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Synthesis and crystallographic analysis of new sulfonamides incorporating NO-donating moieties with potent antiglaucoma action ☆

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ABSTRACT

Several aromatic/heterocyclic sulfonamide scaffolds have been used to synthesize compounds incorporating NO-donating moieties of the nitrate ester type, which have been investigated for the inhibition of five physiologically relevant human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms: hCA I (offtarget), II, IV and XII (antiglaucoma targets) and IX (antitumor target). Some of the new compounds showed effective in vitro inhibition of the target isoforms involved in glaucoma, and the X-ray crystal structure of one of them revealed factors associated with the marked inhibitory activity. In an animal model of ocular hypertension, one of the new compounds was twice more effective than dorzolamide in reducing elevated intraocular pressure characteristic of this disease, anticipating their potential for the treatment of glaucoma.

Inhibitors of zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) have various clinical applications as diuretic, antiglaucoma, antiobesity or antitumor drugs.[1] Various CA isoforms are responsible for specific physiological functions, and drugs with such a diversity of actions target different isozymes of the 15 presently known in humans.[2–6] Sulfonamides and sulfamates constitute the principal type of classical CA inhibitors (CAIs),[1] whereas other chemotypes (coumarins, polyamines and phenols), were only recently investigated in detail for such an action.[7–10] The inhibitors of the sulfonamide/sulfamate type bind as anions to the catalytically critical Zn(II) ion from the enzyme active site, also participating in many other interaction (hydrogen bond networks; van der Waals contacts, π-stacking, etc.) with amino acid residues from the hydrophobic and hydrophilic halves of the enzyme active site, as shown by X-ray crystallographic studies of enzyme–inhibitor complexes.[1–6] X-ray crystal structures are available for many adducts of several isozymes (i.e., CA I, II, IV, VA, VII, IX, XII, XIII and XIV) mostly with sulfonamides, and with several sulfamates/sulfamides.[11] Recently, the various classes of non-classical inhibitors have also been investigated from the crystallographic viewpoint, allowing for a better understanding of the various inhibition mechanisms of these enzymes.[11]

Several sulfonamide CAIs such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** or dichlorophenamide **DCP**, clinically have been used as systemically acting antiglaucoma agents for more than 50 years.[12] Although glaucoma treatment with such CAIs is effective in reducing elevated intraocular pressure (IOP), systemic administration of such drugs leads to a wide range of side-effects due to inhibition of the enzyme present in other tissues (kidneys, red cells, stomach, lungs, etc.) than the eye.[12] Indeed, these sulfonamides are potent inhibitors of most of the 13 catalytically active isoforms described so far in mammals.[1] The possibility of the topical administration of classical drugs from this class (**AAZ, MZA, EZA** or **DCP**) was investigated extensively in the 50s and 60s, but negative results have been constantly obtained, and for more than 40 years it was considered that CAIs could only be given systemically.[1,12] In the mid 90s the first clinically used, topically effective antiglaucoma sulfonamide, dorzolamide **DZA**, has been discovered.[12] The approach for achieving this compound (and also brinzolamide **BRZ**, the second such clinically used pharmacological agent)[12] is known as the 'ring approach', as it involved the exploration of a wide range of ring systems to which sulfamoyl moieties were incorporated.[1–6] We have explored thereafter an

☆ Coordinates and structure factors have been deposited in the Protein Data Bank as entry 3NI5.

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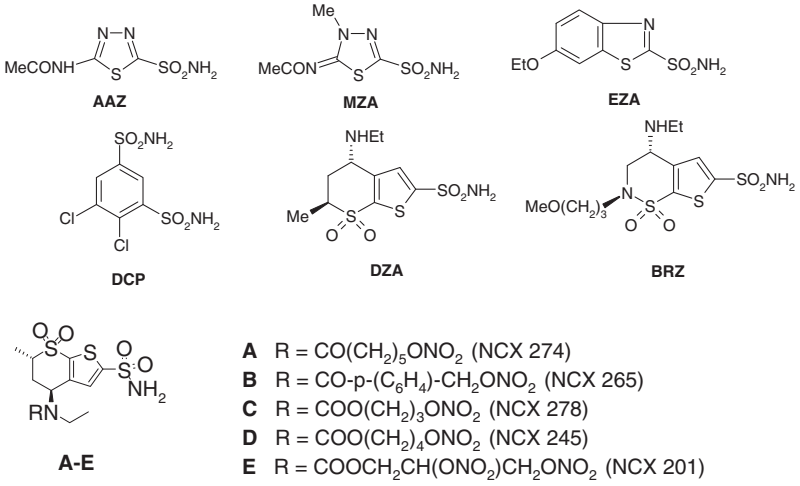
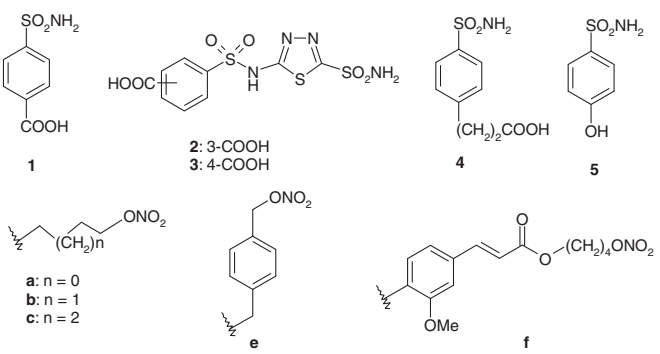


alternative approach (the 'tail approach') for the design of topically acting antiglaucoma sulfonamides, which consisted in attaching tails that will induce the desired physico-chemical properties (such as for example water solubility, enhanced penetrability through the cornea, etc.) to scaffolds of simple aromatic/heterocyclic sulfonamides incorporating derivatizable moieties of the amino/hydroxy/carboxy type.[6]

We recently applied this approach to investigate some dorzolamide derivatives incorporating NO-donating moieties of types **A–E** (NCX201–NCX278).[13] NO-donors are in fact a relatively underexplored class in the field of ocular therapeutics, despite the ubiquitous presence of NO targets in all eye compartments devoted to aqueous humor production and drainage.[14] It has been observed that hypertensive glaucoma patients have a decreased NO/cGMP content in the aqueous humor[15] and that some NO-donors have been shown to decrease IOP in normal and pathological conditions.[12,15,16] Compounds of types **A–E** investigated earlier[13] showed interesting CA inhibitory and IOP lowering effects in normotensive rabbits. A similar activity has been reported for prostaglandins derivatized with NO-donating moieties by NicOx in collaboration with Pfizer.[14b]

In the present study our aim was to prepare new sulfonamide CAIs incorporating NO-releasing moieties. Sulfonamides have been chosen for this purpose as they are the most investigated class of CAIs. The hydroxyl or carboxyl moieties present in some aromatic/heteroaromatic sulfonamides were chosen to be derivatized by means of ester moieties which can release NO and sulfonamides in vivo, both of which have beneficial effects in controlling glaucoma.[12–15]

Five different sulfonamide scaffolds have been used to prepare the new CAIs incorporating NO-donating moieties reported here:



4-carboxy-benzenesulfonamide **1**, 3- or 4-carboxy-benzolamides **2** and **3**, 4-(2carboxyethyl)-benzenesulfonamide **4** and 4-hydroxy-benzenesulfonamide **5**.[17] The carboxylic or phenol moieties of **1–5** have been derivatized to introduce NO-containing donor groups of types **a–e**, which incorporate either aliphatic C3–C5 moieties or the aromatic ones of type **e** and **f** (ferrulic acid derivative).[18]

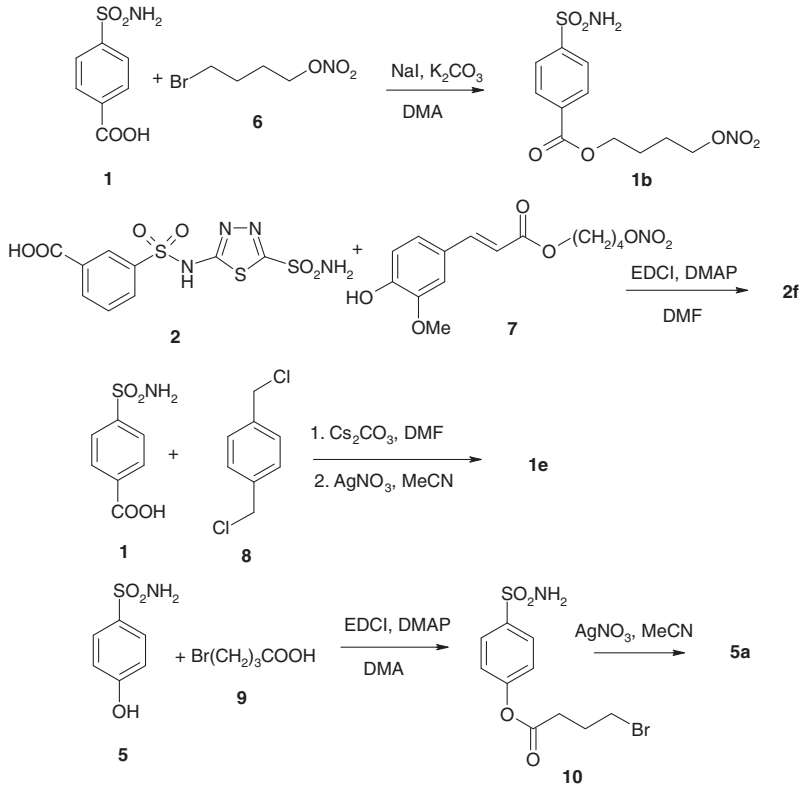
The preparation of some of the new compounds reported here is depicted in Scheme 1. For simplicity, the sulfonamide incorporating the NO-donating moieties will be denominated by a combination of figures (indicating the original sulfonamide **1–5** from which it has been prepared) and letters, indicating the NO-donating moiety which has been used to derivatize the sulfonamide. For example, **1e** is the ester of the sulfonamide **1** with the mononitrate of 1,4-di(hydroxymethyl)-benzene **e**.[19]

The aliphatic nitrate esters, such as **1b**, were prepared by reaction of the carboxylate potassium salt of sulfonamide **1** and the bromo nitrate ester **6** (in the presence of sodium iodide),[18] whereas esterification of carboxylic acids (of types **1–4**) with the ferrulic acid nitrate ester **7** (NCX 2057)[18] in the presence of carbodiimides, afforded derivatives such as **2f**, shown in Scheme 1. Alternatively, some of these esters have been prepared by reaction of the carboxylate caesium salt of **1** with 1 equiv of 1,4-di(chloromethyl)benzene, leading to a monochlorinated intermediate which by reaction with silver nitrate in acetonitrile was transformed to the nitrate ester **1e** (Scheme 1). The phenol **5** has been derivatized in a rather similar manner: reaction with 4-bromobutanoic acid in the presence of carbodiimides led to the bromo derivative **10** which has been converted to the nitrate ester **5a** by reaction with silver nitrate in acetonitrile (Scheme 1).[19] All compounds reported here were characterized by physico-chemical methods which confirmed their structures.[19]

Inhibition data against five physiologically relevant CA isoforms (i.e., hCA I and II—cytosolic isoforms; hCA IV—membrane-associated one; hCA IX and XII; transmembrane enzymes)[1] with compounds **1b–5c** reported here and standard sulfonamide CAIs (**AAZ, DCP** and **DZA**) are shown in Table 1.[20]

It may be observed that against the slow cytosolic isoform hCA I (an offtarget enzyme for antiglaucoma agents)[1] compound **1f** was a strong inhibitor (K_I of 15 nM), **2b, 3b, 3f** and **5b** showed effective inhibition (K_Is in the range of 32–43 nM) whereas the remaining compounds were less effective inhibitors (K_Is in the range of 137–950 nM) similar to the clinically used sulfonamides.

The main target isoform for antiglaucoma sulfonamides is hCA II.[1] Data of Table 1 show that several of the new sulfonamides reported here, such as **1b, 1f, 2b, 2f, 3b, 3f** and **5b** acted as very good inhibitors of this isozyme, with K_Is in the range of 10–33 nM, in the



Scheme 1. Synthesis of some of the sulfonamides incorporating NO-donating moieties of type **1–5(a–f)**. DMA, dimethylacetmide; EDCI, ethyl-diisoprylaminecarbodiimide, DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide.

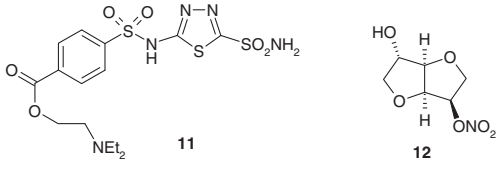
Table 1
hCA I, II, IX and XII inhibition data with sulfonamides **1b–5c** and **AAZ, DCP** and **DZA** as standard inhibitors, by a stopped-flow CO_2 hydration assay method[20]

Compd	K_i^* (nM)				
	hCA I[a]	hCA II[a]	hCA IV[a]	hCA IX[b]	hCA XII[b]
1b	189	18	44	165	30
1e	242	198	345	253	147
1f	15	10	47	11	19
2b	40	28	154	235	31
2f	137	33	225	177	42
3b	32	10	46	10	31
3f	43	14	38	11	43
4f	395	101	139	93	87
5a	587	88	148	83	83
5b	41	12	76	25	29
5c	950	71	89	85	116
AAZ	250	12	74	25	5.7
DCP	1200	38	15000	50	50
DZA	50000	9	8500	52	3.5

[a] Full length, cytosolic isoform.
[b] Catalytic domain, recombinant enzyme.
[*] Errors in the range of ±5–10% of the reported value, from three different determinations.

same range as those of the systemically used antiglaucoma sulfonamides **AAZ** and **DCP** or the topically acting one **DZA**.[1] It may be observed that all sulfonamide scaffolds **1–5** and most NO-containing tails may lead to effective CAIs targeting hCA II. However, the esters **1e, 4f, 5a** and **5c** were less active as hCA II inhibitors compared to the above mentioned derivatives, showing K_Is in the range of 71–198 nM (Table 1).hCA IV, which is also found in the ciliary processes within the eyes,[1,12] was also effectively inhibited by some of these new compounds reported here, such as **1b, 1f, 3b** and **3f** (K_Is in the range of 38–47 nM). All these compounds were thus much more effective hCA IV inhibitors compared to the clinically used drugs from Table 1. The remaining new compounds were on the other hand less effective as inhibitors of this isoform, with K_Is in the range of 76–345 nM.

The tumor associated isoforms hCA IX and XII were also inhibited by all these compounds. Thus, against hCA IX the best inhibitors were **1f, 3b**, **3f**, and **5a**, which showed K_Is in the range of 10–25 nM. Another group of derivatives (**4f, 5a** and **5c**) were medium efficiency inhibitors (K_Is in the range of 83–93 nM) whereas the remaining ones were medium efficacy compounds (K_Is in the range of 165–253 nM). hCA XII has been shown to be overexpressed in the eyes of glaucomatous patients,[21] and inhibition of this enzyme may be relevant for developing novel antiglaucoma agents. Indeed, several of the new sulfonamides reported here (**1b, 1f, 2b, 2f, 3b, 3f** and **5b**) showed K_Is in the range of 19–43 nM for inhibiting this isoform. The remaining derivatives were less effective hCA XII inhibitors (K_Is in the range of 83–147 nM).



In order to better understand the inhibition of CAs with these compounds, we resolved the X-ray crystal structure of the target isoform hCA II complexed with sulfonamide **2b** (Figs. 1 and 2). Crystallographic parameters and refinement statistics for the hCA II—**2b** adduct are shown in Table 2. The electron density of almost all atoms present in the sulfonamide **2b** bound to hCA II is shown in Figure 1, whereas the interactions that the compound makes with the active site of the enzyme are detailed in Figure 2.[22–26]

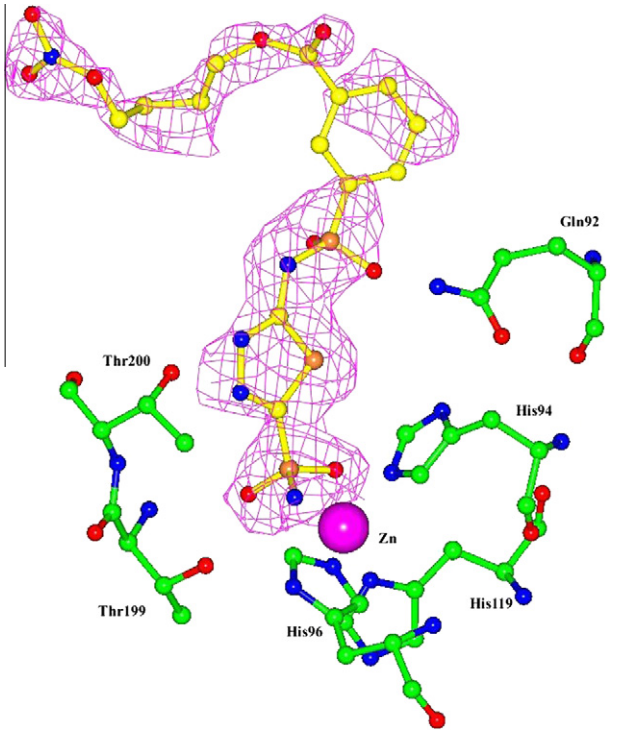


Figure 1. Stick representation of compound **2b** (yellow) bound in the active site of hCA II. The electron density is represented by a 2σ-weighted $2F_o - F_c$ Fourier map (pink mesh). The Zn(II) ion (violet sphere), its three His ligands, and residues Thr199, Thr200 and Gln92 are also shown.

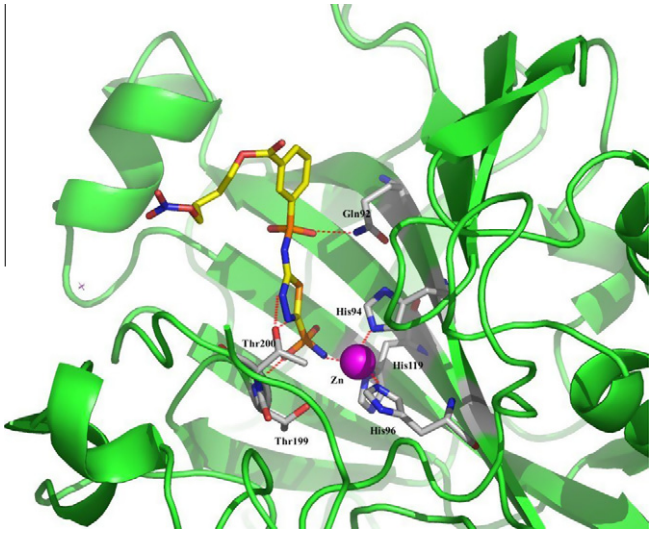


Figure 2. View of compound **2b** (yellow) bound within the hCA II active site. The Zn(II) ion (violet sphere), its three His ligands (His94, 96 and 119), and residues Thr199, Thr200 and Gln92 with which the inhibitor interacts, are also shown (CPK colors). The protein scaffold is shown as the green ribbon.

As for other hCA II-sulfonamide adducts investigated earlier,[3a,11,27,28] the deprotonated sulfonamide moiety of the inhibitor is coordinated to the Zn(II) ion at a distance of 1.96 Å. The same NH moiety makes a hydrogen bond with the OH of Thr199 (of 2.86 Å). Furthermore, the two endocyclic nitrogen of the 1,3,4-thidiazole ring participate in two hydrogen bonds (of 2.50–2.80 Å) with the OH of Thr200, as reported earlier for a structurally related compound, derivative **11**, investigated by Abbate et al.[5d] One oxygen of the secondary SO_2 moiety of inhibitor **2b** makes a hydrogen bond (of 2.97 Å) with the NH_2 of Gln92. However, due to the

Table 2
Crystallographic parameters and refinement statistics for the hCA II—**2b** adduct

Parameter	Value
Crystal parameter	
Space group	$P2_1$
Cell parameters	a = 41.8 Å
	b = 42.1 Å
	c = 72.0 Å
	β = 104.4°
Data collection statistics (20.0–2.1 Å)	
No. of total reflections	37945
No. of unique reflections	13657
Completeness[a] (%)	97.3 (98.5)
F2/sig(F2)	18.9 (4.0)
R-sym (%)	4.2 (31.5)
Refinement statistics (20.0–2.1 Å)	
R-factor (%)	22.9
R-free[b] (%)	27.0
Rmsd of bonds from ideality (Å)	0.013
Rmsd of angles from ideality (°)	1.7

[a] Values in parenthesis relate to the highest resolution shell (2.2–2.1 Å).
[b] Calculated using 5% of data.

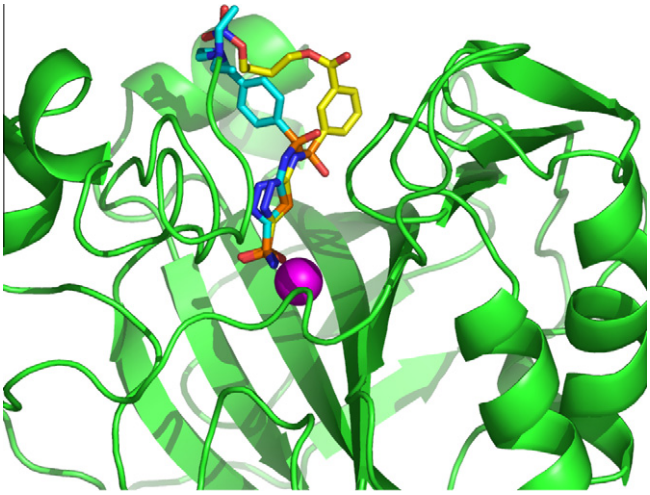


Figure 3. Superposition of the hCA II adducts of sulfonamides **2b** (in yellow) and **11** (sky blue).[5d] The Zn(II) ion is the violet sphere and the enzyme backbone is shown as green ribbon. Only the 1,3,4-thiadiazolyl-sulfamoyl fragments of the two inhibitors are superposable.

meta-substituent of the phenyl moiety present in **2b** (compared to the *para*-one in **11**) the conformation of **2b** and **11** are quite different when bound to the hCA II active site (Fig. 3). Indeed, in the superposition shown in Fig. 3 of the hCA II—**2b** and hCA II—**11** adducts, it may be observed that the amino-1,3,4-thiadiazolyl-2-sulfamoyl moieties of the two inhibitors **2b** and **11** are almost completely superposable. However the terminal fragments of the two inhibitors (5-SO_2NH moiety and 3-substituted phenyl in **2b** vs 4-substituted phenyl in **11**) bind in completely different regions of the enzyme active site. This allows the 1,4-phenylene moiety of inhibitor **11** to participate in an edge-to-face stacking with residue Phe131 from the hCA II active site, whereas this interaction is absent for the inhibitor **2b**, which due to the *meta*-substituent is orientated in a diverse region of the active site, being too far from the phenyl moiety of Phe131 for participating in the edge-to-face stacking interaction. This probably explains why **11** with a K_I of 1.4 nM against hCA II[5d] is 12.8 times a more potent inhibitor compared to **2b** (K_I of 18 nM).

Compound **1b** incorporating a simple 4-carboxybenzenesulfonamide scaffold and the C4 nitrate ester moiety was chosen for in vivo studies, due to the good inhibition observed against the antiglaucoma target isoforms hCA II, IV and XII (K_Is in the range of 18–44 nM, Table 1) and high water solubility (in the range of 2%), compared to other sulfonamides prepared in this study (e.g., **1f** was a better in vitro CAI compared to **1b** but had a very low water solubility (>0.3%), which made it difficult to formulate the drug for topical administration). Furthermore, **1b** is not a strong inhibitor of hCA I and IX, enzymes not involved in glaucoma. In the previous work[13] we have showed that in vivo the sulfonamides incorporating nitrate ester moieties release NO and sulfonamides which both have beneficial effects on the control of intraocular pressure (IOP).

Table 3 shows the IOP data of hypertensive New Zealand rabbits treated topically with vehicle, the standard drug dorzolamide (2%), the NO-donating nitrate ester without CA inhibitory properties isosorbide mononitrate **12** (2%), as well as the NO-donating sulfonamide **1b** reported here (2%). It may be observed that after induction of ocular hypertension with carbomer, the IOP of the experimental animals raised from basal values of 14.7–18.2 mm Hg to values of 32.4–35.4 mm Hg. Treatment with vehicle had no effect on the IOP, whereas treatment with **DZA** led to a maximal IOP decrease of 7.4 mm Hg, at 60 min post-administration of the drug directly into the eye (Table 3). Treatment with the nitrate ester **12** had a similar effect, with an IOP reduction of 6.8 mm Hg, but the maximal effect was observed at 30 min post-administration of the eye drops. The NO-donating sulfonamide **1b** at 2% showed a very strong IOP lowering, of 15.7 mm Hg, the maximal effect being observed at 1 h post-administration, as for the standard drug **DZA**. These data clearly show that the NO-donating sulfonamide **1b** is very effective in reducing elevated IOP in this animal model of glaucoma, with an IOP reduction more than twice that shown by the clinically used drug **DZA**. Its effect is a summation of the sulfonamide CA inhibition effect (as exemplified by the dorzolamide IOP reduction) and the NO-mediated effects (as exemplified by the non-CA inhibiting nitrate **12**).

In conclusion, we report here a new series of sulfonamides incorporating NO-donating moieties of the ester type in their molecule. Several sulfonamide scaffolds have been used to synthesize these derivatives, which have been investigated for the inhibition of five physiologically relevant isoforms, hCA I (offtarget), II, IV and XII (antiglaucoma targets) and IX (antitumor target). Some of the new compounds showed effective in vitro inhibition of the target isoforms involved in glaucoma, and the X-ray crystal structure of one of them also revealed factors associated with the strong inhibitory activity. In an animal model of ocular hypertension, one of the new compounds was twice more effective than dorzolamide in reducing elevated intraocular pressure typical of this disease, anticipating the potential of such compounds for the treatment of glaucoma or elevated IOP.

Table 3
Intraocular pressure (IOP) lowering effects of 2% dorzolamide **DZA**, isosorbide mononitrate **12**, and sulfonamide **1b** in carbomer-induced ocular hypertensive New Zealand white rabbits

Compound*	Basal IOP mm Hg	After carbomer mm Hg	Post-treatment IOP# mm Hg	ΔΔ IOP** (−)mm Hg	T_{max}*** min
Vehicle	17.9 ± 0.3	32.4 ± 1.3	31.8 ± 1.9	0 ± 0.9	—
DZA	14.7 ± 0.9	35.4 ± 0.8	27.1 ± 1.2	7.4 ± 1.9	60
12	16.8 ± 0.3	35.3 ± 1.2	28.4 ± 1.6	6.8 ± 0.5	30
1b	18.2 ± 0.7	34.2 ± 1.4	20.6 ± 1.8	15.7 ± 1.8	60

* All drugs were administered at the final concentration of 2% in a volume of 50 μl. IOP was recorded before treatment (basal IOP) and at 30, 60, 90, 180 and 240 min thereafter, using an applanation tonometer (Tono-Pen XL-Medtronic, Solan). Values are reported as mean ± SEM of 8 rabbits per group. Carbomer 0.25% (THEA Pharmaceutical S.R.) 0.1 ml was introduced bilateraly into anterior chamber of preanesthetized rabbits (Zoletil 100, 0.10 mg/Kg b.w.) for inducing the ocular hypertension, 2–3 weeks before the treatment.

\# Post-treatment IOP is that reflecting the lowest measurement recorded over the observation period.

** ΔΔ IOP reflects the maximal difference recorded in drug-treated versus vehicle (Cremophor EL 5% and DMSO 0.4% in phosphate buffer pH 6.00 at room temperature).

*** The time after administration when the maximal IOP lowering was achieved.

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17. Sulfonamides **2, 3** were prepared as reported earlier[5d] whereas **1, 4** and **5** are commercially available from Sigma–Aldrich (Milan, Italy).
18. The nitrate esters **6** and **7** were prepared as described earlier (Lazzarato, L.; Donnola, M.; Rolando, B.; Marini, E.; Cena, C.; Corazzi, G.; Guaita, E.; Morini, G.; Fruttero, R.; Gasco, A.; Biondi, S.; Ongini, E. *J. Med. Chem.* **2008**, *51*, 1894), whereas the remaining ones were obtained in a similar manner from commercially available halogeno derivatives and silver nitrate (all reagents were from Sigma–Aldrich, Milan, Italy).
19. 4-(Nitrooxy)butyl 4-(aminosulfonyl)benzoate **1b**: 4-Carboxybenzenesulfonamide (200 mg, 0.99 mmol) was dissolved in 10 ml of DMA. The mixture was stirred and sodium iodide (149 mg, 1.0 mmol) and potassium carbonate (276 mg, 2.0 mmol) were added, followed by a solution of 4-bromobutyl nitrate (20% w/w in CH_2Cl_2). This solution was added dropwise for 2 h, then the suspension was stirred at room temperature for 24 h. 100 ml of AcOEt was added and the organic solution was extracted with H_2O, HCl 5% and again water. The organic layer was dried over anhydrous sodium sulfate and evaporated. The solid obtained was purified by flash-chromatography (CH_2Cl_2/MeOH 10:1). ^{1}H NMR (300 MHz, DMSO-d_6), δ: 1.82–2.14 (m, 4H, $CH_2CH_2ONO_2$); 4.32 (t, 2H, OCO–CH_2, $^3J_{HH}$ = 7.1 Hz); 4.60 (t, 2H, –CH_2ONO_2; $^3J_{HH}$ = 6.2 Hz); 7.56 (2H, d, *J* 8.8, 2 × 3-H), 7.98 (2H, d, *J* 8.8, 2 × 2-H), 8.13 (2H, s, SO_2NH_2); ^{13}C NMR (300 MHz, DMSO-d_6), δ 22.1, 30.2, 71,8, 123,4, 127.8, 129.2, 137.7, 143.8, 182.9 (C═O); ESI$^+$ *m/z* = 319 (M+H$^+$).
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22. The hCA II-**2b** complex was crystallized as previously described.[13] Diffraction data were collected under cryogenic conditions (100 K) on a CCD Detector KM4 CCD/Sapphire using Cu Kα radiation (1.5418 Å). The unit cell dimensions were determined to be: a = 41.8 Å, b = 42.1 Å, c = 72.0 Å and $\alpha = \gamma = 90°$, β = 104.4° in the space group $P2_1$ Data were processed with CrysAlis RED (Oxford Diffraction 2006).[23] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2 as starting model. The refinement was carried out with the program REFMAC5;[24] model building and map inspections were performing using the COOT program.[25] The final model of the complex had an *R*-factor of 22.9% and *R*-free 27.0% in the resolution range 20.0–2.1 Å. The correctness of stereochemistry was finally checked using PROCHECK.[26] Coordinates and structure factors have been deposited within the Protein Data Bank, accession code 3NI5.
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