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Soft cannabinoid analogues as potential anti-glaucoma agents

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Cannabinoids are able to reduce elevated intraocular pressure; however, their use in glaucoma treatment is not approved due to severe systemic side effects. New cannabinoid derivatives have been designed based on a retrometabolic/soft drug approach; they were expected to have local effect, but not systemic side effects. Lead compounds and soft analogues were prepared using Pechmann condensation. In agreement with the SAR hypothesis used for the present soft drug design, all the compounds that were successfully synthesized had IOP lowering effect, but the common metabolite of soft analogues that was found to be inactive. Accordingly, when the soft analogue **8** was administered i.v., its biological effect lasted just for 15 minutes; nevertheless, when administered topically, its effect lasted significantly longer. Its metabolite, though, was inactive when applied either i.v. or topically. Thus, the designed soft analogues proved to be good candidates for topical control of glaucoma without producing systemic side effects. The preliminary i.v. experimental data could be successfully described by an indirect response PK/PD model.

1. Introduction

Cannabinoids are able to reduce elevated intraocular pressure (IOP), which is the only controllable major risk factor of open angle glaucoma. The use of cannabinoids, however, in glaucoma treatment is not approved due to their central nervous system (CNS) and cardiovascular side effects. Since 1964, when the most active member of the cannabinoid family, Δ^9-tetrahydrocannabinol (Δ^9-THC), was identified by the Israeli chemist Raphael Mechoulam [1], numerous cannabinoid derivatives have been synthesized. However, they all had undesired side effects. In this paper, we describe the synthesis and pharmacologic activity of new cannabinoid derivatives designed based on retrometabolic drug design approaches aimed to avoid the mentioned systemic side effects.

The use of various retrometabolic drug design approaches for ocular-specific delivery has been reported previously [2–9]. The present design of anti-glaucoma drugs with topical but no systemic effects involves the inactive metabolite approach of soft drug design [10]. First, known biologically active lead compounds were selected. Then, metabolically vulnerable moieties that provide an exposed spot for enzymatic attack, such as ester groups, were built in the structure of the most active lead compound. The lead compounds used in the design of the new soft cannabinoid analogues were closely related to SP−1, a nitrogen-containing cannabinoid derivative that was synthesized and tested by the Abbott Laboratories [11–14]. The vulnerable group was built in the structure of the lead so that the overall physical, physicochemical, steric, and complementary properties of the new soft compounds remained very close to those of the lead compound. The resulting topically applied soft drugs, thus, were expected to have similar IOP-lowering effect as the lead because of their isosteric/isoelectronic properties, and were expected to be readily metabolized into an inactive molecule once they reach the systemic circulation.

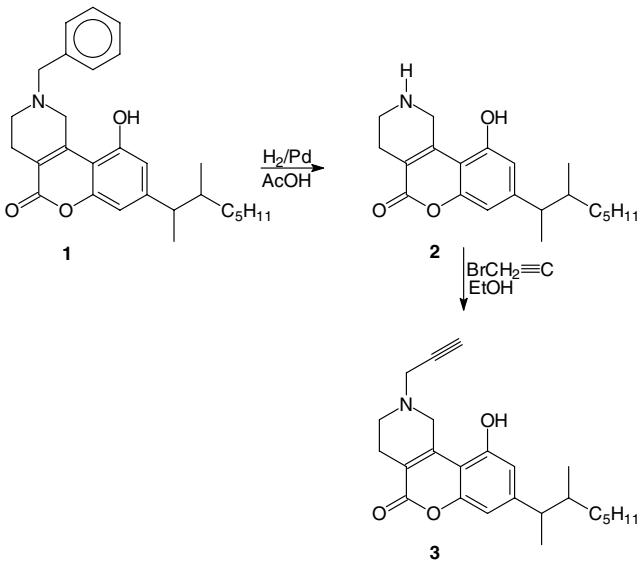
2. Investigations, results and discussion

2.1. Chemistry

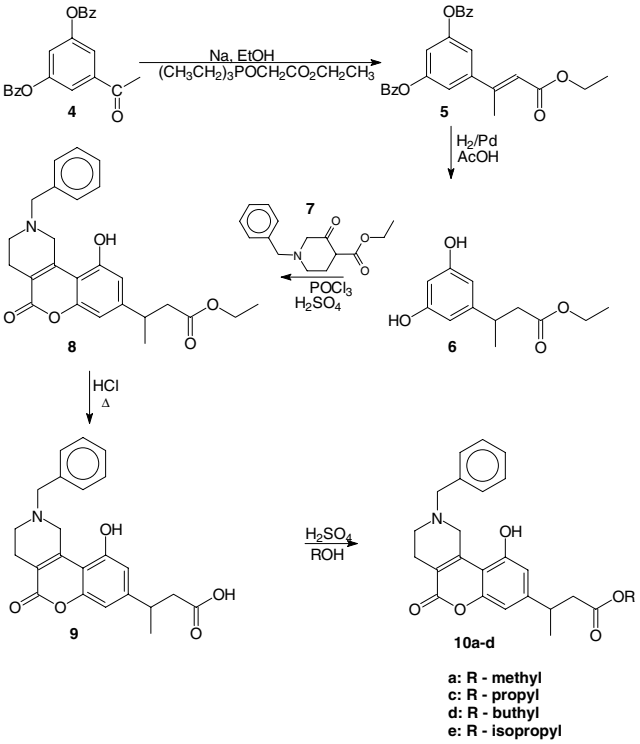
The general synthetic scheme developed in the synthesis of nitrogen containing cannabinoid derivatives that was reported already in a previous paper was suitable for the preparation of the new cannabinoid analogues [9]. Accordingly, the appropriate keto ester (ethyl-1-benzyl-3-oxo-4-piperidinecarboxyate hydrochloride) was allowed to condense with previously prepared substituted resorcinols under Pechmann conditions to give the corresponding pyrones. The pyrone obtained was debenzylated and subsequently alkylated with propargyl bromide to prepare three different lead compounds (**1**, **2** and **3**) as shown in Scheme 1.

In the corresponding soft analogue series, Horner-Emmons reaction was used to prepare the substituted resorcinol needed in the Pechmann condensation (Scheme 2). The pyrone **8** was hydrolyzed into **9** by a procedure similar to

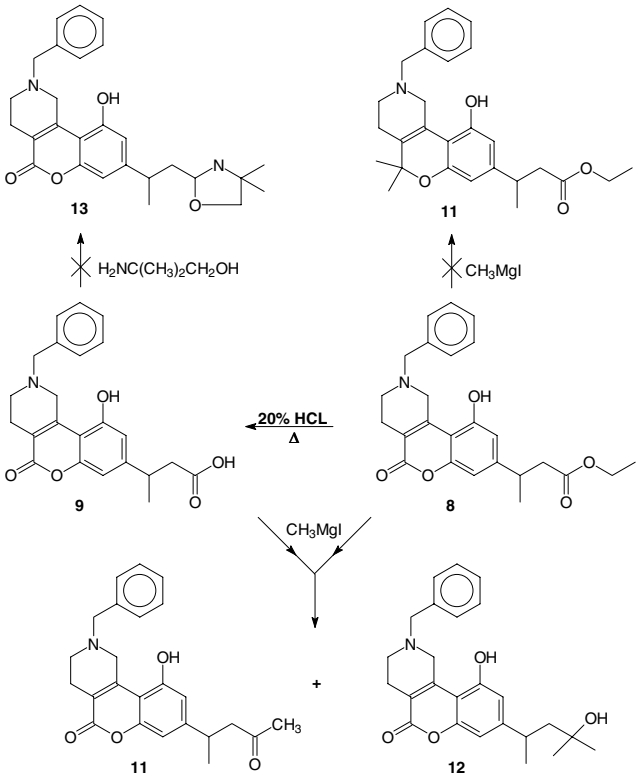
Scheme 1



Scheme 2



Scheme 3



that used by Marvel et al. [15]. However, preparation of **9** in one step directly from the Pechmann reaction gave a much better yield. The different esterpyrones (soft compounds) were synthesized by Fisher esterification.

We also tried to synthesize the corresponding soft esterpyrans. As reported previously, the synthesis of cannabinoids is quite challenging. The synthesis of the N-benzylpyran from the pyrone **1** was successful only when a new procedure was followed [9]. The Grignard reaction as described by Pars et al. [16] failed. Unfortunately, our attempts to prepare the corresponding soft benzylpyran analogues by the same method or new ones were all unsuccessful (Scheme 3). The Grignard reagent, as expected, was reacting not just with the lactone, but with the ester group as well. A five-fold excess of the Grignard reagent gave mainly a product identified as the corresponding ketone. A somewhat less, 4.5-fold excess of the Grignard reagent, resulted mainly in three different spots on thin-layer chromatography (TLC) with very low yield.

Since the Grignard reagent seemed to attack the ester group as or even more easily as the lactone, we hydrolyzed the esterpyrone into the corresponding acidpyrone. Grignard reagents are known to be less likely to attack carboxylic acids than esters. However, the Grignard reaction with the carboxylic acid failed as well mainly due to the low solubility of the carboxylic acid in anisole, ether, or THF. With a four-fold excess of the Grignard reagent, only the starting material was detected even though the reaction was run for several days at $\sim$60 °C. With a ten-fold excess of the Grignard reagent, however, both the corresponding ketone and tertiary alcohol were obtained.

It seemed very likely that there is a barrier of solubility under which the Grignard reaction could not take place. Above the barrier, the product with the two methyl groups inserted, became more soluble than the starting compound and thus, the formation of the corresponding ketone and alcohol could not be avoided. A new synthetic strategy was required. Protection of the acid group seemed to be a viable solution for both insolubility and avoidance of the creation of ketone or alcohol products. Hence, masking of the carboxylic group as its oxazoline derivative, which is inert to Grignard reagents, was attempted. The acid chloride was prepared following the general procedure of Meyers et al. [17]. The 2-amino−2-methyl propanol was added as described there. After work-up, three different products were separated with column chromatography (95% CH_2Cl_2 and 5% MeOH). Unfortunately, we were unable to identify with complete certainty the three different compounds either by NMR or by IR. Two of the three compounds most probably were the amide and the oxazoline, but the yield was very poor.

Additionally, solubilization of the acidpyrone was attempted by attaching tetrabutylammoniumhydrogen sulphate or tetrabutylammonium hydroxide to its side chain. The mass spectrometer analysis of the product of the Grignard reaction indicated that the side chain did not survive the reaction conditions.

Due to the difficulties related to the synthesis of the N-containing pyrane derivatives, the *in vivo* experiments were run just with the soft benzylpyrone analogues synthesized; however, the soft pyrane analogues seem to be very promising as well.

2.2. Pharmacokinetics in rabbits

The pharmacokinetics of one of the soft analogues synthesized (**8**) was evaluated *in vivo* following i.v. administration to one rabbit. The results of the noncompartmental data after a single intravenous injection of 5 mg/kg of **8** are listed in the Table. The initial plasma concentration was approximately 37 μg/ml, which declined with a rate of elimination of 0.038 min^{-1}. The concentration time profile along with the fitted bi-exponential curve is pre-

Table: Pharmacokinetic parameters after intravenous bolus administration of 5 mg/kg of soft drug 8 to one rabbit

	5 mg/kg
C_0 (μg/ml)	37
k_e (min^{-1})	0.038
$t_{1/2}$ (min)	18
AUC_∞ (μg/ml×min)	86
$AUMC_\infty$ (μg/ml×min^2)	1158
MRT (min)	13
CL (l/min/kg)	0.06
Vc (l/kg)	0.14
Vd_{ss} (l/kg)	0.78
Vd_{area} (l/kg)	1.52
A^a	27.55
α^a (min^{-1})	0.85
B^a	1.67
β^a (min^{-1})	0.039
$t_{1/2}$ α^a (min)	0.82
$t_{1/2}$ β^a (min)	18
MSC^a	3.85
$r^{2\,a}$	0.99

[a] Compartmental analysis using a two-compartment body model

sented in Fig. 1. A two-compartment body model described the concentration-time profile the best. The coefficient of determination was 0.999, and the model selection criteria was 3.85. The resulting parameters are listed in the Table. Both compartmental and noncompartmental analysis of the preliminary data indicated short elimination half-life, which ensure rapid inactivation in the systemic circulation.

2.3. Pharmacological effects

The pharmacodynamic effects of the lead compounds and soft analogues synthesized were evaluated in a number of *in vivo* experiments following both i.v. and topical administration in rabbits. Following i.v. administration of 1 mg/kg of the soft analogue **8,** a statistically significant decrease in IOP was observed already at the first observation point at 3 min. This IOP-drop was parallel in both eyes and lasted for 15 min (Fig. 2). This is in agreement with a soft drug

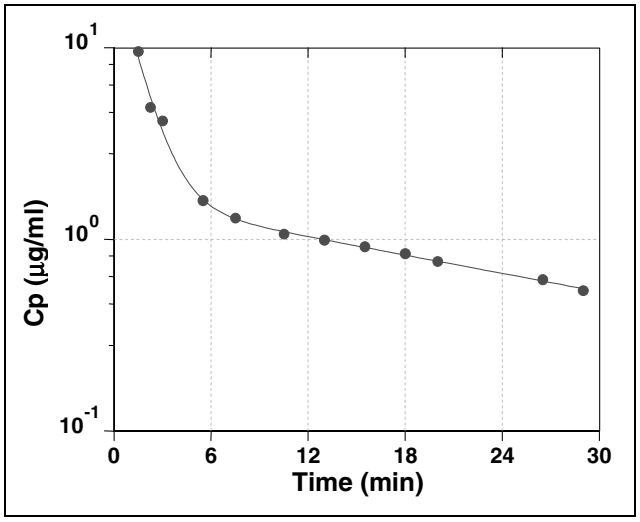


Fig. 1: The concentration-time profile after intravenous bolus administration of 5 mg/kg **8** to one rabbit. The line represents the two-compartment model predicted concentration-time curve with the parameters given in the Table.

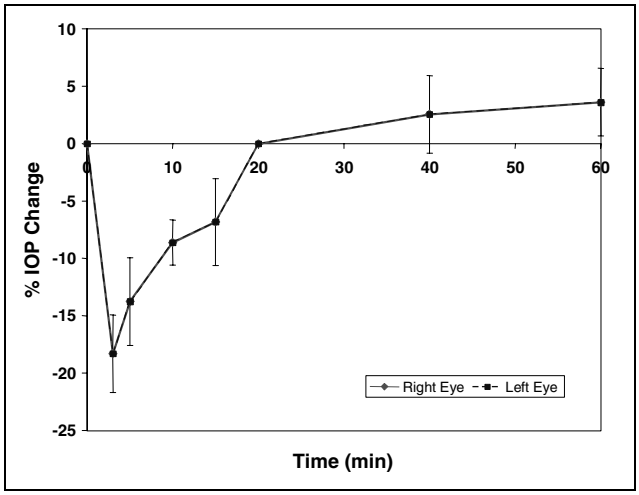


Fig. 2: Percent drop of IOP following i.v. administration of 1 mg/kg of the soft drug **8** to rabbits. Data are mean ± standard deviation for four determinations (mean ±SD, n = 4).

that is active and rapidly inactivated. The maximal reduction was 18 ± 3% (p = 0.005). Following administration of similar doses of classical cannabinoids, the maximum IOP-lowering effect reported was 24–38% [18]. Part of the difference might be due to the rapid inactivation of **8** in the systemic circulation. It is possible that a sufficient concentration needed to produce the same IOP lowering effect could not be reached at the site of action. The metabolite **9** showed essentially no activity when administered i.v., as expected based on the soft design principles (Fig. 3).

Following topical administration of one 50 μl drop of drug-free vehicle, mineral oil or emulphor, there was no significant IOP lowering effect. However, following a single dose of 1% solution in emulphor, but not in mineral oil of the lead **1** in one eye of the rabbit the IOP of the treated eye was statistically different from the control eye (p < 0.005) (Fig. 4). The IOP reduction was detected only at 1 h post-instillation, after which the IOP returned to its normal value. Lead **2** produced similar but less pronounced IOP reduction in emulphor (Fig. 4). Interestingly, lead **3** did not show consistent IOP reduction: it produced significant effect in just one of the four rabbits. From the synthesized soft compounds, the methyl ester **10a** produced a significant IOP reduction at t = 0.25 h (p < 0.1), and 0.5 and 1 h (p < 0.05) (Fig. 4). The IOP-lowering effect of the ethyl ester **8** was significant (p < 0.05) at t = 1 h. That of the propyl ester **10c** and isopropyl ester **10d** was significant (p < 0.1) at t = 1 h post drug installation

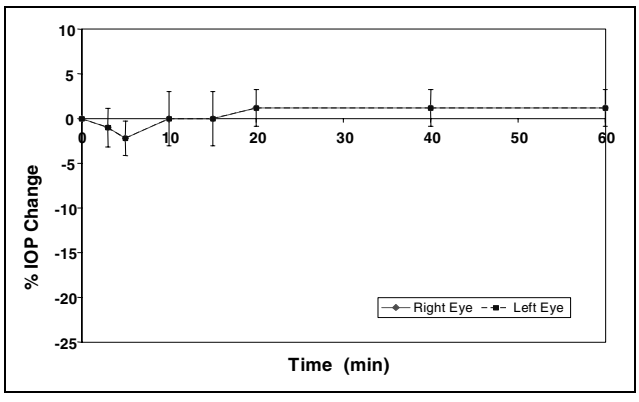


Fig. 3: Percent drop of IOP following i.v. administration of 1 mg/kg of the metabolite **9** to rabbits (mean ±SD, n = 4).

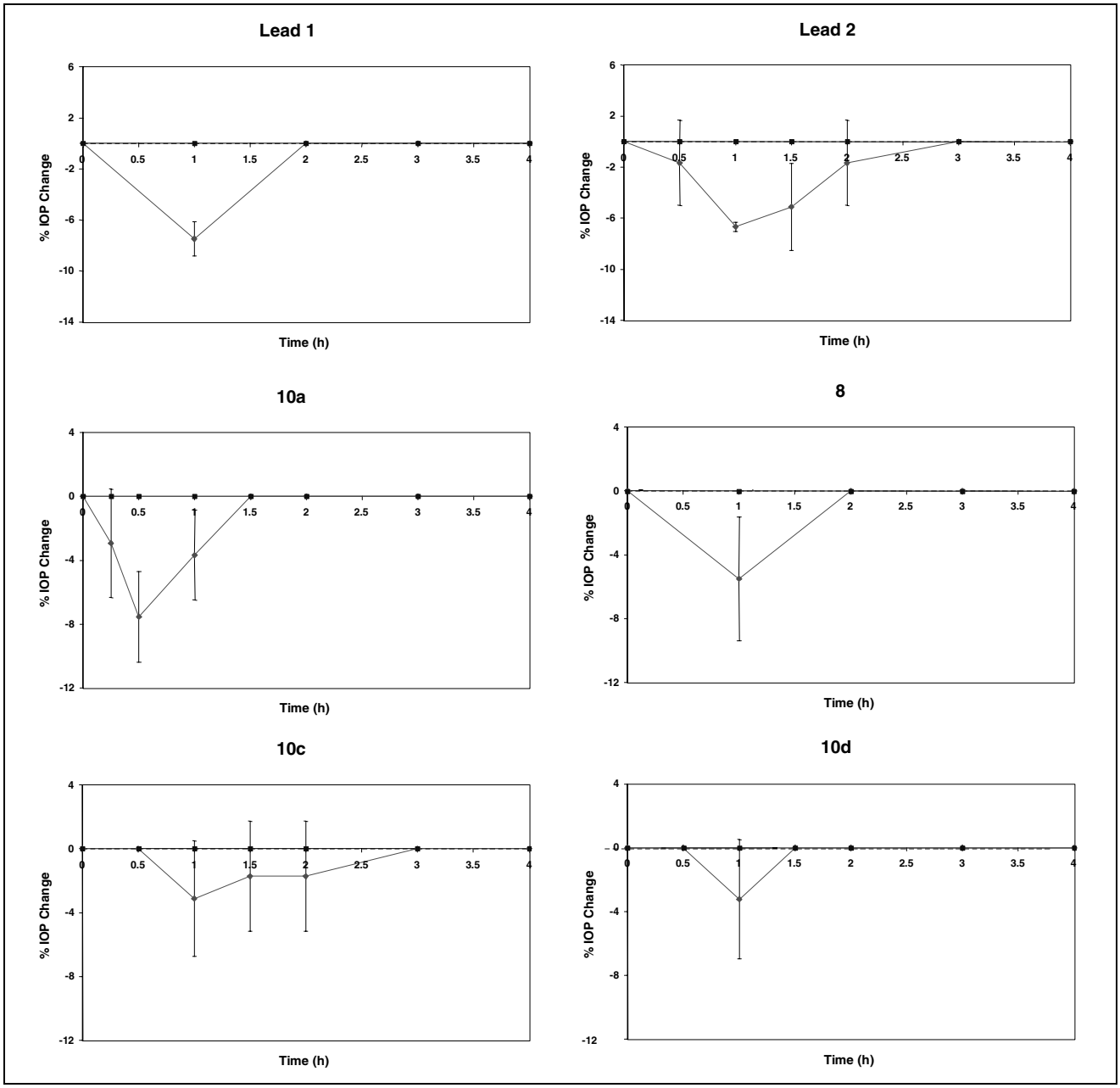


Fig. 4: Percent drop of IOP following topical administration of 1% emulphor solution of the lead **1**, **2** and different soft drugs in rabbits. Data are mean ± standard deviation for four determinations (mean ±SD; n = 4). Untreated eye (◆) treated eye (■).

(Fig. 4). These significant IOP reductions were observed when the soft drugs were dissolved in EL−719. The common metabolite **9**, however, in agreement with the SAR hypothesis used for the present soft drug design, was found to be inactive.

In conclusion, synthesis of a series of N-containing cannabinoid analogues designed on the basis of soft drug design approaches was successfully accomplished. *In vivo* testing in rabbits confirmed that, in agreement with the design principles, these compounds have moderate IOP-lowering activity, whereas their common hydrolytic metabolite is inactive. Following single-dose topical administration, the soft compounds produced less pronounced pharmacological effects than following i.v. administration, but a trend toward a longer lasting IOP-lowering effect was clearly present. The equivocal results obtained probably are due to the extremely low aqueous solubility of the compounds tested. None of the compounds tested exhibited uncommon eye irritation when emulphor was used as vehicle.

2.4. PK/PD analysis

The dose-concentration relationship from the pharmacokinetic analysis was combined with the concentration-effect relationship gained from the pharmacodynamic studies. The model describing the concentration-effect relationship when 1 mg/kg of **8** was administered i.v. was used for the pharmacodynamic component (Fig. 2). Since there seemed to be a delay between the maximum concentration measured in plasma and maximum effect, the IOP-lowering effect was linked to the pharmacokinetics of the soft drug administered by an indirect response model that is described in Fig. 5. This indirect response E_{max} model explained well the experimental results (MSC = 2.94, $r^2 = 0.95$); however, more data is needed for further validation.

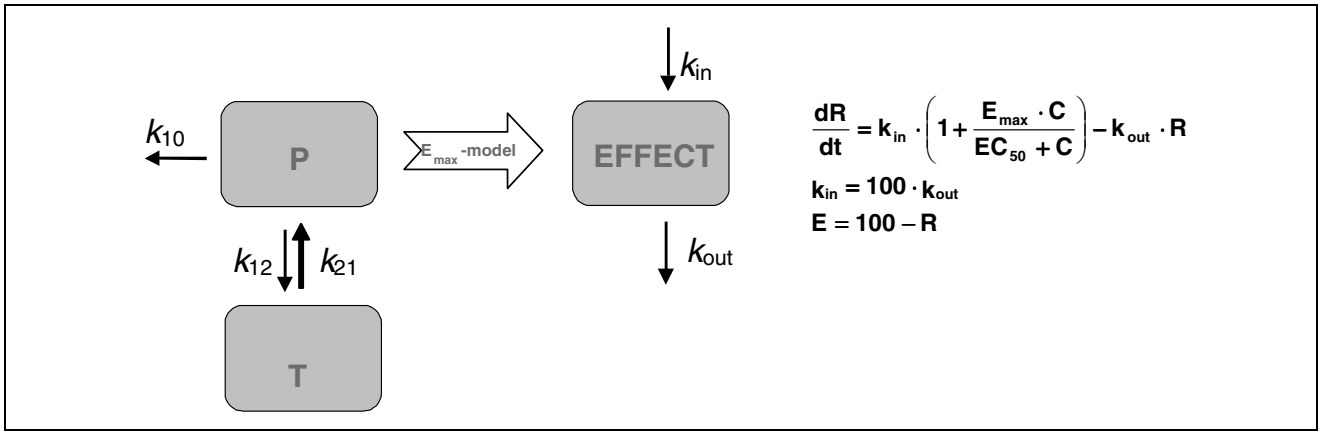


Fig. 5: PK/PD model with the differential equation used to describe the IOP-lowering after i.v. administration of **8** in rabbits. R, response; k_{in}, rate constant of an input process that is assumed to cause the IOP-lowering effect; k_{out}, rate constant of the corresponding output process; E is the measured effect (IOP change in percentage (Fig. 6); E_{max}, maximum effect; EC_{50}, plasma concentration of the drug causing 50% of maximum IOP-lowering effect.

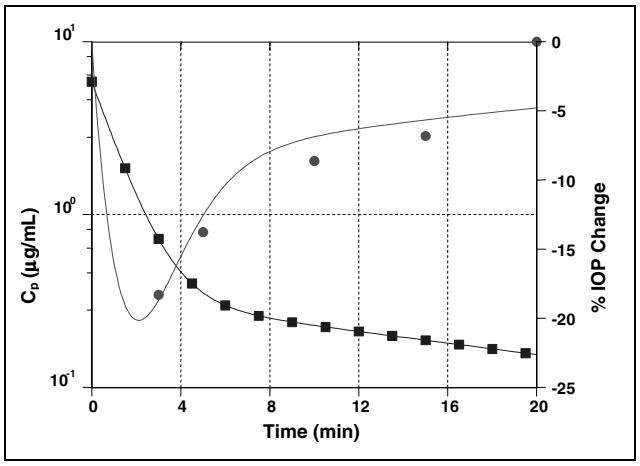


Fig. 6: Pharmacokinetic and pharmacodynamic relationship between the concentration of soft drug (**8**) (■) and IOP-lowering effect (●) in rabbits. The curves represent the best fit of the data obtained with an indirect response model with the following parameters EC_{50} 1.2 µg/ml; E_{max} 40%; k_{out} 0.75.

3. Experimental

3.1. Chemistry

All chemicals, reagents, pH standards, and solvents used were of HPLC or A.C.S. analytical grade, and were used as supplied from Fisher Scientific (Pittsburgh, PA) or Aldrich (Milwaukee, WI). Melting points were taken on a Fisher-Jones (Fisher Scientific, Pittsburgh, PA) apparatus and are uncorrected. All synthesized products were characterized by ESI-MS (electrospray ionization) with a Quattro LC (Micromass, Beverly, MA) mass spectrometer. ^{1}H NMR spectra were recorded on a Varian VXR−300 (Varian Association Inc., Sunnyvale, CA) 300 MHz instrument. Elemental analyses of compounds synthesized were performed by Atlantic Microlab, Inc. (Necroses, GA). All the results were in an acceptable range. TLC (thin layer chromatography) was carried out on silica gel coated glass (Whatman MK6F, 250 µm thickness) or on silica-gel coated aluminum plates (Kieselgel 60 F254, 0.2 mm thickness). Column chromatography (CC) using silica gel was performed for separation or purification of the compounds. Distillations were performed with Aldrich Kugelrohr ball-tube distillation apparatus or short-path distillation apparatus.

3.1.1. [8-(1,2-Dimethylheptyl)-10-hydroxy-5-oxo-1,2,3,4-tetrahydro-5H-[1]benzopyrano[3,4-d]pyridine] (**2**)

The general method reported by Pars et al. was followed [16]. The product was identified by MS m/z = 344, (M + 1); and ^{1}H NMR ($CDCl_3$) δ 0.68 (3 H, d, $CHCH(CH_3)CH_2$), 0.81−0.90 (3 H, m, CH_2CH_3), 1.18 (3 H, d, $PhCHCH_3$), 0.92−1.40 (8 H, br, $(CH_2)_4$), 1.45−1.55 (1 H, m, $CH(CH_3)CH_2$), 2.35−2.50 (1 H, m, $PhCHCH_3$), 2.76 (2 H, t, CH_2CH_2N), 3.22 (2 H, t, CH_2CH_2N), 4.55 (2 H, s, $C{=}CCH_2N$), 6.41 (1 H, s, OH*Ph*CH), 6.56 (1 H, s, OCO*Ph*CH) ppm. Yield 63%.
$C_{21}H_{29}O_3N_1 \times 0.8\ H_2O$

3.1.2. [8-(1,2-Dimethylheptyl)-10-hydroxy-2-(2-propynyl)-5-oxo-1,2,3,4-tetrahydro-5H-[1]benzopyrano[3,4-d]pyridine] (**3**)

The method reported by Pars et al. was followed [16]. The product was identified by MS m/z = 380.34, (M−1); and ^{1}H NMR (DMSO-d_6) δ 0.71 (3 H, d, $CHCH(CH_3)CH_2$), 0.79−0.89 (3 H, m, CH_2CH_3), 1.12 (3 H, d, $PhCHCH_3$), 1.14−1.22 (8 H, br, $(CH_2)_4$), 2.15−2.23 (1 H, m, $CH(CH_3)CH_2$), 2.27−2.36 (1 H, m, $PhCHCH_3$), 2.53 (2 H, t, CH_2CH_2N), 2.59 (1 H, t, $CH{\equiv}C$), 2.95 (2 H, t, CH_2CH_2N), 3.36 (2 H, s, $C{=}CCH_2N$), 3.52 (2 H, s, $HC{\equiv}CCH_2N$), 6.07 (1 H, s, OH*Ph*CH), 6.19 (1 H, s, OCO*Ph*CH), 9.25 (1 H, s, OH) ppm, Yield 50%.

3.1.3. [Ethyl-3-(3,5-dibenzyloxyphenyl)-2-butanoate] (**5**)

The Horner-Emmons reaction was used following the general method reported by Huffman et al. in the synthesis of 3,5-dimethoxyphenyl acids [19]. To a solution of sodium ethoxide prepared from 2.55 g (111 mmol) of sodium in 200 ml of dry ethanol, 18.7 ml (94.3 mmol) of triethylphosophonoacetate was added. After stirring the solution for 2.5 h at 25 °C, 18.6 g (56 mmol) 3,5-dibenzyloxy-acetophenone was added. The reaction mixture was stirred for another 72 h at room temperature and then quenched with glacial acetic acid. The solvent was removed by a rotavapor then made basic, filtered, and dried. The gained product 20.32 g, m.p. 63–66 °C, was identified by MS m/z = 403, (M + 1); and ^{1}H NMR ($CDCl_3$) δ 1.32 (3 H, t, CH_2CH_3), 2.52 (3 H, s, $CH{=}CCH_3$), 4.21 (2 H, q, CH_2CH_3), 5.05 (4 H, s, CH_2Ph), 6.11 (1 H, s, C=CH), 6.62 (1 H, t, $B_2OPhOBz$), 6.71 (2 H, d, BzO*Ph*CH=CH), 7.32–7.43 (10 H, m, Ph) ppm. Yield: 90%.
$C_{26}H_{26}O_4$

3.1.4. [Ethyl-3-(3,5-dihydroxyphenyl)-butanoate] (**6**)

A solution of 50 mmol (20 g) of the ester **5** dissolved in 60 ml of ethanol and 150 ml of ethyl acetate, was mixed with 16 ml of 10% acetic acid solution of palladium on activated carbon. The solution mixture was shaken under hydrogen at 30 psi (2×10^5 Pa) for 48 h, and then the mixture was filtrated; the filtrate was evaporated, and the residue was distilled. The product (10.2 g) was identified by MS m/z = 224, (M); and ^{1}H NMR ($CDCl_3$) δ 1.16 (3 H, t, CH_2CH_3), 1.20 (3 H, d, $CHCH_3$), 2.44–2.59 (1 H, m, CH), 3.05–3.17 (2 H, m, $CHCH_2CO$), 4.06 (2 H, q, OCH_2CH_3), 6.20 (1 H, t, HO*Ph*OH), 6.27 (2 H, d, HO*Ph*CH=CH), 8.83 (2 H, s, OH) ppm. Yield: 91%.

3.1.5. [2-Benzyl-8-(1-methylethoxylcarbonylethyl)-10-hydroxy-5-oxo-1,2,3,4-tetrahydro-5H-[1]benzopyrano[3,4-d]pyridine] (**8**)

Ethyl-1-benzyl-3-oxo-4-piperidinecarboxylate hydrochloride (54 mmol, 16.16 g) was mixed with 50 mmol (11.20 g) of resorcinol (**6**). To the mixture, first 23.2 ml of concentrated sulfuric acid was added dropwise and then 7 ml of phosphorus oxychloride. The reaction mixture was stirred under argon atmosphere for 70 h after which it was made basic by addition of aqueous potassium hydrogen carbonate. The free base was extracted into methylene chloride, washed several times with water, then dried on sodium sulfate, and concentrated. The pure product, which was gained by crystallization from methylene chloride with hexane (6.3 g) m.p. 147–150 °C, was identified by MS m/z = 420, (M−1), and ^{1}H NMR (THF-d_6) δ 1.11 (3 H, t, CH_2CH_3), 1.22 (3 H, d, $CHCH_3$), 2.47–2.53 (2 H, m, $CHCH_2CO$), 2.55 (2 H, t, CH_2CH_2N), 2.61 (2 H, t, CH_2CH_2N), 3.08–3.25 (1 H, m, CH), 3.67 (2 H, s, $PhCH_2N$), 3.94 (2 H, t, $C{=}CCH_2N$), 3.98 (2 H, q, OCH_2), 6.42 (1 H, d, OH*Ph*CH), 6.64 (1 H, d, OCO*Ph*CH), 7.12–7.41 (5 H, m, Ph) ppm. Yield: 29%.
$C_{25}H_{27}O_5N_1$

3.1.6. [2-Benzyl-8-(1-methylcarboxyethyl)-10-hydroxy-5-oxo-1,2,3,4-tetrahydro-5H-[1]benzopyrano[3,4-d]pyridine] (**9**)

Method A: Pyrone **8** (1.3 g, 3 mmol) was gently refluxed with 1.35 ml of 20% hydrochloric acid for 5 h. The mixture was cooled overnight and then dissolved in acetonitrile. The precipitate was filtered and made basic with potassium carbonate aqueous solution. Organic impurities were extracted by methylene chloride. The product, which was precipitating out from the neutral aqueous layer, was purified with acetic acid and methylene chloride.
Method B (directly from **6** and **7**): Ethyl-1-benzyl-3-oxo-4-piperidinecarboxylate hydrochloride (50 mmol, 14.86 g) and 61 mmol (13.68 g) resorcinol (**13**) were used. During work-up, to the dried product 30 ml ethanol and 167 ml 5% sodium hydroxide aqueous solution were added. The solution mixture was stirred for 3–4 h. After hydrolysis of the ester, the ethanol solution was stripped off, and the aqueous layer was made neutral by dropwise addition of 50% sulfuric acid aqueous solution. It was stirred overnight. The precipitate was filtered and crystallized with acetic acid and methylene chloride. The product, m.p. 220–225 °C, was identified by MS m/z = 392, (M–1), and ^{1}H NMR (THF-d_6) δ 1.26 (3 H, d, CHC*H*$_3$), 2.4–2.57 (2 H, m, CHC*H*$_2$CO), 2.57–2.65 (2 H, br, CH$_2$C*H*$_2$N), 2.65–2.8 (2 H, br, C*H*$_2$CH$_2$N), 3.08–3.22 (1 H, m, CH), 3.7–3.9 (2 H, br, PhC*H*$_2$N), 3.9–4.16 (2 H, br, C=CCH$_2$N), 6.5 (1 H, s, OH*Ph*CH), 6.68 (1 H, s, OCO*Ph*CH), 7.1–7.3 (5 H, m, Ph) ppm. Method A: Yield: 94%. Method B: Yield: 81%.
$C_{23}H_{23}O_5N_1 \times 0.3\ H_2O$

3.1.7. [2-Benzyl-8-(1-methylmethoxycarbonylethyl)-10-hydroxy-5-oxo-1,2,3,4-tetrahydro-5H-[1]benzopyrano[3,4-d]pyridine] (**10a**)

To a solution of 2.5 mmol (1 g) of **9** in 10 ml of methanol, 0.2 ml concentrated sulfuric acid was added. The mixture was stirred for 1.5 h, then the solvent was evaporated, and the residue was made neutral with 3% sodium bicarbonate aqueous solution. The organic layer was extracted with methylene chloride, and the residue was purified by CC using methylene chloride and methanol (20 : 1) as solvent. The product (0.65 g) m.p. 148–150 °C, was identified by MS m/z = 406, (M–1); and ^{1}H NMR (THF-d_6) δ 1.26 (3 H, d, CHC*H*$_3$), 2.52–2.58 (2 H, m, CHC*H*$_2$CO), 2.6 (2 H, t, CH$_2$C*H*$_2$N), 2.66 (2 H, t, C*H*$_2$CH$_2$N), 3.12–3.25 (1 H, m, CH), 3.6 (2 H, s, OCH$_3$), 3.72 (2 H, s, PhC*H*$_2$N), 3.99 (2 H, t, C=CC*H*$_2$N), 6.47 (1 H, d, OH*Ph*CH), 6.69 (1 H, d, OCO*Ph*CH), 7.18–7.3 (5 H, m, Ph) ppm. Yield: 64%.
$C_{24}H_{25}O_5N_1$

3.1.8. [2-Benzyl-8-(1-methylpropyloxycarbonylethyl)-10-hydroxy-5-oxo-1,2,3,4-tetrahydro-5H-[1]benzopyrano[3,4-d]pyridine] (**10c**)

To a solution of 2.5 mmol (1 g) of **9** in 10 ml of 1-propanol, 0.2 ml concentrated sulfuric acid was added. After the reaction was completed, the solvent was evaporated. The residue was made neutral with 3% sodium bicarbonate aqueous solution. The organic layer was extracted with methylene chloride, and the residue was purified by CC. The solvent mixture used was methylene chloride and methanol in a ratio of 5 : 1. The purified product (0.65 g) m.p. 92–95 °C, was identified by MS m/z = 434, (M–1); and ^{1}H NMR (CD$_3$OD) δ 0.83 (3 H, t, CH$_2$C*H*$_3$), 1.27 (3 H, d, CHC*H*$_3$), 1.54 (2 H, q, C*H*$_2$CH$_3$), 2.46–2.7 (2 H, m, CHC*H*$_2$CO), 2.62 (2 H, t, CH$_2$C*H*$_2$N), 2.79 (2 H, t, C*H*$_2$CH$_2$N), 3.1–3.27 (1 H, m, CH), 3.82 (2 H, s, PhC*H*$_2$N), 3.94 (2 H, t, OCH$_2$), 4.11 (2 H, s, C=CC*H*$_2$N), 6.57 (1 H, d, OH*Ph*CH), 6.69 (1 H, d, OCO*Ph*CH), 7.24–7.5 (5 H, m, Ph) ppm. Yield: 60%.
$C_{26}H_{29}O_5N_1 \times 0.6\ H_2O$

3.1.9. [2-Benzyl-8-(1-methylbuthyloxycarbonylethyl)-10-hydroxy-5-oxo-1,2,3,4-tetrahydro-5H-[1]benzopyrano[3,4-d]pyridine] (**10d**)

To a solution of 2.5 mmol (1 g) of **9** in 10 ml of butanol, 0.2 ml concentrated sulfuric acid was added. The reaction mixture was stirred overnight. After the solvent was evaporated, the residue was made neutral with 3% sodium bicarbonate aqueous solution. The obtained material was purified by CC using methylene chloride and methanol (30 : 1) (0.7 g) m.p. 98–101 °C, and identified by MS m/z = 448, (M-1); and ^{1}H NMR (CD$_3$OD) δ 0.82 (3 H, t, CH$_2$C*H*$_3$), 1.17–1.24 (2 H, m, CH$_2$C*H*$_2$CH$_3$), 1.27 (3 H, d, CHC*H*$_3$), 1.4–1.54 (2 H, m, CH$_2$C*H*$_3$), 2.51–2.67 (2 H, m, CHC*H*$_2$CO), 2.6 (2 H, t, CH$_2$C*H*$_2$N), 2.72 (2 H, t, C*H*$_2$CH$_2$N), 3.1–3.23 (1 H, m, CH), 3.75 (2 H, s, PhC*H*$_2$N), 3.98 (2 H, m, OCH$_2$), 4.06 (2 H, s, C=CC*H*$_2$N), 6.56 (1 H, d, OH*Ph*CH), 6.68 (1 H, d, OCO*Ph*CH), 7.22–7.46 (5 H, m, Ph) ppm. Yield: 62%.
$C_{27}H_{31}O_5N_1$

3.1.10. [2-Benzyl-8-(1-methylisopropyloxycarbonylethyl)-10-hydroxy-5-oxo-1,2,3,4-tetrahydro-5H-[1]benzopyrano[3,4-d]pyridine] (**10e**)

To a solution of 2.5 mmol (1 g) of **9** in 20 ml of 2-propanol, 0.4 ml concentrated sulfuric acid was added. The reaction mixture was stirred overnight. The residue was purified by hexane (0.75 g) m.p. 125–127 °C, and identified by MS m/z = 434, (M–1); and ^{1}H NMR (CD$_3$OD) δ 1.09 (3 H, t, OCHC*H*$_3$), 1.13 (3 H, t, OCHC*H*$_3$), 1.26 (3 H, d, CHC*H*$_3$), 2.5–2.65 (2 H, m, CHC*H*$_2$CO), 2.59 (2 H, t, CH$_2$C*H*$_2$N), 2.74 (2 H, t, C*H*$_2$CH$_2$N), 3.1–3.24 (1 H, m, CH), 3.76 (2 H, s, PhC*H*$_2$N), 4.06 (2 H, s, C=CC*H*$_2$N), 4.8–5 (H, m, OCH), 6.56 (1 H, s, OH*Ph*CH), 6.68 (1 H, s, OCO*Ph*CH) ppm. Yield: 69%.
$C_{26}H_{29}O_5N_1 \times 0.2\ H_2O$

3.2. Pharmacokinetics in rabbits

One rabbit was anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg). A dose of 5 mg/kg of **8** was administered in the ear vein with a dosing volume of 6 ml/kg over one minute. For data treatment, the mid-time of the injection was used as 0 time. Blood samples were collected into heparinized syringes through the ear vein at appropriate time intervals. The collected blood samples of 100 µl were added into centrifuge tubes containing 200 µl of 5% dimethylsulfoxide in acetonitrile. The samples were mixed, then vortexed and centrifuged for 5 min. The supernatants were injected directly into the HPLC system for analysis.

3.3. Analytical methods

A HPLC method was developed to assay the different soft analogues and their possible metabolites. The samples used to generate calibration curves were prepared in mobile phase. Appropriate concentrations of the analytes of interest were immediately injected onto the HPLC system. The calibration range for **8** was 0.1–100 µg/ml. The slopes and intercepts obtained from the calibration curves of the study were used for the estimation of concentration. The retention time was 10 : 30 min.

3.4. Data treatment

Data were analyzed both by noncompartmental and compartmental pharmacokinetic approaches. The noncompartmental pharmacokinetic analysis was performed with WinNonlin and/or calculated in Excel worksheet. The terminal elimination rate constant (k_e) was obtained directly from the linear regression software, or if not appropriate, the time range was selected manually. The terminal half-live was calculated as $\ln(2)/k_e$. The initial concentration C_0 was determined by logarithmic back-extrapolation to $t = 0$ using the first two data points. The total area under the plasma concentration-time curve (AUC_∞) and area under the first moment curve ($AUMC_\infty$) were calculated using the linear trapezoidal rule up to the last data point (C_x) to which the extrapolated terminal areas calculated as C_x/k_e and $C_xt_x/k_e + C_x/k_e^2$ were added, respectively. The mean residence time (MRT) was calculated as the ratio between the two total area ($AUMC_\infty/AUC_\infty$). The total clearance was obtained as the ratio of dose (D) to AUC_∞, (D/AUC_∞), and the volume of distribution for the central compartment (V_c) was determined by dividing the dose by the initial plasma concentration (D/C_0). The volume of distribution at steady state (Vd_{ss}) was calculated as the product of total clearance (Cl) and mean residence time (MRT). The volume of distribution at pseudo-steady state (Vd_{area}) was defined as the ratio of total clearance to the terminal elimination rate constant (Cl/k_e).
The compartmental pharmacokinetic analysis was performed with the nonlinear regression program Scientist®. Initial estimates for the parameters were obtained with the Simplex search method, and the final fitting was performed with the nonlinear least square method. Minimization of the sum of squares of the residuals was achieved with the Powell variant of the Levenberg-Marquardt algorithm. The drug concentration in blood was weighted at $1/C_p^2$, where C_p is the drug concentration in blood. The highest model selection criteria was obtained with a two-compartment body model with elimination from the central compartment. The equation used, thus, was the bi-exponential equation: $C_p = A \times e^{-\alpha t} + B \times e^{-\beta t}$, where A and B are the intercepts on the y-axis for each exponential segment of the curve, and α and β are the hybrid constants of the distribution phase and the terminal elimination phase, respectively. The pharmacokinetic parameters obtained from the noncompartmental and compartmental analyses were compared by Student's t test. A significant difference was assumed for $p < 0.05$. For the compartmental analysis, both the coefficient of determination and the model selection criteria, a modified Akaike function, were calculated and used to determine the goodness of the resulting curve fittings.

3.5. In vivo IOP testing

The IOP measurements were performed on unrestrained, conscious, healthy, normotensive, male New-Zealand rabbits of about 3.5–4 kg weight, obtained from Harlan (Indianapolis, IN). The rabbits were kept in individual cages with free access to food and water. Procaine hydrochloride (Ophthetic-Allergen Pharmaceuticals, Inc. CT) 0.5% was used as local anesthetic. On each day of the experiment, the control IOP of each eye was taken before the drug administration. One drop of anesthetic was administered immediately prior to each measurement. Drugs were prepared freshly prior to the experiments. In order to perform the investigation on healthy animals, a recovery period of at least 4 days was allowed in between experiments. The same person using the same instrument under the same environmental conditions carried out all the experiments. Digilab

Model 30R Pneuma-Tonometer (Digilab, Inc., Cambridge, MA) was used to measure the intraocular pressure (IOP). The rabbits were also observed for local irritation signs of the drugs on the eye, e.g., congestion, redness, lacrimation, etc.

For intravenous administration a stock solution was prepared from **8** and **9** respectively, dissolved in 10% dimethyl sulfoxide and 90% of aqueous solution with 30% β-hydroxycyclodextrin. A dose of 3 mg/kg of **8** and **9** with a dosing volume of 1 ml/kg was injected into the rabbits ear vein over half a minute. For the data treatment, the mid-time of the injection was used as 0 time, assuming i.v. bolus injection. The average IOP measured before drug administration was considered as baseline. Thus, the percentage change of the IOP of eyes relative to the baseline was calculated.

For topical administration, 1% solutions in mineral oil or EL-719 (Emulphor, Stepan Canada Inc. Ontario, Canada) were prepared. After the control pressure was taken, the drug solution was administered in one eye of the rabbits as a single 50 µl droplet; the other eye served as control. The IOP of both eyes were measured at appropriate time intervals after drug administration. The reduction in IOP was calculated by comparing the measured value of the two eyes in mmHg at each time point. The statistical analysis was performed using paired t-test.

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