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Carbonic Anhydrase Inhibitors: Synthesis of *N*-Morpholyl-thiocarbonylsulfenylamino Aromatic/Heterocyclic Sulfonamides and their Interaction with Isozymes I, II and IV

Andrea Scozzafava and Claudiu T. Supuran*

Università degli Studi, Laboratorio di Chimica Inorganica e Bioinorganica, Via Gino Capponi 7, I-50121 Florence, Italy

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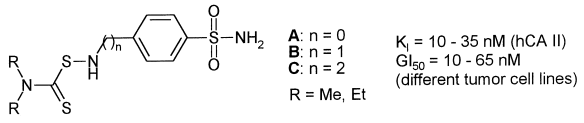
Abstract—Several aromatic/heterocyclic sulfonamides possessing free amino, imino or hydrazino moieties were transformed into the corresponding *N*-morpholylthiocarbonylsulfenyl derivatives, by reaction with *N*-morpholyldithiocarbamate in the presence of oxidizing agents (NaClO or iodine). These compounds showed nanomolar inhibition against three CA isozymes, and interesting in vitro tumor cell growth inhibitory properties, against several leukemia, non-small cell lung, ovarian, melanoma, colon, CNS, renal, prostate and breast cancer cell lines.

Introduction

In a recent work,[1] we showed that *N,N*-dialkylthiocarbonylsulfenylamino derivatives of simple aromatic sulfonamide carbonic anhydrase (CA, EC 4.2.1.1) inhibitors, such as **A**–**C**, in addition to possessing good CA inhibitory properties, also acted as very powerful inhibitors of growth for many types of tumor cells, in vitro and in vivo.[1] (GI_{50} represents the molarity of inhibitor producing a 50% inhibition of growth of the tumor cells after 48 h exposure to variable concentrations (10^{-4}–10^{-8} M) of the test compound).



Thus, in addition to their application as antiglaucoma,[2,3] antiepileptic,[4] antiulcer,[5] or diuretic drugs,[6] CA inhibitors of the sulfonamide type might also lead to the development of new types of anti-tumor agents.[1]

In previous works,[7–9] we showed that by attaching water-solubilizing tails (such as pyridinecarboxamido-; quinolinesulfonylamido, amino acyl, etc.) to the molecules of well-known sulfonamide inhibitors of the type **1**–**20**, it is possible to obtain water soluble compounds with long lasting topical antiglaucoma activity in an animal model of this disease. Here we extend our previous study, proving that other types of 'tails' can be attached to these molecules, leading to interesting derivatives with putative new pharmacological properties.

In this paper, we report the preparation of some new derivatives, using the previously mentioned anti-tumor derivatives **A**–**C** as leads. Thus, reaction of aromatic/heterocyclic sulfonamides **1**–**20** containing amino, imino, or hydrazino moieties with sodium *N*-morpholyldithiocarbamate in the presence of oxidizing agents such as sodium hypochlorite or iodine, led to the *N*-morpholylthiocarbonylsulfenamido-sulfonamides **A1**–**A20** (Scheme 1).[1,10,11]

The thiocarbamylation of compounds **1**–**20** generally occurred with good yields for the aliphatic derivatives **5** and **6**, incorporating H_2N–$(CH_2)_n$ moieties, whereas yields were definitely lower for the aromatic/heterocyclic derivatives possessing H_2N-aryl- or H_2N-heteroaryl moieties, the imine **14**, and the hydrazine **4**. The main complication of this reaction consisted in the formation of the thiuram derivative **22**. In order to avoid this problem, which would produce impure compounds **A(1**–**20)**,[1] the following synthetic strategies have been used: (i) the molar ratio amine **1**–**20**: dithiocarbamate **21**: oxidizing agent was generally of 1:1:1, but using an excess of 2–2.5 mols of amine (when possible, due to the limitation of costs and availability of these derivatives) led to higher yields and decreased formation of the side product **22**; (ii) the temperature had to be maintained at 0–4 °C when iodine was used as oxidizing agent, and at 25–30 °C when sodium hypochlorite has been employed;

*Corresponding author. Tel.: +39-055-2757551; fax: +39-055-2757555; e-mail: cts@bio.chim.unifi.it

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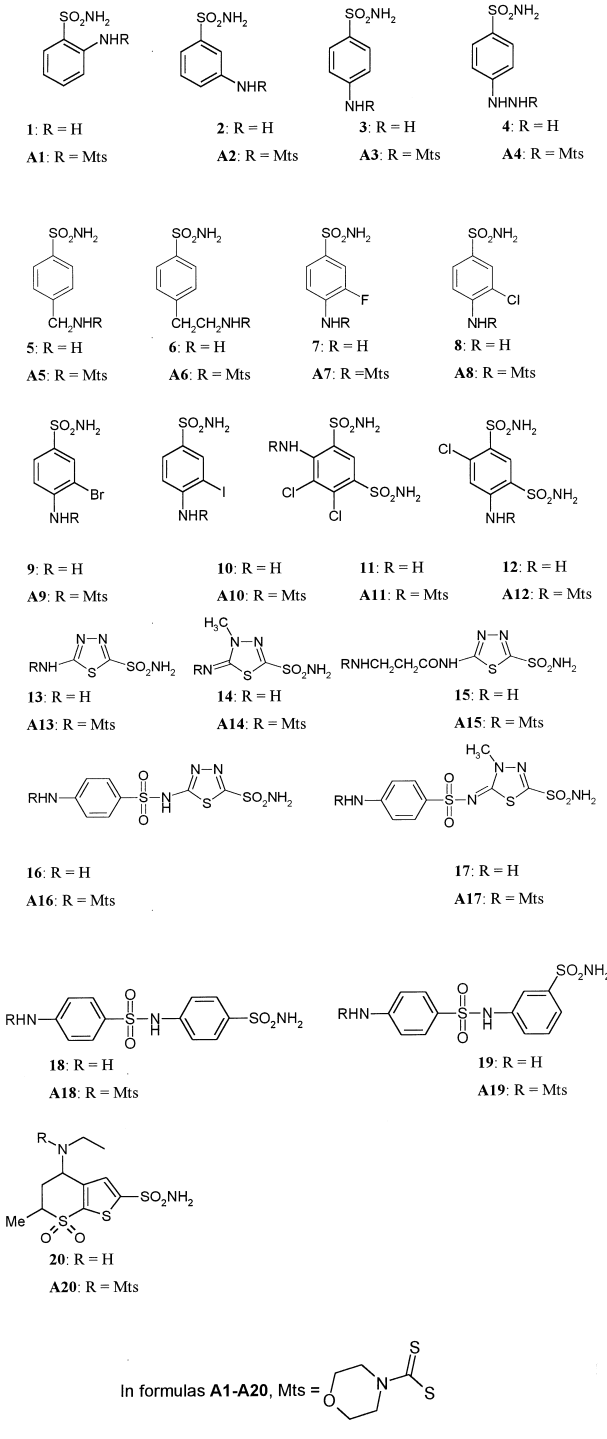
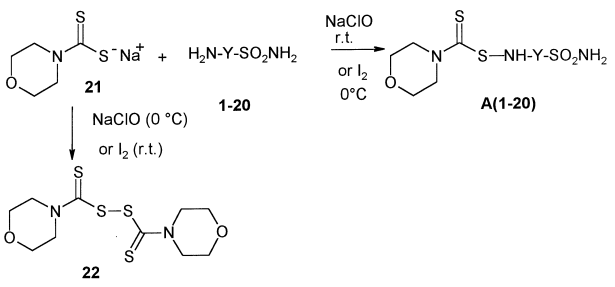


(iii) the dithiocarbamate **21** (as water solution) and the oxidizing agents were dropped slowly and *concomitantly* into the alkaline solution of amine **1**–**20**. This was in fact the most important factor leading to the formation of small amounts of thiuram **22**, and acceptable yields in thiocarbonylsulfenamides. Anyhow, the purification of the obtained derivatives **A(1–20)** (even when variable amounts of **22** were concomitantly formed during the synthesis) was greatly facilitated by the fact that the obtained thiocarbamylated sulfonamides **A(1–20)** are soluble at cold (4 °C) in diluted (0.03–0.05 M) alkaline solutions (NaOH or KOH), whereas the thiuram **22** is not.[12]



Scheme 1.

Inhibition data against three CA isozymes, hCA I, hCA II and bCA IV (h = human; b = bovine isozyme) with the new derivatives **A1**–**A20** (Table 1) proved that the thiocarbamylsulfenyl containing derivatives reported here behave as strong inhibitors, with increased affinities as compared to the parent compounds from which they were prepared (data for the sulfonamides **1**–**20** are given in parenthesis). The affinities of the obtained inhibitor generally varied in the following way, based on the parent sulfonamide from which it was prepared: the derivative of *p*-hydrazino-benzenesulfonamide **A4** < the orthanilamide **A1**≅the metanilamide **A2** < the sulfanilamide **A3** < the homosulfanilamides **A5** < the *p*-aminoethyl-benzenesulfonamides **A6**≅the halogeno-substituted sulfanilamides **A7–A10**≅the 1,3-benzene-disulfonamides **A11** and **A12**≅the sulfanilyl-sulfanilamide **A18** and the sulfanilyl-metanilamide **A19** < the 1,3,4-thiadiazole-2-sulfonamides **A13**, **A15** and **A16**≅4-methyl-δ^2-1,3,4-thiadiazoline-2-sulfonamide **A14** and **A17**≅the dorzolamide derivative **A20** (for the last derivative, the affinities for isozymes II and IV are very high, whereas that for CA I is extremely low). All three CA isozymes investigated here were susceptible to inhibition with this type of sulfonamides, with hCA II and bCA IV the most sensitive, whereas hCA I was generally less susceptible to inhibition as compared to the first two isozymes.

As mentioned in the introductory section, the leads used for the design of the CA inhibitors reported here, **A**–**C**, were shown to act as efficient inhibitors of tumor cell growth, both in vitro as well as in vivo (in a hollow fiber assay),[1] against a variety of leukemia, non-small cell lung cancer, ovarian, melanoma, colon, CNS, renal, prostate and breast cancer cell lines (GI_{50} around 10–65 nM). Since the mechanism of antitumor action with these new sulfonamides is unknown, it is of crucial importance to design novel, structurally-related such compounds, and this is precisely what we report here. Tumor cell growth inhibition data with several of the new compounds reported here are shown in Table 2.

The following should be noted regarding the in vitro tumor cell growth inhibition data with the test compounds **A3**, **A5** and **A6**: (i) different cancer cell lines, of the same tumor type, possessed a very variable response to inhibition of growth in the presence of the new derivatives. For example, the SR leukemia cells were very susceptible to inhibition at nanomolar concentrations of sulfonamides **A3**, **A5**, **A6**, whereas other leukemia lines (MOLT-4) showed the same level of inhibition only at micromolar concentrations of inhibitor; (ii) all the

Table 1. CA inhibition data with the several standard, clinically used inhibitors, the leads **A**, **B**, **C**, and the new derivatives **A1**–**A20** reported in the present study, against isozymes I, II and IV[13]

Inhibitor	K_I[a,b] (nM)		
	hCA I[a]	hCA II[c]	bCA IV[d]
A	15,000	48	140
B	950	13	41
C	630	11	30
A1	11,500 (45,400)	780 (295)	500 (1310)
A2	10,000 (25,000)	150 (240)	290 (2200)
A3	9000 (28,000)	36 (300)	100 (3000)
A4	24,000 (78,500)	790 (320)	650 (3200)
A5	620 (25,000)	10 (170)	35 (2800)
A6	360 (21,000)	8 (160)	24 (2500)
A7	760 (8300)	33 (60)	120 (180)
A8	800 (9800)	40 (110)	94 (320)
A9	2400 (6500)	48 (40)	96 (66)
A10	3000 (6000)	55 (70)	170 (125)
A11	500 (6100)	18 (28)	49 (175)
A12	560 (8400)	15 (75)	90 (160)
A13	240 (8600)	6 (60)	25 (540)
A14	200 (9300)	6 (19)	26 (355)
A15	105 (455)	2 (3)	21 (125)
A16	5 (6)	1.4 (2)	4 (5)
A17	2 (1)	0.5 (0.6)	1.3 (0.8)
A18	30 (42)	3 (6)	10 (50)
A19	31 (44)	7 (9)	10 (53)
A20	>50,000 (50,000)	6 (9)	22 (45)

[a]Data in parentheses represent the inhibition constants of the corresponding parent sulfonamides **1**–**20**.
[b]Mean from at least three determinations by the esterase method.[13] Standard error was in the range of 5–10%.
[c]Human cloned isozyme.
[d]Purified from bovine lung microsomes.[14]

investigated cancer lines were generally inhibited by the investigated sulfonamide, but some types of tumors, such as the leukemia, colon, renal or ovarian ones, were generally more susceptible to inhibition, whereas others, such as the CNS, melanoma, breast (except for the MCF7 line) or prostate cancer cell lines were slightly less susceptible; (iii) no important differences of activity between the three investigated sulfonamides were detected. Still, generally, the most active compounds were **A6** and **A5**, whereas **A3** was slightly less active; (iv) the inhibition of growth of tumor cells was dose-dependent of the concentration of sulfonamide inhibitor used in the experiments (data not shown), with growth inhibition increasing at increasing sulfonamide concentrations.

The mechanism of antitumor action of these sulfonamides is unknown, but it might involve inhibition of some recently isolated CA isozymes (such as CA IX, CA XII, CA XIV)[15–17] present predominantly in tumor cell membranes, or a reduced provision of bicarbonate needed for the nucleotide synthesis mediated by carbamoyl phosphate synthetase II.[18] Alternatively, the acidification of the intracellular milieu as a consequence of CA inhibition by the sulfonamide,[19,20] or the uncoupling of mitochondria and the concomitant inhibition of the mitochondrial isozyme CA V might also be effective.[21,22] A combination of several such mechanisms may also be operative. Optimization of these derivatives from the SAR point of view, might lead to the development of effective novel types of anticancer agents.

Table 2. In vitro tumor growth inhibition data with some of the new CA inhibitors synthesized in the present work (**A3**, **A5** and **A6**)

Tumor	Cell line	GI_{50} (μM)[a] (**A3**	**A5**	**A6**)
Leukemia	HL-60 (TB)	0.086	0.090	0.070
	MOLT-4	10	9.0	54
	SR	0.20	0.15	<0.010
Non-small cell lung cancer	A549/ATCC	46	38	11
	HOP-62	78	15	62
	NCI-H522	0.42	0.019	0.075
Colon cancer	COLO-205	32	30	21
	HCT-15	68	24	>100
CNS cancer	SF-268	58	16	90
	SF-295	44	5.4	>100
Melanoma	LOX IMVI	34	12	5
	M14	14	10	8
	SK-MEL-2	31	12	5
Ovarian cancer	IGROV1	70	52	0.09
	OVCAR-4	>100	>100	61
Renal cancer	768-0	12	10	11
	ACHN	31	15	15
	CAKI-1	30	17	9
Prostate cancer	PC-3	24	12	11
	DU-145	80	13	10
Breast cancer	MCF7	0.033	0.030	0.026
	MDA-MB-435	30	13	12
	MDA-N	61	15	13

[a]Molarity of inhibitor producing a 50% inhibition of growth of the tumor cells after 48 h exposure to variable concentrations (10^{-4}–10^{-8} M) of the test compound. Errors were in the range of ±5–10% (from two determinations).[23]

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12. For example, by using sodium hypochlorite as oxidizing agent, the followed procedure was: an amount of 10 mMols sulfonamide **1–20** was treated with 100 mL of a solution containing 0.8 g (20 mMols) sodium hydroxide (KOH could also be used). After dissolution of the sulfonamide, into the magnetically stirred reaction mixture were slowly (1–2 h) and concomitantly dropped the following two aqueous solutions: (i) 50 mL of a solution containing 10 mMols of sodium/potassium *N*-morpholyldithiocarbamate **21**; and (ii) 50 mL of NaClO solution, containing the stoichiometric amount (10 mMols) of oxidizing agent. The temperature was maintained in the range of 25–30 °C (generally cooling of the reaction mixture had to be done), with strong magnetic stirring, for 1–3 h (TLC control; the thiocarbamylsulfenamide and the thiuram **22** started to precipitate immediately after the addition of the oxidizing agent). A double–2.5-fold amount of initial sulfonamide could be used in the synthesis (for relatively inexpensive raw materials, such as **3–6**, for example), case in which the yields in thiocarbamylsulfenamides were increased. In order to purify the obtained thiocarbamylsulfenamides, the obtained crude precipitate was filtered and treated with an excess (100–150 mL) of a 0.03–0.05 M NaOH (or KOH) and magnetically stirred for 30 min at 4 °C. The insoluble thiuram was filtered and the sodium/potassium salts of the thiocarbamylsulfenamides acidified with a 10% HCl solution (till pH 5.5), when the pure **A(1–20)** precipitated. They were then filtered, thoroughly washed with water and air dried. Yields were generally in the range of 25–40%, but for some derivatives they were much higher (around 70–75 % for **A3**, **A5** and **A6**).
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23. Inhibition of tumor cell growth was determined as follows: stock solutions of inhibitor (1 mM) were prepared in DMSO, and dilutions up to 10 nM prepared with distilled deionized water. The percentage growth (PG) of the cell lines in the presence of five concentrations (10^{-8}–10^{-4} M) of inhibitor was calculated according to one of the following two expressions (1) or (2):

$$PG = 100 \times (\text{Mean OD}_{\text{test}} - \text{Mean OD}_0)/(\text{Mean OD}_{\text{ctrl}} - \text{Mean OD}_0), \quad \text{when } (\text{Mean OD}_{\text{test}} - \text{Mean OD}_0) \geq 0, \tag{1}$$

$$PG = 100 \times (\text{Mean OD}_{\text{test}} - \text{Mean OD}_0)/(\text{Mean OD}_0), \quad \text{when } (\text{Mean OD}_{\text{test}} - \text{Mean OD}_0) < 0, \tag{2}$$

where Mean OD_0 = the average optical density measurements of sulforhodamine B (SRB)-derived color just before exposure of cells to the test compounds; Mean OD_{test} = the average optical density measurements of SRB-derived color after 48 h exposure of cells to the test compounds; Mean OD_{ctrl} = the average optical density measurements of SRB-derived color after 48 h with no exposure of cells to the test compounds. GI_{50} represents the molarity of inhibitor producing a 50% inhibition of growth of the tumor cells after 48 h exposure to variable concentrations (10^{-4}-10^{-8} M) of the test compound, measured as outlined before, and this parameter was obtained by interpolation. GI_{50} is in fact the molarity of inhibitor at which PG = 50%. The standard sulforhodamine B (SRB) protein assay has been used to estimate cell viability or growth (cf. Teicher, B. A. (Ed.). *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval*; Humana Press Inc.: Totowa, NJ, 1997; pp 7–125).