4-Substitutedbenzenesulfonamides incorporating bi/tricyclic moieties act as potent and isoform-selective carbonic anhydrase II/IX inhibitors.

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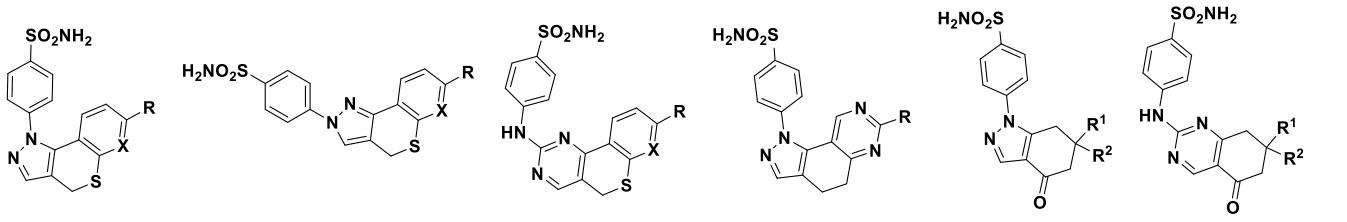
ABSTRACT: As a part of our efforts to expand chemical diversity in the Carbonic Anhydrases Inhibitors (CAIs), three small series of polyheterocyclic compounds (**4-6**) featuring the primary benzenesulfonamide moiety linked to bi/tricyclic scaffolds were investigated. Highly effective inhibitors against the target tumor-associated hCA IX (low nanomolar/subnanomolar potency levels), showing significant functional selectivity profile towards hCA I, II and IV isozymes were identified. Molecular docking studies clarified the reasons behind the activity and selectivity of the new compounds.

Introduction

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) is widespread in organisms all over the phylogenetic tree and catalyzes a simple physiological reaction, the interconversion between CO_2 and bicarbonate, with the generation of a proton.[1] CA inhibition finds pharmacological applications in several fields including diuretics, antiglaucoma, anticonvulsant, and anticancer agents.[2] Sulfonamides and their bioisosteres (sulfamates, sulfamides), were reported as pharmacologically relevant CA inhibitors (CAIs).[3]

Due to the ubiquity of such enzymes, the selective inhibition and polypharmacology of inhibitors is an important aspect of all drug design campaigns.[3] Several drug design strategies have been reported ultimately based on the tail approach[4] for obtaining sulfonamides/dithiocarbamates, which exploit more external binding regions within the enzyme active site (in addition to coordination to the metal ion), thus leading to isoform-selective compounds. Thus, the exploration of novel scaffolds may expand chemical diversity aiding the development of novel classes of CAIs with the desired pharmacologic properties. In this connection, we recently reported a novel class of tricyclic benzenesulfonamides **1a-e** featuring the benzothiopyrano[4,3-*c*]pyrazole (**1a–c**) and pyridothiopyrano[4,3-*c*]pyrazole (**1d–e**) systems (Chart 1),[5] designed as geometrically constrained analogues of celecoxib (CLX) and valdecoxib (VLX), originally launched as cyclooxygenase 2 (COX-2) specific inhibitors, and later shown also to act as potent CAIs.[6]

Compounds **1a-e** behaved as selective CAIs without any inhibitory effect on COX-2. In addition, the most interesting feature was their capability to predominantly exert strong inhibition of only human (h) isoforms hCA I and II, whereas their inhibitory activity against hCA III, IV, VA, VB, VI, VII, IX, XII, XIII, and XIV was up to 2 orders of magnitude lower, potentially resulting in a lower occurrence of side effects. X-ray crystallographic studies performed on **1e** (Chart 1) in complex with hCA II, combined with homology modeling, rationalize this inhibition profile: compound **1e** is buried deep into the active site with the sulfonamide nitrogen directly bound to the zinc ion; the sulfur atom is out of the active site and not directly involved in any interaction with the enzyme, only producing a pucker in the ring geometry; the hydrophobic tricyclic scaffolds protrudes out of the active site, where it is stabilized by van der Waals interactions with hydrophobic residues lining the active site cavity. According to this interaction mode, the differences in affinity for hCA I and II, with respect to hCA IX and XII, might be ascribed to point mutations in amino



1 a-e

a: X = CH, R = OCH_3
b: X = CH, R = Cl
c: X = CH, R = CF_3
d: X = N, R = H
e: X = N, R = CH_3

2 a-d

a: X = CH, R = OCH_3
b: X = CH, R = Cl
c: X = CH, R = CF_3
d: X = N, R = H
e: X = N, R = CH_3

3 a-e

a: X = CH, R = H
b: X = CH, R = OCH_3
c: X = CH, R = Cl
d: X = N, R = H
e: X = N, R = CH_3

4 a-c

a: R = H
b: R = CH_3
c: R = C_6H_5

5 a-c

a: R^1= R^2 = H
b: R^1= R^2 = CH_3
c: R^1 = H, R^2= C_6H_5

6 a-c

a: R^1= R^2 = H
b: R^1= R^2 = CH_3
c: R^1 = H, R^2= C_6H_5

Chart 1. Structure of previously described (**1-3**) and newly synthesized (**4-6**) CAIs.

acid sequences that produce a reduction in hydrophobicity in the hydrophobic pockets within the active site of hCA IX and XII.[5] More recently, class **1** was further investigated by moving the benzenesulfonamide function from position 1 to position 2 of the pyrazole system (**2a-d**) (Chart 1).[7] Contemporaneously, two diverse heteropolycyclic scaffolds from our in-house database, structurally similar to the pyrazoles of types **1** and **2**, that is benzothiopyranopyrimidine and pyridothiopyrano-pyrimidine, were decorated with a benzenesulfonamide moiety at the 2-position of the tricyclic system by an NH linker, yielding compounds **3a–e** (Chart 1)[7] An excellent inhibitory activity against isoforms hCA II, IX, and XII was shown by most of the new sulfonamides **2** and **3**, although without high selectivity profile among the various isoforms.[7]

As a part of our efforts to expand chemical diversity in the CAIs with the aim of identifying potent selective inhibitors, in the present work, three small series of polyheterocyclic compounds (**4-6**) featuring the classical primary benzenesulfonamide moiety crucial for the binding to the catalytically metal ion, were synthesized and biologically evaluated. Specifically, in all the novel classes the sulfur bridge was replaced with the bioisosteric methylene as our previous studies highlighted that the sulfur atom was unnecessary for the interaction of **1e** with the enzyme.[5] The 7-substituted-4-(4,5-dihydro-1*H*-pyrazolo[3,4-*f*]quinazolin-1-yl)benzenesulfonamides **4a-c** (Chart 1) maintained a 5,6,6 tricyclic system analogous to derivatives **1-3**, but an additional nitrogen atom was introduced in the pyridine nucleus, converting it into a pyrimidine, possibly providing an additional or alternative interaction point with the enzyme.

The bicyclic tetrahydroindazole (**5a-c**, Chart 1) and tetrahydroquinazole (**6a-c**, Chart 1) derivatives were conceived as a structural simplification of compounds **1** and **3**, respectively, in which the phenyl- or pyrido-fused moiety was removed and a carbonyl group was added in the non-aromatic ring. The crucial benzenesulfonamide function was maintained, directly linked at 1-position of the pyrazole ring (**5a-c**, Chart 1) or linked at 2-position of the pyrimidine ring by an NH spacer (**6a-c**, Chart 1), in strict similarity to derivatives **1** and **3**, respectively.

Finally, tricyclic compounds **4a-c** and bicyclic derivatives **5a-c** and **6a-c** were decorated with methyl or phenyl groups (R, R^1, and R^2) to evaluate the effect of these substituents on the efficacy and selectivity towards different CA isoenzymes.

Chemistry

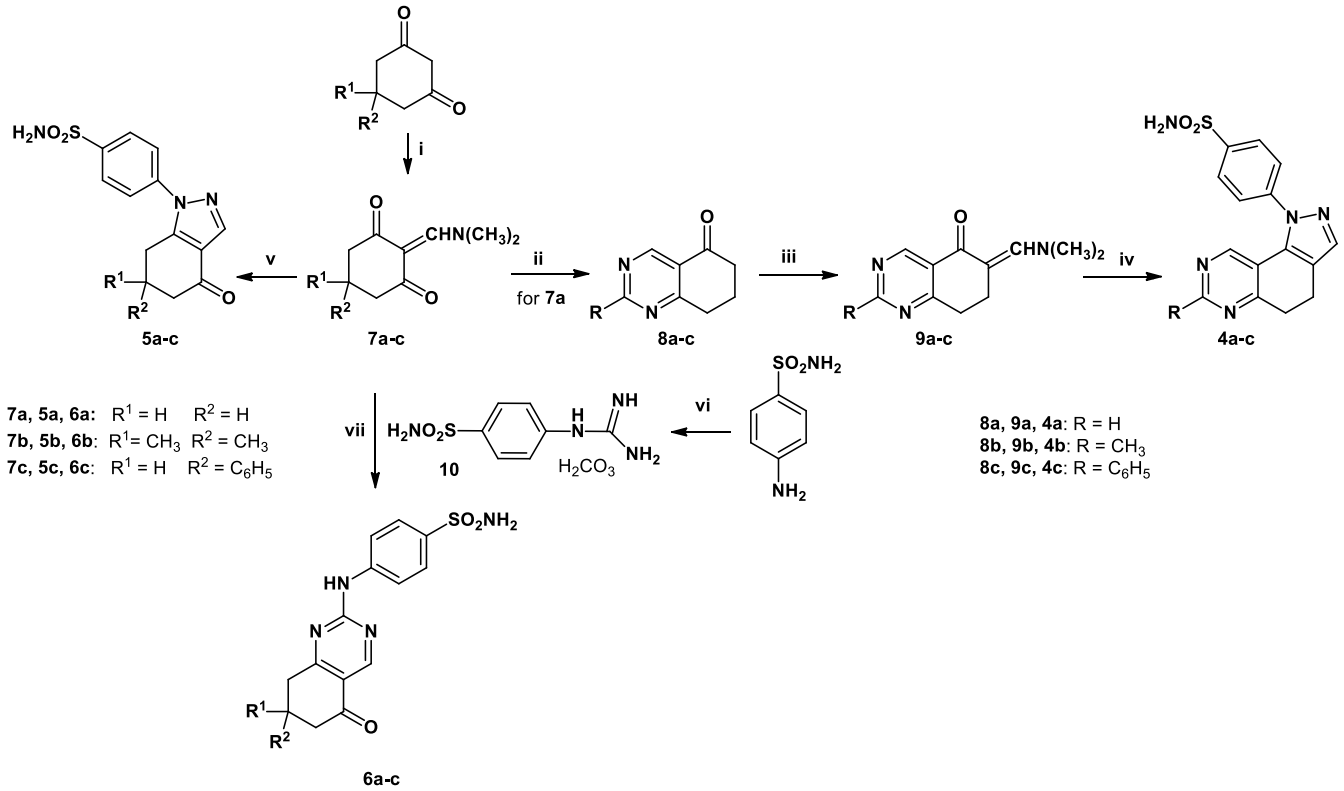
The key intermediates for the synthesis of the target compounds **4a-c**, **5a-c** and **6a-c** were the dimethylaminomethylene derivatives **7a-c**, which were prepared by a previously reported procedure with slight modifications (Scheme 1).[8,9] Briefly, the commercially available 5-substituted-1,3-cyclohexandiones were reacted with an excess of *N*,*N*-dimethylformamide dimetylacetal (DMF-DMA) at 100 °C for one hour to furnish the intermediates **7a-c** in quantitative yield.

The 1,3-bielectrophilic reaction of **7a** with nucleophilic reagents, formamidine, acetamidine or benzamidine hydrochloride, in refluxing ethanol for 15-30 hours (TLC analysis) led to the pyrimidine derivatives **8a-c**,[9] that were then reacted with an excess of DMF-DMA at 100 °C for one hour to obtain the intermediates **9a-c** in very good yields.[10,11] Derivatives **9a-c** were then solubilized in ethanol and reacted with a slight excess of commercial4-hydrazinebenzenesulfonamide hydrochloride, basically according to described procedures.[12-14] The reaction mixture was heated to 80 °C for 20 hours and, after cooling, the obtained crude compounds **4a-c** were purified by flash chromatography (Scheme 1). Similarly, the synthesis of the new bicyclic tetrahydroindazoles **5a-c** and tetrahydroquinazoles **6a-c** took advantage of the reactivity of derivatives **7a-c**[8] (Scheme 1). When **7a-c** were reacted with a slight excess of the commercially available 4-hydrazinebenzenesulfonamide hydrochloride in ethanol at 80 °C for 5-20 hours (TLC analysis), compounds **5a-c** were obtained and purified by recrystallization from ethanol (Scheme 1). Instead, reaction of intermediates **7a-c** with the guanidine **10**[7] in refluxing *n*-butanol, in the presence of sodium hydroxide for 16 hours, furnished compounds **6a-c**, then purified by recrystallization from ethanol (Scheme 1).

Results and Discussion

All the newly synthesized compounds **4a-c**, **5a-c** and **6a-c** were investigated for their enzyme inhibitory capacity against four physiologically relevant CA isoforms, the human (h) hCA I, II, IV, and IX (Table 1), by a stopped-flow CO_2 hydrase assay.[15] Acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide) was used as standard drug

Scheme 1.

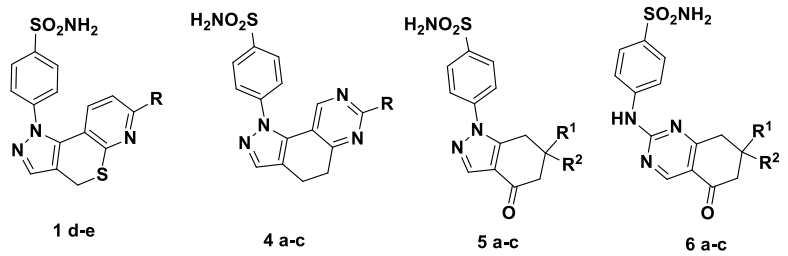


Reagents and conditions: (**i**) DMF-DMA, 100 °C; (**ii**) formamidine, acetamidine or benzamidine hydrochloride, refluxing ethanol; (**iii**) DMF-DMA, 100 °C; (**iv**) 4-hydrazinebenzenesulfonamide hydrochloride, ethanol, 80 °C; (**v**) 4-hydrazinebenzenesulfonamide hydrochloride, refluxing ethanol; (**vi**) concentrated HCl/50% cyanamide water solution, 100 °C; (**vii**) NaOH, refluxing *n*-butanol.

in the assay.[15] From a general point of view, the data listed in Table 1 show that the bioisosteric substitution of the sulfur bridge with a methylene moiety leads to potent CAIs, confirming our previous findings of the absence of a direct involvement of the sulfur in the interaction with the enzyme. All the newly synthesized compounds are highly effective inhibitors against the target tumor-associated hCA IX, exhibiting potency levels in the low nanomolar/subnanomolar range (K_i range from 7.3 nM to 0.55 nM), with an activity gain of several times compared to the reference AAZ (K_i 25.8 nM); the cytosolic isoform hCA I was moderately inhibited by compounds **4-6**, whereas diverse activities against isozymes II and IV were observed. All derivatives showed to act as preferential inhibitors of hCA IX, with selectivity ratios ranging from 61.3 to >1370 against hCA I, from 7.12 to 577 for hCA II and from 1.8 to 359 for hCA IV. Specifically, switching the pyridothiopyranopyrimidine of **1d-e** to the pyrazolo-dihydroquinazoline of **4a-c** affected hCA inhibition depending on the substituent at 7-position: the unsubstituted compound **4a** showed a gain in activity towards all the isoenzyme tested, but particularly for the isoform IX. Insertion of a 7-methyl or 7-phenyl group on **4a**, yielding compounds **4b-c**, resulted in a modest decrease in potency hCA IX inhibition (**4a** K_i 0.57 nM, **4b** K_i 7.0 nM, **4c** K_i 7.3 nM). However, the installation of the 7-phenyl substituent conferred a high degree of selectivity to hCA IX when compared the other tested hCAs. Sub-nanomolar inhibition against the isoform IX was achieved by all the compounds featuring a simplified scaffold (**5b-c**, **6a-c**, K_i values ranging from 0.55 to 0.89 nM), independent by the decoration of the central nucleus, with the only exception of **5a** (K_i 5.2 nM). The isoforms hCA I and II were scarcely (hCA I) or effectively (hCA II) inhibited by compounds **5-6** [K_i values spanning between 272.2 nM to 746.3 nM (the sole exception to this trend is **6a**, K_i 63.9 nM) and 5.2 nM to 8.6 nM (the sole exception to this trend is **5a**, K_i 78.2 nM), for hCA I and hCA II, respectively), while sparing K_i values were obtained for the membrane-bound hCA IV.

To clarify the reasons behind the displayed activity and selectivity of the newly designed compounds, molecular docking studies were undertaken for ligands **4c**, **5c**, and **6c** as representative compounds of the three series. For this purpose, the latest version of AutoDock4.2 (AD4)[16] was employed in combination with the available high-resolution crystal structure of hCA IX bound to an inhibitor (PDB code 5FL4, 1.82 Å).[17] Taking into account that a zinc atom participates to the binding event, the recently released AutoDock4(Zn) force field was employed,[18] which greatly enhances the accuracy of the pose prediction when docking zinc-chelating compounds. In particular, a specialized potential was introduced into the standard AutoDock force field to describe both the geometric and energetic components of the interactions of zinc-

Table 1. Inhibition of hCA Isoforms I, II, IV, IX with Sulfonamides **4a-c**, **5a-c** and **6a-c**, and AAZ as reference standard by a Stopped-Flow CO_2 Hydrase Assay.



cpd	R	R^1	R^2	Ki (nM)[a] hCA I [hCA I/hCA IX][b]	hCA II [hCA II/hCA IX][b]	hCA IV [hCA IV/hCA IX][b]	hCA IX
1d[c]	H	-	-	193	72	328	2340
1e[c]	CH_3	-	-	155	49	7500	3250
4a	H	-	-	73.5 [129]	4.6 [8.1]	7.1 [12.5]	0.57
4b	CH_3	-	-	429.0 [61.3]	68.6 [9.8]	581.3 [83]	7.0
4c	C_6H_5	-	-	>10000 [>1370]	4209.2 [577]	7228.7 [990]	7.3
5a	-	H	H	746.3 [143.5]	78.2 [15]	9.5 [1.8]	5.2
5b	-	CH_3	CH_3	272.2 [309]	8.6 [9.8]	39.7 [45]	0.88
5c	-	H	C_6H_5	295.7 [405]	5.2 [7.1]	213.7 [293]	0.73
6a	-	H	H	63.9 [116]	5.2 [9.45]	73.0 [132.7]	0.55
6b	-	CH_3	CH_3	633.4 [712]	8.2 [9.2]	319.4 [359]	0.89
6c	-	H	C_6H_5	686.3 [869]	6.0 [7.6]	78.0 [98.7]	0.79
AAZ				250	12.1	74	25.8

[a]Mean from three different assays. Errors were in the range of ± 5-10% of the reported values (data not shown). [b] K_i ratio for the indicated enzyme isoforms. [c] Data from ref 5.

coordinating ligands. To test the predictive power of the protocol, the co-crystal ligand of the selected X-ray complex, a sulfonamide inhibitor, was re-docked into its cognate receptor structure. Indeed, AD4 yielded a pose which is virtually superimposable to the experimental one (root mean squared deviation of 0.72 Å). The encouraging docking results prompted the employment of the aforementioned AD4 force field for the docking of the selected hCAs inhibitors into the hCA IX structure. Analysis of the achieved results revealed that **4c**, **5c**, and **6c**, chelate the catalytic zinc in the active site through their negatively charged nitrogen of the sulfonamide moiety, which is consistent with the pose of all the other sulfonamides complexed to CAs. Furthermore, the sulfonamide in each compound is well positioned to form an H-bond with the backbone NH of T200. Apart from these shared interactions, the different core structure of the three selected CA inhibitors induces different interaction patterns with the rest of the protein structure. Specifically, **4c** (Figure 1a) would preferentially adopt a conformation in which the two nitrogens of the pyrimidine ring accept a double H-bond from the Q71 and Q92 side-chains. Supposedly, the tight anchoring provided by the double H-bonding interaction might explain the high affinity of **4c** for hCA IX. Interestingly, the pendant phenyl ring of **4c** is lodged in a hydrophobic gorge lined by L91, L123 and V130 residues. These residues belong to a so-called "hot spot" region which plays a key role in the compound selectivity being highly variable in terms of sequence and, consequently, three-dimensional conformation, within each CA isoenzyme.[19] In this respect, it could be argued that the bulky and lipophilic phenyl substituent may not be easily allocated in the rather narrow "hot spot" gorge of hCA I and hCA II, accounting for the lower inhibitory activity displayed by **4c** (Figures 1b, 1c) against these latter two isoenzymes. On the other hand, hCA IV features a "hot spot" region lined by rather hydrophilic residues: N79, K95, and E127, in which the phenyl substituent would not engage favorable contacts.[20] Compounds **5c** and **6c**, regardless of their own chirality, share a common binding mode (Figure 1d and Figure S1 in Supporting Information) taking favorable van der Waals contacts with Q92, V130, L199, T201, and P203 residues. These data would indicate the absence of an enantio-discriminating binding of these compounds. Interestingly, a similar binding pose was also experimentally verified for the other co-crystal ligands of hCA IX.[17,19] Moreover, their phenyl moiety points towards the same hydrophobic "hot spot" cleft described earlier. Thus, the selectivity of **5c** and **6c** to hCA IX could also be ascribed to a difficulty in allocating bulky and lipophilic substituents in the cleft of other hCAs. The strong enhancement of potency displayed by most of the newly synthesized derivatives against hCA IX,

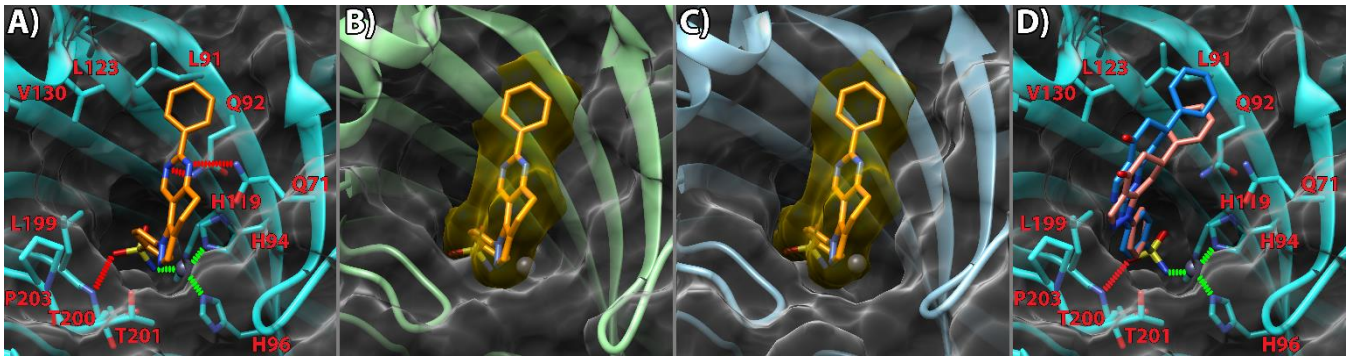


Figure 1. (A) **4c**/hCA IX (PDB 5FL4)[17] complex. The protein is depicted as cyan ribbons and sticks while the ligand as orange sticks. (B) **4c** hCA IX docked binding pose within the hCA I (PDB 4WUP)[21] structure. The protein is depicted as green ribbons and its molecular surface in gray. The ligand is represented as orange sticks and its molecular surface in yellow. (C) **4c** hCA IX docked binding pose within the hCA II (PDB 3QYK)[22] structure. The protein is depicted as light blue ribbons and its molecular surface in gray. The ligand is represented as orange sticks and its molecular surface in yellow. (D) Superimposition of the docked poses of **5c** (pink sticks) and **6c** (blue sticks) in the hCA IX active site. The protein is depicted as cyan ribbons and sticks. In panels A and D important residues are labeled. H-bonds are shown in red dashed lines while coordination bonds in green dashed lines. The images were generated using the UCSF Chimera software.[23]

combined with their significant functional selectivity profiles, could be seen with interest for the design of novel anti-cancer agents with limited side effects. Indeed, due to its key role as a survival factor for tumor cells and its involvement in insurgence of resistance to classical anticancer treatments, CA IX represents an excellent drug target for the development of novel cancer therapeutic approaches.

Experimental Section

Chemistry. General directions are in the SI. The purity of tested compounds is ≥95% (HPLC analysis).

The following intermediates were obtained according to methods previously described: 2-[(dimethylamino)methylene]cyclohexan-1,3-dione **7a**, 2-[(dimethylamino)methylene]-5,5-dimethylcyclohexan-1,3-dione **7b**, and 2-[(dimethylamino)methylene]-5-phenylcyclohexan-1,3-dione **7c**;[8] 2-methyl-7,8-dihydroquinazolin-5(6*H*)-one **8b**, 2-phenyl-7,8-dihydroquinazolin-5(6*H*)-one **8c**;[9] 6-[(dimethylamino)methylene]-2-methyl-7,8-dihydroquinazolin-5(6*H*)-one **9b**;[10] 6-[(dimethylamino)methylene]-2-phenyl-7,8-dihydroquinazolin-5(6*H*)-one **9c**;[11] *N*-(4-aminosulfonyl)phenylguanidine carbonate **10**.[7]

General procedure for the synthesis of 7-substituted 4-(4,5-dihydro-1*H*-pyrazolo[3,4-*f*]quinazolin-1-yl)benzenesulfonamides 4a-c. The appropriate derivative **9a-c** (7.0 mmol) was solubilized in ethanol (3-5 mL) and added with 1.851 g (8.3 mmol) of 4-hydrazinebenzenesulfonamide hydrochloride. The reaction mixture was heated to 80 °C for 20 hours. After cooling, the obtained solid was purified by flash chromatography on silica gel column (petroleum ether 40–60 °C/ethyl acetate 1:9 as the eluting system) to furnish the desired compounds 4a-c (yields, physical, and spectral data are reported in SI).

General procedure for the synthesis of 6-substituted 4-(4-oxo-4,5,6,7-tetrahydro-1H-indazol-1-yl)benzenesulfonamides 5a-c. The appropriate derivative **7a-c**[8] (6.0 mmol) was solubilized in ethanol (3-5 mL) and added with 1.606 g (7.2 mmol) of 4-hydrazinebenzenesulfonamide hydrochloride. The reaction mixture was heated to 80 °C for 5-20 hours (TLC analysis). After cooling, the obtained solid was purified by recrystallization from ethanol to give the desired compounds **5a-c** (yields, physical, and spectral data are reported in SI).

General procedure for the synthesis of 7-substituted 4-[(5-oxo-5,6,7,8-tetrahydroquinazolin-2-yl)amino]benzenesulfonamides 6a-c. Compounds **7a-c**[8] (1.0 mmol) were solubilized in *n*-BuOH (3-5 mL) and added with 0.300 g (1.0 mmol) of 4-guanidinobenzenesulfonamide carbonate 10 and 0.080 g (2.0 mmol) of NaOH. The reaction mixture was heated up to 120 °C and left to stir for 16 hours (TLC analysis). After cooling, the obtained crude solid compounds 6a-c were purified by recrystallization from ethanol (yields, physical, and spectral data are reported in SI).

7,8-Dihydroquinazolin-5(6H)-one 8a. 2-[(Dimethylamino)methylene]cyclohexan-1,3-dione **7a** (1.00 g, 6.0 mmol) was solubilized in 10 mL of EtOH and added with 0.480 g (6.0 mmol) of formamidine hydrochloride. The reaction mixture was heated to 100 °C for 5 hours. After cooling the solid obtained was purified by flash-chromatography on silica gel (petroleum ether 40-60/ethyl acetate 1:9 as the eluting system) to furnish **8a** as a yellow oil (yield 25%; spectral data are reported in SI).

6-((Dimethylamino)methylene)-7,8-dihydroquinazolin-5(6H)-one 9a. Compound **8a** (0.355 g, 2.4 mmol) was dissolved in 8 mL (3.6 mmol) of DMF-DMA and heated to 100 °C for about 1 hour. After cooling, the obtained suspension was triturated with ethyl ether to yield **9a** as a yellow solid, which was used in the next step without further purification (yield 95%; physical, and spectral data are reported in SI).

ASSOCIATED CONTENT

Supporting Information. General chemistry directions, yields, physical, and spectral data of compounds **4a-c**, **5a-**

c, and **6a-c**, spectral data of compounds **8a** and **9a**, CA inhibition assay and molecular modeling methods. SMILES molecular formula strings (CSV), coordinates of the predicted ligand/hCA IX complexes (PDB). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ▽S.S. and E.B. contributed equally to this work.

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ABBREVIATIONS

CA, carbonic anhydrase; CAIs, carbonic anhydrase inhibitors; CLX, celecoxib; VLX, valdecoxib; COX-2, cyclooxygenase 2; AAZ, Acetazolamide; AD4, AutoDock4.2.

REFERENCES

1 Supuran, C. T. Structure and function of carbonic anhydrases. *Biochem. J.* **2016**, *473*, 2023–2032.

2 Supuran, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat. Rev. Drug Discov.* **2008**, 7, 168–181.

3 Supuran, C. T. How many carbonic anhydrase inhibition mechanisms exist? *J. Enzyme Inhib. Med. Chem.* **2016**, *31*, 345–360.

4 Hou, Z.; Lin, B.; Bao, Y.; Yan, H. N.; Zhang, M.; Chang, X. W.; Zhang, X. X.; Wang, Z. J.; Wei, G. F.; Cheng, M. S.; Liu, Y.; Guo, C. Dual-tail approach to discovery of novel carbonic anhydrase IX inhibitors by simultaneously matching the hydrophobic and hydrophilic halves of the active site. *Eur. J. Med. Chem*. **2017**, *132*, 1–10.

5 Marini, A. M.; Maresca, A.; Aggarwal, M.; Orlandini, E.; Nencetti, S.; Da Settimo, F.; Salerno, S.; Simorini, F.; La Motta, C.; Taliani, S.; Nuti, E.; Scozzafava, A.; McKenna, R.; Rossello, A.; Supuran, C. T. Tricyclic sulfonamides incorporating benzothiopyrano[4,3-c]pyrazole and pyridothiopyrano[4,3-c]pyrazole effectively inhibit α- and β-carbonic anhydrase: X-ray crystallography and solution investigations on 15 Isoforms. *J. Med. Chem.* **2012**, 55, 9619–9629.

6 Supuran, C. T.; Casini, A.; Mastrolorenzo, A.; Scozzafava, A. COX-2 selective inhibitors, carbonic anhydrase inhibition and anticancer properties of sulfonamides belonging to this class of pharmacological agents. *Mini Rev. Med. Chem. 2004*, **4**, 625–632.

7 Barresi, E.; Salerno, S.; Marini, A. M.; Taliani, S.; La Motta, C.; Simorini, F.; Da Settimo, F.; Vullo, D.; Supuran, C. T., Sulfonamides incorporating heteropolycyclic scaffolds show potent inhibitory action against carbonic anhydrase isoforms I, II, IX and XII. *Bioorg. Med. Chem.* **2016**, *24*, 921–927.

8 Schenone, P.; Mosti, L.; Menozzi, G. Reaction of 2-dimethylaminomethylene-1,3-diones with dinucleophiles. I. Synthesis of 1,5-disubstituted 4-acylpyrazoles. *J. Heterocycl. Chem.* **1982**, *19*, 1355–1361.

9 Mosti, L.; Menozzi, G.; Schenone, P. Reaction of 2-dimethylaminomethylene-1,3-diones with dinucleophiles. III. Synthesis of 5-acylpyrimidines and 7,8-dihydroquinazolin-5(6H)-ones. *J. Heterocycl. Chem.* **1983**, 20, 649–654.

10 Mosti, L. Menozzi, G; Schenone, P. Reaction of ketenes with N,N-disubstituted α-aminomethyleneketones. XX. Synthesis of 2H-pyrano[2,3-f]quinazoline derivatives. *J. Heterocycl. Chem.* **1987**, 24, 603–608.

11 Pyrimido[4,5-f]quinazolines. Tonkikh, N. N.; Strakovs, A.; Petrova, M. V. *Chem. Heterocycl. Compd. (N. Y., NY, U. S.)* **2003**, *39*, 520-524.

12 Da Settimo, A.; Primofiore, G.; Da Settimo, F.; Marini, A. M.; Taliani, S.; Salerno, S.; Dalla Via, L. Synthesis of pyrimido[1,2-α]benzimidazol-4(10H)-one derivatives and evaluation of their interactions with DNA. *J. Heterocycl. Chem.* **2003**, *40*, 1091–1096.

13 Primofiore, G. M., A. M.; Salerno, S.; Da Settimo, F.; Bertini, D.; Dalla Via, L. Synthesis and antiproliferative evaluation of new aryl substituted pyrido[3',2':5,6]thiopyrano[4,3-c]pyrazoles. *J. Heterocycl. Chem.* **2005**, *42*, 1357-1361.

14 Marini, A. M.; Da Settimo, F.; Salerno, S.; La Motta, C.; Simorini, F.; Taliani, S.; Bertini, D.; Gia, O.; Dalla Via, L. Synthesis and in vitro antiproliferative activity of new substituted benzo[3',2':5,6]thiopyrano[4,3-d]pyrimidines. *J. Heterocycl. Chem.* **2008**, *45*, 745–749.

15 Khalifah, R. G. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **1971**, *246*, 2561–2573.

16 Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. *J. Comp. Chem.* **2009**, *30*, 2785–2791.

17 Leitans, J.; Kazaks, A.; Balode, A.; Ivanova, J.; Zalubovskis, R.; Supuran, C. T.; Tars, K. Efficient expression and crystallization system of cancer-associated carbonic anhydrase isoform IX. *J. Med. Chem.* **2015**, *58*, 9004–9009.

18 Santos-Martins, D.; Forli, S.; Ramos, M. J.; Olson, A. J., AutoDock4(Zn): an improved AutoDock force field for small-molecule docking to zinc metalloproteins. *J. Chem. Inf. Model.* **2014**, *54*, 2371–2379.

19 Alterio, V.; Hilvo, M.; Di Fiore, A.; Supuran, C. T.; Pan, P.; Parkkila, S.; Scaloni, A.; Pastorek, J.; Pastorekova, S.; Pedone, C.; Scozzafava, A.; Monti, S. M.; De Simone, G. Crystal structure of the catalytic domain of the tumor-associated human carbonic anhydrase IX. *Proc. Natl. Acad. Sci. USA*. **2009**, *106*, 16233–16238.

20 Zubriene, A.; Smirnoviene, J.; Smirnov, A.; Morkunaite, V.; Michailoviene, V.; Jachno, J.; Juozapaitiene, V.; Norvaisas, P.; Manakova, E.; Grazulis, S.; Matulis, D. Intrinsic thermodynamics of 4-substituted-2,3,5,6-tetrafluorobenzenesulfonamide binding to carbonic anhydrases by isothermal titration calorimetry. *Biophys. Chem.* **2015**, *205*, 51–65.

21 Hen, N.; Bialer, M.; Yagen, B.; Maresca, A.; Aggarwal, M.; Robbins, A. H.; McKenna, R.; Scozzafava, A.; Supuran, C. T. Anticonvulsant 4-aminobenzenesulfonamide derivatives with branched-alkylamide moieties: X-ray crystallography and inhibition studies of human carbonic anhydrase isoforms I, II, VII, and XIV. *J. Med. Chem*. **2011**, *54*, 3977–3981.

22 10.2210/pdb3qyk/pdb.

23. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E., UCSF Chimera--a visualization system for exploratory research and analysis. *J. Comp. Chem*. **2004**, *25*, 1605–1612.

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