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Carbonic anhydrase inhibitors: The first selective, membrane-impermeant inhibitors targeting the tumor-associated isozyme IX

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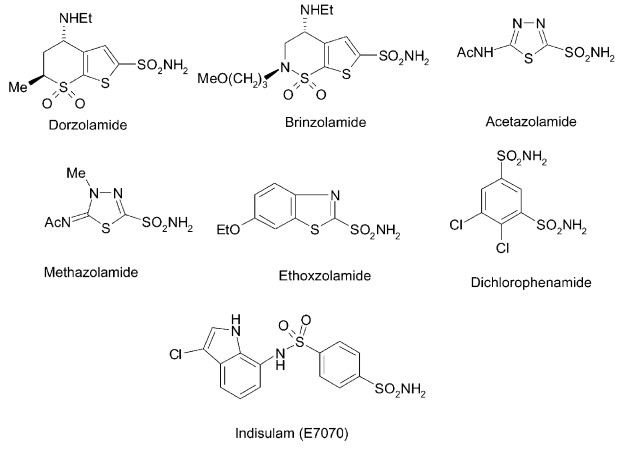
Abstract—The inhibition of the tumor-associated transmembrane carbonic anhydrase IX (CA IX) isozyme possessing an extracellular active site has been investigated with a series of positively-charged, pyridinium derivatives of sulfanilamide, homosulfanilamide and 4-aminoethylbenzenesulfonamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) were also provided for comparison. A very interesting inhibition profile against CA IX with these sulfonamides has been observed. Several nanomolar (K_i's in the range of 6–54 nM) CA IX inhibitors have also been detected. Because CA IX is a highly active isozyme predominantly expressed in tumor tissues with bad prognosis of disease progression, this finding is very promising for the potential design of CA IX-specific inhibitors with applications as anti-tumor agents. This is the first report of inhibitors that may selectively target CA IX, due to their membrane-impermeability and high affinity for this clinically relevant isozyme.

1. Introduction

Sulfonamide inhibitors of the metallo-enzyme carbonic anhydrase (CA, EC 4.2.1.1) are extensively used in clinical medicine and as diagnostic tools, for the treatment of glaucoma and macular edema, diverse neuro-muscular disorders, or investigated as potential antitumor drugs.[1–5] Six such drugs were or are still used clinically, such as the topically acting antiglaucoma drugs dorzolamide and brinzolamide, the systemic inhibitors acetazolamide, methazolamide, ethoxzolamide and dichloro phenamide, whereas indisulam (E7070) is in phase II clinical trials as an antitumor sulfonamide with a complex mechanism of action also involving CA inhibition of several isozymes involved in tumorigenesis.[4,5]



As mentioned above, most of these compounds are systemically acting inhibitors showing several undesired side effects due to inhibition of many of the different CA isozymes present in the target tissue/organ (14 isoforms are presently known in humans).[1,2] Therefore, many attempts to design and synthesize new sulfonamides were recently reported, in order to avoid such side effects.[6–10] At least four CA isozymes (CA IV, CA IX, CA XII and CA XIV) are associated to cell membranes, with the enzyme active site generally oriented extra-

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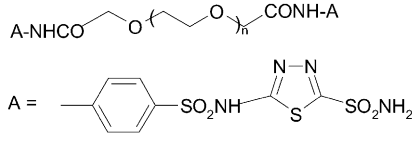
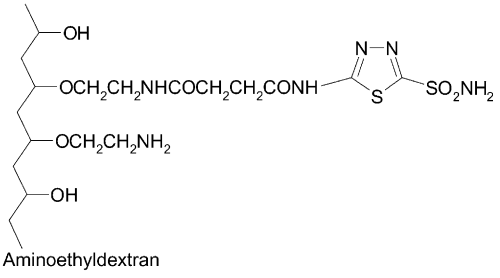
cellularly.[1,2] Some of these isozymes were shown to play pivotal physiological roles (such as for example CA IV and XII in the eye, lung and kidney, CA IX in the gastric mucosa and many tumor cells)[1–5] whereas the function of other such isozymes (CA XIV) is for the moment less well understood.[1] Due to the extracellular location of these isozymes, it would be possible to design membrane-impermeant CA inhibitors (CAIs), which in this way would become specific inhibitors for the membrane-associated CAs. This possibility has been recently explored in this laboratory, by designing positively-charged sulfonamides,[11] whereas an alternative approach consisted in designing polymeric (high molecular weight) inhibitors, but such compounds were not very useful in vivo due to the usual problems connected with polymers (i.e., allergic reactions, problems of bioavailability, etc).[12]

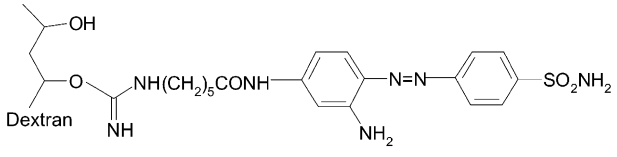
The first approach towards introducing the membrane-impermeability to CAIs from the historical point of view was that of attaching aromatic/heterocyclic sulfonamides to polymers, such as polyethyleneglycole, aminoethyldextran, or dextran.[12] Compounds such as **1–3**, possessing molecular weights in the range of 3.5–99 kDa, prepared in this way, showed indeed membrane-impermeability due to their high molecular weights, and selectively inhibited in vivo only CA IV and not the cytosolic isozymes (primarily CA II), being used in several renal and pulmonary physiological studies.[12] Due to their macromolecular nature, such inhibitors could not be developed as drugs/diagnostic tools, since in vivo they induced potent allergic reactions.[1,12] A second approach for achieving membrane-impermeability is that of using highly polar, salt-like compounds. Only one such sulfonamide has till recently been used in physiological studies, QAS (quaternary ammonium sulfanilamide) **4**, which has been reported to inhibit only extracellular CA-s in a variety of arthropods (such as the crab *Callinectes sapidus*) and fish.[13] The main draw-back of QAS is its high toxicity in higher vertebrates.[13]



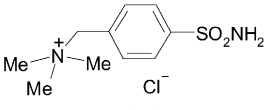
1: M_r = 3.5 kDa



2: M_r = 6.7 - 99 kDa

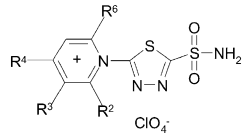


3: Mr = 5, 100 and 1000 kDa

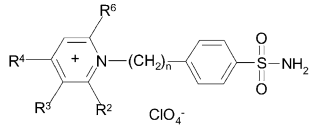


4: QAS

Thus, a program of developing cationic sulfonamides has been initiated in our laboratory using QAS **4** as a lead molecule, which is also a relatively weak CAI, with micromolar affinity for hCA II.[14–17] Such compounds, of types **5–8**, were prepared by reaction of aromatic/heterocyclic sulfonamides containing free NH_2 groups with pyrylium salts, affording pyridinium derivatives. The inhibitors **5–8** obtained in this way, showed nanomolar affinities both for CA II, as well as CA IV, and more importantly, they were unable to cross the plasma membranes in vivo.[15–18] In two model systems (human red cells, and perfusion experiments in rats, respectively), this new class of potent, positively charged CAIs, was able to discriminate for the membrane-bound versus the cytosolic isozymes, selectively inhibiting only CA IV (the only membrane-bound isozyme present in the investigated systems).[15–17] Such data constituted the proof-of-concept for the specific in vivo inhibition of membrane-associated CA isozymes, but also for the eventual development of novel anticancer therapies, since it has been shown that some tumor cells predominantly express only some membrane-associated CA isozymes, such as CA IX and CA XII.[1,4,5] This type of selective CAI may also be of great relevance for different physiological studies. For example, Sterling et al.[18] investigated the functional and physical relationship between the downregulated in adenoma bicarbonate transporter and CA II, by using membrane-impermeant inhibitors of type **5** (in addition to the classical inhibitors such as acetazolamide), which could clearly discriminate between the contribution of the cytosolic and membrane-associated isozymes in these physiological processes.



5



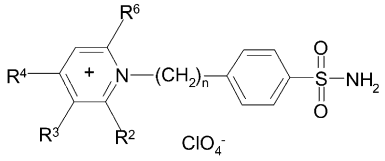
6: n = 0
7: n = 1
8: n = 2

Up to now no CA IX inhibition studies with this type of membrane-impermeant CAIs have been reported. Thus, we decided to explore some of the pyridinium derivatives of type **6–8** for their interaction with the catalytic domain of tumor-associated isozyme IX, recently cloned and purified by this group.[19–21]

2. Chemistry

Reaction of sulfanilamide, homosulfanilamide or 4-(2-aminoethyl)-benzenesulfonamide with 2,6-di-, 2,4,6-tri- or 2,3,4,6-tetrasubstituted pyrylium salts afforded the pyridinium salts **6–8** investigated here, by the general Bayer–Piccard synthesis.[15,22] The synthesis and characterization of these CAIs have been described in detail in a previous paper.[15]

Table 1. Inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX with the pyridinium salts **6–8**



6-8

Compd	R^2	R^3	R^4	R^6	K_i*			
					hCA I[a] (μM)	hCA II[a] (nM)	bCA IV[b] (nM)	hCA IX[c] (nM)
6a	Me	H	Me	Me	10	150	290	165
6b	Me	H	Ph	Me	7	60	211	48
6c	Et	H	Ph	Et	6	60	182	43
6d	*n*-Pr	H	Ph	*n*-Pr	10	120	194	178
6e	*i*-Pr	H	Ph	*i*-Pr	5	50	90	160
6f	Me	H	Ph	Ph	40	210	852	280
6g	Et	H	Ph	Ph	43	400	1300	450
6h	*n*-Pr	H	Ph	Ph	140	580	1483	> 500
6i	*i*-Pr	H	Ph	Ph	125	440	2102	> 500
6j	*n*-Bu	H	Ph	Ph	305	620	2155	> 500
6k	Ph	H	Ph	Ph	290	510	2500	> 500
6m	Me	Me	Me	Me	5	40	61	72
7a	Me	H	Me	Me	7	50	92	38
7b	*i*-Pr	H	Me	Me	6	50	80	42
7c	*i*-Pr	H	Me	*i*-Pr	11	80	144	54
7d	Me	H	Ph	Me	4	20	70	26
7e	Et	H	Ph	Et	2	21	52	29
7f	*n*-Pr	H	Ph	*n*-Pr	24	90	163	230
7g	*i*-Pr	H	Ph	*i*-Pr	12	61	101	100
7h	Me	H	Ph	Ph	32	121	161	64
7i	Et	H	Ph	Ph	42	314	983	79
7j	*n*-Pr	H	Ph	Ph	130	390	1260	85
7k	*i*-Pr	H	Ph	Ph	112	370	1214	80
7m	*n*-Bu	H	Ph	Ph	300	595	2104	135
7n	*t*-Bu	H	Ph	Ph	110	321	1070	> 500
7o	Ph	H	Ph	Ph	280	472	1956	120
7p	Ph	H	H	Ph	280	493	1954	106
7q	Me	Me	Me	Me	3	30	51	35
8a	Me	H	Me	Me	4	21	60	14
8b	*i*-Pr	H	Me	Me	2	15	32	31
8c	*i*-Pr	H	Me	*i*-Pr	3	20	70	49
8d	Me	H	Ph	Me	1	8	20	6
8e	Et	H	Ph	Et	1	9	21	8
8f	*n*-Pr	H	Ph	*n*-Pr	7	42	82	205
8g	*i*-Pr	H	Ph	*i*-Pr	6	21	70	89
8h	Me	H	Ph	Ph	18	103	144	37
8i	Et	H	Ph	Ph	40	220	761	70
8j	*n*-Pr	H	Ph	Ph	112	270	1055	84
8k	*i*-Pr	H	Ph	Ph	94	350	864	78
8m	*n*-Bu	H	Ph	Ph	290	544	2008	120
8n	*t*-Bu	H	Ph	Ph	92	275	1000	> 500
8o	Ph	H	Ph	Ph	270	419	1830	95
8p	Ph	H	H	Ph	265	420	1905	81
8q	Me	Me	Me	Me	2	10	21	8
Acetazolamide					0.25	12	70	25
Methazolamide					0.05	14	36	27
Dichlorophenamide					1.2	38	380	50
Indisulam					0.03	15	65	24

*Errors in the range of ±10% of the reported value, from three different determinations.
[a] Human (cloned) isozymes.
[b] From bovine lung microsomes.
[c] Catalytic domain of the human, cloned isozyme. Data for isozymes I, II and IV are from ref 15.

3. CA inhibition

Inhibition data against isozymes I, II and IV with compounds **6–8** were previously published,[15] and will not be discussed here. We shall focus on the CA IX inhibition with these membrane-impermeant compounds and the eventual isozyme-selectivity issues for this class of CAIs. Data of Table 1 clearly show that most of the compounds **6–8** act as efficient CA IX inhibitors, and that their affinity for this isozyme differs considerably as compared to affinities for the cytosolic isozymes CA I and II, and the other membrane-associated isozyme investigated, CA IV. The following SAR can be drawn from data of Table 1: (i) for a given substitution pattern of the pyridinium ring, the 4-aminoethyl-benzenesulfonamide derivatives **8** were more active than the corresponding homosulfanilamide derivatives **7**, which in turn were more active than the corresponding sulfanilamides **6**. This behavior has also been observed for the other three investigated isozymes;[15] (ii) some of the derivatives possessing bulky substitutents at the pyridinium ring (mainly phenyls, *tert*-butyls; *n*-butyl, *n*-propyl or *iso*-propyl), such as **6h–6k**, **7n** and **8n**, were very ineffective CA IX inhibitors, showing inhibition constants >500 nM; (iii) another group of compounds, including **6a**, **6d–6g**, **7f**, and **8f** showed a moderate inhibitory power towards the tumor-associated isozyme IX, showing K_i values in the range of 160–450 nM. Most of these compounds are sulfanilamide derivatives (except **7f** and **8f**), and the substitution pattern at the pyridinium ring includes (with one exception, **6a**) at least one phenyl group in 4, or two phenyls in the 2 and 4 positions. It should be noted that the corresponding homosulfanilamides and 4-aminoethylbenzenesulfonamides incorporating the same substitution pattern as the compounds mentioned above (of type **6**), lead to much better CA IX inhibitors (see later in the text); (iv) a third group of derivatives, including **6m**, **7g–7m**, **7o**, **7p**, **8g**, **8i–8m**, **8o** and **8p**, showed good CA IX inhibitory properties, with K_i values in the range of 64–135 nM. As mentioned above, except for the tetramethyl-pyridinium-substituted derivative **6m**, most of these compounds incorporate 4-phenyl-pyridinium or 2,4-diphenylpyridinium moieties, whereas the group in position 6 is generally quite variable (alkyls or phenyl are tolerated). The most interesting observation regarding this subtype of CA IX inhibitors is constituted by the fact that the 2,4,6-triphenyl-pyridinium- and 2,6-diphenyl-pyridinium derivatives of homosulfanilamide and 4-aminoethylbenzenesulfonamide (**7o**,**p** and **8o**,**p**) efficiently inhibit isozyme IX, although they act as very weak inhibitors for isozymes I, II and IV (Table 1). As it will be discussed shortly, this may be due to the fact that the hCA IX active site is larger than that of the other investigated isozymes, notably CA II, I and IV; (v) a last group of derivatives (**6b**,**c**; **7a–7e**; **7q**; **8a–8e**; **8h** and **8q**) showed very good CA IX inhibitory properties, these compounds possessing K_i values in the range of 6–54 nM, similarly to the clinically used inhibitors acetazolamide, methazolamide, dichlorophenamide and indisulam, for which the inhibition data are provided for comparison. It should be noted that three derivatives **8d**, **8e** and **8q** showed inhibition constants <10 nM, these being the most potent CA IX inhibitors ever reported up to now. Correlated with their membrane-impermeability,[15,18] it may be assumed that in vivo such compounds may lead for the first time to a selective CA IX inhibition. Thus, the best substitution pattern at the pyridinium ring includes either only compact alkyls (**7a–c**, **7q**, **8a** and **8q**), or 2,6-dialkyl-4-phenyl-pyridinium substituents (all compounds mentioned above except **8h**, which incorporates a 2-methyl-4,6-diphenylpyridinium ring); (vi) the number of the substitutents at the pyridinium ring seems to be less important for the activity of this series of CAIs, since both di-, tri- or tetrasubstituted derivatives showed good inhibitory potency. The nature of these groups on the other hand—as discussed in detail above—is the most important parameter influencing CA inhibitory properties (together with the linker between the benzenesulfonamide moiety and the substituted pyridinium ring); (vii) the isozyme most similar to hCA IX regarding the affinity for these inhibitors was hCA II (which has 33% homology with hCA IX)[23] whereas the affinities of isozymes I and IV were rather different.

At this point the most important questions are: how can one explain these inhibition data and what is their relevance for the possibility to obtain CA IX specific inhibitors? For replying to them, one must consider first that no X-ray crystal structure of isozyme IX is available up to now, although many attempts have been done to crystallize this protein (unpublished data from this laboratory). This is in strong contrast with hCA II, for which many X-ray crystal structures are available, alone or in complexes with inhibitors and activators.[24–26] Examining the active site residues of these two isozymes and the architecture of hCA II, may afford some answers to the above questions. First of all, the zinc ligands and the proton shuttle residue of these two isozymes are identical.[2,19–21,23] An important difference is constituted by the amino acid in position 131, which is Phe for hCA II and Val for hCA IX. Phe 131 is known to be very important for the binding of sulfonamide inhibitors to hCA II:[25b,27] in many cases this bulky side chain limits the space available for the inhibitor aromatic moieties, or it may participate in stacking interactions with groups present in it (for recent examples see refs[25b,27]). Thus, the presence of a less bulky such residue in hCA IX (i.e., a valine) which is also unavailable for participation to stacking interactions has as a consequence the fact that the hCA IX active site is larger than that of hCA II. A second residue that drew our attention is 132, which is Gly in hCA II and Asp in hCA IX. This residue is situated on the rim of the hydrophilic half of the entrance to the active site of hCA II (and presumably also of hCA IX) and it is critical for the interaction with inhibitors possessing elongated molecules, as recently shown by us.[25c] Strong hydrogen bonds involving the CONH moiety of Gly 132 were shown to stabilize the complex of this isozyme with a *p*-aminoethylbenzenesulfonamide derived inhibitor.[25c] In the case of hCA IX, the presence of aspartic acid in this position at the entrance of the active site may signify that: (i) stronger interactions with polar moieties of the inhibitor bound within the active site should be possible, since the COOH moiety possesses more donor atoms; (ii) this residue may

have flexible conformations, fine-tuning in this way the interaction with inhibitors. Thus, the stronger hCA IX inhibition with some of these inhibitors (as compared to their affinity for isozyme II), such as for example **7h–m**, **7o**, **7p**, **8a**, **8d**, **8h** and **8o–q**, might be explained just by the different interactions with the two active site residues mentioned above. Anyhow, the final answers may arrive only after the report of the X-ray crystal structure of this isozyme and its complexes with inhibitors.

4. Conclusions

In a large series of substituted-pyridinium derived sulfanilamides, homosulfanilamides and *p*-aminoethylbenzenesulfonamides, a large number of effective hCA IX inhibitors were detected. Some low nanomolar CA IX inhibitors were reported for the first time. Since these compounds are membrane-impermeant due to their salt-like character, and as hCA IX is present on the extracellular side of many aggressive tumor cells, compounds of this type target specifically this tumor-associated CA isozyme without affecting the cytosolic CAs known to play important physiological functions. Thus, compounds of this type may constitute the basis of new anticancer therapies based on CA inhibitors.

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