Bioorganic & Medicinal Chemistry 17 (2009) 3212–3215

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Carbonic anhydrase inhibitors. The nematode α-carbonic anhydrase of *Caenorhabditis elegans* CAH-4b is highly inhibited by 2-(hydrazinocarbonyl)-3-substituted-phenyl-1*H*-indole-5-sulfonamides

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ARTICLE INFO

Article history:
Received 9 January 2009
Accepted 22 January 2009
Available online 27 January 2009

Keywords:
Carbonic anhydrase
Nematode
Caenorhabditis elegans
Sulfonamides
Indole-5-sulfonamide

ABSTRACT

A series of 2-(hydrazinocarbonyl)-3-substituted-phenyl-1*H*-indole-5-sulfonamides possessing various 2-, 3- or 4-substituted phenyl groups with methyl-, halogeno- and methoxy-functionalities, as well as the perfluorophenyl moiety, have been evaluated as inhibitors of an α-carbonic anhydrase (CA, EC 4.2.1.1) of the nematode model organism *Caenorhabditis elegans* (CAH-4b, or ceCA). The substitution pattern at the 3-phenyl ring highly influenced the ceCA inhibitory activity of these heterocyclic sulfonamides, with best inhibitors (K_Is in the range of 6.0–13.4 nM) incorporating 3-methyl-, 4-methyl-, 2-/3-/4-fluoro-, 4-chloro- and 3-/4-bromo-phenyl such moieties. Some of these sulfonamides also showed a good selectivity profile for the inhibition of the nematode over the human isozymes CA I and II (selectivity ratios in the range of 1.78–4.95 for the inhibition of ceCA over hCA II). These data can be used for the design of possibly new antihelmintic drugs, since the genome of many parasitic nematodes encode for a multitude of orthologue CA isozymes to ceCA investigated here.

1. Introduction

Helminths that include the nematodes infect 25% of the world's population.[1] In addition to nematode species that have a global distribution, such as *Onchocerca volvulus*, *Wuchereria bancrofti* and *Brugia malayi*, for which few effective therapies are presently known,[2] *Mansonella perstans* filariasis is widely present in Africa and equatorial America,[2a] *Strongyloides stercoralis*, an intestinal nematode acquired in the tropics or subtropics, is emerging as an important cause of infection in the Western countries too.[2b] Although there are effective drugs for some of these infections (thiabendazole is effective against larva migrans, mebendazole for ascariasis, trichiuriasis and hookworms, albendazole for inoperable cases of cystic hydatid disease caused by *Echinococcus granulosus*, diethylcarbamazine for *Toxocara* induced visceral larva migrans and loiasis, ivermectin for onchocerciasis, praziquantel for schistosomiasis and niridazole for *Dracunculus medinensis*)[1,2] the widespread resistance to many of them,[3] both in humans and animals, may lead to serious medical problems. It is thus pivotal to design agents targeting other metabolic pathways in these organisms, which may circumvent the resistance/toxicity problems of the currently used antihelmintics. One of the enzymes present in many worm species listed above is the carbonic anhydrase, CA (EC 4.2.1.1).[2c] Indeed, CAs are widespread all over the phylogenetic tree, with several different evolutionarily unrelated gene families encoding them.[4–7] CAs are catalysts for the interconversion of carbon dioxide and bicarbonate and are involved in pH regulation and function in several metabolic pathways.[4–7] In mammals there are 16 CAs known to date. These include several cytosolic isoforms (CA I-III, CA VII, and CA XIII), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial forms (CA VA and VB), as well as one secreted CA isozyme, CA VI. Three acatalytic isozymes are also known, that is, CA VIII, CA X and CA XI.[4–7] In mammals, these enzymes are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumourigenicity and several other physiologic/pathologic processes.[8–10]

Sulfonamides represent the main class of clinically used CA inhibitors (CAIs).[4] Such agents include acetazolamide **AZA** or ethoxzolamide **EZA** among others, and are clinically employed for the management of a variety of disorders connected to CA disbalances, such as glaucoma;[4,5] in the treatment of edema due to congestive heart failure,[8] or for drug-induced edema; as mountain

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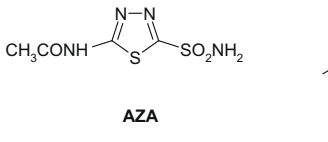
0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.01.048



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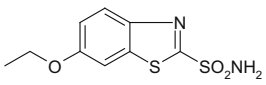

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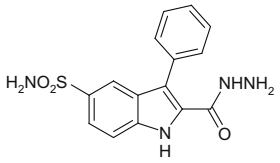

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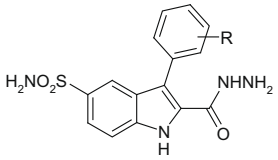


2

sickness drugs,[8] whereas other agents of this pharmacological class show applications as anticonvulsants,[9] antiobesity[4] or antitumour drugs/tumour diagnostic agents.[4,7] As there are few isoforms-selective inhibitors to date, many new sulfonamides are constantly being reported in the search of derivatives with better inhibition profiles as compared to the promiscuous, first generation inhibitors such as **AZA** or **EZA**.[4]

There are only a few reports regarding the CAs present in helminths and in particular nematode species.[2c] An α-CA, subject to environmental pH regulation denominated CAH-4b (or ceCA) was recently cloned and characterized in the model organism *Caenorhabditis elegans* (which contains at least 6 CA isoforms) by us.[10] In addition, DeRosa et al.[11] investigated another α-CA isoform homologous to *C. elegans* CAH-6 (78% homology) and human CA (hCA) III (55% homology) for its involvement in exsheathment of *Ostertagia ostertagi* nematodes, that infect intestines of cattle. DeRosa et al. did not directly confirm that this CA isoform was involved in the process of exsheathment, but the transcriptional regulation of the enzyme suggests that it may function in developmental processes related to this phenomenon.[11] Indeed, the strong α-CA inhibitor **EZA** was shown to be useful as an anti-infective in cattle infected with *Ostertagia ostertagi*.[11] CAH-4b was shown by our group to be susceptible to inhibition by sulfonamides such as **AZA** and **EZA** among others but highly effective such inhibitors were not yet detected.[10] Thus, considering the fact that nematodes contain many CAs in their genome (although scantly investigated to date) and the fact that these enzymes seem to be susceptible to inhibition by sulfonamides, we decided to investigate other classes of sulfonamide derivatives as ceCA inhibitors, which may be useful as lead molecules to design possibly new antihelmintic therapies. In this work we investigate the inhibition of *C. elegans* CA with a series of 2-(hydrazinocarbonyl)-3-phenyl-1*H*-indole-5-sulfonamides reported earlier to act as efficient inhibitors of several mammalian α-CAs.[12]

2. Results and discussion

2.1. Chemistry

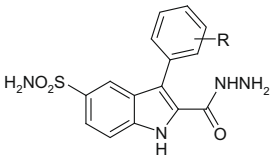
In recent work from this group,[12] we investigated the interaction between 2-(hydrazinocarbonyl)-3-phenyl-1*H*-indole-5-sulfonamides of types **1** and **2** with the 13 CA isoforms of mammalian (human or murine) origin, some of which are established medicinal chemistry targets for obtaining diuretics, antiglaucoma, antiobesity, antiepileptic or anticancer drugs/diagnostic tools.[4–6] These sulfonamide behaved as very potent inhibitors of several such isoforms (in the low nanomolar range)[12] but also showed an inhibition profile quite distinct of those of the first generation, promiscuous CAIs such as **AZA** and **EZA**, making them interesting candidates for obtaining isoform-selective inhibitors.[12b] In fact, the high resolution X-ray crystal structure of **1** in adduct with hCA II[12a] allowed us to design the newer generation inhibitors **2**, which showed a more interesting inhibition profile against several mammalian isoforms (such as CA I, II, IX, XIV and XV among others) as compared to the lead molecule **1**.[12b] The main chemical difference between **1** and **2** consists in the presence of various substituents at the 3-phenyl group of the indole rings of **2.** Such moieties may increase lipo- or hydrosolubility of the compounds incorporating them, and eventually also interact in a positive/negative manner with amino acid residues present in the CA active site region where this moiety of the inhibitor lies (such as among others Trp5, Asn62, His64 and Pro201, as shown for the hCA II—**1** adduct).[12a,13,14] Thus, incorporation of 2-, 3- or 4-substituted phenyl groups possessing methyl-, halogeno- and methoxy-functionalities in the 3-position of the indolesulfonamides **2**, not only influence the physico-chemical properties of these compounds but also modulates their interaction with the active site of various CA isozymes.[12] Since the α-CA of the nematode *C. elegans* (ceCA) belongs to the same family of enzymes as the mammalian CAs investigated earlier,[12] our working hypothesis was to investigate these sulfonamides possessing a new ring system/substitution pattern, of types **1** and **2**, as well as various moieties which can modulate their physico-chemical properties and interaction with the active site, for the inhibition of the nematode CA from the model organism.

2.2. Carbonic anhydrase inhibition

Inhibition data with the new sulfonamides **1**, **2a–2n** as well as the standard drugs **AZA** and **EZA** against ceCA as well as the ubiquitous,[4] human CA isoforms hCA I and II[12b] are shown in Table 1.[15]

The following structure–activity relationship (SAR) can be drawn by considering data of Table 1:

Table 1
Inhibition of human CA (hCA) isozymes I and II and nematode CA of *Caenorhabditis elegans* (ceCA) with sulfonamides **1**, **2a**–**n**, acetazolamide (**AZA**) and etoxzolamide (**EZA**) as standards[15]



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Inhibitor	R	K_I (nM)[#]			Selectivity ratio
		hCA I[a]	hCA II[a]	ceCA[b]	K_I(hCA II)/K_I(ceCA)
1	H	7.5	7.2	339	0.02
2a	2-Me	107	11.6	326	0.03
2b	3-Me	730	48.4	13.3	3.63
2c	4-Me	104	60.5	12.2	4.95
2d	2-F	621	36.0	12.5	2.88
2e	3-F	116	8.6	9.9	0.86
2f	4-F	108	15.5	6.0	2.58
2g	2-Cl	640	38.8	372	0.10
2h	3-Cl	311	9.2	10.1	0.91
2i	4-Cl	112	11.6	6.5	1.78
2j	2-Br	110	48.5	260	0.18
2k	3-Br	510	54.1	13.4	4.03
2l	4-Br	659	40.8	11.5	3.54
2m	3-OMe	342	7.4	330	0.02
2n	F_5	10	7.0	260	0.02
AZA	—	250	12	35	0.34
EZA	—	5	8	40	0.20

[#] Errors in the range of ±5% of the reported data from three different assays by a stopped-flow CO_2 hydration method.[15]
[a] From Ref. 12b.
[b] This work.

(i) Several investigated sulfonamides, such as **1**, **2a**, **2g**, **2j**, **2m** and **2n** showed weak ceCA inhibitory activity, with inhibition constants in the range of 260–372 nM. These compounds include the parent, unsubstituted compound **1**, as well as some of its derivatives substituted with 2-methyl-, 2-chloro-, 2-bromo-, 3-methoxy-phenyl as well as pentafluorophenyl moieties. It can also be observed that most of these compounds act as much better hCA II inhibitors, whereas their behavior towards hCA I is more variable, with some of them being low nanomolar inhibitors (e.g., **1**, which shows a K_I of 7.5 nM against this isoform) whereas others are weaker hCA I than ceCA inhibitors (e.g., **2g** and **2m**). It is thus obvious that small structural changes in the scaffold of these sulfonamides greatly influence their interaction with the various CA active sites, leading to a very diverse inhibition profile.

(ii) Derivatives **2b2f**, **2h**, **2i**, **2k** and **2l** showed excellent ceCA inhibition, possessing K_Is in the range of 6.0–13.4 nM, being thus 2.61–5.83-times more effective nematode CA inhibitors than acetazolamide **AZA**, the compound showing the best activity among sulfonamides/sulfamates tested up to now for their interaction with this enzyme.[10] Indeed, both acetazolamide and ethoxzolamide are medium potency ceCA inhibitors, with K_Is in the range of 35–40 nM (Table 1). It is clear that the best substitution pattern of the phenyl ring in position 3 of the indole of sulfonamides **2** includes moieties such as 3- and 4-Me-; 2-, 3- and 4-F-; 3- and 4-Cl-/Br-. Thus, in the *ortho* position of this phenyl ring only a small substituent such as fluorine is well tolerated and leads to an effective inhibitor (**2d** with a K_I of 12.5 nM), whereas the corresponding 2-Me-, 2-Cl- and 2-Br-derivatives are an order of magnitude weaker at inhibiting ceCA (K_Is of 260–372 nM) as compared to **2d**. However, the same moieties in the 3- or 4-position lead to much better ceCA inhibitors, proving that a clash between the *ortho*-phenyl substituent and an amino acid residue from the ceCA active site may be responsible for the weaker activity of these derivatives. On the other hand, some of these weaker ceCA inhibitors (e.g., **2a**, **2m**, etc.) act as much better, low nanomolar hCA II inhibitors, showing that the SAR is quite diverse for the mammalian and nematode enzymes with this class of sulfonamide inhibitors.

(iii) A main problem when developing CAIs with possible pharmacological applications resides in the large number of isoforms present in the host (human) organism and the undesired side effects ensued by their inhibition by means of the compound targeting the parasitic enzyme. Indeed, in this case both the host (human) and nematode enzymes belong to the α-CA class and are thus rather susceptible by inhibition with this class of compounds. Data of Table 1 show in fact that the clinically used compounds **AZA** and **EZA** are much better hCA II than ceCA inhibitors, showing a selectivity ratio for the inhibition of the nematode over the human enzyme in the range of 0.20–0.34. Many of the indole sulfonamides **1** and **2** investigated here show the same inhibition profile, with selectivity ratios of 0.02–0.91 (e.g., **1**, **2a**, **2e**, **2g**, **2h**, **2j**, **2m** and **2n**). However, some of the newly investigated derivatives, such as **2b**–**2d**, **2f**, **2i**, **2k** and **2l** show selectivity ratios of 1.78–4.95, being thus much more efficient ceCA than hCA II inhibitors. Most of these compounds are on the other hand only medium-weak hCA I inhibitors (Table 1), and since hCA I and II are the most abundant CA isozymes in the blood and gastro-intestinal tract of humans, it may be concluded that these derivatives might indeed selectively inhibit the nematode CA over the human CAs.

3. Conclusions

In this paper, we investigated a series of 2-(hydrazinocarbonyl)-3-substituted-phenyl-1*H*-indole-5-sulfonamides possessing various 2-, 3- or 4-substituted phenyl groups with methyl-, halogeno- and methoxy-functionalities, as well as the perfluorophenyl moiety for the inhibition of an α-CA isozyme present in the nematode *C. elegans* (CAH-4b, or ceCA). The substitution pattern at the 3-phenyl ring of the investigated sulfonamides highly influenced the ceCA inhibitory activity of these compoounds, with the best inhibitors (K_Is in the range of 6.0–13.4 nM) incorporating 3-methyl-, 4-methyl-, 2-/3-/4-fluoro-, 4-chloro- and 3-/4-bromo-phenyl moieties. Not only did these newly designed inhibitors display good inhibition profiles, but some showed increased selectivity for the nematode CA over the human isozymes CA I and II (selectivity ratios in the range of 1.78–4.95). Therefore, these data suggest that CAs have the potential to be used for the design of new antihelmintic drugs, which would selectively target parasitic isoforms, reducing the potential side effects of using such an abundant enzyme. This may be of considerable importance considering that the genome of many parasitic nematodes encode for a multitude of orthologue isozymes to the ceCA investigated here.

4. Experimental

4.1. Chemistry

Buffers, and chemicals, including sulfonamides AZA and EZA were from Sigma–Aldrich (Milan, Italy) of highest purity available, and were used without further purification. Sulfonamides **1** and **2** were prepared as reported earlier.[12]

4.2. CA catalytic/inhibition assay

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes as reported by Khalifah.[15] 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 7.4) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration),[16] following the CA-catalyzed CO_2 hydration reaction for a period of 5–10 s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO–water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. At least 7 different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[12] and represent the mean from at least three different determinations. Mammalian[16–18] and nematode[10] CA isozymes were prepared in recombinant form as reported earlier by our groups.

Acknowledgements

This research was financed in part by a grant of the 6th Framework Programme of the European Union (DeZnIT project). Ö.G. is grateful to TUBITAK (Ankara, Turkey) for the providing financing under the contract no. 2219/2008. Work in the FAM lab is funded

by the bbsrc and MRC. F.A.M. and R.A.H. thank Kara Turner for technical support.

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