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Carbonic anhydrase inhibitors. N-Cyanomethylsulfonamides—a new zinc binding group in the design of inhibitors targeting cytosolic and membrane-anchored isoforms

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Abstract
A series of N-cyanomethyl aromatic sulfonamides and bis-sulfonamides was prepared by reaction of arylsulfonyl halides with aminoacetonitrile. The obtained derivatives incorporated various aryl moieties, such as 4-halogeno/alkyl/aryl/nitro-substituted-phenyl, pentafluorophenyl or 2-naphthyl. Moderate inhibitory activity was detected for some compounds against the cytosolic human isoform II of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), hCA II, with inhibition constants of 90, 180 and 560 nM for the 4-nitrophenyl-, 4-iodophenyl- and pentafluorophenyl-N-cyanomethylsulfonamides, respectively. Other derivatives acted as weak inhibitors of isoforms hCA I (K_Is of 720 nM–45 μM), hCA II (K_Is of 1000–9800 nM) and hCA IX (K_Is of 900–10200 nM). Thus, the N-cyanomethylsulfonamide zinc binding group is less effective than the sulfonamide, sulfamate or sulfamide ones for the design of effective CA inhibitors.

Keywords: *Carbonic anhydrase, CA I, CA II, CA IX, inhibition, N-cyanomethyl sulfonamides*

Introduction

The α-carbonic anhydrases (CAs, EC 4.2.1.1) constitute a family of metalloenzymes that catalyze the reversible hydration of CO_2 to bicarbonate and a proton [1–5]. We have been working with the molecular cloning of some of the 16 presently known human CA (hCA) isoforms [6–8], as well as screening for inhibitory effects of a variety of compounds on most of them, showing that such various isozymes (e.g., hCA I, II, IV, VA, VB, VII, IX, XII, XIII and XIV) constitute valid targets for the development of novel antiglaucoma, antitumor, antiobesity or anticonvulsant drugs [9–13]. Furthermore, very recently representatives of the α- or β-CA class have been cloned and characterized in other organisms, such as *Plasmodium falciparum* [14], *Mycobacterium tuberculosis* [15], *Cryptococcus neoformans* [16] or *Candida spp.* [17], some of them being also investigated from the inhibition point of view [14,16], as it has been proved that these enzymes are critical for the growth or virulence of these pathogens [14–17]. Since many of these organisms are highly pathogenic, and present different degrees of resistance to the currently available drugs targeting them, inhibition of their CAs may constitute novel approaches to fighting such diseases [14–17]. Thus, there is a constant need for the design of novel classes of CA inhibitors (CAIs) [1–5], belonging to different classes of compounds, as such derivatives may lead to interesting pharmacological applications.

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In addition to the classical sulfonamide zinc binding group (ZBG) for the design of CAIs [1–5], recently we also explored alternative ZBGs, discovering that N-substituted sulfonamides [18], sulfamates and their derivatives [19,20], as well as sulfamides and their derivatives [21–23], may lead to effective inhibitors targeting several physiologically relevant isoforms.

Continuing our work in exploring different ZBGs for the design of CAIs, we report here that N-cyanomethyl aromatic sulfonamides and bis-sulfonamides show interesting inhibitory activity against the cytosolic isoforms CA I and II, and the transmembrane, tumor-associated isozyme CA IX.

Materials and methods:

General

Melting points were determined on a Büchi Melting Point 510 and are uncorrected. ^{1}H-NMR spectra were recorded on a Bruker AC-250 spectrometer using DMSO-d_6 as solvent and tetramethylsilane as internal standard. Chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (J) are expressed in Hertz. Electron Ionization mass spectra (30 eV) were recorded in positive or negative mode on a Waters MicroMass ZQ. Arylsulfonyl halides, aminoacetonitrile, solvents, buffers and inorganic reagents were from Sigma-Aldrich, Milan, Italy and were used without further purification. Recombinant human CA isoforms I, II and IX were prepared as reported earlier by our group [9–13,20–23].

General procedure

N-Cyanomethylsulfonamides were prepared as previously described by our group for obtaining N-cyanosulfonamides, by reaction of arylsulfonyl halides with 2-amino acetonitrile in aqueous acetone [24].

N-Cyanomethyl-4-fluorophenyl sulfonamide (1). mp 72°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.6 (t, 1H, $J = 5.7$ Hz), 7.9 (m, 2H), 7.4 (t, 2H, $J = 9$ Hz), 4.1 (d, 2H, $J = 2$ Hz); MS ESI$^+$ m/z 237 $(M + H)^+$. ESI$^-$ m/z 213 $(M - H)^-$.

N-Cyanomethyl-4-chlorophenyl sulfonamide (2). mp 111°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.6 (t, 1H, $J = 6$ Hz), 7.8 (d, 2H, $J = 8.5$ Hz), 7.7 (d, 2H, $J = 8.5$ Hz), 4.1 (d, 2H, $J = 3.5$ Hz); MS ESI$^-$ m/z 229 $(M - H)^-$.

N-Cyanomethyl-4-bromophenyl sulfonamide (3). mp 126°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.7 (t, 1H, $J = 6$ Hz), 7.8 (d, 2H, $J = 8.5$ Hz), 7.7 (d, 2H, $J = 8.5$ Hz), 4.1 (d, 2H, $J = 3.2$ Hz); MS ESI$^+$$m/z$ 299 $(M + Na)^+$. ESI$^-$ m/z 275 $(M - H)^-$.

N-Cyanomethyl-4-iodophenyl sulfonamide (4). mp 147°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.7 (t, 1H, $J = 5.7$ Hz), 8 (d, 2H, $J = 7$ Hz), 7.6 (d, 2H, $J = 7$ Hz), 4.1 (d, 2H, $J = 2.5$ Hz); MS ESI$^-$ m/z 321 $(M - H)^-$.

N-Cyanomethyl-4-methylphenyl sulfonamide (5). mp 101°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.5 (s, 1H), 7.7 (d, 2H, $J = 7.5$ Hz), 7.4 (d, 2H, $J = 7.5$ Hz), 4.06 (s, 2H), 2.4 (s, 3H); MS ESI$^-$ m/z 209 $(M - H)^-$.

N-Cyanomethyl-4-tertbutylphenyl sulfonamide (6). mp 96°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.5 (t, 1H, $J = 5.9$ Hz), 7.7 (d, 2H, $J = 7$ Hz), 7.6 (d, 2H, $J = 7$ Hz), 4.06 (s, 2H), 1.3 (s, 9H); MS ESI$^+$$m/z$ 275 $(M + Na)^+$.

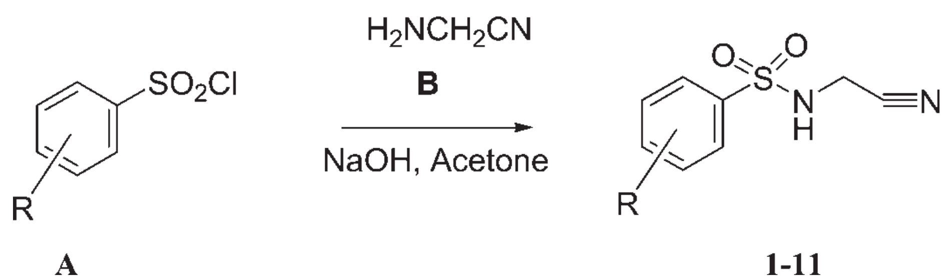
N-Cyanomethyl-4-biphenyl sulfonamide (7). mp 95°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.6 (s, 1H), 7.9–7.4 (m, 9H), 4.1 (s, 2H); MS ESI$^-$ m/z 271 $(M - H)^-$.

N-Cyanomethyl-4-nitrophenyl sulfonamide (8). mp 93°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 9 (s, 1H), 8.4 (d, 2H, $J = 7$ Hz), 8.1 (d, 2H, $J = 7$ Hz), 4.2 (s, 2H); MS ESI$^-$ m/z 240 $(M - H)^-$.

N-Cyanomethyl-pentafluorophenyl sulfonamide (9). mp 98°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 4.3 (s, 2H); MS ESI$^-$ m/z 285 $(M - H)^-$.

N-Cyanomethyl-2-naphthyl sulfonamide (10). mp 93°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.68 (t, 1H, $J = 6$ Hz), 8.5 (s, 1H), 8.2 (m, 2H), 8.1 (d, 1H, $J = 7.5$ Hz), 7.8 (d, 1H, $J = 7.5$ Hz), 7.7 (m, 2H), 4.15 (d, 2H, $J = 3.2$ Hz); MS ESI$^+$$m/z$ 269 $(M + Na)^+$. ESI$^-$ m/z 245 $(M - H)^-$.

Bis-N-cyanomethyl-1,3-benzene-di-sulfonamide (11). mp 87°C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.9 (s, 1H), 8.2–8.1 (m, 3H), 4.1 (s, 4H); MS ESI$^-$ m/z 313 $(M - H)$.



Scheme 1. Preparation of N-cyanomethylsulfonamides **1**–**11** by reaction of arylsulfonyl halides **A** with aminoacetonitrile **B**.

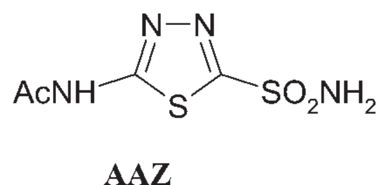
CA inhibition assay

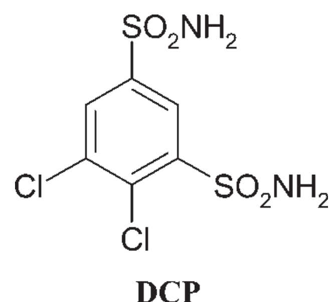
An Applied Photophysics (Oxford, UK) stopped-flow instrument was used for assaying the CA-catalysed CO_2 hydration activity [25]. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7–17 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 (1 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, from Lineweaver-Burk plots, as reported earlier, and represent the mean from at least three different determinations [9–13]. Human CA I and CA II were from Sigma-Aldrich (Milan, Italy) whereas recombinant hCA IX (catalytic domain) were prepared as reported earlier by us [10,13].

Results and discussion

N-Cyanosulfonamides of the type RSO_2NHCN were shown by our group to lead to effective CAIs targeting isoforms CA II and IV [24]. It is known that CAIs (of the sulfonamide, sulfamate or sulfamide type) bind to the Zn(II) ion within the enzyme active site in the deprotonated form, at the same time participating in numerous hydrogen bonds and hydrophobic interactions with amino acid residues from the cavity, such multiple interactions explaining the high affinity of many inhibitors for various CA isozymes [1–6,9–13]. Considering RSO_2NHCN [24] compounds as lead molecules, we investigate here whether a bulkier substituent at the sulfonamide moiety than the cyano one, i.e., the cyanomethyl group, may still be used for the design of effective CAIs, which in this way would incorporate a new ZBG, the N-cyanomethylsulfonamide one. Thus, by using the same chemistry as reported earlier for the preparation of N-cyanosulfonamides [24], we obtained a series of N-cyanomethylsulfonamides by reaction of arylsulfonyl halides **A** with aminoacetonitrile **B**, under Schotten-Baumann conditions, as shown in Scheme 1 [24]. A group of derivatives of type **1**–**11** have been prepared in this way, which incorporate various aryl moieties, such as 4-halogeno/alkyl/aryl/nitro-substituted-phenyl, pentafluorophenyl or 2-naphthyl. A bis-N-cyanomethyl-sulfonamide (compound **11**) has also been obtained, as it is known that benzene-1,3-disulfonamides act as efficient CAIs, with one of the clinically used drugs (dichlorophenamide, **DCP**) belonging to this class of derivatives [1–5].

Derivatives **1**–**11** have been investigated as inhibitors of three physiologically relevant CA isoforms, the ubiquitous human cytosolic hCA I and II, as well as the transmembrane, human, tumor-associated hCA IX (Table I).





As seen from the data of Table I, the N-cyanomethylsulfonamides **1**–**11** investigated here show inhibitory activity against the three investigated

Table I. Inhibition data for isozymes hCA I, II and IX with N-cyanomethylsulfonamides **1**–**11** prepared in the present study, and the clinically used sulfonamides acetazolamide (**AAZ**) and dichlorophenamide (**DCP**) as standard inhibitors.

RSO_2NHCH_2CN **1-10** $R(SO_2NHCH_2CN)_2$ **11**

No.	R	K_I* (nM) hCA I[a]	hCA II[a]	hCA IX[b]
1	4-FC_6H_4	5600	3200	nt
2	4-ClC_6H_4	3800	1000	5200
3	4-BrC_6H_4	7300	2300	nt
4	4-IC_6H_4	8300	180	2500
5	4-$CH_3C_6H_4$	3580	3200	nt
6	4-*t*-BuC_6H_4	2340	3500	nt
7	4-PhC_6H_4	5650	9000	nt
8	4-$O_2NC_6H_4$	10200	90	900
9	C_6F_5	720	560	10200
10	2-Naphthyl	7200	7600	nt
11	1,3-C_6H_4	45000	9800	nt
AAZ[c]		250	12	25
DCP[c]		1200	38	50

nt = not tested.
*Errors in the range of 5–10% of the shown data, from 3 different assays.
[a] Human isozyme, from Sigma-Aldrich. [b] Catalytic domain of the human recombinant isozyme, obtained as reported in refs. [10,13] CO_2 hydrase assay method [25]. [c] From ref. [26].

CA isoforms. The following structure–activity relationship correlations were observed for this group of derivatives: (i) against the slow cytosolic isozyme hCA I [1–4], the N-cyanomethylsulfonamides **1**–**11** showed weak inhibitory activity, with inhibition constants in the range 720 nM–45 μM, being thus much less effective inhibitors as compared to the unsubstituted sulfonamides used clinically acetazolamide or dichlorophenamide (which showed K_Is in the range 250–1200 nM). Only the pentafluorophenyl-substituted derivative **9** showed interesting activity against this isozyme, being in fact a more efficient inhibitor than the aromatic unsubstituted sulfonamide **DCP** (but less effective as compared to the heterocyclic sulfonamide **AAZ**). Otherwise, the substitution pattern at the benzene nucleus slightly influenced activity, except for the bis-substituted derivative **11** which was the least effective CAI (K_I of 45 μM). The weak inhibitory activity of these derivatives is presumably due to the rather bulky nature of the N-cyanomethyl-sulfonamide ZBG, which may hinder its access in the neighborhood of the Zn(II) ion of the enzyme active site for efficient binding. In fact the bis-sulfonamide **11** investigated here, which is also the most bulky one in the series of investigated compounds, was also the least effective hCA I inhibitor; (ii) against the physiologically most relevant and ubiquitous isoform hCA II, the N-cyanomethyl-sulfonamides **1**–**11** showed inhibition constants in the range 90–9800 nM. Thus, two derivatives, **4** and **8**, may be considered as effective hCA II inhibitors, as their K_Is, of 90–180 nM, are only 2.3–4.7 times higher as that of the aromatic disulfonamide DCP (K_I of 38 nM), which has clinical applications as an antiglaucoma drug [1–4]. These two compounds incorporate the 4-nitrophenyl- and the 4-iodophenyl moiety. The best hCA II inhibitor is the former derivative, in which the nitro group in the *para* position probably enhances the acidification of the SO_2NH proton, which is beneficial for the inhibition of CA due to the fact that inhibitors generally bind the Zn(II) ion as monoanions [1–4]. However, small variations in the moieties substituting the phenyl ring lead to a pronounced decrease of hCA II inhibitory activity, such as for example in the perfluorophenyl compound **9** (which is a medium potency inhibitor, with a K_I of 560 nM) or the other derivatives **1**–**3**, **5** and **6**, which showed K_Is in the range 1000–3500 nM. On the other hand, in the case of derivatives incorporating bulkier aryl groups (biphenyl, 2-naphthyl—compounds **7** and **10**) as well as for the bis-sulfonamide derivative **11**, the hCA II inhibitory activity is even more decreased, such compounds possessing K_Is in the range 7600–9800 nM. It is thus obvious that generally the N-cyanomethylsulfonamides are less effective hCA II inhibitors as compared to the unsubstituted sulfonamides (such as for example **AAZ** and **DCP**, Table I), the sulfamates [19,20] or the sulfamides [21–23]; (iii) due to the rather weak hCA I and II inhibitory activity in this group of derivatives and because hCA IX is rather difficult to prepare [26], we investigated the inhibition of this transmembrane, tumor-associated isoform only with the most effective hCA II inhibitors detected so far (the active sites of hCA II and hCA IX are rather similar) [10]. Indeed, the four compounds showed weak hCA IX inhibitory activity, with K_Is in the range 900–10200 nM, being thus much less effective as compared to the unsubstituted sulfonamides (**AAZ** and **DCP** show K_Is of 25–50 nM against this isoform).

In conclusion, in a series of N-cyanomethylsulfonamides incorporating substituted-phenyl or naphthyl moieties, moderate hCA II inhibitory activity has been detected for some compounds (inhibition constants of 90–560 nM for the 4-nitrophenyl-, 4-iodophenyl- and pentafluorophenyl-N-cyanomethylsulfonamides, respectively), whereas other such derivatives acted as weak inhibitors of isoforms hCA I (K_Is of 720 nM–45 μM), hCA II (K_Is of 1000–9800 nM) and hCA IX (K_Is of 900–10200 nM). Thus, the N-cyanomethylsulfonamide zinc binding group is less effective than the sulfonamide, sulfamate or sulfamide ones for the design of effective CA inhibitors.

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