Discovery of β-Adrenergic Receptors Blocker-Carbonic Anhydrase Inhibitor Hybrids for Multitargeted Antiglaucoma Therapy

Discovery of β-adrenergic receptors blocker - carbonic anhydrase inhibitor hybrids for multitargeted anti-glaucoma therapy.

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Abstract

The combination of a β-adrenergic receptors (AR) blocker and a carbonic anhydrase (CA, EC 4.2.1.1) inhibitor in eye drops formulations is one of the most clinically used treatment for glaucoma. A novel approach consisting of single-molecule, multi-targeted compounds for the treatment of glaucoma is proposed here by designing compounds which concomitantly interact with the β-adrenergic and CA targets. Most derivatives of the two series of benzenesulfonamides incorporating 2-hydroxypropylamine moieties reported here exhibited striking efficacy against the target hCA II and XII, whereas a subset of compounds also showed significant modulation of β_1- and β_2-ARs. X-ray crystallography studies provided rationale for the observed hCA inhibition. The best dual-agents decreased IOP more effectively than clinically used dorzolamide, timolol, and the combination of them in an animal model of glaucoma. The reported evidence supports the

proof-of-concept of β-ARs blocker - CAI hybrids for anti-glaucoma therapy with an innovative mechanism of action.

Keywords: glaucoma, carbonic anhydrase; sulfonamide; β-adrenergic receptor; hybrids; linker.

Introduction

Glaucoma consists of a cluster of optic neuropathies characterized by a broad spectrum of clinical presentations and etiologies, leading to a progressive, irreversible vision loss. This causes blindness, affecting over 60 million people globally.[1-3] An increasing number of affected people of up to 80 million is expected by 2020 due to both population increase and aging.[3,4] The diverse types of glaucoma feature an enhanced intra-ocular pressure (IOP).[1,5] The increase in IOP results from the malfunction of ciliary processes and the trabecular meshwork in the anterior chamber of the eye. These tissues physiologically support an adequate pressure in the eye by regulating aqueous humor secretion and its drainage. The main ionic constituent of the aqueous humor is bicarbonate. This fluid is present in the region between cornea and the lens (Figure 1), and its secretion and flow from the ciliary body to the anterior chamber leads to the homeostatic control of the IOP. [1-2] Glaucoma occurs when an increase in IOP occurs, which is due to either an excessive retention of aqueous humor within the anterior chamber or to an excessive secretion of the fluid (Figure 1).[1,5-7] Open-angle glaucoma (OAG) and angle-closure glaucoma (ACG) are the two most common types of primary glaucoma, with OAG being the most common in the Americas and Europe.[8-9] ACG is due to the impaired drainage of the aqueous humor from the anterior chamber. Conversely, this angle is constitutively open in OAG, but the drainage of the humor is diminished. The cause of the altered flow is unknown in OAG and under extensive investigation.[1,2,5] Because of the asymptomatic nature of chronic glaucoma, up to 50% of people in the industrialized world are unaware of their diagnosis and do not receive the required treatment.[6]

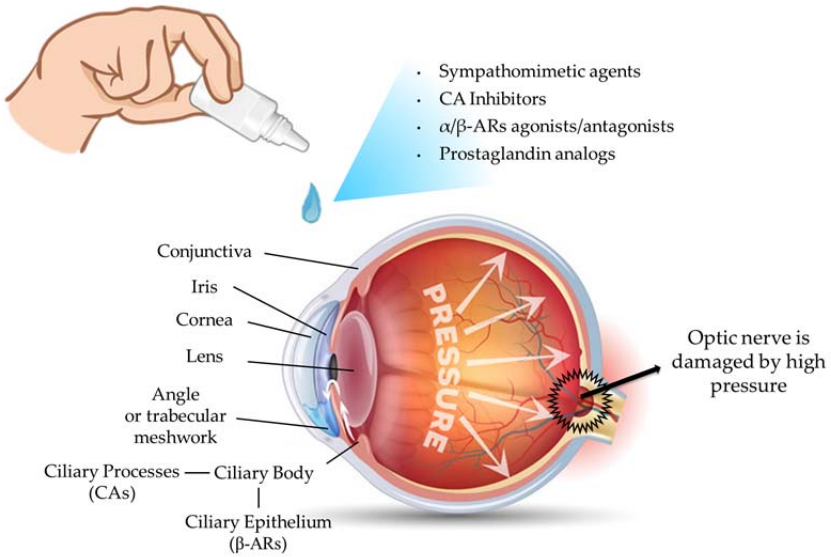


Figure 1. Mechanism and available pharmacologic treatments for glaucoma.

Multiple treatments for glaucoma exist and are chiefly separated into pharmacologic, laser, and surgical therapies.[5-10] In most individuals, pharmacologic therapy is the first therapy of choice for IOP reduction and generally includes the use of topically acting agents, such as eye drops, that reduce aqueous humor production, as well as agents that raise the outflow facility.[7-10] Lowering IOP is the cornerstone of glaucoma therapy, since each additional millimeter of mercury IOP increase can lead to an 11% increase in the risk of glaucoma progression.[11]

The clinically available drugs include sympathomimetic stimulants (epinephrine), parasympathomimetic agents (pilocarpine), β-blockers (timolol), carbonic anhydrase (CA) inhibitors (CAIs, acetazolamide and dorzolamide), and prostaglandin derivatives (latanoprost and travoprost) (Figure 2). These classes of drugs can be used alone or in various combinations.[7-12]

β-Blockers reduce IOP via blockade of the sympathetic nerve endings in the ciliary epithelium, reducing the production of aqueous humour.[13] Among the topically-acting β-blockers available for the treatment of glaucoma, there are non-selective agents, which target both β_1- and β_2-adrenoceptors, and cardio-selective drugs, which block only the β1-receptors.[12] In the past, β-blockers were the most common first line topical glaucoma medication, however the use of more efficient prostaglandin analogues became the primary course of treatment during the 1990s.[10]

When monotherapy alone is not effective in controlling IOP, other drugs with different mechanisms of action can replace or be added in conjunction with beta-blockers or prostaglandin analogues.[12] Commonly used second-line agents include topical CAIs. Inhibition of CAs in the ciliary processes reduces aqueous humor secretion, probably by slowing the rate of bicarbonate production and therefore reducing the transport of water and osmotically obligated sodium within the fluid. As a result, the aqueous humour secretion decreases, leading to a reduction of IOP up to 25-30%.[7,14,15] If necessary, CAIs such as acetazolamide, methazolamide, ethoxzolamide, and dichlorophenamide can still be used as systemic antiglaucoma drugs, though they may show a wide range of undesired side effects in some patients.[14,15]

Compliance is the major challenge with adding multiple drops. It has been demonstrated that increasing the number of drop bottles to a patient's treatment results into a negative influence on patient adherence.[12] Fixed combination therapies have been developed and are currently available in the clinic. For example, the combination of a β-blocker (timolol) and CAI (dorzolamide) represents one of the most used therapeutic options.[12]

The current therapies are often inadequate given that topical glaucoma therapy is burdened by the need for multiple classes of medications to control IOP,[16] undesired side effects,[10] and barriers such as patient compliance[17] and difficulty with proper drop instillation.[18] New needed pharmacotherapies for glaucoma should exhibit favorable benefit-risk profiles and alternative mechanisms of action relative to current therapies.[8] This can be achieved by either increasing efficacy (ocular hypotensive efficacy), decreasing adverse events, or both.

Herein a multi-targeted approach for the treatment of glaucoma is proposed by design, synthesis, crystallography and biological *in vitro / vivo* evaluation of a series hybrid drugs which concomitantly affect the β-adrenergic receptors and carbonic anhydrases in the eye.

Results and Discussion

Compounds Design and Chemistry.

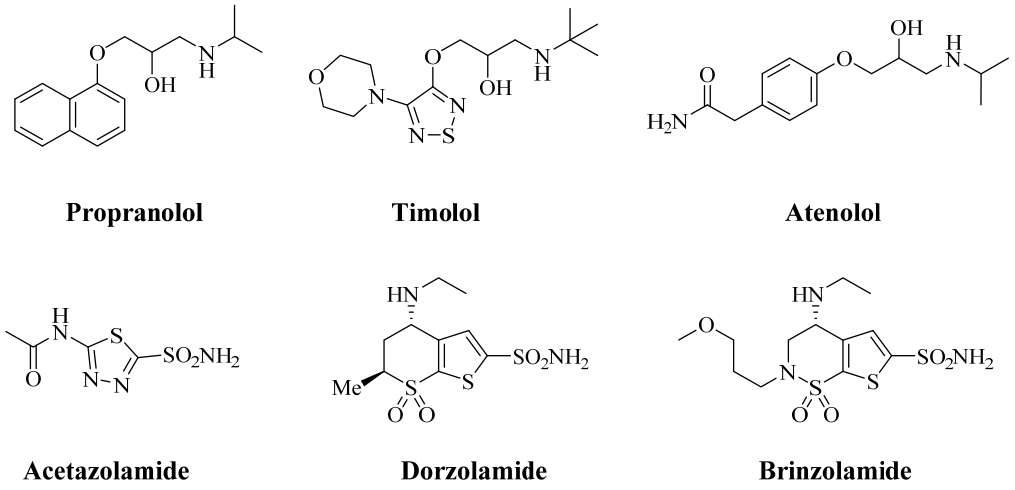


Figure 2. Molecular structures of clinically used β-blockers and anti-glaucoma CAI drugs.

An approach consisting of single-molecule, multi-targeted compounds was chosen over the co-administration of single drugs due to potential therapeutic benefits.[19-22] Indeed, it is likely that single target agents cannot sufficiently affect the intricate biochemical processes involved in disease pathology, such as the above depicted for glaucoma. Most biological targets interact in a complex network of enzymes/receptors whose equilibrium could be more likely influenced by drugs that affect multiple targets.[19-22] Moreover, combinatorial drug therapies could be replaced by multi-functional molecules, minimizing the pharmacokinetic and metabolic issues that arise from multiple drug intake. Patients are more likely to remember to take a single drug; thus, compliance is enhanced. Finally, chemical or metabolic drug-drug interactions are avoided by means of multi-targeted molecules.[21]

The hybrid drug strategy has been previously applied to target glaucoma by carbonic anhydrase inhibition. CAI – nitric oxide (NO) donor hybrids stood out amongst the most effective topically active agents.[23-25] Since hypertensive glaucoma patients show a decreased content of NO/cGMP in the aqueous humor, it has been shown that NO-donors can decrease IOP in normal and pathological

conditions. One such compounds was twofold more efficient than dorzolamide to reduce high IOP characteristic of this disease in an animal model.[24]

The herein reported derivatives feature a benzene sulfonamide moiety, representing the CA inhibitory fragment, and the aryloxy-2-hydroxypropylamine portion of β-blockers such as propranolol and timolol (Figure 2, 3).

Sulfonamides are the most effective zinc binding group (ZBG) commonly used to design CAIs.[26-29] Primary sulfonamides (Figure 2), such as acetazolamide (**AAZ**) or brinzolamide (**BRZ**), represent the first-generation CAIs and have been clinically used for almost 70 years as anti-glaucoma agents, diuretics, anti-epileptics, or anti-obesity drugs.[27,28] Dorzolamide (**DZA**), a second-generation CAI, was the first topically acting sulfonamide used clinically as an anti-glaucoma medication. It is indicated for the reduction of elevated IOP in patients with open-angle glaucoma or ocular hypertension that do not sufficiently respond to β-blockers.[7,14,15]

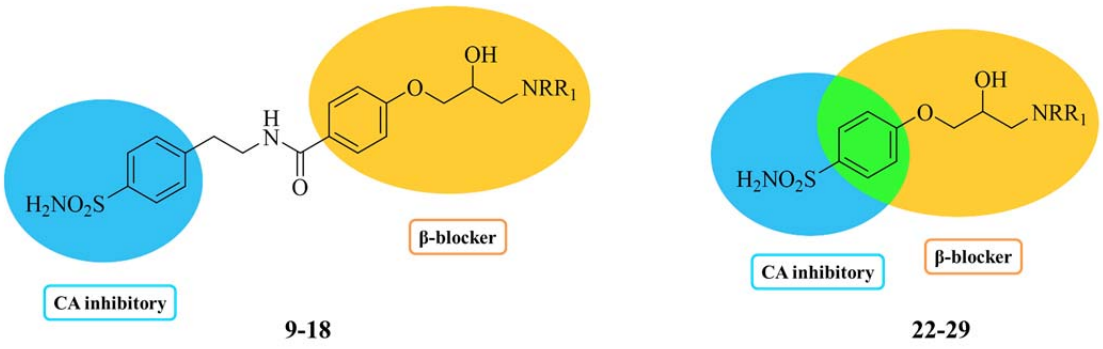
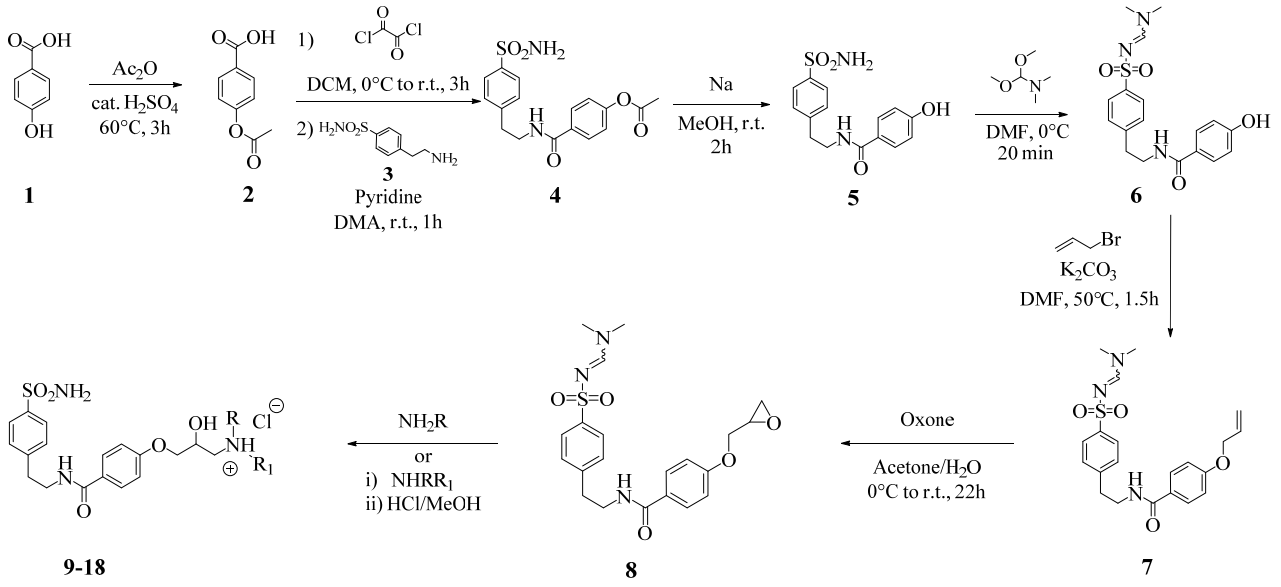


Figure 3. Design of aryloxy-2-hydroxypropylamine sulfonamides dual-targeted agents.

Modulation of the β-ARs was investigated by swapping the substituents appended at the aryloxy-2-hydroxypropylamine moiety, which is a rather common hallmark of β-blockers and thus maintained in the hybrids structure (Figure 2, 3). The aryloxy-2-hydroxypropylamine portion was directly appended at the benzenesulfonamide scaffold (derivatives **22-29**) or alternatively detached by means of an ethylbenzamide spacer (derivatives **9-18**). In this second case, uniquely small aliphatic amines were considered in the β-blocker portion, owing to the hydrophobic nature of the spacer.

The incorporation of such 2-hydroxypropilamine moieties at the benzenesulfonamide scaffold (both direct and spaced) represents an application of the CAI "tail approach",[30-32] the most common method to develop isoform selective inhibitors within the zinc-binders class. This approach explores the modulation of moieties appended at the aromatic/heterocyclic ring present in the scaffold of the CAIs in order to selectively promote interactions with isoform unique residues at the entrance of the active site cavity.

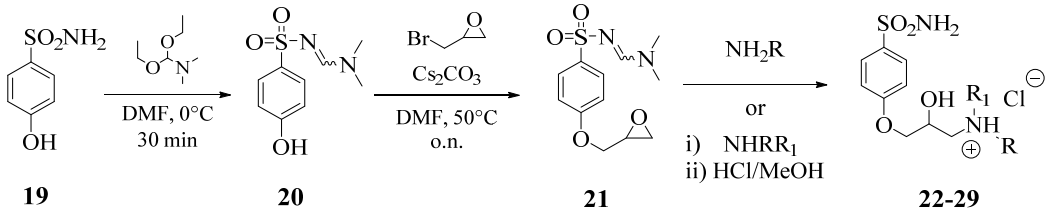


Scheme 1. General synthetic procedure for compounds **9-18**.

Two synthetic strategies were planned to obtain the hybrid compounds. Preparation of the first series molecules **9-18** shared a common key epoxy intermediate **8,** for which the synthetic route is illustrated in *Scheme 1*. Coupling of ethylamminobenzenesulfonamide **2** with freshly prepared 4-acetoxybenzoyl chloride **2** generated amide **4**, which was therefore deacetylated with sodium methoxide and protected on the sulfonamide group with N,N-dimethylformamide dimethyl acetal. Reaction of the resulting phenol **6** with allyl bromide followed by epoxidation of the olefinic function in presence of Oxone and acetone provided for intermediate **8.** Epoxide ring opening (**8**) to afford 2-hydroxypropylamine derivatives **9-18** was achieved by treatment with an excess of varied primary and secondary amines. Reaction with primary amines occurred with an additional and unexpected cleavage of the sulfonamide protecting group, resulting directly in the 2-

hydroxypropylamine derivatives **9-16**. Because secondary amines were not able to carry out the deprotection process, treatment in acidic media was necessary to free the sulfonamide group and generate compounds **17-18**.

In a similar manner, preparation of the second series of molecules **22-29** (*Scheme 2*) shared the common epoxy intermediate **21**, which was obtained by reacting the N-protected 4-hydroxybenzenesulfonamide **20** with epibromohydrin in presence of Cs_2CO_3. Epoxide **21** was reacted with different amines and subsequently treated in acidic media in the case of secondary amines to give the 2-hydroxypropylamine derivatives **22-29**. These synthetic strategies were adopted to provide **9-18** and **22-29** as racemates, since the mixture of both enantiomers was deemed more suitable to initially seek for biological activity.



Scheme 2. General synthetic procedure for compounds **22-29**.

Carbonic Anhydrase Inhibition.

The CA inhibition profiles of compounds **9-18** and **22-29** were evaluated, in addition to acetazolamide (**AAZ**) as standard inhibitor, against four physiologically relevant isoforms, hCA I II, IX, and XII. The choice of these isoforms was based upon: hCA II and XII are upregulated in the eyes of glaucoma patients [5,7,27] and might be responsible for increased blood flow and thus the oxygen supply in hypoxic neovascular retinic tissues; hCA IX has been found to be upregulated in the hypoxia-suffering cells of the retinal pigment epithelium;[26-28] and hCA I is the main off-target isoform for the therapeutic application of CAIs in the reported ocular diseases.[27] The following structure–activity relationship (SAR) can be gathered from the inhibition data reported in Table 1:

Table 1. Inhibition data of human CA isoforms hCA I, II, IX and XII with sulfonamides **9-18, 22-29** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO2 hydrase assay.[33]

Cmpd			K_I (nM)[a]			
	R	R_1	hCA I	hCA II	hCAIX	hCA XII
9	-CH_3	H	20.2	14.1	1.7	1.5
10	-CH_2CH_3	H	9.3	48.8	22.1	1.9
11	-$CH_2CH_2CH_3$	H	52.2	18.4	7.4	1.5
12	-$CH(CH_3)_2$	H	8.9	3.0	61.8	18.1
13	- $CH(CH)_3CH_2CH_3$	H	45.8	55.7	53.6	2.8
14	-$C(CH_3)_3$	H	59.1	16.1	83.1	23.8
15	-CH_2Ph	H	35.6	1.9	74.7	2.4
16	-CH_2CH_2OH	H	33.2	16.6	5.8	27.7
17	-$(CH_2CH_2)O(CH_2CH_2)$-		6.9	1.5	7.0	4.1
18	- $CH(CH_3)_2$	- $CH(CH_3)_2$	6.6	1.2	6.0	1.6
22	-CH_3	H	750.8	1174.3	23.4	4.9
23	-$CH(CH_3)_2$	H	350.4	155.5	47.6	44.4
24	- $CH(CH)_3CH_2CH_3$	H	74.5	240.6	107.7	58.6
25	-$C(CH_3)_3$	H	234.3	174.1	95.4	41.9
26	-CH_2Ph	H	85.4	44.1	93.1	72.4
27	-CH_2CH_2Ph	H	145.6	75.0	59.2	39.8
28	-CH_2CH_2OPh	H	183.1	15.2	84.6	82.2
29	-$(CH_2CH_2)N(CH_3)(CH_2CH_2)$-		230.6	48.9	126.5	164.1
AAZ	-	-	250	12	25	5.7
DZA[b]	-	-	50000	9	52	3.5

a. Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10 % of the reported values).
b. From ref. 27.

(i) The cytosolic isoform hCA I was effectively inhibited by most of the sulfonamides of the first series with inhibition constants (K_Is) ranging in the low nanomolar range, between 6.6 and 59.1 nM. Conversely, the benzenesulfonamides directly bearing the 2-hydroxypropylamine (**22-29**) moieties were found to act as weaker inhibitors with K_Is spanning between 74.5 and 750.8 nM. The sec-butyl and benzyl bearing derivatives **24** and **26** stood out from the others with K_Is less than 100 nM. All the first series compounds and **24, 26-28** from the second series were stronger inhibitors compared to the clinically used **AAZ** (K_I value of 250 nM).

(ii) The physiologically dominant isoform hCA II was strongly inhibited by all reported sulfonamides (K_I values ranging between 1.5 and 75.0 nM, Table 1), apart from the second series compounds incorporating small substituents (methyl, isopropyl, sec-butyl and tert-butyl) on the amine moiety (**22-25**), whose K_Is spanned from 155.5 to 1174.3 nM. The hCA II inhibition profiles showed the efficacy of the spacer-containing compounds. It is worth mentioning that the bulkiest derivatives of the first series **15** (benzylamine, K_I 1.9 nM), **17** (morpholine, K_I 1.5 nM) and **18** (diisopropylamine, K_I 1.2 nM) as well as those of the second series **26-29** (K_Is ranging between 15.2 and 75.0 nM) exhibited the strongest hCA II inhibition profiles within each subset. Despite a generally comparable efficacy with the standard **AAZ** (K_I value of 12 nM), only compounds **12**, **15, 17** and **18** exhibited a stronger effectiveness.

(iii) The data in Table 1 depicted similar inhibitory trends against the membrane-associated isoforms hCA IX and XII, which were strongly inhibited by most derivatives. In detail, the K_Is for the first series of derivatives against hCA IX spanned from 1.7 (**9**, methylamine) to 83.1 nM (**14**, tert-butylamine), whereas the efficacy of compounds **22-29** was weaker with inhibition constants ranging between 23.4 (**22**, methylamine) and 126.5 nM (**29**, N-methylpiperazine), not permitting a rationale for a SAR. Again, only compounds **9-11**, **16-18** among the benzamide-bearing derivatives and compound **22** in series two were found to possess at least comparable efficacy to the **AAZ** (K_I of 25 nM).

(iv) Most of the spacer-supplied compounds demonstrated strong hCA XII inhibitory effectiveness (K_Is ranging between 1.5 and 4.1 nM), except for **12** (isopropylamine), **14** (tert-butylamine) and **16** (ethanolamine), which showed a 10-fold diminished activity (K_Is of 18.1-27-8 nM). Among the spacer-devoid derivatives, compound **22**, that incorporated a methyl group on the amine moiety, exhibited low nanomolar efficacy (K_I of 4.9 nM), whereas the remaining compounds were significantly weaker with inhibition constants ranging between 39.8 and 164.1 nM.

(v) Noteworthy, the first series of derivatives were shown to be generally more efficacious against the hCAs in comparison to the directly linked dual-derivatives, to a greater extent against the cytosolic isozymes hCA I and II. The second series compounds **22** and **23**, which bear a methyl and isopropyl group appended at the amine moiety, were able to target hCA IX and XII with selectivity over the cytosolic isozymes (selectivity ratio hCA II / IX of 50.2 and 3.2 and hCA II / XII of 240.5 and 3.5, respectively). The inhibition profiles of compounds **22** and **23** indicate a potential to target several CAs-associated diseases without causing the typical side-effects of non-selective inhibitors in systematically administered treatments.

β-ADR Binding assay

The β-ADRs binding properties of compounds **9-18** and **22-29** were evaluated, in addition to propranolol and atenolol [34] as standard β-blockers, against the human cloned β_1- and β_2-ADRs receptors expressed in HEK293T cell membranes. The following remarks can be drawn from the data reported in Table 2:

Table 2. Inhibition binding constants (pK_i) of the tested compounds for human cloned β_1- and β_2-ADRs expressed in HEK293T cell membranes.

Compound			**pK_i** [a]	
	R	R_1	β_1-ADR	β_2-ADR
9	-CH_3	H	<4	<4
10	-CH_2CH_3	H	<4	<4
11	-$CH_2CH_2CH_3$	H	<4	<4
12	-$CH(CH_3)_2$	H	<4	<4
13	- $CH(CH)_3CH_2CH_3$	H	<4	<4
14	-$C(CH_3)_3$	H	<4	<4
15	-CH_2Ph	H	<4	<4
16	-CH_2CH_2OH	H	<4	<4
17	-$(CH_2CH_2)O(CH_2CH_2)$-		<4	<4
18	- $CH(CH_3)_2$	- $CH(CH_3)_2$	<4	<4
22	-CH_3	H	<4	<4
23	-$CH(CH_3)_2$	H	4.98±0.06	4.50±0.05

24	- $CH(CH)_3CH_2CH_3$	H	4.81±0.03	4.52±0.13
25	$-C(CH_3)_3$	H	4.87±0.06	5.30±0.08
26	$-CH_2Ph$	H	4.10±0.03	<4
27	$-CH_2CH_2Ph$	H	4.57±0.02	4.40±0.02
28	$-CH_2CH_2OPh$	H	5.18±0.05	4.55±0.03
29	$-(CH_2CH_2)N(CH_3)(CH_2CH_2)-$		<4	<4
Propranolol [b]	-		7.93±0.03	8.76±0.02
Atenolol [b]	-		5.82±0.14	5.02±0.20
Timolol [c]			8.27±0.08	9.68±0.02

a. Values are reported as mean ± SEM of 3-5 experiments, each one performed in duplicate.
b. Racemate.
c. From ref. 34

The competition binding experiments did not show striking affinities of the multi-targeted derivatives. The data in Table 2 highlight the absence of binding affinity reported towards both the β_1 and β_2-ADRs for the spacer-endowed derivatives **9-18**, which were not able to compete with [^{3}H]-CGP12177 at the receptor subtypes below 100 μM. Conversely, interesting pK_i values can be gathered from the binding curves measured for the second series compounds (**22-29**). Most derivatives were found to exhibit low micromolar affinities for both β-ADRs subtypes (except **22** and **29**).

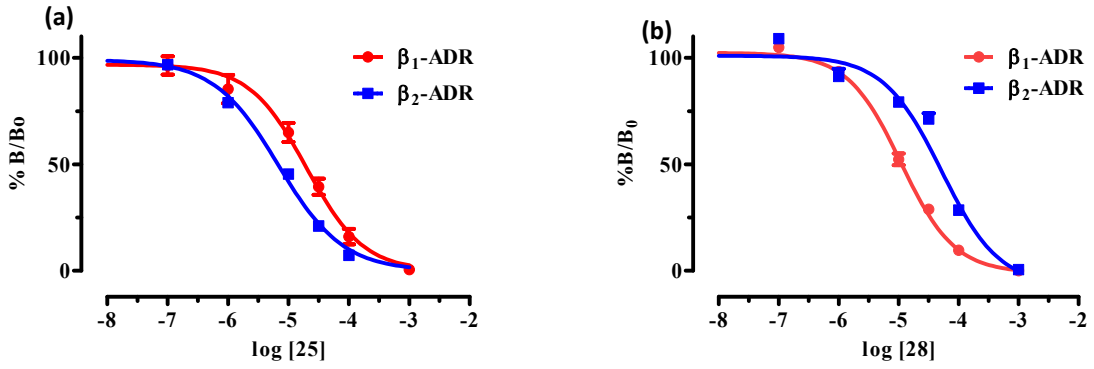


Figure 4. Inhibition of [^{3}H]-CGP12177 specific binding to membrane homogenates of HEK293T cells which stably express the β_1- and β_2-adrenergic receptors by increasing concentration of compound (a) 25 and (b) 28. The curves were fitted using the standard four parameter logistic equation and are the mean ± S.E.M. from 3-4 independent experiments. Non- specific binding was determined in the presence of 10 μM propranolol. Y-axis: normalized Bound/Total bound.

When the *tert*-butylamine bearing **25** showed approximately a three-fold β_2/β_1 selectivity (below 10 μM for the β_2-AR subtype), the remaining derivatives showed preferential affinity for the β_1-AR, with the highest selectivity (over four-fold) reported for **28.** The incorporation of a simple methyl group or *N*-methylpiperazine moiety in the 2-hydroxylpropyl portion deprives respectively **22** and **29** of any affinity for both receptor subtypes up to 100 μM.

The K_i values measured for most compounds **23-28** were comparable to the racemic standard β-blocker atenolol (low micromolar range).[34] Moreover, the K_i of **25** against the β_2-subtype exceed that reported for the clinically used drug. Such results are of interest in terms of single enantiomer binding constants evaluation for future developed derivatives.

Although the similarity between derivatives **22-29** (sulfonamide-bearing) and clinically used β-blockers, such as atenolol, practolol and celiprolol (which possess an amide or urea group appended at the same position of the aromatic scaffold) indicates analogue functional tendency, further investigations with this respect are currently ongoing.

X-ray Crystallography

The X-ray crystal structure of hCA II was determined in complex with compounds **12** and **23** (Table 3). Compounds **12** (R enantiomer) and **23** (S enantiomer) were observed to bind directly to the active site zinc and displace the catalytic water, as is expected for sulfonamide-based compounds (Figure 5A, 5B). Hydrogen bonds were observed between the nitrogen and oxygen atoms of the sulfonamide and Thr199 (2.7-2.8 and 3.0Å, respectively) (Figure 6A, 6B).

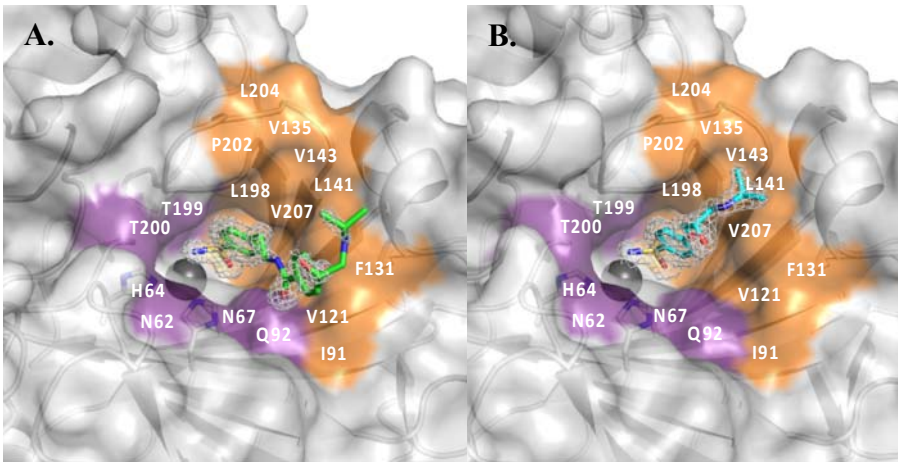


Figure 5. Surface representation of hCA II in complex with inhibitors. A. hCA II in complex with compound **12** (represented as green sticks). **B.** hCA II in complex with compound **23** (represented as cyan sticks). Hydrophobic and hydrophilic residues are labeled and coloured (orange and purple, respectively).

The presence of the spacer in compound **12** resulted in the formation of an additional hydrogen bond between the carbonyl and Q92 side chain (3.0Å). This observation rationalizes the stronger binding affinity of compound **12** for CA II in relation to the direct compound **23** (Figure 6A). Both inhibitors were further stabilized by van der Waals interactions with active site residues I91, V121, F131, L141, and L198 in the case of compound **12** and residues Q92, V121, F131, V135, L198, and P202 for compound **23** (Figure 6A, 6B).

Steric hindrance between the benzene ring in spacer-containing compound **12** and F131 shifted the tail of this compound toward the interface between the hydrophobic and hydrophilic regions of the active site, allowing a second molecule to bind between monomers within the crystal lattice (Supplemental Figure 1A). This secondary inhibitor molecule interacts directly with hCA II through hydrogen bonding with D72 (2.6 and 2.7Å) and is further stabilized through an interaction with the NH group of the active site-bound **12** (2.9Å) (Supplemental Figure 2). Similarly, two additional molecules of compound **23** were observed to bind outside the hCA II active site, thought to bind due to crystal packing (Supplemental Figure 1B).

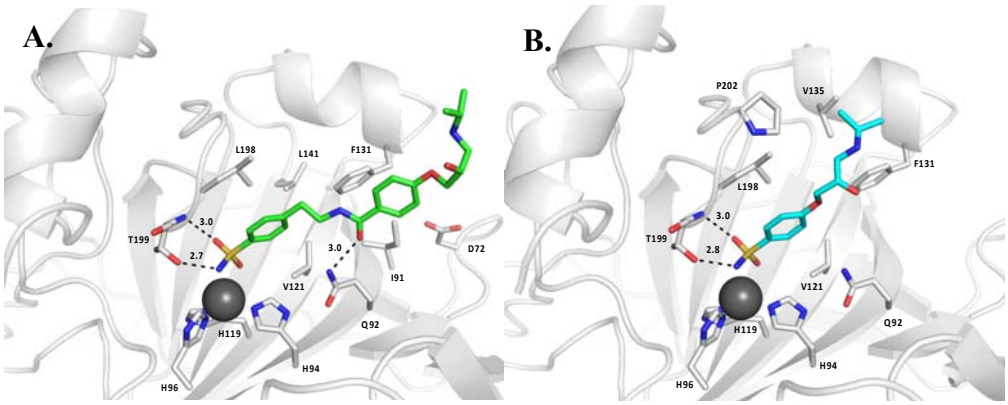


Figure 6. Active site binding interactions. hCA II in complex with **A.** compound **12** (green) and **B.** compound **23** (cyan). Hydrogen bonds are represented as black dashes with distances labelled in angstroms.

Table 3. X-ray crystallography statistics.

Sample	CA II_12	CA II_23
PDB Accession Code	5WLV	5WLT
Space Group Cell Dimensions (Å;°)	$P2_1$ a = 42.6, b = 41.6, c = 72.5; β= 104.3	$P2_1$ a = 42.7, b = 41.6, c = 72.7; β= 104.5
Resolution (Å)	30.8 – 1.4 (1.42 – 1.40)	40.3 – 1.6 (1.60 – 1.57)
Total Reflections	69,038	53,038
I/Iσ	21.3 (2.8)	17.1 (2.2)
Redundancy	3.2 (3.1)	4.0 (4.0)
Completeness (%)	99.4 (99.5)	90.2 (92.7)
Rcryst (%)	14.5 (22.3)	14.5 (18.9)
Rfree (%)	16.7 (25.5)	17.6 (25.0)
Rsym (%)	5.2 (40.4)	6.8 (54.8)
Rpim	3.4 (27.6)	3.5 (28.7)
# of Protein Atoms	2099	2094
# of Water Molecules	234	213
# of Ligand Atoms	60	76
Ramachandran stats (%): Favored, allowed, generously allowed	97.3, 2.7, 0	96.9, 3.1, 0
Avg. B factors (Å^2): Main-chain, Side-chain, Ligand	15.2, 19.5, 40.0	14.4, 18.7, 41.3
rmsd for bond lengths, angles (Å,°)	0.006, 0.939	0.006, 0.918

Antiglaucoma Activity (IOP Lowering Activity)

In spite of not striking β-ADR-binding affinities, several molecules within the second series demonstrated a multi-targeted action and excellent and suitable water solubility to be formulated as 1% eye drops at the neutral pH value. We investigated the intraocular pressure (IOP) lowering

properties of some of these compounds, more precisely, **23** (iso-propylamine, for which the X-ray crystal structure in complex with hCA II was reported), **25** (tert-butylamine) and **27** (phenethylamine) in a rabbit model of glaucoma. The morpholine-bearing compound **17** of the first subset was included in the study to evaluate the IOP lowering efficacy of a uniquely CA inhibitory derivative.[35,36]

DZA hydrochloride and timolol, as well as their combination (1% + 0.25%, in the clinically used ratio) were used for comparison as a standard drugs, with a control using the vehicle (hydroxypropylcellulose at 0.05%). The 1% compounds eye drops 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. The benzamide derivative **17** was a strong, low nanomolar inhibitor for hCA II, IX and XII (K_Is of 1.5, 7.0 and 4.1 nM), but lacked activity on the β-ADR. On the contrary, compounds **23**, **25** and **27** showed a significant, low micromolar action against the β_1- and β_2-ADR, at the expense of a weaker efficacy against target hCAs (Figure 7).

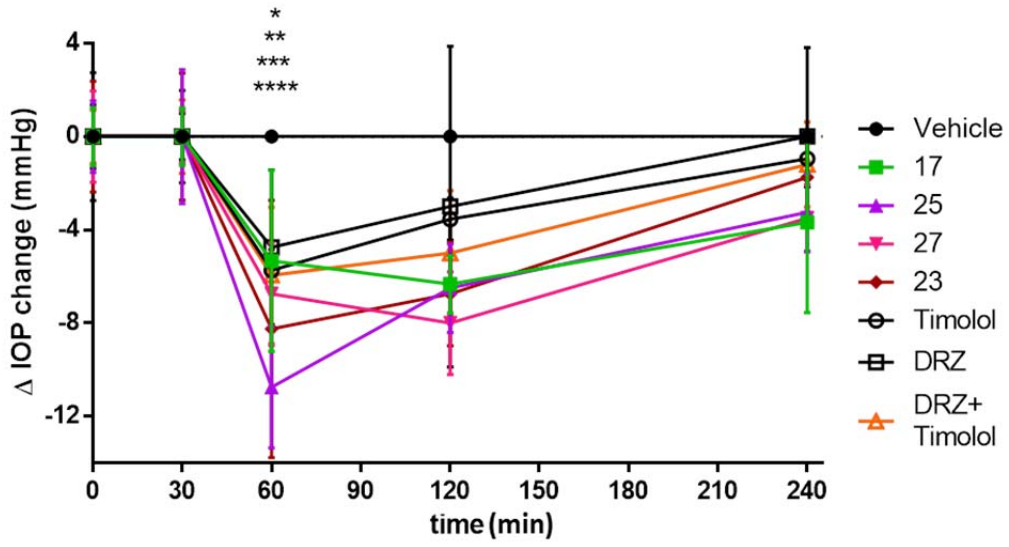


Figure 7. Drop of intraocular pressure (ΔIOP, mmHg) versus time (min) in hypertonic saline-induced ocular hypertension in rabbits, treated with 50 μL of 1 % solution of compounds **17, 23, 25, 27. Timolol** 0.25%, **DRZ** 1% and their association (**DRZ+timolol**) were used as reference drugs. Data are analyzed with 2way Anova followed by Dunnett's multiple comparison test. * $p<0.05$ **17** and **DRZ** *vs* vehicle at 60'; ** $p<0.005$ **timolol** and **DRZ+timolol** *vs* vehicle at 60'; *** $p<0.001$ **27** *vs* vehicle at 60'; ****$p< 0.0001$ **23** and **25** *vs* vehicle at 60'.

The dual agents **23, 25, 27** were more effective than standard **DRZ** and timolol with IOP decreases of 8.25, 10.75 and 6.75 mmHg at 60 min post-administration, respectively (Figure 7). Compound **25** stood out as the most efficient one, with a twofold enhanced efficacy in comparison to **DRZ** and timolol, which caused an IOP decrease of 4.75 mmHg and 5.73 at 60 min post-administration, respectively. Noteworthy, 1% eye drops of the multi-targeted derivatives were more effective than the combination of the CAI and β-blocker leads in the ratio 1% + 0.25%. CAI **17** showed to lower IOP at 60 min post-administration in a comparable manner to the standards. Compounds **17**, **23** and **25** showed similar IOP lowering effectiveness after 2 hr post-administration in the range 6.33-6.75 mmHg, with **27** excelling among the others producing a IOP drop of 8.0 mmHg. The IOP drops produced by all assayed derivatives were greater that those occurring using the standard drugs and their combination (3.0, 3.54, 5.0 mmHg, respectively). When compounds **23** and **25** were more active after 60 min post-administration, compounds **17** and **27** reached the greater efficacy after 120 min. Derivatives **17**, **23** and **25** maintained IOP lowering action at 4 hr post-administration (3.25-3.67 mmHg), when timolol and the drugs combination were barely active and **DRZ** lost its efficacy. A significant enhancement in the IOP lowering efficacy of dual-agents **23, 25, 27** with respect to **17** (that uniquely inhibit the CAs) was observed at 60 min post-administration, whereas the range thinned after 2-hours, with the multi-target **27** standing out anyhow as the most efficient compound. It is worth stressing that **17** inhibits hCA II and XII much more potently (K_Is of 1.5 and 4.1 nM) than the **23**, **25**, **27 (**K_Is in the range 75.0-174.1 and 39.8-44.4 nM, respectively). As a result, it is reasonable to ascribe the powerful IOP lowering efficacy of **17**, that did not possess a dual action, to the orders of magnitude greater hCA II and XII inhibition than **23**, **25**, **27.** Hence, the more effectual IOP lowering action of the hybrids compounds, in spite of a remarkably weaker CA inhibition than derivative **17**, witnesses the concomitant, although unbalanced (nanomolar vs micromolar) modulation of two physiological systems, namely CAs and β-ADRs.

Conclusions

With the incidence of glaucoma steadily growing because of both demographic expansion and population aging, new pharmacologic therapies that possess more favourable benefit-risk profiles are needed. These can be achieved by either increasing efficacy (ocular hypotensive efficacy), decreasing adverse events, or both. The combination of a β-blocker and a CAI included in eye drops is one of the clinically-available options to treat glaucoma and is extensively used. In the present work, a novel single molecule, multi-targeted approach was chosen over the co-administration of single drugs due to varied potential therapeutic benefits, such as an improved effect onto the intricate biochemical processes involved in disease pathology. Concomitant modulation of β-adrenergic and CA systems present in the eye was achieved by combining benzenesulfonamide fragments of classical CAIs with 2-hydroxypropylamine fragments of known, clinically used β-blockers, such as propranolol or timolol. The resulting two series of molecules, which differ by the spacer incorporated between the pharmacophores, were investigated for their inhibitory activity against target (II, IX and XII) and off-target (I) hCAs as well as for their effectiveness to modulate the β_1- and β_2-ARs. A first subset of derivatives reported no multi-targeted modulatory efficacy, with a remarkable CA inhibitory potency at the expense of a void affinity to β-ARs. The second subset of hybrid molecules exhibited a slightly worsening of CA inhibition profiles, with the affinity to β-ARs raising to micromolar range, which is comparable to racemic β-blocker atenolol.

The X-ray crystal structure of hCA II was determined in complex with compounds **12** and **23,** namely the isopropyl-substituted derivatives of both series. Multi-target derivatives **23, 25, 27** were shown to possess more effective IOP lowering properties than the lead, clinically used dorzolamide and timolol, and their combination based onto the balanced multi-targeted modulation. The reported evidence supports the proof-of-concept of adrenergic receptors blocker - carbonic anhydrase inhibitor hybrids for anti-glaucoma therapy with an innovative mechanism of action. These spread the way of the research of more effective anti-glaucoma agents acting on the two systems in a more powerful and balanced manner. Identification of eutomers within the

racemic mixtures will be of help in enhancing efficacy onto β-ARs without significantly affecting the compounds CA inhibitory activity.

Experimental protocols

Chemistry

Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Alfa Aesar and TCI. All r eactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphe re 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 D MSO-d6. 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, sept et; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; dd, double of doubles. The assignment o f exchangeable protons (OH and NH) was confirmed by the addition of D_2O. Analytical thin-layer c hromatography (TLC) was carried out on Sigma Aldrich silica gel F-254 plates. Flash chromatograp hy purifications were performed on Sigma Aldrich Silica gel 60 (230-400 mesh ASTM) as the statio nary phase and ethyl acetate/n-hexane or MeOH/DCM were used as eluents. Melting points (mp) w ere measured in open capillary tubes with a Gallenkamp MPD350.BM3.5 apparatus and are uncorre cted. HPLC was performed by using a Waters 2690 separation module coupled with a photodiode ar ray detector (PDA Waters 996) and as column a Nova-Pak C18 4 μm 3.9 mm × 150 mm (Waters), s ilica-based reverse phase column. Sample was dissolved in acetonitrile 10%, and an injection volum e of 45 μL was used. The mobile phase, at a flow rate of 1 mL/min, was a gradient of water + trifluo roacetic 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 wer e >96% HPLC pure. Derivatives **9-18** and **22-29** were obtained and evaluated as racemates. None of

the screened derivatives reported PAINS alerts determined by SwissADME server (www.swissadme.ch).

4-Acetoxybenzoic acid (2).[37]

A suspension of 4-hydroxy benzoic acid **1** (1.0 g, 1.0 eq) in Ac_2O (5 ml, 7.3 eq) was treated with four drops of H_2SO_4 95% and the obtained solution was stirred 3h at 60°C under a nitrogen atmosphere. The reaction mixture was cooled to r.t., quenched with slush (50g) and then stirred for 1.5 h to obtain a white precipitate, that was filtered and washed with water to afford the titled compound **2**. 82% yield; silica gel TLC R_f *0.23* (EtOAc/*n*-Hex 50 % *v/v*); δ_H (400 MHz, DMSO-d_6): 2.34 (s, 3H, CH_3), 7.30 (d, J = 8.8, 2H, Ar), 8.02 (d, J = 8.8, 2H, Ar), 13.05 (bs, 1H, exchange with D_2O, COO*H*); δ_C (100 MHz, DMSO-d_6): 21.8, 123.0, 129.3, 131.8, 154.9, 167.6, 169.8. Experimental in agreement with reported data.[37]

4-(2-(4-Acetoxybenzamido)ethyl)benzenesulfonamide (4).[38]

Oxalyl chloride (4.15 eq) and DMF (0.02 eq) were added to a solution 4-acetoxy benzoic acid **2** (0.9 g, 1.0 eq) in CH_2Cl_2 (18 ml), under a nitrogen atmosphere. The reaction mixture was stirred 0.5h at r.t. and at reflux temperature for 2.5h. The volatiles were removed under vacuum and the obtained viscous oil is dissolved in DMA (4 ml). Pyridine (10.0 eq) and 4-(2-aminoethyl)-benzensulfonamide **3** (1.05 eq) were added to the obtained solution under a nitrogen atmosphere, the reaction mixture was stirred for 1h at r.t. and then was quenched with slush (30g). The pH was taken to 2 with HCl 6M, at ice bath temperature to obtain a precipitate that was filtered and washed with water and Et_2O to afford the titled compound **7**. 83% yield; silica gel TLC R_f *0.30* (MeO*H*/DCM 10 % *v/v*); δ_H (400 MHz, DMSO-d_6): 2.32 (s, 3H, CH_3), 2.97 (t, J = 7.2, 2H, CH_2), 3.55 (q, J = 7.2, 2H, CH_2), 7.25 (d, J = 8.4, 2H, Ar), 7.32 (s, 2H, exchange with D_2O, SO_2NH_2), 7.47 (d, J = 8.0, 2H, Ar), 7.78 (d, J = 8.0, 2H, Ar), 7.89 (d, J = 8.4, 2H, Ar), 8.64 (t, J = 5.2, 1H, N*H*CO); δ_C (100 MHz, DMSO-d_6): 21.8, 35.7, 41.4, 122.6, 126.6, 129.5, 130.1, 133.0, 143.0, 144.7, 153.5, 166.4, 169.9. Experimental in agreement with reported data.[38]

4-Hydroxy-N-(4-sulfamoylphenethyl)benzamide (5).[38]

4-(2-(4-Acetoxybenzamido)ethyl) benzenesulfonamide **4** (1.35 g, 1.0 eq) was added to a freshly prepared solution of sodium (1.0 eq) in dry methanol (40 ml) under a nitrogen atmosphere and the resulting solution was stirred for 2h at r.t. The reaction mixture was quenched with slush (40 g) and acidified to pH 1-2 with HCl 6M, to obtain a precipitate that was filtered and washed with water and Et_2O to afford the titled compound **4**. 82% yield; silica gel TLC R_f *0.22* (MeOH/DCM 10 % *v/v*); δ_H (400 MHz, DMSO-d_6): 2.94 (t, J = 7.2, 2H, CH_2), 3.51 (q, J = 7.2*,* 2H, CH_2), 6.81 (d, J = 8.8, 2H, Ar), 7.31 (s, 2H, exchange with D_2O, SO_2NH_2), 7.45 (d, J = 8.8, 2H, Ar), 7.71 (d, J = 8.8, 2H, Ar), 7.77 (d, J = 8.8, 2H, Ar), 8.35 (t, J = 5.2, 1H, N*H*CO), 9.97 (s, 1H, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6): 35.9, 41.3, 115.7, 126.2, 126.7, 130.0, 130.1, 143.0, 144.9, 161.0, 166.9.

Experimental in agreement with reported data.[38]

N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-hydroxy-benzamide (6) .

N,N-Dimethylformamide dimethyl acetal (2.0 eq) was added to a solution of 4-(2-(4-hydroxybemzamido)ethyl)benzenesulfonamide **5** (0.94 g, 1.0 eq) in DMF (5 ml) cooled to 0°C. The reaction mixture was stirred 20' at r.t. and then quenched with H_2O (40 ml) to obtain a precipitate, that was filtered and washed with water and Et_2O to afford the titled compound **6**. 84% yield; silica gel TLC R_f *0.30* (MeOH/DCM 10 % *v/v*); δ_H (400 MHz, DMSO-d_6): 2.92 (m, 5H, CH_2 + CH_3), 3.17 (s, 3H, CH_3), 3.50 (q, J = 6,8 , 2H, CH_2), 6.81 (d, J = 8.8, 2H, Ar), 7.41 (d, J = 8.8, 2H, Ar), 7.71 (m, 4H, Ar), 8.23 (s, 1H), 8.35 (t, J = 5.2, 1H, N*H*CO), 9.97 (s, 1H, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6): 35.8, 35.9, 41.3, 41.8, 115.7, 126.2, 126.9, 129.9, 130.1, 141.8, 144.8, 160.6, 161.0, 166.9.

4-Allyloxy-N-{2-[4-(1-dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-benzamide (7).

K_2CO_3 (2.0 eq) and allyl bromide (1.2 eq) were added to a solution of N-{2-[4-(1-dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-hydroxy-benzamide **6** (1.0 g, 1.0 eq) in dry DMA (4 ml) under a nitrogen atmosphere. The suspension was stirred for 1.5h at 50°C and then quenched with water (40 ml). The obtained solid was filtered and purified by silica gel chromatography eluting with 10% MeOH/DCM to afford the titled compound **7**. 74% yield; silica gel TLC R_f *0.50* (MeOH/DCM 10 % *v/v*); δ_H (400 MHz, DMSO-d_6): 2.93 (m, 5H, CH_2 + CH_3), 3.17 (s, 3H, CH_3), 3.51 (q, *J* = 6,8 , 2H, CH_2), 4.65 (d, *J* = 5.2, 2H, CH_2), 5.31 (dd, *J* = 1.6, 10.4, 1H, C*H*), 5.44 (dd, *J* = 2, 17.4, 1H, C*H*), 6.08 (m, 1H, C*H*), 7.02 (d, *J* = 8.4, 2H, Ar), 7.42 (d, *J* = 8.8, 2H, Ar), 7.72 (d, *J* = 8.4, 2H, Ar), 7.82 (d, *J* = 8.8, 2H, Ar), 8.24 (s, 1H), 8.46 (t, *J* = 5.2, 1H, N*H*CO); δ_C (100 MHz, DMSO-d_6): 35.8, 35.9, 41.3, 41.8, 69.2, 115.1, 118.6, 126.9, 127.8, 129.8, 130.1, 134.3, 141.8, 144.7, 160.6, 161.3, 166.6.

N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide (8).[39]

$NaHCO_3$ (3.5 eq) and oxone (1.0 eq) were added to a solution of 4-allyloxy-N-{2-[4-(1-dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-benzamide **7** (1.60 g, 1.0 eq) in a mixture aceton/water 3:2 (40 ml) at 0°C and the resulting suspension was stirred 15' at the same temperature. Additional $NaHCO_3$ (3.5 eq) and oxone (1.0 eq) were added and the temperature was raised to r.t. The reaction mixture was stirred at r.t. for 4h, then the temperature was reduced again to 0°C and other two portions of $NaHCO_3$ (3.5 eq x 2) and oxone (1.0 eq x 2) were added, the second 15' after the first. The reaction mixture stirred at r.t. for 18h, then filtered to remove the undissolved salts and treated with an aqueous solution of $Na_2S_2O_4$ (5.0 eq). The obtained suspension was concentrated in vacuum and then extracted with ethyl acetate (4 x 25 ml). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give a residue purified by silica gel chromatography eluting with 10% MeOH/DCM to afford the titled compound **8**. 76% yield; silica gel TLC R_f *0.50* (MeOH/DCM 10 % *v/v*); δ_H (400

MHz, DMSO-d_6): 2.76 (dd, J = 2.6, 5.2, 1H, C*H*), 2.89 (t, J = 4.8, 1H, C*H*), 2.92 (m, 5H, CH_3 + CH_2), 3.17 (s, 3H, CH_3), 3.38 (m, 1H, C*H*), 3.52 (q, J = 6,8 , 2H, CH_2), 3.93 (dd, J = 6.6, 11.4, 1H, C*H*), 4.43 (dd, J = 2.7, 11.4, 1H, C*H*), 7.05 (d, J = 8.8, 2H, Ar), 7.42 (d, J = 8.4, 2H, Ar), 7.72 (d, J = 8.4, 2H, Ar), 7.82 (d, J = 8.8, 2H, Ar), 8.24 (s, 1H), 8.46 (t, J = 5.2, 1H, N*H*CO); δ_C (100 MHz, DMSO-d_6): 35.8, 35.9, 41.3, 41.8, 44.7, 50.5, 70.0, 114.9, 126.9, 128.0, 129.8, 130.1, 141.8, 144.7, 160.6, 161.4, 166.6.

General synthetic procedure of compounds 4-(3-alkylamino-2-hydroxy-propoxy)-N-[2-(4-sulfamoyl-phenyl)-ethyl]-benzamide 9-16, 22-29.[40,41]

Procedure 1

The proper epoxide **8** or **21** (0.25 g, 1.0 eq) was suspended in variable volume of primary amine or, alternatively in EtOH and treated with the appropriate primary amine (10 eq), and the reaction mixture was stirred at r.t. or under heating until starting materials were consumed (TLC monitoring). EtOH and excess of amine were removed under vacuum and the residue was triturated with water (**9-16**) or with Et_2O (**22-26**). A solution of the obtained deprotected free base in HCl 1.25M in MeOH was stirred at r.t. for 20' and then concentrated under vacuum to give a residue that was triturated with aceton to give the titled compounds **9-16, 22-26**.

Procedure 2

The proper secondary amine (3 eq) was added to a suspension of the proper epoxide **8** or **21** (0.25 g, 1.0 eq) in EtOH (8 ml) and reaction mixture stirred at 70°C until starting materials were consumed (TLC monitoring). EtOH and excess of amine were removed under vacuum and the obtained residue was triturated with water (**17-18**) or with Et_2O (**27-29**). A solution of the resulting protected free base in HCl 1.25 in MeOH was stirred at 80 °C in a sealed tube until starting materials were consumed (TLC monitoring). The solvent was removed under vacuum and the obtained residue was triturated with aceton to give the titled compounds **17-18**, **27-29.**

4-(2-Hydroxy-3-methylamino-propoxy)-N-[2-(4-sulfamoyl-phenyl)-ethyl]-benzamide hydrochloride (9).

Compound **9** was obtained according the general *procedure 1* earlier reported. N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was suspended in EtOH and treated with a 35% aqueous solution of methylamine overnight at r.t. and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **9**. 55% yield; silica gel TLC R_f *0.10* (TFA/MeO*H*/DCM 3/15/82% *v/v*); δ_H (400 MHz, DMSO-d_6): 2.64 (m, 3H, CH_3), 2.95 (t, J = 6.8, 2H, CH_2), 3.03 (m, 1H, C*H*), 3.15 (m, 1H, C*H*), 3.53 (q, J = 7.2, 2H, CH_2), 4.05 (d, J = 5.2, 2H, CH_2), 4.21 (m, 1H, C*H*), 5.95 (bs, 1H, exchange with D_2O, O*H*), 7.04 (d, J = 8.8, 2H, Ar), 7.33 (s, 2H, exchange with D_2O, SO_2NH_2), 7.46 (d, J = 8.4, 2H, Ar), 7.78 (d, J = 8.4, 2H, Ar), 7.85 (d, J = 8.8, 2H, Ar), 8.53 (t, J = 5.6, 1H, CON*H*), 8.73 (bd, exchange with D_2O, 2H, NH_2^+); δ_C (100 MHz, DMSO-d_6): 33.93, 35.78, 41.31, 51.79, 65.62, 70.78, 114.98, 126.61, 127.98, 129.89, 130.06, 142.95, 144.79, 161.42, 166.56; *m/z* (ESI positive) 408.2 $[M\text{-}Cl^-]^+$

4-(2-Hydroxy-3-(methylamino)propoxy)-N-(4-sulfamoylphenethyl)benzamide hydrochloride (10).

Compound **10** was obtained according the general *procedure 1* earlier reported. N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was treated with ethylamine in EtOH overnight at r.t. and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **10**. 54% yield; silica gel TLC R_f *0.15* (TFA/MeO*H*/DCM 3/15/82% *v/v*);δ_H (400 MHz, DMSO-d_6): 1.27 (t, J = 7.2, 3H, CH_3), 2.95 (t, J = 6.8, 2H, CH_2), 3.03 (m, 3H, C*H* + CH_2), 3.17 (m, 1H, C*H*), 3.52 (q, J = 7.2, 2H, CH_2), 4.06 (d, J = 5.2, 2H, CH_2), 4.22 (m, 1H, C*H*), 5.95 (bs, 1H, exchange with D_2O, O*H*), 7.04 (d, J = 8.8, 2H, Ar), 7.33 (s, 2H, exchange with D_2O, SO_2NH_2), 7.46 (d, J = 8.4, 2H, Ar),

7.78 (d, *J* = 8.4 Hz, 2H, Ar), 7.85 (d, *J* = 8.8, 2H, Ar), 8.51 (t, *J* = 5.6, 1H, exchange with D_2O, CON*H*), 8.71 (bd, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 11.70, 35.75, 41.28, 43.30, 49.80, 65.77, 70.78, 114.95, 126.59, 127.95, 129.87, 130.03, 142.93, 144.76, 161.40, 166.52; *m/z* (ESI positive) 422.2$[M\text{-}Cl^-]^+$

4-(2-Hydroxy-3-propylamino-propoxy)-N-[2-(4-sulfamoyl-phenyl)-ethyl]-benzamide hydrochloride (11).

Compound **11** was obtained according the general *procedure 1* earlier reported. N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was treated with propylamine (8 ml) for 8h at 50°C and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **11**. 64% yield; silica gel TLC R_f *0.24* (TFA/MeO*H*/DCM 3/15/82% *v/v*); δ_H (400 MHz, DMSO-d_6): 0.95 (t, *J* = 6.0, 3H, CH_3), 1.68 (m, 2H, CH_2), 2.94 (m, 4H, 2 x CH_2), 3.03 (m, 1H, C*H*), 3.17 (m, 1H, C*H*), 3.53 (m, 2H, CH_2), 4.06 (d, *J* = 5.2, 2H, CH_2), 4.21 (m, 1H, C*H*), 5.94 (bs, 1H, exchange with D_2O, O*H*), 7.04 (d, *J* = 8.8, 2H, Ar), 7.33 (s, 2H, exchange with D_2O, SO_2NH_2), 7.46 (d, *J* = 8.4, 2H, Ar), 7.77 (d, *J* = 8.4, 2H, Ar), 7.84 (d, *J* = 8.8, 2H, Ar), 8.50 (t, *J* = 5.6, 1H, CON*H*), 8.61 (bs, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 11.87, 19.68, 35.76, 41.30, 49.71, 50.28, 65.74, 70.80, 114.98, 126.61, 127.95, 129.89, 130.05, 142.94, 144.78, 161.44, 166.58; *m/z* (ESI positive) 436.2 $[M\text{-}Cl^-]^+$

4-(2-Hydroxy-3-isopropylamino-propoxy)-N-[2-(4-sulfamoyl-phenyl)-ethyl]-benzamide hydrochloride (12).

Compound **12** was obtained according the general *procedure 1* earlier reported. N-{2-[4-(1-dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was treated with isopropylamine (5 ml) for 6h at 50°C and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **12**. 54%

yield; silica gel TLC R_f *0.40* (TFA/MeO*H*/DCM 3/15/82% *v/v*); δ_H (400 MHz, DMSO-d_6): 1.29 (t, *J* = 6.0, 6H, 2 x CH_3,), 2.96 (t, *J* = 7.2, 2H, CH_2,), 3.01 (m, 1H, C*H*), 3.16 (m, 1H, C*H*), 3.53 (q, *J* = 6.4, 2H, CH_2,), 4.08 (d, *J* = 5.2, 2H, CH_2,), 4.25 (m, 1H, C*H*), 5.95 (d, *J* = 5.2, 1H, exchange with D_2O, O*H*), 7.05 (d, *J* = 8.8, 2H, Ar), 7.33 (s, 2H, exchange with D_2O, SO_2NH_2), 7.46 (d, *J* = 8.4, 2H, Ar), 7.78 (d, *J* = 8.4, 2H, Ar), 7.85 (d, *J* = 8.8, 2H, Ar), 8.52 (t, *J* = 5.6, 1H, exchange with D_2O, CON*H*), 8.70 (bd, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 24.01, 35.94, 49.27, 50.99, 69.42, 72.04, 115.05, 126.75, 126.97, 127.69, 129.96, 130.19, 143.09, 144.93, 162.07, 166.78; *m/z* (ESI positive) 436.2 $[M\text{-}Cl^-]^+$.

4-(2-Hydroxy-3-sec-butylamino-propoxy)-N-[2-(4-sulfamoyl-phenyl)-ethyl]-benzamide hydroclhoride (13).

Compound **13** was obtained according the general *procedure 1* earlier reported. N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was treated with sec-butylamine (8 ml) overnight at r.t. and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **13**. 49% yield; silica gel TLC R_f *0.31* (TFA/MeO*H*/DCM 3/15/82% *v/v*); δ_H (400 MHz, DMSO-d_6): 0.94 (td, *J* = 2.0, 7.6, 3H, CH_3), 1.27 (t, *J* = 6.4, 3H, CH_3), 1.51 (m, H, C*H*), 1.82 (m, 1H, C*H*), 2.96 (t, *J* = 7.2, 2H, CH_2), 3.03 (m, 1H, C*H*), 3.17 (m, 2H, CH_2), 3.53 (q, *J* = 6.4, 2H, CH_2), 4.08 (d, *J* = 5.2, 2H, CH_2), 4.26 (m, 1H, C*H*), 5.94 (d, *J* = 2.4, 1H, exchange with D_2O, O*H*), 7.05 (d, *J* = 8.8, 2H, Ar), 7.33 (s, 2H, exchange with D_2O, SO_2NH_2), 7.46 (d, *J* = 8.4, 2H, Ar), 7.77 (d, *J* = 8.4, 2H, Ar), 7.84 (d, *J* = 8.8, 2H, Ar), 8.52 (t, *J* = 5.6, 1H, CON*H*), 8.77 (bt, exchange with D_2O, 2H, NH_2^+); δ_C (100 MHz, DMSO-d_6): 10.66, 15.89, 25.90, 35.77, 41.31, 47.51, 55.88, 65.97, 70.80, 114.98, 126.61, 127.94, 129.90, 130.05, 142.95, 144.79, 161.45, 166.55; *m/z* (ESI positive) 450.2 $[M\text{-}Cl^-]^+$

4-(2-Hydroxy-3-t-butylamino-propoxy)-N-[2-(4-sulfamoyl-phenyl)-ethyl]-benzamide hydrochloride (14).

Compound **14** was obtained according the general *procedure 1* earlier reported. N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was treated with t-butylamine (8 ml) overnight at r.t. and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **14**. 47% yield; silica gel TLC R_f *0.25* (TFA/MeO*H*/DCM 3/15/82% *v/v*); δ_H (400 MHz, DMSO-d_6): 0.94 (td, *J* = 2.0, 7.6, 3H, CH_3), 1.35 (s, 9H, 3 x CH_3), 2.96 (m, 3H, C*H* + CH_2), 3.17 (m, 1H, C*H*), 3.52 (q, *J* = 6.4, 2H, CH_2), 4.10 (d, *J* = 5.2 , 2H, CH_2), 4.26 (m, 1H, C*H*), 5.95 (d, *J* = 4.8, 1H, exchange with D_2O, O*H*), 7.06 (d, *J* = 8.8, 2H, Ar), 7.33 (s, 2H, exchange with D_2O, SO_2NH_2), 7.46 (d, *J* = 8.4, 2H, Ar), 7.78 (d, *J* = 8.4, 2H, Ar), 7.85 (d, *J* = 8.8, 2H, Ar), 8.53 (t, *J* = 5.6, 1H, CON*H*), 8.74 (bt, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 25.90, 35.76, 41.29, 44.91, 57.31, 66.35, 70.76, 114.99, 126.60, 127.94, 129.88, 130.04, 142.94, 144.77, 161.44, 166.54; *m/z* (ESI positive) 450.2 $[M\text{-}Cl^-]^+$

4-(2-Hydroxy-3-benzylamino-propoxy)-N-[2-(4-sulfamoyl-phenyl)-ethyl]-benzamide (15).

Compound **15** was obtained according the general *procedure 1* earlier reported. N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was treated with benzylamine in EtOH for 7h at 50°C to afford the titled compound **15** as a free base. 68% yield; silica gel TLC R_f 0.35 (TFA/MeO*H*/DCM 3/15/82% *v/v*);δ_H (400 MHz, DMSO-d_6): 2.19 (bs, 1H, exchange with D_2O, N*H*), 2.62 (m, 2H, CH_2), 2.95 (m, 2H, CH_2), 3.53 (q, *J* = 6.4, 2H, CH_2), 3.76 (s, 2H, CH_2), 3.97 (m, 2H, CH_2), 4.07 (m, 1H, C*H*), 5.04 (d, *J* = 4.4, 1H, exchange with D_2O , O*H*), 7.01 (d, *J* = 8.8, 2H, Ar), 7.32 (m, 7H, partial exchange with D_2O, Ar + SO_2NH_2), 7.46 (d, *J* = 8.4, 2H, Ar), 7.78 (d, *J* = 8.4, 2H, Ar), 7.81 (d, *J* = 8.8, 2H, Ar), 8.46 (t, *J* = 5.6, 1H, CON*H*); δ_C (100 MHz, DMSO-d_6): 35.79, 41.31, 52.62, 54.01, 69.07, 71.82, 114.90, 126.61,

127.43, 127.55, 128.80, 129.02, 129.82, 130.04, 141.82, 142.94, 144.79, 161.92, 166.64; *m/z* (ESI positive) 484.2 $[M+H]^+$.

4-(2-Hydroxy-3-((2-hydroxyethyl)amino)propoxy)-N-(4-sulfamoylphenethyl)benzamide hydrochloride (16).

Compound **16** was obtained according the general *procedure 1* earlier reported. N-{2-[4-(1-dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was treated with ethanolamine in EtOH overnight at r.t. and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **16**. 46% yield; silica gel TLC R_f *0.09* (TFA/MeO*H*/DCM 3/15/82% *v/v*) (MeOH/DCM 10 % *v/v*); δ_H (400 MHz, DMSO-d_6): 2.96 (t, *J* = 6.8, 2H, CH_2), 3.03 (m, 3H, C*H* + CH_2), 3.17 (m, 1H, C*H*), 3.52 (q, *J* = 7.2, 2H, CH_2), 3.74 (m, 2H, CH_2), 4.06 (d, *J* = 4.8, 2H, CH_2), 4.27 (m, 1H, C*H*), 5.30 (bs, 1H, exchange with D_2O, O*H*), 5.93 (bs, 1H, exchange with D_2O, OH,), 7.04 (d, *J* = 8.8, 2H, Ar), 7.33 (s, 2H, exchange with D_2O, SO_2NH_2), 7.46 (d, *J* = 8.4, 2H, Ar), 7.78 (d, *J* = 8.4, 2H, Ar), 7.85 (d, *J* = 8.8, 2H, Ar), 8.53 (t, *J* = 5.6, 1H, exchange with D_2O, CON*H*), 8.80 (bd, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 35.75, 41.28, 50.27, 50.42, 65.65, 70.84, 114.96, 126.59, 127.93, 129.87, 130.03, 142.93, 114.76, 161.41, 166.53; *m/z* (ESI positive) 438.2 $[M\text{-}Cl^-]^+$.

4-(2-Hydroxy-3-morpholinopropoxy)-N-(4-sulfamoylphenethyl)benzamide hydrochloride (17).

Compound **17** was obtained according the general *procedure 2* earlier reported. N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was treated with morpholine in EtOH for 5h at 70°C and then the obtained protected free base was treated with HCl 1.25M in MeOH in sealed tube to afford the titled compound **17** as hydrochloride salt. 43% yield; silica gel TLC R_f *0.20* (TFA/MeO*H*/DCM 3/15/82% *v/v*); δ_H (400 MHz, DMSO-d_6): 2.96 (t, *J* = 6.8, 2H, CH_2), 2.28 (m, 4H, 2 x CH_2), 3.17 (m, 1H, C*H*), 3.53 (m,

4H, 2 x CH_2), 3.85 (m, 2H, CH_2), 4.03 (m, 2H, CH_2), 4.06 (d, J = 4.8, 2H, CH_2), 4.43 (m, 1H, CH), 6.04 (bs, 1H, exchange with D_2O, OH), 7.04 (d, J = 8.8, 2H, Ar), 7.33 (s, 2H, exchange with D_2O, SO_2NH_2), 7.46 (d, J = 8.4, 2H, Ar), 7.78 (d, J = 8.4, 2H, Ar), 7.85 (d, J = 8.8, 2H, Ar), 8.51 (t, J = 5.6, 1H, exchange with D_2O, CONH), 10.37 (bs, 1H, exchange with D_2O, NH^+); δ_C (100 MHz, DMSO-d_6): 35.76, 51.93, 53.56, 59.69, 63.98, 64.32, 71.10, 115.01, 126.61, 128.02, 129.91, 130.05, 142.95, 144.78, 161.41, 166.54; *m/z* (ESI positive) 464.2 $[M\text{-}Cl^-]^+$.

4-(2-Hydroxy-3-diisopropylamino-propoxy)-N-[2-(4-sulfamoyl-phenyl)-ethyl]-benzamide hydrochloride (18).

Compound **18** was obtained according the general *procedure 2* earlier reported. N-{2-[4-(1-Dimethylamino-ethylidenesulfamoyl)-phenyl]-ethyl}-4-oxiranylmethoxy-benzamide **8** (0.25 g, 1.0 eq) was treated with diisopropylamine in EtOH for 24h at 70°C and then the obtained protected free base was treated with HCl 1.25M in MeOH in sealed tube to afford the titled compound **18** as hydrochloride salt. 41% yield; silica gel TLC R_f *0.36* (TFA/MeO*H*/DCM 3/15/82% *v/v*); δ_H (400 MHz, DMSO-d_6): 1.30 (m, 12H, 4 x CH_3), 2.96 (t, J = 6.8, 2H, CH_2), 3.19 (m, 1H, CH), 3.33 (m, 1H, CH), 3.53 (m, 2H, CH_2), 3.74 (m, 2H, 2 x CH), 4.13 (m, 2H, CH_2), 4.27 (m, H, CH), 5.93 (d, J = 4.8, 1H, exchange with D_2O, OH), 7.06 (d, J = 8.8, 2H, Ar), 7.33 (s, 2H, exchange with D_2O, SO_2NH_2), 7.46 (d, J = 8.4, 2H, Ar), 7.78 (d, J = 8.4, 2H, Ar), 7.85 (d, J = 8.8, 2H, Ar), 8.51 (t, J = 5.6, 1H, exchange with D_2O, CONH), 9.01 (bs, 1H, exchange with D_2O, NH^+); δ_C (100 MHz, DMSO-d_6): 17.07, 17.94, 35.76, 51.08, 55.70, 56.55, 66.53, 70.49, 114.95, 126.60, 128.01, 129.90, 130.03, 142.95, 144.76, 161.32, 166.52; *m/z* (ESI positive) 478.2 $[M\text{-}Cl^-]^+$.

N,N-Dimethylaminomethylene-4-hydroxy-benzenesulfonamide (20).[42]

N,N-Dimethylformamide diethyl acetal (1.2eq) was added to a solution of 4-hydroxybenzenesulfonamide **19** (1.5 g, 1.0 eq) in DMF (1.5 ml) at 0°C and that was stirred for 0.5h at r.t. The reaction mixture was treated with EtOAc (40ml) and the obtained solid was filtered and

purified by silica gel chromatography eluting with 10% MeOH/DCM to afford the titled compound **20.** 76% yield; silica gel TLC R_f 0.22 (MeOH/DCM 5 % *v/v*); δ_H (400 MHz, DMSO-d_6): 2.92 (s, 3H, CH_3), 3.16 (s, 3H, CH_3), 6.88 (d, *J* = 8.4. 2H, Ar), 7.61 (d, *J* = 8.4. 2H, Ar), 8.18 (s, 1H, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6): 35.9, 41.7, 116.2, 129.1, 134.2, 160.3, 161.3. Experimental in agreement with reported data.[42]

N,N-Dimethylaminomethylene-4-oxiranylmethoxy-benzenesulfonamide (21).

Epibromohydrin (1.2 eq) was added dropwise to a suspension of N,N-dimethylaminomethylene-4-hydroxy-benzenesulfonamide **20** (0.5 g, 1.0 eq) and Cs_2CO_3 (1.5 eq) in dry DMF under a nitrogen atmosphere and that was stirred o.n. at 50°C. The reaction mixture was quenched with slush and extracted with EtOAc (2x20ml). The organic layers were washed with brine (4x15ml), dried over anhydrous Na_2SO_4 and concentrate under vacuum to give a residue that was triturated with Et_2O to afford the titled compound **21**. 76% yield; m.p. °C; silica gel TLC R_f *0.45* (MeOH/DCM 5 % *v/v*); δ_H (400 MHz, DMSO-d_6): 2.75 (dd, *J* = 2.6, 5.0, 1H, C*H*), 2.89 (t, *J* = 4.8, 1H, C*H*), 2.90 (s, 3H, CH_3), 2.92 (s, 3H, CH_3), 3.38 (m, 1H, C*H*), 3.93 (dd, *J* = 6.6, 11.4, 1H, C*H*), 4.45 (dd, *J* = 2.7, 11.4, 1H, C*H*), 7.11 (d, *J* = 7.0, 2H, Ar), 7.72 (d, *J* = 7.0, 2H, Ar); δ_C (100 MHz, DMSO-d_6): 35.9, 41.8, 44.7, 50.4, 70.2, 115.6, 128.9, 136.3, 160.5, 161.5.

4-(2-Hydroxy-3-methylamino-propoxy)-benzenesulfonamide hydrochloride (22).

Compound **22** was obtained according the general *procedure 1* earlier reported. N,N-dimethylaminomethylene-4-oxiranylmethoxy-benzenesulfonamide **21** (0.25 g, 1.0 eq) was suspended in EtOH and treated with a 35% aqueous solution of methylamine overnight at r.t. and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **22**. 42% yield; silica gel TLC R_f *0.12* (TFA/MeO*H*/DCM 3/15/82% *v/v*); δ_H (400 MHz, DMSO-d_6): 2.46 (s, 3H, CH_3), 2.95 (m, 1H, C*H*), 3.08 (m, 1H, C*H*), 4.02 (d, *J* = 4.4, 2H, CH_2), 4.16 (m, 1H, C*H*), 5.91 (bs, 1H, exchange with D_2O , O*H*), 7.07 (d, *J* =

7.8, 2H, Ar), 7.19 (s, 2H, exchange with D_2O, SO_2NH_2), 7.72 (d, *J* = 7.8, 2H, Ar), 8.83 (bd, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 33.88, 51.71, 65.54, 70.98, 115.51, 128.59, 137.46, 161.56; *m/z* (ESI positive) 261.1 $[M\text{-}Cl^-]^+$.

4-(2-Hydroxy-3-isopropylamino-propoxy)-benzenesulfonamide hydrochloride (23). Compound **23** was obtained according the general *procedure 1* earlier reported. N,N-Dimethylaminomethylene-4-oxiranylmethoxy-benzenesulfonamide **21** (0.25 g, 1.0 eq) was treated with isopropylamine (5 ml) for 6h at 50°C and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **23**. 40% yield; silica gel TLC R_f *0.24* (TFA/MeO*H*/DCM 3/15/82% *v/v*); δ_H (400 MHz, DMSO-d_6): 1.30 (t, *J* = 6.0, 6H, 2 x CH_3), 3.01 (m, 1H, C*H*), 3.16 (m, 1H, C*H*), 3.35 (m, 2H, 2 x C*H*), 4.12 (d, *J* = 4.4, 2H, CH_2), 4.29 (m, 1H, *CH*), 5.97 (bs, 1H, exchange with D_2O ,O*H*), 7.15 (d, *J* = 7.0, 2H, Ar), 7.27 (s, 2H, exchange with D_2O, SO_2NH_2), 7.80 (d, 2H, *J* = 7.0, Ar), 8.89 (bd, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 19.11, 19.56, 47.51, 50.77, 65.93, 71.03, 115.52, 128.60, 137.45, 161.59; *m/z* (ESI positive) 289.1 $[M\text{-}Cl^-]^+$.

4-(3-(Sec-butylamino)-2-hydroxypropoxy)benzenesulfonamide hydrochloride (24). Compound **24** was obtained according the general *procedure 1* earlier reported. N,N-Dimethylaminomethylene-4-oxiranylmethoxy-benzenesulfonamide **21** (0.25 g, 1.0 eq) was treated with sec-butylamine (5 ml) for 6h at 50°C and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **24**. 40% yield; silica gel TLC R_f *0.32* (TFA/MeO*H*/DCM 3/12/85% *v/v*); δ_H (400 MHz, DMSO-d_6): 0.94 (t, *J* = 7.6, 3H, CH_3), 1.27 (t, *J* = 6.4, 3H, CH_3), 1.53 (m, H, C*H*), 1.87 (m, 1H, C*H*), 3.03 (m, 1H, C*H*), 3.17 (m, 2H, CH_2), 4.12 (d, *J* = 5.2, 2H, CH_2), 4.30 (m, 1H, C*H*), 5.97 (d, *J* = 2.4, 1H, exchange with D_2O, O*H*), 7.15 (d, *J* = 8.4, 2H, Ar), 7.27 (s, 2H, exchange with D_2O, SO_2NH_2), 7.80 (d, 2H, *J* = 8.4, Ar),

8.90 (bt, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 10.7, 15.9, 25.9, 47.5, 55.9, 65.9, 71.0, 115.5, 128.6, 137.4, 161.6; *m/z* (ESI positive) 303.1 $[M\text{-}Cl^-]^+$.

4-(3-(Tert-butylamino)-2-hydroxypropoxy)benzenesulfonamide hydrochloride (25).

Compound **25** was obtained according the general *procedure 1* earlier reported. N,N-dimethylaminomethylene-4-oxiranylmethoxy-benzenesulfonamide **21** (0.25 g, 1.0 eq) was treated with tert-butylamine (5 ml) for 6h at 50°C and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **25**. 43% yield; silica gel TLC R_f *0.30* (TFA/MeO*H*/DCM 3/12/85% *v/v*); δ_H (400 MHz, DMSO-d_6): 1.36 (s, 9H, 3 x CH_3), 2.97 (m, 1H, C*H*), 3.16 (m, 1H, C*H*), 4.15 (m, 2H, CH_2), 4.30 (m, 1H, C*H*), 5.98 (bs, 1H, exchange with D_2O, O*H*), 7.16 (d, *J* = 8.4, 2H, Ar), 7.28 (s, 2H, exchange with D_2O, SO_2NH_2), 7.80 (d, 2H, *J* = 8.4, Ar), 8.99 (bdt, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 25.9, 45.0, 57.3, 66.3, 71.0, 115.5, 128.6, 137.4, 161.6; *m/z* (ESI positive) 303.1 $[M\text{-}Cl^-]^+$.

4-(3-(Benzylamino)-2-hydroxypropoxy)benzenesulfonamide hydrochloride (26).

Compound **26** was obtained according the general *procedure 1* earlier reported. N,N-dimethylaminomethylene-4-oxiranylmethoxy-benzenesulfonamide **21** (0.25 g, 1.0 eq) was treated with benzylamine (3 ml) for 8h at 50°C and then the obtained free base was treated with HCl 1.25M in MeOH (10 ml) to afford the corresponding hydrochloride salt **26**. 54% yield; silica gel TLC R_f *0.34* (TFA/MeO*H*/DCM 3/12/85% *v/v*); δ_H (400 MHz, DMSO-d_6): 2.97 (m, 1H, C*H*), 3.16 (m, 1H, C*H*), 4.09 (d, *J* = 5.2, 2H, CH_2), 4.24 (m, 2H, CH_2), 4.33 (m, 1H, C*H*), 5.98 (bs, 1H, exchange with D_2O ,O*H*), 7.12 (d, *J* = 7.0, 2H, Ar), 7.26 (s, 2H, exchange with D_2O, SO_2NH_2), 7.47 (m, 3H, Ar), 7.62 (m, 2H, Ar), 7.79 (d, 2H, *J* = 7.0, Ar), 9.43 (bd, 2H, exchange with D_2O, NH_2^+); δ_C (100 MHz, DMSO-d_6): 49.7, 51.1, 65.6, 70.9, 115.5, 128.5, 129.5, 129.8, 131.1, 132.6, 137.4, 161.5; *m/z* (ESI positive) 337.1 $[M\text{-}Cl^-]^+$.

4-(2-Hydroxy-3-(phenethylamino)propoxy)benzenesulfonamide hydrochloride (27).

Compound **27** was obtained according the general *procedure 2* earlier reported. N,N-dimethylaminomethylene-4-oxiranylmethoxy-benzenesulfonamide **21** (0.25 g, 1.0 eq) was treated with phenethylamine in EtOH for 6h at 70°C and then the obtained protected free base was treated with HCl 1.25M in MeOH in sealed tube to afford the corresponding hydrochloride salt **27**. 35% yield; silica gel TLC R_f *0.37* (TFA/MeO*H*/DCM 3/12/85% *v/v*); δ_H (400 MHz, DMSO-d_6): 3.05 (m, 3H, CH_2 + C*H*), 3.24 (m, 3H, CH_2 + C*H*), 4.09 (d, *J* = 5.2, 2H, CH_2), 4.31 (m, 1H, C*H*), 6.02 (d, *J* = 4.8, 1H, exchange with D_2O, O*H*), 7.14 (d, *J* = 8.8, 2H, Ar), 7.29 (m, 6H, partial exchange with D_2O, SO_2NH_2 + Ar), 7.38 (m, 1H, Ar), 7.80 (d, 2H, *J* = 8.8, Ar), 9.19 (bs, 2H, exchange with D_2O, NH_2^+) ; δ_C (100 MHz, DMSO-d_6): 32.3, 49.1, 50.3, 65.7, 71.0, 115.5, 127.6, 128.6, 129.5, 129.6, 137.5, 138.3, 161.6; *m/z* (ESI positive) 351.1 $[M\text{-}Cl^-]^+$.

4-(2-Hydroxy-3-((2-phenoxyethyl)amino)propoxy)benzenesulfonamide hydrochloride (28).

Compound **28** was obtained according the general *procedure 2* earlier reported. N,N-dimethylaminomethylene-4-oxiranylmethoxy-benzenesulfonamide **21** (0.25 g, 1.0 eq) was treated with 2-(phenoxy)ethylamine in EtOH for 6h at 70°C and then the obtained protected free base was treated with HCl 1.25M in MeOH in sealed tube to afford the corresponding hydrochloride salt **28**. 29% yield; silica gel TLC R_f *0.35* (TFA/MeO*H*/DCM 3/12/85% *v/v*); δ_H (400 MHz, DMSO-d_6): δ_H (400 MHz, DMSO-d_6): 3.15 (m, 1H, C*H*), 3.32 (m, 1H, C*H*), 4.95 (m, 2H, CH_2), 4.12 (d, *J* = 5.2, 2H, CH_2), 4.34 (m, 3H, CH_2 + C*H*), 5.98 (d, *J* = 4.8, 1H, exchange with D_2O, O*H*), 7.02 (m, 3H, Ar), 7.14 (d, *J* = 8.8, 2H, Ar), 7.27 (s, 2H, exchange with D_2O, SO_2NH_2), 7.37 (t, *J* = 8.0, 1H, Ar), 7.81 (d, 2H, *J* = 8.8, Ar), 9.09 (bd, 2H, exchange with D_2O, NH_2^+) ; δ_C (100 MHz, DMSO-d_6): 47.1, 50.6, 64.1, 65.6, 71.0, 115.5, 115.6, 122.1, 128.6, 130.5, 137.5, 158.6, 161.5; ; *m/z* (ESI positive) 367.1 $[M\text{-}Cl^-]^+$.

4-(2-Hydroxy-3-(4-methylpiperazin-1-yl)propoxy)benzenesulfonamide (29).

Compound **29** was obtained according the general *procedure 2* earlier reported. N,N-dimethylaminomethylene-4-oxiranylmethoxy-benzenesulfonamide **21** (0.25 g, 1.0 eq) was treated with 1-methylpiperazine in EtOH for 6h at 70°C and then the obtained protected free base was treated with HCl 1.25M in MeOH in sealed tube. The di-hydrochloride salt of **29** was washed with aceton and thus treated with a $NaHCO_{3(aq)}$ saturated solution, which was extracted with EtOAc (3x20ml) and the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give the titled compound **29**. 36% yield; silica gel TLC R_f *0.08* (TFA/MeO*H*/DCM 3/12/85% *v/v*); δ_H (400 MHz, DMSO-d_6): 2.17 (s, 3H, CH_3), 2.38 (m, 4H, 2 x CH_2), 2.48 (m, 6H, 2 x CH_2 + 2 x C*H*), 3.96 (m, 2H, CH_2), 4.09 (m, 1H, C*H*), 4.93 (bs, 1H, exchange with D_2O ,O*H*), 7.12 (d, *J* = 8.8, 2H, Ar), 7.23 (s, 2H, exchange with D_2O, SO_2NH_2), 7.77 (d, 2H, *J* = 8.8, Ar); δ_C (100 MHz, DMSO-d_6): 46.7, 54.3, 55.7, 61.8, 67.3, 72.4, 115.4, 128.5, 137.0, 162.1; *m/z* (ESI positive) 330.1 $[M\text{-}Cl^-]^+$.

CA inhibition.

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[33] 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. 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,[43] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[44]

β-ADR -Binding assay.

Plasmids

The coding region sequences (CDSs) of β_1 and β_2 adrenergic receptor were cloned inside AID-express-puro2 plasmid [45] replacing the coding sequence of Activation Induced Deaminase (AID). We used this plasmid for the presence of an Internal Ribosome Entry Site (IRES) sequence, which allows the expression of a reporter gene (GFP) under the same promoter of our CDS, producing only one mRNA but two different proteins; this let us analyze the presence and the amount of the β adrenergic receptors by FACS analysis for GFP positive cells.

The CDS of β_1 adrenergic receptor was cloned by digestion using BamHI and ApaI from pcDNA3 Flag β_1 adrenergic receptor (a gift from Robert Lefkowitz -Addgene plasmid # 14698); this fragment was then blunted (using Cloned Pfu DNA Polymerase AD from Agilent Technologies at 72ºC for 15min) and cloned inside AID-express-puro2 digested by NheI and BglII.

Differently, for β_2 adrenergic receptor we extracted RNA from HEK293T cells (using TRIzol – Thermo Fisher Scientific) to obtain cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). After, we amplified the receptor by PCR adding NheI and BamHI restriction sites to the ends of the fragment to clone it inside AID-express-puro2 digested by NheI and BglII (forward primer: aaaGCTAGCatggggcaacccgggaacg; reverse primer: aaaGGATCCttacagcagtgagtcattt). These plasmids were used to transfect HEK293T cells, for stably expression of β_1 and β_2 adrenergic receptors.

Cell culture and transfections

HEK293T cells were cultured at 37 °C, 5% CO_2, in Dulbecco's modified eagle medium (DMEM, EuroClone S.p.A Pero, Milano, Italy) supplemented with 10% fetal bovine serum (FBS; Carlo Erba Reagents, Cornaredo, Milano, Italy), 2 mM L -glutamine (Carlo Erba Reagents, Cornaredo, Milano, Italy), and 1 mM penicillin/streptomycin (Carlo Erba Reagents, Cornaredo, Milano, Italy). Transfections were performed in six-well plates (5 x 10^5 cells) using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) or GeneJuice (Novagen s.r.l., Podenzano, Piacenza, Italy) according to the manufacturer's instructions. 48h after transfections cells were plated in 96-well plates in medium supplemented with puromycin (1.5 μg/ml), in several dilution to obtain single clones. Colonies were picked after 10–14 days and only wells bearing single colonies were expanded for FACS analysis for the presence of GFP. Clones GFP positive were then utilized for further analysis.

Membrane preparation.

HEK293T cells stably expressing the human cloned β_1 and β_2 ADRs were grown at approximately 80% confluence. Then, they were harvested by scraping the culture plate with a cell scraper, washed by centrifugation at 500 g at room temperature and homogenized in ice-cold buffer (50 mM Tris HCl, pH 7.4) with an Ultra-Turrax (IKA Labortechnik, Staufen, Germany) twice for 20 s at half speed. The homogenates were centrifuged for 10 min at 50,000 g at 4°C in Avanti J-26XP centrifuge (Beckman Coulter S.p.A., Cassina de' Pecchi, Milano, Italy). The resultant membrane pellet was resuspended in buffer and stored frozen at -80°C. Protein concentration was determined colorimetrically using a commercial protein determination kit based on the BCA reaction (Thermo Scientific, Rockford, IL, USA).

Radioligand binding experiments

Saturation binding experiments were performed by incubating cell membranes (about 20 μg/ml of protein) in a total volume of 1 mL incubation buffer (50 mM Tris HCl pH 7.4), containing increasing concentrations (approximately 0.03-0.1-0.3-1-3 nM) of [^{3}H]-CGP12177 (Perkin-Elmer Life and Analytical Science, Monza, Italy). Incubations were carried out at 25°C for 90 min. Non-specific binding was determined in the presence of 10 μM propranolol.

Reactions were terminated by rapid filtration through glass fiber filters grade MGB (Sartorius Italy S.r.l., Bagno a Ripoli, Firenze, Italy) that had been soaked for 60 min in 0.5% polyethyleneamine using a Brandell cell harvester (Biomedical Research and Development Laboratory, Inc Atlas Drive, Gaithersburg, MD, USA).

Filters were washed three times with 4 ml aliquots of ice-cold milliQ water and dried before the addition of 4.5 ml of scintillation cocktail (Filter Count, Perkin-Elmer Life and Analytical Science, Monza, Italy). The radioactivity bound to the filters was measured using TRICARB 1100 scintillation counter (Perkin-Elmer Life and Analytical Science, Monza, Italy).

Clones expressing about 29 pmol/mg protein and 12 pmol/mg protein of the β_1 and β_2 ADRs respectively were used for all subsequent experiments (Supplemental Figure 3).

Competition experiments were performed by incubating 20 μg/ml of protein with increasing amounts of test compound (from 1 nM-100 μM) and 0.2 nM [^{3}H]-CGP12177 for β_1-ADR and β_2-ADR binding assay, in a final incubation volume of 250 μL in 96 well plates (Sarstedt s.r.l., Verona, Italy). Non-specific binding was determined in the presence of 10 μM propranolol. The incubations were terminated by rapid vacuum filtration over WhatmanGF/B using a FilterMate harvester (Perkin-Elmer Life and Analytical Science, Monza, Italy). Each filter was abundantly washed with ice-cold milliQ water.

Radioactivity adherent to the filters was quantified in a Topcount NXT Microplate Scintillation Counter (Perkin-Elmer Life and Analytical Science, Monza, Italy) using Microscint20 (Perkin-Elmer Life and Analytical Science, Monza, Italy) scintillator after 4 hours.

Stock solutions of tested compounds were made in DMSO, and dilutions were usually made in the incubation buffer. DMSO at the highest concentration used had no effect on binding.

Data analysis

All experiments are conducted in duplicate and data are presented as mean ± S.E.M., unless otherwise noted. Saturation radioligand binding experiments were analyzed by fitting rectangular hyperbolic to the experimental data to obtain Bmax (the maximal binding capacity) and Kd (the dissociation constant). Data from equilibrium binding studies were corrected for non-specific binding and were analyzed by computer-aided nonlinear regression analysis using a four parameter logistic equation. IC_{50} values were converted to binding constants K_i using the Cheng-Prusoff correction. All curve fitting were performed using the Prism programme 5.02 (Graphpad Software, San Diego, CA, USA).

X-ray Crystallography.

The expression and purification of hCA II and hCA IX-mimic were performed as previously described.[46] hCA IX-mimic is a molecule of hCA II with seven active site mutations (A65S, N67Q, E69T, I91L, F131V, K170E and L204A) to mimic the residues in wild type hCA IX. The hCA IX-mimic is utilized due to the ease of expression and crystallization in relation to wild type. Protein was expressed in BL21(DE3) competent cells and purified utilizing the affinity chromatography technique on a p-(aminomethyl)benzenesulfonamide column. Purity was verified via SDS-PAGE. The formation of the protein-inhibitor complex was initiated utilizing the co-crystallization technique. Crystals were grown via the hanging drop vaporization method with a 1:1 protein to precipitant solution ratio (1.6 M Na-Citrate, 50 mM Tris, pH 7.8) and growth was observed after 2 weeks. The crystals were additionally soaked in ~10mM inhibitor for 5 min prior to freezing.

X-ray diffraction data was collected on a Pilatus 6M detector at the Cornell High Energy Synchrotron Source (CHESS) F1 beamline with a wavelength of 0.977 Å. A crystal-to-detector distance of 270 mm, 1° oscillation angle, and exposure time of 2 sec per image were utilized to collect data. A total of 270 images were collected. The data was indexed, integrated, and scaled to the $P2_1$ monoclinic space group in *HKL2000*.[47] Molecular replacement (search model PDB: 3KS3) was used to determine phases. Refinement of the structure and ligand restraint files were generated in *Phenix*.[48] Interactions between the inhibitor and protein were analyzed in Coot and figures generated in *PyMol*.[49]

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 Model 30™Pneumatonometer (Reichert Inc. Depew, NY, USA) prior to hypertonic saline injection (basal) at 1, 2, 3, and 4 h after administration of the drug. Vehicle (phosphate buffer pH 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%).[43,44] 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.[50] All efforts were made to minimize animal suffering and to reduce the number of animals used.

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■ ABBREVIATIONS USED

CA, carbonic anhydrase; CAI, CA inhibitor; K_I, inhibition constant; β-ADR, β-adrenoreceptor; IOP, intra ocular pressure; DCM, dichloromethane; TFA, trifluoroacetic acid; DMF, dimethylformammide; DMA, dimethylacetammide.

■ ASSOCIATED CONTENT

Coordinates and structure factors for hCA II and hCA IX-mimic complexes with **12** and **23** have been deposited in the Protein Data Bank (PDB) with accession codes: hCAII_12: 5WLV, hCA IX-

mimic_12: 5WLU, hCA II_23: 5WLT, hCA IX-mimic_23: 5WLR. Authors will release the atomic coordinates and experimental data upon article publication.

■ **SUPPORTING INFORMATION.** Supporting information is available free of charge on the ACS Publications website: SMILES representation for compounds (CSV), additional X-ray crystallography figures (crystal contact inhibitors) and [^{3}H]-CGP12177 saturation binding experiments to β_1 and β_2-ADRs.

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