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Nitric oxide-donating carbonic anhydrase inhibitors for the treatment of open-angle glaucoma ☆

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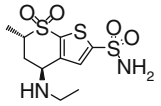
ABSTRACT

Novel bi-functional compounds with a nitric oxide (NO)-releasing moiety bound to a dorzolamide scaffold were investigated. Several compounds were synthesized and their activity as selective carbonic anhydrase inhibitors (CAI) evaluated in vitro on recombinant hCA type I, II and IV enzyme isoforms where they showed different degrees of potency and selectivity to hCA II. A high resolution X-ray crystal structure for the CA II adduct with **8** confirmed the high affinity of this class of compounds for the enzyme. Compounds **4**, **6**, and **8** showed highly potent and efficacious NO-mediated properties as assessed by their vascular relaxant effect on methoxamine-precontracted rabbit aortic rings. Finally, compounds **4** and **6** exerted potent intraocular pressure (IOP) lowering effects in vivo in normotensive rabbits thereby anticipating their potential for the treatment of hypertensive glaucoma.

Open-angle glaucoma is a common multifactorial disease of the eye which is often associated with elevated intraocular pressure (IOP) leading to damage of the optic nerve and consequently loss of vision.[1,2] Indeed, targeting IOP with pressure lowering agents still remains the main treatment strategy for this disease.[3]

IOP depends on the delicate balance between production of aqueous humor by the ciliary body and its drainage through the conventional trabecular meshwork outflow facility and the unconventional uveoscleral pathway.[4] Selective activation of the carbonic anhydrase type-II isozyme (CA II), which is expressed in the ciliary bodies, or elicited via β-adrenergic stimulation, facilitates aqueous formation and transport through cell membranes, as well as its release into the posterior chamber and, thereby unregulated CA can increase IOP.[5–8] By contrast, alleviation of IOP by prostaglandins, specifically $F_{2\alpha}$ ($PGF_{2\alpha}$) receptors modulate uveoscleral outflow.[9] Emerging understanding of conventional outflow through the trabecular meshwork has recently revealed therapeutic roles by a selective prostamide receptor subtype[10] and cytoskeletal-acting kinases.[11] Interestingly, the nitric oxide (NO)/soluble guanylyl cyclase (sGC) signaling pathway[12] is known to enhance local cyclic guanosine monophosphate (cGMP) levels, presumably beneficially for aqueous humor homeostasis.[13]

Accordingly, topical carbonic anhydrase inhibitors (CAI's), such as dorzolamide (**1**; Trusopt®)[14–17], or brinzolamide (**2**; Azopt®)[18,19] together with β-blockers like timolol, (Timoptol®)[20] and $PGF_{2\alpha}$ analogues[21] latanoprost (Xalatan®) and travoprost (Travatan®), are widely used drugs for controlling aqueous humor dynamics and IOP in hypertensive glaucoma. Systemic CAI's, such as acetazolamide (**3**; Diamox)[5] see limited use due to unpleasant side effects that arise, such as fatigue, paresthesias, and kidney stones and emphasise the advantages of containing exposure via topical dosing to the eye.[6–8]



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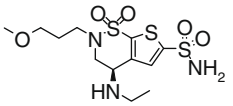

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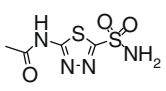

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☆ The X-ray coordinates of the CA II—**8** adduct are available in PDB with the ID 3K2F.

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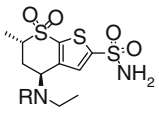
NO-donors are a relatively underexplored class in the field of ocular therapeutics despite the ubiquitous presence of NO targets in all eye compartments devoted to aqueous humor production and drainage.[22] It has been observed that hypertensive glaucoma patients have a decreased NO/cGMP content in the aqueous humor[23] and that some NO-donors have been shown to decrease IOP in normal and pathological conditions.[12,24,25]

As part of our research activities, we decided to explore NO-donating CAI, for topical administration, which would combine a dual mechanism of action in a single molecule, and be capable of enhancing NO/sGC/cGMP signaling while inhibiting CA enzymes. These NO-donating CAI could represent an interesting new class of drugs, with improved activity over existing treatments, to reduce IOP more efficiently in hypertensive glaucoma patients.



1-8

1 R = H (Dorzolamide)
4 R = $CO(CH_2)_5ONO_2$ (NCX 274)
5 R = CO-p-(C_6H_4)-CH_2ONO_2 (NCX 265)
6 R = $COO(CH_2)_3ONO_2$ (NCX 278)
7 R = $COO(CH_2)_4ONO_2$ (NCX 245)
8 R = $COOCH_2CH(ONO_2)CH_2ONO_2$ (NCX 201)

Our intended chemical strategy for attaining these new derivatives foresaw attachment of an NO-donor linker (incorporating a nitrate ester) to the amine functionality of dorzolamide. Thus, five new NO-donating CAI **4–8** (see Scheme 1) were synthesized: amides[26] **4** and **5**, and carbamates, **6**, **7** and **8**.

The NO-donating moieties included were mainly alkyl nitrates, with the exception of the benzylic nitrate **5**. The dinitrate ester **8** was included to eventually measure the effect of increasing the theoretical deliverable amount of NO to eye tissues.

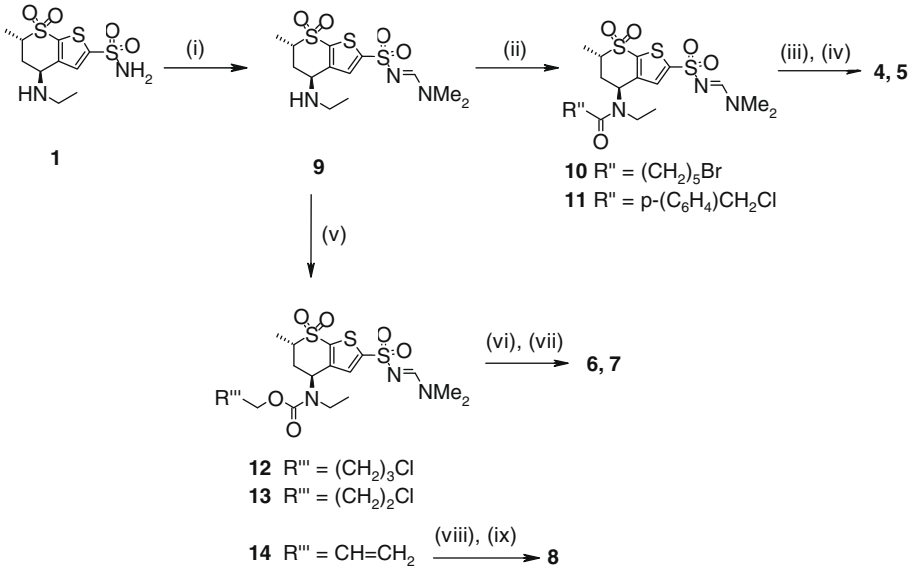
It was suspected that these derivatives would undergo hydrolysis by the enzymes present in the eye compartments to release dorzolamide and the NO-donating moiety. Carbamates are known to be readily hydrolyzed to amines[27] and were explored as a potential NO-donor connection moiety. However, the initial tests carried out on compounds **4–8** to assess hydrolysis in rabbit corneal homogenate[21,28] documented that they are stable to eye esterases, thus suggesting that these compounds are novel, stable NO-donating CAI that exert their activity as such.

Table 1
Inhibitory potency and selectivity of different compounds on recombinant human carbonic anhydrase (CA) isoforms I, II and IV

NCX	Compound	K_i (nM)			$K_{i\ CAII}$/ $K_{i\ CAIV}$
		hCA I	hCA II	hCA IV	
Dorzolamide	**1**	50,000	9	43	4.7
ISMN	**15**	NE	NE	NE	
274	**4**	2950	14	4360	311.1
265	**5**	470	71	46	0.6
278	**6**	410	13	181	13.9
245	**7**	705	76	339	4.5
201	**8**	1520	63	3905	62.0

Carbonic anhydrase (CA) activity was monitored using a CO_2 hydrase stopped flow assay.[29]
NE, not effective at concentrations up to 100 μM.

As shown in Table 1, the new CAIs were initially assayed for their inhibitory activity and selectivity on CA enzymes by exposing an increasing concentration of test drug to the human recombinant type I, II and IV isoforms.[29] Dorzolamide (**1**) was used as the reference standard, with CA II K_i = 9 nM and $K_{i\ CAII}/K_{i\ CAIV}$ = 4.7, as we wanted to maintain a similar CA enzyme inhibition profile with the novel NO-donating CAI.[15] Compounds **4–8** inhibited CA II with the following order of potency: **6** ⩾ **4** > **8** ⩾ **5** ⩾ **7** with K_i ranging from 13 to 76 nM (Table 1). The alkyl nitrate linkers proved to be more selective for CA II than the benzyl nitrate **5**. Shortening the carbamate chain from 4 to 3 carbon atoms (compounds **7** and **6**), increased significantly both potency on CA II and selectivity over CA IV ($K_{i\ CAII}$ = 13 nM and $K_{i\ CAII}/K_{i\ CAIV}$ = 13.9 for compound **6**). Notably, compounds **4** and **6** (K_i = 14 and 13 nM, respectively) were



Scheme 1. Synthesis of amine derivatives **4–8**.[26] Reagents and conditions: (i) *N*,*N*-dimethylformamide dimethyl acetal, Et_3N, DMF, rt, 3 h, 82%; (ii) for compound **10**, 6-bromocaproyl chloride, Et_3N, DCM, rt, 22 h, 83%. For compound **11**, 4-(chloromethyl)benzoyl chloride, Et_3N, DCM, rt, 70 h, 86%; for compound **4**: (iii) $AgNO_3$, MeCN, 110 °C, μW, 20 min, 71%; (iv) 37% HCl, THF, 110 °C, μW, 28 min, 75%. For compound **5**: (iii) 37% HCl, THF, 110 °C, μW, 28 min, 79%; (iv) $AgNO_3$, MeCN, 110 °C, μW, 20 min, 77%. (v) For compound **12**, 3-chloropropyl chloroformate, Et_3N, DCM, rt, 66 h, 72%; For compound **13**, 4-chlorobutyl chloroformate, Et_3N, DCM, rt, 91 h, 60%; For compound **14**, allyl chloroformate, pyridine, DMAP, DCM, rt, 18 h, 33%. For compound **6**: (vi) (a) NaI, MeCN, 150 °C, μW, 30 min, (b) $AgNO_3$, MeCN, 110 °C, μW, 20 min, 75% over two steps; (vii) 37% HCl, THF, 110 °C, μW, 28 min, 68%. For compound **7**: (vi) 37% HCl, THF, 110 °C, μW, 28 min, 94%; (vii) (a) NaI, MeCN, 150 °C, μW, 30 min, (b) $AgNO_3$, MeCN, 130 °C, μW, 4 min, 64% over two steps. For compound **8**: (viii) 10% HCl, MeOH, Δ, 18 h, 69%; (ix) (a) I_2, MeOH, −15 °C, 1 h, (b) $AgNO_3$, MeCN, Δ, 20 h, then $AgNO_3$, MeCN, 120 °C, μW, 10 min, 21% from **14**.

equipotent to dorzolamide (K_i = 9 nM) for CA II and comparably selective inhibitors of CA II over CA IV ($K_{i\ CAII}/K_{i\ CAIV}$ = 311 and 13.9, respectively, for compound **4** and **6**) to dorzolamide ($K_{i\ CAII}/K_{i\ CAIV}$ = 4.7).

To better understand the interaction of these new NO-donating CAI with the CA active site, the X-ray crystal structure for the complex of CA II with racemic dinitrate **8** was elucidated (Figs. 1–4, and Table 2). The structure of the complex was similar to that of the native enzyme. Inspection of the electron density maps (Fig. 1) showed the presence of one molecule of inhibitor **8** bound to the active site (Fig. 2). The analysis of the structure showed that the

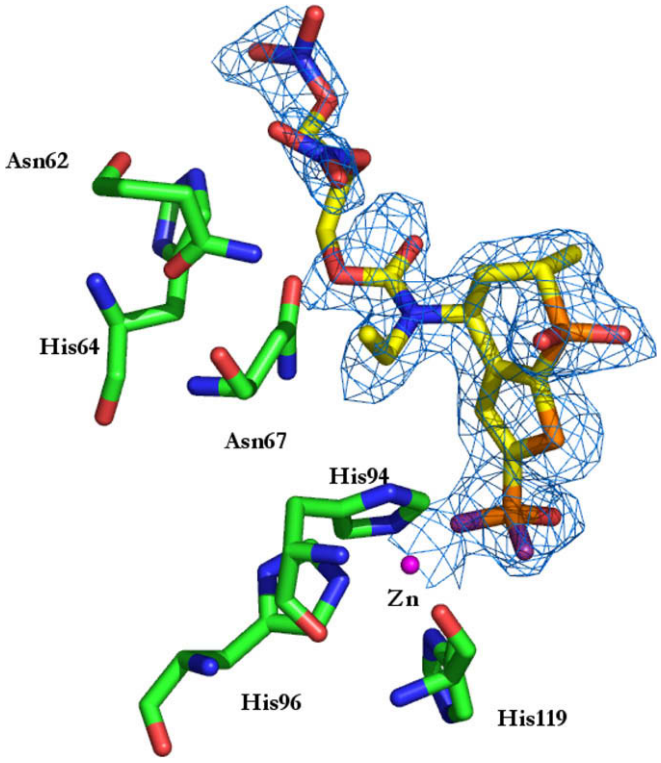


Figure 1. Omit electron density map of the inhibitor **8** (in yellow) bound within the human CA contoured at 1.0 σ level and amino acid residues/Zn(II) ion involved in the binding.

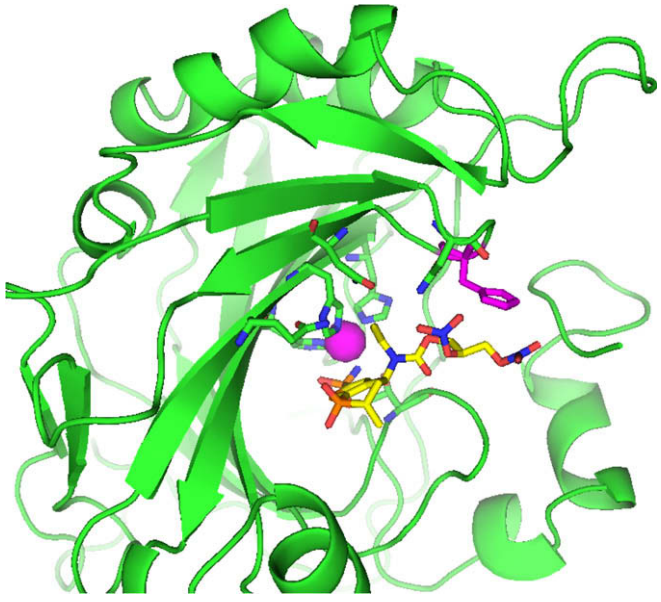


Figure 2. Sulfonamide **8** (yellow) bound within the hCA II active site. The Zn(II) ion (violet sphere), its three histidine ligands (His94, 96 and 119, in green) and proton shuttle residue (His64, in violet) as observed. The inhibitor completely fills the active site channel. The enzyme backbone is shown as green ribbon.

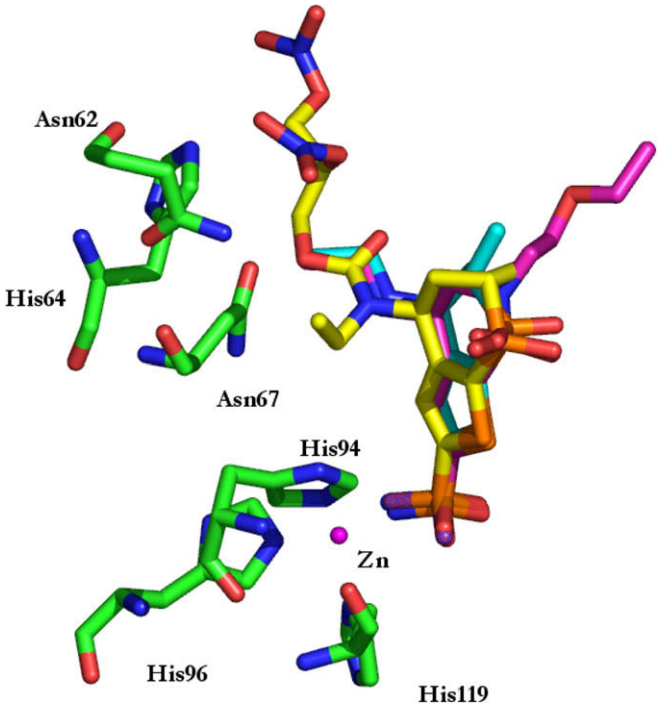


Figure 3. Superposition of the hCA II—**8** adduct (inhibitor **8** in yellow, PDB file 3K2F) with the hCA II—dorzolamide (sky blue) adduct (PDB file 1cil) and hCA II—brinzolamide (magenta) adduct (PDB file 1a42). The heterocyclic scaffolds of the three inhibitors are superimposable whereas the tails (mainly the NO-donating one of **8**) adopt a different conformation and interact with various amino acid residues.

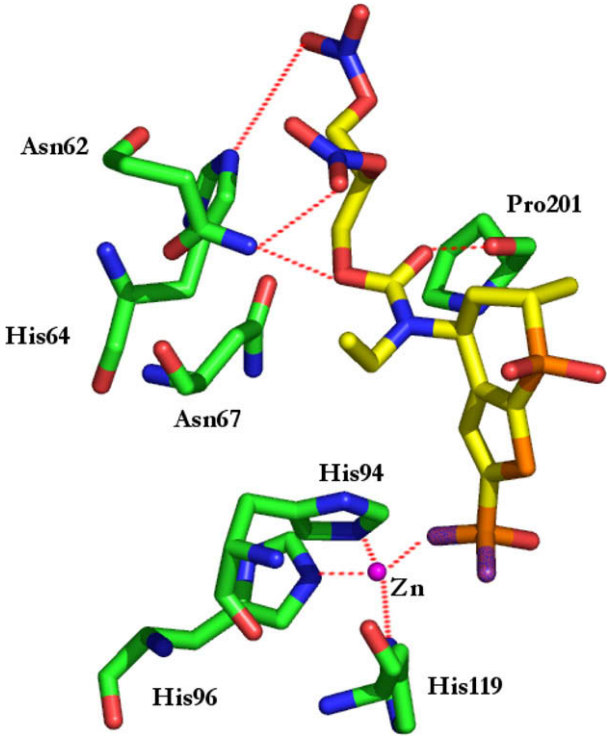


Figure 4. Interactions between sulfonamide **8** and amino acid residues/metal ion within the hCA II active site.

geometry of the zinc^{+} binding site and the key hydrogen bonds between the sulfonamide moiety of the inhibitor **8** and the enzyme active site were all retained with respect to other hCA II-sulfonamide complexes (Figs. 1 and 4). The ligand **8** was involved in multiple hydrogen bonds with several amino acid residues of the active site: Asn62, His64, Asn67, Gln92, Thr199 and Pro201 (distances provided in Table 3). Thus, ligand **8** makes three particularly interesting and previously unobserved hydrogen bonds: secondary nitrate and ester oxygen with the Nδ2 carboxamide hydrogens of Asn62 and the primary nitrate oxygen with the imidazolic NH moiety of His64. The thienothiopyran scaffold of **8** makes the same type of interaction with hCA II as its ancestral CA inhibitors, dorzolamide **1** or brinzolamide **2,** as shown in the superposition of the three hCA II—sulfonamide ligands as presented in Figure 3.

Table 2
Crystallographic parameters and refinement statistics for the hCA II—**8** adduct

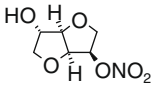
Parameter	Value
Crystal parameter	
Space group	$P2_1$
Cell Parameters	a = 41.63Å
	b = 42.01 Å
	c = 72.46 Å
	β = 104.40°
Data collection statistics (20.0–2.0 Å)	
No. of total reflections	54,133
No. of unique reflections	16,290
Completeness (%)[a]	98.2 (90.6)
F2/sig(F2)	20.9 (3.0)
R-sym (%)	14.4 (41.6)
Refinement statistics (20.0–2.0 Å)	
R-factor (%)	22.3
R-free (%)[b]	27.0
Rmsd of bonds from ideality (Å)	0.012
Rmsd of angles from ideality (°)	1.54

[a] Values in parenthesis relate to the highest resolution shell (2.1–2.0 Å).
[b] Calculated using 5% of data.

Table 3
Interactions between various groups of the inhibitor **8** and amino acid residues within the hCA II active site

Compound **8**	hCA II residue	Distance (Å)
NAJ	Zn	1.96
OAL	Zn	3.08
OAT	O Pro201	3.78
OAL	N Thr199	2.86
CAS	N Asn67	3.05
CAS	N Gln92	3.05
OBB	Nδ2 Asn62	2.97
OAV	Nδ2 Asn62	3.34
OBG	Nδ2 His64	3.70

Compounds **4** and **6** and **8** were further characterized for NO-donor capability by analyzing their cGMP-mediated vasorelaxant properties on methoxamine-precontracted rabbit aortic rings.[30] In this set of experiments, isosorbide mononitrate (ISMN, **15**) was used as a positive reference standard and dorzolamide **1** as the comparative CA inhibitory compound (Table 4).[30]



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The release of NO was clearly demonstrated for all three compounds studied (see Fig. 5). More specifically, compounds **4**, **6** and **8** were potent and effective stimulators of NO/sGC/cGMP signaling with an EC_{50} of 4.25 ± 0.80 μM, 2.05 ± 0.41 μM, and 0.37 ± 0.05 μM, respectively, compared to ISMN **15**, which gave an EC_{50} of 10.8 ± 2.4 μM and dorzolamide which displayed little effect.

Normotensive rabbit eyes have been shown to respond readily to IOP lowering agents with CA inhibitory activity[34] as well as to standard NO-donors[24], thus these were used in the model to address the in vivo IOP lowering potentials of two CA II inhibitors from our series, namely compounds **4** and **6** (Table 3). In this set of experiments, ISMN and dorzolamide were also used as comparators at clinically relevant doses. Both **4** and **6**, despite their poor solubility (0.25 and 15.1 mg/mL, respectively), performed as well as an equimolar dose of dorzolamide **1** (solubility >20 mg/mL) in lowering the IOP in normotensive rabbits (Table 3), reaching the maximal response between 30 and 90 min post-dosing followed by a slow decay over the next 180 min to reach basal IOP levels at 360 min post-dosing. However, in this experiment, both **4** and **6** resulted in greater IOP lowering and over a longer duration than the NO-donor ISMN **15** (Table 3), which attained a minimum of 2 mm below baseline at 30 min post-dosing and rapidly decayed to basal level in a few hours.

In conclusion, a new class of NO-donating CAI were synthesized and tested as potential IOP-lowering agents for the treatment of open-angle glaucoma. These are new chemical entities with relevant CA inhibitory properties via direct binding, as confirmed by means of X-ray crystallographic work. Importantly **4** and **6** inhibited CA II isozymes similarly to dorzolamide, and with selectivity

Table 4
Intraocular pressure (IOP) lowering effects of dorzolamide, ISMN and compounds **4** and **6** in normotensive New Zealand white rabbits

Compound[a]		IOP lowering effect in normotensive New Zealand white rabbits				Solubility[d] (mg/mL)
		Basal IOP (mmHg)	Post-treatment [b] IOP (mmHg)	$\Delta\Delta_{IOP}$ [c] (mmHg)	T_{max} (min)	
Vehicle		20.1 ± 0.6	19.9 ± 0.7	0 ± 0.6	0	
Dorzolamide	**1**	19.7 ± 0.7	16.7 ± 0.8	−3.8 ± 0.5**	60	>20
ISMN	**15**	19.2 ± 0.5	16.6 ± 0.5	−1.9 ± 0.5*	30	<20
NCX 274	**4**	19.8 ± 0.7	15.9 ± 0.7	−4.7 ± 0.7**	60	0.25
NCX 278	**6**	19.2 ± 0.5	16.5 ± 0.4	−3.7 ± 0.3**	120	15.1[e]

[a] All drugs were administered at the final concentration of 55 mM in 50 μL using a positive displacer; IOP was recorded before treatment (pre-treatment) and at 30, 60, 90, 180 and 300 min thereafter using an applanation tonometer (Model 30 Classic, Medtronic, USA); Values are reported as mean ± SEM; N = 8/10 rabbits per group.
[b] Post-treatment IOP is that reflecting the lowest measurement recorded over the observation period.
[c] $\Delta\Delta_{IOP}$ reflects the maximal difference recorded in drug-treated versus vehicle (Cremophor EL 5%, DMSO 0.3%, BAK 0.02% in phosphate buffer, pH 6 at room temperature) whereas T_{max} is the time at which the maximal differences were recorded.
[d] Solubility in Cremphor EL 5%, DMSO 0.3%, BAK 0.02%, pH 6 at room temperature.
[e] Milky appearance even after microfiltration.
* p <0.05 versus vehicle (Student t-test).
** p <0.01 versus vehicle (Student t-test).

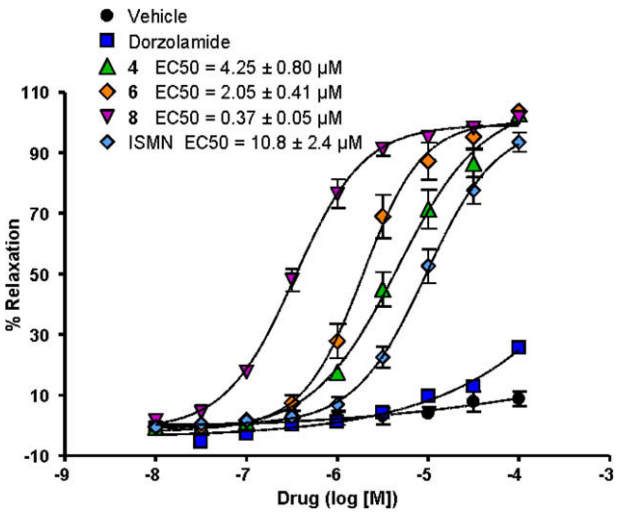

Figure 5. Vasorelaxant potency of vehicle (closed circle), dorzolamide (**1**, closed square), ISMN (**15**, closed diamond) and compounds **4** (open triangle), **6** (open diamond) and **8** (closed triangle).[33]

towards the CA II isoform known to be consistent with beneficial IOP lowering ability. Interestingly, compounds **4**, **6** and **8** also released NO in vitro, showing higher potency than ISMN in the vasorelaxation of pre-contracted rabbit aorta rings. Compounds **4** and **6** proved to be promising in an in vivo IOP-lowering model, performing as well as an equimolar dose of dorzolamide, despite their lower solubility, and were superior to ISMN. Additional studies are necessary to elucidate the detailed mechanism of NO donation for this class of NO-donating CAI, as NO donation via hydrolysis of the amide or carbamate bond by corneal homogenate esterases is apparently not involved. The NO release of these and other such bi-functional compounds (some of them in advanced clinical trials) probably involves the reaction of the NO-releasing prodrug with reducing agents, most probably thiol containing derivatives such as cysteine residues from protein, or glutathione, a compound found in high concentration in the eye tissues.[35] Further studies of novel NO-donating CAI in animal models of glaucoma are also under consideration to further investigate this class.

References and notes

1. (a) Boland, M. V.; Quigley, H. A. *J. Glaucoma* **2007**, *16*, 406; (b) Leske, M. C. *Ophthalmic Epidemiol.* **2007**, *14*, 166.
2. (a) Lutjen-Drecoll, E.; Futa, R.; Rohen, J. W. *Invest. Ophthalmol. Vis. Sci.* **1981**, *21*, 563; (b) Rohen, J. W. *Ophthalmology* **1983**, *90*, 758; (c) Alvarado, J.; Murphy, C.; Juster, R. *Ophthalmology* **1984**, *91*, 564.
3. (a) Whitson, J. T. *Exp. Opin. Pharmacother.* **2007**, *8*, 3237; (b) Woodward, D. F.; Gil, D. W. *Trends Pharmacol. Sci.* **2004**, *25*, 238.
4. (a) McLaren, J. W. *Exp. Eye Res.* **2009**, *88*, 641; (b) Gabelt, B. T.; Kaufman, P. L. *Prog. Retin. Eye Res.* **2005**, *24*, 612; (c) Civan, M. M.; Macknight, A. D. C. *Exp Eye Res.* **2004**, *78*, 625.
5. Becker, B. *Am. J. Ophthalmol.* **1955**, *39*, 177.
6. Supuran, C. T. *Nat. Rev. Drug Disc.* **2008**, *7*, 168.
7. Wistrand, P.J., Lindqvist, A. In *Carbonic Anhydrase—From Biochemistry and Genetics to Physiology and Clinical Medicine*, Proceedings of the International Workshop on Carbonic Anhydrase, held in Spoleto, Italy in March 1990; Botrè, F., Gros, G., Storey, B.T., Eds.; VCH: Weinhein, 1991; pp 352–378.
8. Supuran, C. T. In *Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism*; Puscas, I., Ed.; Helicon: Timisoara, 1994; pp 29–111.
9. Toris, C. B.; Gabelt, B. T.; Kaufman, P. L. *Surv. Ophthalmol.* **2008**, *53*, S107.
10. Liang, Y.; Woodward, D. F.; Guzman, V. M.; Li, C.; Scott, D. F.; Wang, J. W.; Wheeler, L. A.; Garst, M. E.; Landsverk, K.; Sachs, G.; Krauss, A. H.; Cornell, C.; Martos, J.; Pettit, S.; Fliri, H. *Br. J. Pharmacol.* **2008**, *154*, 1079.
11. Acott, T. S.; Kelley, M. J. *Exp. Eye Res.* **2008**, *86*, 543.
12. Nathanson, J. A. *J. Pharmacol. Exp. Ther.* **1992**, *260*, 956.
13. (a) Dismuke, W. M.; Mbadugha, C. C.; Ellis, D. Z. *Am. J. Physiol. Cell Physiol.* **2008**, *294*, C1378; (b) Ellis, D. Z.; Dismuke, W. M.; Chokshi, B. M. *Invest. Ophthamol. Vis. Sci.* **2009**, *50*, 1808.
14. Ponticello, G. S.; Freedman, M. B.; Habecker, C. N.; Lyle, P. A.; Schwam, H.; Varga, S. L.; Christy, M. E.; Randall, W. C.; Baldwin, J. J. *J. Med. Chem.* **1987**, *30*, 591.
15. Ponticello, G. S.; Sugrue, M. F.; Plazonnet, B.; Durand-Cavagna, G. In *Integration of Pharmaceutical Discovery and Development: Case Studies*; Borchardt, R. T., Freidinger, R. M., Sawyer, T. K., Smith, P. L., Eds.; Pharmaceutical Biotechnology; Plenum: New York, 1998; Vol. 11, pp 555–574.
16. Balfour, J. A.; Wilde, M. I. *Drugs Aging* **1997**, *10*, 384.
17. Sugrue, M. F. *Prog. Ret. Eye Res.* **2000**, *19*, 87.
18. Silver, L. H. *Am. J. Ophthalmol.* **1998**, *126*, 400.
19. Silver, L. H. *Surv. Ophthalmol.* **2000**, *44*, S147.
20. Cheng, J. W.; Cai, J. P.; Wei, R. L. *Ophthalmology* **2009**, *116*, 1243.
21. Perry, C. M.; McGavin, J. K.; Culy, C. R.; Ibbotson, T. *Drugs Aging* **2003**, *20*, 597.
22. Krauss, A.H., Toris, C.A.; Kallberg, M.E.; Gelatt, K.; Prasanna, G.; Impagnatiello, F.; Chiroli, V.; Chong, W.K.M.; Carreiro, S.; Ongini, E. Abstract of Papers, The Association for Research in Vision and Ophthalmology Annual Meeting, Fort Lauderdale, FL, May 2009; Abstract 1471.
23. Galassi, F.; Renieri, G.; Sodi, A.; Ucci, F.; Vannozzi, L.; Masini, E. *Br. J. Ophthalmol.* **2004**, *88*, 757.
24. Kotikoski, H.; Vapaatalo, H.; Oksala, O. *Curr. Eye Res.* **2003**, *26*, 119.
25. Becquet, F.; Courtois, Y.; Goureau, O. *Surv. Ophthalmol.* **1997**, *42*, 71.
26. Synthesis of compound **4** (NCX 274):To a solution of dorzolamide hydrochloride (**1**; 5.00 g, 13.85 mmol; US Pharmacopeia) in dry N,N-dimethylformamide (10 mL), under nitrogen, was added triethylamine (2.12 mL, 15.23 mmol) followed by *N*,*N*-dimethylformamide dimethyl acetal (2.21 mL, 16.62 mmol). The resulting solution was stirred at room temperature for 3 h. The solution was cooled to 0 °C and water (25 mL) added slowly. The mixture was extracted with ethyl acetate, the combined organic extracts were washed with water, dried (Na_2SO_4) and the solvent was removed under reduced pressure to give sulfonyl-amidine **9** as an off-white foam (4.35 g, 82%).
To a solution of crude **9** (100 mg, 0.26 mmol) in dry dichloromethane (2.00 mL), at 0 °C, under nitrogen, was added triethylamine (73 μL, 0.53 mmol) followed by dropwise addition of 6-bromocaproyl chloride (79 μL, 0.53 mmol). The solution was allowed to warm to ambient temperature and stirred for 22 h. The solution was diluted with dichloromethane and washed with water. The organic layer was dried (Na_2SO_4) and the solvent was removed under reduced pressure. Purification by flash chromatography, eluting with ethyl acetate gave the bromo-amide **10** as a white foam (122 mg, 83%).
To a solution of bromide **10** (370 mg, 0.66 mmol) in acetonitrile (16 mL), in a microwave vial, was added silver nitrate (456 mg, 2.66 mmol). The mixture was pre-stirred at ambient temperature in the microwave, for 2 min, then heated at 110 °C with stirring, for 20 min. The salts were removed by filtration over a pad of celite and the solvent was removed under reduced pressure. The crude residue was dissolved in dichloromethane and washed with water. The organic layer was dried (Na_2SO_4) and the solvent was removed under reduced pressure. Purification by flash chromatography, eluting with ethyl acetate gave the nitrate **10** as a white foam (253 mg, 71%).
To a solution of the amidine **10** (253 mg, 0.469 mmol) in THF at 0 °C, in a microwave vial, was added 37% hydrochloric acid (1.873 mL) dropwise. The solution was allowed to warm to ambient temperature, then heated in the microwave at 110 °C, with stirring, for 28 min. The mixture was allowed to cool to ambient temperature, then a saturated aqueous $NaHCO_3$ solution added slowly, until pH 8. The THF was removed under reduced pressure. The aqueous residue was extracted with dichloromethane, the combined organics dried (Na_2SO_4) and the solvent removed under reduced pressure. Purification by flash chromatography, eluting with a gradient of 50% EtOAc/hexane to 100% EtOAc gave sulfonamide **4** as a white solid (170 mg, 75%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.99 (2H, s), 7.31 and 7.18 (1H, s), 5.40 and 5.00 (1H, s), 4.49 (2H, t, J = 6.5 Hz), 3.92 (1H, m), 3.53–3.11 (2H, m), 2.75 (1H, m), 2.44–2.25 (3H, m), 1.80–1.30 (9H, m), 1.15 (3H, t, J = 7.2 Hz); ^{13}C NMR (300 MHz, DMSO-d_6) δ 173.1, 150.0, 145.0, 129.1, 74.6, 56.2, 50.0, 42.8, 33.2, 26.8, 25.6, 25.1, 16.2, 12.4; ESI$^+$ m/z = 484 (M+H$^+$).
27. Simplício, A.; Clancy, J.; Gilmer, J. *Molecules* **2008**, *13*, 519.
28. Chang, S. C.; Bundgaard, H.; Buur, A.; Lee, V. H. *Invest. Ophthalmol. Vis. Sci.* **1987**, *28*, 487.
29. Khalifah, R. G. *J. Biol. Chem.* **1971,** *246*, 2561. An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction. 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 (10 mM) were prepared in distilled–deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled–deionized water. 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 represent the mean from at least three different determinations. CA isozymes I, II and IV were recombinant ones obtained as reported earlier.[31,32]

30. Chiroli, V.; Batugo, M. R.; Biondi, S.; Bonfanti, A.; Brambilla, S.; Gale, D. C.; Li, L.; Miglietta, D.; Nicoli, F.; Prasanna, G. A.; Ronchetti, D.; Vernier, W. F.; Chong, W. K. M. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2785.
31. Scozzafava, A.; Menabuoni, L.; Mincione, F.; Briganti, F.; Mincione, G.; Supuran, C. T. *J. Med. Chem.* **1999**, *42*, 2641.
32. Nishimori, I.; Minakuchi, T.; Morimoto, K.; Sano, S.; Onishi, S.; Takeuchi, H.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2006**, *49*, 2117.
33. Vasorelaxant potency was determined in rabbit aortic rings pre-contracted with 3 μM methoxamine. EC_{50} (effective concentration giving 50% of response) values were estimated for each test compound from the logic curve obtained by plotting the percentage of vasorelaxant effects as a function of concentration. The EC_{50} could not be calculated for compounds with efficacy below 50% at the highest testable concentration of 100 μM (NC). Results are expressed as mean ± SEM of three independent experiments.
34. Reitsamer, H. A.; Bogner, B.; Tockner, B.; Kiel, J. W. *Invest. Ophthalmol. Vis. Sci.* **2009**, *50*, 2301.
35. Scozzafava, A.; Casini, A.; Supuran, C. T. *Curr. Med. Chem.* **2002**, *9*, 1167.