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Carbonic anhydrase inhibitors. Inhibition of the cytosolic human isozymes I and II, and the transmembrane, tumor-associated isozymes IX and XII with substituted aromatic sulfonamides activatable in hypoxic tumors

Franciszek Sączewski,[a] Jarosław Sławiński,[a] Anita Kornicka,[a] Zdzisław Brzozowski,[a] Elżbieta Pomarnacka,[a] Alessio Innocenti,[b] Andrea Scozzafava[b] and Claudiu T. Supuran[b,*]

[a]*Department of Chemical Technology of Drugs, Medical University of Gdansk, 80-416 Gdansk, Poland*
[b]*Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy*

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Abstract—Some 2-mercapto-substituted-benzenesulfonamides and their disulfides/sulfones were prepared and investigated as inhibitors of four isoforms of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), that is, CA I and II (cytosolic enzymes), and the tumor-associated CA IX and XII. Some mercaptans led to a consistent increase of inhibitory power (52.8- to 243-fold) over the corresponding oxidized (S-S type) derivatives, acting as potential hypoxia-activatable drugs.

Hypoxia (O_2 concentration less than 3 μM) constitutes a challenging clinical problem, being common in many cancer types due to the presence of an imperfect blood vessel network in solid tumors.[1,2] As a consequence, a significant proportion of hypoxic cells are present in such tumors, which are not accessible to radio- and chemotherapy. Acidic extracellular pH (pHe) is also associated with hypoxia, supporting tumor progression by different molecular mechanisms,[3–6] and both these parameters influence the uptake of anticancer drugs and modulate the response of tumor cells to conventional chemo- and radiotherapy.[2,3] One of the genes highly upregulated by hypoxia is that encoding isozyme IX of carbonic anhydrase (CA, EC 4.2.1.1), that is, CA IX.[7] Indeed, the levels of this enzyme, which efficiently catalyzes CO_2 hydration to bicarbonate and H^+ ions, dramatically increase in response to hypoxia via a direct transcriptional activation of the *CA9* gene by the hypoxia inducible factor HIF-1.[8] It has also been proven that expression of CA IX in tumors is generally associated with poor prognosis.[9] We have recently showed that CA IX is one of the main players in the tumor acidification processes, and that the CA IX-mediated acidification is reversed by potent (and somehow selective) sulfonamide CA inhibitors (CAIs), not only in transfected cell lines, but also in tumor cell lines naturally expressing CA IX.[10,11] This effect was observed only under hypoxia and not in normoxic cell cultures.[10,11] In addition, a fluorescein-labeled sulfonamide CAI designed by us was shown to bind only to hypoxic cell expressing CA IX, whereas it was not bound either to normoxic, or to control cells lacking CA IX, offering thus the potential of novel diagnostic/therapeutic tools for the management of hypoxic tumors.[10,11] A second CA isozyme associated with tumors and hypoxia (but slightly different of CA IX) is CA XII,[7] which was also recently shown to be a target for antitumor sulfonamides by this group.[11b]

As hypoxia essentially occurs only in solid tumors, this physiological condition may be exploited for the design of hypoxia-activated prodrugs.[2,12,13] Indeed, a non-toxic, diffusible prodrug that may be activated in the reducing, acidic conditions of the solid tumor, leading

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* Corresponding author.Tel.: +39 055 4573005; fax: +39 055 4573385; e-mail: claudiu.supuran@unifi.it

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to a potent toxin, should theoretically kill only the cancer cells, showing thus appreciably less toxicity and side effects, as the active drug should be absent from the non-cancerous tissues. Many recent examples of this strategy have been reported in the literature, in which nitro derivatives, quinones, tetraalkyl ammonium halides, N-oxides or transition metal complexes among others have been used due to their possibility to be reduced to more toxic species which are being delivered to the tumor.[2,12,13] However, none of the reported[2,12,13] approaches takes advantage of a protein abundantly present only in hypoxic tumors, such as CA IX and/or CA XII. Here, we report the first study for designing hypoxia-activatable sulfonamide CA IX/XII inhibitors. Since these enzymes are highly overexpressed in tumors, their active site is extracellular, and their role in tumor acidification is essential,[10,11] we estimate that blocking their activity by a hypoxia activatable CAI may lead to a completely novel approach for developing less toxic anticancer therapies.

The clinically used sulfonamide CAIs acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND**, employed as standard inhibitors in the enzyme assays, are commercially available from Sigma–Aldrich or have been prepared as previously described.[7] Compounds **1–16** investigated in the present study generally belong to the 2,4,5-substituted-benzenesulfonamide class. Compounds **1**, **2**,[14a] and **11–16**[14b] have been reported earlier,whereas **3–10** are new and were prepared according to the procedures described in Schemes 1 and 2. Thus, hydrochlorothiazide **I** was treated with carbon disulfide in the presence of strong base (KOH), leading to the tricyclic intermediate **II** which by hydrolysis in alkaline medium followed by treatment with hydrochloric acid leads to the key intermediate **4** (the 2-mercapto analog of hydrochlorothiazide). Oxidation of **4** with DMSO or alkylation at the SH moiety with methyl sulfate or alkyl halides leads to the disulfide **7** or the substituted mercaptans **5** and **6** (Scheme 1).[14] 2-Mercapto-4-chloro-5-carboxy-benzenesulfonamide **III**[14] was treated with thionyl chloride with formation of the key intermediate, the acyl chloride disulfide **IV**, which was treated either with amines or alcohols with formation of the corresponding amides **8**, **9** or ester, **10**, respectively (Scheme 2).[14]

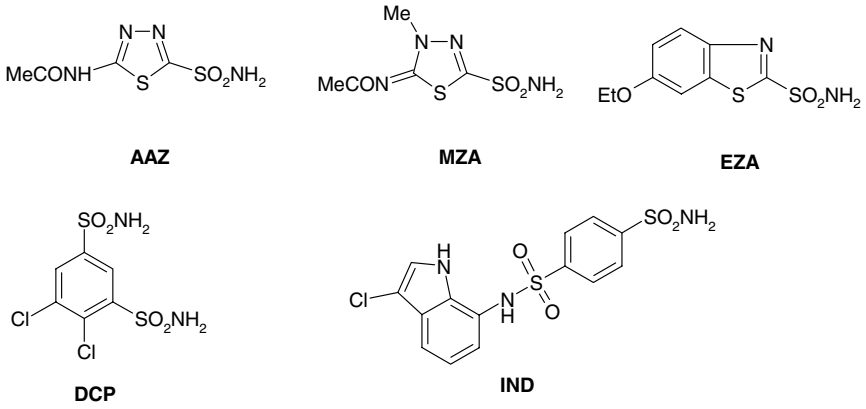
In order to prove that some of these new derivatives investigated here (such as, for example the disulfides **7–10**) may be activated in the reducing conditions present in hypoxic tumors, they have been reduced in situ in the assay buffer with sodium dithionite, with formation of the corresponding 2-mercapto-benzenesulfonamides **7H–10H** (Scheme 3). The reaction was complete in 10–15 min (TLC control).

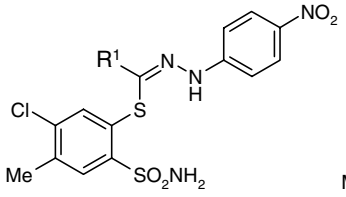
It should be observed that compound **4** is in fact identical with **7H**, and the enzyme assay of these two preparations of the same derivative may also be considered as a control experiment showing that the hypoxia activatable disulfides effectively inhibit the tumor-associated isoforms CA IX and CA XII (see discussion later in the text).

Inhibition data against the human isozymes hCA I and II,[7] as well as the transmembrane, tumor-associated isozymes hCA IX and hCA XII with the sulfonamides **1–16** investigated here, the reduced derivatives **7H–10H**, as well as standard, clinically used CAIs are shown in Table 1. Among the last such compounds are acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND**, previously investigated by us for targeting the tumor-associated CA isoforms.[15–17]

It is well known that ortho-substitution of benzenesulfonamides with bulky moieties is detrimental to the CA inhibitory properties of such derivatives,[18] but few recent studies investigated this ortho effect in detail. As most of the derivatives **1–16** possess just this type of substitution pattern, our study represents the most detailed investigation regarding the role of the ortho substituent on the CA inhibitory properties of benzenesulfonamides, against four physiologically relevant isoforms, that is, hCA I, II, IX, and XII.

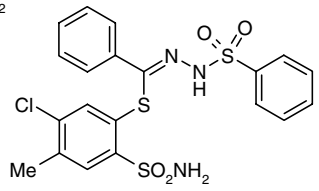
Thus, the slow cytosolic isoform hCA I was moderately inhibited by compounds **1–16** investigated here, with inhibition constants in the range of 2.6–392 μM (whereas the clinically used inhibitors presented in Table 1 showed much more potent inhibitory activity against this isoform, with K_Is in the range of 25 nM–1.20 μM). Indeed, the compounds possessing bulky moieties in ortho to the sulfamoyl group, such





1 $R^1 = C_2H_5$

2 $R^1 = C_6H_5$



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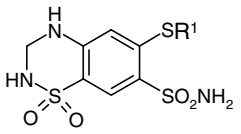
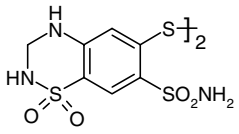


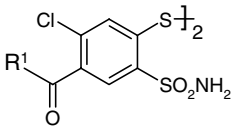
4 $R^1 = H$

5 $R^1 = CH_3$

6 $R^1 = i\text{-}C_4H_9$



7

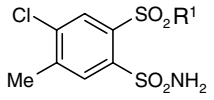


R^1

8 

9 $PhCH_2NH$

10 C_2H_5O



R^1

11 CH_2COOH

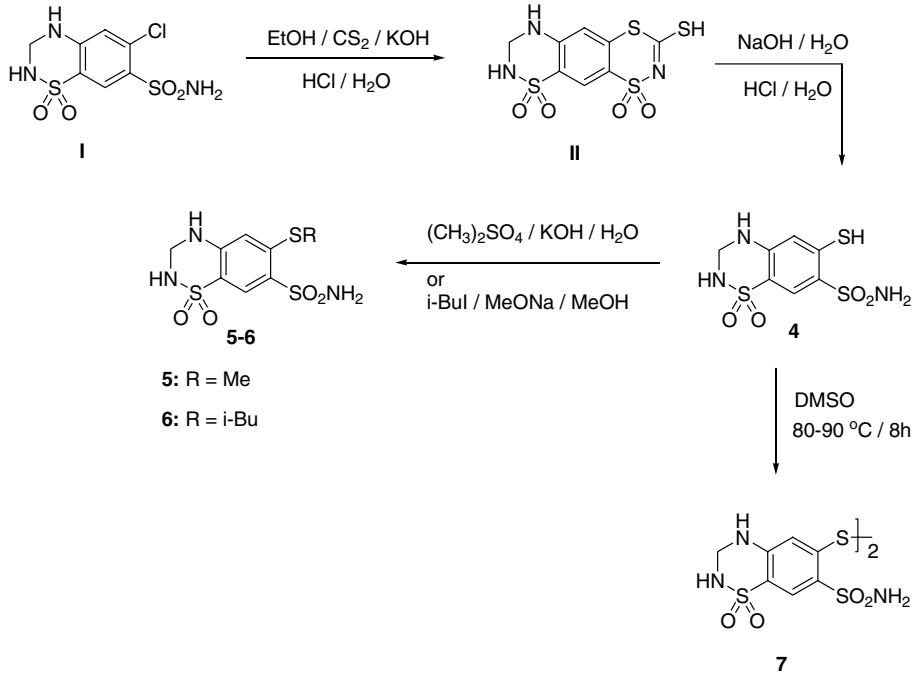
12 $CH(Me)COOH$

13 CH_2COOMe

14 $CH(Me)COOMe$

15 $CH_2CONHNH_2$

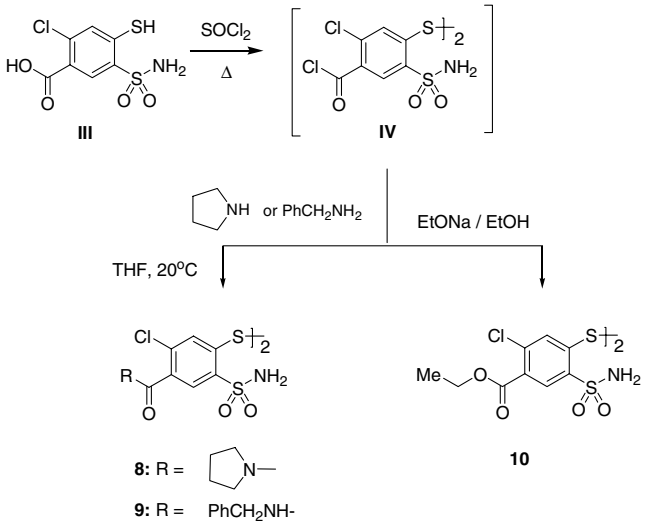
16 $CH(Me)CONHNH_2$



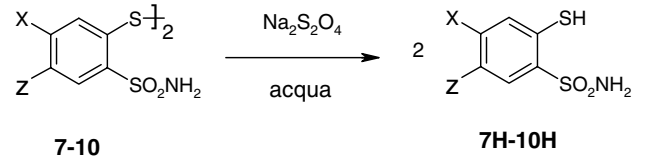
5: R = Me

6: R = i-Bu

Scheme 1.



Scheme 2.



Scheme 3. For the nature of X and Z see structures **7**–**10** in Schemes 1 and 2.

as **1**–**3**, **6**, **7**, and **9**, were the least effective inhibitors (K_Is in the range of 82.1–392 μM). Compounds **8**, **9H**, **12**, and **16** were on the other hand more effective hCA I inhibitors, with K_Is in the range of 14.2–27.3 μM. The remaining derivatives (**4**, **5**, **7H**, **8H**, **10**, **10H**, **11**, and **13**–**15**) showed much better hCA I inhibitory activity, with K_Is in the range of 2.6–11.6 μM. It is noteworthy that all the reduced compounds **7H**–**10H** (possessing 2-mercaptobenzenesulfonamide functionalities) were much more effective inhibitors as compared to the corresponding disulfides **7**–**10**, obviously due to their less bulky character, associated with a facilitated access to the Zn(II) ion within the enzyme active site. It can also be observed that pure **4** as well as **7H** (which is **4** generated in situ by dithionite reduction) had the same biological activity. It should also be observed that in addition to the ortho-SH moiety which leads to the best CA I inhibitors in this library of substituted sulfonamides, some other 2-substituents lead to effective inhibitors, such as the esters present in **13** and **14**. It is interesting to note that the corresponding carboxylic acids **11** and **12** were much worse inhibitors, whereas the carbohydrazides **15** and **16** showed an intermediate behavior between that of the esters and the carboxylic acids. It is quite difficult to rationalize these results at this moment, since no X-ray crystallographic structures of such adducts are available.

The rapid, physiologically most relevant cytosolic isozyme hCA II showed also a very interesting inhibition

Table 1. Inhibition data of sulfonamides **1**–**16** reported in the present paper and standard CA inhibitors, against isozymes I, II, IX, and XII, by a stopped-flow, CO_2 hydration assay[17]

Inhibitor	K_I[c]			
	hCA I[a] (μM)	hCA II[a] (nM)	hCA IX[b] (nM)	hCA XII[b] (nM)
AAZ	0.90	12	25	5.7
MZA	0.78	14	27	3.4
EZA	0.025	8	34	22
DCP	1.20	38	50	50
IND	0.031	15	24	3.4
1	220	>10,000	>10,000	>10,000
2	379	>10,000	>10,000	>10,000
3	113	>10,000	>10,000	>10,000
4	7.3	8.7	115	118
5	10.9	3800	139	108
6	392	2675	>10,000	1000
7	310	>10,000	780	1970
7H	7.5	8.6	113	117
8	22.1	4620	766	190
8H	2.6	19.6	14.5	20
9	82.1	6100	>10,000	>10,000
9H	19.7	19.9	40.9	35.6
10	7.8	5570	>10,000	>10,000
10H	6.4	18.5	56	53
11	11.6	547	5440	2404
12	27.3	806	4995	3045
13	5.1	9.6	1840	1348
14	5.8	244	1613	1103
15	7.7	9.2	1440	1390
16	14.2	536	3415	2417

[a] Human (cloned) isozymes, by the CO_2 hydration method.
[b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.[17]
[c] Errors in the range of 5–10% of the reported value (from three different assays).

profile with the compounds investigated here. Thus, a number of such derivatives, among which **1**–**3**, and **5**–**10**, showed very weak inhibitory activity, with K_Is either above 10 μM, or in the range of 2675–6.1 μM. It may be observed that all these compounds either possess very bulky ortho-substituents (such as **1**–**3**, **6**) or are disulfides, and as thus, possess a very bulky structure, which is not favorable to their binding within the restricted cavity of the hCA II active site.[19–24] However, reduction of the disulfide to the corresponding mercapto derivatives, leads to an impressive increase of the inhibitory activity. Indeed, derivatives **4** (**7H**), **8H**, **9H**, and **10H** behave as potent hCA II inhibitors, with inhibition constants in the range of 8.6–19.9 nM, in the same range as the clinically used sulfonamides **AAZ**–**IND** (K_Is in the range of 8–38 nM). Among the derivatives **11**–**16**, it is again interesting to observe that the ester **13** and the corresponding carbohydrazide **15** are also quite potent hCA II inhibitors (K_Is in the range of 9.2–9.6 nM), whereas the other compounds are weak inhibitors (K_Is in the range of 244–806 nM). Thus, SAR is very strict for this small group of compounds: for example, the additional methyl group present in **14** with respect to **13** leads to a 25.4-fold decrease of inhibitory activity, whereas for the pair **15** and **16** (which again differ only by a methyl group), this loss of activity is 58.2-fold.

Against the tumor-associated isoform hCA IX, again the compounds incorporating bulky 2-substituents, such as **1**–**3**, **6**, **9**, and **10**–**16**, showed weak inhibitory activity, with K_Is in the range of 1.6 to >10 μM. The same situation observed for hCA II is in fact also present here. Again by reduction of the disulfides **7**–**10**, much more effective inhibitors are generated. Indeed, derivatives **4** (**7H**), **8H**, **9H**, and **10H** showed inhibition constants in the range of 14.5–115 nM, in a broader range as compared to hCA II, which is in fact a positive finding for the potential design of hypoxia activatable, CA IX-selective inhibitors. Thus, for the pairs disulfide/mercaptan of types **7**–**10** and **7H**–**10H**, respectively, only a moderate inhibitory activity has been observed for **7H** (a 6.9-fold increase of inhibitory activity of **7H** over **7**), whereas for the remaining derivatives this increase was quite consistent (52.8-fold for **8H** over **8**; 243-fold for **9H** over **9**, and 178-fold for **10H** over **10**, respectively). The best CA IX inhibitor was **8H**, which is also a good hCA II and XII inhibitor, but its highest affinity is anyhow for hCA IX. It is also interesting to note that the nature of the R1 moiety in **8**–**10** (and the corresponding reduced derivatives) greatly influences their hCA IX inhibitory capacity, and many other such derivatives should be investigated in the future. The mercaptobenzenesulfonamides **8H**–**10H** showed hCA IX inhibitory activity in the same range as the clinically used compounds **AAZ**–**IND** (Table 1).

Similarly with hCA IX, hCA XII was modestly inhibited by the bulky derivatives **1**–**3**, **6**, **7**, **9**, and **10**–**16**, which showed inhibition constants in the range of 1.1 to >10 μM. The mercapto-derivatives **4** (**7H**), **8H**, **9H**, and **10H** showed inhibition constants in the range of 20–118 nM. Thus, the SAR of this library of sulfonamides for the inhibition of this isozyme is quite similar to what is described above for hCA IX.

In conclusion, a series of 2-mercapto-substituted-benzenesulfonamides, and their corresponding disulfides and sulfones has been prepared by an original procedure starting from hydrochlorothiazide or 2-mercapto-4-chloro-5-carboxy/methyl-benzenesulfonamides, respectively. The new derivatives were investigated as inhibitors of four CAs, that is, the cytosolic, ubiquitous isozymes CA I and II, as well as the transmembrane, tumor-associated isozymes CA IX and XII. All the derivatives incorporating bulky moieties in ortho to the sulfonamide group, including the disulfides, showed very weak inhibitory activity against all investigated isozymes (K_Is in the range of 1–10 μM), whereas compounds possessing such more compact groups (SH, SMe, SO_2CH_2COOMe, etc.) were much better inhibitors (K_Is in the range of 14.5–190 nM). Some disulfide/mercaptan pairs of compounds lead to a consistent inhibitory power increase (52.8- to 243-fold) of the reduced (SH type of compound) over the oxidized (S-S type) derivative, allowing thus for the design of hypoxia-activatable drugs. Indeed, both CA IX and CA XII are highly overexpressed in hypoxic tumors, and this type of compound may allow the development of less toxic antitumor therapies. The best inhibitor detected here (hypoxia-activatable compound) **8H**, showed an inhibition constant of 14.5 nM against CA IX, of 20 nM against CA XII, of 19.6 nM against CA II, and of 2.6 μM against CA I.

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