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Carbonic anhydrase inhibitors: 2-Substituted-1,3,4-thiadiazole-5-sulfamides act as powerful and selective inhibitors of the mitochondrial isozymes VA and VB over the cytosolic and membrane-associated carbonic anhydrases I, II and IV

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ABSTRACT

A series of 2-substituted-1,3,4-thiadiazole-5-sulfamides was prepared and assayed as inhibitors of several carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the cytosolic CA I and II, the membrane-associated CA IV and the mitochondrial CA VA and VB. The new compounds showed weak inhibitory activity against hCA I (K_Is of 102 nM–7.42 μM), hCA II (K_Is of 0.54–7.42 μM) and hCA IV (K_Is of 4.32–10.05 μM) but were low nanomolar inhibitors of hCA VA and hCA VB, with inhibition constants in the range of 4.2–32 nM and 1.3–74 nM, respectively. Furthermore, the selectivity ratios for inhibiting the mitochondrial enzymes over CA II were in the range of 67.5–415, making these sulfamides the first selective CA VA/VB inhibitors.

Among the sixteen α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms found in animals, two CA isozymes, VA and VB, are present in mitochondria.[1–3] These isozymes are involved in several biosynthetic processes, such as ureagenesis, gluconeogenesis, and lipogenesis, both in vertebrates as well as in invertebrates.[1,4,5] The provision of enough of the substrate, bicarbonate, in several biosynthetic processes involving pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC), and carbamoyl phosphate synthetases I and II, is assured mainly by the catalysis involving the mitochondrial isozymes CA VA and CA VB, probably assisted by the high activity cytosolic isozyme CA II, as shown schematically in Figure 1.[1,2] CAs thus play a key role in fatty acid biosynthesis. Mitochondrial pyruvate carboxylase (PC) is needed for efflux of acetyl groups from the mitochondria to the cytosol where fatty acid biosynthesis takes place.[1] Pyruvate is carboxylated to oxaloacetate in the presence of bicarbonate and under the catalytic influence of the mitochondrial isozymes CA VA and/or CA VB. The mitochondrial membrane is impermeable to acetyl-CoA which reacts with oxaloacetate, leading to citrate, which is thereafter translocated to the cytoplasm by means of the tricarboxylic acid transporter. As oxaloacetate is unable to cross the mitochondrial membrane, its decarboxylation regenerates pyruvate which can be then transported into the mitochondria by means of the pyruvate transporter (Fig. 1). The acetyl-CoA thus generated in the cytosol is in fact used for the de novo lipogenesis, by carboxylation in the presence of ACC and bicarbonate, with formation of malonyl-CoA, the conversion between CO_2 and bicarbonate being assisted by CA II. Subsequent steps involving the sequential transfer of acetyl groups lead to longer chain fatty acids.[1–5] Therefore, several CA isozymes are critical to the entire process of fatty acid biosynthesis: VA and/or VB within the mitochondria (to provide enough substrate to PC), and CA II within the cytosol (for providing sufficient substrate to ACC). Inhibition of CAs by clinically used sulfonamides such as acetazolamide **AZA**, zonisamide **ZNS** or the sulfamate topiramate, **TPM**, can decrease lipogenesis in adipocytes in cell culture.[1–3] Furthermore, among some of the side effects of these drugs is also the weight loss,[6] which may in fact lead to novel anti-obesity therapies.[7]

In previous contributions from our laboratories[8–10] we have shown that both CA VA and CA VB are druggable targets. Rather large libraries of various sulfonamides and sulfamates have been assayed as inhibitors of these mitochondrial enzymes, with several low nanomolar inhibitors being detected.[8–10] However, an important drawback of most of these compounds is represented by the rather low selectivity for inhibiting the mitochondrial CAs over

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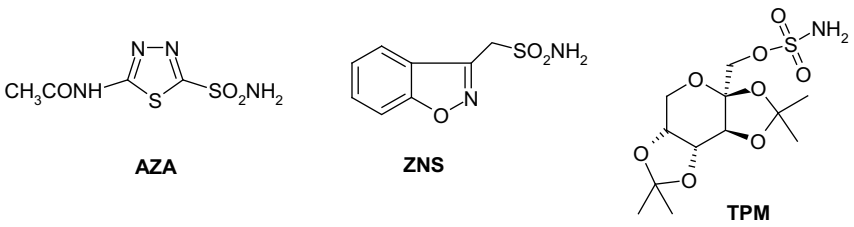
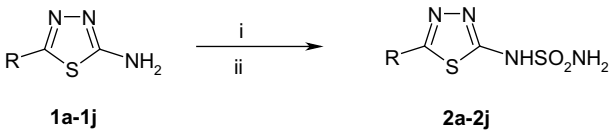


the cytosolic/membrane-bound ubiquitous isoforms such as CA I, II (cytosolic) and CA IV (extracellular, membrane-associated isozyme).[1,8–10] Considering the interest in CA VA/VB—selective inhibitors which might be developed as antiobesity agents, we explore here a less investigated class of CA inhibitors (CAIs) for obtaining compounds targeting the mitochondrial CAs, that is, the sulfamides.[11,12] Indeed, aromatic, heterocyclic and sugar sulfamides have been reported earlier to generate effective inhibitors of several CA isozymes (mainly CA I, II and IX),[11,12] and the X-ray crystal structures for two such compounds complexed within the CA II active site are also available,[12c,12d] proving the sulfamide moiety to be an effective zinc binding group for obtaining CAIs, similarly to the bioisosteric sulfonamide and sulfamate ones.[11]

Here we report the synthesis of 1,3,4-thidiazole sulfamides possessing various 2-substitutents.[13,14] This scaffold has been chosen as it is present in one of the most investigated and powerful CAI, acetazolamide **AZA**, used clinically since 1956,[1] and also because its binding to the enzyme is effective, as shown for many **AZA** derivatives for which the X-ray crystal structure has been resolved in adduct with different CA isoforms[15] (Scheme 1).

Starting with the commercially available 2-substituted-5-amino-1,3,4-thiadiazoles **1**, which have been sulfamoylated with in situ generated sulfamoyl chloride, by a method reported earlier by one of this groups,[14] the sulfamides **2a–j** have been prepared with excellent yields.[13] The various substituents in position 2 of the heterocyclic ring of the new compounds **2a–j** were chosen in such a way as to have a rather comprehensive SAR insight for this



Scheme 1. Synthesis of 2-substituted-1,3,4-thiadiazole-5-sulfamide **2a–2j**: Reagents and conditions: (i) *t*-BuOH, $ClSO_2NCO$, NEt_3, CH_2Cl_2. (ii) TFA 95% in CH_2Cl_2.

class of CAIs. Thus, starting with the unsubstituted parent compound ($R = H$, **2a**), both aliphatic (Et, *t*-Bu, etc), aromatic (Ph, substituted phenyl, etc) as well as sulfide- and sulfone incorporating such moieties have been introduced in this position. X-ray crystallographic data on many CA—sulfonamide/sulfamate/sulfamide adducts revealed that in addition to the zinc binding group (a sulfamide one for derivatives **2**) and organic scaffold (1,3,4-thiadiazole for **2**), the tails present in CAIs are critical both for the isozyme inhibition profile as well as for modulationg the physico-chemical properties of such inhibitors, which may thus lead to various pharmacological applications.[1,15–18] This explains the choice of the tail groups present in derivatives **2a–j** reported here.

Inhibition data against five physiologically relevant CA isozymes, that is, the cytosolic hCA (human CA) I and II, the membrane-associated hCA IV as well as the two mitochondrial isoforms hCA VA and VB, are presented in Table 1.[19] Three standard CAIs, that is, **AZA**, **ZNS** and **TPM**, have been assayed in the same conditions in order to allow a better understanding of the inhibi-

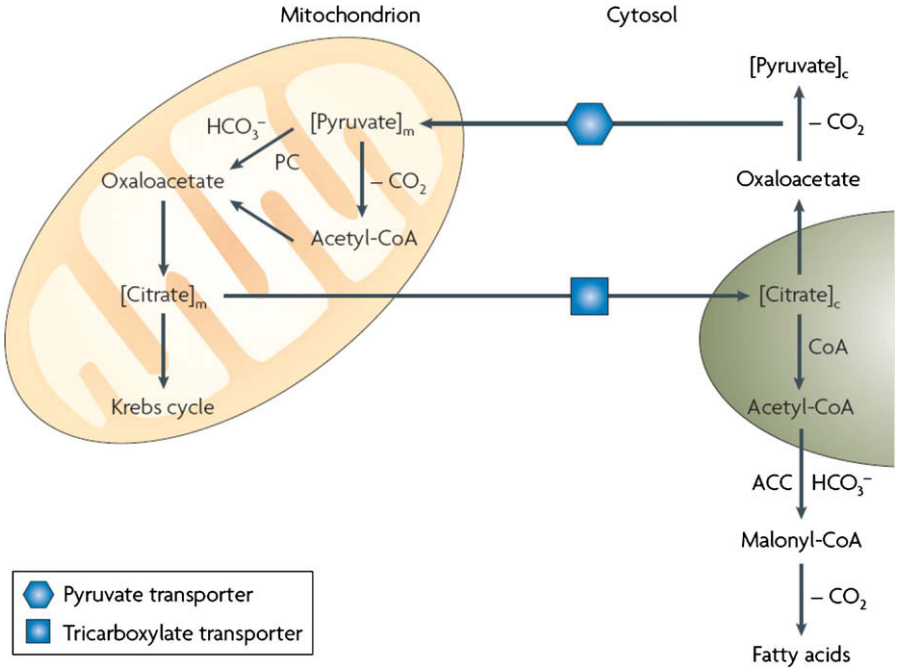
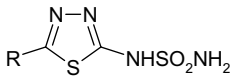


Figure 1. The transfer of acetyl groups from the mitochondrion to the cytosol (as citrate) for the provision of substrate for *de novo* lipogenesis.[1] All steps involving bicarbonate need the presence of at least two CA isozymes: CA VA/VB in the mitochondrion and CA II in the cytosol (see discussion in the text).

Table 1
Inhibition data of human CA isozyms I, II (cytosolic), IV (membrane-associated) and VA, and VB (mitochondrial) with compounds **2a–2j** and standard inhibitors (acetazolamide **AZA**, zonisamide **ZNS** and topiramate **TPM**), by a stopped-flow, CO_2 hydration assay.[19]



2a-2j

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No	R	K_I*				
		hCA I[a](μM)	hCA II[a](μM)	hCA IV[b](μM)	hCA VA[c](μM)	hCA VB[c](μM)
2a	H	7.42	0.97	8.91	28.3	74
2b	Et	7.01	0.95	8.64	18.7	63
2c	*t*-Bu	5.54	0.90	8.40	10.4	2.8
2d	CF_3	6.86	1.08	4.32	7.3	3.9
2e	MeS	2.40	0.92	8.30	32	2.9
2f	EtS	1.89	1.13	7.58	9.3	23.1
2g	Ph	1.66	0.87	5.54	9.2	7.5
2h	4-$MeOC_6H_4$	0.102	0.54	8.76	8.0	1.3
2i	4-Br-C_6H_4	5.85	0.82	10.05	4.2	4.5
2j	$MeSO_2$	0.103	0.94	6.10	8.7	2.7
AZA	–	0.250	0.012	0.074	63	54
ZNS	–	0.056	0.035	8.59	20	6033
TPM	–	0.250	0.010	4.90	63	30

* Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[19]
[a] Human, recombinant isozymes.
[b] Truncated cloned human isoform lacking the first 20 aminoterminal amino acids.
[c] Full length, recombinant human isoforms.[8–10]

tion profile of the new compounds **2a–2j** investigated here. Data of Table 1 allow the following SAR to be evidenced for the new sulfamides **2** reported here:

(i) Against hCA I, the sulfamides **2** showed a moderate-weak inhibitory activity, with inhibition constants in the range of 102 nM–7.42 μM, bing thus, with two exceptions (compounds **2h** and **2j**) much weaker inhibitors as compared to the clinically used drugs **AZA-TPM** (K_Is in the range of 56–250 nM). The least active hCA I inhibitor was the parent, unsubstituted compound **2a**, whereas introduction of various substituents in position 2 of the thiadiazole ring enhances activity. The groups leading to best activity were 4-methoxyphenyl and methylsulfonyl (**2h** and **2j**).

(ii) Isozyme hCA II was also weakly inhibited by the new sulfamides **2**, with K_Is in the range of 0.54–1.13 μM, whereas the clinically used drugs were much stronger inhibitors of this ubiquitous isoform (K_Is in the range of 10–35 nM). It may be observed a very flat SAR for sulfamides **2** in inhibiting hCA II, with the nature of groups substituting in 2 the thiadiazole ring, having a small influence on the inhibitory power (Table 1).

(iii) Sulfamides **2** act as quite weak inhibitors of the membrane-associated isoforms hCA IV, with K_Is in the range of 4.32–10.05 μM, unlike **AZA** which is a strong inhibitor (K_I of 74 nM) but similarly to **ZNS** and **TPM** (K_Is of 4.90–8.59 μM).

(iv) Excellent inhibitory properties were evidenced for derivatives **2** against the two mitochondrial CA iozymes, hCA VA and hCA VB. Indeed, these compounds showed K_Is in the range of 4.2–28.3 nM against hCA VA, and of 1.3–74 nM against hCA VB, respectively. It may be observed that compounds **2** are much better inhibitors of the mitochondrial CAs as compared to the three clinically used drugs (K_Is in the range of 20–63 nM against hCA VA, and of 30–6033 nM against hCA VB, respectively, Table 1). For hCA VA, the substitution patterns of the heterocyclic ring leading to the best inhibitors included the trifluoromethyl, thioethyl-, aryl and methylsulfonyl moieties (K_Is < 10 nM) whereas the remaining ones generated slightly less effective inhibitors. For hCA VB, only three compounds (**2a, 2b** and **2f**) showed K_Is > 10 nM, all the other substitution patterns leading to derivatives with excellent activity (K_Is < 7.5 nM). These are in fact the compounds with the best inhibitory activity ever reported against the mitochondrial enzymes CA VA and CA VB.

(v) Another very interesting property of the newly described sulfamides **2** is related to their selective inhibition of the mitochondrial isozymes (CA VA and VB) over the cytosolic and membrane asociated isoforms (CA I, II and IV). Especially CA II constitutes a problem when designing various CAIs targeting other izoymes, because CA II has generally a very high affinity for sulfonamides, sulfamates and sulfamides, and it is also a ubiquitoous enzyme in vertebrates, including humans.[1–3] As seen from data of Table 1, the three clinically used compounds mentioned here (but also the other CAIs in clinical use)[1] are generally much better CA II than CA VA/VB inhibitors (except for zonisamide against CA VA). For example the selectivity ratio of **AZA** for inhibiting CA VA over CA II is of 0.19, and for inhibiting CA VB over CA II is of 0.22. Basically **AZA** has a much higher affinity for the cytosolic isoform CA II than for the mitochondrial ones. However, all compounds **2** reported here showed a much better inhibitory activity against the mitochondrial isozymes than against the cytosolic (or membrane-associated) ones. Thus, for example, **2h** has a selectivity ratio for inhibiting CA VA over CA II of 67.5, and for inhibiting CA VB over CA II of 415. For **2i**, these ratios are of 195 and of 182, respectively. Thus, these compounds are 67.5–415-times better inhibitors of the mitochondrial over the cytosolic isozymes, which is a very interesting result, never evidenced earlier for any other class of CAIs.

In conclusion, we prepared a small series of 2-substituted-1,3,4-thiadiazole-5-sulfamides and assayed them for the inhibition of five physiologically relevant isozymes, the cytosolic CA I and II, the membrane-associated CA IV and the mitochondrial CA VA and VB. The new compounds showed weak inhibitory activity against hCA I (K_Is of 102 nM–7.42 μM), hCA II (K_Is of 0.54–7.42 μM) and hCA IV (K_Is of 4.32–10.05 μM) but were low nanomolar inhibitors of hCA VA and hCA VB, with inhibition constants in the range of 4.2–32 nM and 1.3–74 nM, respectively. Furthermore, the selectivity ratios for inhibiting the mitochondrial enzymes over CA II were in the range of 67.5–415, making these sulfamides the first selective CA VA/VB inhibitors.

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13. General procedure for the preparation of thiadiazole-sulfamides **2**: To a solution of 2-substituted-5-amino-1,3,4-thiadiazole **1** in methylene chloride and 1.1 equiv of triethylamine was added dropwise a solution of *tert*-butoxycarbonylamino sulfonyl chloride (prepared ab initio by reacting 1 equiv of *tert*-butanol and 1 equiv of chlorosulfonyl isocyanate in methylene chloride at 0 °C).[14] The mixture was stirred 1 h at room temperature, and then concentrated under vacuum. The residue is purified on silica gel column chromatography using ethyl acetate-petroleum ether 7–3 as eluent to give the Boc-protected sulfamide in good yield (75–80%). This compound was then deprotected using a solution of trifluoroacetic acid in methylene choride 50–50 v–v. **2a**: mp 151–154 °C; MS (ESI^+, 20eV): *m/z* 203.17 $[M+Na]^+$, 383.10 $[2M+Na]^+$ ^1H NMR (400 MHz, DMSO-d_6) δ 8.63 (s, 1H), 6.77 (s, 2H), 3.16 (s, 1H). **2b**: mp 100–110 °C; MS (ESI^+, 20eV): *m/z* 231.1 $[M+Na]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 6.71 (s, 2H), 3.16 (s, 1H), 2.78 (q, 2H), 1.2 (t, 3H).**2c**: mp 150–153 °C; MS (ESI^+, 20eV): *m/z* 259.23 $[M+Na]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 6.73 (s, 2H), 3.16 (s, 1H), 1.30 (s, 9H). **2d**: mp 179-180°C; MS (ESI^-, 20eV): *m/z* 247.06 $[M-H]^-$; ^1H NMR (400MHz, DMSO-d_6) δ 7.23 (s, 2H), 3.16 (s, 1H). **2e**: mp 150–153 °C; MS (ESI^+, 20 eV): *m/z* 249.09 $[M+Na]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 6.85 (s, 2H), 3.16 (s, 1H), 2.61 (s, 3H). **2f**: mp 145–148 °C; MS (ESI^+, 20 eV): *m/z* 263.20 $[M+Na]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 6.86 (s, 2H), 3.16 (s, 1H), 3.12 (q, 2H), 1.31 (t, 3H). 2 g: mp 170–172 °C; MS (ESI^+, 20 eV): *m/z* 279.15; $[M+Na]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 7.78 (s, 2H), 7.53 (s, 3H), 6.88 (s, 2H), 3.16 (s, 1H). **2h**: 171–174 °C; MS (ESI^+, 20 eV): *m/z* 309.17 $[M+Na]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 7.72 (d, 2H), 7.06 (d, 2H), 6.82 (s, 2H), 3.81 (s, 3H), 3.16 (s, 1H). **2i**: mp 173–176 °C; MS (ESI^+, 20 eV): *m/z* 359.06 $[M+Na]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 7.74 (m, 4H), 6.89 (s, 2H), 3.16 (s, 1H). **2j**: 154–156 °C; MS (ESI^+, 20 eV): *m/z* 281.15 $[M+Na]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 7.14 (s, 2H), 3.5 (s, 3H), 3.16 (s, 1H).
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19. Khalifah, R.G. *J. Biol. Chem.*, **1971**, *246*, 2561. An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction.[18] The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, and represent the mean from at least three different determinations.