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Carbonic anhydrase inhibitors; Fluorinated phenyl sulfamates show strong inhibitory activity and selectivity for the inhibition of the tumor-associated isozymes IX and XII over the cytosolic ones I and II

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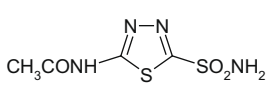
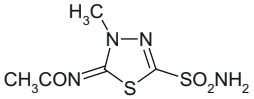
ABSTRACT

A series of fluorinated-phenylsulfamates have been prepared by sulfamoylation of the corresponding phenols and the inhibition of four physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isozymes, the cytosolic CA I and II (off-targets), and the transmembrane, tumor-associated CA IX and XII is investigated. Unlike the lead molecule (phenylsulfamate), a very potent CA I and II inhibitor and a modest CA IX/XII inhibitor, the fluorinated sulfamates were stronger inhibitors of CA IX (K_Is of 2.8–47 nM) and CA XII (K_Is of 1.9–35 nM) than of CA I (K_Is of 53–415 nM) and CA II (K_Is of 20–113 nM). Some of these compounds were selective CA IX over CA II inhibitors, with selectivity ratios in the range of 11.4–12.1, making them interesting candidates for targeting hypoxic tumors overexpressing CA IX and/or XII.

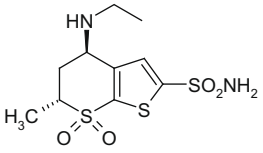
Sulfamates/bis-sulfamates[1–3] were shown recently to act as inhibitors of several carbonic anhydrase (CA, EC 4.2.1.1) isozymes involved in fundamental physiologic/pathologic states.[4,5] In mammals, this family of metalloenzymes comprises 16 different isoforms, of which several are cytosolic (CA I-III, CA VII, and CA XIII), five are membrane-bound (CA IV, CA IX, CA XII, CA XIV, and CA XV), two mitochondrial (CA VA and VB), and one (CA VI) is secreted into saliva/milk.[6–13] Three acatalytic forms are also known, that is, CA VIII, CA X, and CA XI.[9] These proteins are involved in crucial physiological processes connected with respiration and transportation 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, tumorigenicity, and several other physiologic/pathologic processes.[5–13] Many CAs are well-established drug targets.[4] In fact, sulfonamide CA inhibitors (CAIs), such as acetazolamide **AAZ**, methazolamide **MZA,** and dorzolamide **DZA** or brinzolamide **BRZ** among others, are clinically used agents for the management of a variety of disorders connected to CA disbalances, such as glaucoma, in the treatment of edema due to congestive heart failure,[7,8] or for drug-induced edema,[7] in the management of mountain sickness,[7,8] or as diuretics.[5–7] Other agents of this pharmacological class show applications as anticonvulsants,[12] antiobesity[13], or antitumor drugs/tumor diagnostic agents.[5,6,9,11,14]



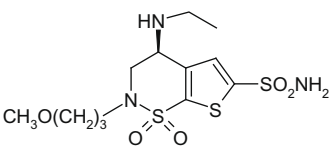
AAZ



MZA



DZA



BRZ

Two of the CA isozymes mentioned above, CA IX and XII, are overexpressed in many tumor types, contributing to the extracellular acidification and intracellular alkalinization of these cells.[15–17] It has been shown by several groups[17,18] that these isoforms play a role in acidosis, pH regulation, and survival of hypoxic tumor cells, and that their inhibition with sulfonamide CAIs leads to a perturbation of these processes, reducing the cell capability to adapt to hypoxic stress and causing its death. This leads to a strong antitumor effect of such sulfonamide CA IX/XII inhibitors.[15–18]

Various classes of sulfonamides and sulfamates have been investigated to date in the search of potent and isoform-selective CAIs,

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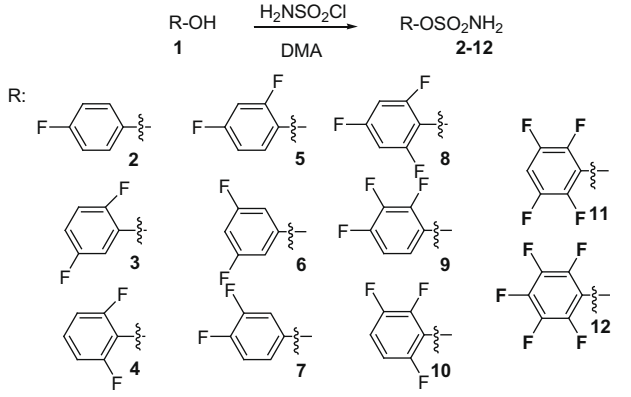

Scheme 1. Preparation of fluorophenyl sulfamates **2**–**12** by sulfamoylation of the corresponding phenols **1**.

with potential applications as diagnostic tools/antitumor lead candidates targeting CA IX and/or XII.[4,5,15–18] However, considering the potential of this class of derivatives to design new therapies for the management of malignant diseases, much effort is being dedicated by many laboratories to obtain novel such compounds.[4] Here we report a new class of such derivatives based on a very simple lead molecule, phenyl-sulfamate, investigated earlier by our group.[1]

A series of fluorophenylsulfamates (**2**–**12**) was prepared as previously described[1,2] by reacting the corresponding fluoro-phenol **1** with sulfamoyl chloride in dimethylacetamide (Scheme 1).[19] This procedure proved to be very efficient and the different derivatives were obtained in high yields after purification either by crystallization or by silica-gel chromatography. Sulfamoyl chloride was prepared from chlorosulfonyl isocyanate and formic acid as described earlier.[1,2] The rationale for introducing fluorine atoms in the molecule of lead **1** is based on our previous observations[20] that sulfonamides incorporating functionalities possessing one or more fluorine atoms bound to an aromatic ring/aliphatic chain possess enhanced CA inhibitory activity but also lipo- and hydrosolubilities, compared to structurally similar derivatives containing hydrogen instead of fluorine. On the other hand, fluorine-containing sulfamates were scarcely investigated, with only the perfluorophenyl derivative **12** being prepared earlier and studied for its interaction with isoforms CA I, II, and IX by our group.[1a] We thus decided to obtain compounds containing between 1 and 5 fluorine atoms in the aromatic ring of the lead **1**, in order to investigate how the presence of the halogen atoms, their number, and positions in the various isomers of the difluoro- (5 isomers) and trifluoro-substituted (3 isomers) compounds influences the inhibition of various CA isozymes. The different compounds **2–12** reported here were obtained in very good yields (>90%) by the above-mentioned reaction (Scheme 1) and their structures confirmed by spectroscopic and spectrometric analyses.[19]

Compounds **2–12**, the lead molecule Ph-OSO_2NH_2 as well as standard CAIs (acetazolamide **AAZ** and dorzolamide **DZA**) were tested for their inhibitory activity against two cytosolic, widespread isozymes (CA I and II) as well as the two transmembrane, tumor-associated isoforms CA IX and XII (Table 1).

The following SAR is obvious from the data shown in Table 1:

(i) Whereas the lead molecule Ph-OSO_2NH_2 is a very potent inhibitor of the cytosolic isoform hCA I (K_I of 2.1 nM), all the fluorine-containing congeners **2–12** act as much weaker inhibitors, with inhibition constants in the range of 53–415 nM. The mono-, di-, tri-, and tetrafluorophenyl-substituted sulfamates **2–11** show a rather compact behavior of medium potency hCA I inhibitors (K_Is in the range of 53–84 nM) whereas the five fluorine atoms present in **12** lead to a drastic diminution of the inhibitory capacity of this sulfamate, with a K_I of only 415 nM. Thus, in the case of hCA I, the fluorinated sulfamates behave contrary to many analogous sulfonamide classes, for which perfluoro-substituted derivatives act as much more potent inhibitors than the corresponding perhydro derivatives.[20] It is impossible to rationalize this behavior without a thorough X-ray crystallographic analysis of hCA I-fluorophenyl sulfamate adducts. In fact Christianson's group[22] reported that some fluorophenyl-substituted sulfonamides possess a rather variable binding pattern within the hCA II active site, by means of an elegant X-ray crystallographic analysis.

(ii) As for hCA I, the second cytosolic isoform hCA II was highly inhibited by the lead Ph-OSO_2NH_2 (K_I of 1.3 nM) whereas the fluorinated analogues **2–12** showed weaker potency, with inhibition constants in the range of 20–113 nM. The best hCA II inhibitor (inhibition similar to that of the potent, clinically used compounds **AAZ** and **DZA**) was the 2,3,4-trifluorophenyl-sulfamate **9** (K_I of 20 nM) together with the difluoroderivatives **3** and **5** (K_Is in the range of 24–25 nM). The weakest hCA II inhibitor was the pentafluorophenylsulfamate 12 (K_I of 113 nM) whereas the remaining derivatives showed an intermediate behavior, of medium-potency hCA II inhibitors (K_Is of 33–74 nM). Thus, small variations in the number of fluorine atoms substituting the phenyl ring as well as their positions strongly influence the hCA II inhibitory activity for this congeneric series of sulfamates, with the fluorinated derivatives **2–12** being 15.4–86.9 times less inhibitory compared to the lead molecule Ph-OSO_2NH_2. This feature, difficult to explain without X-ray crystal structures of these adducts, is however a beneficial one for compounds that should inhibit less the house-keeping, ubiquitous isozyme hCA II compared to the target ones involved in tumorigenesis, that is, hCA IX and XII (see discussion later in the text).

(iii) The transmembrane, tumor-associated isoform hCA IX, was moderately inhibited by the lead Ph-OSO_2NH_2 (K_I of 63 nM) and was much better inhibited by the fluorinated sulfamates **2–12**, which showed inhibition constants in the range of 2.8–47 nM. Thus, the situation is completely reverted for the transmembrane isozyme (hCA IX) compared to the cytosolic ones hCA I and II discussed above. The best

Table 1
Inhibition of hCA isoforms I, II, (cytosolic) IX and XII (transmembrane, tumor-associated) with sulfamates **2–12**, the lead (phenylsulfamate) and standard sulfonamide inhibitors (**AAZ** and **DZA**), by a stopped-flow CO_2 hydrase assay.[21]

Compound	K_I (nM)[#]			Selectivity ratio	
	hCA I[a]	hCA II[a]	hCA IX[b]	hCA XII[b]	K_I(hCA IX)/K_I(hCA II)
Ph-OSO_2NH_2	2.1[*]	1.3[*]	63[*]	56	0.02
2	75	74	6.5	3.9	11.38
3	85	24	9.5	5.9	2.52
4	55	44	17	13	2.58
5	84	25	9.6	6.3	2.60
6	56	33	9.1	7.4	3.62
7	53	35	10	5.8	3.50
8	54	35	21	14	1.66
9	54	20	11	8.1	1.81
10	57	41	16	3.9	2.56
11	61	34	2.8	1.9	12.14
12	415[*]	113[*]	47[*]	35	2.40
AAZ[**]	250	12	25	5.7	0.48
DZA[**]	50000	9	52	3.5	0.17

[a] h = human cloned full length enzyme.
[b] Catalytic domain of human recombinant enzyme.
[#] Mean value was from at least three different measurements.[21] Errors were in the range of ±5% of the obtained value (data not shown).
[*] From Ref. 1a.
[**] From Ref. 4.

hCA IX inhibitor was the tetrafluorophenylsulfamate 11 (K_I of 2.8 nM), whereas several other compounds (e.g., **2, 3, 5, 6,** and **7**) showed K_Is <10 nM, thus being very effective hCA IX inhibitors (better than the clinically used **AAZ** and **DZA**, which possess inhibition constants of 25–52 nM against this isozyme). Again the least effective inhibitor was the pentafluorophenyl derivative **12** (K_I of 47 nM), as for hCA I and II. The difference of hCA IX inhibitory activity between the tetra- and pentafluorophenyl derivatives **11** and **12** is 16.8, which is probably the highest one evidenced so far for two compounds from a congeneric series differing by only one atom. It is indeed really puzzling to explain this phenomenon but in the absence of X-ray crystal structures this is virtually impossible. The remaining derivatives (**4** and **8–10**) showed a compact behavior of strong hCA IX inhibitors, with inhibition constants of 11–21 nM (Table 1).

(iv) hCA XII was also moderately inhibited by the lead PhOSO$_2$NH$_2$ (K_I of 56 nM) and was much better inhibited by the fluorinated compounds **2**–**12** (K_Is in the range of 1.9–35 nM). Thus there is a close parallelism between the inhibition of hCA XII and IX with this class of inhibitors. Indeed, the best hCA XII inhibitor was again the tetrafluorophenylsulfamate **11** (K_I of 1.9 nM) whereas the least effective one was the pentafluorophenylsulfamate **12** (K_I of 35 nM). Compounds **2, 3, 5–7,** and **10** also showed highly effective hCA XII inhibitory properties (K_Is in the range of 3.9–7.4 nM), whereas **4, 8,** and **9** were slightly less effective (K_Is of 8.1–14 nM). Thus, most of these compounds showed very strong hCA XII inhibition, similarly to the clinically used drugs **AAZ** and **DZA**.

(v) Many of the effective CA IX inhibitors reported so far[1-5] have as the main drawback the lack of selectivity for inhibiting the tumor-associated isozyme (CA IX) over the cytosolic, ubiquitous one CA II. Data in Table 1 show, for example, that the selectivity ratios CA IX/CA II for the two clinically used, standard drugs **AAZ** and **DZA** are in the range of 0.17–0.48, thus the two sulfonamides being much better CA II inhibitors than CA IX inhibitors. The same is true for the lead compound, for which this ratio is even less favorable (i.e., of 0.02). However, many of the fluorinated sulfamates reported here show selectivity ratios >2.5 for inhibiting CA IX over CA II. Thus, the most CA IX-selective inhibitors were the monofluorophenyl derivative **2** and the tetrafluoro-substituted one **11** (selectivity ratios of 11.38–12.52), whereas the remaining derivatives had this parameter in the range of 1.66–3.62.

In conclusion, we report here a series of simple fluorinated phenylsulfamates which have been investigated for the inhibition of four physiologically relevant CA isozymes, the cytosolic CA I and II (off-targets) and the transmembrane, tumor-associated CA IX and XII. Unlike the lead molecule (phenylsulfamate), a very potent CA I and II inhibitor and a modest CA IX/XII inhibitor, the fluorinated sulfamates act as stronger inhibitors of CA IX (K_Is of 2.8–47 nM) and CA XII (K_Is of 1.9–35 nM) than of CA I (K_Is of 53–415 nM) and CA II (K_Is of 20–113 nM). Some of these compounds are selective CA IX inhibitors over CA II inhibitors, with selectivity ratios in the range of 11.4–12.1, making them interesting candidates for targeting hypoxic tumors overexpressing CA IX and/or XII.

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19. *4-Fluorophenylsulfamate* **2**: mp 78–80 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) d 8.01 (s, 2H), 7.32 (s, 2H), 7.3 (d, 2H, J = 2.8 Hz); 2,6-difluorophenylsulfamate **4**: mp 99–101 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) d 8.42 (s, 2H), 7.39 (m, 1H), 7.27 (m, 2H); 2,4-difluorophenylsulfamate **5**: mp 83–85 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) d 8.26 (s, 2H), 7.49 (m, 2H), 7.16 (m, 1H); 3,5-difluorophenylsulfamate **6**: mp 68–70 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) d 8.25 (s, 2H), 7.30 (m, 1H), 7.08 (m, 2H); 3,4-difluorophenylsulfamate **7**: mp 69–72 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) d 8.12 (s, 2H), 7.32 (s, 2H), 7.56 (m, 1H), 7.42 (m, 1H), 7.16 (m, 1H); 2,3,4-trifluorophenylsulfamate **9**: mp 70–72 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) d 8.50 (s, 2H), 7.57 (m, 1H), 7.43 (m, 1H); 3,4,6-trifluorophenylsulfamate **10**: mp 86–88 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) d 8.46 (s, 2H), 7.41 (t, 2H, J = 9.2 Hz), 7.3 (d, 2H, J = 2.8 Hz); 2,3,5,6-tetrafluorophenylsulfamate **11**: mp 68–70 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) d 8.97 (s, 2H), 7.21 (m, 1H); pentafluorophenylsulfamate **12**: mp 104−106 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) 9.2 (s, 2H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 138.3, 136, 133.6, 131.6; IR (KBr) 3381, 3305, 3094, 1527, 1370, 1223, 1141 cm^{-1}; MS m/z 262 (M−H)$^-$.

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21. Khalifah, R. G.. *J. Biol. Chem.* **1971,** *246*, 2561. An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. 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 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining the ionic strength constant), following the CA-catalyzed CO_2 hydration reaction. The CO_2 concentrations ranged from 1.7 to 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 have been 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 (10 mM) were prepared in distilled–deionized water with 5–10% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled–deionized water. The inhibitors were tested in concentrations from 0.01 nM to 100 μM, in subsequent 10-fold dilutions. 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 nonlinear least-squares methods using PRISM 3, and represent the mean from at least three different determinations. CA isozymes were recombinant ones obtained as reported earlier.[14,15,18] Enzyme concentrations used in these assays were hCA I: 10.4 nM; hCA II: 12 nM; hCA IX: 8.9 nM: hCA XII: 15.3 nM.
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