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Benzenesulfonamides Incorporating Flexible Triazole Moieties are Highly Effective Carbonic Anhydrase Inhibitors: Synthesis, Kinetic, Crystallographic, Computational and Intraocular Pressure Lowering Investigations

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Abstract. Herein we report the synthesis of two series of benzenesulfonamide containing compounds which incorporate the phenyl-1,2,3-triazole moieties. We explored the insertion of appropriate linkers, such as ether, thioether and amino type, into the inner section of the molecules with the intent to confer additional flexibility. All obtained compounds were screened in vitro as inhibitors of the physiologically relevant human (h) isoforms of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). Many of them were low nanomolar/subnanomolar hCA II, IX and XII inhibitors, whereas they did not potently inhibit hCA I. Computational and X-ray crystallographic studies of the enzyme-inhibitor adducts helped us to rationalize the obtained results. Some of the sulfonamides reported here showed significant intraocular pressure lowering activity in an animal model of glaucoma.

Keywords: carbonic anhydrase; inhibitor; sulfonamide; glaucoma; click chemistry

Introduction.

Up to now, different approaches have been pursued with the intent to develop sulfonamide-like carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs)[1-3] which possess better selectivity profiles towards the different human (h) isoforms of the enzyme. Typically, the sulfonamide moiety coordinates the Zn(II) ion present within the hCAs active sites and establishes two additional hydrogen bonds with a residue nearby (Thr199). Such binding features are common among the active site architectures of all the 15 human isozymes,[1] which belong to the α-class (seven distinct genetic families have been identified so far, the α-, β-, γ-, δ-, ζ-. η- and θ-CAs). These CA isoforms differ by many molecular features, including the oligomeric organization (mono- or homo-dimeric), cellular localization, distribution in organs and tissues, expression levels, and kinetic properties/affinities for the main classes of inhibitors.[1,2]

In the last decades one of the research trends on hCAs, has tackled the design of isoform-selective sulfonamide-inhibitors by applying two principal methods: the ring and the tail approaches.[1,4,5] The first consists in modulating the ring (mainly its chemical nature) directly linked to the sulfonamide group, whereas the latter resides in appending different tails to the aromatic/heterocyclic ring bearing the zing-binding-group (ZBG). This allows to modulate the possible interactions the ligand establishes with the middle/outer parts of the active site cavity, which are the most variable among the 15 isoforms mentioned above.[1c]

The many synthetic efforts greatly contributed in the CA field mainly by the introduction of large sets of substitution patterns at the aromatic/heterocyclic ring.[4,5] These allowed to explore compounds possessing a variety of physico-chemical properties as well as to carry-out detailed structure-activity relationship (SAR) studies.[1] It should be stressed that, although the tail approach has been extensively applied to aromatic sulfonamides and led to many novel potent CAIs, only a rather limited number of such derivatives showed relevant isoform-selectivity inhibition profiles.[4d]

The role of the CAs in vertebrates, that is, the catalysis of the hydration reaction of carbon dioxide to bicarbonate and a proton,[1c,2a] is pivotal for a variety of physiological-pathological

processes,[6-12] including respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH andCO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiological and pathological processes in humans.[1-3] Indeed atypical expression levels and/or activities of these enzymes were proved connected with several human diseases, such as glaucoma (hCAs I, II, IVand XII) , oedema (hCA II, IV, XII and XIV), osteoporosis (hCA IV and XIV), obesity (hCAs VA VB), cancer (hCAs IX and XII) and some central-nervous system affecting diseases (hCAs I, II and VII).[1-3]

Primary sulfonamides such as sulfanilamide (**SA**), acetazolamide (**AAZ**), ethoxzolamide (**EZA**) and dichlorphenamide (**DCP**) are clinically used for almost 70 years as antiglaucoma agents for systematic administration (first generation CAIs; figure 1A), although they show a range of side effects due to the lack of enzymatic as well as tissue selectivity. Consecutively they were also approved for the treatment of oedema.[1c,2a,7-10] The second generation antiglaucoma drugs, such as dorzolamide (**DZA**) and brinzolamide (**BRZ**), act topically, thus leading to significant reduction of the side effects (Figure 1B).

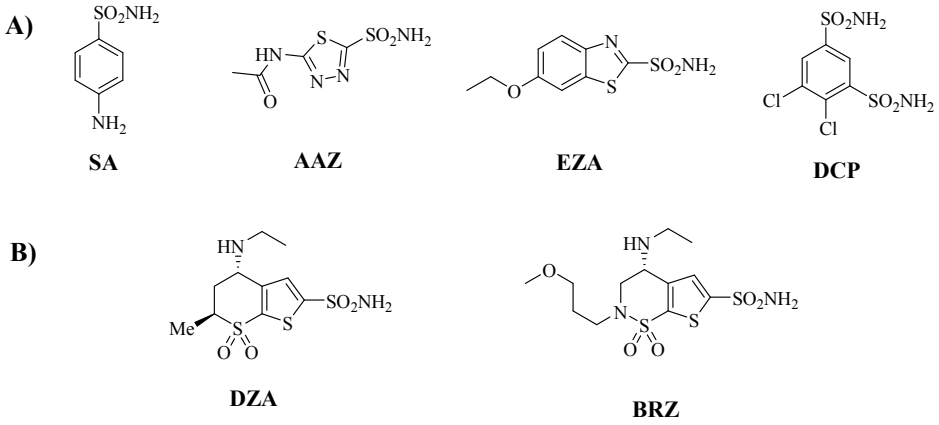


Figure 1: Molecular structures of the first (**A**) and second (**B**) generation antiglaucoma CAI drugs.

Nowadays X-ray crystal structures are available for many CA complexes with inhibitors,[1c,13] including the majority of the twelve catalytically active hCAs. Nevertheless the rationalisation of

these data did not lead to the expected breakthrough in rational drug design of more selective enzyme inhibitors, since too many factors influence the interaction between the rather large active site of the enzyme and the various classes of inhibitors. It should be also stressed that most of the reported co-crystallized inhibitor complexes are with the model isozyme II, displaying a lack of detailed knowledge about the interaction between inhibitors and the remaining human isoforms.

The copper-catalyzed azide−alkyne cycloadditions (CuAAC), better known as "Click Chemistry", have acquired a prominent role in medicinal chemistry,[5,14] thus becoming a versatile and useful synthetic tool to generate 1,4-disubstituted-1,2,3-triazoles to be used as biologically uncleavable linker between valuable molecular fragments and/or as moiety that can actively contribute to additional enzyme-inhibitor interaction points. The 1,2,3-triazole ring is an amide bioisoster endowed with a moderate dipole character, hydrogen bonding capability, rigidity, stability in the *in vivo* environment and an aromatic character that may allow π-stacking interactions with appropriate aminoacid residues within the enzymatic cavity sites.

In the last years, click chemistry has often been used to obtain CAIs belonging to the sulfonamide or coumarin classes. For example, aromatic sulfonamides incorporating glycosyl moieties as well as aromatic-heteroaromatic or aliphatic groups by means of a triazole linker, were obtained and showed to be potent inhibitors against physiologically/pathologically relevant isoforms such as hCA I, II, IX, and XII.[5] Noteworthy, Pala et al. recently reported two series of benzene and tetrafluorobenzene sulfonamides bearing aliphatic or aromatic moieties through the Click Chemistry approach. [5d]

Herein we continue the exploration of this type of derivatives, including a molecular flexibility element in the compounds structure by adopting two different synthetic strategies for the benzene sulfonamide scaffold. The two series of triazole derivatives were investigated for the inhibition of the physiologically dominant, cytosolic isoforms hCA I and II, as well as the transmembrane, tumor-associated ones hCA IX and XII. In addition, we examined the enzyme inhibitory properties of such compounds at the molecular level by reporting the high resolution X-

ray crystal structure for two derivatives bound to hCA II as well as molecular docking studies on hCAII and IX. Finally, some of the new compounds were screened for their antiglaucoma activity in an animal model of the disease.

RESULTS AND DISCUSSION

Compound Design and Synthesis. Herein we report a deeper investigation based on the benzenesulfonamide-containing 1,2,3-triazolyl moieties previously reported from Pala et al.[5d] Compounds of the phenyltriazolylbenzenesulfonamide (**PTB**)-type, shown in figure 2, were considered as analogs of **SLC-0111**,[4d,e] since they incorporate the 1,2,3-triazolyl moiety as bioisoster of the ureido group. Such derivatives were all characterized by the direct connections between three main molecular fragments: the benzenesulfonamide head (**α**), the triazolyl spacer (**β**) and the aromatic tailing portion (**γ**). PTB-based structures possess a rather low conformational freedom which is limited only to the torsion angles between the single portions (Figure 2).

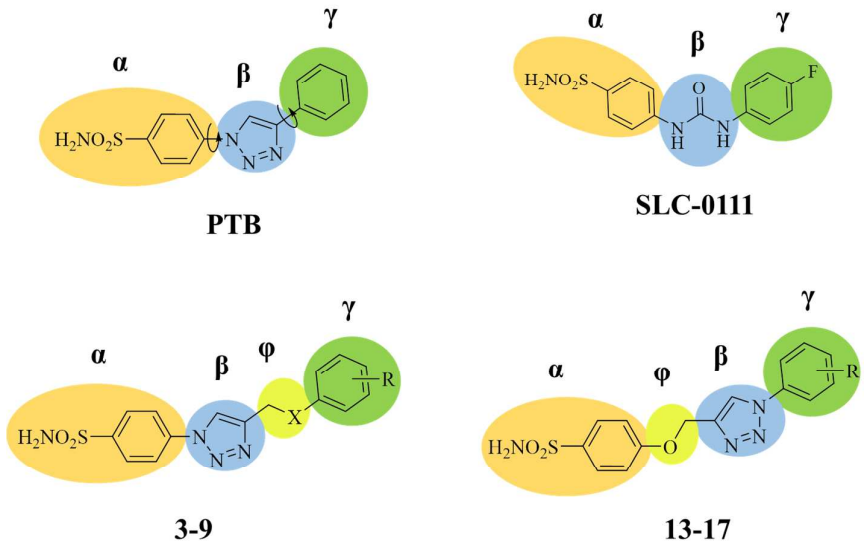
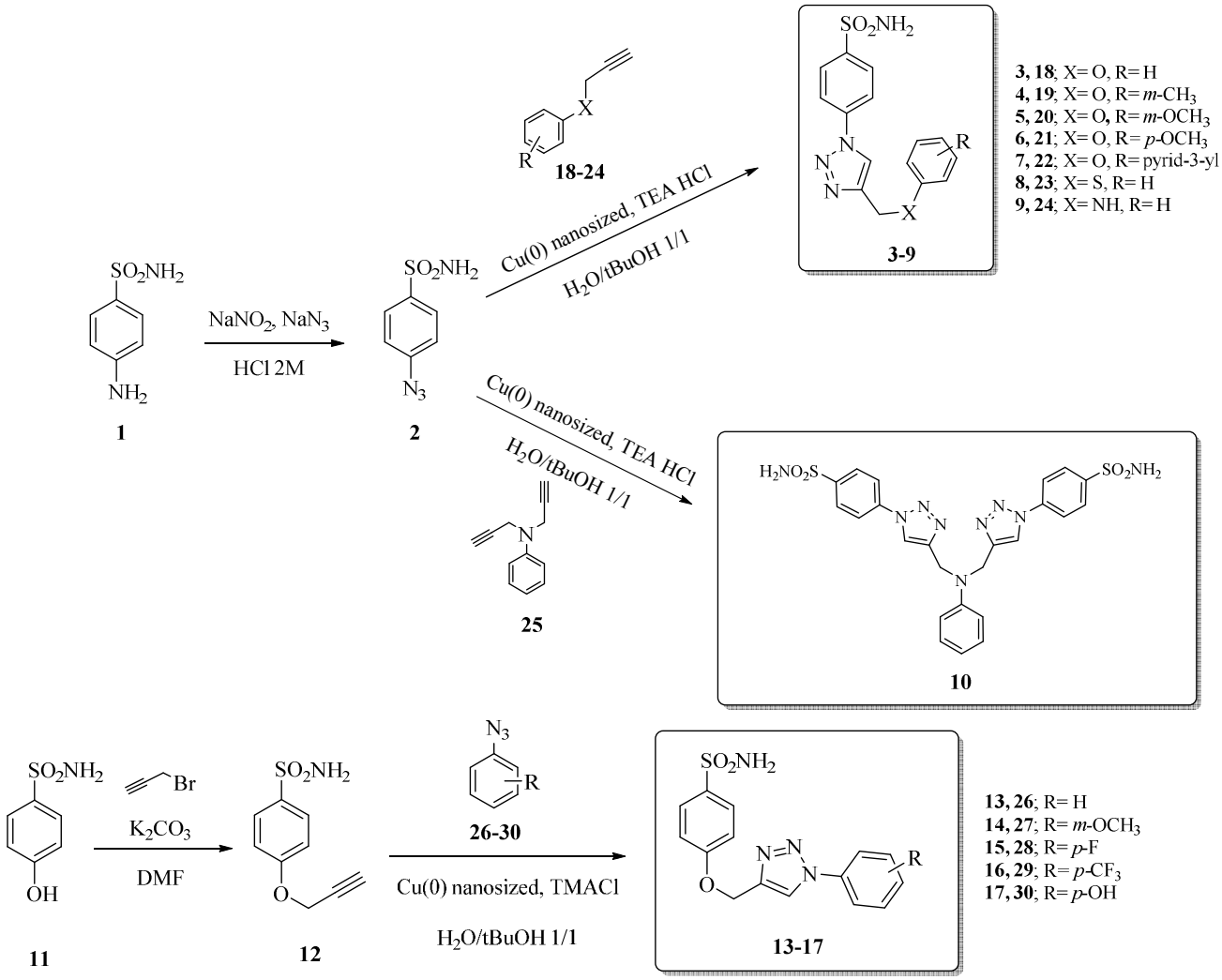


Figure 2. General structures of the designed benzenesulfonamides in the present study.

Hence, considering these facts, the present work was conducted with the aim to investigate whether an enhanced flexibility between these three molecular fragments will produce better interactions within the CAs active sites, and thus will reinforce inhibitory properties, as well as the

selectivity profiles for the different isoforms. The enhanced flexibility will reduce the molecular tension and increase the degree of freedom, thus allowing the aromatic tail to better interact with the most energetically favourable enzymatic sub-pocket(s).[13f]

We proceeded by means of introduction of a CH_2X (X = O, S, NH) linker (**φ**) between **β** and **γ** (derivatives **3-9**) and **α** and **β** (derivatives **13-17**). In comparison to the reference PTB, a larger set of substituents on the aromatic tail has been explored, obtaining two novel series of benzenesulfonamide not investigated as CAIs up to now.



Scheme 1. General synthetic procedure for compounds **3-10, 13-17**.

Two different synthetic strategies were used for obtaining the above-mentioned derivatives, and both involved the application of the Click chemistry approach (Scheme 1). For the first series, the key intermediate 4-azidobenzenesulfonamide **2** was obtained from sulfanilamide treated with $NaNO_2$ and NaN_3 in acid aqueous media. Reaction of **2** with the propargyl amino, thioether or ethers derivatives **18-24** afforded the first series of triazoles, **3-9**, incorporating diverse aryl groups at the triazole-benzenesulfonamide moiety by means of the linker CH_2X. A double propargylation which occurred at aniline NH_2 suggested us to react the dipropargyl derivate **25** with key intermediate **2** in order to obtain the novel double head, twin-tailed sulfonamide **10**.

For the second series, starting from 4-hydroxy-benzenesulfonamide, the propargyl ether key intermediate **12** was obtained as an alkyne by using click chemistry. Reaction of **12** with freshly prepared aromatic azides **26-30** afforded the second series of triazoles, **13-17**, that on the contrary to the preceding one, incorporated diverse aryl-triazole tails appended to the zinc-binding group (ZBG) scaffold through a CH_2O linker.

Carbonic Anhydrase Inhibition. We investigated the CA inhibitory activities of compounds **3-10**, and **13-17** by means of the stopped flow carbon dioxide hydrase assay,[15] in comparison to the lead compound **PTB**[5d] and acetazolamide (**AAZ**) as standard CAI, against four physiologically significant isoforms, the cytosolic, hCA I and II, as well as the transmembrane, tumor-associated hCA IX and XII. The rational for the choice of these four isoforms is that hCA II and XII (upregulated in the eyes of glaucomatous patients)[7b-d, 10b] are targets for antiglaucoma drugs,[1,4,7] whereas hCA IX (and XII) have been validated as targets for the treatment and prognosis of hypoxic cancers.[2b,12] Otherwise hCA I is one of the main off-target isoforms both for the antiglaucoma or anticancer CAIs therapeutic application.[1]

Table 1: Inhibition data of human CA isoforms hCA I, II, IX and XII with sulfonamides **3-10**, **13-17** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO_2 hydrase assay.[15]

			K_I (nM)			
Compound	X	R	hCA I	hCA II	hCA IX	hCA XII
3	O	H	349.9	1.0	2.1	1.1
4	O	*m*-CH_3	406.8	1.5	2.3	5.4
5	O	*m*-OCH_3	351.1	1.5	9.3	1.1
6	O	*p*-OCH_3	512.6	4.3	14.3	1.0
7	O	Pyridyl-3-yl	123.0	1.4	17.2	6.0
8	S	H	195.7	1.5	2.4	4.9
9	NH	H	7.9	0.83	2.2	1.1
10	-	-	>10000	>10000	12.1	10.2
13	-	H	565.6	1.2	2.6	1.1
14	-	*m*-OCH_3	278.1	12.4	20.5	101.6
15	-	*p*-F	949.8	2.6	16.2	3.2
16	-	*p*-CF_3	>10000	15.7	23.6	4.3
17	-	*p*-OH	92.8	1.0	18.7	7.1
PTB			342.0	43.9	25.9	6.0
AAZ			250.2	12.0	25.2	5.7

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10 % of the reported values).

The following SAR can be unveiled from the inhibition data shown in Table 1:

i) The cytosolic isoforms hCA I was moderately inhibited by most of the sulfonamides of the first series with inhibition constants (K_Is) ranging between 123.0 and 512.6 nM and comparable to the lead **PTB** (K_I 342.0 nM) and standard **AAZ** (K_I 250 nM). The only exceptions were derivative **9**, which was an effective hCA I inhibitor (K_I of 7.9 nM), and the double head derivative **10** which resulted inactive (K_I > 10000 nM). We may hypothesize that the presence of hydrophilic moieties in these molecular structures is well tolerated within the hCA I active site. Indeed the substitution of the phenyl with a more hydrophilic pyridyl ring (**7**) or the substitution of the oxymethylene linker with a strongly hydrophilic aminomethylene one (**9**) increased the inhibitory activity up to 123.0 and 7.91 nM respectively.

The sulfonamides belonging to the second series showed a diminished hCA I inhibitory action. Indeed only derivate **17** which incorporates a hydroxy group to the phenyl ring acted as a strong hCA I inhibitor, with K_I of 92.8 nM, whereas the remaining compounds possessed inhibition constants ranging between 278.1 and 949.8 nM, whereas derivative **16** did not inhibit hCA I up to 10000 nM.

ii) The physiologically dominant isoform hCA II was very potently inhibited by all the investigated compounds (K_I values ranging between 0.83 and 15.7 nM, Table 1), with the exception of the double head derivative **10** which did not inhibit hCA II up to 10000 nM. The definition of a proper SAR for hCA II is not feasible, considering that the remaining derivatives showed rather similar affinities. However it could be stressed that the presence of a hydrophilic aminomethylene linker in the first series of derivatives led to the strongest inhibitor reported here (**9**) with its subnanomolar activity of 0.83 nM for this isoform, whereas the presence of a *para* substituent on the phenyl ring, represented herein by the methoxy group of **6**, reduced the inhibitory potency by four times.

Within the second series of derivatives, the incorporation of a methoxy or trifluoromethyl group in *meta* or *para* positions to the sulfamoyl ZBG, respectively, led to a ten fold decrease of the inhibitory activity. Generally all the investigated compounds (except derivative **10**) were better

inhibitors compared to the lead **PTB** or the clinically used **AAZ** (K_I values of 43.9 and 12.1 nM), thus proving that compounds endowed with enhanced flexibility between their molecular fragments act as more effective hCA II inhibitors compared to the rigid compound **PTB**.

iii) All the reported derivatives acted as very efficient inhibitors for the tumor-associated isoform hCA IX. The general inhibitory tendency was slightly less compact compared to the behaviour observed against hCA II, with K_Is ranging between 2.1 and 23.6 nM, but the data in Table 1 allowed us to draw a very clear-cut SAR: for both reported series, the derivates bearing an unsubstituted phenyl ring are the most efficient inhibitors regardless on the nature of the XCH_2 linker. Indeed derivatives **3**, **8**, **9** and **13** were low nanomolar hCA IX inhibitors, with K_Is of 2.1, 2.4, 2.2 and 2.6 nM respectively. Only compound **4** represented the exception to this general rule with a K_I of 2.34 nM, although it has a methyl substituent at position 3 of the aromatic tail. Conversely, for the first series of derivatives, the insertion of a methoxy group on the ring or its substitution with a pyridyl moiety decreased the inhibitory potency to the range of 9.3-17.3 nM. Within the second series f CAIs, the incorporation of *p*-F-, *p* -OH-, *m*-MeO- and *p* -CF_3- moiety on the tail drove to K_Is ranging between 16.2 and 23.6 nM. It shoud be also highlighted the potent inhibitory activity of the double head derivate **10** against this isoform with a K_I of 12.1 nM, in comparison to its ineffective activity on the cytosolic isoforms hCA I and hCA II, making this compound selective for the tumor-associated over the cytosolic isoforms.

All the reported derivatives were more potent inhibitors compared to the lead **PTB** or the clinically used **AAZ** (K_I values of 25.9 and 25.0 nM), especially the unsubstituted compounds **3**, **8** ,**9** and **13** which showed a more than ten times better inhibitory activity, confirming the importance of the flexible features to better interact with the CA IX active site.

iv) The reported derivatives showed a very efficient inhibitory activity also against the other tumor-associated isoform hCA XII (which is also involved in glaucoma). However in this case the outline of a straightforward SAR, as for CA IX, was not observed. Indeed within the first series, compounds **3** and **9**, unsubstituted on the external phenyl ring and compounds **5** and **6**,

incorporating a methoxy group respectively at the *meta* and *para* positions, acted as low nanomolar inhibitors with K_Is ranging between 1.0 and 1.1 nM. On the contrary, derivative **8**, bearing a -CH_2S- linker, showed in this case a five-fold reduction in the inhibitory activity. The insertion of a methyl group at the *meta* position (**4**) or the substitution of the phenyl ring with a pyridyl one (**7**) reduced the activity by five and six times respectively.

Conversely, for the second group of compounds, the unsubstituted derivative **13** acted as the most potent inhibitor with a K_I of 1.1 nM, whereas the insertion of substituents on the phenyl ring generally reduced the inhibitory efficiency. In particular derivative **17**, bearing a *meta* methoxy group showed a K_I increased by a hundred times compared to its unsubstituted counterpart.

Differently from the previously discussed isoforms, not all the new sulfonamides inhibited CA XII better than the lead **PTB** or the clinically used **AAZ** (K_I values of 6.0 and 5.7 nM). These data suggested that the enhancement of the molecular flexibility did not generally increase the interaction with the CA XII active site, but depending on the nature and position of the substituents on the external phenyl ring, a more intricate interaction was probably in act between the enyme and the inhibitor.

Finally it should be stressed the potent inhibitory activity of the double head derivate **10** against this isoform too, with a K_I of 10.2 nM. Although its inhibitory potency was slightly reduced in comparison to lead **PTB** or **AAZ**, this data was surprising and extremely interesting when merged with the K_I of compound **10** against CA I, II and IX. In fact **10** acted as a strong and selective inhibitor of the tumor-associated isoform hCA IX and XII over the cytosolic isoforms hCA I and II.

Molecular modeling. In order to gain a better understanding on the binding modes of the synthesized compounds, docking studies were carried out for the inhibitors **3-9** and **13-17** with hCA II (PDB ID: 4Q6E) and hCA IX (PDB ID: 3IAI)[13a]. In hCA II, all compounds were predicted to orient the aromatic sulfonamide moiety deeply into the catalytic cleft of the active site forming a hydrogen bond with the hydroxyl group of T199 side chain, while the NH^- coordinates the zinc ion

and the phenyl ring is neighboured by several hydrophobic residues (V121, H94 and L198). The 1,2,3-triazole ring, linked to the position 4 of the benzosulfonamide directly (first series: compounds **3-9**), or through an oxymethylene linker (second series: compounds **13-17**), mostly forms edge-to-face π- π interactions with the phenyl group of the F131side chain. The flexible hydrophobic tail of the compounds is oriented towards the lipophilic half of the CA active site, in the region delimited by residues F131, G132, V135, Q136, P202 and L204 (Figure 3).

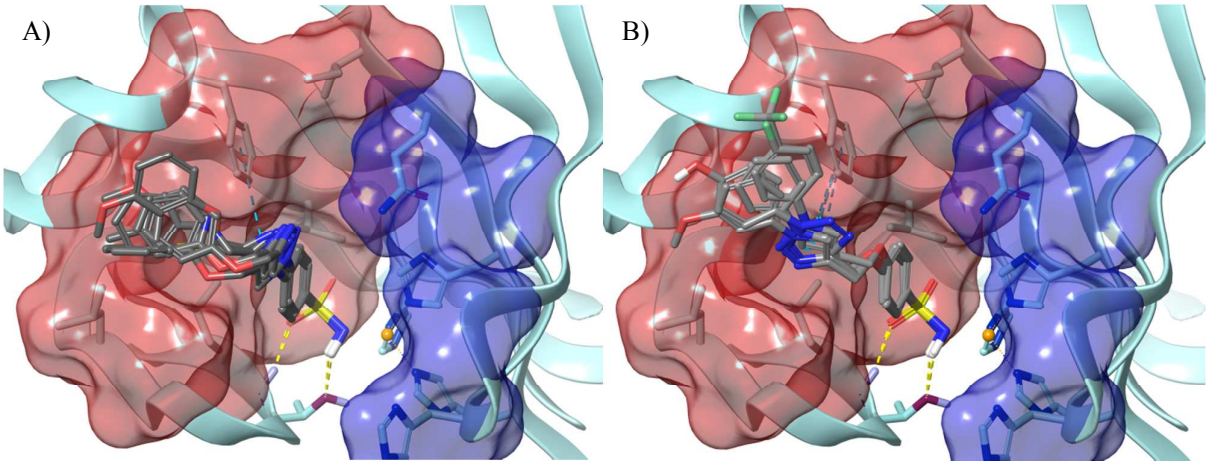


Figure 3. Representation of the binding orientation of compounds A) **3-9** and B) **13-17** in hCA II (PDB code 4Q6E). The hydrophobic half of the active site cleft is shown as a red surface, while the hydrophilic half as a blue one.

An experimental validation of the docking procedure was achieved by the X-ray crystallographic structures of the adducts formed by hCA II with compounds **3** and **13** (at 1.0 Å atomic resolution)**.** Indeed the superposition of the *in silico* and the experimental poses highlight a very good matching (Figure 4).

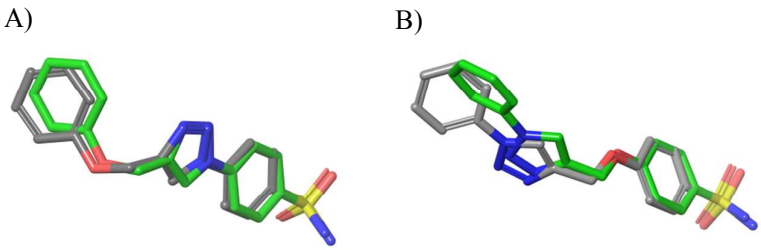


Figure 4. Docking (grey) vs. X-ray (green) orientation in hCAII active site for compounds **3** (A) and **13** (B).

As mentioned above, docking studies were also performed in the hCA IX active site (PDB ID: 3IAI).[13a] Compounds **3-9** oriented themselves in quite similar poses as observed for hCA II. The triazole ring established hydrophobic interactions with V131, whereas the tail aryl groups extended in a partly exposed pocket formed by hydrophobic residues. The different orientation of the aromatic tails could be correlated to the mutation of F131 replaced by V131 in hCA IX active site. Conversely compounds **13-17**, which bear the aryltriazole moieties linked to benzenesulfonamide through an oxymethylene linker, extend their tails towards the hydrophilic region delimited by residues N62, H64, Q67 and Q92, where the triazole forms polar interactions with Q67 and Q92. The different orientation assumed by such moieties within hCA IX active site is likely due to the F131V CAII/IX mutation. In absence of the π- π interaction driving force the aromatic tails were not driven near the hydrophobic side anymore, but instead found good interaction points on the hydrophilic region (Figure 5B).

The different set of interactions established by the aromatic tails of the two series of compounds within hCA II and hCA IX active sites could explain the general reduced inhibitory activity against the tumor-associated isoforms compared to the cytosolic one.

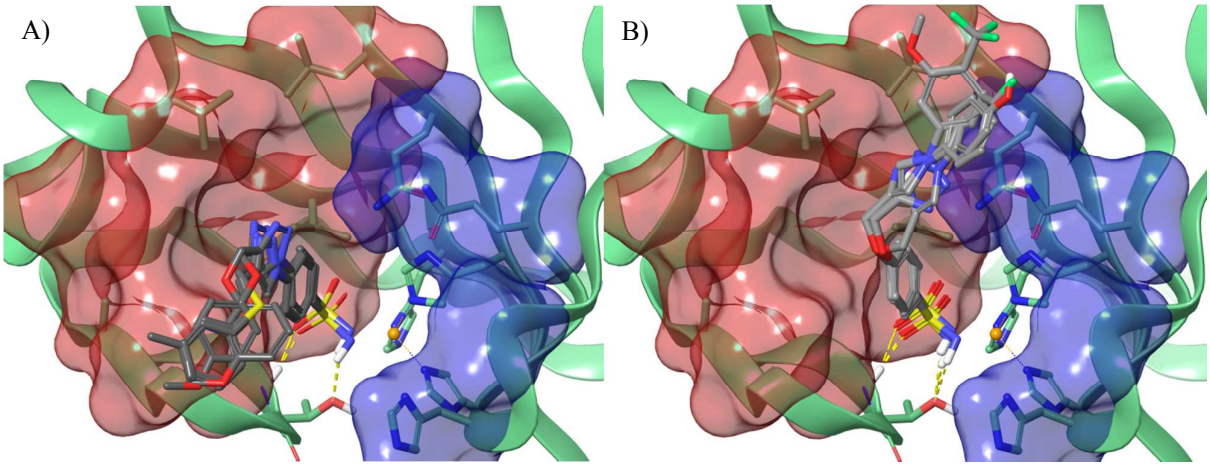


Figure 5. Representation of the binding orientation of compounds A) **3-8** and B) **13-17** in hCAIX (PDB code 3IAI). The hydrophobic half of the active site cleft is shown as a red surface, while the hydrophilic half as a blue one.

X-ray Crystallography. The Fo -Fc electron density maps (Figure 6) show a well defined density for all the atoms of the inhibitors **3** and **13** bound within the hCA II active site, which are coordinated to the Zn(II) ion by means of the sulfonamide moiety. The sulfonamide nitrogen coordinated to the zinc ion also makes a strong H-bond with the OH of Thr199. These interactions are common to all CA - sulfonamide adducts reported to date.[1,13] The scaffold of the inhibitors occupied the entire available space inside the cavity and were oriented toward the hydrophobic part of the active site cleft, establishing strong van der Waals interactions. In particular the phenyl ring attached to the sulfonamide group made hydrophobic interactions with residues V121 and L198. The 1,2,3-triazole ring forms hydrogen bonds with water molecules and in the inhibitor **13** an edge-to-face π- π stacking interaction with the phenyl group of the F131 side chain. Furthermore the terminal phenyl ring of inhibitors **3** and **13** forms strong hydrophobic interactions with V135 and F131.

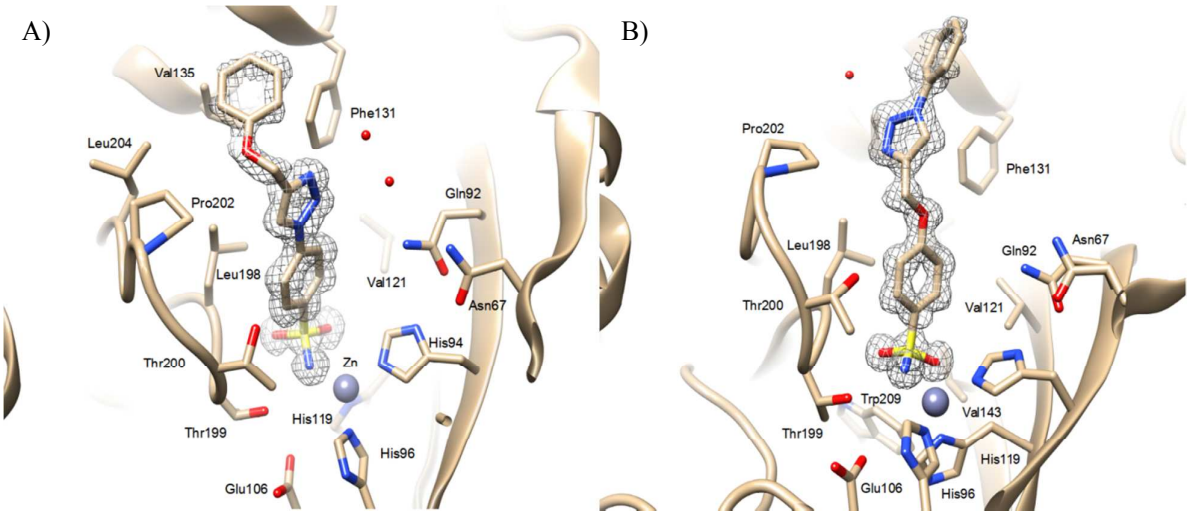


Figure 6. Active site region in the hCA II-**3** (A) and **13** (B) complexes. The omit Fo-Fc electron density maps (contoured at 2.0 σ) for the two inhibitors are also shown.

Table 2.

Summary of Data Collection and Atomic Model Refinement Statistics.[a]

	HCAII + 3	**HCAII + 13**
PDB ID	5LJQ	5LJT
Wavelength (Å)	0.976	0.976
Space Group	$P2_1$	$P2_1$
Unit cell (a,b,c,β) (Å, °)	42.37, 41.44, 72.22, 104.45	42.36, 41.40, 72.17 104.41
Limiting resolution (Å)	40.0-1.05 (1.11-1.05)	41.0-1.00 (1.06-1.00)
Unique reflections	109788	121936
Rsym (%)	6.1 (57.9)	6.0 (67.2)
Redundancy	4.75 (3.77)	3.5 (2.6)
Completeness overall (%)	97.1 (86.1)	93.7 (75.6)
<I/(I)>	12.61 (2.07)	10.22 (1.30)
Refinement statistics		
Resolution range (Å)	30.0-1.05	30.0- 1.00
Unique reflections, working/free	104237/5466	115805/6012
Rfactor (%)	13.35	13.82
Rfree(%)	14.85	15.72
No. of protein atoms	4176	4328
No. of water molecules	418	439
No. of heterogen atoms	52	66
r.m.s.d. bonds(Å)	0.0046	0.0047
r.m.s.d. angles (°)	1.176	1.189
Average B factor (Å^2)		
All atoms	12.48	11.84
inhibitor	14.33	10.81
solvent	26.55	26.07

[a]Values in parentheses are for the highest resolution shell.

IOP Lowering Activity. We have investigated the intraocular pressure (IOP) lowering properties of some of the herein reported derivatives, more precisely **3**, **13** (for which the X-ray structures of the adducts with hCA II was reported) and **9** (which showed a unique subnanomolar inhibitory activity against hCA II) in an animal model of glaucoma.[16] The three compounds showed a sufficient water solubility to be formulated as 1% eye drops at the neutral pH value (dorzolamide, **DRZ**, the clinically used drug is a hydrochloride salt with a pH of the eye drops of 5.5 which produces eye irritation and stinging as side effects).[7b-d, 10b] The drugs were administered to rabbits with high IOP, induced by the injection of 0.1 mL of hypertonic saline solution (5% in distilled water) into the vitreous of both eyes (Figure 6). **DZA** hydrochloride was used as a standard drug, whereas the control experiments were done by using the vehicle (hydroxypropylcellulose at 0.05%). The selected derivatives were very potent inhibitors of isoforms hCA II (responsible for aqueous humor secretion; K_I of 0.83-1.2 nM, Table 1) and hCA XII (isoform that is overexpressed in the eyes of glaucomatous patients;[17] K_I of 1.1 nM, Table 1).

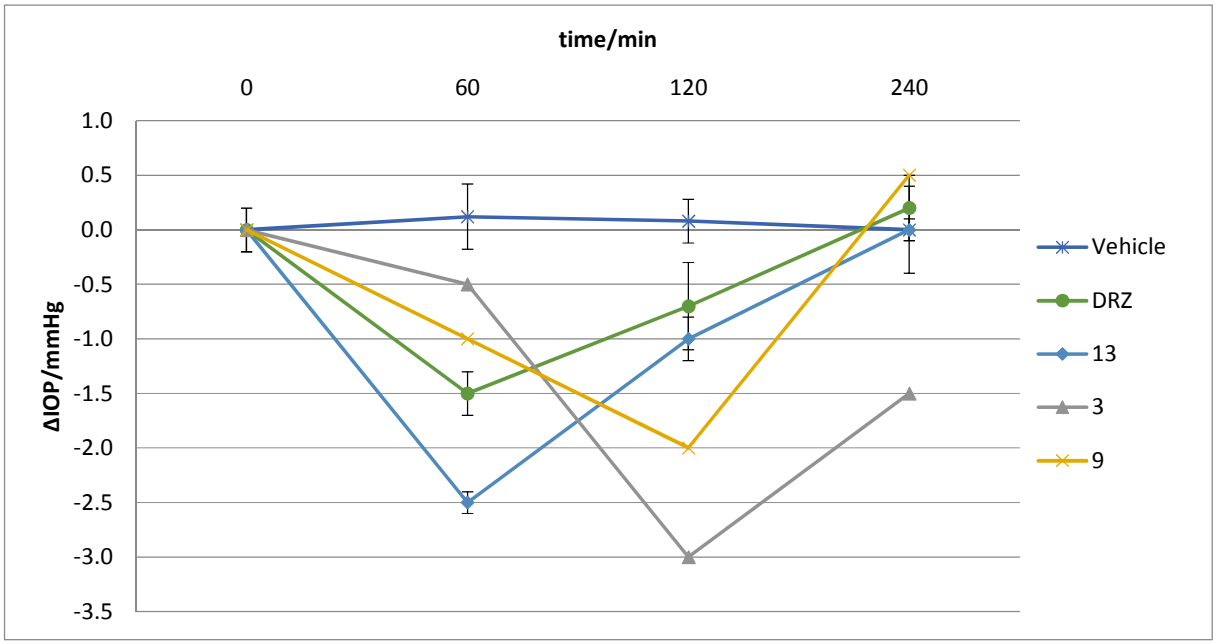


Figure 7. Drop of intraocular pressure (ΔIOP, mmHg) versus time (min) in hypertonic saline-induced ocular hypertension in rabbits, treated with 50 μL of 1 % solution of compounds **3**, **9** and **13** and **DRZ** as the standard drug and vehicle. Errors were within 10−15% of the reported IOP

values (from three different measurements for each of the four animals in the study group) and were statistically significant ($p = 0.045$ by the Student's t test).

As seen from the data of Figure 7, all three compounds proved to be more effective than **DRZ**, even if at different times post-administration. Indeed, a comparable behaviour to **DRZ**, which caused an IOP drop of 1.5 mm Hg at 60 min post-administration, emerged only for derivative **13**, that effected an IOP drop increased to 2.5 mm Hg after the same time. Another analogy between **13** and **DRZ** was the IOP rise in short-term, so that after 4 h no IOP decrease was seen. Conversely for derivatives **3** and **9**, the peak of IOP drop, that reached 3.0 and 2.0 mm Hg respectively, was at 2 h post-administration, whereas their IOP lowering activity after 1h was reduced in comparison to DRZ. In addition, while for compound **9** after 4 h no IOP decrease was seen, **3** showed the ability to protract its IOP lowering action up to the same time point.

It should be mentioned that the animal model employed here is of normotensive rabbits, and this is why the absolute IOP drops are not very high, but the advantage of this model is that the measurements can be done rapidly and are highly reproducible.

Conclusions. We report two new series of benzenesulfonamides incorporating phenyl-1,2,3-triazole moieties, which have been prepared by means of click chemistry. The sulfonamides, designed in order to be endowed with an enhanced flexibility between their molecular fragments in comparison to their lead **PTB**, has been screened for the inhibition of four physiologically relevant CA isoforms: hCA I, II, IX, and XII. Hence, interesting SAR have been extrapolated and afterwards the X-ray crystal structures of two of them bound to hCA II shed light on their inhibitory behaviour at the molecular level. The crystallographic results supported a deeper molecular modeling analysis concerning all the reported derivatives and both hCA II and hCA IX. Three of these compounds also showed highly effective *in vivo* antiglaucoma activity in an animal model of the disease being more effective compared to the clinically used drug dorzolamide, even if at different time post-

administration. The attempt to functionalize the undesired dipropargylaniline, obtained during the synthetic pathway, with benzenesulfonamide moieties has been rewarded with the most surprising and excellent inhibitory profile, that belongs to compound **10.** Indeed this double head sulfonamide act as the unique totally selective inhibitors within the two series. Preliminary hypothesis about such a selectivity have to be underpinned with crystallographic studies (currently ongoing), but it is clear that this innovative substitution pattern for the benzensulfonamide-like inhibitors is worth to be deeper investigated with additional similar derivatives.

Experimental protocols

Chemistry. Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Alfa Aesar and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) spectra were recorded using a Bruker Advance III 400 MHz spectrometer in DMSO-d_6. Chemical shifts are reported in parts per million (ppm) and the coupling constants (*J*) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; sept, septet; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; dd, double of doubles, appt, aparent triplet, appq, aparent quartet. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck Silica gel 60 (230-400 mesh ASTM) as the stationary phase and ethyl acetate/*n*-hexane were used as eluents. Melting points (mp) were measured in open capillary tubes with a Gallenkamp MPD350.BM3.5 apparatus and are uncorrected. HPLC was performed by using a Waters 2690 separation module coupled with a photodiode array detector (PDA Waters 996) and as column a Nova-Pak C18 4 μm 3.9 mm × 150 mm (Waters), silica-based reverse phase column. Sample was dissolved in acetonitrile 10%, and an injection volume of 45 μL was used. The mobile

phase, at a flow rate of 1 mL/min, was a gradient of water + trifluoroacetic acid (TFA) 0.1% (A) and acetonitrile + TFA 0.1% (B), with steps as follows: (A%:B%), 0−10 min 90:10, 10−25 min gradient to 60:40, 26:28 min isocratic 20:80, 29−35 min isocratic 90:10. TFA 0.1% in water as well in acetonitrile was used as counterion. All compounds reported here were >96% HPLC pure. The solvents used in MS measures were acetone, acetonitrile (Chromasolv grade), purchased from Sigma-Aldrich (Milan - Italy), and mQ water 18 MΩ, obtained from Millipore's Simplicity system (Milan-Italy). The mass spectra were obtained using a Varian 1200L triple quadrupole system (Palo Alto, CA, USA) equipped by Electrospray Source (ESI) operating in both positive and negative ions. Stock solutions of analytes were prepared in acetone at 1.0 mg mL^{-1} and stored at 4°C. Working solutions of each analyte were freshly prepared by diluting stock solutions in a mixture of mQ H_2O/ACN 1/1 (*v/v*) up to a concentration of 1.0 μg mL^{-1} The mass spectra of each analyte were acquired by introducing, via syringe pump at 10 μL min^{-1}, of the its working solution. Raw-data were collected an processed by Varian Workstation Vers. 6.8 software.

General synthetic procedure of compounds 3-10, 13-17.[5d]

The appropriate alkyne **12, 18-24** (1.0 eq) was added to a suspension of aryl azide **2, 26-30** (1.1 eq) in H_2O/t-BuOH 1/1 (4 ml) at r.t., followed by copper (0) nanosized (0.1 eq) and TEA HCl or TMACl (1.0 eq). The suspension was stirred at r.t. or 60°C until starting materials were consumed (TLC monitoring), then quenched with H_2O (20 ml) and the formed precipitate was filtered-off and washed with H_2O. The residue was purified by silica gel column chromatography eluting with the appropriate mixture of ethyl acetate in *n*-hexane, or alternatively was dissolved in a minimal amount of acetone. Thus the obtained solution was filtered through Celite 521® and concentrated under *vacuo* to give a residue that was triturated with Et_2O to afford the titled compounds **3-10, 13-17.**

4-(4-Phenoxymethyl-[1,2,3]triazol-1-yl)-benzenesulfonamide **(3).**

Compound **3** was obtained according the general procedure earlier reported using **18** (1.0 eq), **2** (0.12 g, 1.1 eq) in t-BuOH/H_2O 1/1 (4 ml), TEA HCl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred for 27h at r.t. to give the titled compound **3** as a light yellow solid.

72% yield; m.p. 200-202°C; silica gel TLC R_f 0.22 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 5.29 (s, 2H, CH_2), 7.01 (t, J = 7.2, 1H), 7.12 (d, J = 7.6, 2H), 7.37 (t, J = 7.6, 2H), 7.57 (s, 2H, exchange with D_2O, SO_2NH_2), 8.07 (d, J = 8.8, 2H), 8.19 (d, J = 8.8, 2H), 9.10 (s, 1H); δ_C (100 MHz, DMSO-d_6): 61.7, 115.6, 121.3, 121.9, 124.0, 128.4, 130.5, 139.5, 144.8, 145.3, 158.9; *m/z* (ESI positive) 331.0 $[M+H]^+$

4-(4-m-Tolyloxymethyl-[1,2,3]triazol-1-yl)-benzenesulfonamide **(4).**

Compound **4** was obtained according the general procedure earlier reported using **19** (1.0 eq), **2** (0.12 g, 1.1 eq) in t-BuOH /H_2O 1/1 (4 ml), TEA HCl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred for 24h at r.t. to give the titled compound **4** as a yellow solid.

68% yield; m.p. 176-178°C; silica gel TLC R_f 0.13 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 2.33 (s, 3H, CH_3), 5.27 (s, 2H, CH_2), 6.83 (d, J = 7.6, 1H), 6.91 (d, J = 7.6, 1H), 6.94 (s, 1H), 7.23 (t, J = 7.6, 1H), 7.57 (s, 2H, exchange with D_2O, SO_2NH_2), 8.07 (d, J = 8.8, 2H), 8.19 (d, J = 8.8, 2H), 9.09 (s, 1H); δ_C (100 MHz, DMSO-d_6): 22.1, 61.8, 112.6, 116.3, 121.3, 122.7, 124.0, 128.5, 130.3, 139.5, 140.0, 144.8, 145.4, 158.9; *m/z* (ESI positive) 345.0 $[M+H]^+$

4-[4-(3-Methoxy-phenoxymethyl)-[1,2,3]triazol-1-yl]-benzenesulfonamide **(5).**

Compound **5** was obtained according the general procedure earlier reported using **20** (1.0 eq), **2** (0.12 g, 1.1 eq) in t-BuOH/H_2O 1/1 (4 ml), TEA HCl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred for 17h at r.t. to give the titled compound **5** as a yellow solid.

70% yield; m.p. 159-161°C; silica gel TLC R_f 0.14 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 3.78 (s, 3H, CH_3), 5.28 (s, 2H, CH_2), 6.60 (dd, J = 2.0, 8.0, 1H), 6.68 (s, 1H), 6.70 (dd,

J = 2.0, 8.0, 1H), 7.25 (t, *J* = 8.0, 1H), 7.59 (s, 2H, exchange with D_2O, SO_2NH_2), 8.07 (d, *J* = 8.8, 2H), 8.19 (d, *J* = 8.8, 2H), 9.11 (s, 1H); δ_C (100 MHz, DMSO-d_6): 56.1, 61.9, 102.0, 107.7, 107.8, 121.3, 124.0, 128.5, 131.0, 139.5, 144.9, 145.2, 160.1, 161.5; *m/z* (ESI positive) 361.0 $[M+H]^+$

4-[4-(4-Methoxy-phenoxymethyl)-[1,2,3]triazol-1-yl]-benzenesulfonamide **(6).**

Compound **6** was obtained according the general procedure earlier reported using **21** (1.0 eq), **2** (0.12 g, 1.1 eq) in t-BuOH/H_2O 1/1 (4 ml), TEA HCl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred for 24h at r.t. to give the titled compound **6** as a yellow solid.

68% yield; m.p. 209-210°C; silica gel TLC R_f 0.18 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 3.74 (s, 3H,CH_3), 5.22 (s, 2H,CH_2), 6.92 (d, *J* = 8.8, 2H), 7.05 (d, *J* = 8.8, 2H), 7.58 (s, 2H, exchange with D_2O, SO_2NH_2), 8.06 (d, *J* = 8.0, 2H), 8.19 (d, *J* = 8.0, 2H), 9.09 (s, 1H); δ_C (100 MHz, DMSO-d_6): 56.3, 62.4, 115.6, 116.7, 121.3, 123.9, 128.5, 139.5, 144.8, 145.5, 152.9, 154.6; *m/z* (ESI positive) 361.0 $[M+H]^+$

4-(4-((Pyridin-3-yloxy)methyl)-1H-1,2,3-triazol-1-yl)benzenesulfonamide **(7).**

Compound **7** was obtained according the general procedure earlier reported using **22** (1.0 eq), **2** (0.12 g, 1.1 eq) in t-BuOH/H_2O 1/1 (4 ml), TEA HCl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred for 48h at r.t. to give the titled compound **7** as a yellow solid.

51% yield; m.p. 277-280d; silica gel TLC R_f 0.09 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 5.29 (s, 2H, CH_2), 6.31 (t, *J* = 6.4, 1H), 6.45 (d, *J* = 9.4, 1H), 7.48 (t, *J* = 9.4, 1H), 7.55 (s, 2H, exchange with D_2O, SO_2NH_2), 7.90 (d, *J* = 6.4, 1H), 8.04 (d, *J* = 8.8, 2H), 8.17 (d, *J* = 8.8, 2H), 8.89 (s, 1H); δ_C (100 MHz, DMSO-d_6): 44.1, 106.5, 120.6, 121.2, 123.2, 128.4, 139.4, 140.1, 141.2, 144.8, 152.8, 162.1; *m/z* (ESI negative) 330.0 $[M-H]^-$

4-(4-Phenylsulfanylmethyl-[1,2,3]triazol-1-yl)-benzenesulfonamide **(8).**

Compound **8** was obtained according the general procedure earlier reported using **23** (1.0 eq), **2** (0.12 g, 1.1 eq) in t-BuOH/H_2O 1/1 (4 ml), TEA HCl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred for 24h at r.t. to give the titled compound **8** as a yellow solid.

63% yield; m.p. 202-204°C; silica gel TLC R_f 0.62 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 4.43 (s, 2H, CH_2), 7.25 (t, J = 7.2, 1H), 7.37 (t, J = 7.6, 2H), 7.45 (d, J = 7.6, 2H), 7.53 (s, 2H, s, 2H, exchange with D_2O, SO_2NH_2), 8.04 (d, J = 8.4, 2H), 8.12 (d, J = 8.4, 2H), 8.82 (s, 1H); δ_C (100 MHz, DMSO-d_6): 28.1, 121.1, 122.6, 127.0, 128.4, 129.8, 130.0, 136.4, 139.4, 144.7, 146.2; *m/z* (ESI positive) 347.0 $[M+H]^+$

4-(4-Phenylaminomethyl-[1,2,3]triazol-1-yl)-benzenesulfonamide **(9).**

Compound **9** was obtained according the general procedure earlier reported using **24** (1.0 eq), **2** (0.12 g, 1.1 eq) in t-BuOH/H_2O 1/1 (4 ml), TEA HCl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred for 20h at r.t. to give the titled compound **9** as a yellow solid.

78% yield; m.p. 221-223°C; silica gel TLC R_f 0.57 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 4.42 (d, J = 5.6, 2H, CH_2), 6.20 (t, J = 5.6, 1H, exchange with D_2O, N*H*) ,6.59 (t, J = 7.2, 1H), 6.71 (d, J = 8.0, 2H), 7.12 (t, J = 8.0, 2H), 7.57 (s, 2H, exchange with D_2O, SO_2NH_2), 8.04 (d, J = 8.4, 2H), 8.16 (d, J = 8.4, 2H), 8.85 (s, 1H); δ_C (100 MHz, DMSO-d_6): 39.5, 113.3, 117.1, 121.0, 122.2, 128.5, 129.8, 139.6, 144.6, 148.3, 149.2; *m/z* (ESI positive) 330.0 $[M+H]^+$

N,N-Di-(1-(4-sulfamoylphenyl)-[1,2,3]triazol-4-yl-methyl)-aniline **(10).**

Compound **10** was obtained according the general procedure earlier reported using **25** (1.0 eq), **2** (0.12 g, 2.2 eq) in t-BuOH/H_2O 1/1 (4 ml), TEA HCl (2.0 eq) and copper nanosize (0.2 eq). The reaction mixture was stirred for 72h at r.t. to give the titled compound **10** as a yellow solid.

65% yield; m.p. 253-254°C; silica gel TLC R_f 0.14 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 4.83 (s, 4H, 2 x CH_2) ,6.59 (t, J = 7.6, 1H), 6.96 (d, J = 7.8, 2H), 7.16 (t, J = 7.8, 2H), 7.52 (s, 4H, exchange with D_2O, 2 x SO_2NH_2), 8.05 (d, J = 8.4, 4H), 8.17 (d, J = 8.4, 4H), 8.94 (s,

2H); δ_C (100 MHz, DMSO-d_6): 46.6, 113.9, 117.8, 121.1, 122.5, 128.4, 130.0, 139.6, 144.7, 147.2, 148.6; *m/z* (ESI negative) 564.0 $[M-H]^-$

4-(1-Phenyl-1H-[1,2,3]triazol-4-ylmethoxy)-benzenesulfonamide **(13).**

Compound **13** was obtained according the general procedure earlier reported using **12** (0.05g, 1.0 eq), **26** (1.1 eq) in t-BuOH/H_2O 1/1 (3.5 ml), TMACl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred at 60°C for 2.5h and the obtained residue was purified by silica gel column chromatography eluting with 50% ethyl acetate in *n*-hexane to afford **13** as a white solid.

70% yield; m.p. 200-202°C; silica gel TLC R_f 0.24 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 5.38 (s, 2H, CH_2), 7.26 (s, 2H, exchange with D_2O, SO_2NH_2) 7.28 (m, 2H, overlap with signal at 7.26), 7.55 (t, *J* = 7.6, 1H), 7.65 (t, *J* = 7.6, 2H), 7.81 (d, *J* = 8.4, 2H), 7.95 (d, *J* = 7.6, 2H), 9.03 (s, 1H); δ_C (100 MHz, DMSO-d_6): 62.3, 115.7, 121.1, 124.0, 128.6, 129.8, 130.9, 137.5, 137.6, 144.3, 161.2; *m/z* (ESI positive) 331.0 $[M+H]^+$

4-[1-(3-Methoxy-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-benzenesulfonamide **(14).**

Compound **14** was obtained according the general procedure earlier reported using **12** (0.05g, 1.0 eq), **27** (1.1 eq) in t-BuOH/H_2O 1/1 (3.5 ml), TMACl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred at 60°C for 3h and the obtained residue was purified by silica gel column chromatography eluting with 50% ethyl acetate in *n*-hexane to afford **14** as a white solid.

72% yield; m.p. 184-186°C; silica gel TLC R_f 0.21 (EtOAc/n-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 3.90 (s, 3H, CH_3), 5.37 (s, 2H, CH_2), 7.11 (d, *J* = 6.8, 1H), 7.27 (s, 2H, exchange with D_2O, SO_2NH_2), 7.28 (m, 2H, overlap with signal at 7.27), 7.54 (m, 3H), 7.82 (d, *J* = 8.8, 2H), 9.05 (s, 1H); δ_C (100 MHz, DMSO-d_6): 56.6, 62.3, 106.7, 113.1, 115.5, 115.7, 124.1, 128.6, 131.8, 137.6, 138.5, 144.2, 161.1, 161.2; *m/z* (ESI positive) 361.0 $[M+H]^+$

4-[1-(4-Fluoro-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-benzenesulfonamide **(15).**

Compound **15** was obtained according the general procedure earlier reported using **12** (0.05g, 1.0 eq), **28** (1.1 eq) in t-BuOH/H_2O 1/1 (3.5 ml), TMACl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred at 60°C for 2h and the obtained residue was purified by silica gel column chromatography eluting with 50% ethyl acetate in *n*-hexane to afford **15** as a white solid.

70% yield; m.p. 211-213°C; silica gel TLC R_f 0.29 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 5.37 (s, 2H, CH_2), 7.27 (s, 2H, exchange with D_2O, SO_2NH_2), 7.28 (m, 2H, overlap with signal at 7.27), 7.51 (at, J = 8.4, 2H), 7.81 (d, J = 8.4, 2H), 8.00 (m, 2H), 9.00 (s, 1H); δ_F (376 MHz, DMSO-d_6): -112.97 (s, 1F); δ_C (100 MHz, DMSO-d_6): 62.2, 115.8, 117.8 (d, J^2_{CF} = 23.1), 123.6 (d, J^3_{CF} = 8.8), 124.4, 128.7, 134.1, 137.6, 144.3, 161.2, 162.7 (d, J^1_{CF} = 244.5); *m/z* (ESI positive) 349.0 $[M+H]^+$

4-[1-(4-Trifluoromethyl-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-benzenesulfonamide **(16).**

Compound **16** was obtained according the general procedure earlier reported using **12** (0.05g, 1.0 eq), **29** (1.1 eq) in t-BuOH/H_2O 1/1 (3.5 ml), TMACl (1.0 eq) and copper nanosize (0.1 eq).The reaction mixture was stirred at 60°C for 1.5h and the obtained residue was purified by silica gel column chromatography eluting with 50% ethyl acetate in *n*-hexane to afford **16** as a white solid.

69% yield; m.p. 198-199 °C; silica gel TLC R_f 0.16 (EtOAc/n-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 5.36(s, 2H, CH_2), 7.23 (s, 2H, exchange with D_2O, SO_2NH_2), 7.24 (m, 2H, overlap with signal at 7.23), 7.78 (d, J = 8.8, 2H), 8.00 (d, J = 8.6, 2H), 8.18 (d, J = 8.6, 2H), 9.14 (s, 1H); δ_F (376 MHz, DMSO-d_6): -61.03 (s, 3F); δ_C (100 MHz, DMSO-d_6): 62.2, 115.8, 121.6, 124.3, 124.7 (d, J^1_{CF} = 270.6), 128.2 (q, J^3_{CF} = 3.8), 128.6, 129.8 (q, J^2_{CF} = 32.2), 137.6, 140.3, 144.7, 161.2; *m/z* (ESI positive) 399.0 $[M+H]^+$

4-[1-(4-Hydroxy-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-benzenesulfonamide **(17).**

Compound **17** was obtained according the general procedure earlier reported using **12** (0.05g, 1.0 eq), **30** (1.1 eq) in t-BuOH/H_2O 1/1 (3.5 ml), TMACl (1.0 eq) and copper nanosize (0.1 eq). The

reaction mixture was stirred at 60°C for 1h and the obtained residue was purified by silica gel column chromatography eluting with 60% ethyl acetate in *n*-hexane to afford **17** as a white solid.

49% yield; m.p. 229-231°C; silica gel TLC R_f 0.09 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 5.34 (s, 2H, CH_2), 6.97 (d, J = 8.8, 2H), 7.26 (m, 2H, overlap with signal at 7.27), 7.27 (s, 2H, exchange with D_2O, SO_2NH_2), 7.71 (d, J = 8.8, 2H), 7.81 (d, J = 8.8, 2H), 8.84 (s, 1H), 10.02 (s, 1H, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6): 62.3, 115.8, 117.0, 123.0, 123.9, 128.6, 129.6, 137.6, 143.9, 158.8, 161.3; *m/z* (ESI positive) 347.0 $[M+H]^+$

General synthetic procedure of alkynes 12, 18-24.[18]

Propargyl bromide (1.2 eq) was added to a suspension of the proper phenol, thiophenol or aniline (0.5 g, 1.0 eq) and K_2CO_3 (2.0 eq) in dry DMF (4 ml) under a nitrogen atmosphere and that was stirred at r.t. until starting material was consumed (TLC monitoring). The reaction mixture was quenched with H_2O (20 ml) and extracted with Et_2O or EtOAc (25 ml). The organic layer was washed with brine (4x15ml), dried over Na_2SO_4 filtered-off and concentrated under *vacuo* to give the titled compounds **12**, **18-24.**

Prop-2-ynyloxy-benzene **(18).**

Compound **18** was obtained according the general procedure earlier reported. The reaction mixture was stirred at r.t. for 2h to give the titled compound **18.**

74% yield; silica gel TLC R_f 0.68 (EtOAc/*n*-hexane 20 % v/v); δ_H (400 MHz, $CDCl_3$): 2.58 (t, J = 2.4, 1H), 4.76 (d, J = 2.4, 2H), 7.06 (m, 3H), 7.37 (t, J = 8.8, 2H); δ_C (100 MHz, $CDCl_3$): 56.1, 75.8, 79.0, 115.3, 121.9, 129.8, 157.9.

Experimental in agreement with reported data.[18]

1-Methyl-3-prop-2-ynyloxy-benzene **(19).**

Compound **19** was obtained according the general procedure earlier reported. The reaction mixture was stirred at r.t. for 2h to give the titled compound **19.**

81% yield; silica gel TLC R_f 0.48 (EtOAc/*n*-hexane 20 % v/v); δ_H (400 MHz, DMSO-d_6): 2.45 (s, 3H, CH_3), 2.52 (t, J = 2.4, 1H), 4.69 (d, J = 2.4, 2H), 6.81 (m, 3H), 7.20 (t, J = 7.2, 1H); δ_C (100 MHz, $CDCl_3$): 21.9, 56.0, 75.7, 79.1, 112.0, 116.1, 122.8, 129.5, 139.9, 157.9.

Experimental in agreement with reported data.[19]

1-Methoxy-3-prop-2-ynyloxy-benzene **(20).**

Compound **20** was obtained according the general procedure earlier reported. The reaction mixture was stirred at r.t. for 2h to give the titled compound **20.**

78% yield; silica gel TLC R_f 0.78 (EtOAc/n-hexane 50 % v/v); δ_H (400 MHz, $CDCl_3$): 2.53 (t, J = 2.4, 1H), 3.80 (s, 3H, CH_3), 4.68 (d, J = 2.4, 2H), 6.57 (m, 3H), 7.20 (t, J = 7.4, 1H); δ_C (100 MHz, $CDCl_3$): 55.6, 56.2, 75.8, 78.9, 101.9, 107.2, 107.6, 103.2, 159.2, 161.2.

Experimental in agreement with reported data.[19]

1-Methoxy-4-prop-2-ynyloxy-benzene **(21).**

Compound **21** was obtained according the general procedure earlier reported. The reaction mixture was stirred at r.t. for 2h to give the titled compound **21.**

71% yield; silica gel TLC R_f 0.65 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, $CDCl_3$): 2.51 (t, J = 2.4, 1H), 3.78 (s, 3H, CH_3), 4.64 (d, J = 2.4, 2H), 6.85 (d, J = 9.2, 2H), 6.93 (d, J = 9.2, 2H); δ_C (100 MHz, $CDCl_3$): 56.0, 56.9, 75.6, 79.2, 114.9, 116.5, 152.0, 154.8.

Experimental in agreement with reported data.[19]

2-Prop-2-ynyloxy-pyridine **(22).**

Compound **22** was obtained according the general procedure earlier reported. The reaction mixture was stirred at r.t. for 3h to give to an oil that was purified by silica gel column chromatography eluting with 5% MeOH in DCM to afford **22.**

50% yield; silica gel TLC R_f 0.49 (MeOH/CH_2Cl_2 10 % v/v); δ_H (400 MHz, $CDCl_3$): 2.49 (t, J = 2.4, 1H), 4.76 (d, J = 2.4, 2H), 6.25 (t, J = 6.8, 1H), 6.61 (d, J = 9.2, 1H), 7.35 (m, 1H), 7.64 (d, J = 6.8, 1H); δ_C (100 MHz, $CDCl_3$): 31.3, 41.2, 73.5, 79.0, 116.6, 121.0, 129.6, 147.5.

Experimental in agreement with reported data.[20]

Prop-2-ynylsulfanyl-benzene **(23).**

Compound **23** was obtained according the general procedure earlier reported. The reaction mixture was stirred at r.t. for 2h to give the titled compound **23**.

96% yield; silica gel TLC R_f 0.61 (EtOAc/*n*-hexane 10 % v/v); δ_H (400 MHz, $CDCl_3$): 2.30 (t, J = 2.4, 1H), 3.67 (d, J = 2.4, 2H), 7.32 (m, 1H), 7.39 (t, J = 7.6, 2H), 7.52 (m, 2H); δ_C (100 MHz, $CDCl_3$): 22.9, 71.9, 80.2, 127.3, 129.3, 129.4, 130.4.

Experimental in agreement with reported data.[21]

Phenyl-prop-2-ynyl-amine **(24)** and *phenyl-di-prop-2-ynyl-amine* **(25).**

Compound **24** and compound **25** were obtained according the general procedure earlier reported. The reaction mixture was stirred at r.t. for 4h and the obtained oil was purified by silica gel column chromatography eluting with EtOAc/petroleum ether 1/20 to afford the titled compounds **24** and **25** as two yellow liquids.

24: 35% yield; silica gel TLC R_f 0.76 (EtOAc/*n*-hexane 20 % v/v); δ_H (400 MHz, DMSO-d_6): 2.24 (t, J = 2.4, 1H), 3.96 (d, J = 2.4, 2H), 6.78 (d, J = 7.4, 2H), 6.85 (t, J =7.4, 1H), 7.31 (t, J =7.4, 2H); δ_C (100 MHz, $CDCl_3$): 34.4, 72.1, 80.8, 114.5, 119.7, 129.6, 146.3.

Experimental in agreement with reported data.[22a]

25: 19% yield; silica gel TLC R_f 0.88 (EtOAc/*n*-hexane 20 % v/v); δ_H (400 MHz, DMSO-d_6): 2.27 (t, *J* = 2.4, 2H), 4.14 (d, *J* = 2.4, 4H), 6.93 (t, *J* = 7.6, 1H), 7.02 (d, *J* =7.6, 2H), 7.24 (t, *J* = 7.6, 2H); δ_C (100 MHz, $CDCl_3$): 41.1, 73.4, 79.1, 116.5, 120.8, 129.6, 147.6.

Experimental in agreement with reported data.[22b]

4-Prop-2-ynyloxy-benzenesulfonamide **(12).**

Compound **12** was obtained according the general procedure earlier reported. The reaction mixture was stirred at 60°C for 5h and then was quenched with H_2O (20 ml). The obtained suspension was stirred overnight at r.t.. The formed precipitate was filtered-off and purified by silica gel column chromatography eluting with 50 % EtOAc in *n*-hexane to afford the title compound **12**.

71% yield; m.p. 131-132°C; silica gel TLC R_f 0.47 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 3.66 (t, *J* = 2.4, 1H), 4.94 (d, *J* = 2.4, 2H), 7.17 (d, J= 8.8, 2H), 7.26 (s, 2H, exchange with D_2O, SO_2NH_2), 7.80 (d, *J* = 8.8, 2H); δ_C (100 MHz, DMSO-d_6): 56.7, 79.6, 79.7, 115.9, 128.6, 137.8, 160.4.

Experimental in agreement with reported data.[23]

General synthetic procedure of phenylazides 2, 26-30.[24]

The proper aniline (0.5g, 1.0eq) was dissolved in a 4M HCl aqueous solution (5 ml) at 0°C. $NaNO_2$ (1.2 eq) was slowly added and the resulting solution was stirred at the same temperature for 0.5h. Then NaN_3 (1.5 eq) was added portion-wise and the mixture was stirred at r.t. for 0.5h. Reaction mixture was filtered-off or extracted with Et_2O (2 x 15 ml) and the combined organic layers were dried over Na_2SO_4, filtered-off and the solvent evaporated in *vacuo* to afford the corresponding phenylazide which was used without further purification.

4-Azido-benzenesulfonamide **(2).**

Compound **2** was obtained according the general procedureearlier reported. Sulfanilamide was treated with $NaNO_2$ and NaN_3 in a HCl 2M aqueous solution and the formed precipitate was filtered-off to afford the titled compound **2** as a yellow solid.

60% yield; m.p. 120-121°C; silica gel TLC R_f 0.47 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 7.33 (d, J = 8.8, 2H), 7.41 (s, 2H, exchange with D_2O, SO_2NH_2), 7.87 (d, J = 8.8, 2H); δ_C (100 MHz, DMSO-d_6): 120.5, 128.6, 141.5, 143.9.

Experimental in agreement with reported data.[5d]

Phenylazide **(26).**

Compound **26** was obtained according the general procedure earlier reported.

60% yield; silica gel TLC R_f 0.76 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6):7.12 (d, J = 7.6, 2H), 7.20 (t, J = 7.6, 1H), 7.42 (t, J = 7.6, 2H); δ_C (100 MHz, DMSO-d_6): 120.0, 126.1, 131.0, 140.3.

Experimental in agreement with reported data.[24]

3-Methoxyphenylazide **(27).**

Compound **27** was obtained according the general procedure earlier reported.

74% yield; silica gel TLC R_f 0.78 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 3.80 (s, 3H, CH_3), 6.66 (t, J = 2.4, 1H), 6.74 (ddd, J = 0.8, 2.4, 8.2, 1H), 6.81 (ddd, J = 0.8, 2.4, 8.2, 1H), 7.36 (t, J= 8.2, 1H); δ_C (100 MHz, DMSO-d_6): 56.3, 105.7, 112.0, 112.1, 131.7, 141.5, 161.5.

Experimental in agreement with reported data.[24]

4-Fluorophenylazide **(28).**

Compound **28** was obtained according the general procedure earlier reported.

89% yield; silica gel TLC R_f 0.79 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 67.19 (m, 2H), 7.29 (t, J = 8.8, 2H); δ_F (376 MHz, DMSO-d_6): -117.77 (s, 1F); δ_C (100 MHz, DMSO-d_6): 117.7 (d, J^2_{CF} = 23), 121.8 (d, J^3_{CF} = 9.0), 136.4, 160.3 (d, J^1_{CF} = 241.0).

Experimental in agreement with reported data.[25]

4-Trifluoromethyl-phenyl azide **(29).**

Compound **29** was obtained according the general procedureearlier reported.

67% yield; silica gel TLC R_f 0.84 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6): 7.35 (d, J = 8.8, 2H), 7.78 (d, J = 8.8, 2H); δ_F (376 MHz, DMSO-d_6): -56.18 (s, 3F); δ_C (100 MHz, DMSO-d_6): 120.8, 125.0 (d, J^1_{CF} = 269.6), 126.2 (q, J^2_{CF} = 32.0), 130.0, 144.7.

Experimental in agreement with reported data.[26]

4-Hydroxy-phenyl azide **(30).**

Compound **30** was obtained according the general procedure earlier reported.

46% yield; silica gel TLC R_f 0.84 (EtOAc/*n*-hexane 50 % v/v); δ_H (400 MHz, DMSO-d_6):6.84 (d, J = 8.8, 2H), 6.97 (d, J = 8.8, 2H), 9.56 (bs, 1H, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6): 117.5, 121.1, 130.6, 156.0.

Experimental in agreement with reported data.[27]

Molecular modeling.

Crystal structures of hCA II (4Q6E) and hCA IX (3IAI) were used in docking computation. Input 3D ligand structures were prepared by LigPrep (LigPrep, version 3.3, Schrödinger, LLC, New York, NY, 2015) and Epik (Epik, version 3.1, Schrödinger, LLC, New York, NY, 2015) for the evaluation of their ionization states. The target structures were prepared according to the recommended Protein Preparation module in Maestro - Schrödinger suite [Maestro, version 10.1, Schrödinger, LLC, New York, NY, 2015], assigning bond orders, adding hydrogens, deleting water

molecules, and optimizing H-bonding networks Finally, energy minimization with a root mean square deviation (RMSD) value of 0.30 was applied using an Optimized Potentials for Liquid Simulation (OPLS_2005, Schrödinger, New York, NY, USA) force field. Docking experiments were carried out with Glide standard precision (SP) (Glide, version 6.6, Schrödinger, LLC, New York, NY, 2015) using grids prepared with default settings and centered in the centroid of the complexed ligand. All computations were performed on an Intel® 2xCPU Xeon® 6-core E5-2620 v2 @ 2.10GHz (1200 MHz) 15MB processor running Linux.

CA inhibition.

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 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.[15] 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 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as reported earlier, and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[5a-d]

Co-crystallization and X-ray data collection.

Crystals of hCA II complexed with compounds **3** and **13** were obtained using the sitting drop vapor

diffusion method. An equal volume of 0.8 mM solution of hCA II in Tris pH=8.0 and 1.6 mM of the inhibitors in Hepes 20mM pH=7.4 was mixed and incubated for 15 minutes. 2 µl of the complex solution were mixed with 2 µl of 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 in 24 well Linbro plate. Crystals of the complexes grew in a few 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 with compounds **3** and **13** were collected using synchrotron radiation at the ID-23-1 beamline at ESRF (Grenoble, France) with a wavelength of 0.976 Å and a PILATUS 6M-F detector, to a maximum resolution of 1.05 and 1.00, respectively. Data were integrated and scaled using the program XDS.[28] Data processing statistics are showed in Table 2.

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.[29] 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 the inhibitors were calculated and energy minimized using the program JLigand 1.0.39. Geometrical restraints for the inhibitors were generated using the Grade Web Server (http://grade.globalphasing.org). During the refinement, anisotropic temperature factors were introduced and hydrogen atoms were added to the models. Manual building of the models was performed using COOT.[30] Solvent molecules were introduced automatically using the program ARP.[31] The quality of the final models were assessed with COOT and Rampage.[32] Crystal parameters and refinement data are summarized in Table 2. Atomic coordinates were deposited in the Protein Data Bank (PDB accession codes: 5LJQ, 5LJT). Graphical representations were generated with Chimera.[33]

Hypertensive Rabbit IOP Lowering Studies.

Male New Zealand albino rabbits weighing 1500−2000 g were used in these studies. Animals were anesthetized using zoletil (tiletamine chloride plus zolazepam chloride, 3 mg/kg body weight, im) and injected with 0.1 mL of hypertonic saline solution (5% in distilled water) into the vitreous of both eyes. IOP was determined using a tonometer (Tono-pen Avia tonometer, Reichhert Inc., Depew, NY 14043, USA) prior to hypertonic saline injection (basal) at 1, 2, 3, and 4 h after administration of the drug. Vehicle (phosphate buffer 7.00 plus DMSO 2%) or drugs were instilled immediately after the injection of hypertonic saline. Eyes were randomly assigned to different groups. Vehicle or drug (0.50 mL) was directly instilled into the conjunctive pocket at the desired doses (1−2%).[16] The IOP was followed for 4 h after drug administration. Four different animals were used for each tested compound. All animal manipulations were carried out according to the European Community guidelines for animal care [DL 116/92, application of the European Communities Council Directive of 24 November 1986 (86/609/EEC)]. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE - Animal Research: Reporting of in Vivo Experiments–guidelines.[34] All efforts were made to minimize animal suffering and to reduce the number of animals used.

■ ASSOCIATED CONTENT

Coordinates and structure factors for hCA II complexes with **3** and **13** have been deposited in the Protein Data Bank (PDB) accession code: 5LJQ and 5LJT. Authors will release the atomic coordinates and experimental data upon article publication.

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Notes

The authors declare the following competing financial interest(s): CTS and FC are authors on several patents claiming sulfonamide CAIs. The other authors do not declare any conflict of interest.

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■ ABBREVIATIONS USED

CA, carbonic anhydrase; CAI, CA inhibitor; K_I, inhibition constant; TEA HCl, triethylamine hydrochloride; TMACl, tetramethylammonium chloride.

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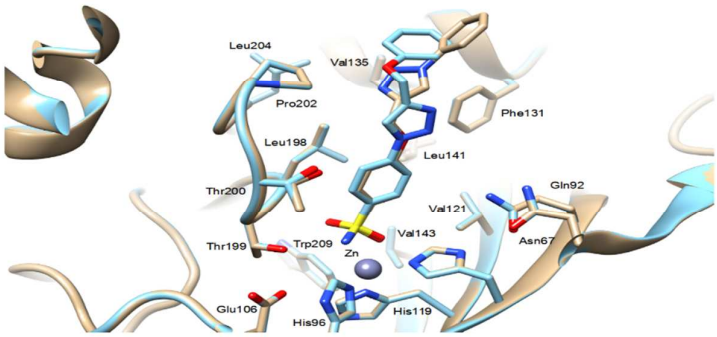
TOC Graphic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