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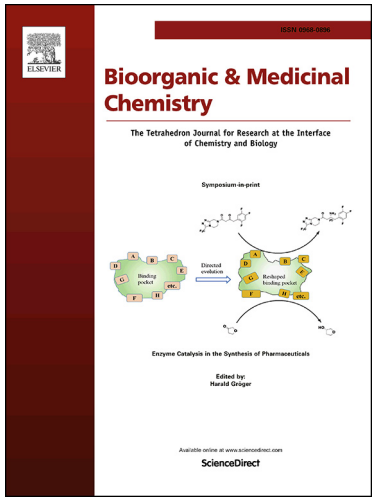

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Highly Hydrophilic 1,3-Oxazol-5-yl Benzenesulfonamide Inhibitors of Carbonic Anhydrase II for Reduction of Glaucoma-Related Intraocular Pressure

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ABSTRACT

Four inhibitors of human carbonic anhydrase II (*h*CA II) were designed based on the previously reported subnanomolar 1,3-oxazole-based sulfonamide inhibitors of the enzyme to incorporate primary and secondary amine functionality in the carboxamide side chain. The new hydrophilic compounds were found to inhibit the target isoform in sub-nanomolar to low nanomolar range with a good degree of selectivity to several other *h*CA isoforms. The hydrophilic character of these compounds is advantageous for intraocular residence time but not for corneal permeability which generally requires that a drug be sufficiently lipophilic. Two of the four compounds investigated, however, were found to exert comparable efficacy as 1% eye drops in PBS to that of the clinically used 2% dorzolamide (Trusopt®) eye drops. This indicated that the absorption of the compounds may occur *via* alternative route across conjunctiva and sclera.

Key words: glaucoma; intraocular pressure; hydrophilicity; intraocular delivery; non-corneal absorption; carbonic anhydrase inhibitors

1. Introduction

Intraocular pressure is decreased in glaucoma treatment with eye drops of prostaglandin analogues, beta blocking agents and carbonic anhydrase inhibitors (CAI).[1] The topical CAIs in the glaucoma treatment include dorzolamide (**1**) and brinzolamide (**2**), compounds that are relatively lipophilic and non-selective as inhibitors of carbonic anhydrase isoforms.[2] Acetazolamide (**3**) and methazolamide (**4**) are also used as anti-glaucoma agents (Fig. 1), but they are delivered as per oral tablets; an approach that causes frequent adverse drug reactions.[3] Selective and potent inhibition of carbonic anhydrase II isoform (*h*CA II) is desirable in drug discovery, because this enzyme is critically important in the reduction of intraocular pressure.[4] Potent and selective *h*CA II inhibitors are expected to be effective drugs in the glaucoma treatment.

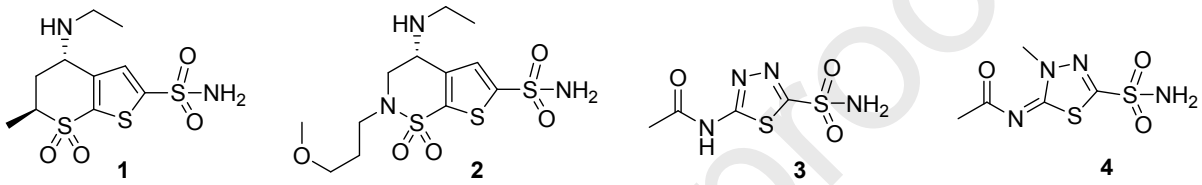


Fig. 1. Examples of clinically used antiglaucoma carbonic anhydrase inhibitors.

Topical ocular drugs are usually lipophilic, because they are delivered to the eye via permeation across the cornea.[5] Lipophilicity tends to decrease water solubility and maximal attainable soluble drug concentration in the tear fluid, whereas higher concentration gradient between tear fluid and the eye can be achieved with hydrophilic compounds. Furthermore, instead of ocular absorption the topically applied drugs are effectively absorbed to the systemic blood stream across the conjunctiva lining the inner side of the eyelids.[6,7] Systemic drug loss reduces ocular bioavailability, especially in the case lipophilic compounds.[7,8,9] In addition, it is known that ocular drug absorption can take place also via non-corneal route, across the bulbar conjunctiva (on the ocular surface) and sclera.[10] Hydrophilic compounds can utilize this route, because the conjunctiva is less tight membrane than the cornea, and the loss of hydrophilic compounds to the blood stream in the conjunctiva and sclera is slower than in the case of lipophilic compounds.[10,11] The site of action of CAIs is in the ciliary body, a tissue that is located next to sclera, making non-corneal absorption an interesting approach for highly potent, but hydrophilic derivatives. Compared to the cornea, the conjunctiva has a wider inter-cellular space available for permeation of hydrophilic compounds.[12]

Previously, we described a series of 1,3-oxazol-5-yl benzenesulfonamides **5a-c** which displayed a remarkably potent inhibition profile toward human carbonic anhydrase (CA, EC 4.2.1.1) and, in particular, its *h*CA II isoform[13] which is primarily targeted by intraocular pressure-reducing antiglaucoma drugs.[4] The most *h*CA II-selective compound (**5a**) has been shown to be

efficacious *in vivo* in lowering ocular hypertension in rabbits. Furthermore, its high potency and the pronounced selectivity toward the CA isoform in question has been rationalized by X-ray crystallographic structure of its complex with the protein.[14] In light of these findings, it seemed worthwhile to select **1a** as the scaffold for the introduction of peripheral functional groups which would increase the resulting compounds' hydrophilicity and provide also a reactive 'handle' for subsequent chemical conjugation to polymer nanoparticles.

Anti-glaucoma eye drop treatment has poor patient compliance as many patients do not use the eyedrops properly, leading to the progression of the disease and loss of vision.[15] Therefore, increasing interest has been focused on the long acting intraocular drug delivery with polymeric systems.[16] The new compounds presented in this work were designed with this goal in mind, since their structure, on one hand, allows convenient conjugation of the compounds to the polymeric carriers *via* amide and other potentially biodegradable linkages. On the other hand, the hydrophilicity of these compounds was seen as beneficial in this context as hydrophilic compounds have slower clearance from the intraocular spaces.[17-18] Therefore, similar drug cargo of hydrophilic drug in controlled release system may extend the dosing interval as compared to the use of lipophilic compound with similar potency. Yet, hydrophilic compounds might be useful as traditional eye drop medications if they would be delivered across the conjunctiva-sclera route to the ciliary body.

If both of the latter design requirements are taken into account, synthesis and subsequent biological investigation of amides **6** prepared from dibasic amines and analogous to compounds **5b-c** became our goal (Figure 2). Foreseeable risks associated with such a structural modification included potential loss of potency and/or selectivity toward *h*CA II compared to distinctly hydrophobic lead compound **5a**. Indeed, the active site of carbonic anhydrase is known to have a very characteristic topology where a hydrophobic half of the protein surface is clearly delineated from the hydrophilic one.[19] Therefore, replacing the small hydrophobic 1,3-oxazole methyl substituent with a large hydrophilic carboxamides group bearing a basic amine could, in principle, lead to a loss of the desired affinity to *h*CA II. Despite these potential risks we set off to synthesize a set of compounds **6** for investigation of their carbonic anhydrase inhibitory potency *in vitro* and subsequent efficacy characterization as glaucoma-associate intraocular pressure-lowering agents *in vivo*. Herein, we report the results of these studies.

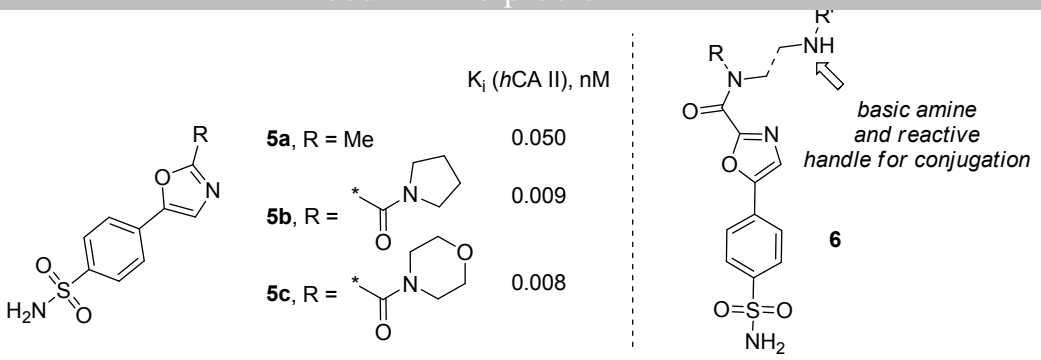
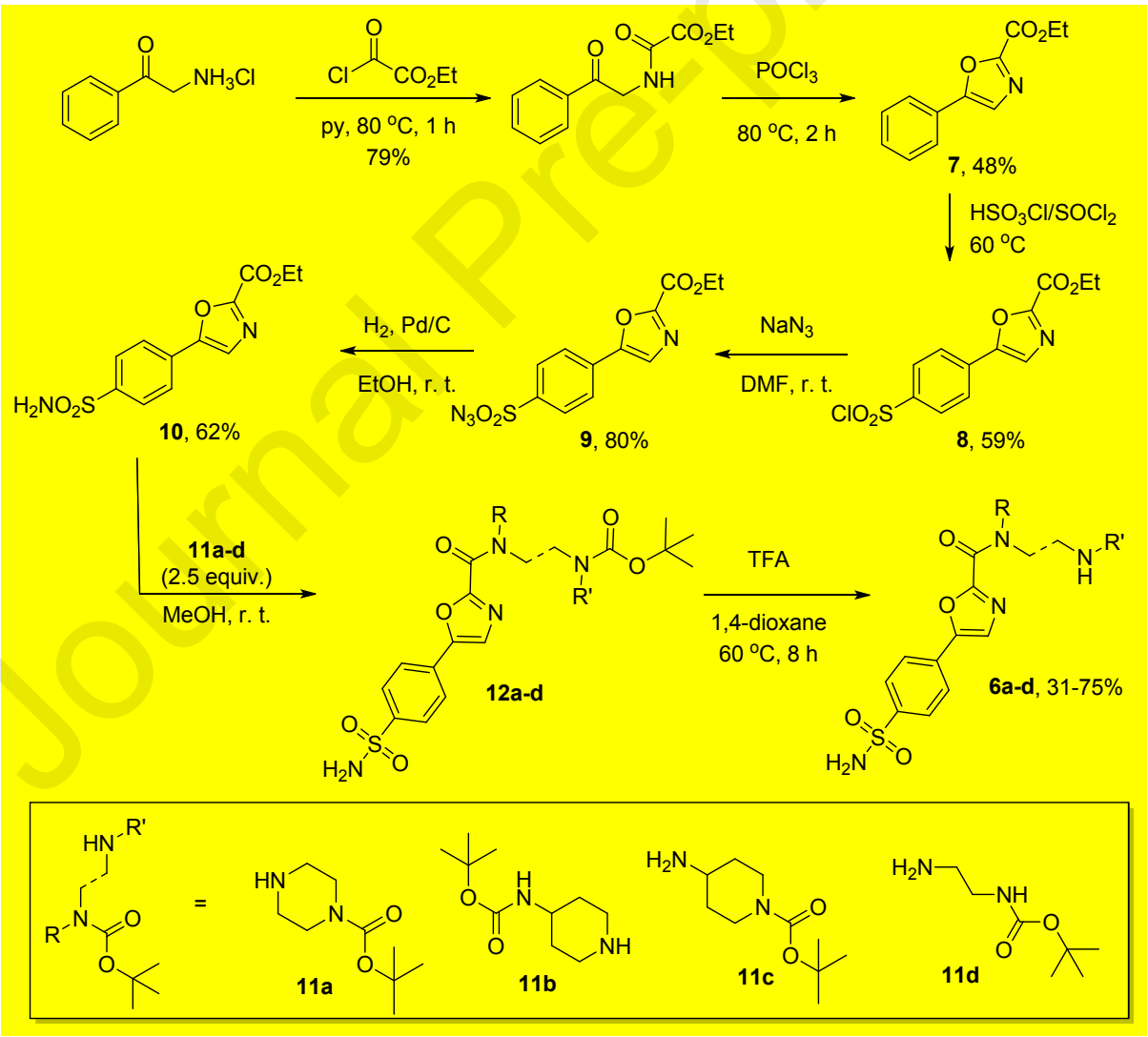


Fig. 2. Earlier reported potent *h*CAII inhibitors **5a-c** and their modified hydrophilic analogs **6** designed and investigated in this work.

2. Results and Discussion

2.1 Chemistry

Scheme 1. Synthesis of hydrophilic sulfonamides **6a-d** investigated in this work.



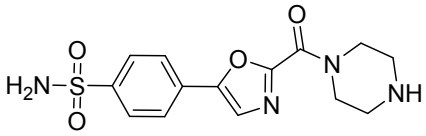
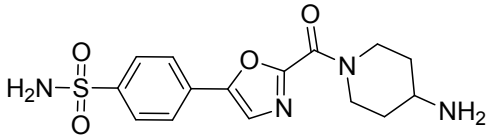
The key building block - ethyl 5-phenyloxazole-2-carboxylate (**7**) was synthesized in two steps from α-aminoacetophenone hydrochloride as described previously.[13] This compound was

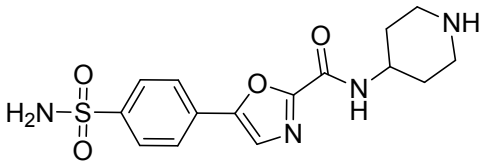
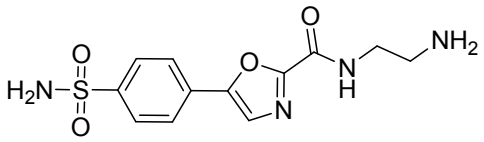
chlorosulfonylated under somewhat harsh conditions ($SOCl_2$/HSO_3Cl, 60 °C) to obtain sulfonyl chloride **8** in respectable 59% yield. Direct conversion to respective sulfonamide **10** by treatment with aqueous ammonia failed. Despite our efforts to reduce the reaction temperature and apply slow addition technique, the carboxylic acid in **7** was non-selectively converted to primary amide, apparently, being sufficiently activated by the primary sulfonamide group which also formed at the same time. This obstacle was circumvented *via* a two-step procedure entailing a high-yielding synthesis of sulfonyl azide **9** followed by hydrogenation of the latter over Pd/C to give the target sulfonamide **10** in good yield over two steps. The electron-withdrawing influence of the sulfonamide group which complicated the preparation of **10** as described above, was a desirable feature in subsequent synthesis of the target compounds **6a-d**. Indeed, on reaction with 2.5-fold excess of mono-Boc-protected dibasic amines **11a-d** at r. t. in MeOH, respective amides **12a-d** were obtained in moderate to good yields, deprotected with TFA in 1,4-dioxane at 60 ºC and purified chromatographically to give the target compounds **6a-d** (Scheme 1).

2.2 Carbonic anhydrase inhibition

The inhibitory profile obtained for sulfonamides **6a-d** in a stopped-flow kinetics assay against human CA I, II, IV and XII is shown in Table 1. In addition to *h*CA II, the other three isoforms were selected to arbitrarily evaluate the off-target profile of the compounds intended to inhibit the target isoform.

Table 1. Inhibitory activity of compounds **6a-d** against the target (*h*CA II) as well as selected off-target (*h*CA I, IV and XII) isoforms.

Compound	Structure	K_i (nM)[a]			
		*h*CA I	*h*CA II	*h*CA IV	*h*CA XII
6a		108.7	5.0	419.8	68.5
6b		61.9	0.54	256.6	91.5

6c		50.8	0.91	204.3	87.3
6d		101.4	4.4	39.1	90.1
	3[b]	250	12	75	5.7

[a] Mean from 3 different assays by stopped flow technique (errors were in the range of ± 5-10 % of the reported values). [b]Sulfonamide inhibitor acetazolamide (AAZ) used as a reference pan-CA inhibitor in stopped flow CO_2 hydrase assay.

To our delight, all four inhibitors **6a-d** preserved the potent inhibition profile against the target *h*CA II isoform (although taking some toll on potency compared to the less hydrophilic leads 5a-c) and a clearly better isoform selectivity profile compared to acetazolamide (**4**) employed as a reference inhibitor. Interestingly, sub-nanomolar inhibitory activity was displayed by compounds **6b** and **6c** which are isomeric (and nearly isosteric) to each other.

Of course, the ultimate efficacy profile of these inhibitors on the reduction of glaucoma-related intraocular pressure (IOP) would depend on a multitude of factors among which permeability characteristics (intrinsically linked to a favorable set of molecular parameters) will have a superior significance.

2.3 Docking studies

In order to rationalize the one-order *h*CA II potency gap between **6a,d** and **6b,c** docking studies were performed. Figure 3 shows the docking poses of **6a** and **6b** in the active site of *h*CA II. In the case of both compounds, the phenylsulfonamide moiety acts as a zinc binding group showing orientation well documented in a wide range of crystallographic studies.[20] Another common feature is ionic interaction of the free amino function with Glu69. However the conformation of **6a** enabling the latter interaction is somewhat tense due to the shorter spacer present between the carboxamide and amino groups in comparison to that of **6b**. Additionally, the presence of larger aliphatic chain in **6b** favors enhanced lipophilic interaction of the inhibitor molecule with the spacy hydrophobic cleft lined up with the residues Leu141, Ile91, Val121, Val143, Trp209 and Phe131. It is impact of these interactions what we consider to be the crucial factor explaining higher inhibitory activity of **6b** comparing to **6a**. Moreover, we strongly believe this conclusion

to be relevant for compounds **6c** and **6d** (docking poses not shown) exhibiting similar activity trends.

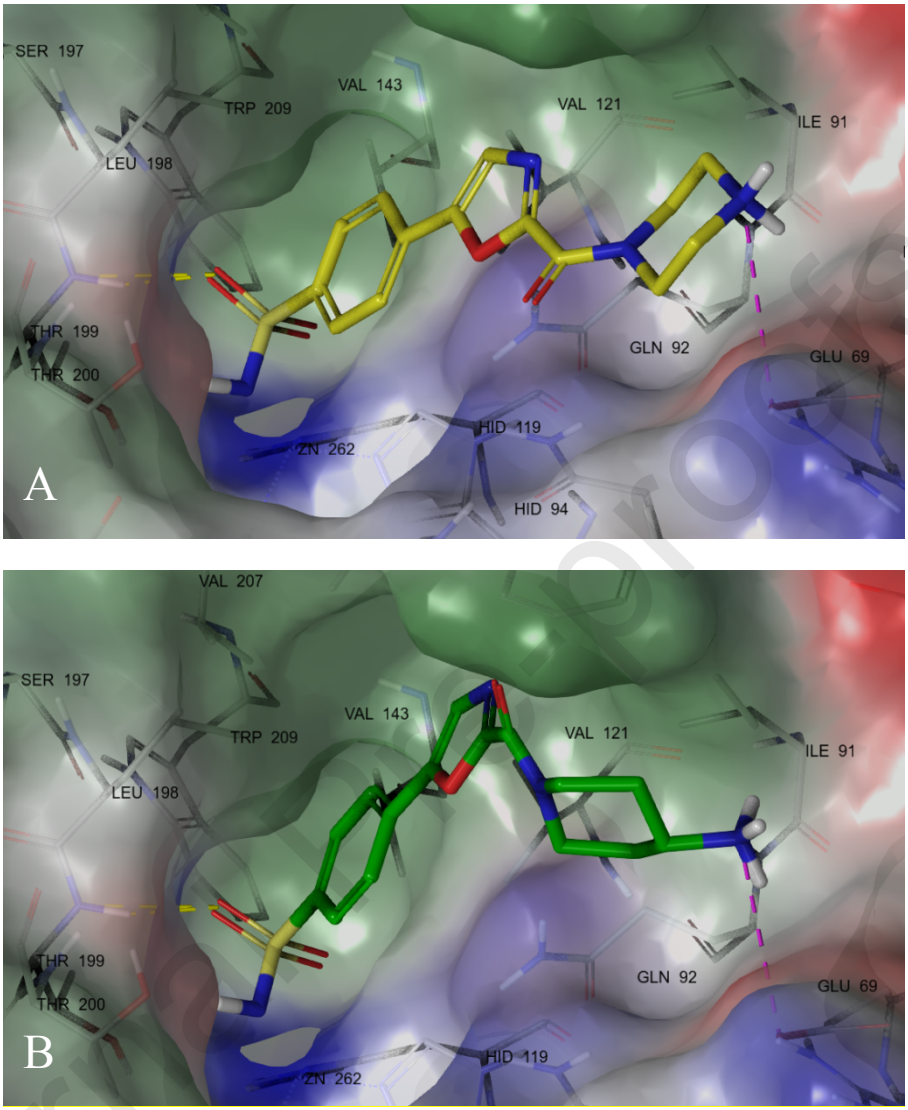


Figure 3. Binding poses of **6a** (A) and **6b** (B) in the *h*CAII active site. Hydrophobic, positively and negatively charged regions of the catalytic cleft are surface shown in green, red, and blue correspondingly. Hydrogen bonds are represented in yellow dashed lines. Salt bridges are represented as purple dashed lines.

2.4 In silico ADME properties

ADME descriptors of compounds **6a-d** were evaluated and compared to those of commercially available carbonic anhydrase inhibitors (Table 2). Based on the calculated $logD_{7.4}$ and logP values, it can be concluded that **6a-d** are indeed much more hydrophilic relative to the topical carbonic anhydrase inhibitors in the clinical use (**1** and **2**) whereas orally used drugs (**3** and **4**) are similarly hydrophilic.

Table 2. Chemical descriptors of carbonic anhydrase inhibitors **6a-d** and **1-4** (calculated using ACDLabs 12.0).[a]

Compound	MW	HBa	HBd	HBtot	LogP	$LogD_{7.4}$	$LogD_{8.0}$	PSA	logPSA
1	324.4	6	3	9	1.67	1.53	1.62	151.33	2.180
2	383.5	8	3	11	4.30	4.19	4.26	163.80	2.214
3	222.3	7	3	10	-0.26	-0.69	-1.13	151.66	2.181
4	236.3	7	2	9	0.13	-0.21	-0.62	138.87	2.143
6a	336.4	8	3	11	0.07	-0.73	-0.30	126.91	2.103
6b	350.4	8	4	12	0.11	-1.56	-1.00	140.90	2.149
6c	350.4	8	4	12	0.53	-1.78	-1.29	135.70	2.133
6d	310.3	8	5	13	-0.21	-1.89	-1.79	149.69	2.175

[a] MW = molecular weight, HBa = hydrogen bond acceptors, HBd = hydrogen bond donors, HBtot = total amount of hydrogen bond formers, LogP = logarithmic value of partition coefficient, $LogD_{7.4}$/$LogD_{8.0}$ = logarithmic value of distribution coefficient at pH 7.4/8.0, PSA = polar surface area, LogPSA = logarithmic value of polar surface area.

Computational molecular descriptors presented above were used to further calculate the conjunctival and corneal permeability values for the same compounds using an earlier reported approach.[21] In general, the calculated corneal and conjunctival permeability values (P_{app}) of the new compounds (**6a-d**) were similar or lower than those of dorzolamide (**1**) and brinzolamide (**2**) (Table 3).

Table 3. Calculated permeability (P_{app}) values of compounds **1-4** and **6a-d**.

Compound	Cornea (rabbit)		Cornea (porcine)		Conjunctiva (porcine)	
	P_{app} (cm/s)	% of **1**	P_{app} (cm/s)	% of **1**	P_{app} (cm/s)	% of **1**
1	7.79E-06	100	1.75E-07	100	1.86E-06	100
2	1.83E-05	235	1.64E-07	94	1.77E-06	95
3	1.24E-06	16	1.74E-07	100	1.85E-06	100
4	2.57E-06	33	2.54E-07	146	2.35E-06	126

6a	7.94E-07	10	2.00E-07	114	2.07E-06	111
6b	3.07E-07	4	1.35E-07	77	1.62E-06	87
6c	2.67E-07	3	1.39E-07	80	1.65E-06	89
6d	1.63E-07	2	9.47E-08	54	1.30E-06	70

2.5 Pharmacodynamic effect of intraocular pressure (IOP) in vivo

The newly developed hydrophilic *h*CAII inhibitors were tested in normotensive rabbits for their ability to lower the intraocular pressure (IOP). The results are shown as pressure readings (in mmHg) and percentage changes in Fig. 4 and 5, respectively. Compounds **6a** and **6d** showed a clear effect in the treated rabbit eye relative to the untreated control eye. Compounds **6c** and **1** (dorzolamide) had some effect in both eyes, whereas the compound **6b** did not have any detectable effects on the IOP.

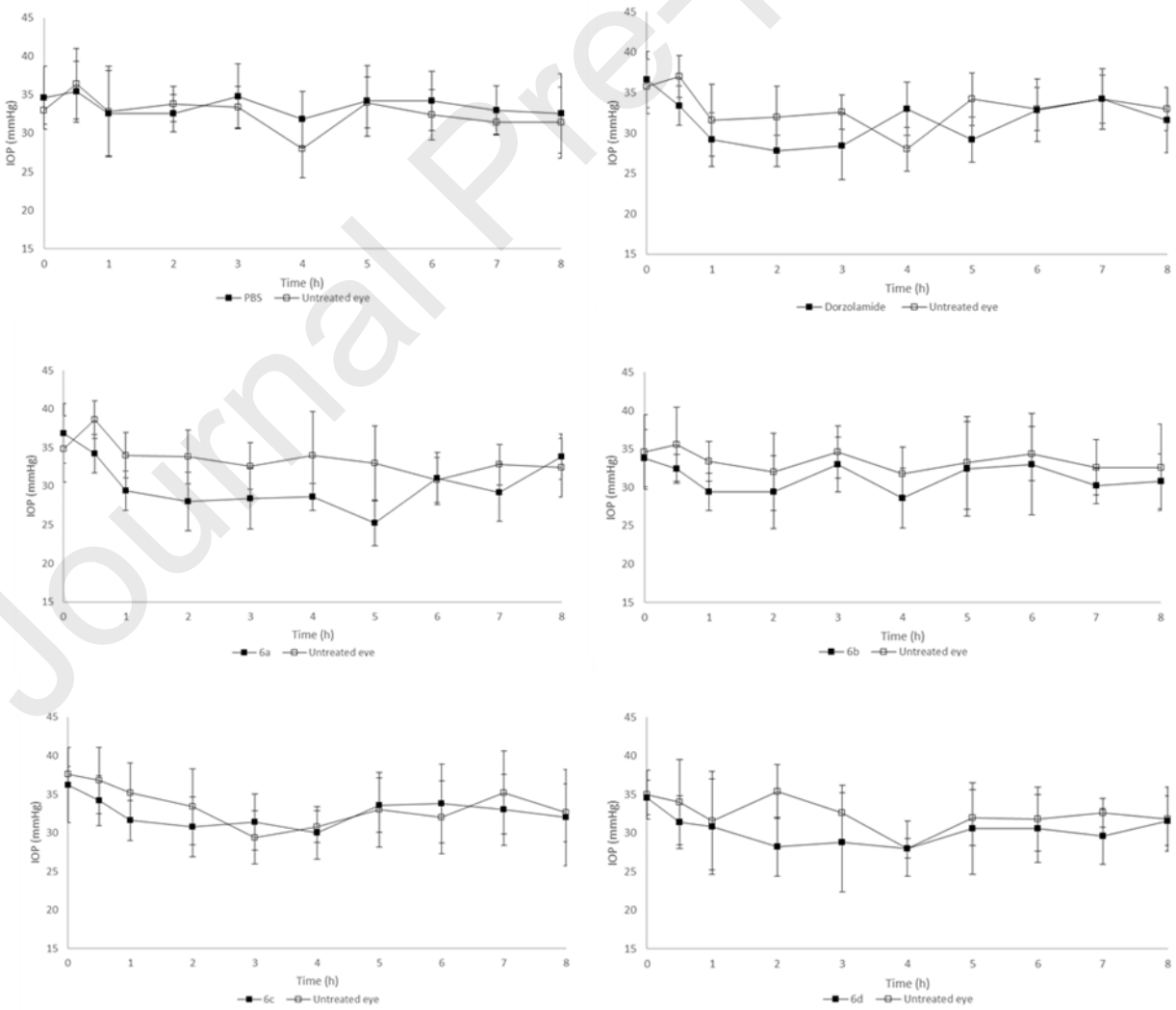


Figure 4. IOP ± SD (mmHg) after administration of **6a-d** and **1** (dorzolamide, DRZ) as well as phosphate buffered saline (PBS) as negative control, compared to fellow untreated eye in albino rabbits (n=5).

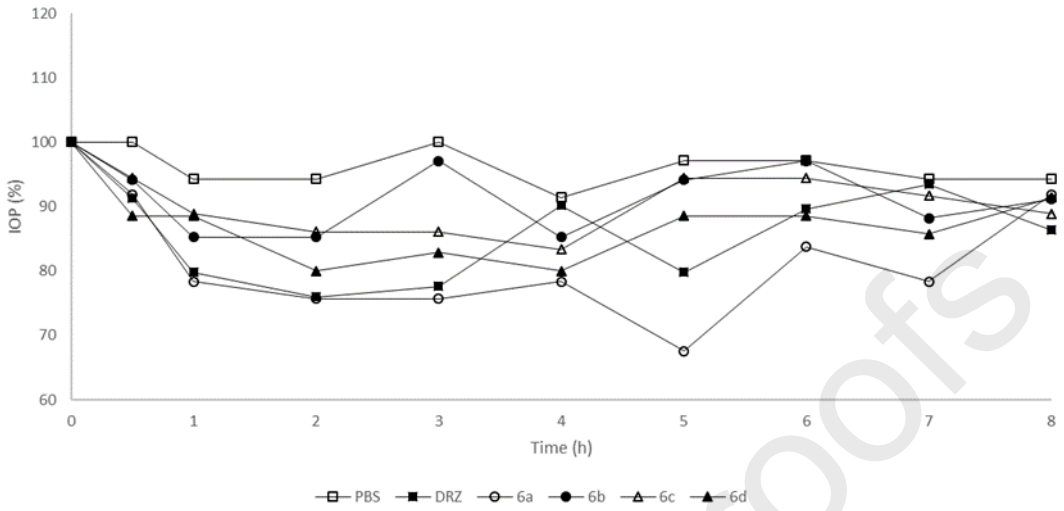


Figure 5. Percentage change in IOP ± SD after administration of **6a-d**, and **1** (dorzolamide, DRZ) as well as phosphate buffered saline (PBS) as negative control, compared to fellow untreated eye in albino rabbits (n=5).

Based on the results presented in Fig. 4, the area under curve (AUC) for IOP *vs*. time curves and maximum responses (E_{max}) were determined for each compound (Table 4). For compound **6a**, the percentage decrease of AUC was comparable with the statistical significance of P=0.056. However, the power of the performed test (0.450) was below the desired power of 0.800, which resulted in the absence of statistically significant difference in AUC values. On the contrary, the maximum effects produced by compounds **6a**, **6d** and **1** (dorzolamide) were significant in comparison to PBS vehicle. At the same time compounds **6b** and **6c** did not produce a statistically significant effect on the IOP in the rabbits. Notably, **6a** appeared to have a longer action in the rabbit eye compared to **1** (dorzolamide) which might be due to the slow clearance from the eye.

Table 4. Effect of carbonic anhydrase inhibitors **6a-d** and **1** on IOP in albino rabbits (n=5).[a]

Compound	E_{max} ±SD	$AUC_{0\text{-}8h}$ ± SD
6a	**35.60** ± 4.88**	159.79 ± 51.95
6b	22.00 ± 5.76	66.92 ± 43.36
6c	19.40 ± 10.89	84.04 ± 96.66
6d	**29.60* ± 7.79**	113.57 ± 57.57
1 (dorzolamide)	**27.80* ± 4.96**	124.36 ± 35.57
PBS	12.00 ± 6.81	25.90 ± 59.86

[a] All values presented are mean ±SD; decrease in area under curve (AUC) was calculated from the curve of decrease in IOP (%) *vs.* time as compared to the baseline; * = statistically significant P<0.05; ** = statistically significant P<0.001.

Possible eye irritation caused by the administration of carbonic anhydrase inhibitors was followed visually throughout the experiment. Compounds **6a**, **6c**, **6d** and **1** (dorzolamide) did not induce blinking or head shaking after drug administration to the rabbits' eyes, whereas compound **6b** caused mild irritation (eye lid closure for 5 to 10 seconds). All the eye drops of new compounds were close to neutrality (pH 6.80 – 7.36) and isotonicity (osmolality 254-337 mOsm/kg).

*h*CA II inhibitors **6a** and **6d** (administered as 1% eye drops) displayed the IOP lowering efficacy that comparable to that of the clinically used dorzolamide 2% eye drops (**1**). Due to the low quantity of the compounds **6b** and **6c** they were formulated as 0.5% eye drops and the lack of efficacy observed with these hCA II inhibitors can potentially be overcome at higher doses. The compounds which displayed efficacy (at half the dose compared to **1**) are hydrophilic and were predicted to have 10-50 times lower corneal permeability in the rabbits than **1** (dorzolamide) (Table 3). The comparable pharmacological effect displayed by hydrophilic compounds **6a** and **6d** and more lipophilic **1** may indeed be attributed to the conjunctival permeation (not feasible for lipophilic compounds) and reduced systemic loss which is characteristic for hydrophilic compounds.

3. Conclusion

We have described next-generation 1,3-oxazole-based carbonic anhydrase inhibitors endowed with a primary or secondary amine periphery. The compounds were designed with a dual goal of increasing compounds' hydrophilicity and provide a reactive 'handle' for potential conjugation to sustained-release nanoparticles. Increased hydrophilicity, while desirable for increased drug residence in the intraocular space was generally viewed as an obstacle for corneal drug absorption. However, hydrophilic compounds may be efficiently absorbed *via* conjunctiva and thus have greater efficacy which may be expected if corneal absorption alone is considered. Compounds described herein displayed a potent and selective inhibition of *h*CA II isoform, a glaucoma target and two of these compounds (**6a** and **6d**) showed comparable efficacy as 1% eye drops in reducing the intraocular pressure in normotensive rabbit to that of clinically used 2% dorzolamide eye drops. This is despite the fact that the corneal permeability of these hydrophilic compounds was predicted to be significantly lower than that of dorzolamide. The data support the concept of hydrophilic compounds permeating across the conjunctiva and sclera into the ciliary body.

3. Experimental section

4.1 Synthetic chemistry

NMR spectroscopic data were recorded with 400 spectrometer (400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C) in DMSO-d_6 or in $CDCl_3$ and were referenced to residual solvent proton signals (δ_H = 2.50 and 7.26 ppm, respectively) and solvent carbon signals (δ_C = 39.52 and 77.00 ppm, respectively). Mass spectra were recorded with a Bruker Maxis HRMS-ESI-qTOF spectrometer (electrospray ionization). Melting points were determined with a Stuart SMP50 instrument in open capillary tubes and are uncorrected. Column chromatography was carried out with silica gel grade 60 (0.040–0.063 mm) 230–400 mesh. Preparative HPLC was carried out on Shimadzu LC-20AP chromatograph, equipped with spectrophotometric detector. Column: Agilent Zorbax prepHT XDB-C18, 5 lm, 21.2 150 mm. Eluent: A) 0.1% TFA in water, B) 0.1% TFA in acetonitrile. All reagents and solvents were used as received from commercial sources unless otherwise noted.

*4.1.1. Ethyl 5-(4-(chlorosulfonyl)phenyl)oxazole-2-carboxylate (**8**)*

A mixture of chlorosulfonic acid (6.13 mL, 92.1 mmol) and thionyl chloride (0.67 mL, 9.21 mmol) was cooled in an ice bath and substrate **7**[13] (2.00 g, 9.21 mmol) was added portionwise. The mixture was stirred for 30 minutes, then warmed to 60°C and stirred for 12 hours. The course of the reaction was monitored by TLC. The reaction mass was then poured into chloroform over crashed ice. Organic layer was separated, washed with 10% $NaHCO_3$ aqueous solution (15.0 ml), H_2O (3*15.0 ml) and brine (15.0 ml), dried over Na_2SO_4, filtered and concentrated. The mixture was purified via column chromatography to give 1.92 g (66%) of product as yellow powder. M.p. 85-88°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.00 (s, 1H, $CH_{Oxazole}$), 7.80 (d, J = 8.4 Hz, 2H, H_{Ar}), 7.74 (d, J = 8.4 Hz, 2H, H_{Ar}), 4.40 (q, J = 7.1 Hz, 2H, CH_2), 1.35 (t, J = 7.1 Hz, 3H, CH_3) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.3, 153.0, 151.5, 144.5, 132.9, 128.0 (2C), 127.0, 125.9 (2C), 63.1, 14.2 ppm. HRMS (ESI), m/z calcd for $C_{12}H_{11}ClNO_5S^+$ 316.0041 $[M+H]^+$ found 316.0039.

*4.1.2. Ethyl 5-(4-sulfamoylphenyl)oxazole-2-carboxylate (**10**)*

To a solution of **8** (1.50 g, 4.75 mmol) in dimethylformamide (5.0 mL) NaN_3 (0.32 g, 5.0 mmol) was added. The reaction mixture was stirred for 2 hours. The course of the reaction was monitored by TLC. The reaction mixture was poured onto H_2O, the precipitate formed was filtered and dissolved in hot ethanol (20.0 ml) followed by addition of 10% Pd/C (0.15 g). The suspension was stirred under H_2 atmosphere at 80°C and the course of the reaction was monitored by TLC. After the reaction completion, the catalyst was filtered off and the solvent

was removed by rotary evaporation. The residue was purified by flash chromatography to provide sulfonamide **10** (49% over 2 steps, 0.69 g; 2.3 mmol) as white powder. M.p. 180-183°C. ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.05 (s, 4H, H_{Ar}), 7.98 (s, 1H, $CH_{Oxazole}$), 6.72 (br.s, 2H, SO_2NH_2), 4.48 (q, J = 7.1 Hz, 2H, CH_2), 1.43 (t, J = 7.1 Hz, 3H, CH_3) ppm. ^{13}C NMR (100 MHz, DMSO) δ 155.5, 152.4, 152.3, 145.2, 129.7, 127.1 (2C), 127.0, 125.7 (2C), 62.7, 14.5 ppm. HRMS (ESI), m/z calcd for $C_{12}H_{13}N_2O_5S^+$ 297.0540 $[M+H]^+$ found 297.0536.

*4.1.3. Preparation of compounds **6a–d**; General Procedure (GP)*

To a solution of **10** (0.10 g, 0.34 mmol) in methanol corresponding mono-*Boc*-protected diamine **11** (0.68 mmol) was added and the mixture was refluxed for 12 hours. The solution was then removed by rotary evaporation, the residue was dissolved in chloroform (5.0 mL), washed by 10% solution of HCl (2x2.5 mL) and H_2O (3x2.5 mL), dried over Na_2SO_4 and concentrated. The residue was dissolved in 1,4-dioxane (3.0 mL) and trifluoroacetic acid (3.0 mL) was added slowly. The mixture was stirred for 4 hours. The formation of amine salt was monitored by TLC. The white precipitate formed was filtered off, washed with 1,4-dioxane and additionally purified by reverse-phase HPLC using acetonitrile:H_2O system containing trifluoroacetic acid (0.1%).

*4.1.3.1. 4-(2-(4-Sulfamoylphenyl)oxazole-5-carbonyl)piperazin-1-ium 2,2,2-trifluoroacetate (**6a**)*

Prepared according to GP1. Yield: 0.049 g (32%). M.p. 165-168°C. ^{1}H NMR (400 MHz, DMSO) δ 8.99 (s, 2H, NH_2^+), 8.10 (s, 1H, $H_{Oxazole}$), 8.00 (d, J = 8.5 Hz, 2H, H_{Ar}), 7.96 (d, J = 8.5 Hz, 2H, H_{Ar}), 7.48 (br.s, 2H, SO_2NH_2), 4.33 (t, J = 5.0 Hz, 2H, CH_2), 3.87 (t, J = 5.0 Hz, 2H, CH_2), 3.28 – 3.21 (m, 4H, CH_2) ppm. ^{13}C NMR (126 MHz, DMSO, the signal of TFA not included) δ 155.1, 154.1, 151.1, 145.0, 129.8, 127.2 (2C), 125.5 (2C), 125.5, 43.9 (2C), 43.4, 43.0 ppm. HRMS (ESI), m/z calcd for $C_{14}H_{17}N_4O_4S^+$ 337.0971 $[M+H]^+$ found 337.0980.

*4.1.3.2. 1-(5-(4-Sulfamoylphenyl)oxazole-2-carbonyl)piperidin-4-aminium 2,2,2-trifluoroacetate (**6b**)*

Prepared according to GP1. Yield: 0.117 g (75%). M.p. 158-161°C. ^{1}H NMR (400 MHz, D_2O) δ 7.86 – 7.79 (m, 2H, H_{Ar}), 7.76 – 7.68 (m, 2H, H_{Ar}), 7.60 (s, 1H, $H_{Oxazole}$), 4.89 – 4.79 (m, 1H, CH_2), 4.70 (s, SO_2NH_2, NH_3^+ in exchange with water, 5H), 4.62 – 4.52 (m, 1H, CH_2), 3.56 (tt, J = 11.0, 4.0 Hz, 1H, CH), 3.42 – 3.27 (m, 1H, CH_2), 3.08 – 2.97 (m, 1H, CH_2), 2.26 – 2.10 (m, 2H, CH_2), 1.83 – 1.61 (m, 2H, CH_2) ppm. ^{13}C NMR (100 MHz, D_2O, the signal of TFA was not included) δ 156.2, 153.6, 151.2, 141.6, 130.2, 126.6 (2C), 125.4 (2C), 124.4, 47.8, 45.3, 41.6, 29.9, 29.0 ppm. HRMS (ESI), m/z calcd for $C_{15}H_{19}N_4O_4S^+$ 351.1127 $[M+H]^+$ found 351.1124.

*4.1.3.3. 4-(5-(4-Sulfamoylphenyl)oxazole-2-carboxamido)piperidin-1-ium 2,2,2-trifluoroacetate (**6c**)*

Prepared according to GP1. Yield: 0.097 g (62%). M.p. 156-159°C. ^{1}H NMR (400 MHz, D_2O) δ 7.83 – 7.77 (m, 2H, H_{Ar}), 7.73 – 7.68 (m, 2H, H_{Ar}), 7.55 (s, 1H, $H_{Oxazole}$), 4.70 (s, SO_2NH_2, NH_2^+,

COONHCH in exchange with water, 5H), 4.11 (tt, J = 11.0, 4.1 Hz, 1H, CH), 3.51 (dt, J = 13.5, 3.8 Hz, 2H, CH_2), 3.15 (td, J = 12.8, 3.0 Hz, 2H, CH_2), 2.21 (dd, J = 14.8, 3.8 Hz, 2H, CH_2), 1.94 – 1.80 (m, 2H, CH_2) ppm. ^{13}C NMR (100 MHz, D_2O, the signal of TFA not included) δ 155.7, 154.0, 151.8, 141.6, 130.2, 126.6 (2C), 125.3 (2C), 125.2, 45.0, 42.9 (2C), 27.5 (2C) ppm. HRMS (ESI), m/z calcd for $C_{15}H_{19}N_4O_4S^+$ 351.1127 $[M+H]^+$ found 351.1139.

*4.1.3.4. 2-(5-(4-Sulfamoylphenyl)oxazole-2-carboxamido)ethan-1-aminium 2,2,2-trifluoroacetate (**6d**)*

Prepared according to GP1. Yield: 0.045 g (31%). M.p. 160-163°C. ^{1}H NMR (400 MHz, D_2O) δ 7.90 – 7.83 (m, 2H, H_{Ar}), 7.83 – 7.76 (m, 2H, H_{Ar}), 7.66 (s, 1H, $H_{Oxazole}$), 4.71 (s, SO_2NH_2, NH_3^+, COONHCH in exchange with water, 6H), 3.77 – 3.67 (m, 2H, CH_2), 3.31 – 3.23 (m, 2H, CH_2) ppm. ^{13}C NMR (100 MHz, D_2O, the signal of TFA not included) δ 156.9, 153.8, 151.8, 141.5, 130.0, 126.5 (2C), 125.3, 125.2 (2C), 39.0, 37.1 ppm. HRMS (ESI), m/z calcd for $C_{12}H_{15}N_4O_4S^+$ 311.0814 $[M+H]^+$ found 311.0809.

4.2 Carbonic anhydrase inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[22] 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 Tris (pH 8.3) 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 0.005 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as reported earlier, and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house.[23-26]

4.3 Docking studies

The crystal structure of *H*ca II (PDB code 2AW1[27]) was downloaded from the Protein Data Bank.[28] Protein-ligand complex was preprocessed using Schrodinger Protein PrepWizard.[29]

Complexed ligand is removed from active site and the protein structure was minimized using Schrodinger Prime module and OPLS3e force field at 310 K. Glide Grid program was used to prepare docking grid with included positional constraints. As a constraining factor interaction of sulfamide group with zinc ion in active site and hydrogen bond with Thr 200 residue was used. Grid spacing was set up as 0.375Å. Grid box size was selected as 8 x 8 x 8 Å. Docking procedure was carried out with Schrodinger Glide program[30] using OPLS3e force field. Before docking receptor constraints was included, where needed ligand features is marked to use. Energy window of 2 kcal/mol for conformer generation was chosen. Docked compounds subjected to 15 docking runs, with generation of 5000 poses per structure. Reference ligand geometry was used for binding pose control and best docked conformations were taken into account.

4.4 QSPR modeling

The permeability properties of the molecules were calculated using the formulae presented in Table 5.

Table 5. Formulae for estimating permeability properties of carbonic anhydrase inhibitors.[21,31]

Permeability (P_{app}, cm/s)	Formula[a]	Reference
Cornea (rabbit)	LogPapp = -3.885-0.183(HBtot)+0.277(logD7.4)	25
Cornea (porcine)	LogPapp = -4.6823−0.7670(logPSA)−0.1346 (HBd)+3.0024(Halogen ratio)	9
Conjunctiva (porcine)	LogPapp = -4.1594-0.6121(logPSA)-0.0792(HBd)+3.2914(Halogen ratio)	26

[a] LogPapp = logarithmic value of apparent permeability, HBtot = total amount of hydrogen bond formers, LogD7.4 = logarithmic value of distribution coefficient at pH 7.4, LogPSA = logarithmic value of polar surface area, HBd = hydrogen bond donors, Halogen ratio = sum of all halogens divided by the sum of all heavy atoms excluding hydrogen (zero for all compounds in this work).

4.5. Intraocular pressure studies

New Zealand White young adult female rabbits from Envigo Laboratories (UK) were used in the experiments. The animals were housed under standard laboratory conditions of 12-hour dark-light cycles and were provided with normal pellet diet with water *ad libitum*. Animals were handled in accordance with the statement of the Animals in Research Committee of the ARVO

(Association for Research in Vision and Ophthalmology, Rockville, Maryland, USA) and all animal experiments were approved by the Finnish National Animal Experiment Board (Eläinkoelautakunta, ELLA).

Commercial dorzolamide was used as reference drug (Trusopt®, dorzolamide 20 mg/mL, Santen Pharmaceutical Co., Ltd). The compounds investigated (**6a-d**) were dissolved in PBS at 10 mg/mL (**6a** and **6d**) or 5 mg/mL (**6b** and **6c**) concentrations. The pH and osmolality (Auto-Osmometer Osmostat OM-6020, Kagaku Ca. Ltd.) of the eye drop solutions were measured to ensure that the eye drops were close to neutrality and isotonicity.

Before the start of the experiment, validation of animal model and some pre-experiments were done to habituate the animals for measuring and to get background information of the variability in individual animals, fellow eyes, days and the circadian rhythm.

Each drug was applied as single eye drop at volumes of 25 µl to the left eye of the rabbit. The right eye was left untreated. The rabbits were held immobile for one minute after administration. Tonometer (TonoLab®, Icare Finland Ltd.) was used to measure intraocular pressure (IOP). In all experiments, the same eye was measured six times, the smallest and largest values were excluded and the average of four centremost values were used to calculate the value for the time point. The IOP of five normotensive rabbits were measured from both eyes before the administration of the molecule and at the timepoints 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hours after treatment. All experiments were conducted between 8:00am and 5:00pm. The wash-out period maintained between each test was at least four days. The change from baseline value(%) and the corresponding area under curve (AUC) of each molecule were compared with the vehicle and the statistical differences in the maximum effect (E_{max}) and AUC were tested with One-Way repeated measurement ANOVA with Dunnett's method.

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Conflict of Interest

None of the authors declares any conflict of interest.

32.

Highly Hydrophilic 1,3-Oxazol-5-yl Benzenesulfonamide Inhibitors of Carbonic Anhydrase II for Reduction of Glaucoma-Related Intraocular Pressure

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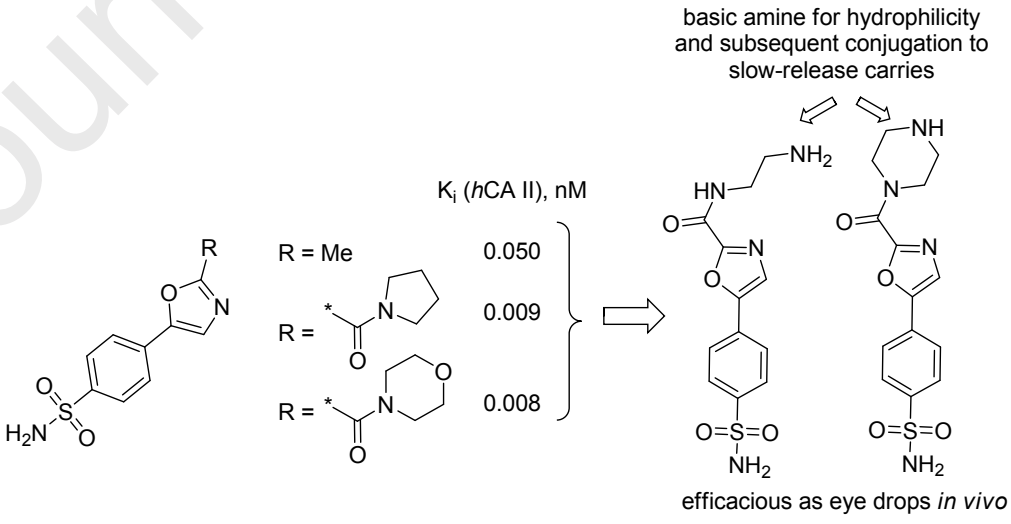

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