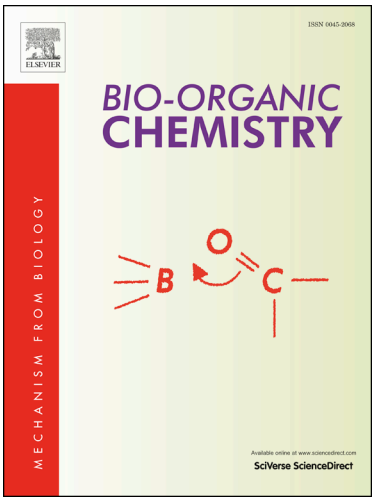

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Sulfonamides incorporating piperazine bioisosteres as potent human Carbonic Anhydrase I, II, IV and IX inhibitors

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Keywords

Carbonic anhydrase, sulfonamides, piperazines, piperazine bioisosteres, carbonic anhydrase inhibitors, isoform selectivity.

Abstract

Starting from the molecular simplification of (*R*) 4-(3,4-dibenzylpiperazine-1-carbonyl)benzenesulfonamide **9a,** a compound endowed with selectivity for human Carbonic Anhydrase (hCA) IV, a series of piperazines and 4-aminopiperidines carrying a 4-sulfamoylbenzamide moiety as Zn-binding group have been designed and tested on human isoforms hCA I, II, IV and IX, using a stopped flow CO_2 hydrase assay. The aim of the work was to derive structure-activity relationships useful for designing isoform selective compounds. These structural modifications changed the selectivity profile of the analogues from hCA IV to hCA I and II, and improved potency. Several of the new compounds showed subnanomolar activity on hCA II. X-ray crystallography of ligand-hCAII complexes was used to compare the binding modes of the new piperazines and the previously synthesized 2-benzyl-piperazine analogues, explaining the inhibition profiles.

1. Introduction

Carbonic Anhydrase (CA) is a metallo-enzyme widely expressed in nature. Eight genetically-different families (α-ι) [1] have been found up until now. In humans, sixteen different isoforms belonging to the α family have been characterized, differing for cellular and tissue distribution, and catalytic activity. The reversible hydration of CO_2 is the most important reaction catalysed by these enzymes, affecting several important physiological processes such as pH and electrolyte balance. An abnormal activity of these enzymes often leads to pathological effects [2]. CA inhibitors (CAI) are used in the clinic for more than 70 years mainly as diuretics, antiglaucoma and antiepileptic agents, although their use may be limited by side effects due to poor isoform selectivity [3]. Some examples of clinically relevant CAI are reported in Figure 1. However, recent findings suggest also other important uses, such as in renal and central nervous system diseases [4], pain [5], and cancer [6]; for the latter use, it is worth mentioning compound SLC-0111 which is under development as an anticancer agent in hypoxic tumors [7]. The wide number of possible therapeutic applications fosters the research for new potent and selective CAI.

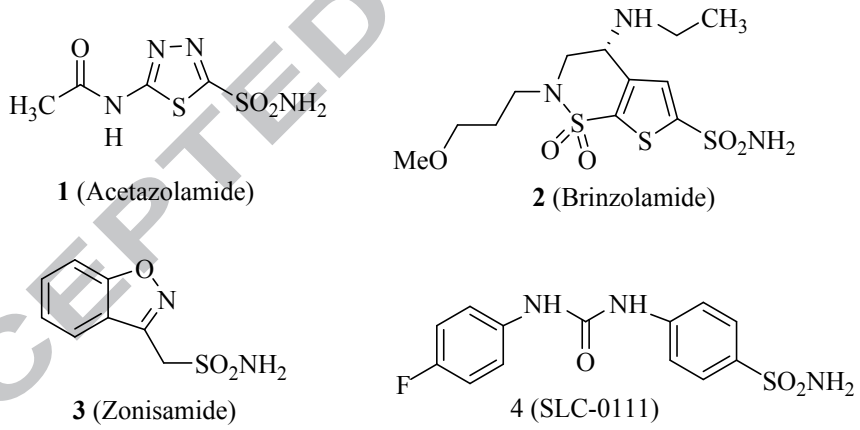


Figure 1. Structure of some CAIs used in the clinic or under clinical development.

The catalytic site of α-CA contains a Zn(II) ion, coordinated to three histidine residues and to the reactive water molecule/hydroxide ion. Inhibitors can block enzymatic activity with different mechanisms [8]. A primary sulfonamide on an aryl moiety is the metal ion-chelator group most studied in the field of CAI, since it potently and irreversibly displaces the active water molecule. The highly conserved amino-acid sequence of the region surrounding the zinc ion does not allow a selective interaction of the sulfonamide group; therefore, isoform selectivity can be achieved by adding structural tails that interact with neighbouring less conserved regions [3].

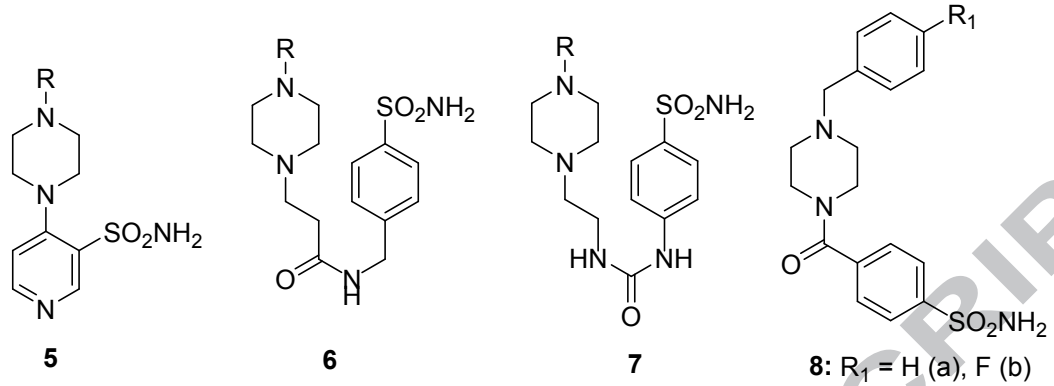


Figure 2. Structure of some piperazine CAIs reported in the literature. R substituents in compounds **5**-**7** are mainly aryl or arylmethyl groups

The piperazine ring is widely used as a scaffold in medicinal chemistry, and it has been used also in the field of CAI [9-11]. The piperazine moiety has been often decorated with aryl or benzyl substituents on one N atom, while the other N atom has been linked to a benzenesulfonamide moiety directly or through a spacer of variable length and flexibility; some examples (compounds **5**-**7**) are shown in Figure 2 [12-15]. The activity of compound **8a** and **8b** as CAI was reported in the literature while the present work was in progress [16].

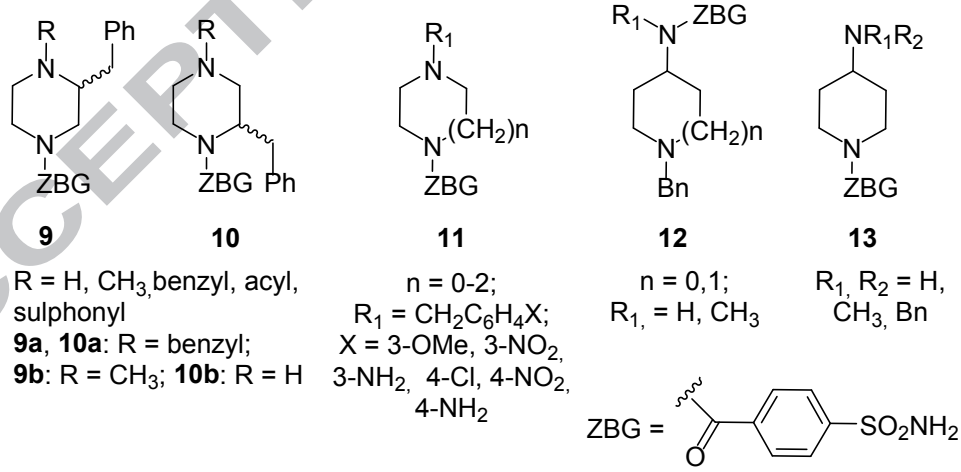


Figure **3**. Structure of the lead compounds and of the newly designed piperazines.

The activity of a series of 2- or 3-benzylpiperazines as CAI (general formulas **9** and **10**, Figure 3) has been recently reported by our group [17]. The compounds carried on one nitrogen atom a sulfamoyl benzamide group as Zn binding group, while the other nitrogen atom was decorated with alkyl/acyl/sulfonyl moieties. In general, all compounds were able to inhibit hCA I, II, IV and IX with

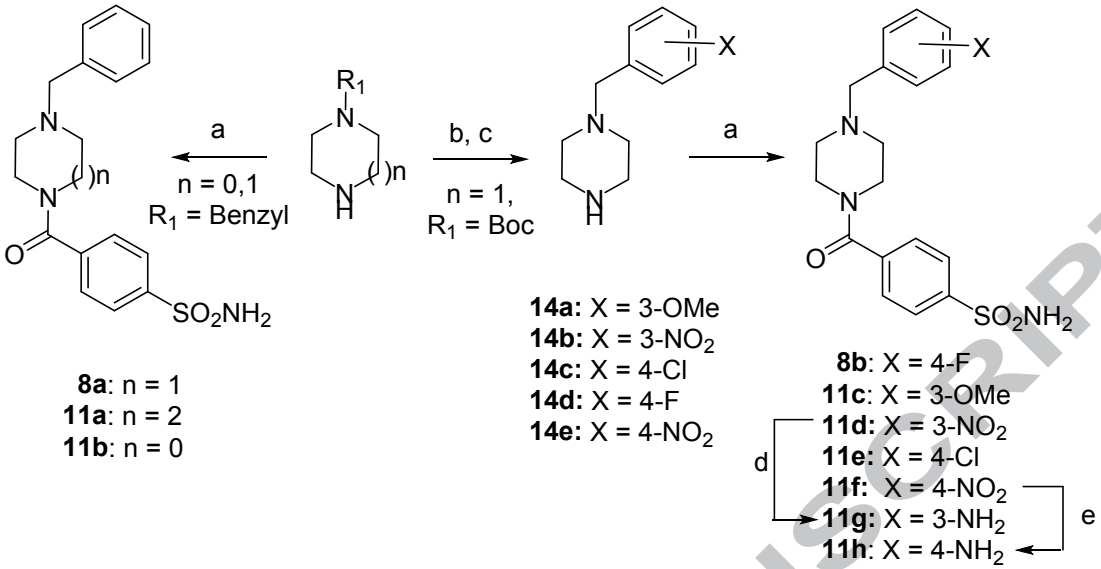
potency ranging from nanomolar to micromolar, hCA IV being the most sensitive and hCA IX the least sensitive isoform. (*R*)-**9a** (Figure 3, R = Bn) resulted particularly interesting for its selectivity ratios: this compound was found to be 165, 26, and 182 times more potent on hCA IV than on hCA I, II and IX, respectively (Table 1). Both the absolute configuration and the position of the benzyl group affected potency and selectivity: in fact, (*S*)-**9a**, the S-enantiomer, showed a slight preference for hCA II over hCA I and hCA IV (3 and 9 times, respectively) while being 30 times less potent on hCA IX. Shifting the position of the benzyl group on the piperazine ring, from C3 to C2, significantly reduced potency and selectivity: as an example, the activity of (*S*)-**10a** (R = Bn) on CA IX was largely improved.

With these premises, in order to expand structure-activity relationships of this class of compounds, the effect of structural changes on the piperazine ring and of different aromatic substituents on activity and selectivity was investigated, hoping to improve potency on CA IV. To this aim, some structural analogues of compounds **9** and **10** (compounds **11**-**13**, Figure 3) were prepared, characterized by the presence of a piperazine or another 5-7-membered ring carrying two endocyclic nitrogen atoms, or one endocyclic and the other one exocyclic. The activity and selectivity of the new compounds was assessed on isoforms hCA I, II, IV and IX, and compared to the previously reported derivatives **9** and **10**.

2. Results and discussion

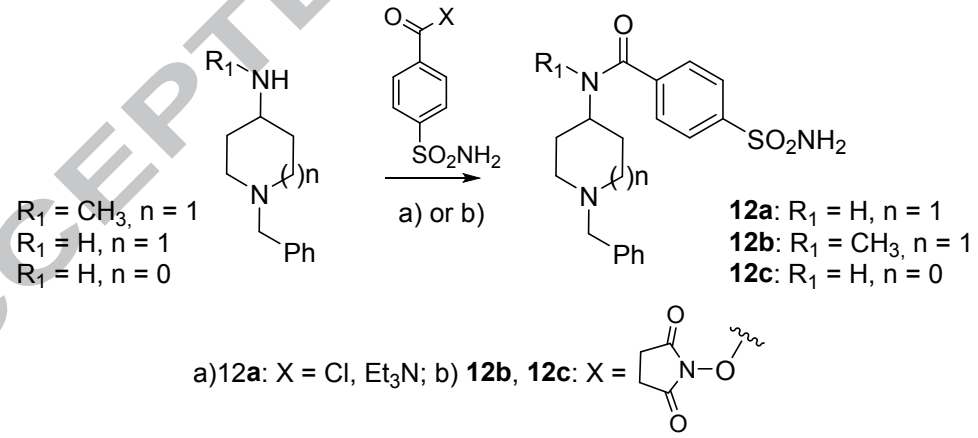
2.1 Chemistry

Compounds **11a,b** were synthesized as reported in Scheme 1. Reaction of commercially available N-benzylhomopiperazine, or of N-benzylimidazolidine [18] with 4-sulfamoylbenzoyl chloride gave compounds **11a,b** in good yields; compound **8a** [16] was prepared in the same way from N-benzylpiperazine. Reaction of N-Boc-piperazine with the suitable benzyl bromide or chloride, followed by deprotection with trifluoroacetic acid gave compounds **14a**-**e** [19-23], which were treated with 4-sulfamoylbenzoyl chloride to obtain compounds **8b** [16], **11c**-**f**. The nitro group of **11d** and **11f** were then reduced to NH_2 using different reagents: in fact, the reaction with $SnCl_2$ was successful only for the 3-nitro derivative **11d**, easily giving **11g**. Catalytic hydrogenation of **11f** gave the 4-NH_2 derivative **11h**.



Scheme 1. Synthesis of compounds **8a**,**b** and **11a-h**. Reagent and conditions: a) 4-sulfamoylbenzoyl chloride, Et_3N; b) substituted benzyl halide, Et_3N; c) TFA; d) $SnCl_2$; e) H_2/Pd/C.

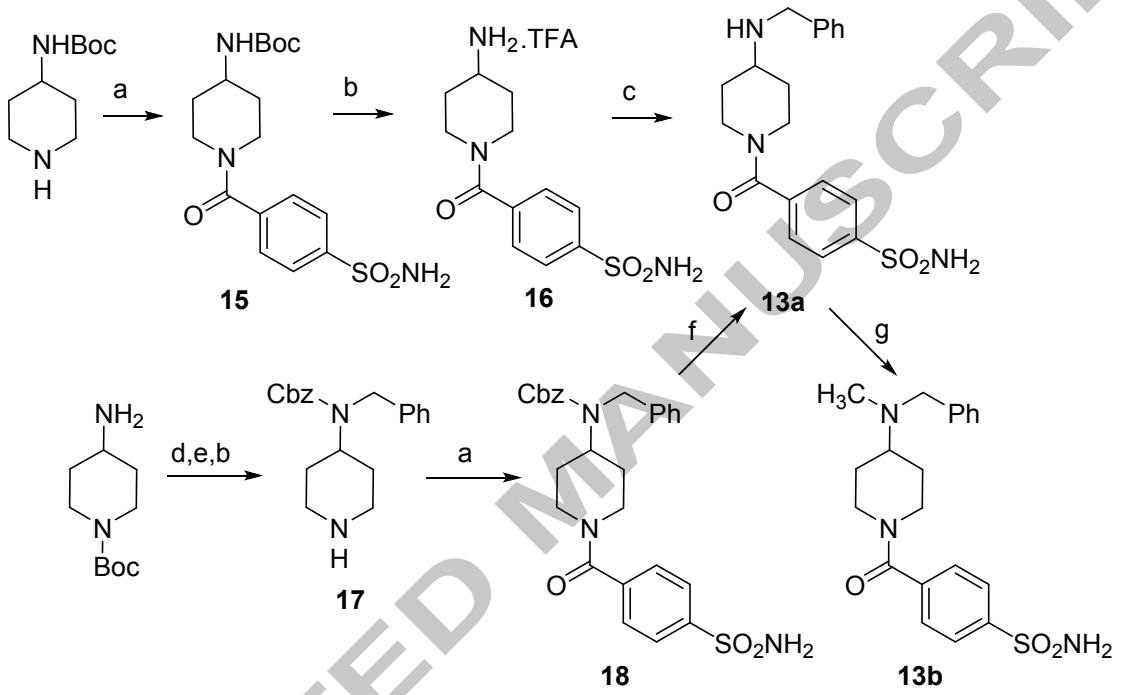
Compounds **12a-c** (Scheme 2) were obtained by reacting 4-sulfamoylbenzoyl chloride or 2,5-dioxopyrrolidin-1-yl 4-sulfamoylbenzoate [24] with commercially available 1-benzylpiperidin-4-amine, 1-benzylpirrolidin-3-amine, or with 1-benzyl-N-methylpiperidin-4-amine, prepared according to Pryde [25].



Scheme 2. Synthesis of compounds **12a-c.**

Scheme 3 shows the synthesis of 4-aminopiperidines **13a**,**b**. At first we thought to obtain the desired compounds starting from commercially available 4-(Boc-amino)piperidine, which was reacted with 2,5-dioxopyrrolidin-1-yl 4-sulfamoylbenzoate to give **15**, then deprotected, obtaining compound **16** as trifluoroacetate salt. However, the reaction of **16** with benzyl bromide gave **13a** in poor yield, and for this reason a new strategy was applied. Commercially available 4-(amino)piperidine-1-Boc was

treated with benzaldehyde, reduced with $NaBH_4$, then protected as Cbz derivative; removal of Boc gave compound **17** [19], which was transformed into **18** by reaction with 2,5-dioxopyrrolidin-1-yl 4-sulfamoylbenzoate. Deprotection with HBr/AcOH gave **13a**, which was transformed into **13b** by reaction with methyl iodide.



Scheme 3. Synthesis of compounds **13a,b**. Reagents and conditions: a) 2,5-dioxopyrrolidin-1-yl 4-sulfamoylbenzoate; b) TFA; c) $PhCH_2Br$, $NaHCO_3$; d) PhCHO, $NaBH_4$: e) ClCOOBn, Et_3N; f) HBr, AcOH; g) MeI, $NaHCO_3$.

All final compounds were characterized through ^1H, ^{13}C-NMR and HRMS spectra; representative NMR spectra have been reported in Appendix A. As far as piperazines **11c-11h** are concerned, in both ^1H and ^{13}C-NMR spectra the presence of the amide group split the aliphatic protons in four unresolved signals, centred around 2.30 and 2.50 ppm (CH_2NBn) and at 3.20 and 3.6 ppm (CH_2NCO). In addition, the ^{13}C-NMR spectra of these compounds showed four peaks for the four piperazine C atoms, suggesting that the symmetry of the molecule has been disrupted by the presence of the amide conformers. The presence of amide conformers was evident also in other compounds: for example, in the ^{13}C-NMR spectrum of **12b** the peaks for the CH_3, CH and the benzylic CH_2 groups were doubled, and the piperidine CH_2 moieties gave each a different signal. In the $CDCl_3$ ^1H-NMR spectrum of **12b**, two signals were found for the NMe group (a singlet at 2.75 ppm and another one enclosed in a multiplet between 2.90 and 3.10 ppm), for the CH proton (two multiplets centred at.

5.13 and 3.29), and for the benzylic CH_2 (two singlets at 3.51 and 3.39 ppm). On the contrary, in the ^{1}H NMR spectrum (CD_3OD) of the isomer **13b** the N-Me and the benzylic methylene groups gave only the expected singlets at 2.22 and 3.63 ppm, respectively, while the piperidine protons gave each a different signal.

Also the ^{1}H NMR spectrum of **11b** clearly showed a mixture of two conformers, as all the aliphatic and the benzenesulfonamide protons gave two sets of similar peaks at different ppm and with half integrals; in addition, the ^{13}C-NMR spectrum showed two peaks for each C atom.

2.2 CA inhibition study

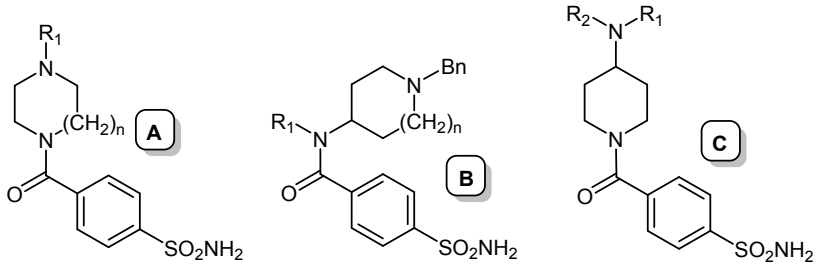
The inhibitory activity of the new compounds on several hCA isoforms was assessed using a stopped flow CO_2 hydrase assay [26]. The ubiquitous cytosolic hCA I and hCA II, the membrane-anchored hCA IV, and the transmembrane, tumor-associated hCA IX were chosen for biological testing. Results are reported in Table 1; the standard sulfonamide inhibitor acetazolamide (AAZ) and both enantiomers of the previously synthesized **9a**,**b** and **10a**,**b** were taken as reference compounds.

The activity of the unsubstituted piperazine **8a** could help to understand the contribution of the C-benzyl moiety of compounds **9** and **10**; its biological activity, already reported for hCA I, II and IX [16], was further measured on hCA IV. Indeed, **8a** was almost equipotent with (*R*)-**9a** on hCA IV (Table 1) but it lost the selectivity for this one with respect to the other tested isoforms. To check the importance of ring size the piperazine moiety was expanded to diazepane (**11a**) or contracted to imidazolidine (**11b**). These modifications brought a substantial reduction of potency on hCA IV (about 12-15 times) and on CA IX (12-23 times). Activity on hCA I and II was not modified by ring expansion while it was improved by ring contraction, since imidazolidine **11b** was, respectively, 8 and 5 times more potent than piperazine **8a**. Therefore, these modifications introduced in **11a** and **11b** some selectivity toward hCA I and II.

Then the attention was focused on the N-benzyl moiety. Compounds **11c-h**, carrying different substituents in position 3 and 4 on the aromatic ring, were prepared aiming to investigate how these modifications could affect potency or selectivity. Compound **8b**, whose activity on hCA I, II and IX had been already reported [16], was tested also on hCA IV. On this isoform only 4-NO_2 and 4-NH_2 groups were tolerated, since **11f** and **11h** were only twice less potent than **8a**; all the other substituents decreased activity (11-53 times). Aromatic substitution did not substantially modify activity on hCA IX, with the exception of the 3-OMe group (**11c**), which was 23 times less active than **8a**. On hCA I only the 4-Cl moiety (**11e**, 8 times less active than **8a**) and the 4-NO_2 group (**11f**, 3 times more active than **8a**) modulated potency. On hCA II aromatic substitution slightly modulated activity (3-4 times

increase or decrease of Ki), with compounds **11c** (3-OMe) and **11f** (4-NO_2) showing subnanomolar Ki values, similarly to the already described **8b**. Therefore, aromatic substitution, irrespective of the position and electronic characteristics of the group, changed the selectivity profile of **8a**, since compounds **11c-h** were more active on CA I and II with respect to IV and IX. Compound **11c**, carrying a 3-OMe group, showed the best selectivity ratios, being 8, 248 and 769 times more active on hCA II than on hCA I, IV and IX, respectively.

Table 1. Inhibitory activity of compounds **11-13, 15** and **18** on human (h) CA isoforms I, II, IV and IX. The standard sulfonamide inhibitor acetazolamide (AAZ), **9a**,**b** and **10a**,**b** were used as reference compounds.[a]



Cmp	Structure	R_1	R_2	n	K_I[a] (nM)			
					hCA I	hCA II	hCA IV	hCA IX
R-**9a**[b]					380.2±12	60.7±4.1	2.3±0.12	418.5±32
S-**9a**[b]					22.1±1.2	8.3±0.37	73.3±3..7	249.2±15
R-**10a**[b]					455.7±24	176±15.4	66.9±2.8	2639±89
S-**10a**[b]					88.9±7.4	46.0±2.1	79.3±5.0	27.9±1.9
8a[c]	A	Bn	-	1	6.8±0.31[c]	3.0 ±0.14[c]	4.2±0.23	33.1±0.98[c]
8b[c]	A	4-F-Bn	-	1	0.69±0.02[c]	0.50±0.01[c]	68.4±3.0	45.1 ±2.8[c]
11a	A	Bn	-	2	7.7±0.40	2.3±0.11	52.9±2.9	407.6±33
11b	A	Bn	-	0	0.8±0.06	0.6±0.02	63.9±5.1	750.7±62
11c	A	3-OMe-Bn	-	1	7.2±0.35	0.9±0.04	224.9±12	761.4±48
11d	A	3-NO_2-Bn	-	1	6.4±0.27	9.5±0.44	48.7±3.5	40.4±2.0
11e	A	4-Cl-Bn	-	1	54.8±2.9	13.3±0.92	89.4±4.7	48.4±2.3
11f	A	4-NO_2-Bn	-	1	2.4±0.13	0.77±0.05	9.7±0.63	52.3±1.6
11g	A	3-NH_2-Bn	-	1	7.4±0.50	6.9±0.30	74.9±4.9	36.3±1.7
11h	A	4-NH_2-Bn	-	1	5.8±0.24	3.5±0.22	9.1±0.75	40.2±0.9
12a	B	H	-	1	0.9±0.07	0.5±0.04	17.5±1.1	19.7±0.86
12b	B	Me	-	1	7.9±0.61	7.9±0.47	272.0±14	353.7±19
12c	B	H	-	0	36.2±1.8	4.4±0.25	25.3±1.9	21.6±1.0
13a	C	H	Bn	-	39.9±2.0	44.5±1.8	92.2±6.7	38.4±3.1
13b	C	Me	Bn	-	9.3±0.48	8.9±0.31	37.4±2.6	129.6±6.9
15	C	H	Boc	-	6.5±0.13	3.3±0.15	42.4±3.1	75.7±3.8
18	C	Cbz	Bn	-	56.1±4.0	86.4±3.8	632.5±24	421.4±26
AAZ	-		-	-	250.0±13	12.0±0.8	74.0±3.5	25.0±1.7

[a] Mean from 3 different assays, by a stopped flow technique. [b] From ref [17]. [c] From ref [16]

The effect of moving one nitrogen atom of **8a** outside the ring was checked by testing 4-aminopiperidine derivatives **12a-c** and **13a,b**. The shift of the sulfamoylbenzamide group outside the six-membered ring gave a compound (**12a**) which showed subnanomolar potency on CA I and II and 2-digit nanomolar activity on CA IV and IX. Its N-methyl derivative **12b** showed a selectivity profile similar to **12a** but a decreased potency, particularly on hCA IV and IX; this effect may be due to the removal of a H-bond donor group, or to steric hindrance. Decreasing ring size was not productive, since **12c** was less potent than **12a** and showed only a slight preference for CA II (5-8 times) over the other tested isoforms.

An exocyclic N-benzyl group was detrimental for activity on hCA I, II and IV; in fact, on these isoforms the activity of **13a** was decreased, respectively, 6, 15 and 22 times when compared to **8a**, resulting in a loss of selectivity. By adding a Me group of the NH moiety of **13a** (compound **13b**) potency was restored on CA I and II. It seems that the addition of a NMe group, possibly by increasing lipophilic interactions, improves activity on hCA I, II and IV, but not hCA IX. Synthetic intermediates **15** and **18** were also tested: the good activity of **15** suggests that a basic N atom in this part of the molecule is not required, at least on hCA I and II, but a Cbz group may be too large thus producing steric hindrance on all the four isoforms. The structure-activity relationships are summarized in Figure 4.

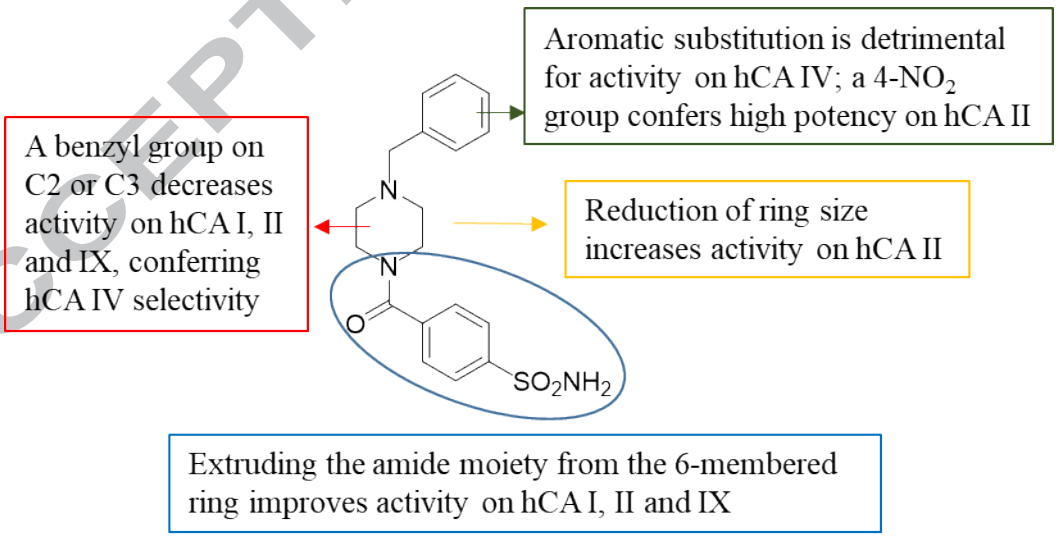


Figure 4. Summary of the structure-activity relationships found in this study.

2.3 X-ray crystallography

To study the differences in binding mode between the new derivatives and the previously synthesized 2-benzylpiperazine analogues, compounds **11a, 11b**, (*R*)-**9b** and (*R*)-**10b** were crystallized with hCA II. Compounds (*R*)-**9b** and (*R*)-**10b** were chosen among those having higher potency on this isoform (Ki 5.4 nM and 8.1 nM, respectively [17]; see their structures in Figure 3). Crystal parameters and refinement data of the four complexes are summarized in Table 2.

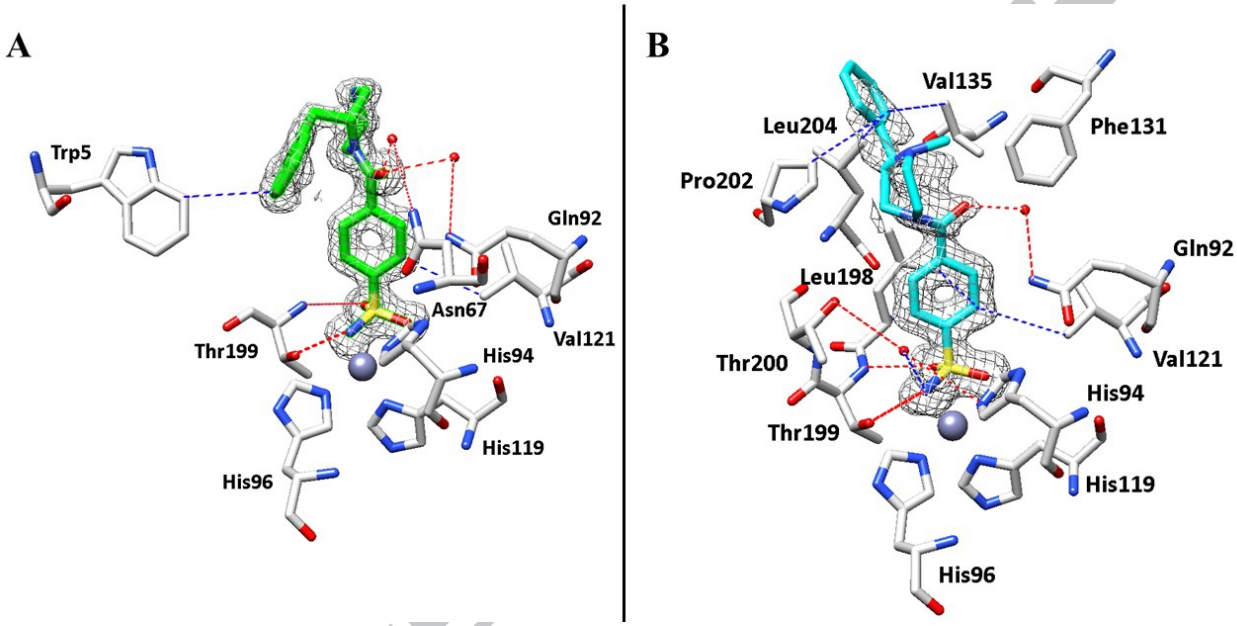


Figure 5. Active site region of hCA II/(*R*)-**10b** (PDB: 6RG3) (A) and hCA II/(*R*)-**9b** (PDB: 6RG4) (B) adducts. Inhibitors showed as σA-weighted |Fo−Fc| density map at 2.0 σ. Hydrogen bonds, van der Waals interactions and water bridges are also shown (see text for explanation).

In all the four complexes, the sulfonamide moiety was directly bound to the zinc ion in the active-site. Two additional hydrogen bond interactions, evidenced in Figures 5 and 6, between the sulfonamide and Thr199 contributed to stabilize the complexes (NH^- with the OH in the side chain and the sulfonamide oxygen with the peptide nitrogen). These interactions are typical of this zinc binding group [27-29]. The binding of (*R*)-**9b** and (*R*)-**10b** was further stabilized through a water molecule bridging the benzoate carbonyl group and Gln92; a second water bridge was observed between the carbonyl moiety of (*R*)-**10b** and the NH_2 group of Asn67. On the contrary, compound (*R*)-**9b** showed an additional water molecule linking the hydroxyl portion of the side-chain of Thr200 with the NH of the sulfonamide group (Figure 5). The tail portion of (*R*)-**10b** showed limited interaction with hCA II active site and, in particular, only a hydrophobic interaction of the benzyl group with residues Trp5 was observed (Figure 5A). On the other hand, compound (*R*)-**9b** showed

different hydrophobic interactions with hCA II arranging the benzyl portion in a different pocket of the active-site *via* interactions with Val135, Pro202, and Leu204 (Figure 5B).

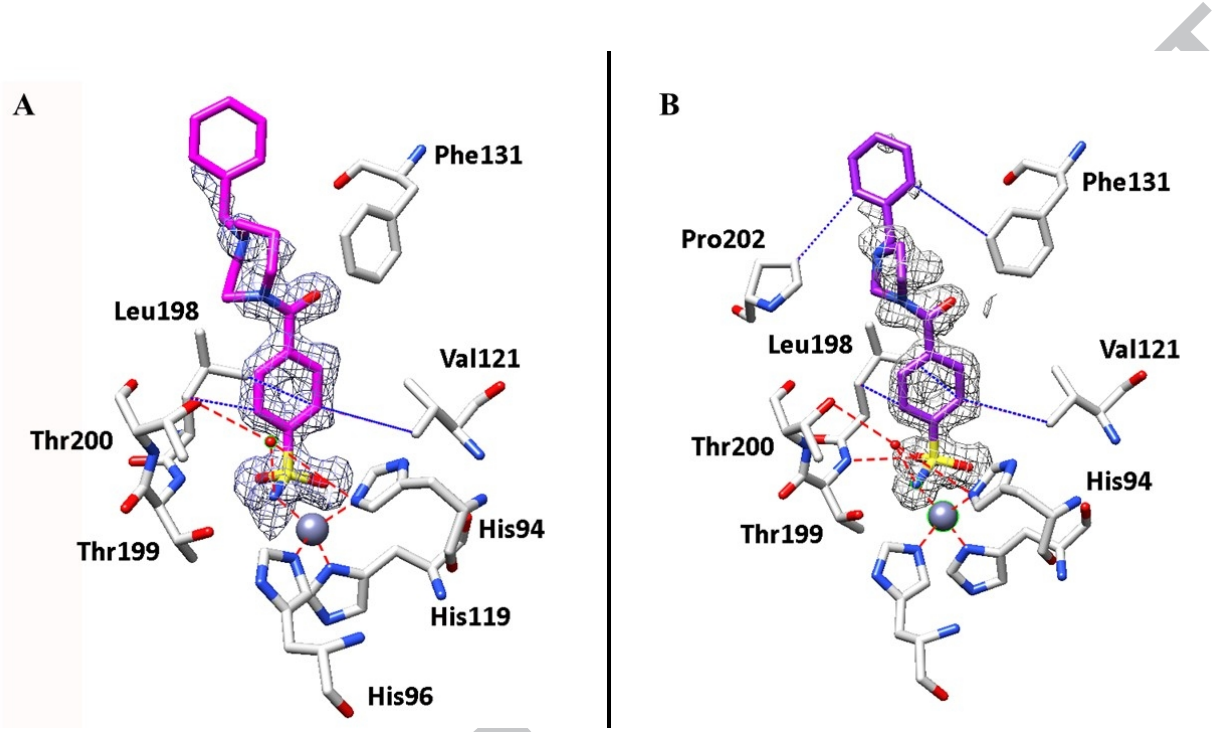


Figure 6. Active site region of hCA II/**11a** (PDB:6RHJ) (A) and hCA II/**11b** (PDB:6RHK) (B) adducts. Inhibitors are showed as σA-weighted |Fo−Fc| density map at 2.0 σ. Hydrogen bonds, van der Waals interactions and water bridges are also shown.

The benzyl portion located on the N atom of diazepane **11a** or imidazolidine **11b** scaffolds moved to the opposite side of the cavity with respect to (*R*)-**10b** (Figure 3); the higher potency of compounds **11a** and **11b** (Ki 2.3 and 0.6 nM, respectively, Table 1) for hCA II could result from this different orientation. Both **11a** and **11b** showed a water bridge linking Thr200 and the sulfonamide NH group (Figure 6A and 6B) as found for compound (*R*)-**9b** (Figure 5B). Moreover, the size of the heterocyclic ring could influence the hydrophobic interactions made by the benzyl group. In fact, a bulkier scaffold such as the diazepane ring pushed the benzyl portion away from the hydrophobic region delimited by residues Phe131, Val135, Leu198, and Pro202 (Figure 6A). On the contrary, the less bulky imidazolidine scaffold of **11b** allowed its N-benzyl moiety to form stronger hydrophobic interactions with residues Phe131 and Pro202 (Figure 6B). Unfortunately, the poor electron density for the benzyl groups of both inhibitors made their orientation not easily inferable.

Interestingly, comparison of the binding modes of compounds (*R*)-**10b** and (*R*)-**9b** (Figure 7A) showed that the presence of a methyl group on the piperazine nitrogen atom of (*R*)-**9b** led to a different ring conformation which shifted the carbonyl moiety of 1.8 Å (Figure 7B) and placed the benzyl group in the opposite side of the cavity with respect to (*R*)-**9b**. This orientation was different also with respect to the position of the 4-fluorobenzyl moiety in the structure of the complex hCA II/**8b** [16]. As previously discussed for **11b** and **11a** (Figure 4B), these substantial differences prove the importance of the inhibitor tail in order to modulate its potency.

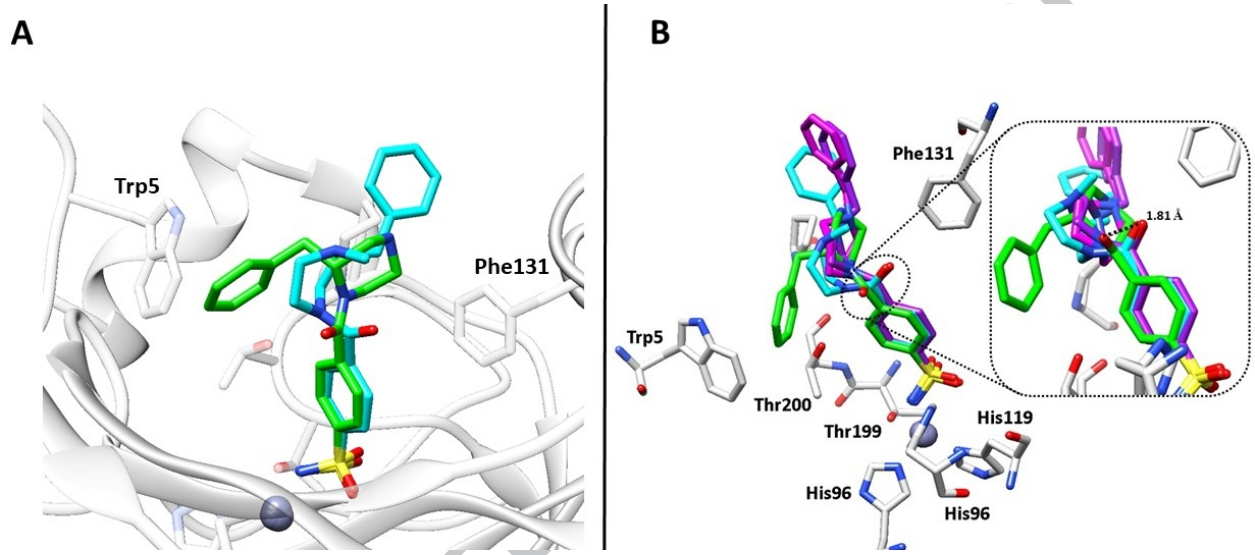


Figure 7. (A) Structural superposition between (*R*)-**10b** (green) and (*R*)-**9b** (cyan) bound to the hCA II active site. (B) Structural superposition between (*R*)-**10b** (green), (*R*)-**9b** (cyan), **11a** (magenta) and **11b** (purple).

Table 2 Summary of Data Collection and Atomic Model Refinement Statistics

	HCAII + 9b	**HCAII + 10b**	**HCAII + 11a**	**HCAII + 11b**
PDB ID	6RG4	6RG3	6RHJ	6RHK
Wavelength (Å)	0.827	0.827	1.000	1.000
Space Group	P21	P21	P21	P21
Unit cell (a, b, c, α, β, γ) (Å,°)	42.37, 41.30, 71.93 90.0, 104.4, 90.0	42.36, 41.26, 71.92 90.0, 104.3, 90.0	42.37, 41.37, 71.76 90.0, 104.4 90.0	42.60, 41.63, 72.35 90.0, 104.54, 90.0
Limiting resolution (Å)	41.07-1.25 (1.25-1.33)	41.08-1.32 (1.41-1.32)	69.49-1.44 (1.44-1.47)	70.03-1.44 (1.44-1.47)
Unique reflections	66753 (11257)	188539 (10131)	43292 (2186)	44092 (2184)

Rsym (%)	5.8 (99.2)	4.7 (77.4)	7.6 (18.5)	5.8 (19.4)
Rmeas (%)	6.9 (118.9)	5.5 (92.1)	9.5 (28.2)	7.1 (24.4)
Redundancy	3.3 (3.2)	3.3 (3.3)	2.7 (2.3)	2.9 (2.4)
Completeness overall (%)	99.1 (99.1)	99.2 (98.7)	99.4 (99.0)	99.3 (98.8)
<I/σ(I)>	9.39 (0.79)	12.39 (1.24)	7.2 (2.2)	10.0 (3.4)
CC (1/2)	99.8 (54.1)	99.9 (64.3)	98.9 (82.9)	99.6 (93.1)
Refinement statistics				
Resolution range (Å)	41.07-1.25	41.08-1.32	50.0-1.44	50.0-1.44
Unique reflections, working/free	62531/3239	53621/2719	41141/2125	41791/2061
Rfactor (%)	15.5	17.7	17.6	15.4
Rfree(%)	20.0	19.7	20.0	17.5
r.m.s.d. bonds(Å)	0.015	0.0127	0.0126	0.0134
r.m.s.d. angles (°)	2.008	1.427	1.8699	1.9184
Ramachandran statistics (%)				
Most favored	97.3	96.9	96.9	97.3
additionally allowed	2.7	2.7	3.1	2.7
outlier regions	0.0	0.4	0.0	0.0
Average B factor (Å^2)				
All atoms	18.16	17.60	13.53	13.36
inhibitors	34.36	17.68	28.17	32.19
solvent	30.31	28.91	21.90	23.29

3. Conclusion

A series of benzenesulfonamides carrying a piperazine nucleus or its bioisosteres have been prepared in order to further investigate the structure-activity relationships of this class of compounds. The removal of the C-benzyl moiety from **9a** to give **8a** resulted in a loss of selectivity for the CA IV isoform, while structural modification on **8a** shifted the selectivity toward the cytosolic hCA I and II isoforms. In particular, the reduction of ring size from 6 to 5 atoms to give **11b**, the introduction of a NO_2 group on the para position of the N-benzyl ring (**7f**) or the extrusion of the sulfamoylbenzamide moiety out the six-membered ring (**8a**) gave compounds showing subnanomolar potency on hCA II.

This information can be useful in the design of selective CAI carrying a piperazine ring; in particular, the decoration of the piperazine C-atoms with a benzyl group seems tolerated only on hCA IV but not on the other tested isoforms. Work is underway to check the contribution of other C-substituents in order to expand the structure-activity relationships in this class of potent sulfonamide CA inhibitors.

4. Experimental

4.1 General remarks and instrumentation. All melting points were taken on a Büchi apparatus and are uncorrected. When reactions were performed under anhydrous conditions, the mixtures were maintained under nitrogen. NMR spectra were recorded on a Brucker Avance 400 spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C). Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063- 0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040-0.063 mm; Merck). Yields are given after purification, unless differently stated. The purity of the tested compounds has been assessed by means of LC-DAD analyses, carried out on an Agilent 1200 system (Agilent, Palo Alto CA, USA). High resolution mass spectrometry (HR-MS) analysis was performed with a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with an electrospray ionization source (ESI). The analysis was carried out in positive ion mode monitoring protonated molecules, $[M+H]^+$ species, and it was used a proper dwell time acquisition to achieve 60,000 units of resolution at Full Width at Half Maximum (FWHM). Elemental composition of compounds was calculated on the basis of their measured accurate masses, accepting only results with an attribution error less than 5 ppm and a not integer RDB (double bond/ring equivalents) value, in order to consider only the protonated species [30]. Compounds were named following IUPAC rules by means of MarvinSketch 18.1.

4.1.1 General procedure for the insertion of the Zinc Binding Group. To a solution of the suitable amine (30-100 mg) in anhydrous CH_3CN (5-30 mL), stirred at room T, Et_3N (2 eq) and 4-sulfamoylbenzoyl chloride (1.5 eq) were sequentially added (Method A); alternatively, (Method B), the amine was treated with 2,5-dioxopyrrolidin-1-yl 4-sulfamoylbenzoate [24]. The mixture was left stirring at room T for 6-20 hrs. After removal of the solvent under vacuum, the residue was partitioned between ethyl acetate and sat. aqueous $NaHCO_3$; drying and removal of the solvent gave a residue which was purified by flash chromatography, when necessary. In this way the following compounds were prepared:

4-(4-Benzylpiperazine-1-carbonyl)benzene-1-sulfonamide 8a [16] (Method A): from commercially-available *N*-benzylpiperazine. White solid, m.p. 204-5 °C, 60% yields. [^{1}H-NMR] (DMSO) δ: 7.84 (d, J=8.2 Hz, 2H, Ar), 7.54 (d, J=8.2 Hz, 2H, Ar), 7.44 (s, 2H, SO_2NH_2), 7.36-7.20 (m, 5H, Ar), 3.61 (bs, 2H), 3.48 (s, 2H, CH_2Ph), 3.40-3.20 (m, 2H+water), 2.41 (bs, 2H), 2.32 (bs, 2H) ppm.

4-{4-[(4-Fluorophenyl)methyl]piperazine-1-carbonyl}benzene-1-sulfonamide 8b [16] (Method A): from **14d** [22] after purification using DCM/MeOH 90:10 as eluent. White solid, m.p.209 °C (d) (lit [16] 206-207 °C), 41% yields. [^{1}H-NMR] (DMSO) δ: 7.84 (d, *J* = 8.4 Hz, 2H, Ar), 7.54 (d, *J* = 8.4 Hz, 2H, Ar), 7.44 (s, 2H, SO_2NH_2), 7.32 (dd, J = 8.3 Hz, 5.8 Hz, 2H, Ar), 7.12 (t, J=8.8 Hz, 2H, Ar), 3.70-3.55 (m, 2H), 3.47 (s, 2H, CH_2Ar), 3.28-3.22 (m, 2H), 2.46-2.28 (m, 4H) ppm.

4-(4-Benzyl-1,4-diazepane-1-carbonyl)benzene-1-sulfonamide 11a (Method A): from commercially-available *N*-benzyl-homopiperazine. White solid, m.p. 175°C (d), 41% yields. [^{1}H-NMR] (DMSO) δ: 7.94-7.82 (m, 2H, Ar), 7.64-7.51 (m, 2H, Ar), 7.45 (s, 2H, NH_2), 7.35-7.23 (m, 5H, Ar), 3.72-3.54 (m, 4H), 3.32 (s, 2H+water), 2.78-2.69 (m, 1H), 2.67-2.53 (m, 3H), 1.89-1.78 (m, 1H), 1.72-1.62 (m, 1H) ppm. [^{13}C NMR] (DMSO, mixture of conformers) δ: 169.57 (CO), 169.50 (CO), 144.88 (C_{Ar}), 140.72 (C_{Ar}), 139.48 (C_{Ar}), 129.03 (CH_{Ar}), 128.90 (CH_{Ar}), 128.67 (CH_{Ar}), 128.64 (CH_{Ar}), 127.53 (CH_{Ar}), 127.43 (CH_{Ar}), 127.35 (CH_{Ar}), 126.29 (CH_{Ar}), 126.26 (CH_{Ar}), 61.57 (CH_2Ph), 61.37 (CH_2Ph), 55.53 (CH_2), 55.19 (CH_2), 54.43 (CH2), 53.84 (CH_2), 49.55 (CH_2), 48.50 (CH_2), 45.70 (CH_2), 45.00 (CH_2), 28.56 (CH_2), 26.99 (CH_2) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{19}H_{24}N_3O_3S$ 374.1533; found 374.1532.

4-(3-Benzylimidazolidine-1-carbonyl)benzene-1-sulfonamide 11b (Method A): from N-benzylimidazolidine [18], after purification using DCM/MeOH 90:10 as eluent. White solid, m.p. 175 °C (d); 10% yields. [^{1}H-NMR] (DMSO, mixture of conformers) δ: 7.85-7.79 (m, 2H, Ar), 7.71 (d, *J*=8.0 Hz, 1H, Ar), 7.65 (d, *J* = 8.0 Hz, 1H, Ar), 7.43 (s, 1H, SO_2NH_2), 7.41 (s, 1H, SO_2NH_2), 7.34-7.20 (m, 5H, Ar), 4.10 (s, 1H), 3.97 (s, 1H), 3.66 (s, 1H), 3.55 (s, 1H), 3.52 (t, *J* = 6.4 Hz, 1H), 3.44 (t, *J* = 6.0 Hz, 1H), 2.86 (t, *J* = 6.4 Hz, 1H), 2.80 (t, *J* = 6.4 Hz, 1H) ppm. [^{13}C-NMR] (DMSO, mixture of conformers) δ: 167.16 (CO), 166.86 (CO), 145.91 (C_{Ar}), 145.79 (C_{Ar}), 139.80 (C_{Ar}), 139.58 (C_{Ar}), 138.64 (C_{Ar}), 138.53 (C_{Ar}), 129.12 (CH_{Ar}), 128.94 (CH_{Ar}), 128.81 (CH_{Ar}), 128.78 (CH_{Ar}), 128.32 (CH_{Ar}), 128.10 (CH_{Ar}), 127.67 (CH_{Ar}), 126.25 (CH_{Ar}), 126.13 (CH_{Ar}), 70.27 (CH_2), 68.52 (CH_2), 57.43 (CH_2), 56.98 (CH_2), 52.87 (CH_2), 51.31 (CH_2), 47.00 (CH_2), 44.34 (CH_2) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{17}H_{20}N_3O_3S$ 346.1220; found 346.1222.

4-{4-[(3-Methoxyphenyl)methyl]piperazine-1-carbonyl}benzene-1-sulfonamide 11c (Method A): from **14a** [19] after purification using DCM/MeOH 90:10 as eluent. White solid, m.p 186°C (d), 47% yields. [^{1}H-NMR] (DMSO) δ: 7.82 (d, *J* = 8.2 Hz, 2H, Ar), 7.52 (d, *J* = 8.2 Hz, 2H, Ar), 7.41 (s,

2H, SO_2NH_2), 7.19 (m, 1H, Ar), 6.87-6.70 (m, 3H, Ar), 3.69 (s, 3H, OCH_3), 3.65-3.55 (m, 2H), 3.43 (s, 2H, CH_2-Ar), 3.30-3.20 (m, 2H+water), 2.45-2.26 (m, 4H) ppm. [^{13}C-NMR] (DMSO) δ: 168.18 (CO), 159.74 (C_{Ar}), 145.22 (C_{Ar}), 139.88 (C_{Ar}), 139.58 (C_{Ar}), 129.73 (CH_{Ar}), 127.95 (CH_{Ar}), 126.31 (CH_{Ar}), 121.52 (CH_{Ar}), 114.81 (CH_{Ar}), 112.90 (CH_{Ar}), 62.19 (CH_2-Ar), 55.41 (OCH_3), 53.13 (CH_2), 52.59 (CH_2), 47.55 (CH_2), 42.07 (CH_2) ppm. ESI-HRMS (*m/z*) [M+H]$^+$: calculated for $C_{19}H_{24}N_3O_4S$ 390.1482 found 390.1480.

4-{4-[(3-Nitrophenyl)methyl]piperazine-1-carbonyl}benzene-1-sulfonamide 11d (Method A): from **14b** [20] after purification using DCM/MeOH/NH_3 85:15:1.5 as eluent. White solid, m.p, 176°C (d), 73% yields. [^{1}H-NMR] (DMSO) δ: 8.15 (s, 1H, Ar), 8.11 (d, *J* = 8.0 Hz, 1H, Ar), 7.85 (d, *J* = 8.4 Hz, 2H, Ar), 7.77 (d, *J* = 7.6 Hz, 1H, Ar), 7.62 (t, *J* = 8.0 Hz, 1H, Ar), 7.56 (d, *J* = 8.4 Hz, 2H, Ar), 7.42 (s, 2H, NH_2), 3.64 (bs, 4H), 3.30-3.20 (bs, 2H+water), 2.50-2.40 (bs, 2H+DMSO), 2.40-2.30 (m, 2H) ppm. [^{13}C-NMR] (DMSO) δ: 168.19 (CO), 148.37 (C_{Ar}), 145.26 (C_{Ar}), 140.95 (C_{Ar}), 139.55 (C_{Ar}), 135.95 (CH_{Ar}), 130.24 (CH_{Ar}), 127.94 (CH_{Ar}), 126.30 (CH_{Ar}), 123.63 (CH_{Ar}), 122.56 (CH_{Ar}), 60.99 (CH_2-Ar), 53.04 (CH_2), 52.49 (CH_2), 47.54 (CH_2), 42.01 (CH_2) ppm. ESI-HRMS (*m/z*) [M+H]$^+$: calculated for $C_{18}H_{21}N_4O_5S$ 405.1227 found 405.1230.

4-{4-[(4-Chlorophenyl)methyl]piperazine-1-carbonyl}benzene-1-sulfonamide 11e (Method A): from **14c** [21] after purification using DCM/MeOH/NH_3 90:10:1 as eluent. White solid, m.p. 253°C (d); 46% yields. [^{1}H NMR] (DMSO) δ: 7.82 (d, *J* = 8.4 Hz, 2H, Ar), 7.52 (d, *J* = 8.4 Hz, 2H, Ar), 7.40 (s, 2H, SO_2NH_2), 7.34 (d, *J* = 8.4 Hz, 2H, Ar), 7.28 (d, *J* = 8.4 Hz, 2H, Ar), 3.65-3.50 (m, 2H), 3.45 (s, 2H, CH_2Ar), 3.30-3.20 (m, 2H+water), 2.42-2.25 (m, 4H) ppm. [^{13}C-NMR] (DMSO) δ: 168.17 (CO), 145.22 (C_{Ar}), 139.55 (C_{Ar}), 137.35 (C_{Ar}), 132.04 (C_{Ar}), 131.12 (CH_{Ar}), 128.67 (CH_{Ar}), 127.95 (CH_{Ar}), 126.30 (CH_{Ar}), 61.30 (CH_2-Ar), 53.07 (CH_2), 52.48 (CH_2), 47.55 (CH_2), 42.02 (CH_2) ppm. ESI-HRMS (*m/z*) [M+H]$^+$: calculated for $C_{18}H_{21}ClN_3O_3S$ 394.0987 found 394.0987.

4-{4-[(4-Nitrophenyl)methyl]piperazine-1-carbonyl}benzene-1-sulfonamide 11f (method A): from **14e** [23] after purification using DCM/MeOH/NH_3 92:8:0.8 as eluent. White solid, m.p. 226 °C (d); 65% yields. [^{1}H NMR] (DMSO) δ: 8.18 (d, *J* = 8.8 Hz, 2H, Ar), 7.85 (d, *J* = 8.4 Hz, 2H, Ar), 7.59 (d, *J* = 8.8 Hz, 2H, Ar), 7.55 (d, *J* = 8.4 Hz, 2H, Ar), 7.42 (s, 2H, SO_2NH_2), 3.66 (bs, 4H), 3.30-3.20 (m, 2H+water), 2.50-2.31 (m, 4H) ppm. [^{13}C-NMR] (DMSO) δ: 168.19 (CO), 147.13 (C_{Ar}), 146.75 (C_{Ar}), 145.24 (C_{Ar}), 139.51 (C_{Ar}), 130.25 (CH_{Ar}), 127.96 (CH_{Ar}), 126.30 (CH_{Ar}), 123.87 (CH_{Ar}), 61.21 (CH_2-Ar), 53.15 (CH_2), 52.59 (CH_2), 47.55 (CH_2), 42.02 (CH_2) ppm. ESI-HRMS (*m/z*) [M+H]$^+$: calculated for $C_{18}H_{21}N_4O_5S$ 405.1227 found 405.1225.

***N*-(1-Benzylpiperidin-4-yl)-4-sulfamoylbenzamide 12a** (Method A): from commercially-available 1-benzylpiperidinyl-4-amine after purification using DCM/MeOH/NH_3 90:10:1 as eluent. White solid, m.p. 220 °C (d); 19% yields. [^{1}H] NMR (DMSO) δ: 8.40 (d, *J* = 7.6 Hz, 1H, NH) 7.92 (d, *J* =

8.4 Hz, 2H, Ar), 7.83 (d, *J* = 8.4 Hz, 2H, Ar), 7.44 (s, 2H, SO_2NH_2), 7.36-7.19 (m, 5H, Ph), 3.80-3.68 (m, 1H), 3.49 (s, 2H, CH_2Ph), 2.77 (d, *J* = 11.3 Hz, 2H), 2.06-1.88 (m, 1H), 1.74 (d, *J* = 11.6 Hz, 2H), 1.65-1.45 (m, 2H) ppm. [^{13}C] NMR (DMSO) δ: 165.12 (CO), 146.62 (C_{Ar}), 138.09 (C_{Ar}), 129.29 (CH-Ar), 128.26 (CH_{Ar}), 128.39 (CH_{Ar}), 127.42 (CH_{Ar}), 125.98 (CH_{Ar}), 62.46 (CH_2), 52.58 (CH_2), 47.52 (CH), 31.76 (CH_2) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{19}H_{24}N_3O_3S$ 374.1533, found 374.1531.

***N*-(1-Benzylpiperidin-4-yl)-N-methyl-4-sulfamoylbenzamide 12b** (Method B): from 1-benzyl-*N*-methylpiperidin-4-amine [25] after purification using DCM/MeOH/NH_3 90:10:1 as eluent. White solid, m.p. 207 °C (d); 38% yields. [^{1}H-NMR] ($CDCl_3$, mixture of conformers) δ: 7.92-7.80 (m, 2H, Ar), 7.50-7.35 (m, 2H, Ar), 7.35-7.15 (m, 5H, Ar), 5.27-5.05 (m, 2H, SO_2NH_2), 4.60-4.43 (m, 0.5 H), 3.58-3.43 (bs, 1H), 3.43-3.37 (bs, 1H), 3.37-3.21 (m, 0.5 H), 3.10-2.90 (m, 2.5 H), 2.90-2.80 (m, 1H), 2.75 (s, 1.5 H), 2.23-2.06 (m, 1H), 1.99-1.62 (m, 4H), 1.62-1.48 (m, 1H) ppm. [^{13}C-NMR] (DMSO, mixture of conformers) δ: 169.72 (CO), 144.92 (C_{Ar}), 140.75 (C_{Ar}), 138.98 (C_{Ar}), 138.71 (C_{Ar}), 129.95 (CH_{Ar}), 128.61 (CH_{Ar}), 127.75 (CH_{Ar}), 127.36 (CH_{Ar}), 127.12 (CH_{Ar}), 126.34 (CH_{Ar}), 62.44 (CH_2), 62.11 (CH_2), 57.01 (CH), 52.90 (CH_2), 52.50 (CH_2), 51.83 (CH), 32.31 (CH_3), 29.57 (CH_2), 28.56 (CH_2), 27.64 (CH_3) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{20}H_{26}N_3O_3S$ 388.1689, found 388.1692.

***N*-(1-Benzylpyrrolidin-3-yl)-4-sulfamoylbenzamide 12c** (Method B): from commercially-available 1-benzylpirrolidin-3-amine after purification using DCM/MeOH/NH_3 90:10:1 as eluent. White solid, m.p. 174-178 °C; 54% yields. [^{1}H-NMR] (MeOD) δ: 7.98-7.86 (m, 4H, Ar), 7.38-7.20 (m, 5H, Ar), 4.60-4.49 (m, 1H), 3.72-3.60 (m, 2H), 2.94- 2.85 (m, 1H), 2.85-2.77 (m, 1H), 2.65-2.50 (m, 2H), 2.41-2.27 (m, 1H), 1.90-1.76 (m, 1H) ppm. [^{13}C NMR] (DMSO) δ: 165.56 (CO), 146.61 (C_{Ar}), 139.41 (C_{Ar}), 137.79 (C_{Ar}), 129.04 (CH_{Ar}), 128.60 (CH_{Ar}), 128.46 (CH_{Ar}), 127.28 (CH_{Ar}), 125.96 (CH_{Ar}), 60.22 (CH_2), 59.86 (CH_2Ph), 52.96 (CH_2), 49.42 (CH_2), 31.12 (CH_2) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{18}H_{22}N_3O_3S$ 360.1376, found 360.1377.

***tert*-Butyl *N*-[1-(4-sulfamoylbenzoyl)piperidin-4-yl]carbamate 15** (Method B): from commercially-available *tert*-butyl piperidin-4-ylcarbamate after purification using AcOEt as eluent. White solid, m.p. 229 °C (d), 65% yields. [^{1}H-NMR] (DMSO) δ: 7.83 (d, *J* = 8.0 Hz, 2H, Ar), 7.49 (d, *J* = 8.0 Hz, 2H, Ar), 7.40 (s, 2H, SO_2NH_2), 6.90-6.84 (m, 1H, NH), 4.32-4.21 (m, 1H), 3.52-3.31 (m, 2H), 3.11-2.98 (m, 1H), 2.98-2.83 (m, 1H), 1.82-1.50 (m, 2H), 1.33 (s, 9H, $C(CH_3)_3$), 1.28-1.25 (m, 2H) ppm. [^{13}C-NMR] (DMSO) δ: 168.17 (CO), 155.29 (CO), 145.19 (C_{Ar}), 139.93 (C_{Ar}), 127.63 (CH_{Ar}), 126.37 (CH_{Ar}), 78.16 (CMe_3), 47.53 (CH), 46.33 (CH_2), 32.66 (CH_2), 31.79 (CH_2), 28.71 (CH_3) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{17}H_{26}N_3O_5S$ 384.1588, found 384.1586.

Benzyl *N*-benzyl-N-[1-(4-sulfamoylbenzoyl)piperidin-4-yl]carbamate 18 (Method B): from **17** [19] after purification using DCM/MeOH/NH_3 90:10:1 as eluent. White solid, m.p. 206-209 °C, 98% yields. [^{1}H-NMR] (DMSO) δ: 7.83 (d, *J* = 8.0 Hz, 2H, Ar), 7.60-7.09 (m, 14 H, Ar + SO_2NH_2), 5.06 (bs, 2H, OCH_2Ph), 4.60-4.40 (m, 3H, NCH_2Ph + 1H), 4.23-4.05 (m, 1H), 3.30-3.20 (m, 1H+water), 3.14-2.98 (m, 1H), 2.82-2.65 (m, 1H), 1.76-1.60 (m, 3H), 1.59-1.47 (m, 1H) ppm. [^{13}C-NMR] (DMSO) δ: 168.10 (CO), 145.07 (C_{Ar}), 140.04 (C_{Ar}), 138.86 (C_{Ar}), 137.25 (C_{Ar}), 130.34 (CH-Ar), 128.73 (CH_{Ar}), 128.24 (CH_{Ar}), 127.92 (CH_{Ar}), 127.60 (CH_{Ar}), 127.13 (CH_{Ar}), 126.95 CH_{Ar}), 126.27 (CH_{Ar}), 66.82 (OCH_2Ph), 54.88 (CH), 46.91 (NCH_2), 41.43 (CH_2) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{27}H_{30}N_3O_5S$ 508.1901, found 508.1903.

4.1.2 General procedure to obtain compounds 14a-e. N-Boc-piperazine and Et_3N (1.2 eq) were dissolved in anhydrous CH_2Cl_2, and the suitable benzyl bromide (1 eq) was added dropwise at room T. After completion of the reaction (TLC), the mixture was partitioned between CH_2Cl_2 and water. Drying and removal of the solvent gave an oily residue used in the following step without further purification: it was dissolved in CH_2Cl_2 (5 mL) and trifluoroacetic acid (10 eq) was added. After 6 hrs stirring at room T, the solvent was removed and the residue was partitioned between ethyl acetate and sat. aqueous Na_2CO_3; drying and removal of the solvent gave a residue which was purified by flash chromatography if necessary.

1-[(3-Methoxy)methyl]piperazine 14a [19]: oil, 98% yields. [^{1}H-NMR] ($CDCl_3$) δ: 7.08 (t, *J* = 8.0 Hz, 1H, Ar), 6.93-6.85 (m, 2H, Ar), 6.66 (d, *J* = 8.0 Hz, 1H, Ar), 3.66 (s, 3H, OCH_3), 3.33 (s, 2H, CH_2Ar), 2.87-2.70 (m, 4H), 2.45-2.20 (m, 4H), 1.90 (bs, 1H, NH) ppm.

1-[(3-Nitrophenyl)methyl]piperazine 14b [20]: purified using DCM/MeOH/NH_3 90:10:1 as eluent. Oil, 96% yields. [^{1}H-NMR] (DMSO) δ: 8.09 (s, 1H, Ar), 8.07 (d, *J* = 8.0 Hz, 1H, Ar), 7.71 (d, *J* = 7.6 Hz, 1H, Ar), 7.57 (t, *J* = 8.0 Hz, 1H, Ar), 3.51 (s, 2H, CH_2Ar), 2.78-2.53 (m, 4H), 2.40-2.17 (m, 4H) ppm.

1-[(4-Chlorophenyl)methyl]piperazine 14c [21]: oil, 75% yields. [^{1}H-NMR] (DMSO) δ: 7.31 (d, *J* = 8.4 Hz, 2H, Ar), 7.25 (d, *J* = 8.4 Hz, 2H, Ar), 3.35 (s, 2H, CH_2Ar), 2.66-2.57 (m, 4H), 2.25-2.15 (m, 4H) ppm.

1-[(4-Fluorophenyl)methyl]piperazine 14d [22]: oil, 70% yields. [^{1}H-NMR] ($CDCl_3$) δ: 7.41-7.25 (m, 2H, Ar), 6.99 (t, *J* = 8.8 Hz, 2H, Ar), 3.49 (s, 2H, CH_2Ar), 3.00-2.88 (m, 4H), 2.50-2.83 (m, 4H), 2.08 (bs, 1H, NH) ppm.

1-[(4-Nitrophenyl)methyl]piperazine 14e [23]: oil, 92% yields. [^{1}H-NMR] (DMSO) δ: 8.16 (d, *J* = 8.8 Hz, 2H), 7.56 (d, *J* = 8.8 Hz, 2H), 3.30-3.20 (m, 2H, $ArCH_2$), 2.73-2.60 (m, 4H), 2.33-2.20 (m, 4H) ppm.

4.1.3 4-{4-[(3-Aminophenyl)methyl]piperazine-1-carbonyl}benzene-1-sulfonamide 11g Compound **11d** (0.075 g, 0.186 mmol) was dissolved in abs EtOH (25 mL); the solution was heated at 75°C, then $SnCl_2 \cdot 2H_2O$ (0.29 g, 1.29 mmol) was added. The mixture was stirred at 80°C for 3 hrs. After cooling, the mixture was concentrated and treated with a saturated solution of Na_2CO_3, producing a white precipitate which was filtered off through celite. The solution was partitioned between H_2O and CH_2Cl_2. Drying (Na_2SO_4) and removal of the solvent gave a residue which was purified by flash chromatography using DCM/MeOH/NH_3 85:15:1.5 as eluent, obtaining the title product (59% yields). White solid, m.p. 204 °C (d). [^{1}H-NMR] (MeOD) δ: 7.94 (d, *J* = 8.4 Hz, 2H, Ar), 7.53 (d, *J* = 8.4 Hz, 2H, Ar), 7.01 (t, *J* = 7.6 Hz, 1H, Ar), 6.68 (s, 1H, Ar), 6.67-6.57 (m, 2H, Ar), 3.80-3.70 (m, 2H), 3.42 (s, 2H, CH_2Ar), 3.40-3.34 (m, 2H), 2.60-2.46 (m, 2H), 2.45-2.34 (m, 2H) ppm. [^{13}C-NMR] (DMSO) δ: 168.15 (CO), 149.03 (C_{Ar}), 145.22 (C_{Ar}), 139.61 (C_{Ar}), 138.73 (C_{Ar}), 129.08 (CH_{Ar}), 127.95 (CH_{Ar}), 126.30 (CH_{Ar}), 116.96 (CH_{Ar}), 114.85 (CH_{Ar}), 113.27 (CH_{Ar}), 62.81 (CH_2-Ar), 53.26 (CH_2), 52.66 (CH_2), 47.60 (CH_2), 42.08 (CH_2) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{18}H_{23}N_4O_3S$ 375.1485, found 375.1486.

4.1.4 4-{4-[(4-Aminophenyl)methyl]piperazine-1-carbonyl}benzene-1-sulfonamide 11h Compound **11f** (0.03 g, 0.0743 mmol) was dissolved in AcOEt (20 mL) and hydrogenated in a Parr apparatus at 30 psi for 4 hrs, using Pd/C (10 mg) as catalyst. Filtration and removal of the solvent under vacuum gave a residue which was purified by flash chromatography using DCM/MeOH/NH_3 85:15:1.5 as eluent, obtaining the title product in 72% yields. White solid, m.p. 180°C (d). [^{1}H-NMR] (MeOD) δ: 7.96 (d, *J* = 7.5 Hz, 2H, Ar), 7.55 (d, *J* = 7.4 Hz, 2H, Ar), 7.05 (d, *J* = 7.4 Hz, 2H, Ar), 6.67 (d, *J* = 7.4 Hz, 2H, Ar), 3.77 (bs, 2H, CH_2), 3.45 (s, 2H, CH_2Ar), 3.39 (bs, 2H, CH_2), 2.55 (bs, 2H, CH_2), 2.41 (bs, 2H, CH_2) ppm. [^{13}C-NMR] (DMSO) δ: 168.14 (CO), 148.18 (C_{Ar}), 145.19 (C_{Ar}), 139.59 (C_{Ar}), 130.36 (CH_{Ar}), 127.94 (CH_{Ar}), 126.29 (CH_{Ar}), 114.09 (CH_{Ar}), 62.15 (CH_2-Ar), 52.88 (CH_2), 52.39 (CH_2), 47.52 (CH_2), 41.99 (CH_2) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{18}H_{23}N_4O_3S$ 375.1485, found 375.1488.

4.1.5 4-[4-(Benzylamino)piperidine-1-carbonyl]benzene-1-sulfonamide 13a Compound **18** (0.030 g, 0.0592 mmol) was suspended in glacial acetic acid (5 mL) and, at 0 °C, treated with HBr (33% solution in acetic acid, 0.32 mL). The mixture was left stirring at room T for 24 hrs, cooled in ice bath, made alkaline (pH 9) with ammonium hydroxide, and extracted with AcOEt. Drying (Na_2SO_4) and removal of the solvent under vacuum gave a residue which was purified by means of flash chromatography (DCM/MeOH/NH_3 90:10:1), obtaining the title compound with 91% yields.

M.p.190°C (d). [^{1}H NMR] ($CDCl_3$) δ: 7.80 (d, *J* = 8.0 Hz, 2H, Ar), 7.35 (d, *J* = 8.0 Hz, 2H, Ar), 7.30-7.16 (m, 5H, Ar), 4.5-4.37 (m, 1H), 3.77 (s, 2H, CH_2 Ph), 3.60-3.42 (m, 1H), 3.05-2.87 (m, 2H), 2.04-1.85 (m, 1H), 1.82-1.70 (m, 1H), 1.50-1.37 (m, 1H), 1.36-1.20 (m, 1H) ppm. [^{13}C] NMR (DMSO) δ: 168.08 (CO), 145.06 (C_{Ar}), 141.48 (C_{Ar}), 140.13 (C_{Ar}), 128.55 (CH_{Ar}), 128.38 (CH_{Ar}), 127.62 (CH_{Ar}), 126.96 (CH_{Ar}), 126.32 (CH_{Ar}), 53.41 (CH), 50.18 (CH_2 Ph), 46.03 (CH_2), 32.58 (CH_2), 31.87 (CH_2) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{19}H_{24}N_3O_3S$ 374.1533, found 374.1531.

4.1.6 4-{4-[Benzyl(methyl)amino]piperidine-1-carbonyl}benzene-1-sulfonamide 13b Compound **13a** (0.030 g, 0.08 mmol), dissolved in CH_3CN, was treated with Et_3N (0.01 mL, 0.08 mmol) and CH_3I (0.01 mL, 0.16 mmol). After stirring at room T for 3 days the solvent was removed under vacuum and the residue purified by means of flash chromatography (DCM/MeOH 91:9). A white solid was obtained in 46% yields, m.p. 164 °C (d). [^{1}H NMR] (MeOD) δ: 7.98 (d, *J* = 8.0 Hz, 2H, Ar), 7.57 (d, *J* = 8.0 Hz, 2H, Ar), 7.33-7.20 (m, 5H, Ar), 4.75-4.66 (m, 1H), 3.75-3.64 (m, 1H), 3.63 (s, 2H, CH_2 Ph), 3.20-3.06 (m, 1H), 2.92-2.71 (m, 2H), 2.22 (s, 3H, NCH_3), 2.10-1.99 (m, 1H), 1,90-1.80 (m, 1H), 1.75-1.50 (m, 2H) ppm. [^{13}C] NMR (MeOD) δ: 169.40 (CO), 144.94 (C_{Ar}), 139.24 (C_{Ar}), 137.33 (C_{Ar}), 129.24 (CH_{Ar}), 128-13 (CH_{Ar}), 127.30 (CH_{Ar}), 127.09 (CH_{Ar}), 126.18 (CH_{Ar}), 60.44 (CH), 57.44 (CH_2 Ph), 41.34 (CH_2), 36.36 (CH_3), 27.28 (CH_2), 26.99 (CH_2) ppm. ESI-HRMS (*m/z*) $[M+H]^+$: calculated for $C_{20}H_{26}N_3O_3S$ 388.1689, found 388.1685.

4.1.7 4-(4-aminopiperidine-1-carbonyl)benzene-1-sulfonamide trifluoroacetate salt 16 Compound **15** (125 mg, 0.33 mmol) was dissolved in trifluoroacetic acid (3 mL) and left strring at room T for 20 hrs. Removal of the solvent under vacuum gave a residue which was crushed with Et_2O. Low melting solid, 82% yields. [^{1}H-NMR] (DMSO) δ: 7.94 (s, 2H, SO_2NH_2), 7.84 (d, *J*=6.8 Hz, 2H, Ar), 7.50 (d, *J*=6.8 Hz, 2H, Ar), 4.42 (bs, 1H), 3.51-3.40 (m, 2H), 3.20-3.00 (m, 1H), 2.95-2.80 (m, 1H), 2.08-1.87 (m, 1H), 1.86-1.77 (m, 1H), 1.60-1.30 (m, 2H) ppm.

4.1.8 Benzyl N-benzyl-N-(piperidin-4-yl)carbamate 17 This compound was prepared following the procedure reported by Lee [19] for a fluorinated analogue. Oil, 94% yields. [^{1}H NMR] ($CDCl_3$) δ: 7.42-7.04 (m, 10H, Ar), 5.25-5.03 (m, 2H, OCH_2), 4.46 (s, 2H, NCH_2), 4.22-4.04 (m, 2H), 3.10-2.98 (m, 2H), 2.70-2.49 (m, 2H), 1.99-1.86 (m, 2H), 1.70-1.50 (m, 4H) ppm.

4.2 CA inhibition studies. An Sx.18Mv-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic activity of various CA isozymes for CO_2 hydration reaction as previously described [17, 26]. Phenol red (at a concentration of 0.2 mM) was used as

indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer and 20 mM Na_2SO_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 (0.1 mM) were prepared in distilled−deionized water, and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 6 hours 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 the Cheng−Prusoff equation, as reported earlier [17], and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier and their concentrations were in the range of 5.7 – 12 nM [31, 32]. Inhibition data are reported as mean (± standard error) of three independent experiments. Significance of differences among the groups was assessed by one-way ANOVA followed by Newman-Keuls post hoc test for multiple comparisons. Calculations were made with Prism 5 statistical software (GraphPad Software, Inc., San Diego, CA, USA). The results were considered to be statistically significant when $p<0.05$.

4.3 Crystallization and X-ray data collection

Crystals were obtained through the hanging drop vapor diffusion method using 24 well Linbro plate. 2 µL of 0.8 mM solution of hCA II in Tris-HCl pH=8.0 were mixed with a solution of 1.5, 1.6 and 1.7 M sodium citrate, 50 mM Tris pH 8.0 and were equilibrated against 500 µL of the same solution at 296 K. Crystals of the protein grew in a few days. hCA II crystals were soaked in 5mM inhibitor solution for 2 days. The crystals were flash-frozen at 100K using a solution obtained by adding 25% (v/v) glycerol to the mother liquor solution as cryoprotectant. Data on crystals of the complexes of hCAII with (*R*)-**9b** and (*R*)-**10b** were collected using synchrotron radiation at the ID-29 beamline at ESRF (Grenoble, France) with a wavelength of 0.827 Å and a Pilatus3 6M Dectris CCD detector. Data on crystals of the complexes of hCAII with **11a** and **11b** were collected using synchrotron radiation at the XRD2 beamline at Elettra (Trieste, Italy) with a wavelength of 1.000 Å and a Pilatus3 6M Dectris CCD detector. Data were integrated and scaled using the program XDS [33].

4.4 Structure determination

The crystal structure of hCA II (PDB accession code: 4FIK) without solvent molecules and other heteroatoms was used to obtain initial phases of the structures using Refmac5 [34]; 5% of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of Rfree calculations. The initial |Fo - Fc| difference electron density maps unambiguously showed the inhibitor molecules. Atomic models for inhibitors were calculated and energy minimized using the program JLigand 1.0.40 [35]. Refinements proceeded using normal protocols of positional, anisotropic atomic displacement parameters alternating with manual building of the models using COOT [36]. Solvent molecules were introduced automatically using the program ARP [37]. The quality of the final models was assessed with COOT and RAMPAGE [38]. Atomic coordinates were deposited in the Protein Data Bank (PDB accession code: 6RG3, 6RG4, 6RHJ, 6RHK). Graphical representations were generated with Chimera [39].

5. Conflict of interest disclosure

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material.

^{1}H and ^{13}C-NMR spectra of **11b**, **11e**, **12b** and **13b**.

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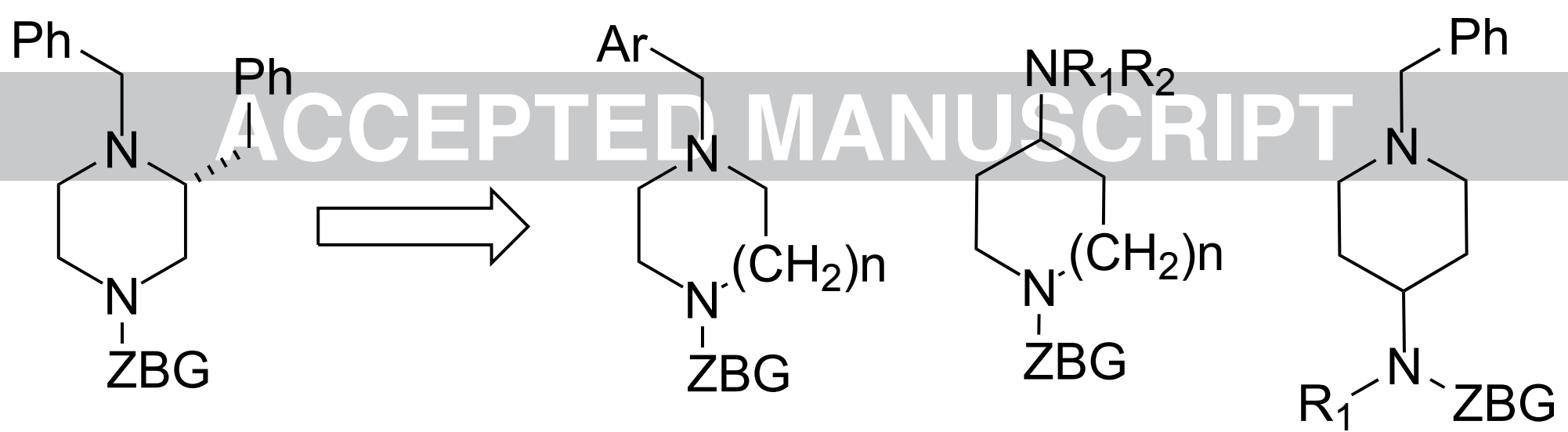

Zn-Binding Group (ZBG) = $COC_6H_4SO_2NH_2$

n = 0,1, 2; Ar = 3 or 4 substituted Ph; R_1, R_2 = H, CH_3, Bn

Highlights

- A series of piperazines and 4-aminopiperidines have been prepared as simplified analogues of a 2-benzylpiperazine lead compound.
- The new compounds have been tested on physiologically relevant hCA isoforms.
- Several compounds are potent inhibitors of hCA I and II.
- Removal of the 2-benzyl group decreased activity and selectivity toward hCA IV.