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Carbonic anhydrase inhibitors. Synthesis of 2,4,6-trimethylpyridinium derivatives of 2-(hydrazinocarbonyl)-3-aryl-1*H*-indole-5-sulfonamides acting as potent inhibitors of the tumor-associated isoform IX and XII

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ABSTRACT

A series of 2-(hydrazinocarbonyl)-3-aryl-1*H*-indole-5-sulfonamides possessing various 2-, 3- or 4- substituted phenyl groups with methyl-, halogeno- and methoxy-functionalities, or a perfluorophenyl moiety, has been derivatized by reaction with 2,4,6-trimethylpyrylium perchlorate. The new sulfonamides were evaluated as inhibitors of four mammalian carbonic anhydrase (CA, EC 4.2.1.1) isoforms, that is, CA I, II (cytosolic), CA IX and XII (transmembrane, tumor-associated forms). Excellent inhibitory activity was observed against hCA IX with most of these sulfonamides, and against hCA XII with some of the new compounds. These compounds were generally less effective inhibitors of hCA II. Being membrane impermeant, these positively-charged sulfonamides are interesting candidates for targeting the tumor-associated CA IX and XII, as possible diagnostic tools or therapeutic agents.

In a recent communication from this group,[1] we investigated the interaction between 2-(hydrazinocarbonyl)-3-phenyl-1*H*-indole-5-sulfonamide (compound **L**), and 12 carbonic anhydrase (CA, EC 4.2.1.1) isoforms of mammalian (human, h or murine, m) origin. This sulfonamide showed a potent inhibition of CA I and II (K_Is of 7.2–7.5 nM), was a medium potency inhibitor of CA VII, IX, XII and XIV and showed weak inhibitory properties against the other isoforms, making it thus an interesting clinical candidate for situations in which a strong inhibition of CA I and II is needed. Furthermore, the inhibition profile of **L** was quite distinct from that of the clinically used compounds such as acetazolamide **AZA**, methazolamide, **MZA**, ethoxzolamide **EZA** or dichlorophenamide **DCP** which promiscuously inhibit most of these mammalian isoforms in the low nanomolar range.[2] Indeed, many of these CAs are medicinal chemistry targets for the development of diuretics, antiglaucoma, antiobesity, anticonvulsant or anticancer drugs/diagnostic tools.[2–4]

The crystal structure of the hCA II adduct with sulfonamide **L** reported earlier,[1] also revealed many favorable interactions between the inhibitor and the enzyme which explain its strong, low nanomolar affinity for isoform II (K_I of 7.2 nM) but may also be exploited for the design of effective inhibitors incorporating such bicyclic moieties.

As shown in Figure 1, where a schematic representation for the binding of sulfonamide **L** to the hCA II active site is presented, the inhibitor is coordinating to the Zn(II) ion within the enzyme active site in deprotonated state, with the SO_2NH^- group participating to a hydrogen bond with the OH of Thr199. The $CONHNH_2$ fragment of the inhibitor participates in a network of three hydrogen bonds with a water molecule (Wat101) and two amino acid residues known to interact with various inhibitors bound to the hCA II active site, that is, Asn62 and Asn67,[5–7] but it is placed in a solvent accessible region of the active site. Several van der Waals interactions between the inhibitor and amino acid residues lining the active site also favorably influence the strong binding of **L** to the enzyme cavity[1] (data not shown). The 3-phenyl moiety of **L** is accommodated in a hydrophobic region of the active site[1] where also enough additional space is available for various moieties to be introduced as substituents, allowing thus for further derivatization of this group. Using this X-ray crystal of the hCA II—**L** adduct structure as starting point, and the observation that probably the 3-phenyl and carboxamido moieties of **L** can be derivatized without losing the potent CA inhibitory properties of the obtained

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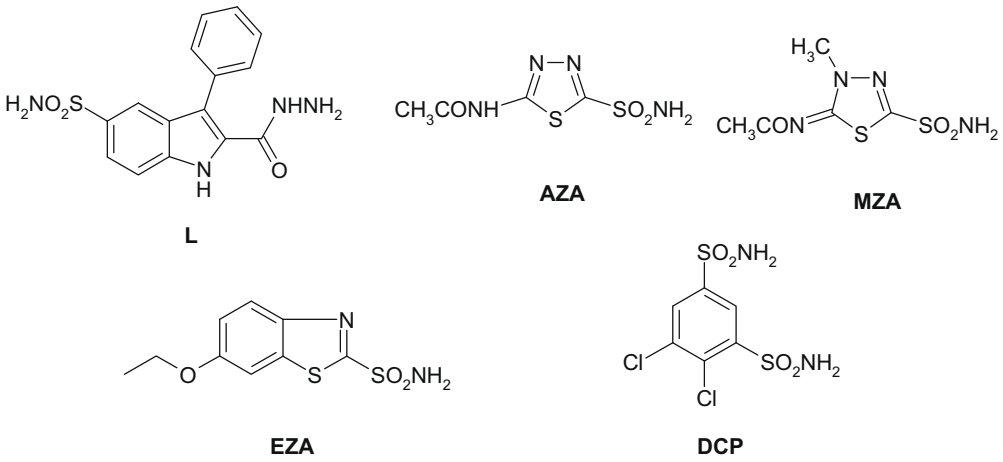


sulfonamides, we report here the synthesis and inhibitory activity against the physiologically relevant isoforms CA I, II (cytosolic, house-keeping enzymes) and CA IX and XII (transmembrane, tumor-associated CAs) of a series of sulfonamides incorporating derivatized phenyl moieties of the lead **L**, as well as pyridinium moieties obtained by reaction of the terminal amino group belonging to the $CONHNH_2$ functionality with 2,4,6-trimethylpyrylium perchlorate. We have in fact demonstrated previously that pyrylium salts can be used to generate positively charged, membrane impermeant pyridinium sulfonamides by reaction with various aromatic/heterocyclic amines also incorporating sulfamoyl moieties, and that many of these derivatives show excellent CA inhibitory properties.[8,9] Furthermore, some of them were crystallized in complex with hCA II,[7d] being thus useful for the drug design of tighter binding CA inhibitors (CAIs). Such a pyridinium sulfonamide CAI was crucially important for demonstrating the critical role that CA IX plays in hypoxic tumor acidification processes, which lead to metastatic spread of these tumors and lack of response to classical chemo- and radiotherapy.[10] In addition, in the same study we also showed that the tumor acidification processes

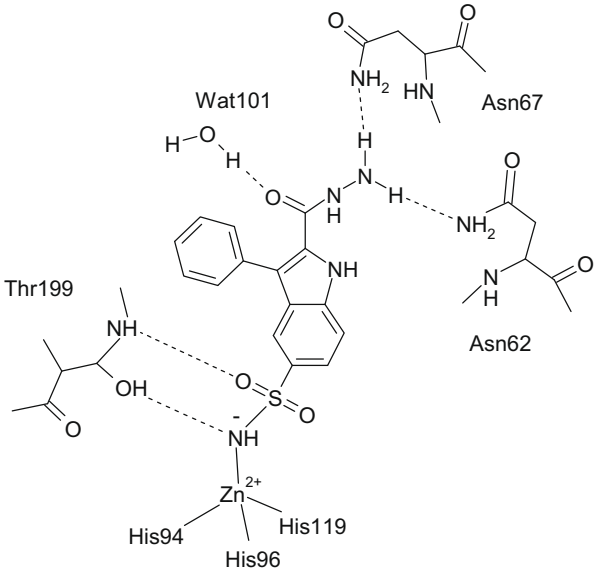


Figure 1. Schematic interactions to which **L** participates when bound within the hCA II active site, as determined by X-ray crystallography (PDB code 3B4F).[1] Hydrogen bonds in which several moieties of the inhibitor participate with amino acid residues Asn62, Asn67 and Thr199 from the enzyme active site and with a water molecule (Wat101) are shown as dotted lines.

can be reverted by inhibiting the CA IX activity with such sulfonamides.[10] Since the positively charged pyridinium sulfonamides are also unable to cross biological membranes,[8,9] and as CA IX has an extracellular active site,[2] this class of membrane-impermeant sulfonamides constitutes a very promising type of compounds for further evaluation and possible development as tumor therapeutics or diagnostic tools (see Scheme 1).[11]

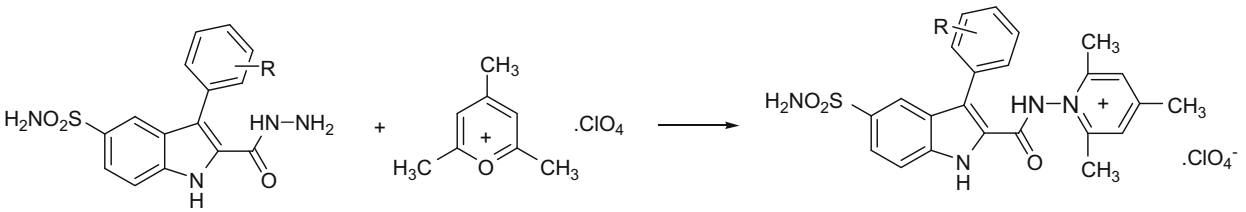
The reaction between pyrylium salts and amines, leading to pyridinium salts, has been discovered by Bayer and Piccard in 1911,[12] and applied thereafter for the preparation of a large number of such derivatives, unaccessible by other synthetic procedures.[13] Many biologically active compounds have been obtained in this way,[13] among which also different classes of sulfonamide CAIs.[8,9,13] The apparently simple reaction between a pyrylium salt and a primary amine, leading to pyridinium salts, is in reality a complicated process, as established by detailed spectroscopic and kinetic data from Balaban's and Katritzky's groups.[14] Thus, the nucleophilic attack of a primary amine RNH_2 on pyrylium cations generally occurs in the α position, with the formation of intermediates which are deprotonated in the presence of bases leading to 2-amino-tetradehydropyran derivatives. In many cases the deprotonation reaction is promoted by the amine itself, when this is basic enough. The obtained unstable 2-amino-tetradehydropyrans are tautomers with ketodieneamines, which are the key intermediates for the conversion of pyrylium into pyridinium salts.[13,14] In acidic media, in the rate-determining step of the whole process, the ketodieneamines are converted to the corresponding pyridinium salts, although other products, such as vinylogous amides with diverse structures have also been isolated in such reactions.[13,14]

The Bayer–Piccard synthesis was applied to sulfonamides **1**,[15] possessing a highly nucleophilic amino moiety incorporated in the carbohydrazide functionality, which were reacted with 2,4,6-trimethylpyrylium perchlorate **2**, leading thus to a series of pyridinium salts of type **3–17**.[16]

Data of Table 1 show the inhibitory activity against four physiologically relevant human CA isozymes (hCA I and II—cytosolic, ubiquitous forms, as well as hCA IX and XII, which are transmembrane, tumor-associated CAs with an extracellular active site)[2,3] of the new compounds **3–17** reported here, the lead molecule **L** as well as standard, clinically used sulfonamides such as **AZA**, **MZA**, **EZA** and **DCP**.

The following should be noted regarding inhibition data of Table 1:[17–20]

(i) Against the cytosolic isoform hCA I, the new pyridinium sulfonamides **3–17** generally showed very good inhibitory activity, with K_Is in the range of 3.2–113 nM, being thus more active than



Scheme 1. Preparation of 1-({[5-(aminosulfonyl)-3-substitutedphenyl-1*H*-indol-2-yl]carbonyl}amino)-2,4,6-trimethylpyridinium perchlorates **3**–**17**. Reagents and conditions: MeOH (reflux), 6% $HClO_4$.

the clinically used sulfonamides **AZA** and **DCP** (K_Is in the range 250–1200 nM). Most of these new derivatives, similarly to the lead **L**, were in fact low nanomolar hCA I inhibitors (K_Is in the range 3.2–30.8 nM), except for **8, 10** and **14** which were slightly less active (K_Is in the range 43.4–113 nM). Structure–activity relationship (SAR) is thus rather flat for most of them, with the various substitution patterns of the phenyl moiety not influencing drastically the activity of these compounds. However, most of them (except **9** and **15**) are slightly less inhibitory as compared to the lead molecule **L**. The very active derivatives (**9** and **15**) with K_Is of 3.2 and 5.1 nM, respectively, possess a substituent in the 4-position of the phenyl ring, which is however also present in other compounds with relatively weaker hCA I inhibitory activity. Overall these compounds are among the most effective hCA I inhibitors ever reported, and this may be a very important feature when inhibition of this isozyme is needed.[21] Recently, Feener's group[21] reported elevated levels of hCA I in vitreous from individuals with diabetic retinopathy, the enzyme being involved in retinal hemorrhage and erythrocyte lysis characteristic of this disease for which no effective cures are available. It was proved[21] that hCA I induced alkalinization of vitreous which increased kallikrein activity and generation of factor XIIa. Such results proved that inhibition of hCA I and/or kallikrein-mediated innate inflammation could provide new therapeutic opportunities for the treatment of hemorrhage-induced retinal and cerebral edema, but the presently available CAIs do not generally show good hCA I inhibitory activity (Table 1).[21] Some of the compounds reported here were 5–7.8 times more effective than ethoxzolamide **EZA**, one of the best clinically used hCA I inhibitors, but **EZA** is also a promiscuous inhibitor of most other CAs.[2]

(ii) Although the lead **L** showed excellent hCA II inhibitory activity (K_I of 7.2 nM), the derivatives **3–17** reported here were generally much less effective inhibitors of this ubiquitous isoform, with K_Is in the range of 38–3380 nM, except for the pentafluorophenyl derivative **17** which was a subnanomolar hCA II inhibitor (K_I of 0.93 nM). These data are indeed very interesting, as they prove that the substitution pattern of the 3-phenyl moiety in the pyridinium salts **3–17** is crucial for their hCA II inhibitory activity. Thus, a very active compound has been detected (**17**), together with moderate inhibitors (such as **3, 4, 6, 7, 9–15**, K_Is in the range 38–106 nM), as well as three very ineffective inhibitors (**5, 8**, and **16**, possessing K_Is in the range 1800–3380 nM). It should be noted that all these ineffective hCA II inhibitors have the substituent of the phenyl moiety in the *meta*-position, probably provoking a clash with some amino acid residues present in the hCA II active site, as already documented by us earlier.[5c] However, in the absence of detailed structural data for this class of inhibitors this remains a hypothesis to be checked. The pentafluorophenyl derivative **17** on the other hand, is 7.7 times more effective as a hCA II inhibitor compared to the lead **L**, and it would be also of great interest to resolve its high resolution X-ray crystal structure in adduct with hCA II for understanding the elements leading to this excellent inhibitory activity. Some of these tasks are being currently pursued in our laboratory.

(iii) The tumor associated isoform hCA IX, one of the important targets for obtaining novel antitumor therapies which ultimately emerged,[2,10,11,22] was generally excellently inhibited by the new compounds reported here, although the lead **L** itself is a moderate hCA IX inhibitor (K_I of 102 nM). Thus, except for **17** which was a quite weak hCA IX inhibitor (K_I of 706 nM) and the methoxy-substituted compound **16** (K_I of 116 nM), which is also a moderate inhibitor, all other pyridinium salts **3–15** reported here showed inhibition constants in the range of 8.1–49.6 nM, being thus much more inhibitory compared to **L**. Furthermore, many of these derivatives (e.g., **5–7, 9–13** and **15**) had K_Is < 10 nM, being thus more than 10 times better hCA IX inhibitors compared to **L**. This is a very important result, since CA IX inhibitors have the potential to be developed both as diagnostic tools[2,11,22] and chemotherapeutic agents[2,3,10] for hypoxic tumors overexpressing CA IX (and also CA XII, see later in the text).[22b] It is also important to note two additional features which make these compounds very attractive for further investigations in the field of antitumor drugs/diagnostic tools: they are membrane impermeant due to the presence of the cationic moieties in their molecules, being thus restricted to the extracellular space in vivo[8–10] (where the CA IX/XII active site is also situated), and many of them show rather modest or weak inhibitory activity towards CA II, an isoform which should preferably be not inhibited by an antitumor sulfonamide targeting CA IX. Indeed, as seen from data of Table 1, the presently available,

Table 1
Inhibition of CA isoforms hCA I, II, IX and XII with sulfonamides **3**–**17**, **L**, as well as clinically used CA inhibitors **SA**–**EZA** as standards

Inhibitor	R	K_I* (nM)			
		hCA I[a]	hCA II[a]	hCA IX[b]	hCA XII[b]
L	—	7.5	7.2	102	110
3	H	9.0	71	47.1	92
4	2-F	8.5	91	49.6	38
5	3-F	11.3	3380	9.4	9.7
6	4-F	7.6	65	8.3	9.5
7	2-Cl	25.1	100	9.3	58
8	3-Cl	113	1800	12.8	97
9	4-Cl	3.2	77	9.5	12.6
10	2-Br	43.4	38	9.6	3980
11	3-Br	30.8	74	9.4	6900
12	4-Br	12.3	85	8.8	5680
13	2-Me	10.5	106	8.1	8360
14	3-Me	110	104	39.8	6670
15	4-Me	5.1	68	8.8	6350
16	3-OMe	8.6	2840	116	8300
17	F_5	9.7	0.93	706	4190
AZA	—	250	10	25	5.7
MZA	—	50	14	27	3.4
EZA	—	25	8	34	22
DCP	—	1200	38	50	50

* Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[17]
[a] Human, recombinant isozymes.
[b] Catalytic domain of human, cloned isoform.[18–20]

clinically used sulfonamides although showing effective CA IX inhibitory activity (K_Is in the range of 25–50 nM), are much more potent hCA II (K_Is in the range of 8–38 nM) than hCA IX inhibitors.

(iv) Against the second tumor-associated isoform, hCA XII (less widespread as compared to hCA IX)[2,3] the new compounds reported here showed a mixed behavior, with three of them acting as very effective inhibitors (**5, 6** and **9**, K_Is in the range of 9.5–12.6 nM), whereas **3, 4, 7** and **8** being moderate inhibitors (K_Is in the range of 38–97 nM). The remaining derivatives acted as very weak hCA XII inhibitors (K_Is in the range of 3980–8360 nM). It is rather difficult to explain this SAR data, but it is obvious that small structural variations in the nature, number and position of substituents of the phenyl moiety in these pyridinium salts cause a large variation of the hCA XII inhibitory activity.

In conclusion, we reported here a series of 2-(hydrazinocarbonyl)-3-aryl-1*H*-indole-5-sulfonamides possessing various 2-, 3- or 4- substituted phenyl groups with methyl-, halogeno- and methoxy-, or the perfluorophenyl moieties in their molecule. They were derivatized by reaction with 2,4,6-trimethylpyrylium perchlorate, leading to the corresponding pyridinium derivatives. These compounds were evaluated as inhibitors of four mammalian CA isoforms, that is, CA I, II (cytosolic), CA IX and XII (transmembrane, tumor-associated forms). Good inhibitory activity was observed against hCA I and less effective inhibitors of hCA II, two cytosolic, house-keeping enzymes. Most of these compounds were excellent hCA IX inhibitors, whereas only some of them showed this feature against hCA XII. Being membrane impermeant due to the presence of the cationic moieties in their molecules, and thus restricted to the extracellular space where the CA IX/XII active sites are situated, these new positively-charged sulfonamides are interesting candidates for targeting the tumor-associated enzymes as possible diagnostic tools or therapeutic agents. In fact, very recently Pouyssegur's group[22b] demonstrated by using siRNA silencing, that the dual inhibition of CA IX and XII leads to an impressive 85% reduction of hypoxic tumor cell growth. Indeed, as concluded by these authors, the hypoxia-induced CA IX and CA XII are major tumor prosurvival pH-regulating enzymes, and their combined targeting by inhibitors as those reported here shows that they hold potential as interesting anticancer targets.

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16. 2,4,6-Trimethyl pyrylium perchlorate **2** (1.5 mM) was dissolved in 20 mL methanol. After addition of 2-(hydrazinocarbonyl)-3-substituted-phenyl-1*H*-indole-5-sulfonamide derivatives **1** (3 mM), the solution was refluxed overnight. The cold mixture was treated with 200 mL of 6% perchloric acid to precipitate the pyridinium salts. The obtained products were recrystallized from water with 6% perchloric acid. *1-({[5-(aminosulfonyl)-3-phenyl-1H-indol-2-yl]carbonyl}amino)-2,4,6 trimethylpyridinium perchlorate* ***3***. Yield 79%; mp > 300 °C (dec.); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.61 (9H, s, pyridinium 2,4,6-$(CH_3)_3$), 7.24 (2H, s, SO_2NH_2), 7.42–7.68 (6H, m, Ar-H), 7.70–7.84 (2H, m, Ar-H), 7.90 (2H, s, Ar-H), 8.05 (1H, s, CONH), 12.50 (1H, s, indole NH); LC/MS: *m/z* 436 $(M+H)^+$.
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