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Cloning, characterization and sulfonamide inhibition studies of an α-carbonic anhydrase from the living fossil sponge *Astrosclera willeyana*

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ABSTRACT

The α-carbonic anhydrase (CA, EC 4.2.1.1) Astrosclerin-3 previously isolated from the living fossil sponge *Astrosclera willeyana* (Jackson et al., *Science* 2007, 316, 1893), was cloned, kinetically characterized and investigated for its inhibition properties with sulfonamides and sulfamates. Astrosclerin-3 has a high catalytic activity for the CO_2 hydration reaction to bicarbonate and protons (k_{cat} of 9.0×10^5 s^{-1} and k_{cat}/K_m of 1.1×10^8 $M^{-1} \times s^{-1}$), and is inhibited by various aromatic/heterocyclic sulfonamides and sulfamates with inhibition constants in the range of 2.9 nM–8.85 μM. Astrosclerin, and the human isoform CA II, display similar kinetic properties and affinities for sulfonamide inhibitors, despite more than 550 million years of independent evolution. Because Astrosclerin-3 is involved in biocalcification, the inhibitors characterized here may be used to gain insights into such processes in other metazoans.

1. Introduction

Sponges, animals belonging to the Phylum Porifera, are the most ancient multicellular organisms.[1] Recently, one of our groups identified three isoforms of the enzyme Astrosclerin, a carbonic anhydrase (CA, EC 4.2.1.1) from the calcareous 'coralline' skeleton of a tropical sponge species, the living fossil *Astrosclera willeyana*. By means of a paleogenomics approach, including gene- and protein-expression techniques phylogenetic reconstruction, the Astrosclerins were demonstrated to be descended from a last common ancestor of the Metazoa that likely possessed a single copy of this enzyme.[1] The CA superfamily is composed of five genetically unrelated enzymes families, the α-CAs (present in vertebrates, bacteria, algae and cytoplasm of green plants),[2–4] the β-CAs (predominantly in bacteria, algae, chloroplasts of monodicotyledons and dicotyledons), the γ-CAs (mainly found in Archaea and some bacteria), the δ- and -CAs (present in marine diatoms).[2–5] In the genomes of all living metazoans the α-CAs are present in multiple copies.[2–5] To date, the best studied CA enzymes are the 16 α-CA isozymes described in mammals, which have various subcellular localizations, a different catalytic activity for the physiologic reaction, as well as a diverse susceptibility to different classes of inhibitors.[2–5] All of these enzymes are effective catalysts for a simple but otherwise slow reaction (at physiologic pH), that is, CO_2 hydration to bicarbonate and protons. α-CAs also display promiscuous catalytic activities, with some acting as effective esterases/phosphatases on a large range of substrates.[6,7] More recently, a β-CA-like enzyme has been isolated from an acidophilic extremophile which acts as a catalyst for the hydrolysis of CS_2 to CO_2 and H_2S. Surprisingly, the active site architecture of this enzyme has much in common with that of β-CAs.[8]

As outlined above, CAs are present in organisms across a broad range of the tree of life, from prokaryotes to eukaryotes. Apart some bacterial/fungal β-CAs[4,9] the most studied CAs are the α-CAs, with the human and rodent enzymes being the most intensively investigated.[2–4,9] These were the first CAs to be discovered and are widely distributed from bacteria, to protozoa, fungi, plants and animals.[1–4,9] Outside of the vertebrates, few prokaryotic α-CAs have been cloned, kinetically characterized and investigated for their interaction with different classes of inhibitors, with the exception of two such enzymes from the coral *Stylophora pistillata*.[10,11] Indeed, in this coral, anion and sulfonamide inhibitors (the main classes of CA inhibitors, CAIs)[2] were shown to be useful tools to better understand the physiological role of the two coral enzymes (STPCA and STPCA-2) in biomineralization.[10,11]

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In an earlier study, Jackson et al.[1] identified an active -CA in *A. willeyana* (which was denominated Astrosclerin-3). However, no detailed kinetic or inhibition studies of this enzyme have been reported. Here we report the cloning of this enzyme by using a GST-tagged protein purification method which allows for the preparation of large amounts of protein for kinetic and inhibition studies. We thereafter investigated the catalytic properties of Astrosclerin-3 for the physiologic reaction and its inhibition profile with a large number of aromatic/heterocyclic sulfonamides.

2. Results and discussion

2.1. Cloning and catalytic activity of Astrosclerin

The *Astrosclerin-3* gene of *Astrosclera willeyana* (National Center for Biotechnology Information (NCBI) accession number EF434878) encodes a 292 amino acid long polypeptide, with a calculated molecular weight of 33.2 kDa (or 31.04 kDa for the mature polypeptide, which contains 272 amino acid residues).[1] For preparing large amounts of soluble protein needed for kinetic and inhibition studies, a GST tagged chimeric protein was constructed (see Section 4) from which the GST tag was subsequently removed as previously reported for other GST-tagged - and -CAs.[12] The SDS–PAGE of the GST-Astrosclerin-3 chimeric protein (Mw of 57 kDa) and that of Astrosclerin-3 alone after the removal of the GST tag (Mw 31 kDa) are shown in Figure 1. Only one band of the two proteins has been observed in reducing and non-reducing conditions, proving that Astrosclerin-3 and the GST-Astrosclerin-3 chimeric protein are monomers. It is obvious that the GST construct reported here allows for the preparation of high amount of Astrosclerin-3 in *E. coli* (see Section 4).

Purified Astrosclerin-3 was assayed for its catalytic activity of the physiologic reaction, that is, CO_2 hydration to bicarbonate and protons, by a stopped flow technique (Table 1). In the previous study[1] it was reported that Astrosclerin-3 has a high catalytic activity (but no kinetic parameters were reported), which we confirm here. As observed in Table 1 where the kinetic parameters of previously characterized -CAs are provided, Astrosclerin-3 has a k_{cat} of 9.0×10^5 s^{-1} and k_{cat}/K_m of 1.1×10^8 $M^{-1} \times s^{-1}$. These values are comparable to those of the human isoform hCA II. Both Astrosclerin-3 and hCA II are almost one order of magnitude more efficient catalysts for the CO_2 hydration compared to the slow isoform hCA I (Table 1). Furthermore, this activity is inhibited by the sulfonamide acetazolamide (see below) with inhibition constants between 12 and 250 nM.

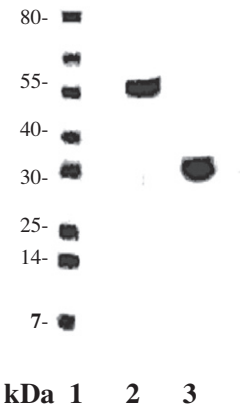

Figure 1. SDS–PAGE of: ladder (lane 1) and GST-Astrosclerin 1 (Mw = 57 kD, lane 2) and Astrosclerin (Mw = 31 kDa, lane 3) enzymes. Only one band of both Astrosclerin and GST-Astrosclerin was present in both reducing (10 mM dithiothreitole) and non-reducing conditions, proving these enzymes to be monomers.

In Fig. 2, an alignment of several CAs is presented, which includes Astrosclerin-3, the human isoforms hCA I–XIV, the vaccinia CA-like protein vaccCAwt[14] as well as two receptor protein tyrosine phosphatases RPTPbeta/gamma, which contain a catalytically inactive CA-like domain.[15,16] Three interesting facts emerged from the data of Fig. 2:

(i) As all catalytically active CAs (hCA I–VII, IX, XII–XIV), Astrosclerin-3 has three zinc-coordinating histidine residues (His 94, 96, 119, hCA I numbering system), without which CO_2 hydrase activity is not possible. Indeed, hCA VIII, X and XI, vaccCAwt and the two RPTPs, which do not have one or more of these His residues, do not show CO_2 hydrase activity.[14–16]

(ii) The gate-keeper residues Glu106 and Thr199, conserved in all catalytically active CAs, are also present in Astrosclerin-3. The two residues participate in a network of hydrogen bonds with the zinc-coordinated water molecule and enhance its nucleophilicity, and probably also contribute to orientating the substrate (and the inhibitors) when they bound in the neighborhood of the catalytic center.[2–5,17]

(iii) The third structural important element in CAs is the proton shuttling residue, which assists water deprotonation and the transfer of the proton from the solvent molecule coordinated to zinc to the environment.[17] In the most active CAs this is a histidine residue placed in the middle of the active site cavity, more precisely His64 (hCA I numbering).[17,18] It may be observed that many but not all catalytically effective CA isoforms have indeed a His in positions 64 (e.g., hCA I, II, VII, XIII among the cytosolic isoforms, hCA IV, VI, IX, XII and XIV among the secreted/extracellular ones). However, some non-catalytic CAs, such as CA VIII, X and XI have His64. Surprisingly, Astrosclerin-3 does not have His64 (hCA I numbering) and no His residues within 15 residues of this position. The nearest His residue appears at position 80 (Astrosclerin-3 numbering), making this the most likely proton shuttling candidate in Astrosclerin-3. Indeed, CAs which do not have a His able to participate in proton shuttling processes (such as CA III which has Lys64 or CA VA/VB which have Tyr64) are characterized by much lower CO_2 hydrase efficacies compared to those possessing a His64 (or His66 in the transmembrane/secreted isoforms numbering, see Figure 2). As far as we know, this is the first report of a highly effective

Table 1
Kinetic parameters for CO_2 hydration reaction catalysed by some human -CA isozymes (hCA I, and II) and the sponge enzyme Astrosclerin, at 20 °C and pH 7.5, and their inhibition data with acetazolamide AAZ (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used drug[13]

Enzyme	Class	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \times s^{-1}$)	K_I (acetazolamide) (nM)
hCA I [a]		2.0×10^5	4.0	5.0×10^7	250
hCA II [a]		1.4×10^6	9.3	1.5×10^8	12
Astrosclerin [b]		9.0×10^5	8.2	1.1×10^8	51

[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method (pH 7.5),[13] from Ref. 12
[b] Recombinant enzymes, stopped flow CO_2 hydrase assay method (pH 7.5),[13] this work.

Figure 2. Alignment of the sequences of Astrosclerin with those of mammalian (hCA I–XV) and vaccinia (vaccCAwt) proteins. The Zn(II) ligands are shown in red and the proton shuttle residue in blue (alignments carried out with ClustalW).

-CA with the putative proton shuttle residue at position 80 instead of 64.

2.2. Sulfonamide inhibition of Astrosclerin

Table 2 shows Astrosclerin inhibition data with a panel of sulfonamides and one sulfamate (obtained for the CO_2 hydration reaction catalyzed by CAs),[13] some of which are clinically used drugs,[2] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, zonisamide **ZNS**, sulpiride **SLP**, indisulam **IND**, celecoxib **CLX**, valdecoxib **VLX**, sulthiame **SLT**, saccharin **SAC** and hydrochlorothiazide **HCT**. The simpler derivatives **1-26** were also included in the study as they represent the most extensively used scaffolds for designing potent or isoform-selective CAIs targeting human/non vertebrate CAs.[19,20]

The data in Table 2 highlights the following:

(i) Simple aromatic/heterocyclic sulfonamides/disulfonamides, such as compounds **1–14, 21--26**, but also the clinically used derivatives **MZA, EZA, DZA, BRZ**, and **ZNS-HCT**, act as

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hCAI            KVLD--ALQAIKTKGKRAPFT-NFDPSTLLPS----SLDFWTYPGSLTHPPLYESVTWII 212
hCAXIII         KITD--TLDSIKEKGKQTRFT-NFDLLSLLPP----SWDYWTYPGSLTVPPLLESVTWIV 212
hCAII           KVVD--VLDSIKTKGKSADFT-NFDPRGLLPE----SLDYWTYPGSLTTPPLLECVTWIV 210
hCAIII          IFLD--ALDKIKTKGKEAPFT-KFDPSCLFPA----CRDYWTYQGSFTTPPCEECIVWLL 210
hCAVII          RLTD--ALYMVRFKGTKAQFS-CFNPKCLLPA----SRHYWTYPGSLTTPPLSESVTWIV 213
hCAVA           RLVD--ILPEIKHKDARAAMR-PFDPSTLLPT----CWDYWTYAGSLTTPPLTESVTWII 209
hCAVB           KLVD--TLPSIKHKDALVEFG-SFDPSCLMPT----CPDYWTYSGSLTTPPLSESVTWII 214
hCAVIII         AVTE--ILQDIQYKGKSKTIP-CFNPNTLLPD--PLLRDYWVYEGSLTIPPCSEGVTWIL 209
RPTPbeta        AIID--GVESVSRFGKQAALD-PFILLNLLP--N-STDKYYIYNGSLTSPPCTDTVDWIV 204
RPTPgamma       PIIH--GLKGVVHHEKETFLD-PFVLRDLLP--A-SLGSYYRYTGSLTTPPCSEIVEWIV 203
hCAIV           PLVE--ALSNIPKPEMSTTMA-ESSLLDLLPKEE-KLRHYFRYLGSLTTPTCDEKVVWTV 219
hCAXII          KIFS--HLQHVKYKGQEAFVP-GFNIEELLP--E-RTAEYYRYRGSLTTPPCNPTVLWTV 213
hCAXIV          HILS--HLHEVRHKDQKTSVP-PFNLRELLP--K-QLGQYFRYNGSLTTPPCYQSVLWTV 211
hCAIX           QLLS--RLEEIAEEGSETQVP-GLDISALLP--S-DFSRYFQYEGSLTTPPCAQGVIWTV 208
hCAVI           NFIS--HLANIKYPGQRTTLT-GLDVQDMLP--R-NLQHYYTYHGSLTTPPCTENVHWFV 215
vaccCAwt        KIVN--QLHSIRSANTSAPFDSVFYLDNLLPS----KLDYFTYLG--TTINHSADAVWII 184
hCAX            RMLNRDTITRITYKNDAYLLQ-GLNIEELYPE----TSSFITYDGSMTIPPCYETASWII 227
hCAXI           RLLNRDTITRISYKNDAYFLQ-DLSLELLFPE----SFGFITYQGSLSTPPCSETVTWIL 227
Astrosclerin    KLSP------VPTGHEDSHSVSDLVYTDLLPR----NRDYYHYEGSLTTPLCDETVQWFV 202
                 .        :                : *        :  * *  :         * :

hCAI            CKESISVSSEQLAQFRSLLSNVEGDNAVP-----MQHNNRPTQPLKGRTVRASF------ 261
hCAXIII         LKQPINISSQQLAKFRSLLCTAEGEAAAF-----LVSNHRPPQPLKGRKVRASFH----- 262
hCAII           LKEPISVSSEQVLKFRKLNFNGEGEPEEL-----MVDNWRPAQPLKNRQIKASFK----- 260
hCAIII          LKEPMTVSSDQMAKLRSLLSSAENEPPVP-----LVSNWRPPQPINNRVVRASFK----- 260
hCAVII          LREPICISERQMGKFRSLLFTSEDDERIH-----MVNNFRPPQPLKGRVVKASFRA---- 264
hCAVA           QKEPVEVAPSQLSAFRTLLFSALGEEEKM-----MVNNYRPLQPLMNRKVWASF------ 258
hCAVB           KKQPVEVDHDQLEQFRTLLFTSEGEKEKR-----MVDNFRPLQPLMNRTVRSSF------ 263
hCAVIII         FRYPLTISQLQIEEFRRLRTHVKGAELVEGCDGILGDNFRPTQPLSDRVIRAAFQ----- 264
RPTPbeta        FKDTVSISESQLAVFCEVLTMQQSGYVMLM--DYLQNNFREQQYKFSRQVFS-------- 254
RPTPgamma       FRRPVPISYHQLEAFYSIFTTEQQDHVKSV--EYLRNNFRPQQRLHDRVVSK-------- 253
hCAIV           FREPIQLHREQILAFSQKLYYDKEQTVS------MKDNVRPLQQLGQRTVIKS------- 266
hCAXII          FRNPVQISQEQLLALETALYCTHM-DDPSP--REMINNFRQVQKFDERLVYTSF------ 264
hCAXIV          FYRRSQISMEQLEKLQGTLFSTEE-E-PS---KLLVQNYRALQPLNQRMVFASF------ 260
hCAIX           FNQTVMLSAKQLHTLSDTLWG------PGD--SRLQLNFRATQPLNGRVIEASF------ 254
hCAVI           LADFVKLSRTQVWKLENSLLD------HRN--KTIHNDYRRTQPLNHRVVESNF------ 261
vaccCAwt        FPTPINIHSDQLSKFRTLLSSSNHDGKPHY----ITENYRNPYKLNDDTQVYYSG----- 235
hCAX            MNKPVYITRMQMHSLRLLSQNQPSQIFLS-----MSDNFRPVQPLNNRCIRTN------- 275
hCAXI           IDRALNITSLQMHSLRLLSQNPPSQIFQS-----LSGNSRPLQPLAHRALRGN------- 275
Astrosclerin    LKNTIKIPKAFLTMLRRVESDEDG--------TLLTFNFRNLQRLNGRQVFEFPPDVDNG 254
                      :    :  :                    :  : *
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Fig. 2 (*continued*)

medium potency—weak Astrosclerin inhibitors, with inhibition constants in the range of 165–8850 nM. The structure activity relationship (SAR) are quite complex but it can be observed that most of these compounds are either simple benznesulfonamide derivatives possessing one-two compact substituents (usually in the para position to the sulfamoyl group), such as amino, halogeno and amino, alkyl, aminoalkyl; hydroxyalkyl, etc.) or simple heterocyclic sulfonamides incorporating 5-membered or fused rings (e.g., 1,3,4-thiadiazole; thienothiopyran, thienothiazine, etc.), or benzene-1,3-disulfonamide derivatives.

(ii) Potent Astrosclerin inhibition was observed with compounds **17–20, AZA, DCP, BZA** and **TPM**, these compounds showing inhibition constants in the range of 16–62 nM (Table 2). Compounds **17–19** belong to the sulfonylated sulfonamides **21** which were reported earlier to strongly inhibit several CAs, probably due to their elongated molecule and possibility to make sufficient favorable contacts with the enzyme active site, as shown in several X-ray crystallographic work from our and other groups.[22,23] A similar shape of the molecule is also observed for **20**, which however belong to a different chemotype compared to the compounds discussed above. Finally, the clinically used strong inhibitors are rather heterogeneous, as **AZA** and **BZA** are 1,3,4-thiadiazole-2-sulfonamides, topiramate is a sugar sulfamate whereas **DCP** is a benzene-1,3-disulfonamide derivative.

(iii) Several very strong, low nanomolar Astrosclerin-3 inhibitors were also detected, that is, compounds **15** and **16**, which are aminobenzolamide (**15**) and its dihalogenated derivative **16**. Thus, benzolamide **BZA** was already a highly effective Astrosclerin inhibitor, as mentioned above, but its further derivatization by means of a 4-amino moiety (with or without halogeno atoms, as in **15** and **16**) leads to an order of magnitude increase of the inhibition constants, from 32 nM (for BZA) to 2.8–3.9 nM for **15** and **16**, respectively.

(iv) the inhibition profiles of Astrosclerin-3 and human isoforms hCA I and II are quite different. hCA I is less susceptible to inhibition by sulfonamides/sulfamates compared to Astrosclerin-3 whereas hCA II is generally better inhibited by these compounds compared to the sponge enzyme.

3. Conclusions

An -CA derived from the calcareous skeleton of the living fossil sponge *Astrosclera willeyana*, was cloned to produce a GST fusion protein in order to generate large amounts of soluble protein. This enzyme (Astrosclerin-3) was kinetically characterized and investigated for its inhibition with sulfonamides and sulfamates. Astrosclerin-3 has a high catalytic activity for the CO_2 hydration reaction to bicarbonate and protons (k_{cat} of $9.0 \times 10^5\ s^{-1}$ and k_{cat}/K_m of $1.1 \times 10^8\ M^{-1} \times s^{-1}$), and is inhibited by various aromatic/heterocyclic sulfonamides and sulfamates with inhibition constants in the range of 2.9 nM–8.85 μM. Astrosclerin-3 and hCA II are highly catalyticlly effective -CAs for the hydration of CO_2 to bicarbonate, although the sponge enzyme it is devoid of a His residue in position 64 as proton shuttle (but it has a putative such residue in position 80). The affinity for sulfonamide inhibitors of Astrosclerin-3

and hCA II are also similar, despite the more than 550 million years of independent evolution. As Astrosclerin-3 is involved in biocalcification processes, the inhibitors described here may be used as pharmacologic tools for studying such processes in other animals, as for two recently described coral -CAs.

4. Experimental protocols

4.1. Chemistry

Compounds **1**–**26** and **AAZ**–**HCT** are either commercially available (Sigma-Aldrich, Milan, Italy) or were prepared as previously described.[20–23]

4.2. Cloning of the GST-Astrosclerin-3 fusion protein

The gene for the CA protein derived from *Astrosclera willeyana* (Astrosclerin-3 – the National Center for Biotechnology Information (NCBI) accession number EF434878) was provided in the pET16b vector. To create a GST-tagged protein, the *astrosclerin-3* was amplified with the primers BamHIAstrosclerins (AAGGATCCATGGGTCGGTGTGATTTCAA) and XhoIAstrosclerina (GATCCTCGAGCAAATGACGACT) that harbored the indicated restriction sites, digested and cloned into the BamHI- and XhoI-linearised pGEX-4T-1 vector (Amersham Biosciences, Milan, Italy), creating a plasmid designated pGEX-4T-1-Astrosclerin. PCR amplification was performed using a proof-reading Phusion polymerase (Finnzymes, Espoo, Finland) using the following thermoprofile: an initial denaturation at 98 °C for 30 s, then denaturation at 98 °C for 10 s, annealing at 64 °C for 30 s and extension at 72 °C for 20 s for a total of 30 cycles, and finally 5 min at 72 °C. The construct was confirmed by sequencing using ABIPrism BigDye terminator V3.1 sequencing kit for fluorescent detection and ABIPrism 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

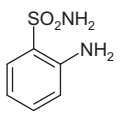
For production of the glutathione *S*-transferase (GST)-CA fusion protein, the plasmid construct pGEX-4T-1-Astrosclerin was transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL cells. Expression of GST-fusion protein was induced with 1 mM IPTG at 20 °C for 4 h.

The bacteria were then harvested and sonicated in phosphate buffer. The sonicated cell extracts were further homogenized twice with the Polytron (Brinkmann) for 30 s, at 4 °C. The lysates were cleared by centrifugation at 30 Kg for 30 min. Cleared supernatants were then applied to prepacked Glutathione Sepharose 4B columns (Amersham Biosciences). Columns were extensively washed with

Table 2
Human (h) hCA I, II, and sponge enzyme (Astrosclerin) inhibition data with compounds **1**–**26** and the clinically used derivatives **AAZ–HCT**[13]

Inhibitor	K_I*		
	hCA I[a] (nM)	hCA II[a] (nM)	Astrosclerin[b](nM)
1	45400	295	7750
2	25000	240	7700
3	28000	300	6081
4	78500	320	675
5	25000	170	973
6	21000	160	715
7	8300	60	890
8	9800	110	884
9	9650	73	965
10	14000	124	879
11	5800	63	552
12	8400	75	820
13	8600	60	370
14	9300	19	393
15	6	2	3.9
16	1.4	0.3	2.8
17	40	5	34
18	164	46	48
19	185	50	16
20	109	33	54
21	95	30	866
22	690	12	792
23	55	80	4260
24	21000	125	1380
25	3000	133	1215
26	24000	125	8850
AAZ	250	12	51
MZA	50	14	326
EZA	25	8	387
DCP	1200	38	62
DZA	50000	9	280
BRZ	45000	3	278
BZA	15	9	32
TPM	250	10	38
ZNS	56	35	165
SLP	1200	40	378
IND	31	15	423
CLX	50000	21	732
VLX	54000	43	517
SLT	374	9	276
SAC	18540	5950	751
HCT	328	290	394

* Errors in the range of 5–10% of the shown data, from 3 different assays.
[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method, from Ref. 19,20
[b] Recombinant Astrosclerin, stopped flow CO_2 hydrase assay method,[13] this work.



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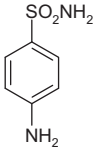

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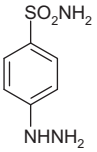

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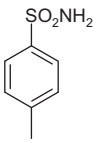

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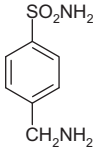

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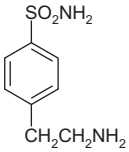

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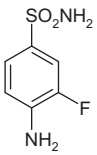

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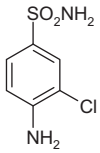

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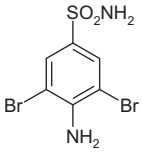

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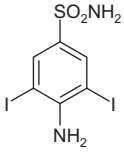

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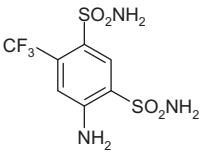

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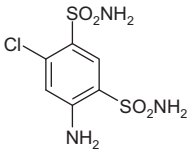

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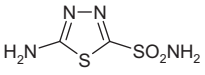

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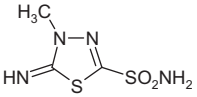

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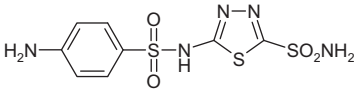

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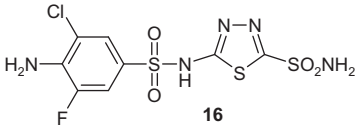

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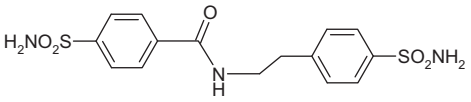

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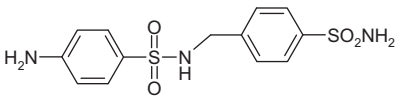

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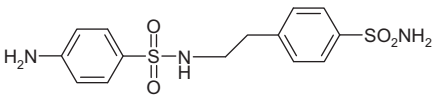

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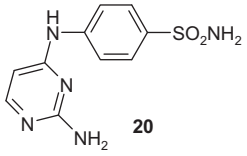

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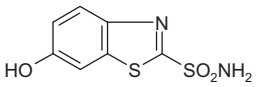

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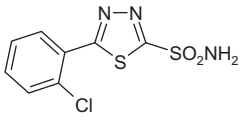

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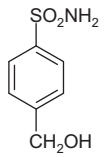

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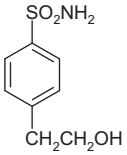

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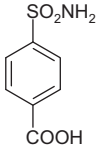

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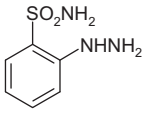

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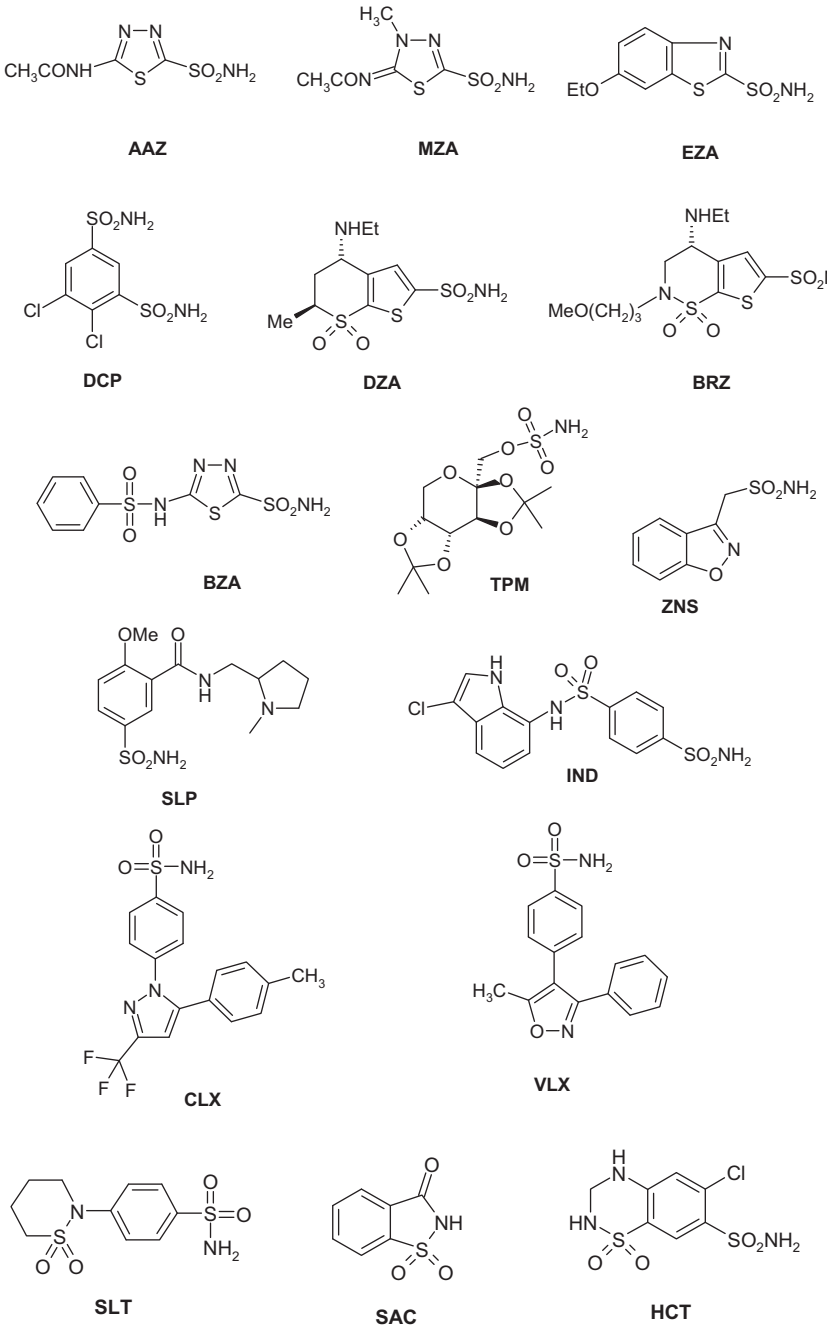

buffer and the GST-CA fusion protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl pH 8.0. Finally the GST domain was cleaved with thrombin (Sigma–Aldrich, Milan, Italy), as previously described.[12] The resulting Astrosclerin-3 recombinant protein was further purified by sulfonamide affinity chromatography.[1,12]

4.3. CA catalytic activity and inhibition

An Applied Photophysics stopped-flow instrument was used for assaying Astrosclerin-3 catalyzed CO_2 hydration activity.[13] Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10–20 mM Hepes (pH 7.5, for -CAs) for maintaining constant the ionic strength, following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. 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 were 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 and dilutions up to 0.01 nM were made with distilled–deionized water. Inhibitor and enzyme solutions were mixed and preincubated 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, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[12] and represent the mean from at least three different measurements.

Acknowledgments

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