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Synthesis and carbonic anhydrase I, II, IX and XII inhibition studies of 4-N,N-disubstituted sulfanilamides incorporating 4,4,4-trifluoro-3-oxo-but-1-enyl, phenacylthiourea and imidazol-2(3*H*)-one/thione moieties

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Abstract: A series of sulfonamides incorporating the sulfanilamide (SA) scaffold were prepared. Reaction of the 4-amino moiety of SA with benzyl chlorides or substituted bromoacetophenones afforded the 4-mono-alkylated derivatives which were then reacted with 1,1,1-trifluoro-4-isobutoxybut-3-en-2-one leading to a series of 4-*N*,*N*-disubstituted SAs. The key intermediates were also reacted with ethoxycarbonyl isothiocyanate leading to thioureas or were cyclized in the presence of potassium cyanate/isothiocyanate to the corresponding imidazol-2(3*H*)-one/thiones. The new compounds were tested as inhibitors of four carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the cytosolic CA I and II, and the transmembrane, tumor-associated CA IX and XII. These sulfonamides were ineffective CA I and II inhibitors but were nanomolar CA IX and XII inhibitors, making them of interest as clinical candidates for antitumor/antimetastasis applications.

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As CO_2 is a crucial molecule in all life processes, being generated in high amounts in most organisms, specific catalysts evolved for its rapid transformation into bicarbonate.[1-3] These catalysts are the enzymes known as carbonic anhydrases (CAs, EC 4.2.1.1), metalloenzymes which catalyze with very high efficiency the reaction (*1*): [1-3]

$$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+ \qquad (1)$$

However, a range of other hydrolytic processes such as COS and CS_2 hydration,[4,5] cyanamide hydration to urea,[6] as well as ester hydrolysis,[7,8] etc. are catalyzed by some members of this enzyme superfamily. Indeed, by an interesting process of convergent evolution, organisms on earth have developed at least five distinct families of such enzymes, the α-, β-, γ-, δ- and ζ-CAs.[1-3,9,10] They provide a means for the organisms to face the high amounts of CO_2 formed in the metabolic processes but also the possible acid-base disequilibria connecting to this, considering the fact that the products formed in the physiologic reaction catalyzed by the CAs are an ion with strong buffering activity (bicarbonate) as well as acid (H^+ ions).[1-3,9,10] It is thus not surprizing that the CAs are involved in many physiologic processes and that perturbation of some of them lead to disequilibria and disease.[11-13] In fact, the CA inhibitors (CAIs) have pharmacologic applications as diuretics, antiglaucoma, anticonvulsant, antiobesity and anticancer agents/diagnostic tools.[1-3,14,15]

There are at least four classes of CAI targeting enzymes from mammals, which belong to the α-CA class: (i) metal ion binders (inorganic anions; sulfonamides and their isosteres (such as the sulfamates, sulfamides, N-hydroxy-sulfonamides);[1-3,14] (ii) compounds which anchor to the zinc-coordinated water molecule/hydroxide ion (phenols, polyamines, sulfocoumarins, etc.;[1-3] (iii) compounds occluding the entrance of the active site (coumarins and their isosteres); [1-3] (iv) compounds which bind in an unknown manner (secondary/tertiary sulfonamides, imatinib, nilotinib, etc).[1-3,14]

The zinc binders coordinate the metal from the center of the CA active site in a tetrahedral or trigonal bipyramydal geometries of the metal ion. [1-3,14] Sulfonamides, sulfamates, sulfamides, which are the main class of clinically used such pharmacologic agents, bind as anions, with the nitrogen atom of the sulfamoyl moiety coordinated to the Zn(II) ion, which is a a tetrahedral geometry.[1-3] The scaffold of the inhibitor also participates in various other favorable interactions with the hydrophilic and/or hydrophobic halves of the active site, as well as ordered water molecules present within it, as shown by extensive X-ray crystallographic work on adducts of various CAs with many representatives of all these classes of inhibitors. [1-3]

Sulfanilamide (SA), the first sulfonamide with clinical applications as a bacteriostatic,[1] was frequently used as a lead molecule for preparing CAIs due to the fact that it is reactive (at the 4-amino moiety), being easily acylated, alkylated or sulfonylated, leading thus to compounds with

effective CA inhibitory properties.[15-18] In fact many such N-4 mono-substituted derivatives were reported as effective and sometimes isoform-selective CAIs.[15,18] However the 4-*N*,*N*-disubstituted such compounds were much less investigated.[15]

Here we report s aseries of such 4-*N*,*N*-disubstituted SAs as well as compounds in which the 4-nitrogen was incorporated into a five-membered heterocycles. We investigated these new sulfonamides as inhibitors[19] of four mammalian (human, h) CA isoforms involved in several pathologies, hCA I and II (antiglaucoma target)[1,14] as well as hCA IX and XII (anti-tumor targets).[12,13]

Scheme 1 here

The rationale for obtaining the new sulfonamides reported here is based on the fact that many mono-*N*-4-substituted SAs, incorporating 4-*N*-acyl- or 4-N-arylsulfonyl moieties in their molecules, showed good inhibitory activity against hCAs of pharmacological interest, such as hCA VA/B, hCA VII, hCA IX and/or hCA XII.[18,20] Recently we also reported a small series of *N*,*N*-disubstituted SAs which were also effective tumor-associated CAIs, with a good selectivity for the inhibition of the transmembrane (hCA IX and XII) over the cytosolic (hCA I and II) isoforms.[15] Thus, we decided to investigate alternative such substitution patterns at the N4 atom from SA.

Reaction of SA with substituted benzyl chlorides **1** afforded the first intermediates **2** and **3** as reported in the literature (Scheme 1).[16] In a similar manner, alkylation of SA with substituted bromoacetophenones **4** afforded the remaining key intermediates **5-9**[15,16] – Scheme 1 (see Supporting Information for the detailed chemistry and characterization of the intermediates and new compounds reported here).[21] We incorporated halogens (F, Cl) and lipophilic (Me, MeO) not very bulky moieties in the key intermediates **2,3** and **5-9**, in order to investigate the structure-activity relationship (SAR) for the newly prepared CAIs reported here.

Indeed, the key intermediates **2, 3, 5-8** were then reacted with 1,1,1-trifluoro-4-isobutoxybut-3-en-2-one leading to a series of 4-*N*,*N*-disubstituted SAs of types **10-15** (Scheme 1) which incorporate the lipophilic trifluoromethylcrabonylethenyl fragment.[21] The trifluoromethylcarbonylethenyl fragment present in compounds **10-15**, was not investigated, as far as we know, for its impact on the CA inhibitory of sulfonamides incorporating it. We decided to use this fragment due to the increased lipophilicity which it can induce in some of the prepared new sulfonamides (Scheme 1).

Some of the key intermediates, such as **5** and **6** were also reacted with ethoxycarbonyl isothiocyanate leading to thioureas **16** and **17** which incorporate an additional hydrophilic moiety (Scheme 1). In order to explore a different chemical space for the SA derivatives, we also cyclized some of the intermediates, such as **5, 7** and **9** in the presence of potassium cyanate or potassium

isothiocyanate, obtaining the corresponding imidazol-2(3*H*)-ones **18, 19** and imidazol-2(3*H*)-thiones **20-22** (Scheme 1).[21] In this way we obtained a series of 13 novel SAs incorpporating either two different 4-*N*,*N*-moieties (compounds **10-17**), or possessing the 4-amino group of SA incorporated in a five-membered heterocyclic ring (compounds **18-22**). The choice of the intermediates which were further derivatized (such as **5, 6, 7** and **9**) was dictated by their availability (and yields of their synthesis) and not by any chemical consideration.

Table 1

CA inhibition data with compounds **10-22** reported here as well as the lead SA and the standard sulfonamide inhibitor acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide, **AAZ**) are shown in Table 1. The following SAR could be observed for the inhibition of the four physiologically significant isoforms hCA I, II, IX and XII with these compounds:

(i) SA is a weak hCA I inhibitor, with an inhibition constant of 28 μM, whereas AAZ is a medium potency inhibitor, with a K_I of 250 nM (Table 1). The new SA derivatives **10-22** reported here showed a very compact behavior of medium potency – weak hCA I inhibitors, with inhibition constants ranging between 302 and 593 nM. Thus, they were more effective compared to SA, but showed weaker inhibitory propertes compared to acetazolamide. As hCA I is basically an offtarget isoform,[22] its weak inhibition with the new sulfonamides reported here may be considered a very positive finding of this study.

(ii) hCA II is the physiologically dominant isoform, being involved in a variety of physiologic functions, house-keeping fuctions, as it is ubiquitous in the vertebrate cells.[1-3] It is also a target for obtaining antiglaucoma CAIs,[1,14] but an offtarget when the tumor-associated isoforms hCA IX and XII should be inhibited.[12,13] The new comppounds **10-22** investigated here behved as weak inhibitors also of hCA II; with K_Is in the range of 274 – 510 nM, being thus quite different from thre strong hCA II inhibitor **AAZ** (K_I of 12 nM) but simlar to the lead **SA** (K_I of 300 nM) – Table 1.

(iii) The tumor associated hCA IX was effectively inhibited by sulfonamides **10-22**, with K_Is in the range of 9.5 – 39 nM (Table 1), being in many cases more effective as inhibitors compared to acetazolamide (K_I of 25 nM) and much more effective when compared to the lead SA (K_I of 294 nM). The SAR is rather straightforward: for the first subseries of compounds **10-17**, the presence of acyl instead of aryl moieties as one of the *N*-4 substituents, leads to more effective hCA IX inhibitors. Indeed, the benzyl derivatives **10** and **11** were the least effective in the subseries, whereas the benzoylated compounds **12-17** showed a better inhibition profile against this isoform. The thioureido fragment present in **16** and **17** led to slightly less effective inhibitors compared to the compounds incorporating the trifluoromethylcarbonylethenyl fragment **12-15**. For the second subseries of derivatives, incorporating the imidazolone or imidazole-thione rings, of types **18-22**,

except for **20** which was a quite potent inhibitor (K_I of 13 nM), the remaining compounds showed a compact behavior of effective hCA IX inhibitors with inhibition constants ranging between 25 and 37 nM. Thus, the presence of the endocyclic oxygen or sulfur seems to be of little importance for the SAR, whereas the nature of the R moiety present in the 4-position of the aryl functionality is the main contributor to the inhibitory power of these derivatives.

iv) hCA XII was also powerfully inhibited by sulfonamides **10-22** with inhibition constants ranging between 12 and 38 nM, comparable to those of acetazolamide and sulfanilamide (Table 1). Again SAR was rather straightforward, as all the substitution patterns present in these compounds led to effective hCA XII inhibitors. The best inhibitors were **17, 18** and **21** (K_Is of 12-14 nM) which incorporated thioureido (**17**), imidazol-2(3*H*)-one (**18**) and imidazol-2(3*H*)-thione (**19**) moieties. For the remaining derivatives the SAR was rather flat (inhibition constants rnging only between 21 and 38 nM, Table 1) being thus difficult to draw detailed conclusions, apart that hCA XII is effectively inhibited by most of these derivatives.

(v) The new sulfonamides reported here were selective hCA IX/XII (over hCA I/II), which is highly significant for the design of isoform-selective CAIs with ptential clinical applications.

In conclusion, we report a series of sulfonamides incorporating the sulfanilamide scaffold, which were prepared by reaction of the 4-amino moiety of SA with benzyl chlorides or substituted bromoacetophenones. The 4-mono-alkylated derivatives were then reacted with 1,1,1-trifluoro-4-isobutoxybut-3-en-2-one leading to a series of 4-*N*,*N*-disubstituted SAs, or they were reacted with ethoxycarbonyl isothiocyanate leading to thioureas. Their cyclization in the presence of potassium cyanate/isothiocyanate led to the corresponding imidazol-2(3*H*)-one/thiones. The new compounds were tested as inhibitors of four CA isoforms, the cytosolic CA I and II, and the transmembrane, tumor-associated CA IX and XII. These sulfonamides were ineffective CA I and II inhibitors but were nanomolar CA IX and XII inhibitors, making them of interest as clinical candidates for antitumor/antimetastasis applications. The disubstitution of the 4 amino group of the parent compound sulfanilamide seems to be essential for the observed selectivity of the sulfonamides reported here acting as CA IX/XII-selective inhibitors.

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Supplementary material: A supplementary file with the detailed description of all compounds reported in the paper is available online.

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21. General Procedure for the Preparation of Trifluoromethyl ketone derivatives **10-15**. A mixture of adduct **2, 3, 5-8** (1 mmol) and 1,1,1-trifluoro-4-isobutoxybut-3-en-2-one (0.30 g, 1,5 mmol) in anhydrous MeCN (5 mL) was refluxed under stirring for 16 h. After evaporation of the solvent in vacuo, the residue was collected with isopropyl ether, filtered off, air dried, and crystallized with *iso*-propyl ether/MeOH to give **10-15**. 4-[Benzyl-((*E*)-4,4,4-trifluoro-3-oxo-but-1-enyl)amino]benzenesulfonamide **10**. Following the general procedure, the title compound was obtained from **2**. 0.28 g, 72%, mp 148-150°C. ^{1}HNMR: δ 5.40 (s, 2H), 6.10 (br, 1H), 7.35-7.60 (m, 7H), 7.74 (s, 2H), 7.96 (d, *J* = 8.8 Hz, 2H), 8.50 (d, *J* = 11.8 Hz, 1H); IR (Nujol) 3388, 3266, 1668, 1600, 1556 cm^{-1}. Anal. Calcd for $C_{17}H_{15}F_3N_2O_3S$: C, 53.12; H, 3.93; N, 7.29. Found: C, 53.10; H, 3.90; N, 7.22.

General Procedure for the Preparation of Thiourea Derivatives 16, 17. A mixture of intermediate **5, 6** (1 mmol) and ethoxycarbonyl isothiocyanate (0.26 g, 2 mmol) in anhydrous MeCN (5 mL) was refluxed under stirring for 6 h. After evaporation of the solvent in vacuo, the residue was collected with *iso*-propyl ether, filtered off, air dried, and crystallized with EtOH to give **16**, **17.** 1-(Ethoxycarbonyl)-3-(4-aminosulfonylphenyl)-3-phenacylthiourea **(16)**. Following the general procedure, the title compound was obtained from **5**. 0.27 g, 63%, mp 102-104 °C. ^{1}HNMR: δ 0.89 (t, *J* = 6.6 Hz, 3H), 4.04 (q, *J* = 6.6 Hz, 2H), 4.24 (d, *J* = 11.2 Hz, 1H), 4.58 (d, *J* = 11.2 Hz, 1H), 7.52 (s, 2H), 7.58-8.02 (m, 10H); IR (Nujol) 3406, 3316, 3268, 1733, 1594 cm^{-1}. Anal. Calcd for $C_{18}H_{19}N_3O_5S_2$: C, 51.29; H, 4.54; N, 9.97. Found: C, 51.25; H, 4.51; N, 9.95.

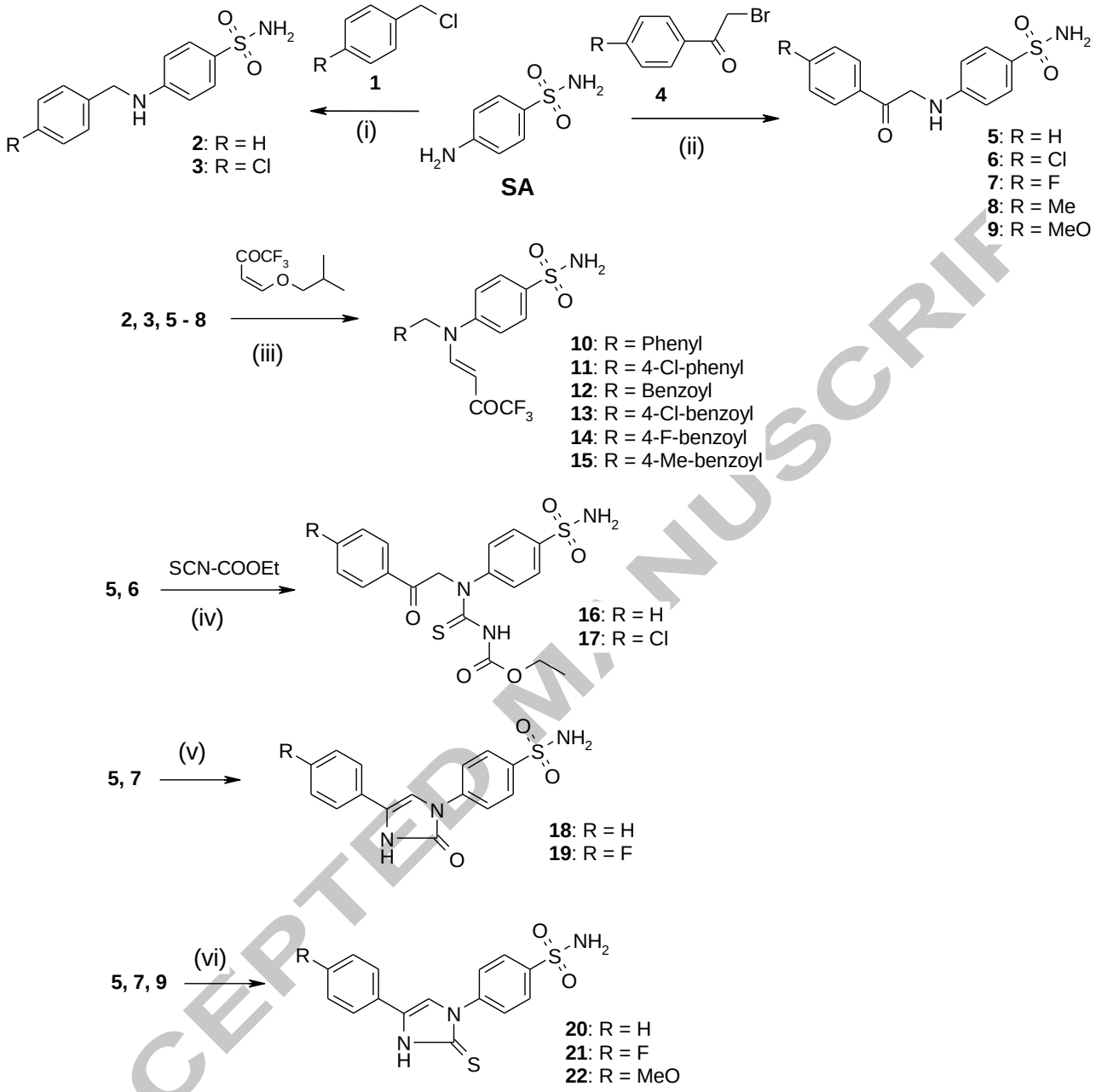
General Procedure for the Preparation of Imidazol-2(3*H*)-one Derivatives 18, 19. A mixture of intermediate **5, 7** (1 mmol) and potassium cyanate (1.62 g, 20 mmol) in acetic acid (5 mL) was stirred for 1 h at 60-65 °C. After cooling, water (20 mL) was added. The insoluble product was filtered off and washed with water, then with cold methanol, and crystallized with MeCN to give **18, 19**. 4-Phenyl-1-(4-aminosulfonylphenyl)-1*H*-imidazol-2(3*H*)-one **(18)**. Following the general procedure, the title compound was obtained from **5**. 0.21 g, 66%; mp >250 °C; ^{1}H NMR: δ 7.27 (m, 1H), 7.36 (s, 2H), 7.40-7.65 (m, 4H), 7.71 (s, 1H), 7.88 (d, *J* = 10.0 Hz, 2H), 8.02 (d, *J* = 10.0 Hz, 2H), 11.21 (s, 1H); IR (nujol) 3344, 3263, 3147, 1683, 1597, 1509 cm^{-1}. Anal. Calcd for $C_{15}H_{13}N_3O_3S$: C, 57.13; H, 4.16; N, 13.32. Found: C, 57.09; H, 4.12; N, 13.28.

22. Supuran, C.T. Carbonic anhydrases: off-targets, add-on activities, or emerging novel targets ? In *Polypharmacology in Drug Discovery*, Peters, J.U., Ed., Wiley, Hoboken, **2012**, pp. 459-491.

Table 1: Inhibition data against hCA I, II (cytosolic) and IX, XII (transmembrane, tumor-associated isoforms) with sulfonmides **10**-**22** by a stopped-flow, CO_2 hydrase assay.[19]

Compound	Ki (nM)*			
	hCA I	hCA II	hCA IX	hCA XII
10	302	276	39	36
11	405	297	33	24
12	397	421	9.5	29
13	336	275	17	27
14	433	419	13	29
15	455	408	18	28
16	498	415	25	24
17	593	510	25	14
18	572	375	34	12
19	497	336	25	38
20	521	296	13	21
21	498	351	37	12
22	411	274	30	35
SA	28000	300	294	37
AAZ	250	12	25	5.7

*Mean from 3 different assays, errors ± 10 % of the reported value



Scheme 1. Reagents and conditions: (i) H_2O, $CaCO_3$, reflux 3 h; (ii) MeOH, reflux 4 h; (iii) MeCN, reflux 16 h; (iv) MeCN, reflux 6; (v) AcOH, potassium cyanate, 60-65 °C 1 h; (vi) 10% HCl, ammonium thiocyanate, reflux 1,5 h.

Synthesis and carbonic anhydrase I, II, IX and XII inhibition studies of 4-N,N-disubstituted sulfanilamides incorporating 4,4,4-trifluoro-3-oxo-but-1-enyl, phenacylthiourea and imidazol-2(3*H*)-one/thione moieties

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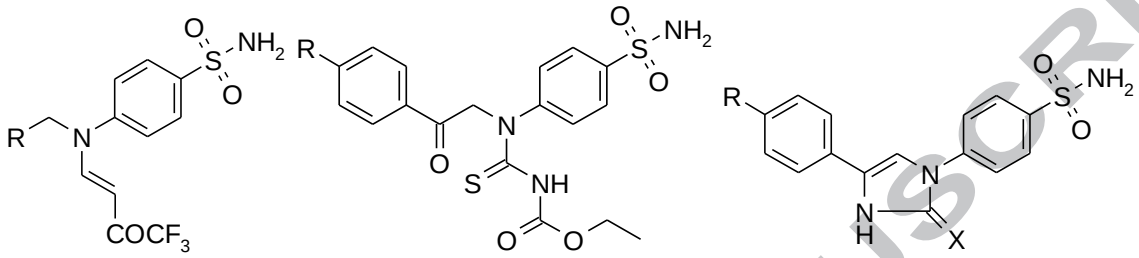

K_I (hCA IX) = 9.5 – 39 nM, K_I (hCA XII) = 12 – 36 nM