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Benzolamide is not a Membrane-impermeant Carbonic Anhydrase Inhibitor

CLAUDIU T. SUPURAN* and ANDREA SCOZZAFAVA

Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino, Italy

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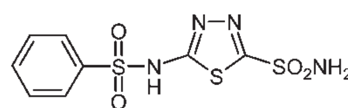
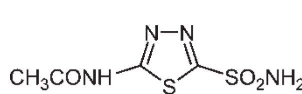
Benzolamide, an orphan drug belonging to the pharmacological class of sulfonamide carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) is widely used in many physiological and pharmacological studies, together with the clinically employed classical drugs, acetazolamide, methazolamide, ethoxzolamide or dichlorophenamide, it being frequently stated that benzolamide is a membrane-impermeant inhibitor. We prove here that this is false: in fact benzolamide is rather similar to acetazolamide from the point of view of penetrability through blood red cell membranes. Unlike these neutral drugs, the cationic, positively-charged CAIs incorporating either tetraalkyl ammonium or pyridinium moieties, due to their salt-like character are indeed membrane-impermeant, being the only type of low molecular weight compound possessing such properties. Selective inhibition of membrane-associated CA isozymes is relevant indeed in many physiological studies and also pharmacologically, since the tumor-associated isozymes (CA IX and XII) are both membrane-bound.

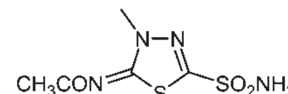
Keywords: Carbonic anhydrase; Isozyme; Sulfonamide; Membrane permeability; Benzolamide; Pyridinium

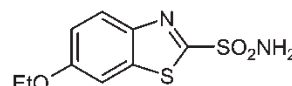
INTRODUCTION

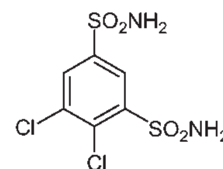
Benzolamide **1**, an orphan drug[1] belonging to the pharmacological class of sulfonamide carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs)[2–5] is widely used in many physiological and pharmacological studies, together with the clinically used classical drugs, acetazolamide **2**, methazolamide **3**, ethoxzolamide **4** or dichlorophenamide **5**. Similar to all the pharmacological agents mentioned here, benzolamide is a potent inhibitor of most CA isozymes, such as CA I, II, IV, V, IX and XIII among others (Table I).[1–5]











With a highly polar molecule and two acidic protons in its molecule—the pKa of the secondary sulfonamide moiety is 3.2 whereas that of the primary one is 7.5,[1] benzolamide is indeed the most hydrophilic and prone to be in anionic form (at neutral pH values) CAI among the derivatives **1–5** discussed here. Probably these physico-chemical properties and the lack of understanding of basic chemical equilibria in which this sulfonamide participates, led to the publication of many papers in which it is stated that this sulfonamide

*Corresponding author. Tel.: +39-055-4573005. Fax: +39-055-4573385. E-mail: claudiu.supuran@unifi.it

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TABLE I Inhibition data with sulfonamides **1–5** against several α-CA isozymes

	K_I (nM)*				
Isozyme	**BZA1**	**AZA 2**	**MZA 3**	**EZA 4**	**DCP 5**
hCA I	15	900	780	25	350
hCA II	9	12	14	8	30
hCA IV	12	220	240	13	120
mCA V	36	60	63	47	640
hCA IX	45	25	27	34	50
hCA XIII	nt	17	nt	nt	nt

* CO_2 hydration assay;[23] h = human; m = murine isozyme; nt = not tested (no data available).

(or some of its close congeners) are membrane-impermeant (or extracellular) CAIs.[6–15] We prove here that this statement is false, and that there are indeed several classes of truely membrane-impermeant CAIs, but which possess different physico-chemical properties and diverse structures. Only one of these studies[15] suggests caution in the use of this agent to inhibit extracellular CAs in excitable brain tissues, as clearly these researchers observed that benzolamide is not that impermeant as annecdotically considered in the scientific literature mentioned above.[6–14] Thus, many of the conclusions from the physiological studies just mentioned should be revisited or corrected.[6–14]

MATERIALS AND METHODS

Sulfonamides **2–5** used in the measurements were commercially available compounds (from Sigma–Aldrich). Benzolamide **1** was prepared as described earlier.[16,17] The positively-charged, pyridinium/tetraalkylammonium-derived sulfonamides **6–8** were prepared as described in the literature.[18–21]

CA Assays

Initial rates of 4-nitrophenylacetate hydrolysis catalysed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC.[22] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2.10^{-2} and 1.10^{-6} M, working at 25°C. A molar absorption coefficient ε of 18,400 $M^{-1} cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, under the conditions of the experiments (pH 7.40), as reported in the literature.[22] Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. 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 0.01 nM were done thereafter with distilled–deionized water. 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. The inhibition constant K_I was determined as described by Pocker and Stone, for isozymes I, II and IV.[22]

A SX.18MV-R Applied Photophysics stopped-flow instrument was used for assaying the CO_2 hydration activity of CA isozymes.[23] 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), and the CA-catalyzed CO_2 hydration reaction was followed for a period of 10–100 s. Saturated CO_2 solutions in water at 20°C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 1–3 mM (in DMSO-water 1:1, v/v) and dilutions up to 0.1 nM done with the assay buffer mentioned above. Enzyme concentration was 0.1 μM, and inhibition constants were calculated as described in reference 23.

Penetrability through Red Cell Membranes

An amount of 10 mL of freshly isolated human red cells thoroughly washed several times with Tris buffer (pH 7.40, 5 mM) and centrifuged for 10 min was treated with 25 mL of a 2 mM solution of sulfonamide inhibitors **1–8**. Incubation was done at 37°C with gentle stirring, for periods of 30–120 min. After the incubation times of 30, 60 and 120 min, respectively, the red cells were centrifuged again for 10 min, the supernatant discarded, and the cells washed three times with 10 mL of the above mentioned buffer, in order to eliminate all unbound inhibitor.[24] The cells were then lysed in 25 mL of distilled water and centrifuged to eliminate membranes and other insoluble impurities. The obtained solution was heated at 100°C for 5 min (in order to denature CA-s) and the sulfonamides possibly present were assayed in each sample by three methods: a HPLC method;[25,26] spectrophotometrically[27] and enzymatically.[22]

HPLC

A variant of the methods of Gomaa[25] and Iyer and Taft[26] has been developed by us, as follows. A commercially available 5 μm Bondapak C-18 column was used for the separation, with a mobile phase consisting of acetonitrile–methanol–phosphate buffer (pH 7.4) 10:2:88 (v/v/v), at a flow rate of 3 mL/min, with 0.3 mg/mL sulphadiazine (Sigma) as internal standard. The retention times were: 12.69 min for acetazolamide; 3.55 min for

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ethoxzolamide; 10.54 min for benzolamide; 4.33 min for dichlorophenamide; 2.11 min for **6**; 2.54 min for **7**; 2.36 min for **8**. The eluent was monitored continuously for absorbance (at 254 nm for acetazolamide, and a wavelength in the range 270–310 nm in the case of the other sulfonamides).

Spectrophotometrically

A variant of the pH-induced spectrophotometric assay of Abdine *et al.*[27] has been used, working for instance at 260 and 292 nm, respectively, for acetazolamide; at 225 and 265 nm, respectively, for benzolamide, etc. Standardized solutions of each inhibitor were prepared in the same buffer as that used for the membrane penetrability experiments.

Enzymatically

The amount of sulfonamide present in the lysate was evaluated based on the extent of hCA II inhibition measured by the esterase method, as described above.[22] Standard inhibition curves had been obtained previously for each sulfonamide, using the pure compound, which were subsequently used for determining the amount of inhibitor present in the lysate. Mention should be made that the three methods presented above led to results in good agreement, within the limits of the experimental errors.

RESULTS AND DISCUSSION

In order to understand the penetrability of the sulfonamide CAIs through membranes, *ex vivo* experiments were performed with freshly isolated human blood. Thus, incubation of human red cells (which contain high concentrations of isozymes I and II, i.e., 150 μM hCA I and 20 μM hCA II, but not the membrane-bound CA IV)[28] with millimolar concentrations of different sulfonamide inhibitors, such as acetazolamide **2**, or methazolamide **3**, ethoxzolamide **4** or dichlorophenamide **5**, almost led to saturation of the two isozymes present in the erythrocytes with inhibitor, even after short periods of incubation (30 min), whereas for benzolamide **1** a similar effect was achieved after somewhat longer periods (60 min) (Table II). This is obviously due to the high diffusibility through the membranes of the first three inhibitors, whereas benzolamide with a pK_a of 3.2 for the second sulfonamido group[1] being present mainly as an (di)anion at the pH at which the experiment was carried out (7.4), is less diffusible and penetrates membranes less rapidly. However, benzolamide does penetrate these membranes quite efficiently, as can be seen from data in Table II. Indeed, the differences between benzolamide and acetazolamide in penetrability are rather low (less than 20% at 30 min, and less than 10% at 60 min), although acetazolamide is generally considered to be membrane-permeant, whereas benzolamide is a membrane-impermeant inhibitor[6–14,17] (a notable exception is a recent paper of Gros's group, in which both benzolamide and acetazolamide are considered poor membrane-permeant CAIs).[29] Mention should be made that working with more dilute solutions of CAIs (i.e., in the concentration range of 5–10 μM), the penetrability of the CAIs through red cell membranes is equally observed, but the levels of sulfonamide inside the cells are much lower, due to the limited amount of sulfonamide initially present in solution (data not shown). These are in fact the usual levels of sulfonamide CAIs used in many physiological experiments mentioned in the introductory section. Thus, caution must be taken in considering benzolamide a membrane-impermeant inhibitor, as this compound achieves 80–90% of

TABLE II Levels of sulfonamide CAIs (μM) in red blood cells at 30 and 60 min, after exposure of 10 mL of blood to solutions of sulfonamide (2 mM sulfonamide in 5 mM Tris buffer, pH 7.4). The concentrations of sulfonamide has been determined by three methods: HPLC; electronic spectroscopy (ES) and enzymatically (EI)—see Materials and Methods for details

	[sulfonamide], μM*					
	t = 30 min			t = 60 min		
Inhibitor	HPLC[a]	ES[b]	EI[c]	HPLC[a]	ES[b]	EI[c]
Benzolamide **1**	110 ± 5	108 ± 3	112 ± 2	148 ± 4	146 ± 6	149 ± 2
Acetazolamide **2**	136 ± 7	139 ± 5	140 ± 4	160 ± 8	167 ± 5	163 ± 5
Methazolamide **3**	168 ± 3	169 ± 5	165 ± 5	168 ± 5	168 ± 4	167 ± 5
Ethoxzolamide **4**	170 ± 5	171 ± 8	170 ± 4	170 ± 5	171 ± 4	170 ± 6
Dichlorophenamide **5**	167 ± 4	165 ± 5	169 ± 3	169 ± 3	168 ± 5	169 ± 5
6	0.6 ± 0.02	0.7 ± 0.04	0.5 ± 0.05	0.9 ± 0.06	0.8 ± 0.03	0.8 ± 0.02
7	0.7 ± 0.02	0.6 ± 0.03	0.8 ± 0.01	0.9 ± 0.03	0.9 ± 0.04	0.9 ± 0.03
8	0.6 ± 0.05	0.5 ± 0.02	0.7 ± 0.04	0.8 ± 0.02	0.6 ± 0.04	0.8 ± 0.05

* Mean ± standard deviation (from 3 determinations) by: [a] the HPLC method[25,26]; [b] the electronic spectroscopic method[27]; [c] the enzymatic method.[22]

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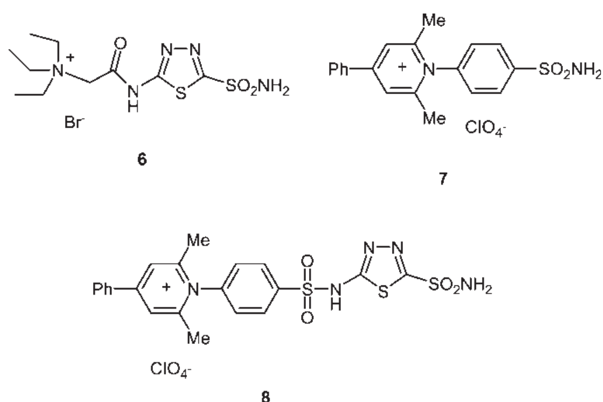
the CA inhibitory effects/penetrability of acetazolamide under similar experimental conditions.



Different cationic sulfonamides described earlier by this group, such as **6–8**, which are equipotent (or more potent, e.g., **8**) inhibitors than benzolamide and acetazolamide against the main isozymes (CA I, II and IV, see Table III), under the same conditions as those described above, were only detected in negligible amounts within the blood red cells (probably due to trace contaminations with membranes) proving that they are indeed unable to penetrate through these membranes, due to their cationic nature. Even after incubation times as long as one hour (and longer, i.e., 2–4 hours, data not shown), only minute traces of such cationic sulfonamides were detected in the supernatants (due to the possible contamination mentioned above), as proved by the three assay methods used for their identification in the cell lysate, which were in good agreement with each other (Table II). These traces are in fact at the detection limit of the assay methods described, and basically show that positively-charged sulfonamides are completely membrane-impermeant, unlike benzolamide or other neutral drugs which penetrate through the membranes (due to the fact that the anionic sulfonamidate anion is in equilibrium with the undissociated sulfonamide drug which may cross the lipid bilayer of the membrane):

$$RSO_2NH_2 \Leftrightarrow RSO_2NH^- + H^+$$

TABLE III CA inhibition data with sulfonamides **6–8** against isozymes I, II and IV

Inhibitor	K_I* (nM)		
	hCA I[a]	hCA II[a]	bCA IV[b]
6	400	10	170
7	250	14	29
8	3	0.2	2

*CO_2 hydration assay;[23] [a] Human (cloned) isozyme; [b] Isolated from bovine lung microsomes.

All this evidence demonstrates that the only approach for achieving membrane impermeability with low molecular weight compounds, is the design of positively-charged sulfonamide CA inhibitors, which being permanently charged are restricted to the extracellular space. As can be seen from the structures of **6–8**, either tetraalkylammonium compounds (**6**) or different pyridinium derivatives (**7** and **8**) are equally membrane-impermeant.

In conclusion, the statement that benzolamide is a membrane-impermeant CAI is false. The only non-polymeric membrane-impermeant CAIs are those incorporating cationic moieties, of the tetraalkyl ammonium or pyridinium type. The lipophilic CAIs methazolamide, ethoxzolamide and dichlorophenamide are the most permeant compounds, saturating blood red cells after a short period (15–30 min under the conditions of our experiments), whereas acetazolamide and benzolamide achieve the same effect at longer periods, usually of around 60 minutes.

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