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Synthesis of novel nitric oxide (NO)-releasing esters of timolol

Valerio Chiroli [a,*], Minerva R. Batugo [b], Stefano Biondi [a], Annalisa Bonfanti [a], Stefania Brambilla [a], David C. Gale [b], Lin Li [b], Daniela Miglietta [a], Fabio Nicoli [a], Ganesh A. Prasanna [b], Daniela Ronchetti [a], William F. Vernier [b], Wesley K. M. Chong [b]

[a] NicOx Research Institute, Via Ariosto 21, 20091 Bresso (Milan), Italy
[b] Pfizer Global Research and Development-La Jolla, 10770 Science Center Drive, San Diego, CA 92121, USA

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ABSTRACT

A novel class of timolol derivatives with nitric oxide (NO)-donating moieties achieved chemical stability yet under physiologically relevant conditions released timolol and NO. Hindered esters **A** were designed and synthesized, whose 'triggered' release relied on enzymatic hydrolysis of the nitrate ester in **A** to **B**, that in turn cyclized to liberate timolol.

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Glaucoma is a degenerative eye disease that in most cases is accompanied by increased intraocular pressure (IOP) within the eye, which might lead to damage to the optic nerve and eventually progress to blindness.[1]

Timolol is a non-selective β-adrenergic receptor blocker widely used for the treatment of glaucoma. Its therapeutic effects depend on the reduction of aqueous humour secretion at the ciliary process level.[2] A major drawback for timolol eyedrops is the relatively high incidence of the cardiovascular and respiratory side-effects[3] due to the fact that 1–2% of the instilled dose penetrates the eye, while the majority reaches the systemic circulation.[4] The poor bioavailability of this compound is largely due to a precorneal process that removes timolol rapidly from the intended absorption site, and because the corneal epithelium acts as a lipophilic barrier restricting corneal drug penetration.[5] To overcome this issue, Bundgaard and Lee reported studies of ester derivatives of timolol with increased lipophilicity and thus improved absorption.[6] Unfortunately these derivatives also showed chemical instability in aqueous solution with short shelf life as eyedrops.[6] Based on these prior studies that also elucidated the ester hydrolysis mechanism,[7] herein an approach for more stable esters was designed, based on benzoates with groups in *ortho* position. As shown in Figure 1, examination of a three-dimensional model[8] of timolol *O*-(2,6-dimethoxybenzoate) shows that the ester carbonyl carbon is flanked on two sides by both methoxy groups, blocking any nucleophile (such as water for hydrolysis) to approach via the expected Bürgi–Dunitz trajectory.[9]

In addition, since NO donors are known to also reduce IOP,[10,11] conjugation of timolol with NO releasing groups was compelling to assess whether the IOP lowering efficacy could be further enhanced. This dual approach has precedent with the agent nipradilol (Hypadil®), an approved glaucoma drug in Japan.[12–14] Herein we report on our preliminary efforts for the design, synthesis and initial pharmacological characterization of chemically stable hybrid molecules able to release both timolol and bioactive NO once instilled in the eye.

A number of derivatives of timolol with varied hindrance were prepared as shown in Table 1. The known simple benzoate **1**,[15] and novel NO releasing derivatives were also evaluated with respect to their stability in aqueous solution at pH 5.7. Lower pH improves ester stability for eyedrop formulation.[15] Table 1 shows derivatives with instability in solution to those with total inertness, even to rabbit corneal homogenate. This tissue was analyzed to contain 0.13 ± 0.02 U/mg protein of esterase activity, as measured from the hydrolytic conversion of *p*-nitro-phenyl acetate to *p*-nitrophenol.[16] Then the liberation of timolol was measured for these derivatives at 37 °C as the displacement over time of labelled L-[4,6-propyl-3H]dihydro-alprenolol[17] from a complex with recombinant human β-receptors from corneal extracts (and compared

* Corresponding author. Tel.: +39 0261036326.
E-mail address: chiroli@nicox.it (V. Chiroli).

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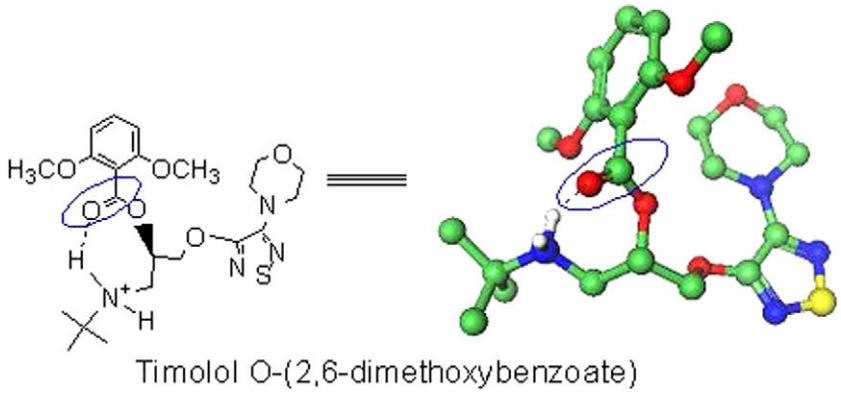


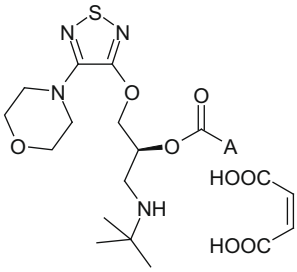
Figure 1. 3-Dimensional model[8] of timolol *O*-(2,6-dimethoxybenzoate), highlighting in the drawn oval, the carbonyl hindered to nucleophilic substitution by the flanking methoxy groups.

to vehicle). Quantification of timolol was thus derived from a standard curve obtained from known concentrations of synthetic timolol.[18]

Also seen in Table 1, the presence of only one substituent at the *ortho*-position relative to the ester moiety (position 2 on the aromatic ring as in esters **1–4**, **6**, **7**, and **9**,)—this was not enough to confer sufficient chemical stability in solution after 1 week (>98% titer was desired). However, the introduction of a second electron rich group at the other *ortho*-site (position 6 on the aromatic ring) exemplified with the di-*ortho*-benzoates **5**, **8**, and **10**, was found to stabilize the compounds in solution and rendered them inert to enzymatic cleavage. These results support the rationale that the benzoate carbonyl moiety is shielded at above and below the ester plane by both *ortho* groups that prevent any nucleophilic attack. Consistent with this interpretation, the aqueous stability of mono-*ortho*-benzoates **3**, **4**, and **6** were relatively poor, with the initial titer in solution dropping to 70–80% in one week as compared to di-*ortho*-benzoates **8** and **10**, which remained above 98% after 12 weeks. Unfortunately at this stage, di-*ortho* benzoates **8** and **10** were stable to esterases in the corneal homogenate, with $t_{1/2}$ > 1400 min. Consequently new analogs needed to be designed with sufficient stability in aqueous solution like di-*ortho* **8** and **10**, but somehow made susceptible to enzymatic hydrolysis (and presumably provide in vivo activity). As shown in Scheme 1, new derivatives were conceived by adapting a triggering reaction release strategy,[19] in which the enzymatically labile group is placed in a more accessible position, such as in conjugate **A**. Metabolic hydrolysis and liberation of NO upon conversion to transient **B**, simultaneously would reveal a trigger (a nucleophilic group; the benzylic alcohol in **B** in this instance), that is then poised to attack the carbonyl of the benzoate ester via intramolecular cyclization and release timolol.

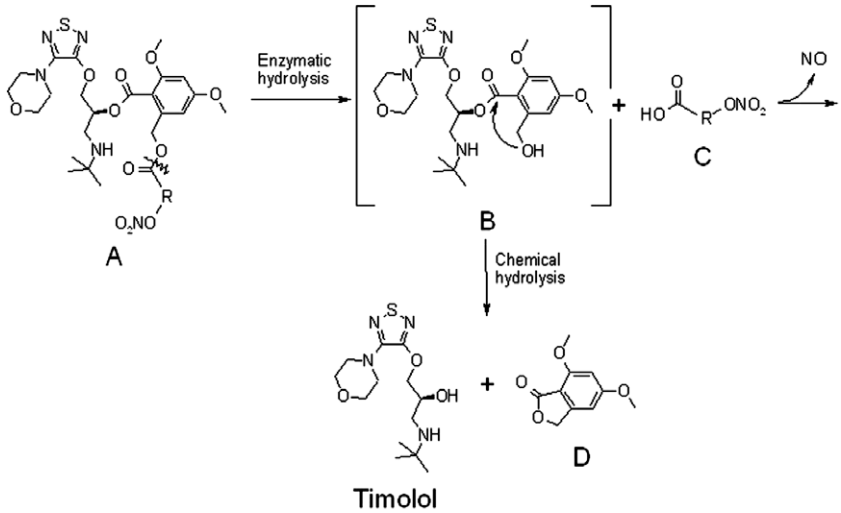
Indeed, the compounds listed in Table 2 showed increased stability in solution and were cleaved in the presence of rabbit corneal homogenate. Of the group in Table 2, the 6-nitroxyhexanoate **11**[20] is stable as it retains 97.7% titer, while its cleavage half-life in pres-

Table 1
Chemical stability in solution (buffer pH 5.7, Tween 80 0.5%, BAK 0.02%, *T* = room temperature) and incubation with rabbit corneal homogenate esterase liability of timolol benzoates **1**–**10**



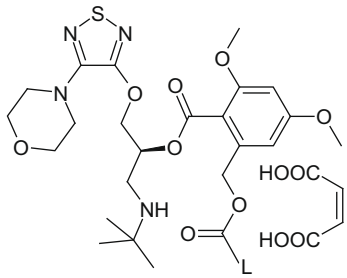
Compound	Linker (A)	Stability in solution (ratio[a])					Incubation with corneal homogenate[b] $t_{1/2}$ (min)
		1d	1w	4w	8w	12w	
1	Ph	96.05	74.8	–	–	–	–
2	Ph 2-OCH_3 5-CH_2ONO_2	Unstable at T_0					
3	Ph 2-$O(CH_2)_2ONO_2$	96.5	82.5	–	–	–	277
4	Ph 2-$O(CH_2)_2ONO_2$3-OCH_3	96.0	72.1	–	–	–	258
5	Ph 2,6-OCH_3 4-$CH_2OCO(CH_2)_5$ ONO_2	100.0	100.2	98.0	96.8	93.2	>1400
6	Ph 2-$OCH_2CH_2ONO_2$,4-$OCH_2CH_2ONO_2$	97.9	84.2	–	–	–	–
7	Ph 2-OCH_3 4-$CH_2OCH_2CH_2ONO_2$	96.7	94.9	–	–	–	–
8	Ph 2-OCH_3 6-CH_3 4-$OCH_2CH_2ONO_2$	101.2	101.4	100.5	99.6	98.5	>1400
9	Ph 2-OCH_3 4-$OCH_2CH_2ONO_2$	98.6	96.3	84.4	–	–	–
10	Ph 2-OCH_3 6-$OCH_2CH_2ONO_2$	99.8	99.3	98.3	98.3	98.3	>1400

[a] Ratio = (Area% t_x/Area% t_0)% by HPLC.
[b] Enzymatic liability was determined in presence of rabbit corneal homogenate (protein concentration was adjusted to 1 mg/mL with MCBD 153 medium obtained from Sigma–Aldrich) through the determination of timolol released into the incubating buffer at 37 °C over time. Specifically, timolol content was determined assessing the displacing activity on recombinant human β-receptors labelled with L-[4,6-propyl-^{3}H]dihydro alprenolol[16] of corneal extracts previously exposed to vehicle of test compounds.



Scheme 1. Hypothetical mechanism for the release of timolol: the triggering strategy undergoing through intramolecular cyclization reaction.

Table 2
Chemical stability in solution (buffer pH 5.7, Tween 80 0.5%, BAK 0.02%, *T* = room temperature) and enzymatic liability of compounds **11**–**15**



Compound	Linker (L)	Stability in solution (ratio[a])					Enz-liab[b] $t_{1/2}$ (min)
		1d	1w	4w	8w	12w	
11	$-(CH_2)_5ONO_2$	99.6	99.6	97.7	92.8	–	10
12	$-CH_2O(CH_2)_2O(CH_2)_2ONO_2$	98.8	93.0	–	–	–	10
13	$-C(CH_3)_2CH_2ONO_2$	98.4	99.1	94.7	94.9	93.2	120
14	$-(CH_2)_2OCH_2CH(ONO_2)CH_2ONO_2$	97.3	96.7	95.8	91.7	86.0	40
15	$-(CH_2)_3ONO_2$	96.5	96.5	90.6	-	–	12

[a] Ratio = (Area % t_x/Area % t_0) %.
[b] Enzymatic liability was determined as specified in Table 1.

ence of corneal homogenate was among the shortest. As expected, a more hindered trigger ester, such as 3-nitroxypivalate **13** was not only stable in buffer but also more stable to corneal homogenate compared to the others (**11**, $t_{1/2}$ = 120 min vs **13**, $t_{1/2}$ = 10–40 min).

The NO releasing properties were determined from vasorelaxation studies in methoxamine-precontracted rabbit aortic rings (EC_{50} values estimated from plots of relaxation at various compound concentrations). NO-donor isosorbide mononitrate (ISMN)[24] was comparator for NO/cGMP-mediated effects, and parallel experiments were performed with highly selective inhibitor of soluble guanylyl cyclase (sGC), 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ),[27] known to abolish NO/cGMP-dependent effects. As shown in Table 3, consistent with previous reports,[28] timolol evoked a vasorelaxant effect with an EC_{50} = 28.8 μM and was not significantly affected by addition of ODQ (EC_{50} = 36.5 μM)—demonstrating that its mechanism of action is independent from the cGMP signalling pathway. For comparison, ISMN[24] had an EC_{50} = 9.6 μM and its effect was completely abolished by 10 μM, ODQ, documenting the responsiveness of the preparation to NO/cGMP signalling and blockage of this pathway with inhibitor. Interestingly, the 'trigger' compounds **11** and **12** gave EC_{50} values comparable with that of ISMN in absence of ODQ (**11**, 6.7 μM

Table 3
Vasodilating potency of timolol, ISMN,[24] trigger compounds **11** and **12** in presence and absence of the guanylate cyclase inhibitor ODQ (10 μM)

Compound	EC_{50} (μM)	EC_{50} (μM) w/10 μM ODQ
ISMN	9.6 ± 2.9	>100*
Timolol	28.8 ± 5.4	36.5 ± 1.3
11	6.7 ± 2.9	56.3 ± 9.3*
12	15.5 ± 6.7	32.7 ± 4.3*

Vasorelaxant potency was determined in rabbit aortic rings precontracted with 3 μM methoxamine. EC_{50} (effective concentration giving 50% of response) 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 four independent experiments.
* $p < 0.05$ versus condition in absence of ODQ.

and **12**, 15.5 μM) and comparable with that of timolol in presence of ODQ (**11**, 56.3 μM and **12**, 32.7 μM), implying that these compounds combine timolol- and NO-mediated properties. Thus, in this biological assay the selected compounds show properties dependent on their expected dual mechanism of action.

In conclusion, new conjugates of timolol with NO-donating capability were made. They are stable in solution and yet also labile to enzymatic hydrolysis, by way of a 'trigger strategy' that allows the release of the core drug (timolol) and NO. These compounds may manifest improved in vivo IOP lowering activity—related studies of the in vivo activity are underway and will be reported in due course.

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20. Representative procedures and sequence for the synthesis of compound **11**.
*(S)-3-(tert-Butylamino)-1-(4-morpholino-1,2,5-thiadiazol-3-yloxy)propan-2-yl 2-(Chloromethyl)-4,6-dimethoxybenzoate (**c**).*To a solution of 2-(chloromethyl)-4,6-dimethoxybenzoic acid[21] (1.5 g, 6.6 mmol) in DCM (10 mL) at 0 °C was added oxalyl chloride (0.61 ml, 7.2 mmol) and stirred 1 h. In turn this mixture was added to a solution of timolol (free base,[22] 1.4 g, 4.4 mmol) in DCM (30 ml) at 0 °C. The mixture was kept to 0 °C for 30 min and then was allowed to warm to ambient temperature and stir for 24 h. The crude mixture was evaporated to dryness. The crude material was purified over silica gel eluting with EtOAc/MeOH/TEA 95:5:0.5 to give 0.850 g of ester **c** as a colorless foam.
*(1S)-3-(tert-Butylamino)-1-{[(4-morpholin-4-yl-1,2,5-thiadiazol-3-yl)oxy]methyl} ethyl 2,4-Dimethoxy-6-({[6-(nitroxy)hexanoyl]oxy}methyl)benzoate (**d**).*To a solution of (*S*)-3-(*tert*-butylamino)-1-(4-morpholino-1,2,5-thiadiazol-3-yloxy)propan-2-yl 2-(chloromethyl)-4,6-dimethoxybenzoate (**c**; 1.1 g, 2.07 mmol) and 6-nitroxyhexanoic acid[23] (0.55 g, 3.1 mmol) in DMSO, was added Cs_2CO_3 (1.0 g, 3.1 mmol) whereupon an exotherm was observed (CAUTION: low molecular weight nitrated compounds, that is, 6-nitroxyhexanoic acid, might be explosive: keep temperature under control and use diluted solution). The mixture stirred at ambient temperature for 8 h, then diluted with EtOAc and washed with water. The separated organic phase was dried over Na_2SO_4 and evaporated in vacuo. The residue was purified over silica gel eluting with EtOAc/Hexane 2:1 to give 0.85 g of the title compound as a colorless oil.
(1*S*)-3-(*tert*-Butylamino)-1-{[(4-morpholin-4-yl-1,2,5-thiadiazol-3-yl)oxy]methyl} ethyl 2,4-dimethoxy-6-({[6-(nitroxy)hexanoyl]oxy}methyl)benzoate maleate (**11**).To a solution of free base **d** (0.55 g, 0.82 mmol) in EtOAc (20 ml) was added a solution of maleic acid (0.095 g, 0.82 mmol) in EtOAc (3 ml). The solution stirred for 30 min and hexane added whereupon an oil separated at the bottom. The upper phase was removed. The lower oil was concentrated under vacuum and crystallized from EtOAc to give 0.45 g of the title compound as a white solid.
Selected data for compound **11**. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32 (s, 11H), 1.45–1.56 (m, 2H), 1.61–1.71 (m, 2H), 2.31 (t, *J* = 7.16 Hz, 2H), 3.39–3.52 (m, 6H), 3.64 (t, *J* = 4.43 Hz, 4H), 3.70 (s, 3H), 4.50 (t, *J* = 6.50 Hz, 2H), 4.58–4.67 (m, 1H), 4.73–4.83 (m, 1H), 5.11 (s, 2H), 5.71 (s, 1H), 6.07 (s, 2H), 6.64 (s, 1H), 6.67 (d, *J* = 1.70 Hz, 1H), 8.45 (br s, 1H), 8.58 (br s, 1H). LC MS: $(M+H)^+$ = 670.2. HRMS Calcd for $(M+H)^+$ = 670.27525, found 670.28691. Anal. Calcd for $C_{33}H_{47}N_5O_{15}S$: C, 50.44; H, 6.03; N, 8.91. Found: C, 50.35; H, 6.13; N, 8.75.
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