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## Graphical Abstract

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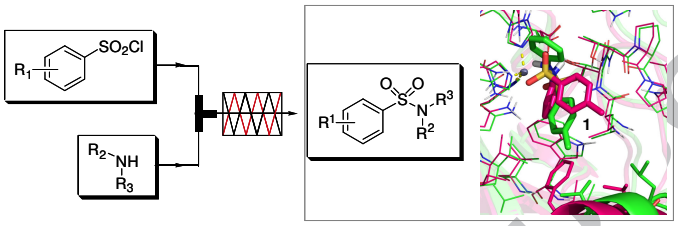

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# Flow Synthesis and Biological Activity of Aryl Sulfonamides as Selective Carbonic Anhydrase IX and XII Inhibitors

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ABSTRACT

A series of secondary and tertiary aryl sulfonamides were synthesized under flow conditions and evaluated for their ability to selectively inhibit tumor-associated carbonic anhydrase isoforms IX and XII. The tested compounds revealed to be highly potent CA IX inhibitors in nanomolar range, and to inhibit CA XII activity with different ranks of potencies. Remarkably, 4-methyl-*N*-phenyl-benzenesulfonamide was a selective nanomolar CA IX inhibitor with an $IC_{50}$ of 90 nM.



Carbonic anhydrases (CAs; also known as carbonate dehydratases EC 4.2.1.1) are metalloenzymes expressed in both prokaryotes and eukaryotes that catalyze the reversible conversion of carbon dioxide to bicarbonate ions and protons ($CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$).[1] CAs are involved in numerous cellular and physiological processes such as respiration, electrolytes and pH homoeostasis, and biosynthetic reactions which require bicarbonate as substrate including lipogenesis, gluconeogenesis, tumorigenicity and ureagenesis, among others.[1] Sixteen human CA isoenzymes belonging to alpha class have been identified. Twelve of them are characterized by a common Zn(II) ion at the catalytic site, while they differ in organ and tissue expression, cellular localization, molecular features, catalytic activity and responsivity to different classes of inhibitors.[1,2]

Among CA isoenzymes, the transmembrane proteins CA IX and XII have recently emerged as promising therapeutic targets for the treatment of human cancer.[3-5] $CO_2$ is the main byproduct of all oxidative processes and is produced in large amount in metabolic active tissues such as tumors, where CA IX and XII are highly overexpressed in response to hypoxia inducible factor (HIF) pathway. Since the spontaneous hydration of $CO_2$ is a very slow process, the catalytic activity of CAs assumes a pivotal role in the maintenance of $CO_2/HCO_3^-$ homeostasis.[6,7] Indeed, in hypoxic tumor cells, both isoforms contribute to extracellular acidification and to maintain intracellular pH more alkaline; thus, promoting tumor cell survival in an acidic environment and low bicarbonate medium. Therefore, considering that in normal tissues the expression levels of CA IX and XII are extremely low, the pharmacological targeting of these tumor-associated isozymes is increasingly asserting as a valuable approach in the search for novel anticancer treatments with reduced or absent side effects.

Numerous compounds have been disclosed to inhibit the diverse CAs.[3] They can be generally classified in two main classes of CA inhibitors (CAi): the metal-complexing anions as the primary aryl sulfonamides, and the corresponding bioisosters including sulfamates and sulfamides.[1-4,6] These compounds are able to coordinate the Zn(II) ion of the enzyme catalytic site and to establish additional interactions promoted by the aryl substituents in the region nearby the catalytic site.[2] However, there are a limited number of compounds that exhibit high selectivity toward CA IX and CA XII. Among these, 7,8-disubstituted coumarins were recently claimed to be nanomolar CA XII selective inhibitors, while the corresponding 6- or 7-modified analogues and the parent 2-thioxo derivatives were described as submicromolar inhibitors of both isoforms (Figure 1).[8,9] Much work is however warranted to discover new compounds with improved specificity and potency.

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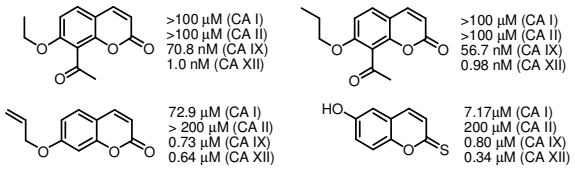


**Figure 1.** Selection of coumarinic derivatives and their inhibitory $K_i$ values towards carbonic anhydrases.

In this work, we report the development of a series of aryl sulfonamides as selective CA IX and XII inhibitors (Figure 2). In this respect, notwithstanding the wide diversification of the sulfonamide derivatives in term of structural features, size, hydrophobic/hydrophilic balance, CA isoform selectivity,[3,10-14] the so far reported compounds have the common structural feature to be primary sulfonamides. In our case, we describe the synthesis and biological evaluation of secondary aryl sulfonamides **1-9** characterized by a diverse substitution both at the amine and aryl sulfonyl portions of the molecule. Moreover, the tertiary sulfonamide **10** has also been prepared.

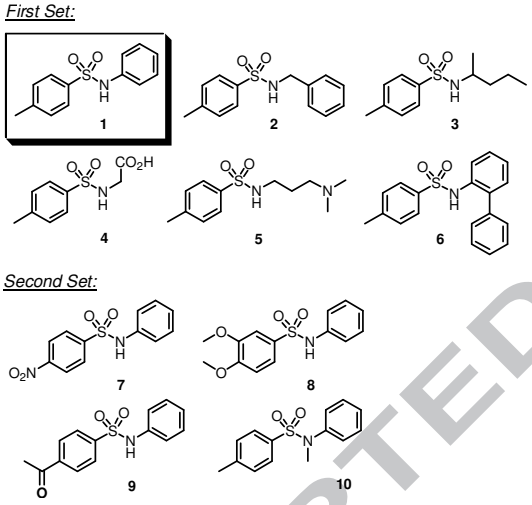


**Figure 2.** Small library of secondary and tertiary sulfonamides.

Products **1-10** were easily generated by an efficient single step flow protocol using a flow meso-reactor apparatus equipped with two loop injection systems, two pumps, a 10 mL reactor heater, a back pressure regulator (BPR), a UV detector and a fraction collector (Figure 3).[15,16] The method consisted in sequentially injecting/switching through the loops and subsequently mixing into the flow stream, two solutions constituted by the sulfonyl chloride (0.20 mmol) in acetone (2 mL) (loop A), and the appropriate primary or secondary amine (0.22 mmol) and $NaHCO_3$ (0.40 mmol) in water/PEG-400 (2 mL, 1:1, v/v) (loop B), respectively (Figure 3). The flow stream was generated by pumping a reservoir of acetone and water at the total flow rate of 0.5 mL·min$^{-1}$ through the coil reactor conditioned at 25 °C (resident time: 15 min.).[15,16] The in line UV detector was instrumental to detect the presence or absence of the product indicating the end of a reaction and the beginning of the next one. The final products were isolated by simple extraction with $Et_2O$/HCl 3 N affording the pure sulfonamides **1-10** in 82-97% yield, ready for biological evaluation.

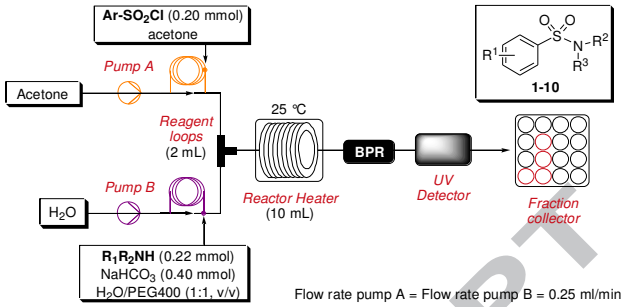


**Figure 3.** Flow synthesis of sulfonamides **1-10**.

The thus obtained aryl sulfonamides **1-10** were tested for their capability to inhibit the tumor-associated CA IX and XII (Table 1).[17] The off-target inhibition of CA isoenzymes I and II was also assessed. Acetazolamide (AAZ) was used as a reference standard. As a result, all the tested compounds were found to be highly potent CA IX inhibitors in nanomolar range, and to inhibit CA XII activity with different ranks of potencies. Interestingly, sulfonamides **1-10** were inactive or maintained a moderate inhibitory profile toward isoforms I and II with compounds **1**, **2**, **4** and **5** showing a remarkable CA IX selectivity (Table 1).

**Table 1.** Carbonic anhydrase inhibition data for compounds **1-10**.

| Compound | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|
| | CA-I | CA-II | CA-IX | CA-XII |
| **AAZ** | 0.295 | 0.015 | 0.073 | 0.060 |
| **1** | > 50 (35%)[a] | > 50 (45%)[a] | 0.090 | 8.442 |
| **2** | > 50 (34%)[a] | > 50 (44%)[a] | 0.076 | 4.280 |
| **3** | > 50 (40%)[a] | 8.071 | 0.070 | 0.248 |
| **4** | > 50 (39%)[a] | 3.623 | 0.217 | 8.570 |
| **5** | > 50 (39%)[a] | 6.672 | 0.094 | 3.302 |
| **6** | > 50 (38%)[a] | 5.100 | 0.090 | 0.226 |
| **7** | > 50 (39%)[a] | 7.321 | 0.128 | 0.174 |
| **8** | > 50 (39%)[a] | > 50 (45%)[a] | 0.079 | 0.179 |
| **9** | > 50 (36%)[a] | > 50 (47%)[a] | 0.074 | 0.255 |
| **10** | > 50 (39%)[a] | > 50 (45%)[a] | 0.093 | 0.266 |

[a]Percent of enzymatic inhibition at 10 μM.

In particular, secondary tolyl sulfonamides **1-6** characterized by both aromatic and alkylic *N*-substituents were endowed with a good inhibitory activity on CA IX enzyme ($IC_{50}$ ranging between 70 and 217 nM). Interestingly, also the tertiary sulfonamide **10** exhibited a good $IC_{50}$ value toward CA IX (93 nM). The biological activity of compounds **1**, **7-9** revealed that while substitution at the aryl nucleus bounded to the sulfamoyl moiety did not have a significant effect on the efficacy of the compounds in inhibiting CA IX, it consistently affected the activity on CA XII. Indeed, 4-methyl-benzenesulfonamido derivative (**1**) inhibited CA XII with an $IC_{50}$ of 8.442 μM, while the 4-nitro- (**7**), 3,4-dimethoxy- (**8**) and 4-acetyl-benzenesulfonamido (**9**) analogues exhibited a good inhibitory profile in nanomolar range ($IC_{50}$= 174 nM, 179 nM and 255 nM, respectively). The best compound in terms of selectivity and potency resulted to be 4-

methyl-*N*-phenyl-benzenesulfonamide (**1**) that was characterized by a nanomolar potency on CA IX ($IC_{50}$= 90 nM), a micromolar activity on CA XII ($IC_{50}$ CA IX/XII ratio= 0.01), and devoted by any activity towards the isoform I and II.

In order to understand the molecular basis responsible for the selectivity profiles displayed by this series of compounds, a computational modelling study was performed. Analyzing the binding sites residues falling within 4 Å from the ligands of the CA I (pdb code: 1AZM),[22] CA II (pdb code: 3K34),[23] CA IX (pdb code: 3IAI)[24] and CA XII (pdb code: 2HL4),[25] it was possible to pinpoint interesting differences that could explain the diverse activities recorded by the tested compounds towards the CA targets (Figure 4). Remarkably, CA II and CA XII are identical in the residue typology and only small differences on the orientation of the side-chains of few residues (e.g. F131 and V135 with an RMSD of 0.75 and 0.57Å, respectively) can be observed after the backbone alignment. Conversely, CA I is the most different isoform being characterized by a unique binding site composition, which includes residues V62, H67, F91, A121, L131, A135.

| | *62* | | *91* | | *119* | | *131* | | *141* |
|---|---|---|---|---|---|---|---|---|---|
| **CA I** | V-HS-H | ~ | FQ-H-H | ~ | H-A | ~ | L---A | ~ | L-V |
| **CA II** | N-HA-N | ~ | IQ-H-H | ~ | H-V | ~ | F---V | ~ | L-V |
| **CA IX** | N-HS-Q | ~ | LQ-H-H | ~ | H-V | ~ | V---L | ~ | L-V |
| **CA XII** | N-HA-N | ~ | IQ-H-H | ~ | H-V | ~ | F---V | ~ | L-V |

**Figure 4.** Alignment of the binding site residues falling within 4 Å from the ligands of the four carbonic anhydrases isoforms. Residue numbering is reported at the first row. Legend: - residue not in the 4 Å range from the ligand, ~ numbering jump.

Next, we directed our efforts on the analysis of the docking results performed by Glide v6.0 of the Schrodinger Suite 2013-2[26] in simple precision mode (SP), relative to the crystallized structure of the CA II and CA IX.[27] Two factors emerged to be relevant for the activity: a) the F131 residue in the CA II isoform is mutated in a valine in the CA IX, and b) the interaction distance between the Zn(II) at the catalytic site and the closest oxygen of the sulfonamide group. In particular, in the most selective 4-methyl-*N*-phenyl-benzenesulfonamide (**1**), the tolyl group displayed a perfect fitting in the V131 region of the CA IX, while a steric clash was present when the molecule adopted the same disposition inside the binding pocket of the CA II (Figure 5). Indeed, the cavity of the CA IX binding site is defined by a small α-helix (a.a. 130 to 137) that is not perfectly aligned with the corresponding portion of the CA II. Moreover, the catalytic site of CA IX may allocate the ligands with a better fitting because of a wider cavity area. All the other poses obtained for compound **1** in the CA II gave a different fitting with even a much higher distance between the interacting oxygen of the sulfonamide moiety and the Zn metal ion (from 5.72 Å to 6.46 Å). Notably, all the other derivatives of the series displayed the same binding feature of **1**, though compounds **3-5** were likely to fit better into the CA II binding site probably because of the higher flexibility of the alkyl groups, while the ortho-phenyl derivative **6** showed a 'compact' disposition into the enzyme cavity. The nitro derivative **7** displayed a disposition in CA II comparable to that adopted by **1** within the CA IX with a good distance from the Zn metal but with a higher exposure of the nitro group to the solvent.

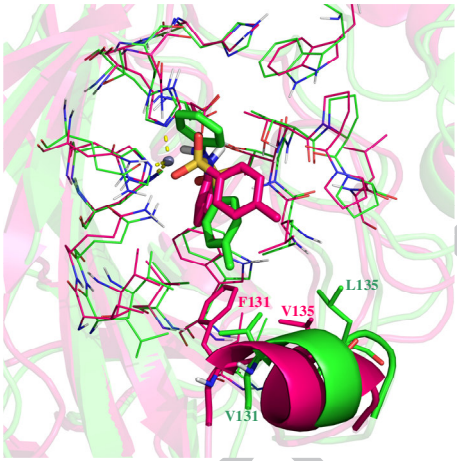


**Figure 5.** Docking pose of compound **1** in CA IX (green) and CA II (pink). The main difference in the binding site is in the α-helix at the bottom of the image, formed by amino acids going from 130 to 137, with sequences RVDEALGR and DFGKAVQQ for CA IX and CA II, respectively. The labelled side chains of the aminoacids shown in sticks belongs to the residues, defining the binding site pocket that are different into the two isoforms.

In summary, we have reported a novel small collection of secondary and tertiary aryl sulfonamides with potent CA IX and CA XII inhibitory activity. The compounds were generated by an efficient, safe, and easily scalable method which can be instrumental for the rapid preparation of additional, refined derivatives and for large scale synthesis of specific lead candidates.[15] Further extension of the structure activity relationships of this class of compounds as well as in vivo characterization of the most potent and selective CA IX inhibitor **1** will be reported in due course.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at …..

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16. Sulfonyl chloride (0.20 mmol) was dissolved in acetone (2 mL) and the resulting solution was injected in the reagent loop A. An aqueous $NaHCO_3$ 0.4 M (1 mL) was added to a solution of the amine (0.22 mmol) dissolved in PEG-400 (1 mL). The $H_2O$/PEG400 solution (2 mL, 1:1, v/v) was then injected in the reagent loop B. A degassed solution of acetone and water were connected with pump A and B, respectively and the flow rate was fixed at 0.5 $mL \cdot min^{-1}$ (0.25 $mL \cdot min^{-1}$ + 0.25 $mL \bullet min^{-1}$). After switching the sample loops, the mixtures exited were joined in a T-piece, entered in a 10 mL PTFE coil reactor warmed at 25 °C, fitted with the back pressure regulator (100 psi), directed in UV detector and the output was recovered in a fraction collector. The reaction mixture was drooped in a tube containing $Et_2O$/HCl 3 N (5 mL, 4:1, v/v). The two phases were separated, and the organic one was washed with $H_2O$ (2 x 1 mL), dried over $Na_2SO_4$, and concentrated under reduced pressure to give the desired pure aryl sulfonamide.
17. An applied photophysics stopped-flow instrument has been used for assaying the CA catalyzed $CO_2$ hydration activity.[18] Phenol red (at a concentration of 0.2 mM) has been used as an indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.4) and 20 mM $NaBF_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed $CO_2$ hydration reaction for a period of 10-100 s. 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 and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. 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 $IC_{50}$s were obtained by non-linear least-squares methods using PRISM 3, as reported earlier[19-21], and represent the mean from at least three different determinations. All CA isoforms were recombinant and obtained in-house as reported earlier.[19-21]
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