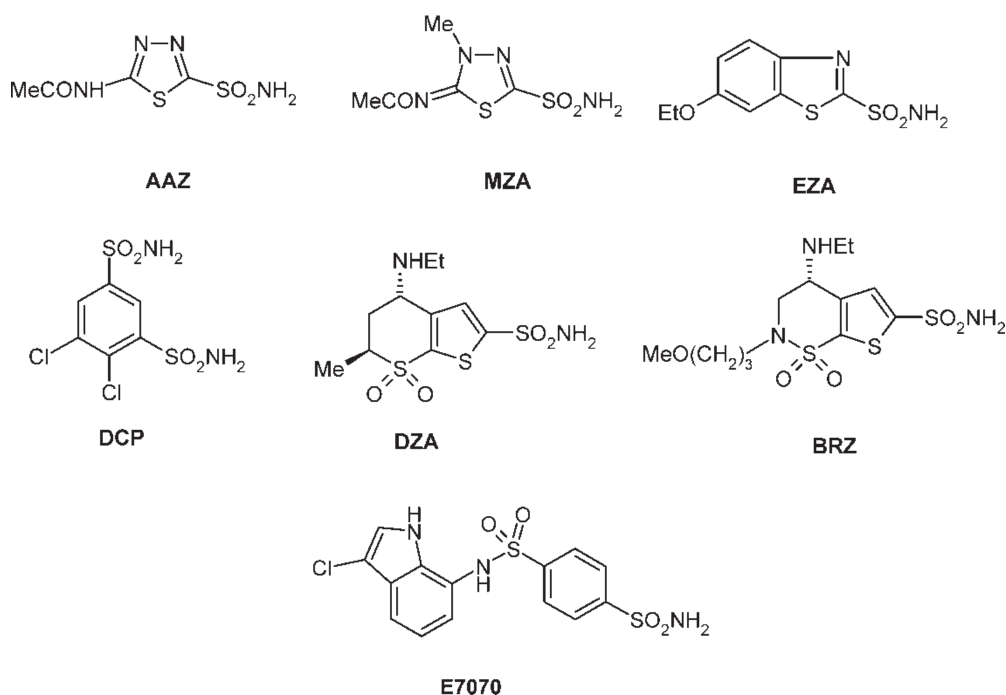
investigated over the past years [2–5]. Thus, isozymes CA I, II and IV are involved in respiration and regulation of the acid/base homeostasy. These complex processes involve both the transport of CO_2/bicarbonate between metabolizing tissues and excretion sites (lungs, kidneys), facilitated CO_2 elimination in capillaries and pulmonary microvasculature, elimination of H^+ ions in the renal tubules and collecting ducts, as well as reabsorption of bicarbonate in the brush border and thick ascending Henle loop in kidneys [2–4]. CA II is also involved in bone development and function, such as the differentiation of osteoclasts, or the provision of acid for bone resorption in osteoclasts [2–4]. Different CAs are involved in the secretion of electrolytes in many other tissues/organs, such as: cerebrospinal fluid formation, by providing bicarbonate and regulating the pH in the choroid plexus; gastric acid production in the stomach parietal cells; bile production, pancreatic juice secretion, intestinal ion transport, etc [1–4]. Some isozymes, such as CA V are involved in molecular signalling processes, such as insulin secretion signalling in pancreas β cells, whereas CA II and V are involved in important metabolic processes, as they provide bicarbonate for gluconeogenesis, fatty acids *de novo* biosynthesis or pyrimidine base synthesis [5]. Finally, some isozymes (such as CA IX, CA XII, CARP VIII) are highly abundant in tumors, being involved in oncogenesis and tumor progression, and generally lacking from the corresponding non-cancerous cells [1–7]. Thus, it is not at all surprising that many CAIs were recently shown to possess potent antitumor properties *in vitro* and *in vivo* [8–10], with one sulfonamide of this type, E7070 in Phase II clinical trials as an antitumor agent [11–13].

We have recently reported the first inhibition studies of the transmembrane, tumor-associated isozyme IX, with both sulfonamide and sulfamates, and detected several low nanomolar inhibitors, potentially useful for the development of novel antitumor therapies [14–18]. Here we continue these investigations, reporting the CA IX and CA XII inhibitory properties of a series of fluorinated sulfonamides. We decided to investigate this type of compounds since in a previous contribution from our laboratory [19], it was shown that by attaching perfluoroalkyl/aryl- carboxamido/sulfonamido tails, such as for example perfluorobutylsulfonyl, perfluorooctyl- carboxamido, perfluorophenylcarboxamido or perfluorophenylsulfonyl, to the aromatic/heterocyclic sulfonamides also incorporating derivatizable amino moieties, of types **1–14**, very effective CAIs could be obtained, which also showed good water solubility and efficacy as topically acting antiglaucoma agents in an animal model of the disease. The best CAIs in that series of derivatives were those incorporating the perfluorinated aromatic moieties (such as C_6F_5CO and $C_6F_5SO_2$), a fact that was then explained after the report of the high resolution X-ray crystal structure of isozyme CA II with one of these agents, the perfluorobenzoylated analog of methazolamide [20]. This compound is almost 10-fold more effective as a CA II inhibitor (K_I of 1.5 nM) compared to methazolamide (K_I of 14 nM). Its binding to the enzyme active site was shown to be similar to that of other sulfonamide inhibitors, when considering the interactions of the sulfonamide zinc anchoring group and thiadiazoline ring contacts [21] but differed considerably when the perfluorobenzoylimino fragment of the molecule was analyzed. Thus, several unprecedented strong



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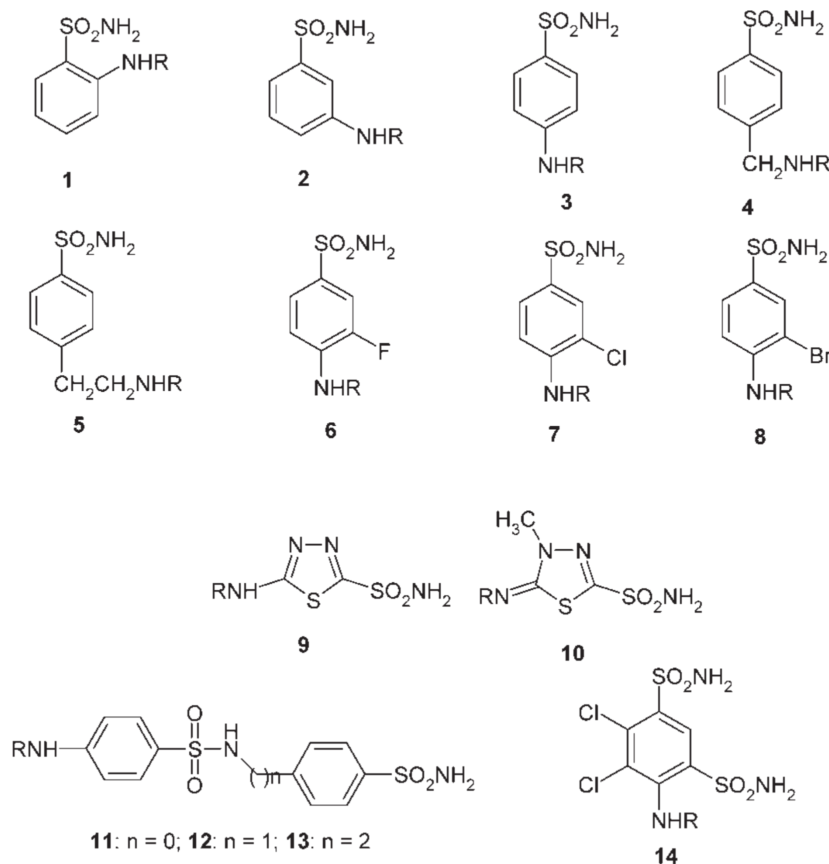
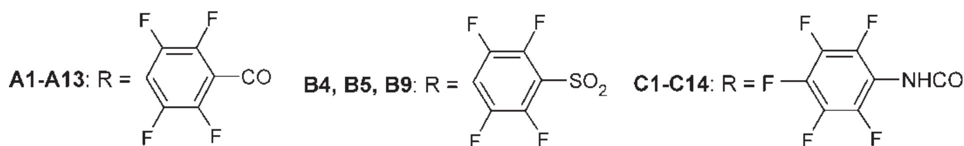
hydrogen bonds involving the imino nitrogen, carbonyl oxygen, a fluorine atom in the *meta-position* belonging to the inhibitor, and two water molecules, as well as Gln 92 of the enzyme active site were seen. A stacking interaction of the perfluorophenyl ring of the inhibitor and the aromatic ring of Phe 131 was also observed for the first time in a CA – sulfonamide adduct [20]. All these findings proved that the polyfluorophenyl tails of such CAIs are indeed beneficial for obtaining very potent and bioavailable CAIs. Here we report an inhibition study of the tumor-associated isozymes CA IX and CA XII with such a series of sulfonamides incorporating structurally related polyfluorinated-phenyl moieties, (2,3,5,6-tetrafluorobenzoyl, 2,3,5,6-tetrafluorophenylsulfonyl- or pentafluoroureido-), recently reported by our group [22,23], but which have not been investigated in detail for their interaction with the tumor-associated isozymes. This is also one of the first CA XII inhibition studies ever reported, since this second tumor-associated isozyme has been much less investigated up to now, as compared to CA IX [24].

Materials and methods

The 22 sulfonamide derivatives incorporating fluorinated moieties, of types **A1-A13, B4, B5, B9** and **C1–C14** were recently reported by this group [22], but were not investigated for their interaction with the tumor-associated isozyme hCA XII, and some of them were also not investigated for their interaction with hCA IX.

The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al. [6]) 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 homogenised in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100, as described earlier [15–18]. The homogenate was extensively centrifuged (11,000 × g) in order to remove soluble and membrane associated proteins as well as other cellular





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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, and 1 mM oxidized glutathione. Active hCA IX was extensively dialysed into a solution of 10mM Hepes (pH 7.5), 10mM Tris HCl, 100mM Na_2SO_4 and 1mM $ZnCl_2$. The protein was further purified by sulfonamide affinity chromatography [25], the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate [25]. The activity of this preparation was identical to that reported in the literature [15–18], with kcat/Km of 55 $\mu M^{-1}\ s^{-1}$.

The CA XII was obtained by working with a CA XII-glutathione-S-transferase (GST) fusion protein construct (Amersham), as follows. The cDNA fragment encoding the open reading frame of hCA XII was obtained by RT-PCR with poly(A)RNA from the human pancreas (Clontech, Palo Alto, CA). The poly(A)RNA (0.1 μg) was reverse-transcribed with random hexamers by using a commercial kit (Takara, Kyoto, Japan). The resultant cDNA was amplified by PCR using adopter primers including *Bam* HI and *Sal* I recognition sequences (underlined in the following sequences, respectively): 5″-TTTGGATCCATGCC-CCGGCGCAGCCTGCAC-3″ and 5″- TTTGTC-GACTCAAGCGTGGGCCTCAGTCTC-3″. The PCR reaction was hot-started with incubation for 2 min at 94°C and consisted of 35 cycles of 30 s at 94°C, 30 s at 56°C and 1.5 min at 72°C. CA XII cDNA was cloned in-frame into the *Bam* HI/*Sal* I site of a pGEX-4T-2 vector to produce a fusion protein with glutathione S-transferase (GST) (Amersham, Piscataway, NJ). The proper cDNA sequence of the CA XII insert included in the vector was reconfirmed by DNA sequencing and then transfected into competent bacteria (*E. coli* JM109) [26].

The PCR products were cleaved with the corresponding restriction enzymes, purified and cloned into a modified pGEX-4T2 vector using T4-ligase (Promega). The constructs were then transfected into *E. coli* strain BL21 for production of the CA XII protein, similarly to the procedure already described for hCA IX [14–18]. The protein expression was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside, the cells were harvested when the OD_{600} reached 1.00 and lysed by sonication in PBS. The cell homogenate was incubated at room temperature for 15 min and homogenized with a Polytron (Brinkmann) twice for 30 s each at 4°C. Centrifugation at 30,000 × g for 30 min afforded a supernatant containing the soluble proteins. The obtained supernatant was then applied to a prepacked Glutathione Sepharose 4B column, extensively washed with buffer and the fusion (GST-CA XII) protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin. The advantage of this method is that CA XII is purified quite easily and the procedure is quite simple. The obtained CA XII was further purified by sulfonamide affinity chromatography [25], the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate [25].

An SX.18MV-R Applied Photophysics stopped-flow instrument was used for activity measurements. Phenol red (at a concentration of 0.2 mM) was 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. 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. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results [25]. Human isozymes CA I and CA II were from Sigma-Aldrich (Milan, Italy).

Results and discussion

As mentioned earlier, CA IX and CA XII are predominantly found in many types of tumor cells, lacking from the corresponding non-cancerous counterparts [1,6,7,24], being thus considered the main target isozymes of the sulfonamides which were reported to possess anticancer effects *in vitro* and *in vivo* [5,8,14–18]. Very recent evidence suggests that CA II may also be involved in tumorigenesis, since the translation inhibitor pdcd4 or a sulfonamide CAI (ethoxzolamide) with low nanomolar affinity for this isozyme, were shown to repress endocrine tumor cell growth by suppression of CA II activity [27]. But it should be mentioned that ethoxzolamide is also a potent inhibitors of other CA isozymes involved in tumorigenesis, such as CA IX [15], and as will be presented shortly, of CA XII too. As this group recently reported the proof-of-concept study [1] that the tumor-associated isozyme CA IX is involved in the extracellular acidification of hypoxic tumors and that this process is reversed by inhibiting the enzyme with sulfonamides, it is particularly important to detect compounds that possess both high affinity for CA IX (and possibly also for the second tumor-associated isozyme, CA XII) as well as appropriate physico-chemical properties

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enabling them to be investigated as antitumor drugs. One such property is the water solubility of such sulfonamides, and we have shown [19,22] that fluorine-containing sulfonamides, as those investigated, here generally possess an enhanced water solubility compared to the corresponding non-fluorinated derivatives.

CA inhibition data against four human isozymes, hCA I, hCA II, hCA IX and hCA XII, with the fluorinated sulfonamides **A1**–**A13**, **B4**, **B5**, **B9** and **C1**–**C14**, as well as standard, clinically used CAIs are shown in Table I. Since the CA I, II and IX inhibition data of the investigated derivatives have been reported previously [23] (except the CA IX data of compounds **C1**–**C14**, which are reported here for the first time), we shall concentrate our discussion on the CA XII inhibitory properties of these derivatives. Very briefly, however, it may be mentioned that all the compounds investigated here act as better CAIs than the parent sulfonamides from which they were obtained (data not shown) [22,23]. The following SAR can be drawn from the data of Table I: (i) with few exceptions (compounds **A6**, **A9**, **A10**, **B9** and **C9**), the CAIs investigated here are more potent CA IX inhibitors than CA II (or CA I) inhibitors, a fact rarely observed up to now for other investigated sulfonamides/sulfamates; [14–18] (ii) the affinity of these sulfonamides for hCA I was medium–low, except for one derivative, **B9**, which behaved as a very potent hCA I inhibitor (K_I of 13–nM). Indeed, the other fluorinated sulfonamides showed K_I values in the range of 250–6100 nM, being as inhibitory (or slightly less) as the clinically used compounds (except dorzolamide which is a very weak CA I inhibitor); (iii) against hCA II, the investigated compounds generally behaved as very potent inhibitors, with K_I values in the range of 0.7–16 nM, except for compounds **A1**, **A2**, **A7**, **A8**, **A9**, **C1** and **C2**, which were slightly less effective inhibitors, with K_I values in the range of 19–84 nM; (iv) against hCA IX, some of these compounds are the most effective inhibitors ever reported up to now [14–18], with the first subnanomolar inhibitor detected (compound **A2**, with a K_I of 0.8 nM) [23]. Other derivatives, such as **A1**, **A4**, **A5**, **A8**, **A11**–**A13**, **B4**, **B5**, **B9**, **C3**, **C4**, **C9** and **C14** showed K_I values in the range 3.2–9.5 nM, being very potent CA IX inhibitors. The less effective CA IX inhibitors were

Table I. CA inhibition data with standard, clinically used inhibitors and the fluorinated sulfonamides investigated in the present study, against human isozymes I, II, IX and XII.

No Inhibitor	K_I* (nM)				Selectivity ratio[b]
	hCA I[a]	hCA II[a]	hCA IX[a]	hCA XII[a]	$K_{I(hCA\ II)}/K_{I(hCA\ XII)}$
AAZ	900	12	25	5.7	2.10
MZA	780	14	27	3.4	4.11
EZA	25	8	34	22	0.36
DCP	1200	38	50	50	0.76
DZA	50000	9	52	3.5	2.57
BRZ	–	3	37	3.0	1.00
E7070	31	15	24	3.4	4.41
A1	1500	38	9.5	46	0.82
A2	1700	21	0.8	3.5	6.00
A3	975	16	15.2	25	0.64
A4	980	13	9.5	18	0.72
A5	900	12	7.3	16	0.75
A6	760	1.5	24	21	0.07
A7	1080	76	16	35	2.17
A8	1870	84	4.8	15	5.60
A9	250	2.1	12.6	1.3	1.61
A10	270	1.4	10.5	0.9	1.55
A11	6300	19	3.2	1.5	12.66
A12	6100	15	5.5	0.8	18.75
A13	6000	13	7.6	0.7	18.57
B4	860	12	8.5	14	0.85
B5	750	9	7.0	11	0.81
B9	13	0.7	4.3	1.0	0.70
C1	1750	44	6.3	59	0.74
C2	1800	30	13	18	1.66
C3	950	8	3.5	13	0.61
C4	1000	15	3.8	20	0.75
C9	270	3.6	4.0	1.6	2.25
C14	740	15	3.1	1.5	10.00

* Errors in the range of 5–10% of the reported value (from 3 different assays).
[a] Human (cloned) isozymes (catalytic domains for hCA IX and hCA XII) [28].
[b] Selectivity ratio towards hCA XII as compared to hCA II.

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A3, **A6**, **A7**, **A9**, **A10**, and **C2**, which possess K_I values in the range 10.5–24 nM, i.e., they appreciably inhibit the tumor-associated isozyme, being more potent than the clinically used sulfonamides, and equipotent to the antitumor sulfonamide in clinical trials, E7070 (Table I). It may be observed that the best CA IX inhibitors incorporate metanilamide, sulfanilyl-sulfanilamide and sulfanilyl-homosulfanilamide moieties (compounds **A2**, **A11** and **A12**) and tetrafluorobenzoyl tails; (v) against CA XII, most of the sulfonamides investigated here showed excellent inhibitory properties. Thus, a group of derivatives, such as **EZA**, **DCP**, **A1**, **A3**, **A4**, **A6**, **A7** and **C1** showed good CA XII inhibitory properties, with inhibition constants in the range 21–59 nM. It may be observed that except **EZA**, all these compounds are aromatic, benzenesulfonamide derivatives. Several other derivatives, such as **A5**, **A8**, **B4**, **B5** and **C2**–**C4** were more effective CA XII inhibitors as compared to the previously discussed compounds, with K_I values in the range 11–20 nM. Finally, a large number of the investigated compounds, such as the clinically used derivatives **AAZ**, **MZA**, **DZA**, **BRZ** and **E7070**0, as well as the fluorinated sulfonamides **A2**, **A9**–**A13**, **B9**, **C9** and **C1**4, showed excellent CA XII inhibitory properties, with K_I values in the range of 0.7–5.7 nM. From all these data it is clear that the most important parameter influencing CA XII inhibitory properties in this series of compounds is the nature of the mother sulfonamide **1**–**14** to which the different fluorinated tails have been attached. It may be observed that particularly strong inhibitors were obtained by using 1,3,4-thiadiazole-2-sulfonamide (**9**), the corresponding thiadiazoline-sulfonamide (**10**) or sulfanilyl-sulfonamides (of types **11**–**13**) scaffolds incorporating such fluorinated tails. It should also be mentioned that in some cases, inhibitors with better affinity for CA XII than for CA II have been obtained. Thus, selectivity ratios in the range of 4.11–18.75 have been observed for **MZA**, **E7070**, **A2**, **A8**, **A11**–**A13** and **C1**4. The most CA XII selective compound was **A1**2, which is a subnanomolar inhibitor of this isozyme, as well as a very good CA IX inhibitor (Table I). Correlated with the fact that it shows good water solubility,[22] this compound seems to be quite promising for *in vivo* investigations as an antitumor sulfonamide. Other investigated compounds showed modest selectivity ratios for inhibiting hCA XII over hCA II (such as **AAZ**, **DZA**, **A7** and **C9**) or were better CA II than CA XII inhibitors; (vi) the compounds incorporating tetrafluorosulfonyl moieties were generally slightly more effective CAIs as compared to the corresponding derivatives incorporating tetrafluorobenzoyl moieties, but the differences in activity were insignificant (also it should be stressed not the entire series was available) Compounds incorporating the pentafluorophenylureido tails also showed effective CA inhibitory properties. Thus, all three tails were good for the design of efficient CA II, CA IX and CA XII inhibitors.

Conclusions

In a series of 2,3,5,6-tetrafluorophenyl-carboxamido/sulfonamido and pentafluoro- phenylureido- aromatic/heterocyclic sulfonamides, very potent inhibitors against the transmembrane, tumor-associated isozymes CA IX and CA XII were detected, some of them showing also selectivity ratios favorable to CA XII over CA II, the other physiologically relevant isozyme with high affinity for sulfonamide inhibitors. The first subnanomolar CA XII inhibitors have been reported in this contribution. The present derivatives constitute valuable candidates for the development of novel antitumor therapies based on the inhibition of tumor-associated CA isozymes, such as CA IX and/or CA XII among others.

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