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Original article

Synthesis, ocular effects, and nitric oxide donation of imidazole amidoximes

L. Oresmaa [a], H. Kotikoski [b], M. Haukka [a], O. Oksala [c], E. Pohjala [c], H. Vapaatalo [d], E. Moilanen [e], P. Vainiotalo [a], P. Aulaskari [a,*]

[a] *Department of Chemistry, University of Joensuu, PO Box 111, 80101 Joensuu, Finland*
[b] *Department of Ophthalmology, Tampere University Hospital, PO Box 2000, 33521 Tampere, Finland*
[c] *Santen Oy, PO Box 33, 33721 Tampere, Finland*
[d] *Institute of Biomedicine, Pharmacology, University of Helsinki, PO Box 63, 00014 Helsinki, Finland*
[e] *The Immunopharmacology Research Group, University of Tampere, Medical School, 33014 Tampere, Finland*

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Abstract

Novel 1-R-imidazole-5-amidoximes and 1-R-5-cyano-imidazole-4-amidoximes (R: H, Me, Bn) were prepared from their corresponding nitriles and were tested for their efficacy to lower intraocular pressure (IOP) in rabbits. The ability of these compounds to donate nitric oxide (NO) was studied by observing the stimulation of formation of cyclic guanosine-3′,5′-monophosphate (cGMP) in the incubation of porcine iris-ciliary body. In the incubation experiments, 1-methylimidazole-5-amidoxime and 1(*H*)-imidazole-4(5)-amidoxime stimulated formation of cGMP indicating NO donating ability of these compounds. 1-Methylimidazole-5-amidoxime lowered IOP significantly after intravitreal injection.

Keywords: Nitric oxide; Cyclic GMP; Intraocular pressure; Imidazole; Amidoxime

1. Introduction

The discovery that nitric oxide (NO) acts as an important mediator of smooth muscle relaxation, has led to the preparation and testing of a wide variety of compounds with the aim of finding suitable new NO-donors [1]. The need for NO releasing compounds is continuously originating from the desire to reveal the mechanisms in different physiological phenomena relating to NO and to find new potential drugs for treatment of various diseases in which NO is involved.

Amidoximes inhibit platelet aggregation [2] and induce vasorelaxation in rat aorta [3,4]. They donate NO in the presence of mild oxidants [5] as well as in the presence of cytochrome P450 [6–8]. Cytochromes P450 (CYP) are a superfamily of enzymes which are found in almost all living organisms. CYP enzymes metabolize a variety of substances both endogenous and exogenous and have an important role in drug metabolism [9]. Several compounds bearing oxime functional group, including amidoximes, have been found to donate NO in the presence of rat liver microsomal cytochromes P450 [6–8]. Several CYP enzymes are known to be expressed also in ocular tissues [10–13]. They might thus provide a pathway for NO donation of amidoximes in the eye. However, amidoximes induce vasorelaxation in rat aorta and some evidence suggests that the NO-donation of amidoximes may also occur in P450-independent pathway [3,4]. The similarities of cytochrome P450 and nitric oxide synthases (NOS) and the knowledge that the biosynthesis of NO from arginine proceeds via oxidative cleavage of the C=NOH group of *N*-hydroxyguanidine, encouraged the study of oxime functionalized compounds as potential substrates for NOS [14]. However, a structure–activity study has shown that monosubstituted *N*-hydroxyguanidine function is obligatory for a compound to act as substrate for NOS [14].

NO is a mediator of physiological and pathophysiological processes in the eye, for example, regulation of aqueous humor dynamics, vascular tone, retinal neurotransmission, retinal ganglion cell death by apoptosis, phototransduction, and ocular immunological responses [15–17]. NO and NO-derived highly reactive radicals have been implicated in various eye diseases such as glaucoma, retinal degeneration, and uveitis [16]. It has

* Corresponding author.
E-mail address: paula.aulaskari@joensuu.fi (P. Aulaskari).

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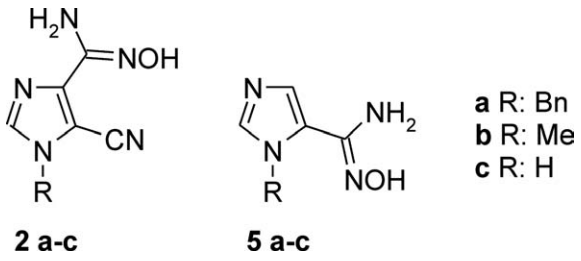


Fig. 1. Prepared and tested imidazole amidoximes.

been found that NO-donating compounds, cyclic guanosine 3′,5′-monophosphate (cGMP) activators, and cGMP itself, lower intraocular pressure (IOP) by reducing the production of aqueous humor and/or by increasing its outflow [18–33].

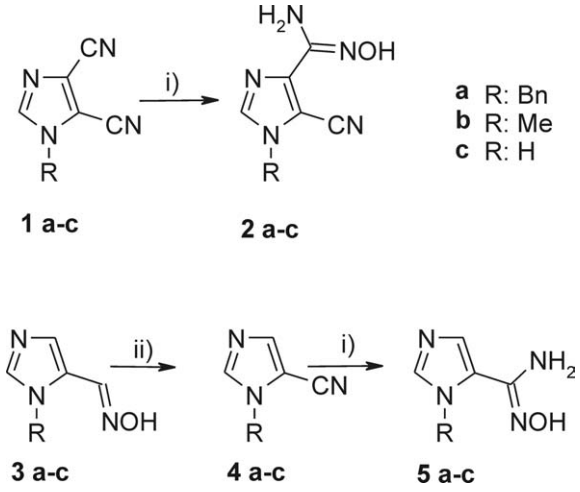
Since imidazole compounds are biologically active, we became interested in attaching potential nitric oxide (NO) donating functionalities to the imidazole scaffold. In our previous study several imidazole nitrolic acids were prepared and tested for their ocular effects [34]. In the view of the strong evidence for the NO-donating ability of various amidoximes [2–8], we synthesized several imidazole amidoximes (Fig. 1) and tested them for their ability to donate NO and for their IOP lowering. We were also interested in to compare the previously tested nitrolic acids and amidoximes tested in the present study, since the only structural difference between these compounds is the group attached to the oxime double bond.

2. Results

2.1. Chemistry

All amidoximes were prepared from their corresponding nitriles. Dicyano compounds **1a** and **1b** were prepared according to literature procedures, starting from commercially available 4,5-dicyanoimidazole **1c** (Scheme 1) [35,36]. Amidoximes **2a**–**c** were obtained by treating the nitriles with neutralized hydroxylamine hydrochloride water or methanol-water solution. Crystal structures of the amidoximes **2a**–**b** confirmed that the reacting cyano group is at the 4 position of the imidazole ring. This is reliable since the position C5 is more sterically hindered due to the 1*N*-substituent.

To prepare structures similar to the previously tested imidazole nitrolic acids, we needed the amidoxime functionality in position C5 of the imidazole ring. Since dicyano compounds reacted at the position C4 we had to find a different way to synthesize the C5 substituted compounds. Functional group modification is sometimes the best way to obtain a desired substituent in the correct position. Direct cyanation of imidazoles leads to 2-substituted compounds. 5-Cyano-1-methylimidazole can be prepared from 1-methylimidazole by anodic cyanation, but the product is a mixture of 5-, 4-, and 2-substituted compounds, which leads to a separation problem [37]. Since we had already synthesized oximes **3a**–**c** in our previous study [34], dehydration of oximes was chosen as the method to prepare the nitriles **4a**–**c**. Dehydration was conducted by refluxing the oximes in acetic anhydride. Compound **4c** had been prepared in this way earlier [38], and can also be prepared as a one pot procedure directly from the aldehyde [39].



Scheme 1. Synthesis of imidazole amidoximes. Reaction conditions i) $NH_2OH{\cdot}HCl$, H_2O; ii) Δ Ac_2O.

According to the NMR spectra amidoximes **2a**–**c** and **5a**–**c** were afforded as single isomers. The compounds **2a**–**c** are (Z)-isomers which can be seen from crystal structures.

2.2. Pharmacology

2.2.1. IOP measurements and nitrite, nitrate and cGMP in aqueous humor and plasma

The tested imidazole amidoximes **2a** (dose 72.4 μg), **2b** (dose 49.6 μg), **2c** (dose 45.3 μg), **5a** (dose 64.9 μg), **5b** (dose 42.1 μg) and **5c** (dose 37.8 μg) had no significant effect on IOP when administered topically on the eye surface in rabbits with normal IOP. The compound **5b** given intravitreally (dose 70.1 μg) lowered IOP maximally 6.6 mmHg from the basal level of 18 mmHg at the time point of 28 h ($P < 0.05$). However, no changes in the levels of nitrite, nitrite + nitrate (NO_x) or cGMP in aqueous humor and plasma were seen at 24 h after intravitreous administration of **5b** (dose 140.2 μg) (Table 1).

Table 1
NO_x, nitrite, and cGMP concentrations in aqueous humor and in plasma 24 h after intravitreal administration of **5b** (dose 140.2 μg) in rabbits (mean ± S.E.M., $n = 4$)

	Aqueous humor ($N = 4$)			Plasma ($N = 4$)	
	Nitrite (μM)	NO_x (μM)	cGMP (pmol/mg protein)	Nitrite (μM)	NO_x (μM)
5b	1.73 ± 0.42	455.73 ± 38.16	15.52 ± 3.15	1.05 ± 0.29	444.29 ± 26.77
Control	1.65 ± 0.36	479.07 ± 61.13	25.34 ± 4.56	0.98 ± 0.15	408.59 ± 47.90

Table 2
Effects of imidazole amidoximes on concentrations of NO_x, nitrite, and cGMP in porcine iris-ciliary body incubation (mean ± S.E.M., $N = 6$). Statistics are calculated on the basis of the respective controls of the experiment

Compound concentration (μM)	NO_x (μM)	Nitrite (μM)	cGMP (pmol/mg protein)
Control	0.34 ± 0.07	0.22 ± 0.05	0.53 ± 0.05
2a			
10	NE	NE	0.43 ± 0.03
100	NE	NE	0.45 ± 0.03*
2b			
10	NA	NA	NE
100	NA	NA	NE
2c			
10	NE	NE	0.55 ± 0.02**
100	NE	NE	0.57 ± 0.0*
5a			
10	NE	NE	0.46 ± 0.04
100	NE	NE	0.49 ± 0.04
5b			
10	2.03 ± 0.05***	2.50 ± 0.04***	0.53 ± 0.06
100	24.74 ± 0.27***	24.71 ± 0.39***	1.08 ± 0.12*
5c			
10	2.65 ± 0.07***	3.62 ± 0.07***	0.51 ± 0.04
100	32.72 ± 0.73***	36.83 ± 0.87***	1.45 ± 0.10**

NE: no effect; NA: not assayed ;*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

2.2.2. Incubation of porcine iris-ciliary bodies

NO_x, nitrite, and cGMP data obtained in incubation of porcine iris-ciliary bodies after administration of test compounds (concentrations 10 and 100 μM) are presented in Table 2. Compounds **5b**–**c** markedly and concentration dependently increased the formation of NO_x, nitrite, and cGMP.

2.2.3. Aqueous humor outflow facility

Aqueous humor outflow facility was measured after the intracameral injection of compound **5b** (dose 0.7 μg, $n = 2$), but it had no significant effect as compared with contralateral control eye (not shown on the tables).

3. Discussion

Various compounds affecting the NO-cyclic GMP pathway lower IOP in animals [18–32]. In the anterior part of the eye, NO-donating compounds and cGMP regulate aqueous humor flow, and thus IOP, at the level of ciliary muscle, trabecular meshwork, and endothelial and vascular smooth muscle cells in the aqueous drainage system [16,40]. Activation of the anterior segment guanylate cyclase elevates cGMP levels, leading to decreased aqueous humor production and thus to reduced IOP [20,22]. In addition, NO-releasing compounds and cGMP analogues increase aqueous humor outflow [24,33]. A nontoxic NO-donating or guanylate cyclase-activating compound would be a lead molecule for a new class of antiglaucomatous drug.

Most of the effects of NO donors are mediated via the activation of guanylate cyclase. We determined these effects by measuring the production of cGMP in porcine iris-ciliary body incubation, a screening method developed by Kotikoski et al. [41]. The iris-ciliary body is the target tissue of NO in the modulation of aqueous humor flow. There is evidence that NO regulates IOP by inducing relaxation of ciliary muscle and trabecular meshwork and thus decreasing the resistance of the uveoscleral outflow pathway. This smooth muscle relaxation in the chamber angle results from an increase in intracellular cGMP [40,42]. Earlier studied NO donors and cGMP activators, which lowered IOP in rabbits in vivo [32], increased the production of cGMP in the porcine iris-ciliary body in vitro [41]. In the present study, amidoximes **5b**–**c** elevated cGMP concentrations in the incubation experiments, indicating that imidazole amidoximes **5b**–**c** are capable of donating NO. Compound **5b** did lower the IOP when injected intravitreally. Even though a distinct relationship between elevated cGMP concentrations and IOP reduction has been recorded for known NO donors [32,41], in the present work compound **5c** and some of the previously studied nitrolic acids [34] failed to lower IOP in vivo even though they raised cGMP levels in vitro. It is possible that these compounds are unable to reach the target tissue in vivo in sufficient concentrations or that the compound **5b** lowers IOP via NO independent mechanism.

Cyano group at the imidazole ring appears to abolish the activities. Compounds **2b** and **5b** differ also with the position of amidoxime functionality but owing to the tautomerization, compounds **2c** and **5c** could be compared (Fig. 2). The fact that amidoxime **5c** increases the cGMP concentration in iris-ciliary body incubation, whereas **2c** fails to do this, suggests that the cyano group is the reason for the lack of the activity. Whether the configuration of the double bond plays a role in the activities is not known. There is no information concerning the isomers in previous studies of the NO donation of amidoximes and in our study the configurations are known only for amidoximes **2a**–**c**.

Poor bioavailability of topically administered ophthalmic drugs is a quite common problem, since eyes are well protected against xenobiotics. In order to reach the target tissue situated in inner parts of the eye, a compound has to pass through precorneal barriers (tear film and conjuctiva) and the cornea. In

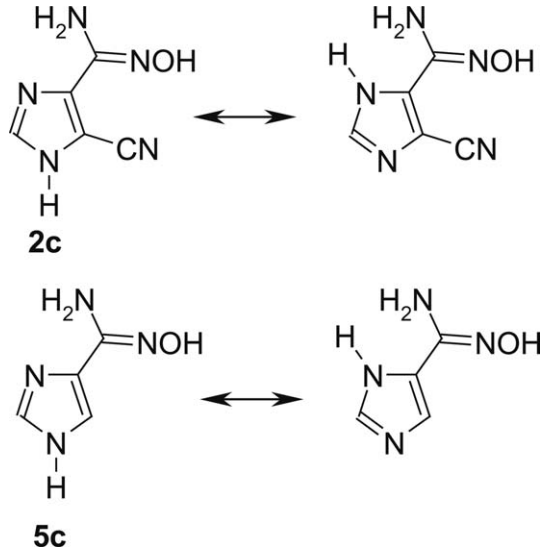


Fig. 2. Tautomers of the compounds **2c** and **5c**.

practice cornea consists three layers, epithelium, stroma, and endothelium. In the epithelium the cells are connected with tight junctions which means that macromolecules are excluded. Structure–activity study shows that the epithelium tends to be the rate limiting barrier for transcorneal absorption [43]. For small, sufficiently lipophilic molecules that penetrate epithelium well, stroma and endothelium may become important [43]. The tested amidoximes failed to lower IOP when administered topically, the same problem that was encountered with previously studied nitrolic acids [34].

4. Conclusion

Novel imidazole amidoximes were prepared from their corresponding nitriles and tested for their ability to lower intraocular pressure (IOP). The nitric oxide (NO) donation of these compounds was studied by following the formation of cyclic guanosine-3′,5′-monophosphate (cGMP) in the incubation of porcine iris-ciliary body. In the incubation experiments 1-methyl-5-amidoxime **5b** and 1(*H*)-imidazole-4(5)-amidoxime **5c** showed elevated concentrations of cGMP, indicating NO-donation of these compounds, and compound **5b** lowered IOP after intravitreal injection.

5. Experimental protocols

5.1. Chemistry

Unless otherwise indicated, all common reagents and solvents together with compound **1c** were obtained from commercial suppliers and were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 250 NMR spectrometer. The mass spectrometry measurements were performed on a Bruker BioApex 47e Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with an Infinity™ cell, 4.7 Tesla 160-mm-bore superconducting magnet (Magnex Scientific Ltd., Abingdon, UK), and an external electron ionization (EI) or electrospray ion source (ESI) (Analytica of Branford Inc., Branford, CT, USA). Elemental analyses were performed on a CE Instruments EA 1110 elemental analyzer. Analyses indicated by the symbols of the elements or functions were within ± 0.4% of the theoretical values.

1-Benzyl-4,5-dicyanoimidazole **(1a)** [35], 1-methyl-4,5-dicyanoimidazole **(1b)** [36], 1-benzylimidazole-5-carbaldehyde oxime **(3a)** [34], 1-methylimidazole-5-carbaldehyde oxime **(3b)** [34] and 1*H*-imidazole-5(4)-carbaldehyde oxime **(3c)** [44] were prepared according to literature procedures.

5.1.1. General procedure for preparation of 1R-5-cyanoimidazoles *4a–c*

Oximes were refluxed in distilled acetic anhydride for 18 h (**4a** and **4b**) or 24 h (**4c**). The reaction mixture was poured over ice and the pH was adjusted to 8 with Na_2CO_3 followed by extraction with chloroform (**4a, 4b**) or ethyl acetate (**4c**). The extract was washed with saturated $NaHCO_3$ solution and with water, dried over $MgSO_4$, and evaporated.

5.1.2. 1-Benzyl-5-cyanoimidazole (*4a*)

Prepared according to the general procedure. Oxime **3a** (1 g, 4.97 mmol), Ac_2O (12 ml). The crude product was purified by flash chromatography, eluting with 5% MeOH in EtOAc (silica gel). Yield 44%. ^{1}H NMR (DMSO-d_6): δ = 5.38 (s, 2H), 7.15–7.42 (m, 5H), 7.88 (*s*, 1H), 8.27 (*s*, 1H). ^{13}C NMR (DMSO-d_6): δ = 49.4 (t), 104.0 (s), 111.5 (s), 127.5 (d), 128.3 (d), 128.9 (d), 135.8 (s), 140.5 (d), 142.2 (d). Anal. ($C_{11}H_9N_3$): C, H, N.

5.1.3. 1-Methyl-5-cyanoimidazole (*4b*)

Prepared according to the general procedure. Oxime **3b** (1 g, 8 mmol), Ac_2O (12 ml). Yield 53%.The crude product was purified by flash chromatography eluting with 10% MeOH/acetone (silica gel). ^{1}H NMR (DMSO-d_6): δ = 3.77 (s, 3H), 7.83 (s, 1H), 8.01 (s, 1H); ^{1}H NMR ($CDCl_3$): δ = 3.82 (s, 3H), 7.63 (s, 1H), 7.66 (s, 1H) in agreement with literature [45]. ^{13}C NMR (DMSO-d_6): δ = 33.6 (q), 106.2 (s), 112.4 (s), 140.0 (d), 143.1 (d).

5.1.4. 1H-4(5)-cyanoimidazole (*4c*)

Prepared according to the general procedure. Oxime **3c** (1 g, 9 mmol), Ac_2O (12 ml). Yield 45%. The product was used for the next step without further purification. ^{1}H NMR (DMSO-d_6): δ = 7.92 (s, 1H), 8.12 (s, 1H), 12.99 (s) in agreement with literature [46]. ^{13}C NMR (DMSO-d_6): δ = 111.8 (s), 116.1 (s), 127.2 (d), 138.2 (d). MS (EI): Calcd for *m*/*z* ($C_4H_3N_3$) 93.03214. Found 93.03241.

5.1.5. General procedure for preparation of the carboxamide oximes *2a–c* and *5a–c*

The cyano compound was added to a solution of $NH_2OH{\cdot}HCl$ and Na_2CO_3 in water (or water/methanol). The resulting mixture was heated under stirring at 70 °C. The mixture was allowed to cool and the precipitate was filtered and washed with water.

5.1.6. (Z)-1-benzyl-5-cyanoimidazole-4-carboxamide oxime (*2a*)

Prepared according to the general procedure. Nitrile **1a** (0.5 g, 2.4 mmol), methanol (1 ml), water (2 ml), $NH_2OH{\cdot}HCl$ (1.7 g, 2.4 mmol), Na_2CO_3 (0.13 g, 1.2 mmol). Reaction time 2 h. Yield 70%. Recrystallization from MeOH. ^{1}H NMR (DMSO-d_6): δ = 5.37 (s, 2 H), 5.70 (s, 2H), 7.26–7.44 (m, 5 H) 8.29 (s, 1H), 9.92 (s, 1 H). ^{13}C NMR (DMSO-d_6): δ = 49.7 (t), 100.1(s), 111.8 (s), 127.6 (d), 128.5 (d), 129.0 (d), 135.6 (s), 141.3 (d), 144.0 (s), 145.6 (s). MS (EI): Calcd for *m*/*z* 214.09581 Found 241.10335. Anal. ($C_{12}H_{11}N_5O$) C, H, N.

5.1.7. (Z)-1-methyl-5-cyanoimidazole-4-carboxamide oxime (*2b*)

Prepared according to the general procedure. Nitrile **1b** (0.5 g, 3.78 mmol) $NH_2OH{\cdot}HCl$ (0.26 g, 3.76 mmol), water

(3 ml), reaction time 2 h. Yield 80%. Raw product was a mixture of **2b** and an unidentified compound which is probably (*E*)-**2b** or the regioisomer of **2b** namely 1-methyl-4-cyanoimidazole-5-carboxamide oxime. Recrystallization from methanol afforded the *(Z)*-**2b**. ^{1}H NMR (DMSO-d_6): δ = 3.75 (s, 3H), 5.66 (s, 2H), 8.01 (s, 1H), 9.90 (s, 1H). ^{13}C NMR (DMSO-d_6): δ = 32.97 (q), 101.02 (s), 111.92 (s), 141.53 (d), 143.29 (s), 145.70 (s). MS (EI): Calcd for *m/z* 165.06451. Found 165.06529. Anal. ($C_6H_7N_5O$): C, H, N.

NMR data for the side product: ^{1}H NMR (DMSO-d_6): 3.76 (s, 3H), 6.11 (s, br, 2H), 7.89 (s, 1H), 10.18 (s, 1H); ^{13}C NMR (DMSO-d_6): δ = 33.53 (q), 112.35 (s), 115.42 (s), 134.55 (s), 141.14 (d), 142.16 (s).

5.1.8. 1H-5(4)-cyanoimidazole-4(5)-carboxamide oxime (2c)

Prepared according to the general procedure. Nitrile **1c** (0.5 g, 4.2 mmol), $NH_2OH{\cdot}HCl$ (0.29 g 4.2 mmol), water (3 ml). Reaction time 1.5 h. Yield 48%. ^{1}H NMR (DMSO-d_6): δ = 5.89 (s, 2H), 7.92 (s, 1H), 10.15 (s, 1H), 13.25 (s, 1H). ^{13}C NMR (DMSO-d_6): δ = 109.7 (s), 115.8 (s), 133.0 (s), 138.1 (d), 143.1 (s). MS (EI): Calcd for *m/z* 151.04886. Found 151.04912. Anal. ($C_5H_5N_5O$): C, H, N.

5.1.9. 1-Benzylimidazole-5-carboxamide oxime (5a)

Prepared according to the general procedure. Nitrile **4a** (0.25 g, 1.4 mmol), $NH_2OH{\cdot}HCl$ (0.19 g, 2.8 mmol), Na_2CO_3 (0.15 g, 1.4 mmol), water (3 ml). Reaction time 5 h. Yield 77%. ^{1}H NMR (DMSO-d_6): δ = 5.51 (s, 2H), 5.76 (s, 2H), 7.11–7.16 (m, 2H), 7.24–7.34 (m, 4H), 7.77 (s, 1H), 9.60 (s, 1H). ^{13}C NMR (DMSO-d_6): δ = 48.73 (t), 124.94 (s), 127.03 (d), 127.31 (d), 128.45 (d), 129.35 (d), 138.35 (s), 140.21 (d), 144.98 (s). MS (ESI): Calcd for $[M + H]^+$ 217.10842. Found 217.10841. Anal. ($C_{11}H_{12}N_4O$) C, H, N.

5.1.10. 1-Methylimidazole-5-carboxamide oxime (5b)

Prepared according to the general procedure. Nitrile **4b** (0.3 g, 2.8 mmol), $NH_2OH{\cdot}HCl$ (0.39 g, 5.6 mmol), water (3 ml). Reaction time 5 h. The reaction mixture was saturated with NaCl and extracted with EtOAc, dried in $MgSO_4$ and evaporated. Yield 52%. ^{1}H NMR (DMSO-d_6): δ = 3.71 (s, 3H), 5.74 (s, 2H), 7.25 (s, 1H), 7.57 (s, 1H), 9.61 (s, 1H). ^{13}C NMR (DMSO-d_6): δ = 34.07 (q), 125.44 (s), 129.24(d), 140.35 (d), 145.12 (s). MS (ESI): Calcd. for $[M + H]^+$ 141.077087. Found 141.07706. Anal. ($C_5H_8N_4O$): C, H, N.

5.1.11. 1H-imidazole-5(4)-carboxamide oxime (5c)

Prepared according to the general procedure. Nitrile **4c** (0.25 g, 2.7 mmol), $NH_2OH{\cdot}HCl$ (0.37 g, 5.4 mmol), Na_2CO_3 (0.29 g, 2.7 mmol) water (2.5 ml). Reaction time 5 h. After the reaction mixture was saturated with NaCl, **5c** precipitated. Yield 53%. ^{1}H NMR (DMSO-d_6): δ= 5.49 (br, 2H), 7.28 (s, 1H), 7.63 (1H), 9.15 (br, 1H), 12.24 (br). MS (ESI): Calcd for $[M + H]^+$ 127.06144. Found 127.06156. Anal. Calcd for $C_4H_5N_4O$: C, 38.09; H, 4.80; N, 44.42. Found: C, 37.99; H, 4.89; N, 43.79.

5.1.12. X-ray structure determinations

The X-ray diffraction data were collected with a Nonius KappaCCD diffractometer using Mo *K*α radiation (λ = 0.71073 Å). The Denzo-Scalepack program package [47] was used for cell refinements and data reduction. All structures were solved by direct methods using the SHELXS-97 or SIR97 programs and the WinGX graphical user interface [48–50]. Structural refinements were carried out with SHELXL-97 [48]. In structure **2c** all hydrogens, and in **2a** NH_2 and OH hydrogens were located from the difference Fourier map and refined with fixed U_{iso} = 0.05. In **2b,** NH_2 and OH hydrogens, were also located from the difference Fourier map, but they were refined isotropically. X-ray data of the compounds **2a**–**c** and **4a** have been deposited in the Cambridge Crystallographic Data Centre as deposition numbers CCDC 282905, CCDC 282904, CCDC 282903, and CCDC 282906, respectively. These data can be obtained free of charge by contacting the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 1223 336033 or e-mail: data_request@ccdc.cam.ac.uk].

5.2. Pharmacology

5.2.1. Intraocular pressure measurements

New Zealand White (NZW) rabbits of both sexes with normal IOP were used in the experiments (four animals for each imidazole amidoxime). The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23) was adhered to the protocol of the study, and the protocol was approved by the local Animal Experimentation Committee. IOP was measured with a pneumatonometer (Modular One Tonometer, Mentor, Cambridge, MA) after topical anesthesia with 0.4% oxybuprocain (Oftan® Obucain, Santen Oy, Tampere, Finland). Measurements were taken at the same time of day by the same investigator. One hour before application of the test compound, a control measurement was taken for both eyes. Thirty microliters of the test compound (experimental eye) or vehicle (control eye) was administered by micropipet in the inferior conjunctival sac, or 50 μl of the test compounds or vehicle was injected by Hamilton precision syringe to the vitreous humor. Different concentrations of imidazole nitrolic acids were tested. IOP was measured three times at each time interval, and the means were reported.

5.2.2. Measurement of nitrite, nitrate, and cyclic GMP in aqueous humor and plasma

In the experiments for the biochemical assays, blood samples were taken and the rabbits were euthanized with 300 mg pentobarbital (Mebunat®, Orion Oy, Espoo, Finland) applied intravenously 24 h after the administration of the test compound, and the aqueous humor was taken by injection syringe for measurements. The procedure has been presented in detail earlier [51].

5.2.3. Incubation of porcine iris-ciliary bodies

Porcine eyes were obtained from an abattoir and were prepared for the experiments within 3 h of enucleation. The eyes

were cut in half at the equator of the bulbus and vitreous and lens were removed. The ciliary body and iris were carefully detached from the sclera by cutting the tissue with a cornea trepan. The tissue samples were pooled and placed in oxygenated modified Krebs solution. After preincubation of 1 h the multidish wells were cleared of the solution and fresh Krebs solution and test compounds or solvent were added. The incubation period of 60 min was used. The data measurements have been described in detail in our recent article [41]. Acetylated cGMP in the samples was assayed with the [^{125}I]-cyclic GMP RIA kit (Amersham International, Little Chalfont, Buckinghamshire, UK). Nitric oxide release from the test compounds was determined spectrophotometrically by measuring nitrate + nitrite (NO_x) in the incubation medium with the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). The concentrations of test compounds were chosen on the basis of our previous studies [34]. The differences in the concentration used are mainly due to the different solubilities of the compounds in the incubation medium.

5.2.4. Measurement of aqueous humor outflow facility

New Zealand White (NZW) rabbits of both sexes with normal IOP were used in the experiments. The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23) was adhered to the protocol of the study and the protocol was approved by the local Animal Experimentation Committee. The outflow facility of aqueous humor in anesthetized rabbits was determined by the two-level constant pressure infusion method [52]. Anesthesia was initiated with an intramuscular injection of a combination of ketamine (Ketalar® 50 mg/ml, Parke-Davis Warner Lambert Nordic AB, Solna, Sweden) and xylazine (Rompun®Vet 20 mg/ml, Bayer AG, Leverkusen, Germany) and maintained by intravenous infusion of the same combination. The eyes of the animals were cannulated with three needles (27 G) connected to polyethylene cannulas after topical anesthesia. One cannula was used for continuous IOP monitoring, one for injection of the test compound or vehicle (5 μl) and one for the infusion of fluid for outflow facility measurements. The outflow facility was measured 60 min after the intracameral administration of the test compound. IOP was increased 5–7 mmHg above the preinfusion level by infusing a mock solution of aqueous humor [53] into the anterior chamber. The infusion rate (F) and the increase in IOP (ΔP) were registered in steady-state conditions. Thereafter, the IOP was raised about 5–7 mmHg above the previous level, and the same procedure was repeated. The outflow facility (C) could then be calculated from the formula $C = F/\Delta P$. Infusion was carried out on both eyes simultaneously from separate reservoirs. Detailed data of the method have been described previously [31].

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