New Zinc Binding Motifs in the Design of Selective Carbonic Anhydrase Inhibitors

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Abstract: The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc enzymes which catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are involved in physiological and pathological processes. The different isozymes have been considered as important targets for inhibitors with clinical applications. Several sulfonamide carbonic anhydrase inhibitors (CAIs) were used for decades as diuretics, anti-glaucoma, anti-epileptic, anti-ulcer agents, or as drugs for treating other neurological/neuromuscular disorders, whereas presently several such agents still find wide applications in therapy, mainly as topically acting anti-glaucoma drugs, anti-cancer, or anti-obesity agents. Although sulfonamides were considered the moiety par excellence to coordinate the catalytic zinc and for designing potent CAIs, in recent years related functional groups such as sulfamate, sulfamide and others have proven to be successful in the design of selective CAIs. The present review will deal with these different zinc binding functions recently reported in literature.

INTRODUCTION

The zinc (II) ion is one of the most important transition metal ions with physiological function. This essential element plays catalytic, structural or regulatory roles in biological systems, with several hundred zinc proteins that have been identified up to now, belonging to each of the fundamental enzyme classes (oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases) [1-3]. Among them, the carbonic anhydrases (CAs, EC 4.2.1.1) are widespread zinc enzymes that catalyze the interconversion between carbon dioxide and bicarbonate at the physiological pH (a proton being also formed in this reaction). Carbonic anhydrase inhibitors (CAIs) are widely used therapeutic agents in the management or prevention of many diseases [4-7]. This is mainly due to the wide distribution of the 16 presently described human CA isozymes in many cells, tissues and organs, where they play crucial physiological functions. Still, the available pharmacological agents are far from being perfect, as they possess many undesired side effects, mainly due to their lack of selectivity for the different isozymes. Thus, development of isozyme-specific or at least organ-selective inhibitors would be highly beneficial both for obtaining novel types of drugs, devoid of major side effects, as well as for physiological studies in which specific/ selective inhibitors may constitute valuable tools for understanding the physiology/ physiopathology of these enzymes [4-7].

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The catalytic and inhibition mechanisms of these enzymes are understood in great detail, and this was helpful for the design of potent inhibitors. For the development of novel CAIs the knowledge of groups that can be incorporated into the inhibitors for coordinating the zinc ion, is critical. This metal coordination both leads to the enzyme inhibition and can provide significant binding energy through supplementary interactions between such zinc-binding functions and amino acid residues from the active site. Further elaboration of these core structures can be also used to develop specific inhibitors for the multitude of CA isoforms presently known in humans, of for the design of inhibitors of CAs belonging to other gene families, such as those present in bacteria, *Archaea*, etc.

Thus, up to recently, only sulfonamides of type $ArSO_2NH_2$ (where Ar is an aromatic or heterocyclic moiety) were known to possess high affinity for CAs. Some recent data indicate that potent CAIs may be designed from many other types of compounds [4-7]. Fig. (**1**) shows the most general structure of a CAI complexed to the enzyme active site. Thus, such a compound must possess: (i) a zinc binding function (ZBF) by which it interacts with the metal ion of the enzyme and the residues Thr199 and Glu106 in its neighbourhood; (ii) the organic scaffold – usually an aromatic or heterocyclic moiety, which may be present or absent in new generation CAIs; (iii) a tail attached to the scaffold, which usually was absent in the first and second generation of sulfonamide CAIs, but which is extremely important (and generally present) for the last generation of such derivatives. All these structural elements interact both with the hydrophobic as well as the hydrophylic halves of the active site, whereas ZBF interacts with Thr199 and Glu106 as shown in the Fig. (**1**). Thus, sulfonamides constitute just a particular case for this type of general interaction.

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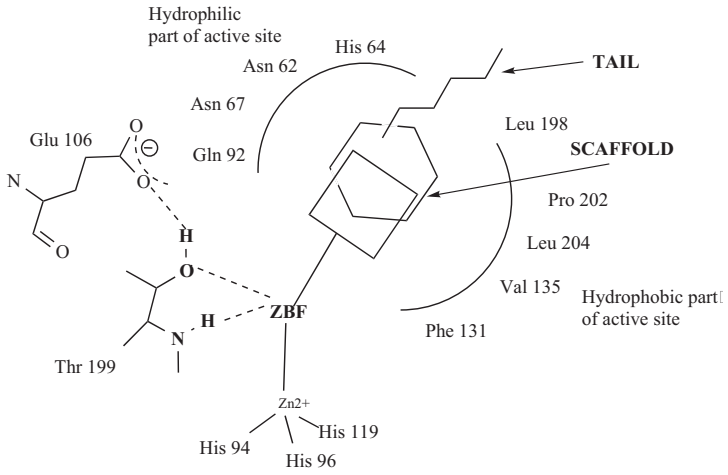


Fig. (1). The general structure of a CAI complexed to the enzyme (α-CA) active site: ZBF = zinc binding function; the organic scaffold may be present or absent; the tail too. These structural elements interact both with the hydrophobic as well as the hydrophilic halves of the active site (representative amino acid residues involved in binding are shown), whereas ZBF interacts with the Zn(II) ion as well as the neighbouring residues Thr 199 and Glu 106.

Incorporation of potent and eventually more selective ZBFs for the active site Zn(II) ion is necessary to improve the development of new generation inhibitors. Herein we highlight new ZBFs that have been proposed as alternative to the classical sulfonamide one: sulfamates, sulfamides, substituted sulfonamide, Schiff's bases, urea and hydroxyurea, as well as hydroxamates (Fig. (**2**)). It may be observed that most of them are structurally related to the sulfonamide moiety, and clearly this was the lead for drug design developments in this field.

I. N-SUBSTITUTED SULFONAMIDES AS CARBONIC ANHYDRASE INHIBITORS

Krebs reported in 1948 that substitution of the sulfonamido moiety in compounds of type $ArSO_2NHR$ (i.e., R different of H) drastically reduces the CA inhibitory properties as compared to the corresponding derivatives possessing primary sulfonamido groups, $ArSO_2NH_2$.[8] As a consequence, other zinc-binding functions except for the SO_2NH_2 one have rarely been taken into consideration in the design of CAIs, although many other zinc enzymes are inhibited by a multitude of derivatives possessing an entire range of zinc binding functions, such as thiols, phosphonates, carboxylates, hydroxamates, etc. [9-10]. Only recently several detailed studies regarding the possible modifications of the sulfonamido moiety, compatible with the retention of strong binding to the enzyme, have been reported [11-16]. Compounds of type (**1-3)** were studied kinetically, for inhibition of reactions catalyzed by CA I and II (CO_2 hydration and ester hydrolysis), but their binding to the enzyme has also

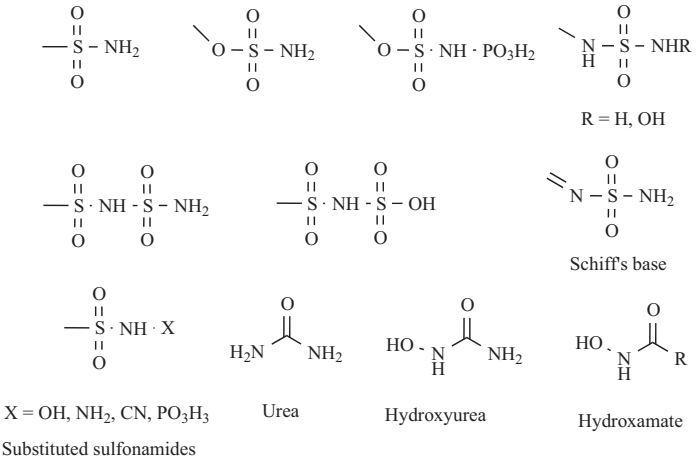


Fig. (2). Zinc binding functions (ZBFs) for the design of potent CAIs: sulfonamides, sulfamates, sulfamides, substituted sulfonamides/sulfamates/sulfamides, Schiff's bases, urea, hydroxyurea and hydroxamates. All these groups bind in deprotonated form, as anions to the Zn(II) ion within the enzyme active site, presumably in monodentate fashion.

Table 1. Inhibition of hCA I and hCA II with Compounds Incorporating Modified Sulfonamide Moieties (1a-t), Sulfamide (2) and Sulfamic Acid (3) (Briganti *et al.* [11])

4-Me-$C_6H_4SO_2$-X	$H_2NSO_2NH_2$	$HOSO_2NH_2$
1a-t	**2**	**3**

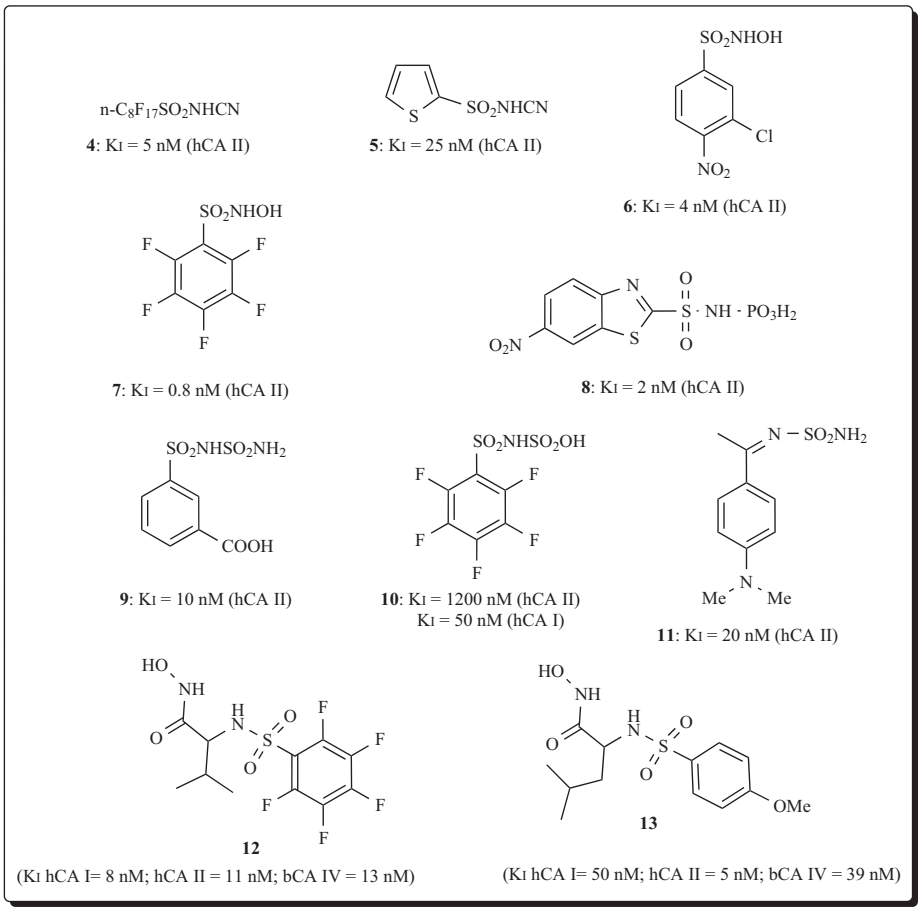
Inhibitor	X	K_I (μM)*	
		hCA I	hCA II
1a	NH_2	50	11
1b	NHOH	41	9
1c	NHOMe	220	173
1d	NO	35	24
1e	NCS	30	18
1f	N_3	27	45
1g	imidazol-1-yl	160	34
1h	$NHNH_2$	70	53
1i	NHNHPh	>1000	120
1j	NHCl	19	2.1
1k	NCl_2	12	3.6
1m	NHCN	210	125
1n	$NHOCH_2COOH$	150	85
1p	OH	130	460
1q	SH	5	10
1r	$NHCONH_2$	>1000	460
1s	$NHCSNH_2$	>1000	410
1t	$NHC(NH)NH_2$	>1000	540
2		310	1130
3		21	390

* Errors in the range of 5-10% of the data shown from 3 different assays.

been monitored spectroscopically, by studying the electronic and ^{1}H-NMR spectra of adducts of such inhibitors with Co(II)-CA II [11].

Thus, for the series of derivatives with modified sulfonamido moieties (**1-3**) (Table 1) it has been observed that the presence of bulky substituents at the sulfonamido moiety (such as phenylhydrazino, ureido, thioureido, guanidino, etc) led to compounds with weak inhibitory properties, whereas moieties present in inorganic anion CAIs (such as NO, NCS, N_3) or compact moieties substituting the sulfonamide nitrogen (such as OH, NH_2, CN, halogeno) led to compounds with appreciable inhibitory properties. Thus, the N-hydroxy sulfonamide (**1b**), the N-chloro-substituted derivatives (**1j,k**), as well as the nitroso- and thiocyanato derivatives (**1d,e**) possessed the same affinity for the two investigated isozymes as the unsubstituted sulfonamide (**1a**). Interestingly, the thiosulfonic acid (as sodium salt) (**1q**) was one of the best inhibitors in this series, in contrast to the sulfonate (as sodium salt) (**1p**), which behaved as a very weak inhibitor. Indeed, by using such compounds as leads, several series of much stronger inhibitors were then reported, possessing modified sulfonamido moieties as zinc-binding functions, of the type SO_2NHOH, SO_2NHCN, $SO_2NHPO_3H_2$, $SO_2NHSO_2NH_2$, SO_2NHSO_3H or $SO_2NHCH_2CONHOH$ among others [12-13, 15-16].

Thus, compounds such as (**4-13**), possessing N-cyano, N-hydroxy, or N-phosphoryl-sulfonamido moieties, or the related modified sulfamide/sulfamic acid zinc-binding functions, and diverse alkyl, aryl or heterocyclic moieties in their molecules, showed affinities in the low nanomolar range for hCA II (except for **10**), being equipotent or better inhibitors than the corresponding unsubstituted sulfonamides [12-13, 15-16]. Compound (**10**) is a weak inhibitor of hCA II (affinity constant of 1.2 μM), but it has a much higher affinity (50



nM) for hCA I, being thus one of the most "selective" hCA I inhibitors reported up to now [15].

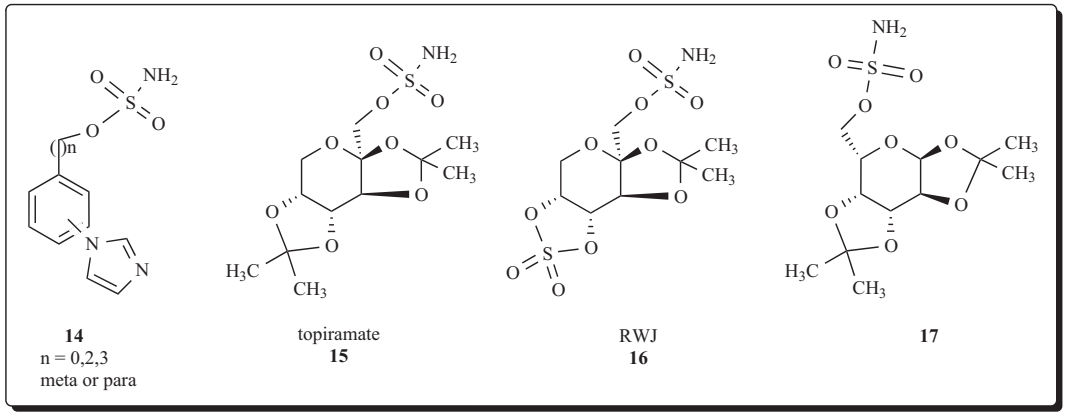
Sulfonylated amino acid hydroxamates were also shown to possess strong CA inhibitory properties [14]. Such hydroxamates generally act as potent inhibitors of metalloproteases containing catalytic zinc ions, such as the matrix metalloproteinases (MMPs) or the bacterial collagenases [9]. They bind to the Zn(II) ions present in these enzymes bidentately, coordinating through the hydroxamate (ionized) moiety [9]. Scolnick *et al.* showed that two simple hydroxamates, of the type RCONHOH (R = Me, CF_3) act as micromolar inhibitors of hCA II, and bind to the Zn(II) ion of this enzyme, as demonstrated by X-ray crystallography [17]. By using these two derivatives as lead molecules, Scozzafava and Supuran [14] designed a series of sulfonylated amino acid hydroxamate derivatives possessing the general formula $RSO_2NHCH(R')CONHOH$ and showed that they bind to the Zn(II) ion of CA, by means of electronic spectroscopic studies on the Co(II)-substituted CA. Some of these compounds, such as (**12**) and (**13**), showed affinity in the low nanomolar range for the major CA isozymes (CA I, II and IV), but substitution of the sulfonamide nitrogen by a benzyl or a substituted-benzyl moiety led to a drastic reduction of the CA inhibitory properties, and to an enhancement of the MMP inhibitory properties. Thus, between the two types of zinc enzymes, the zinc proteases and the CAs, there exist some cross-reactivity from the point of view of the hydroxamate inhibitors, but generally strong MMP inhibitors are weak CAIs, and viceversa.

All these data demonstrate that in addition to the classical CAIs of the aromatic/heterocyclic sulfonamide type, other compounds may be designed with very strong affinity for the active site of different isozymes, a fact that may be relevant for obtaining diverse pharmacological agents that modulate the activity of these wide-spread enzymes.

II. THE SULFAMATE MOTIF IN THE DESIGN OF CARBONIC ANHYDRASE INHIBITORS

Up to recently, only sulfonamides were known to possess high affinity for carbonic anhydrases. Closely related variant, the sulfamate moiety, has demonstrated very attractive possibilities for the design of various pharmacological agents, especially in the carbonic anhydrase field [18].

The first sulfamates which were investigated as CAIs, of type (**14**), were reported by Lo *et al.* [19] in the search of topically acting antiglaucoma agents. Indeed, several *m*- or *p*-imidazolyl-phenyloxyethyl/propylsulfamates (**14**) were



shown to possess CA inhibitory properties (IC_{50} in the range of 23-250 nM, against bovine red cell CA) and to moderately decrease intraocular pressure in albino rabbits after topical administration directly into the eye [19].

But the investigation of the simplest sulfamate, i.e., sulfamic acid (**3**), as CAI has been performed some years later by Briganti *et al.* [11] who showed that sulfamic acid is a moderately weak CAI, with K_I of 21 μM against the human isozyme hCA I, and of 97 μM against hCA II – for the esterase activity of these enzymes. The X-ray crystal structure of the adduct of this compound with the physiologically most important isozyme (hCA II) has been subsequently reported by the same group [20]. It was shown that sulfamic acid binds to the zinc ion of the enzyme as a dianion, *via* its $(NH)SO_3^{2-}$ sulfamate bianionic species (Zn-N distance of 2.07 Å), whereas the NH moiety of the inhibitor also participates in a hydrogen bond with Thr199Oγ (Fig. (**3**)). An additional third hydrogen bond is formed from this NH to an adjacent water molecule at 2.75 Å distance. The second nearest contact of the ligand to Zn, an oxygen atom, leading to extra-coordination, is at a distance of about 3.07 Å from the metal ion, whereas the remaining two oxygens of the SO_3 moiety are involved in two other hydrogen bonds, one with the backbone NH group of Thr 199 at a distance of 2.99 Å and the other to a water molecule at a distance of 2.87 Å. This extended extra-coordination results in a distorted tetrahedral arrangement around the metal ion, the remaining three ligands of zinc being His94, His96 and His119 (as in the uninhibited enzyme) [20]. In summary, this very simple inhibitor showed a large number of favorable contacts in the binding pocket of CA II, and may be used as a lead molecule for the design of tighter-binding CAIs. This has been achieved in several studies which will be presented in the following section.

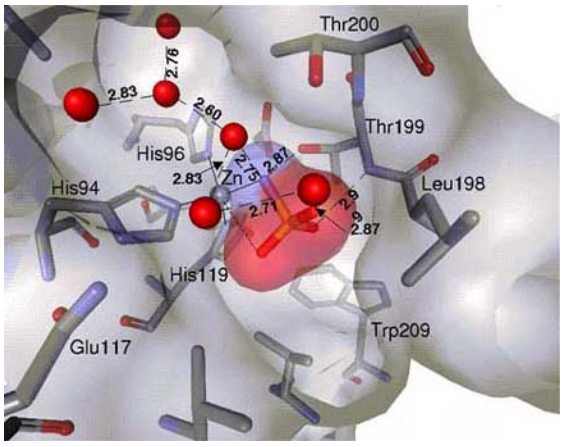


Fig. (3). hCA II adduct with sulfamic acid (**3**) (as dianionic species) as determined by X-ray crystallography. The zinc coordination sphere and amino acid residues in the neighborhood of the bound inhibitor are shown (figures represent distances in Å). (With Permission from American Chemical Society, 2006).

Indeed, two sugar sulfamates [21, 22] topiramate (**15**) and RWJ-37497 (**16**) were recently shown to behave as very potent CAIs and their X-ray crystal structures in complex with isozyme hCA II have been reported [23, 24] (Fig. (**4**)).

Both sugar derivatives (**15**) and (**16**) bind with their sulfamate moiety to zinc, resulting in a tetrahedral coordination of the metal ion (Fig. (**4**)), which is coordinated to the three histidine residues as in the uninhibited enzyme (His94, 96 and 119) as well as the nitrogen atom of the sulfamate moiety, which is presumably in deprotonated state, as for the sulfonamide CAIs complexed within the active site of the enzyme [4, 7, 9, 19, 25-26]. In addition, both compounds make two hydrogen bonds to the side chain oxygen atom of Thr199 and the backbone NH nitrogen atom of the same residue. The hydroxy group of Thr199 forms an additional hydrogen bond with Glu106, such that the Thr199 hydroxyl acts as a hydrogen-bond acceptor for inhibitor binding. A total of eight hydrogen bonds between topiramate bound to the enzyme and amino acid residues from the cavity have been evidenced, which explain the very potent inhibitory activity of topiramate against hCA II (K_I of 5 nM) [24]. Despite the similarity in anchoring to the Zn(II) ion, a surprising difference is observed in the binding mode of the two inhibitors with respect to the ring system. Topiramate also forms several hydrogen bonds to amino acid side chains in a hydrophilic binding pocket (Asn67, Gln92) and to a water molecule that donates a hydrogen bond to Thr200 (Fig. (**4A**)). In addition, this water interacts with the oxygen atom of the six-membered ring of (**15**). RWJ-37497 (**16**) on the other hand, shows a quite different binding mode in which the ring

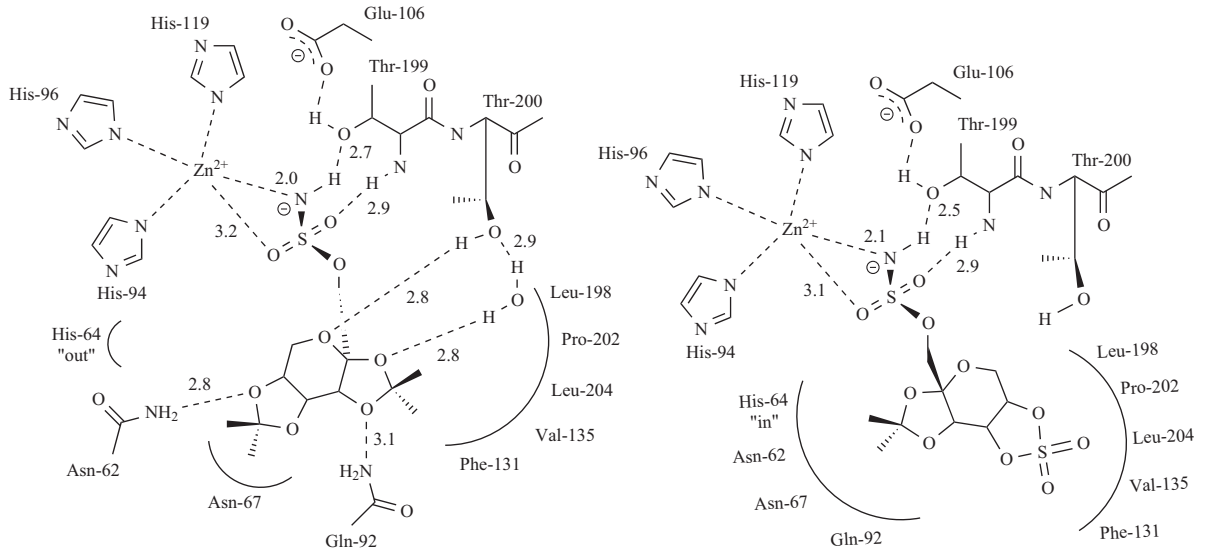


Fig. (4). Schematic representation of the binding mode of topiramate (**15**) (**A**) and RWJ-37497 (**16**) to hCA II (**B**) [23, 24]. Hydrogen bonds between enzyme and inhibitor are shown as dotted lines. Distances are given in Å and are measured between the corresponding non-hydrogen atoms [25].

system is rotated by about 180° in comparison to that of topiramate. Therefore, surprisingly, the cyclic sulfate group points towards a more hydrophobic pocket lined by residues Leu198, Pro202, Phe131 (although this moiety is more hydrophilic as compared to the corresponding diisopropylidene moiety of topiramate), and except for the sulfamate anchoring group no further hydrogen bonds are observed (Fig. (**4B**)). RWJ-37497 was reported to have an IC_{50} of 36 nM against hCA II [22]. It is interesting to note that the topiramate isomer (**17**), possessing the sulfamate-methyl moiety in a different position as compared to (**15**), is a much weaker CAI as compared to topiramate or RWJ-37497, having K_I-s of 400 μM against hCA I, 16 μM against hCA II and 27 μM against bCA IV (h = human, b = bovine; m = murine isozymes) [24].

Potter's and Reed's groups also investigated the CA inhibitory properties of several of the STSIs developed during their research of novel therapies for hormone-dependent tumors (see the preceding section for details) [27-28]. Some of their best STSIs, such as 667COUMATE (**18**), EMATE (**19**), the bis-sulfamate (**20**) (R = Et) as well as the coumarin sulfamate (**36**) (R1 = Me; R2 = cyclohexylethyl) were tested as hCA II inhibitors, being shown to possess IC_{50} values of 17 nM, 9 nM, 290 nM, and 15 nM, respectively [27-28]. It may be seen that except for the bis-sulfamate (**20**) possessing an ethyl group in *ortho* (which presumably interferes with the coordination to the Zn(II) ion within the enzyme active site), the other sulfamates investigated showed very strong CA II inhibitory properties, which may be a beneficial feature for the antitumor effects of such pharmacological agents. The same group also reported docking studies of some of these inhibitors to hCA II [27] and hCA XII [28]. The predictions regarding EMATE binding to hCA II [27] have been soon thereafter shown to be wrong, after the report of the X-ray crystal structure of EMATE with hCA II, by this group [29] (Figs. (**5**) and (**6**)).

Fig. (5). The hCA II – EMATE (**19**) adduct: the enzyme is shown as ribbon diagram, with the zinc ion (central blue sphere) and its protein ligands (His94, 96 and 119) shown. The inhibitor molecule lies towards the hydrophobic half of the enzyme cavity [29]. (With Permission from Elsevier Ltd. 2006).

The binding of EMATE (**19**) to the hCA II is similar to that of other sulfamates/sulfonamides, considering the interactions of the zinc anchoring group (Fig. (**6**)), but differs considerably when the steroidal scaffold of the inhibitor is analyzed. This part of the inhibitor interacts only within the hydrophobic half of the CA active site (Fig. (**5**)), interacting with residues Val121, Phe131, Val135 and Pro202, and leaving the hydrophilic half able to accomodate several water molecules not present in the uncomplexed enzyme (Fig. (**6**)). In addition, a very short bond of 1.78 Å between the zinc ion and the coordinated nitrogen atom of the sulfamate moiety is observed, which may explain the high affinity of this inhibitor for hCA II (K_I of 10 nM) [29].

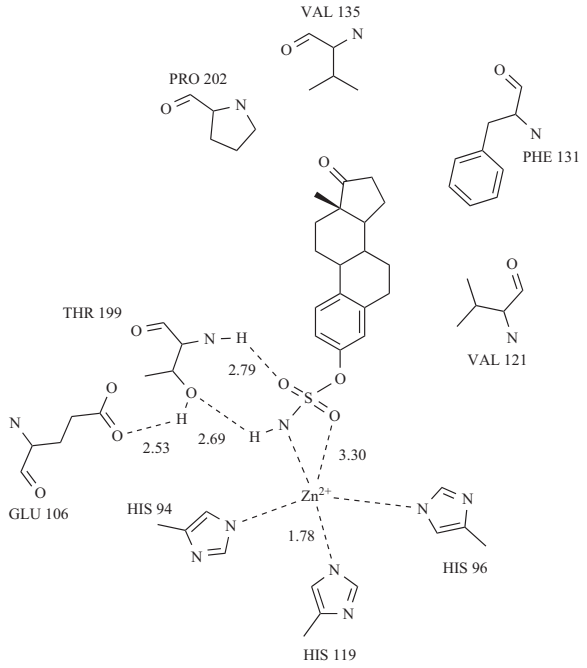


Fig. (6). Detailed schematic representation of EMATE (**19**) bound within the hCA II active site (figures represent distances in Å) [29].

But the most interesting studies regarding the design of CAIs of the sulfamate type with putative antitumor applications have been reported by Winum *et al.* [31, 32] In these studies detailed SAR data were obtained for two series of sulfamates, the first one preponderently including aromatic and steroidal derivatives [31] (Table 2), whereas the second one including mostly aliphatic sulfamates [32] (Table 3), which were tested for their interaction with the red cell isozymes hCA I and II, but also for the first time, with the tumor-associated isozyme CA IX, which is overexpressed in a large range of tumors [30].

As seen from data of Table 2, very potent (nanomolar) inhibitors were detected against the three investigated CA isozymes [31]. Best hCA I inhibitors were phenylsulfamate **21a**, and some of its 4-halogenoderivatives, as well as the aliphatic compound *n*-octyl sulfamate **21r** (K_I-s in the range of 2.1 – 4.6 nM – these are among the most potent hCA I inhibitors ever reported !). Against hCA II, low nanomolar inhibitors (1.1 – 5 nM) were phenylsulfamate (**21a**) and some of its 4-halogeno-/nitro-derivatives, *n*-octyl sulfamate (**21r**), and estradiol 3,17β-disulfamate (**21u**) among others. All the investigated sulfamates showed efficient CA IX inhibitory properties, with inhibition constants in the range of 18-63 nM. The best CA IX inhibitor detected so far was 4-chlorophenylsulfamate (**21d**) (K_I of 18 nM). These data are critical for the design of novel antitumor properties, mainly for hypoxic tumors that overexpress CA IX [30], which are non-responsive to radiation or chemotherapy. The antitumor properties of the STSIs (discussed above in this review) in clinical trials may on the other hand also be due to their potent inhibitory properties of CA isozymes involved in tumorigenicity, such as CA II and especially CA IX, and constitute an attractive dual mechanism of action for such antitumor agents [31, 32].

Seen the excellent (and unexpected) CA inhibitory properties of the only aliphatic sulfamate (**21r**) investigated in the first study [31], the same group reported a large series of aliphatic (and some polycyclic) sulfamates of type (**22**), and their interaction with the same physiologically relevant three CA isozymes, i.e., hCA I, II and IX [31] (Table 3).

For this new series, the best hCA I inhibitor was *n*-tetradecylsulfamate (**22j**) and some (substituted) benzyl/phenethyl sulfamates (inhibition constants in the low micromolar range). Against hCA II, low nanomolar inhibitors (0.7 – 3.4 nM) were *n*-decylsulfamate (**22g**) and the (substituted)benzyl/phenethyl derivatives mentioned above, (**22v-x**). Good CA II inhibition was also observed for the hydroxy/keto-derivatives of dehydroepiandrosterone sulfamate (**22ab**) and (**22ac**). Efficient hCA IX inhibitory properties, with inhibition constants in the range of 9-23 nM, was observed for the aliphatic sulfamates C_{10} – C_{16} (with the best inhibitor the *n*-dodecyl derivative, K_I of 9 nM), and the (substituted)benzyl/phenethyl sulfamates (**22v-x**). The inhibition profile of the three investigated isozymes with this type of compounds was rather different, allowing us to hope that the preparation of CA IX-selective inhibitors is possible. These data are critical for the design of novel antitumor therapies, mainly for hypoxic tumors that overexpress CA IX, which are non-responsive to radiation or chemotherapy [32].

By analogy with the phosphorylated-sulfonamide, with several compounds of the type Ar-SO_2NH-PO_3H_2 (where Ar = Ph, 4-halogeno-C_6H_4- ; 4-Me-C_6H_4- and 4-H_2N-C_6H_4-, etc) recently shown by our group [16] to act as good inhibitors of isozymes CA I and II (K_I values in the range of 8-280 nM against isozyme CA II, and of 42-450 nM against isozyme CA I), sulfamates possessing a phosphoryl moiety substituting the nitrogen atom, of the type R-O-SO_2NH-PO_3H_2 have been reported. We demonstrated that the phosphorylated sulfamate zinc binding group is very efficient for the design of low nanomolar CA inhibitors. Aliphatic compounds incorporating C8-C16 chains lead to inhibitors with affinities of 8-16 nM against hCA I, and 5 – 12 nM against hCA II [33].

III. THE SULFAMIDE MOTIF IN THE DESIGN OF CARBONIC ANHYDRASE INHIBITORS

Sulfamide motif attracts permanent interest as a lot of enzyme inhibitors containing sulfamide fragments exhibit a broad spectrum of activities [34].

The first inhibition study of some CA isozymes with sulfamide ($H_2NSO_2NH_2$) has been reported by this group [11]. It has been shown that this simple compound behaves as a weak inhibitor against the classical cytosolic isoforms CA I and CA II, with inhibition constants of 0.31 mM and 1.13 mM, respectively (for the physiological reaction catalyzed by these enzymes). Furthermore, working with the Co(II)-substituted CA II (in which the active site zinc ion has been replaced by the colored, paramagnetic Co(II) ion) it has been proved by means of electronic- and ^{1}H-NMR spectroscopy (in paramagnetic systems) that the inhibitor directly coordinates to the metal ion within the enzyme active site, which

Table 2. Inhibition Data for Derivatives (21) Investigated by Winum *et al*. [31], Against Isozymes I, II and IX

$RO\text{-}SO_2NH_2$
21a-y

No	Inhibitor 21	R	K_I (nM)		
			hCA I[a]	hCA II[a]	hCA IX[b]
Sulfamic acid (H_2NSO_3H)			21,000	97,000	nt
Topiramate		-	250	5	nt
	a	Ph	2.1	1.3	63
	b	4-Me-C_6H_4	3.8	1.9	59
	c	4-Ph-C_6H_4	113	95	50
	d	4-Cl-C_6H_4	4.6	1.1	18
	e	4-Br-C_6H_4	7.3	1.5	19
	f	4-I-C_6H_4	9.5	3.8	23
	g	4-MeO-C_6H_4	33	1.6	34
	h	4-PhO-C_6H_4	115	98	51
	i	4-AcNH-C_6H_4	37	18	45
	j	4-O_2N-C_6H_4	40	1.5	36
	k	4-NC-C_6H_4	480	149	41
	l	4-*t*-Bu-C_6H_4	43	2.9	33
	m	4-CF_3-C_6H_4	369	138	54
	n	C_6F_5	415	113	47
	o	C_6Cl_5	432	125	39
	p	2,4,6-$Cl_3C_6H_2$	454	138	37
	q	2-naphthyl	103	63	40
	r	*n*-C_8H_{17}	3.5	2.7	25
	s	A	105	76	43
	t	A (**EMATE**)	37	10	30
	u	A	6	5	58
	v	A	15	13	32
	w	A	31	27	44
	x	A	278	15	39
	y	A	28	23	26

[a] Human (cloned) isozymes, by the esterase method; [b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method, nt = not tested. A - see structure in the text

Table 3. Inhibition Data for Sulfamates (22) Investigated by Winum *et al*. [32], Against Isozymes hCA I, II and IX

$RO\text{-}SO_2NH_2$
22a-ac

No	Inhibitor 22	R	K_I		
			hCA I[a] (μM)	hCA II[a] (nM)	hCA IX[b] (nM)
	a	Me	40	6000	>1000
	b	Et	38	5500	>1000

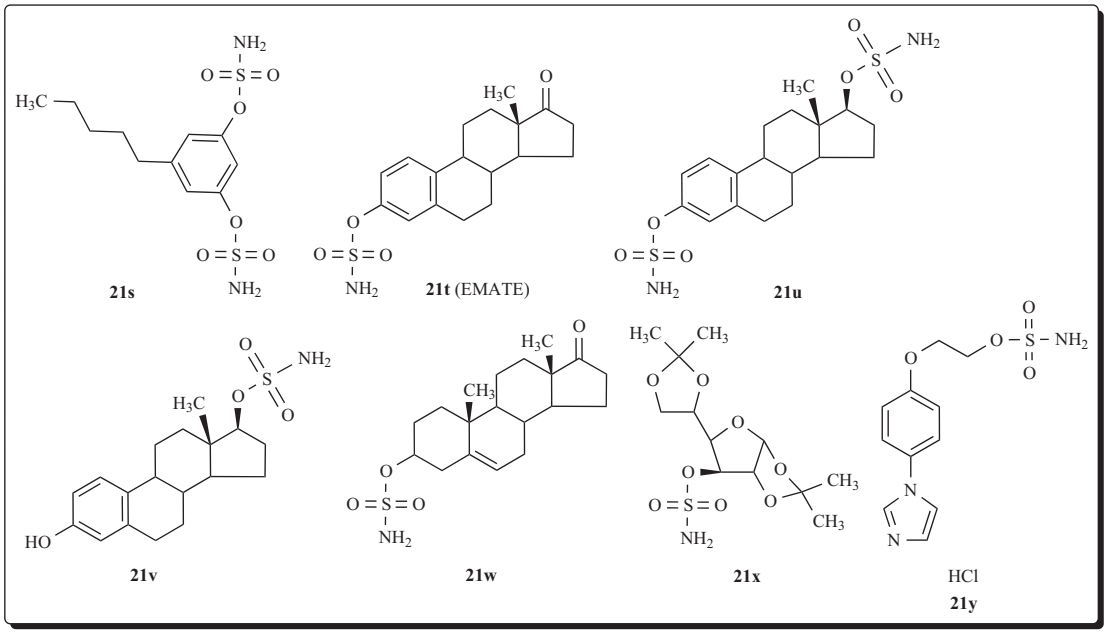
(Table 3. Contd….)

No	Inhibitor 22	R	K_I		
			hCA I[a] (μM)	hCA II[a] (nM)	hCA IX[b] (nM)
	c	*n*-Pr	3.70	750	>1000
	d	*i*-Pr	690	>10000	>1000
	e	*n*-Bu	3.10	70	>1000
	f	*n*-C_5H_{11}	0.71	58	126
	g	*n*-$C_{10}H_{21}$	0.53	0.7	23
	h	*n*-$C_{11}H_{23}$	0.43	4.7	17
	i	*n*-$C_{12}H_{25}$	0.27	10.0	9
	j	*n*-$C_{14}H_{29}$	0.15	87	15
	k	*n*-$C_{16}H_{33}$	58	97	22
	l	*n*-$C_{18}H_{37}$	65	129	120
	m	CF_3-CH_2	7.8	845	458
	n	*n*-$C_6F_{13}CH_2CH_2$	400	8000	335
	o	*n*-$C_8F_{17}CH_2CH_2$	>1000	9000	142
	p	$(CF_3)_2CH$	3.54	1580	279
	q	CH_2=$CHCH_2CH_2$	800	883	386
	r	CH≡CCH_2CH_2	990	5900	633
	s	$ClCH_2CH_2CH_2$	4.62	570	>1000
	t	c-C_5H_9	>1000	>10000	>1000
	u	c-C_6H_{11}	59	60	>1000
	v	$PhCH_2$	0.76	3.4	14
	w	$PhCH_2CH_2$	0.41	1.1	12
	x	*p*-Me-$C_6H_4CH_2$	0.10	2.7	13
	y	*p*-Ph-$C_6H_4CH_2$	>1000	> 10000	>1000
	z	A (Fluorenylmethyl)	38	4500	>1000
	aa	A (Cholesteryl)	>1000	> 10000	>1000
	ab	A	0.40	13	65
	ac	A	0.41	23	76

[a] Human (cloned) isozymes, by the CO_2 hydration method; [b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method. A – See structure in the text.

presumably remains in its tetrahedral geometry, as in the wild type, uninhibited enzyme [11]. This result was thereafter confirmed when the same group reported the high resolution X-ray crystal structure of the adduct of sulfamide with human CA II [20] (Fig. (**7**)).

As observed in Fig. (**7**), the inhibitor molecule, presumably as monoanion, is coordinated to the zinc ion by means of a nitrogen atom (Zn – N distance of 1.76 Å) similarly to the sulfonamides for which such studies have been performed [4, 7, 9]. The same NH moiety coordinated to zinc participates in a hydrogen bond with the OH group of Thr199 (which in turn is hydrogen bonded to the carboxylate moiety of Glu106, these two amino acid residues being known as the "door-keepers" in the CA active site, and are conserved in all α-CAs [4, 9, 7]). Another hydrogen bond then involves one of the oxygen atoms of sulfamide and the backbone NH of Thr200, which in turn participates to another hydrogen bond with this inhibitor: its OH group makes a 3.26 Å hydrogen bond with the second NH_2 moiety of sulfamide (the one non-coordinated to zinc). This second amino moiety also participates in two other hydrogen bonds with water molecules present in the active site, as shown in Figs. **7A** and **7B** [20]. All these data showed for the first time that CAIs may presumably be designed from the sulfamide class of derivatives, as the lead compound (the simple inorganic sulfamide (**2**)), although being a weak inhibitor, makes a lot of favorable interaction with the amino acid residues in the CA II active



site, at the same time possessing a derivatizable NH_2 group positioned in a favorable position for introducing bulkier moieties, in the search of more potent inhibitors. Indeed, in another study [15] it has been then shown that this group can be derivatized by means of reactions with sulfonyl halides, arylsulfonyl isocyanates or aromatic/heterocyclic aldehydes, leading to derivatives of types (**23**-**25**), some of which showed inhibition constants against isozymes CA I, II and IV in the low nanomolar range.

The sulfonylated sulfamides (**23**), the arylsulfonylcarbonylsulfamides (**24**), or the Schiff bases (**25**) incorporated a large variety of moieties R, belonging mainly to the aromatic/heterocyclic class. For derivatives (**23**), some aliphatic derivatives have also been prepared, but their activity was generally weaker as compared to that of the aromatic/heterocyclic compounds [15]. Thus, the main conclusion of this work was that starting from a millimolar lead molecule, sulfamide (**2**), low nanomolar CAIs could be obtained by means of very simple derivatization reactions. Presumably, these compounds bind to the metal ion within the enzyme active site similarly to the lead **1**, as the electronic spectra of the Co(II)-substituted enzyme with some of them were similar to those of the Co(II)-CA II – sulfamide adduct investigated earlier [11, 15].

In another study [35], a series of *N,N*-disubstituted- and *N*-substituted-sulfamides of types (**10**) and (**11**) were prepared from the corresponding amines and N-(*tert*-butoxycarbonyl)-N-[4-(dimethylazaniumylidene)-1,4-dihydropyridin-1-ylsulfonyl]azanide or the unstable N-(*tert*-butoxycarbonyl) sulfamoyl chloride. The disubstituted compounds being too bulky, were ineffective as CAIs (Table **1**), whereas monosubstituted derivatives (incorporating aliphatic, cyclic and aromatic moieties) as well as a bis-sulfamide, behaved as micro – nanomolar inhibitors of two cytosolic isozymes, hCA I and hCA II, responsible for critical physiological processes in higher vertebrates. Aryl-sulfamides were more effective than aliphatic derivatives. Low nanomolar inhibitors have been detected, which generally incorporated 4-substituted phenyl moieties in their molecule (Table 5). This was another interesting example of CAIs in which low

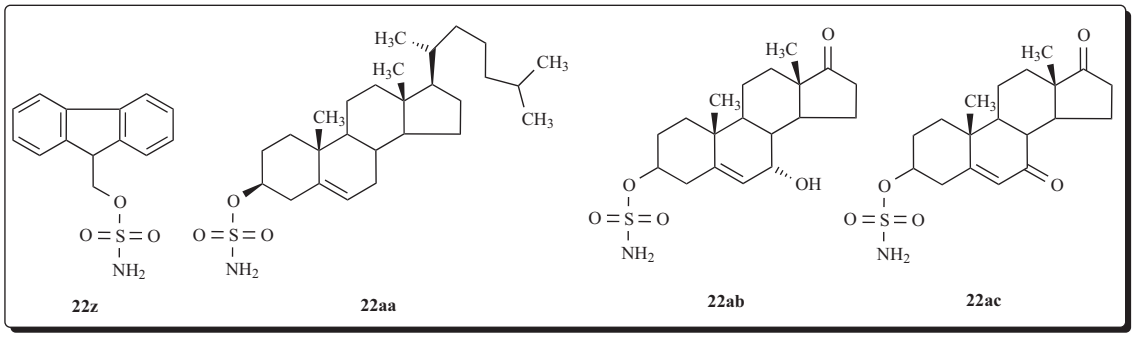


Table 4. CA Inhibition Data with Compounds (23-26) and Standard Inhibitors Against Human Isozymes hCA I and hCA II

Compound		K_I (nM)*	
		hCA I	hCA II
23	*n*-C_8H_{17}-O-SO_2NH-PO_3H_2	8.2	5.3
24	*n*-$C_{12}H_{25}$-O-SO_2NH-PO_3H_2	10.5	9.9
25	*n*-$C_{14}H_{29}$-O-SO_2NH-PO_3H_2	14.6	11.9
26	*n*-$C_{16}H_{33}$-O-SO_2NH-PO_3H_2	16.1	11.2
(acetazolamide)		900	12
(sulfanilamide)		28,000	300
(topiramate)		250	5
21r	*n*-C_8H_{17}-O-SO_2NH_2	3.5	2.7
22i	*n*-$C_{12}H_{25}$-O-SO_2NH_2	270	10
22j	*n*-$C_{14}H_{29}$-O-SO_2NH_2	150	87
22k	*n*-$C_{16}H_{33}$-O-SO_2NH_2	58,000	97

* Errors in the range of 5-10 % of the reported value (from 3 different assays).

nanomolar inhibitors were generated starting from an ineffective lead molecule.

A small library of *N*-hydroxysulfamides (**28**) was then synthesized by an original approach in order to investigate whether this novel zinc binding function is efficient for the design of inhibitors targeting the cytosolic (hCA I and II) and transmembrane, tumor associated (hCA IX and XII) CAs [26]. The parent derivative, N-hydroxysulfamide (**28a**) was a more potent inhibitor as compared to sulfamide or sulfamic acid against all investigated isozymes, with inhibition constants in the range of 473 nm – 4.05 μM. Its substituted *n*-decyl-, *n*-dodecyl-, benzyl- and biphenylmethyl- derivatives were less inhibitory against hCA I (K_I-s in the range of 5.8 – 8.2 μM) but more inhibitory against hCA II (K_I-s in the range of 50.5 – 473 nM) (Table **2**). The same situation was true for the tumor associated isozymes, with K_I-s in the range of 353 – 790 nM against hCA IX and 372 – 874 nM against hCA XII. Some sulfamides/sulfamates of types (**26**) and (**21**), respectively, possessing similar substitution patterns

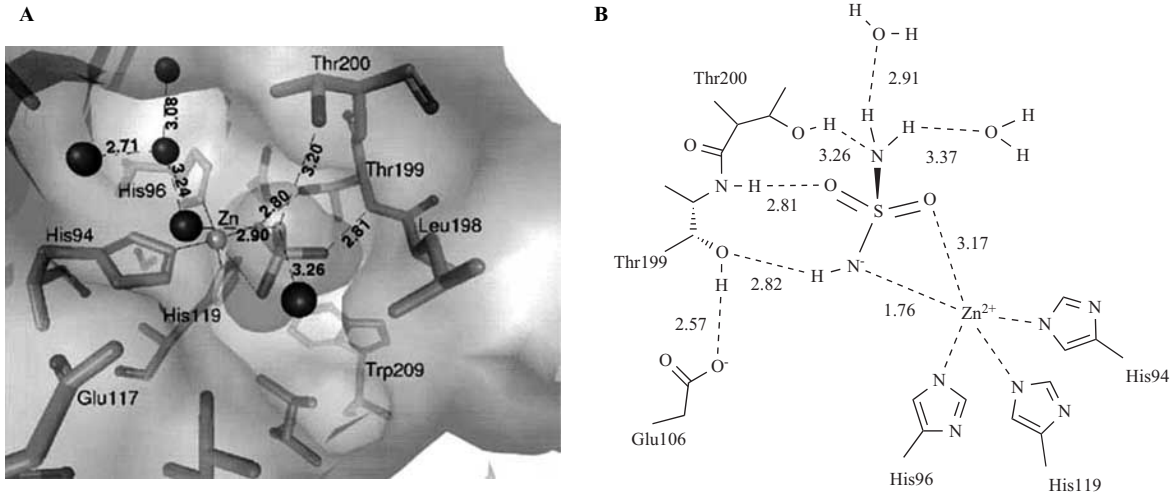
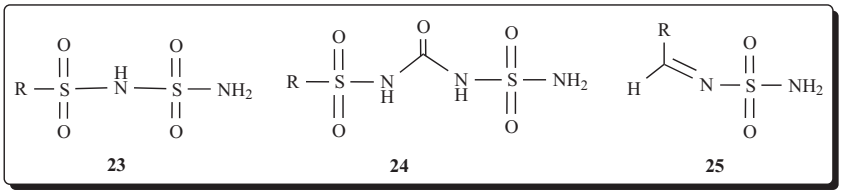


Fig. (7). The hCA II – sulfamide adduct: A – the inhibitor is shown in space fill and stick model, with the zinc ion (gray sphere), its ligands (His94, His96 and His119), water molecules (red balls) and other amino acid residues involved in the binding; B – schematic reprsentation of the inhibitor binding with numbers representing distances in Å [20]. (With Permission from American Chemical Society, 2006).



23 **24** **25**

Table 5. Inhibition Data for Derivatives (10) and (11) Against the Cytosolic, Human Isozymes hCA I and hCA II, by an Esterase Assay Method with 4-Nitrophenyl Acetate as Substrate [35]

$RR'N\text{-}SO_2NH_2$ **26**

$NH_2SO_2\text{-}NH\text{-}RR'\text{-}NH\text{-}SO_2NH_2$ **27**

No	Inhibitor	R	R'	K_I (nM)	
				hCA I[a]	hCA II[a]
	2 $H_2NSO_2NH_2$	-	-	35,000	82,000
	26a	*n*-Bu	H	173	148
	26b	cyclohexyl	H	164	450
	26c	2-adamantyl	H	960	890
	26d	$PhCH_2$	H	133	123
	26d	*i*-Bu	*i*-Bu	> 100,000	> 100,000
	26e	*i*-Pr	*i*-Pr	> 100,000	> 100,000
	26f	cyclohexyl	cyclohexyl	> 100,000	> 100,000
	26g	$PhCH_2$	$PhCH_2$	> 100,000	647
	26h	$-(CH_2)_5-$		155	148
	26i	$-(CH_2)_6-$		163	131
	26j	Ph	H	13	12
	26k	4-Me-C_6H_4	H	15	13
	26l	4-CF_3-C_6H_4	H	8	7
	26m	4-Cl-C_6H_4	H	19	15
	26n	4-Br-C_6H_4	H	23	21
	26p	4-I-C_6H_4	H	18	17
	26k	4-MeO-C_6H_4	H	14	11
	26r	4-HO-C_6H_4	H	16	12
	26s	4-O_2N-C_6H_4	H	18	13
	26t	4-EtO_2C-C_6H_4	H	26	19
	26u	4-NC-C_6H_4	H	20	16
	26v	4-Me_2N-C_6H_4	H	17	21
	26x	C_6F_5	H	34	32
	26y	3-benzoyl-C_6H_4	H	62	49
	26z	2-naphthyl	H	39	36
	27	$(CH_2)_2$-SS-$(CH_2)_2$	149	27	

[a]Human (cloned) isozymes, by the esterase method [34].

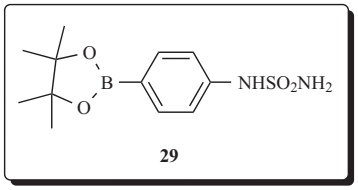
Table 6. Inhibition of Isozymes hCA I, II, IX and XII with Sulfamide, Sulfamic Acid, N-Hydroxysulfamides (28), Sulfamides (26) and the Corresponding Sulfamates (21) [36]

$RNHSO_2NHOH$ **28** $RNHSO_2NH_2$ **26** $ROSO_2NH_2$ **21**

Inhibitor	R	K_I* (nM)			
		hCA I[a]	hCA II[a]	hCA IX[b]	hCA XII[b]
$H_2NSO_2NH_2$	-	$0.31.10^6$	$1.13.\ 10^6$	$9.6.10^3$	$13.2.10^3$
H_2NSO_3H[c]	-	$0.21.10^5$	$0.39.\ 10^6$	$9.2.10^3$	$10.7.10^3$
28a	H	4050	566	865	1340
28b	n-$C_{10}H_{23}$	5800	473	506	874
28c	n-$C_{12}H_{25}$	6000	89.6	485	539
28d	$PhCH_2$	8200	313	790	633
28e	4-$PhC_6H_4CH_2$	8100	50.5	353	372
26a	4-$CF_3C_6H_4$	8	7	26	48
21a	4-$CF_3C_6H_4$	369	138	54	103
26b	4-CNC_6H_4	20	16	30	45
21b	4-CNC_6H_4	480	149	41	76
26c	C_6F_5	34	32	40	19
21c	C_6F_5	415	113	47	34
26d	2-Naphthyl	39	36	38	30
21d	2-Naphthyl	103	63	40	62

* Errors in the range of 5 – 10 % of the shown data, from 3 different assays. [a] Human recombinant isozymes; [b] Catalytic domain of the human recombinant isozyme, CO_2 hydrase assay method [36].

have also been investigated for the inhibition of these isozymes, being shown that in some particular cases sulfamides were more efficient inhibitors as compared to the corresponding sulfamates. Potent CAIs targeting the cytosolic or tumor-associated CA isozymes can thus be designed from various classes of sulfonamides, sulfamides or sulfamates and their derivatives, considering the extensive interactions in which the inhibitor and the enzyme active site are engaged, based on the X-ray crystallographic data showed above and on this study which evaluated comparatively these compounds possessing different zinc binding groups and their relative efficiency in inhibiting various isoforms [36].

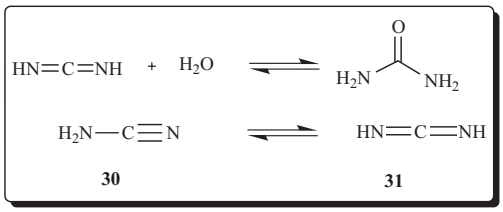


29

Finally, a very interesting sulfamide derivative incorporating boron, of type (**29**), was designed and synthesized in order to obtain compounds with application in boron neutron capture therapy (in the management of tumours). This compound showed only moderate CA inhibitory properties, with inhibition constants in the range of 48 – 92 nM against isoforms hCA I, II and IX [37].

IV. UREA AND HYDROXYUREA AS CARBONIC ANHYDRASE INHIBITORS

Cyanamide (**30**) (with the tautomeric form (**31**)) is a linear molecule isoelectronic to CO_2, the physiologic substrate of the CAs, whereas the structures of the corresponding CAs catalyzed hydration products (urea and bicarbonate ion respectively) are also very similar. In both cases we observe the evolution from a linearly hybridized sp carbon atom in the substrates to a trigonal sp^2 carbon in the reaction products, the cyanamide hydrolysis reaction mimicking very efficiently the physiological one (although cyanamide exists in two tautomeric forms [38]



30 **31**

Due to the chemical simplicity of CO_2, it is difficult to design substrate analogues, as in the case of many other enzymes. Among the few molecules which are isoelectronic (and/or isosteric) with CO_2, N_2O was shown by Khalifah [39] not to act as inhibitor and cyanate is a strong suicide inhibitor being slowly hydrolysed to carbamate which remains bound within the active site [40].

Data from our laboratory clearly showed that cyanamide bound within CA active site subsequently undergoes hydrolysis to ureate as indicated from the time dependence of the electronic spectrum of the adduct of Co(II)-substituted CA with cyanamide, and from the activity measurements data in solution as well as from the X-ray crystallographic studies. X-ray analysis of crystals of hCA II soaked in solutions of cyanamide for different periods of time (3 - 24 hours up to one week) always yielded the structure of the complex of hCA II with urea, instead of the expected cyanamide adduct. This is probably due to the fact that the cyanamide hydrolysis continues within the crystals during data collection (24-36 hours). Thus, cyanamide is one of the first molecules behaving as a suicide inhibitor for CAs, being catalytically transformed to urea. It has to be mentioned that cyanate probably behaves in a similar manner, but in that case the rates of the spontaneous and of the CA catalyzed hydration are of the same order of magnitude [40], whereas in the case of cyanamide, the uncatalyzed rate is practically negligible.

The catalyzed cyanamide hydrolysis presents some interesting features. The hydration process is rather slow, since appreciable amounts of urea started to be evidenced only after several hours of incubation of enzyme with cyanamide. Practically, after 4 hours of incubation, around 5% of the cyanamide is hydrated by hCA I, whereas for the most active isozyme, hCA II, this is around 10 %. After 24 hours of incubation, about 85 % of cyanamide is hydrated by hCA I, and 90-95 % by hCA II. The reaction is practically completed after 40 hours, when an amount of urea equimolar to the enzyme has been detected.

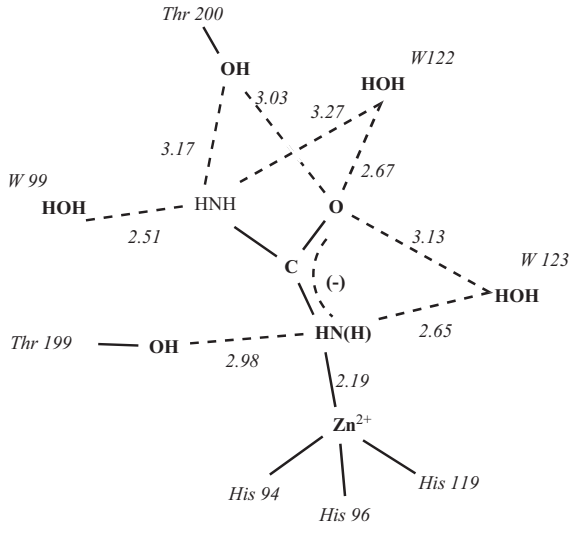


Fig. (8). Binding or urea (urate) within the hCA II active site (Briganti *et al* [41]).

We have observed that the pH at which the reactions have been performed does not influence the hydration rate. Furthermore the spontaneous dissociation rate at the physiological pH of the urea-hCA II adduct is negligible so that we need to heat-denature the enzyme in order to achieve the release of urea into the solution. Behaviour comparable to this has been previously evidenced only for the amide inhibitors of CAs by Khalifah's group, who showed a very slow dissociation process of the E-I adducts, for amides such as ethyl uretane ($H_2NCOOEt$), or oxamate (H_2NCO-COOH) [42].

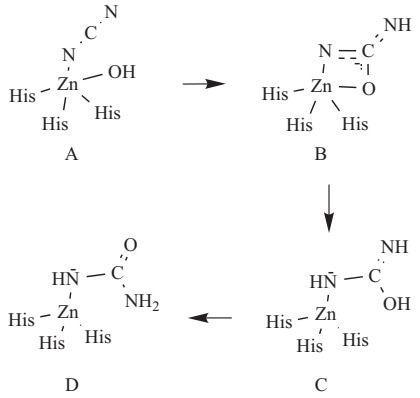
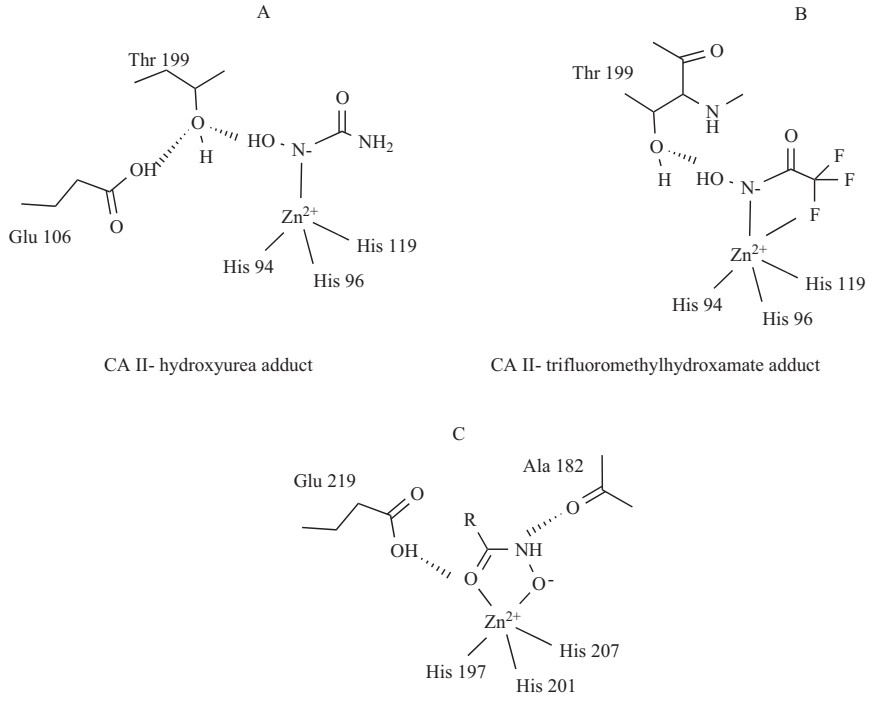


Fig. (9). Proposed mechanism for cyanamide hydration to urea (Briganti *et al.*, [41]).

The proposed catalytic mechanism (Fig. (**9**)) of cyanamide hydration to urea within CA active site is shown below. Since, as mentioned above, the binding mode (and tautomeric form) of cyanamide are unknown, this molecule has been shown schematically (Fig. (**9A**)). We propose a direct attack of the zinc bound hydroxyde ion to the initially bound cyanamide, with formation of a pentacoordinated intermediate (Fig. **9B**), which is similar to the bicarbonate adduct of the Thr-200→His mutant of CA II, described by Lindskog's and Liljas's groups [43]. We hypothesize that the initial pentacoordinated intermediate is then converted to the tetracoordinated adduct shown in (Fig. (**9C**)), which subsequently undergoes an isomerisation reaction, with the production of an ureate ion bound to zinc (Fig. (**9D**)). As suggested by spectroscopic, kinetic and structural data reported in this work, this product is strongly bound into the active site cavity, thus preventing subsequent turnover. Therefore cyanamide is indeed a suicide substrate for carbonic anhydrases. In fact this binding mode of cyanamide to hCA II was thereafter confirmed by cryoscopic X-ray crystallography. In fact, working at very low temperatures and with a short incubation time between enzyme and cyanamide, a frozen intermdiate of the catalytic turnover, of type A has been evidenced and characterized, confirming thus the above mentioned catalytic mechanism of cyanamide hydration to urea(te) [44]. As mentioned above, the ureate thus formed is very strongly fixed within the enzyme active site and cannot be displaced even by very string sulfonamide inhibibitors. It can be thus concluded that the ureate ZBG is one of the most effective oned for the design of CAIs. However, it cannot be



CA II- hydroxyurea adduct

CA II- trifluoromethylhydroxamate adduct

MMP-8 - hydroxamate adduct

Fig. (10). Hypothetic binding of hydroxyurea within the hCA II active site (**A**), based on the hCA II – urea structure, as compared to the binding of trifluoromethyl hydroxamate to hCA II (**B**), and that of a hydroxamate inhibitor within the MMP-8 active site (**C**), both determined by meand of X-ray crystallographic techniques (From Scozzafava and Supuran, [45]).

used in the drug design of inhibitors, since substituted cyanamides are not CA substrates.

The interaction of hydroxyurea ($HONHCONH_2$) with the cytosolic isozymes hCA I and hCA II has also been investigated by means of kinetic and spectroscopic techniques. Hydroxyurea acts as a weak, non-competitive inhibitor of both isozymes, for the 4-nitrophenyl acetate esterase activity, with inhibition constants around 0.1 mM for both isozymes. The spectrum of the adduct of hydroxyurea with Co(II)-hCA II is similar to the spectra of tetrahedral adducts (such as those with sulfamide, acetazolamide or cyanamide), proving a direct interaction of the inhibitor molecule with the metal center of the enzyme, whose geometry remains tetrahedral. Based on the X-ray crystal structure of the adducts of hCA II with ureate and hydroxamate inhibitors, the hypothetical binding of hydroxyurea is proposed to be achieved in deprotonated state, with the nitrogen atom coordinated to Zn(II), and the OH group of the inhibitor making a hydrogen bond with Thr 199 (Fig. (**10**)). This binding may be exploited for the design of both CA as well as matrix metalloproteinase (MMP) inhibitors, since hydroxyurea is the simplest compound incorporating hydroxamate functionality in its molecule. Indeed, such inhibitors of the sulfonylated amino acid hydroxamate type have been generated, with potencies in the low nanomolar range for both type of enzymes, CAs and MMPs [45].

CONCLUSION

The different zinc binding functions presented in this review illustrate the important advances which have been made in the design of potent and sometimes also selective carbonic anhydrase inhibitors during the past few years. Recent developments have led to the discovery of very potent inhibitors in the sulfonamide, N-substituted sulfonamide, sulfamide and sulfamate series, as well as their related compounds.

It is evident that modulation of carbonic anhydrase isozymes activities offers new pharmacological opportunities for the treatment of various pathologies, and that development of selective inhibitors with improved therapeutic potencies is of great interest.

Considering our recent knowledge of the structure-activity relationship for carbonic anhydrase inhibitors, and with respect to the drug design, work in this area may lead to the development of new selective inhibitors with more powerful activity and devoid of serious side effects.

REFERENCES

[1] *The Biological Chemistry of the Elements. The Inorganic Chemistry of Life*, J.J.R. Frausto da Silva; R.J.P. Williams Eds., 2nd ed., Oxford University Press, New York, **2001**.

[2] Hernick, M.; Fierke, C.A. *Arch. Biochem. Biophys.,* **2005**, *433*, 71-84.

[3] Parkin, G. *Chem. Rev.,* **2004**, *104*, 699-767.
[4] *Carbonic anhydrase - Its Inhibitors and Activators*; Supuran, C. T.; Scozzafava, A.; Conway, J., Eds.; CRC Press: Boca Raton, London, New York, **2004**.
[5] Scozzafava, A.; Mastrolorenzo, A.; Supuran, CT. *Expert Opin. Ther. Patents,* **2004**, *14*, 667-702.
[6] Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enz. Inhib. Med. Chem*., **2004**, *19*, 199-229.
[7] Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.*, **2003**, *23*, 146-89.
[8] Krebs, H.A. *Biochem. J.*, **1948**, *43*, 525-528.
[9] Supuran, C.T.; Scozzafava, A. *Expert Opin. Ther. Patents,* **2002**, *12*, 217-242.
[10] Supuran, C.T.; Scozzafava, A. Matrix metalloproteinases (MMPs). In *Proteinase and Peptidase Inhibition: Recent Potential Targets for Drug Development*, Smith, H.J.; Simons, C. Eds., Taylor & Francis, London & New York, pp. 35-61, **2002**.
[11] Briganti, F.; Pierattelli, A.; Scozzafava, A.; Supuran, C.T., *Eur. J. Med. Chem.,* **1996**, *31*, 1001-1010.
[12] Mincione, F.; Menabuoni, L.; Briganti, F.; Mincione, G.; Scozzafava, A.; Supuran, C.T. *J. Enz. Inhib.,* **1998**, *13*, 267-284.
[13] Supuran, C.T.; Scozzafava, A.; Briganti, F. *J. Enz. Inhib.,* **1999**, *14*, 289-306.
[14] Scozzafava, A.; Supuran, C.T. *J. Med. Chem.,* **2000**, *43*, 3677-3687.
[15] Scozzafava, A.; Banciu, M.D.; Popescu, A.; Supuran, C.T. *J. Enz. Inhib.,* **2000**, *15*, 443-453.
[16] Fenesan, I.; Popescu, R.; Scozzafava, A.; Crucin, V.; Mateiciuc, E.; Bauer, R.; Ilies, M.A.; Supuran, C.T. *J. Enz. Inhib.,* **2000**, *15*, 297-310.
[17] Scolnick, L.R.; Clements, A.M.; Liao, J.; Crenshaw, L.; Hellberg, M.; May, J., Dean, T.R.; and Christianson, D.W. *J. Am. Chem. Soc.,* **1997**, *119*, 850-851.
[18] (a) Winum, J-Y.; Scozzafava, A.; Montero, J-L.; Supuran, C.T. *Med. Res. Rev.,* **2005**, *25*, 186-228. (b) Winum, J-Y.; Scozzafava, A.; Montero, J-L.; Supuran, C.T. *Expert Opin. Ther. Patents*, **2004**, *14*, 1273-1308.
[19] Lo, Y.S.; Nolan, J.C.; Maren, T.H.; Welstead, W.J.; Gripshover, D.F.; Shamblee, D.A. *J. Med. Chem*., **1992**, *35*, 4790-4794.
[20] Abbate, F.; Supuran, CT.; Scozzafava, A.; Orioli, P.; Stubbs, M.T.; Klebe, G. *J. Med. Chem.,* **2002**, *45*, 3583-3587.
[21] Maryanoff, B.E.; Nortey, S.O.; Gardocki, J.F.; Shank, R.P.; Dodgson, S.P. *J. Med. Chem*., **1987**, *30*, 880-887.
[22] Maryanoff, B.E.; Costanzo, M.J.; Nortey, S.O.; Greco, M.N.; Shank, R.P.; Schupsky, J.J.; Ortegon, M.P.; Vaught, J.L. *J. Med. Chem*., **1998**, *41*, 1315-1343.
[23] Recacha, R.; Costanzo, M.J.; Maryanoff, B.E.; Chattopadhyay, D. *Biochem. J*., **2002**, *361*, 437-441.
[24] Casini, A.; Antel, J.; Abbate, F.; Scozzafava, A.; David, S.; Waldeck, H.; Schafer, S.; Supuran, C.T. *Bioorg. Med. Chem. Lett*., **2003**, *13*, 841-845.
[25] Antel, J.; Weber, A.; Sotriffer, C.A.; Klebe, G. In *Carbonic anhydrase - Its inhibitors and activators*, Supuran CT, Scozzafava A, Conway J Eds, CRC Press, Boca Raton (FL), USA, **2004**; pp. 45-66.
[26] Nussbaumer, P.; Billich, A. *Expert Opin. Ther. Patents,* **2003**, *13*, 605-625.
[27] Vicker, N.; Ho, Y.; Robinson, J.; Woo, L.L.; Purohit, A.; Reed, M.J.; Potter, B.V. *Bioorg. Med. Chem. Lett*., **2003**, *13*, 863-865.
[28] Ho, Y.T.; Purohit, A.; Vicker, N.; Newman, S.P.; Robinson, J.J., Leese, M.P.; Ganeshapillai, D.; Woo, L.W.L., Potter, B.V., Reed, M.J. *Biochem. Biophys. Res. Commun.*, **2003**, *305*, 909-914.
[29] Abbate, F.; Winum, J.Y.; Potter, B.V.; Casini, A.; Montero, J.L.; Scozzafava, A.; Supuran, C.T. *Bioorg. Med. Chem. Lett*., **2004**, *14*, 231-234.
[30] Pastorekova S, Pastorek J. Cancer-related carbonic anhydrase isozymes. In *Carbonic Anhydrase - Its Inhibitors and Activators*, Supuran, C.T.; Scozzafava, A.; Conway, J.; Eds, CRC Press, Boca Raton (FL), USA, **2004**, pp. 253-280.
[31] Winum, J.Y.; Vullo, D.; Casini, A.; Montero, J-L.; Scozzafava, A.; Supuran, C.T. *J. Med. Chem.,* **2003**, *46*, 2197-2204.
[32] Winum, J.Y.; Vullo, D.; Casini, A.; Montero, J.L.; Scozzafava, A.; Supuran, C.T. *J. Med. Chem*., **2003**, *46*, 5471-5477.
[33] Bonnac, L.; Innocenti, A.; Winum, J.Y.; Casini, A.; Montero, J.L. ; Scozzafava, A. ; Barragan, V.; Supuran, C.T. *J. Enz. Inhib. Med. Chem.,* **2004**, *19*, 287-291.
[34] Winum, J.Y.; Scozzafava, A.; Montero, J.L.; Supuran, C.T. *Expert Opin. Ther. Patents* , **2006**, 16, 27-47.
[35] Casini, A.; Winum, J.Y.; Montero, J.L.; Scozzafava, A.; Supuran, C.T. *Bioorg. Med. Chem. Lett*., **2003**, *13*, 837-840.
[36] Winum, J.Y.; Innocenti, A.; Nasr, J.; Montero, J.L.; Scozzafava, A.; Vullo, D.; Supuran, C.T. *Bioorg. Med. Chem. Lett*., **2005**, *15*, 2353-2358.
[37] Winum, J.Y.; Cecchi, A.; Montero, J.L.; Innocenti, A.; Scozzafava, A.; Supuran, C.T. *Bioorg. Med. Chem. Lett*., **2005**, *15*, 3302-3306.
[38] (a) Kurzer, F.; Douraghi-Zadeh, K. *Chem. Rev*., **1967**, *67*, 107-152; (b) Rabalais, J.W.; McDonald, J.M.; Scherr, V.; McGlynn S.P. *Chem. Rev*., **1971**, *71*, 73 - 95.
[39] Khalifah, R.G. *J. Biol. Chem*., **1971**, *246*, 2561 - 2573.
[40] Supuran, C.T.; Conroy, C.W.; Maren, T.H. *Proteins Struct. Funct. Genet*., **1997**, *27*, 272- 278.
[41] Briganti, F.; Mangani, S.; Scozzafava, A.; Vernaglione, G.; Supuran, CT. *J. Biol. Inorg. Chem.,* **1999**, *4*, 528-36.
[42] Rogers, J.I.; Mukherjee, J.; Khalifah, R.G. *Biochemistry,* **1987**, *26*, 5672-5679.
[43] Xue, Y.; Vidgren, J.; Svensson, L.A.; Liljas, A.; Jonsson, B.H.; Lindskog, S. *Proteins Struct. Funct. Genet*., **1993**, *15*, 80-87.
[44] Guerri, A.; Briganti, F.; Scozzafava, A.; Supuran, C.T.; Mangani, S. *Biochemistry*, **2000**, *39*, 12391-12397.
[45] Scozzafava, A.; Supuran, C.T. *Bioorg. Med. Chem.,* **2003**, *11*, 2241-2246.

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