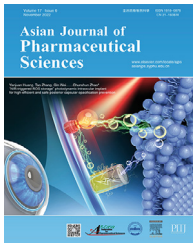
Asian Journal of Pharmaceutical Sciences 17 (2022) 938–948

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/AJPS



Short communication

A novel fixed-combination timolol-netarsudil-latanoprost ophthalmic solution for the treatment of glaucoma and ocular hypertension

Tao Wang[a], *Yunran Zhang*[a,b], *Meiling Chi*[a], *Chen Zhao*[a], *Linlin Cao*[c], *Chutong Tian*[a], *K. Kamei*[a], *Ying Zheng*[d], *Qikun Jiang*[a,*]

[a] *Department of Pharmaceutics, Wuya College of Innovation, Shenyang Pharmaceutical University, Shenyang 110016, China*
[b] *Changzhou pharmaceutical factory co. LTD, Jiangsu 213000, China*
[c] *Department of Pharmaceutics, The Second Hospital of Dalian Medical University, Dalian 116023, China*
[d] *State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau, China*

ARTICLE INFO

Article history:
Received 12 May 2022
Revised 31 August 2022
Accepted 7 November 2022
Available online 17 November 2022

Keywords:
Glaucoma
Intraocular pressure
Homologous salt engineering strategy
Timolol mesylate
Fixed-combination timolol-netarsudil-latanoprost ophthalmic solution

ABSTRACT

Currently commercial fixed-concomitant three agents have multiple problems such as multiple dosing administration, poor efficacy and side effects. Once-daily fixed-combination timolol-netarsudil-latanoprost ophthalmic solution (FC-TNL) has the ability to treat glaucoma by lowering the intraocular pressure (IOP) with great efficacy and improving patient compliance. However, the commercialized netarsudil dimesylate precipitated when the pH of the solution was above 5.4, or when maleic acid, the salt of commercial timolol maleate, was mixed with netarsudil dimesylate. Consequently, the homologous salt engineering strategy was used to make netarsudil dimesylate soluble in pH 4.8–5.2 solution by synthesizing timolol mesylate. Next, the morphology of timolol mesylate was observed by scanning electron microscopy, differential scanning calorimetry, thermogravimetric analysis, and powder X-ray diffraction. The prepared FC-TNL showed good stability during refrigeration storage. Additionally, FC-TNL exerted no influence on the intraocular penetration of each active compounds in the pharmacokinetic study. Importantly, once-daily FC-TNL exerted potent IOP-lowering effect and protective effect on retinal ganglion cells. The FC-TNL was stable, safe and effective, being a promising glaucoma therapeutic.

* Corresponding authors.
E-mail address: jiangqk_student@aliyun.com (Q.K. Jiang).
Peer review under responsibility of Shenyang Pharmaceutical University.

https://doi.org/10.1016/j.ajps.2022.11.001

1. Introduction

Glaucoma is a progressive age-related optic neuropathy characterized by the death of retinal ganglion cells (RGCs), and it is the most common neurodegenerative cause of irreversible blindness worldwide [1]. High intraocular pressure (IOP) is the main risk factor for glaucoma, consequently, lowering IOP is an important aspect in the treatment of glaucoma [2]. The good safety, efficacy and convenience of topical medical therapy remains the first-line treatment for glaucoma [3], and topical ophthalmic solutions are the most commonly used and accepted worldwide [4].

There are some IOP-lowering glaucoma medications, such as prostaglandin F2 analogs, rho-kinase inhibitors, β-adrenergic blocking agents, $\alpha-2$ adrenergic agonists, and carbonic anhydrase inhibitors. These agents are often used in combination due to their complementary mechanisms of action [5]. However, monotherapy and two concomitant agents often have limited action in controlling IOP over time for patients with severe glaucoma [6–10]. Therefore, unfixed-concomitant multiple drugs and fixed-concomitant three agents are often required for an adequate mid- and long-term IOP control [11]. But unfixed-concomitant therapy often results in washout effect, inconvenience and poor patient compliance due to multiple consecutive daily doses. Therefore, fixed-concomitant three agents is the optimal choice. Currently, a triple fixed-combination dorzolamide hydrochloride (2%)-brimonidine tartrate (0.2%)-timolol maleate (0.5%) ophthalmic solution (Krytantek Ofteno®) is the only commercial fixed-concomitant three agents, but it is only available in Latin and South America countries [6]. However, it did not provide a better IOP-lowering effect after twice-daily administration compared with fixed-combination bimatoprost (0.03%)-timolol maleate (0.5%) ophthalmic solution (Ganfort®) demonstrated in phase IV clinical trial [6]. At month 3, 70% of patients treated with Ganfort® had an IOP $<$ 14 mm Hg, but only 38% patients receiving Krytantek Ofteno® had an IOP $<$ 14 mm Hg [6]. Furthermore, another triple fixed-combination bimatoprost (0.01%)-brimonidine tartrate (0.15%)-timolol maleate (0.5%) ophthalmic solution is now in phase III clinical trial, nevertheless, twice-daily treatment obviously increases the incidence of ocular side effects and decreases patient compliance [8]. Therefore, it is necessary to develop an effective, safe, once-daily fixed-concomitant three agents which can potentially decrease dosing frequency, avoid drug washout, limit exposure to preservatives and reduce ocular discomfort.

The β-blocker timolol maleate has a long history of clinical used as monotherapy for primary open-angle glaucoma and ocular hypertension [12,13], which can reduce IOP by inhibiting the production of aqueous humor (AH) [14]. Latanoprost is an ester prodrug of prostaglandin F2 analogs used as the first-line medical therapy for lowering IOP, which can increase the function of the unconventional AH outflow [15,16]. Netarsudil dimesylate, a new rho-kinase inhibitor, is administered once daily and lowers IOP by enhancing the trabecular outflow and reducing AH secretion [17]. Furthermore, netarsudil dimesylate has neuroprotective effect [18]. Various clinical studies demonstrated that once-daily fixed-combination timolol maleate (0.5%)-latanoprost (0.005%) ophthalmic solution (Xalacom®) and fixed-combination netarsudil dimesylate (0.02%)-latanoprost (0.005%) ophthalmic solution (Rocklatan®) (FC–NL) showed favorable clinical tolerability, safety and IOP-lowering effect [19,20]. The mean daytime IOP-lowering efficacy of Xalacom® was approximately 30%−39% and the mean 24-h IOP decrease was 9.8 mmHg (range 7.4–12.2 mmHg) [19]. A drug evaluation showed that the absolute IOP reduction from baseline ranged from 7.2 to 9.2 mmHg, and corresponding to percentage reduction was 30.9%−36.7% in patients who treated with Rocklatan® for 3 months [20]. But these formulations also existed limited efficiency for long-term and severe glaucoma patients.

A potentially effective fixed-combination timolol (0.5%)-netarsudil (0.02%)-latanoprost (0.005%) ophthalmic solution (FC-TNL) could successfully lower IOP by targeting multiple mechanisms involved decreasing AH production and increasing AH outflow [5,21]. Therefore, once-daily FC-TNL can be more potentially to decrease dosing frequency, avoid drug washout, limit exposure to preservatives and reduce ocular discomfort. Consequently, FC-TNL was tried to prepared. It was found that FC-TNL showed poor stability under the following two conditions: (1) pH is an important factor to keep the ophthalmic solution stable. Rhopress® and Rocklatan® were provided as a sterile solution with a pH of 4.8–5.2. Indeed, the commercialized netarsudil dimesylate precipitated when the pH of the solution was above 5.4. (2) Maleic acid, the salt of the commercial timolol maleate, was not compatible with the commercial netarsudil dimesylate, causing white precipitation in pH 4.8–5.2 solution. So far, it has not been reported how to make netarsudil and timolol compatible in pH 4.8–5.2 solution, and prepare FC-TNL.

In the present study, the homologous salt engineering strategy was used to make netarsudil dimesylate soluble in pH 4.8–5.2 solution by synthesizing timolol mesylate. Next, the successful synthesis of timolol mesylate was confirmed using nuclear magnetic resonance spectroscopy, mass spectrograph and fourier transform infrared spectroscopy, the morphology of timolol mesylate was observed by scanning electron microscopy, differential scanning calorimetry, thermogravimetric analysis, and powder X-ray diffraction. The prepared FC-TNL showed good stability during refrigeration storage. Additionally, FC-TNL exerted no influence on the intraocular penetration of each active compounds in the pharmacokinetic study. And FC-TNL did not cause any sign of moderate or severe ocular irritation. More importantly, once-daily FC-TNL exerted great IOP-lowering effect and persistent IOP-controlling effect for 24 h, and it could slow down RGCs death to potentially protect eyesight. The FC-TNL was stable, safe and effective, being a promising formulation in the treatment of glaucoma.

2. Materials and methods

2.1. Materials

Timolol maleate (purity, >99%) was purchased from Kangya of Ningxia Pharmaceutical Co., Ltd. Latanoprost (purity, >98%)

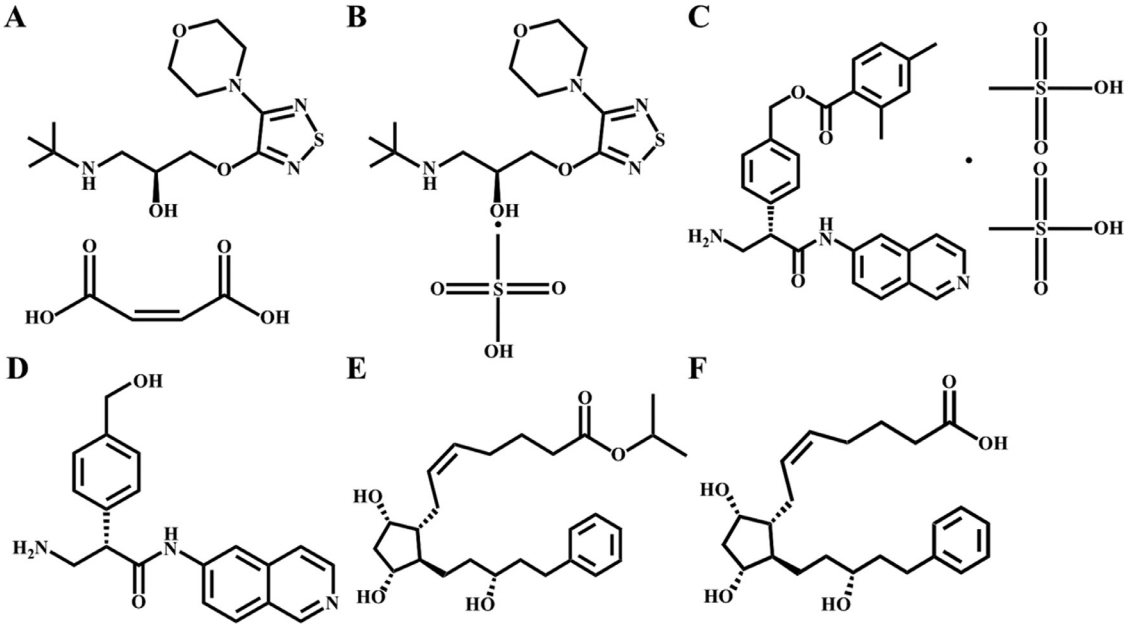


Fig. 1 – Chemical structures of (A) timolol maleate, (B) timolol mesylate, (C) netarsudil dimesylate, (D) netarsudil-M1, (E) latanoprost, and (F) latanoprost acid used in this study.

was purchased from Wuhan Sunrise Technology Development Company Limited. Netarsudil dimesylate (purity, >98%) was purchased from Jiaxing Triview Biochemical Products Co., Ltd. Benzalkonium chloride (BAK) (purity, >99%) was purchased from Taiko Palm-Oleo (Zhangjiagang) Co., Ltd. Latanoprost acid (LA) (purity, >98%) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Netarsudil-M1 was synthesized in our laboratory. Timolol mesylate standard (purity, >99%) was synthesized and purified in our laboratory. Healthy adult male Japanese white rabbits [SYXK (Liao) 2018–0009] (weighing 1.8–2.2 kg) were purchased from the Shenyang aikesaisi Biotechnology Co., Ltd (Shenyang, China). Fixed-combination timolol (0.5%)-netarsudil (0.02%)-latanoprost (0.005%) ophthalmic solution (FC-TNL), fixed-combination netarsudil dimesylate (0.02%)-latanoprost (0.005%) ophthalmic solution (FC–NL), netarsudil dimesylate ophthalmic solution (0.02%), timolol mesylate ophthalmic solution (0.5%), and latanoprost ophthalmic solution (0.005%) were prepared in our laboratory. Timoptic® (timolol maleate ophthalmic solution) (0.5%) was purchased from Wuhan Wujing Pharmaceutical Co. LTD. Xalacom® (fixed-combination timolol maleate-latanoprost ophthalmic solution) (0.5%/0.005%) and Xalatan® (latanoprost ophthalmic solution) (0.005%) were purchased from Pfizer. Boric acid and methanesulfonic acid were purchased from Yuwang Chemical Co., Ltd. (Jinan, China). Mannitol (purity, >99%) was purchased from Dingguo Biotechnology Co., LTD. All other reagents and chemicals used in this study were of analytical grade. The chemical structures are shown in Fig. 1.

2.2. *Preparation of timolol mesylate*

Drug incompatibility can compromise the safety and effectiveness of the combined drugs [22]. Firstly, we mixed timolol maleate and netarsudil dimesylate in pH 4.8–5.2 solution to verify if the two drugs were compatible. Then, timolol mesylate was synthesized by the following methoud. Timolol maleate (1 g, 2.3 mmol) and K_2CO_3 (0.64 g, 4.6 mmol) were dissolved in sterile H_2O (20 ml), the mixture was stirred for 90 min at 35 °C and then cooled to room temperature. The solution was extracted with ethyl acetate (1 time) and the ethyl acetate layer was washed with sterile H_2O (2 times). The organic layer was evaporated and pure timolol was obtained in the form of oil. Methanesulfonic acid (65 µl, 1 mmol) was added to a stirred solution of timolol (0.316 g, 1 mmol) in ethyl acetate (3 ml). The mixture was stirred for 15 min at room temperature. The resulting salt was vacuum suction filtered, washed with ethyl acetate (5 ml), dried at 40 °C under vacuum for 6 h, and timolol mesylate was obtained as a white crystalline powder.

2.2.1. *Characterization of timolol mesylate*

2.2.1.1. *Nuclear magnetic resonance spectroscopy (NMR)* ^{1}H NMR spectra of timolol, timolol maleate and timolol mesylate was recorded in deuterated dimethyl sulfoxide (DMSO–d_6) using a 400 MHz Bruker nuclear magnetic resonance spectrometer (Bruker BioSpin GmbH). The results were interpreted using MestReNova software.

2.2.1.2. *Mass spectrometry (MS)* MS of timolol, timolol maleate and timolol mesylate was performed by AB SCIEX Instruments 4000 Q-Trap (Applied Biosystems, Foster City, CA) under electrospray ionization (ESI) - positive mode with a source temperature of 180 °C. The compounds were dissolved in methanol to obtain a suitable concentration for the analysis in the range of 0.00–1200.00 *m/z*.

2.2.1.3. *Fourier transform infrared spectroscopy (FTIR)* The infrared spectra of timolol, timolol maleate and timolol mesylate was recorded by the KBr disk method using an

FTIR Spectrometer (Broker IFS-55). The spectra were measured under ambient conditions over the range 4000–400 cm^{-1} with a resolution of 2 cm^{-1}.

2.2.1.4. *Scanning electron microscopy (SEM)* Crystals of the formulated timolol maleate and timolol mesylate were examined by SEM (S-4800, Hitachi, Japan). The samples were coated with gold and then examined at a working distance of 8.7 mm and an accelerated voltage of 2 kV.

2.2.1.5. *Differential scanning calorimetry (DSC)* DSC was performed using a Discovery DSC 250 instrument. Samples of timolol maleate and timolol mesylate weighing approximately 4 mg were placed into non-hermetic sealed aluminum pans at a heating rate of 5 °C/min under a nitrogen gas flow of 30 ml/min. The DSC thermograms were recorded under a temperature range of 60–280 °C [23].

2.2.1.6. *Thermogravimetric analysis (TGA)* TGA was carried out using the Discovery TGA 55 equipment. Timolol maleate and timolol mesylate were poured into aluminum oxide pans equipped with lids, then heated from 60 °C to 280 °C at a rate of 5 °C/min. The purge flow rate of dry nitrogen gas was set at 30 ml/min.

2.2.1.7. *Powder X-ray diffraction (PXRD)* The crystallinity of timolol maleate and timolol mesylate was evaluated using an X-ray diffractometer (D/max-r A, Rigaku Denki, Japan) by a Cu Kα radiation ($\lambda = 1.5418$ A°) at room temperature. The voltage and current were set to 40 kV and 40 mA, respectively. The samples were measured under reflection mode during the 2θ range from 5° to 50° at a scan rate of 1°/min [24].

2.3. *Preparation of ophthalmic solutions*

2.3.1. *Preparation of netarsudil dimesylate ophthalmic solution*

Rhopress® can not be available in China. Thus, netarsudil dimesylate ophthalmic solution was prepared in our laboratory referring to the prescribing information of Rhopress® in Table S1.

Briefly, netarsudil dimesylate (28.5 mg), BAK (15 mg) and boric acid (50 mg) were mixed, dissolved in a glass beaker with 95 ml sterile water and stirred using a dry glass stirring rod at room temperature until completely dissolved. The osmolarity was then adjusted to 280–320 mOsmol/kg with mannitol and measured using the STY-1ADK osmometer (Tianjin Tiandatianfa Technology Co. LTD) based on the freezing point depression principle [25]. The pH was adjusted to 4.8–5.2 with sodium hydroxide solution (10%) measured using pH meter (PHS-3C, Eutech Instruments) at 25±0.5 °C. Finally, sterile water was added to bring the solution almost to 100 ml to obtain netarsudil dimesylate ophthalmic solution. The solution was stored in 4 ml opaque white low density polyethylene bottles and closed with white polypropylene caps.

2.3.2. *Preparation of latanoprost ophthalmic solution*

Latanoprost has a low solubility in water (water solubility of 50 mg/l) and a high lipophilicity ($LogD_{(pH7)} = 4.28$) [26]; thus, BAK is not only used as a preservative but also accelerates the dissolution of latanoprost in water [27]. Indeed, BAK is the most frequently used preservative in ophthalmic solutions, typically used in concentrations from 0.004% to 0.02% [28]. Different reaction conditions including BAK concentrations (0.010%, 0.015%, and 0.020%), stirring speed (140±5 rpm and 160±5 rpm) and heating time (1.0–2.5 h) in 80 ± 2 °C water bath [29] were tried to make latanoprost completely dissolve in the water to obtain 0.01% latanoprost stock solution.

Boric acid (50 mg) was dissolved in a glass beaker with 45 ml sterile water and stirred using a dry glass stirring rod at room temperature until completely dissolved, then added in 50 ml of 0.01% latanoprost stock solution. The osmolarity was then adjusted to 280–320 mOsmol/kg with mannitol. The pH was adjusted to 4.8–5.2 with sodium hydroxide solution (10%). Finally, sterile water was added to bring the solution almost to 100 ml to obtain latanoprost ophthalmic solution. The solution was stored in 4 ml opaque white low density polyethylene bottles and closed with white polypropylene caps.

2.3.3. *Preparation of timolol mesylate ophthalmic solution*

Timolol mesylate (650 mg), BAK (20 mg) and boric acid (50 mg) were mixed, dissolved in a glass beaker with 95 ml sterile water and stirred using a dry glass stirring rod at room temperature until completely dissolved. The osmolarity was then adjusted to 280–320 mOsmol/kg with mannitol. The pH was adjusted to 4.8–5.2 with sodium hydroxide solution (10%). Finally, sterile water was added to bring the solution almost to 100 ml to obtain timolol mesylate ophthalmic solution. The solution was stored in 4 ml opaque white low density polyethylene bottles and closed with white polypropylene caps.

2.3.4. *Preparation of FC–NL*

Rocklatan® is fixed-combination netarsudil dimesylate (0.02%)-latanoprost (0.005%) ophthalmic solution, which has been approved by the Food and Drug Administration in 2019 for using in patients with open angle glaucoma and ocular hypertension [5]. Because of the unavailability of Rocklatan® in China, FC–NL was prepared in our laboratory referring to the prescribed information of Rocklatan® shown in Table S1.

Netarsudil dimesylate (28.5 mg) and boric acid (50 mg) were mixed, dissolved in a glass beaker with 45 ml sterile water and stirred using a dry glass stirring rod at room temperature until completely dissolved, then added in 50 ml of 0.01% latanoprost stock solution. The osmolarity was then adjusted to 280–320 mOsmol/kg with mannitol. The pH was adjusted to 4.8–5.2 with sodium hydroxide solution (10%). Finally, sterile water was added to bring the solution almost to 100 ml to obtain FC–NL. The solution was stored in 4 ml opaque white low density polyethylene bottles and closed with white polypropylene caps.

2.3.5. *Preparation of FC-TNL*

The preparation of FC-TNL was performed by referring to the prescribed information of the commercial pruducts summarized in Table S1. Netarsudil dimesylate (28.5 mg), timolol mesylate (650 mg) and boric acid (50 mg) were mixed, dissolved in a glass beaker with 45 ml sterile water and

stirred using a dry glass stirring rod at room temperature until completely dissolved, then added in 50 ml of 0.01% latanoprost stock solution. The osmolarity was then adjusted to 280–320 mOsmol/kg with mannitol. The pH was adjusted to 4.8–5.2 with sodium hydroxide solution (10%). Finally, sterile water was added to bring the solution almost to 100 ml to obtain FC-TNL. The solution was stored in 4 ml opaque white low density polyethylene bottles and closed with white polypropylene caps.

2.4. *In vitro stability studies*

The regulatory expectations for ophthalmic products have always been the most demanding, meanwhile, the stability of ophthalmic drugs is an important parameter related to the storage condition [30]. According to the storage requirements of the commercial three monotherapy agents (Timoptic®, Rhopress®, Xalatan®), three sets of different conditions including refrigeration temperature (5 ± 3 °C), room temperature (25 ± 2 °C), and transportation temperature (40 ± 2 °C), were selected to evaluate the *vitro* stability of FC-TNL.

2.5. *Animal study*

All animal studies were performed in accordance with the ARRIVE guidelines and were conducted according to the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Ethical Care Committee (IAECC) of Shenyang Pharmaceutical University. Healthy male Japanese white rabbits [SYXK (Liao) 2018–0009] (weighting 1.8–2.2 kg) were purchased from the Shenyang aikesaisi Biotechnology Co., Ltd (Shenyang, China). They were kept in individual cages at a temperature of 20–25 °C and relative humidity of 40%−70%. They were fed with balanced diet pellets and had free access to food and water. The eyes of all rabbits were examined prior to the experiment and only the animals without any ocular disease were used for the experiments.

2.5.1. *In vivo pharmacokinetic study*

56 healty male Japanese white rabbits were randomly divided into 4 groups of 14 rabbits in each groups. Each groups was divided into 7 subgroups (2 rabbits for each time point). Each rabbit was treated with 40 μl FC-TNL, Timoptic®, netarsudil dimesylate ophthalmic solution or Xalatan® in the lower conjunctival cul-de-sac of both eyes. The eyelids were kept closed for 10 s after administration to prevent the loss of the instilled drug solution. The animals were sacrificed at 0.083 h, 0.25 h, 0.5 h, 1 h, 4 h, 8 h and 24 h post instillation, and the eyeball was removed and dissected into cornea and AH. Cornea was separately weighted at the time of collection, and all the samples (cornea and AH) separately stored in separated tubes, frozen immediately and stored at −80 °C until analysis.

Latanoprost and netarsudil are ester prodrugs, which are quickly hydrolyzed to LA and netarsudil-M1 by corneal esterases after ocular administration [17,31]. Therefore, the content of timolol, netarsudil, netarsudil-M1, latanoprost and LA was evaluated in all the collected samples by ultraperformance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS). The pharmacokinetic parameters, including time to maximum concentration (T_{max}), maximum observed concentration (C_{max}), and area under the concentration time curve from zero to time of last non-zero concentration (AUC_{0-t}) were calculated using DAS 2.0.

2.5.2. *In vivo pharmacodynamic study*

To verify if the change of timolol from maleate to mesylate affects its pharmacological activity, the IOP-lowering efficacy of timolol mesylate ophthalmic solution was evaluated in a high IOP rabbit model and compared with Timoptic® (timolol maleate ophthalmic solution). Healty male Japanese white rabbits were acclimatized for 1 week before the start of the experiments. Then, all rabbits were first anesthetized with an intravenous injection of urethane, 100 μl AH were removed from the anterior chamber of the right eye using a disposable syringe and then slowly injected 100 μl the compound carbomer solution (0.3%) through disposable syringe [32]. After the procedure, all eyes were treated with a drop of levofloxacin hydrochloride eye drops (0.3%). The IOP was measured using a Tonometer (Icare® TA01i, Finland) at 9:00 AM and 9:00 PM. When the IOP of all the right eyes was higher than 21 mmHg for 3 d consecutively, twice-daily timolol mesylate ophthalmic solution or Timoptic® (40 μl) was administered into the lower conjunctival sac of the right eye in each treatment group at 9:00 AM and 9:00 PM for 10 d consecutively. Rabbits did not receive any treatment in the right eyes of model group. And all the left eyes without any treatment were used as a control. Regarding IOP measurements, baseline measurements were obtained twice daily (at 9:00 AM and 9:00 PM) for 3 d consecutively in all study groups prior to treatment. IOP was measured at 9:00 AM and 9:00 PM after daily administration. IOP change was calculated as the difference in IOP between the value after the treatment and that at the baseline in the same eye.

The IOP-lowering efficacy of FC-TNL was evaluated in a high IOP rabbit model and compared with the three monotherapy agents (Timoptic®, netarsudil dimesylate ophthalmic solution, Xalatan®). Establishment of high IOP rabbit model, related IOP measurements and IOP change were as the same as above. Once-daily FC-TNL, netarsudil dimesylate ophthalmic solution or Xalatan® (40 μl) was administered into the lower conjunctival sac of the right eye in each treatment group at 9:00 PM for 10 d consecutively. Timoptic® (40 μl) was administered into the lower conjunctival sac of the right eye in each treatment group at 9:00 AM and 9:00 PM for 10 d consecutively. Rabbits did not receive any treatment in the right eyes of model group. And all the left eyes without any treatment were used as a control.

The IOP-lowering efficacy of FC-TNL was evaluated in above high IOP rabbit model and compared with FC–NL and Xalacom®. Establishment of high IOP rabbit model, related IOP measurements and IOP change were as the same as above. Once-daily FC-TNL, FC–NL or Xalacom® (40 μl) was administered into the lower conjunctival sac of the right eye in each treatment group at 9:00 PM for 10 d consecutively. Rabbits did not receive any treatment in the right eyes of model group.

And all the left eyes without any treatment were used as a control.

2.5.3. *Histological examination*

Extensive studies reported that rho kinase inhibitors increased ocular blood flow, which may slow down the progression of the glaucomatous optic neuropathy by directly increasing the perfusion of the retina and optic disk [18,33]. Therefore, the rabbits were euthanized after the pharmacodynamic study and the eyeballs were removed and fixed in 4% paraformaldehyde to evaluate the potential effect of FC-TNL on rabbit's optic nerve. The obtained tissue sections of retina sliced using a microtome and stained with hematoxylin and eosin (H&E). The retinas from each animal were imaged and Imaging-Pro-Plus 6.0 software (Media Cybernetics Inc., Silver Spring, MD) was used to quantify the number of RGCs.

2.5.4. *Ocular irritation test*

Hundreds of substances that can damage eyesight are daily used. Therefore, it has become important to ensure the safety of the eyes that are subjected to consumer products, especially ophthalmic preparation since the mid-twentieth century [34]. Thus, healthy male Japanese white rabbits without any ophthalmic disease were used to test the ocular irritation of FC-TNL. 15 rabbits were average divided into 5 groups. Once-daily 40 μl of normal saline, blank ophthalmic solution (contains 0.02% BAK, 0.05% boric acid, mannitol adjusts osmolality to 280–320 mOsmol/kg, sodium hydroxide adjusts pH to 4.8–5.2), Xalacom®, netarsudil dimesylate ophthalmic solution, or FC-TNL was administered into the lower conjunctival sac of the right eye in each groups. The eyelids were gently held together for approximately 10 s to avoid the loss of the administered formulation. The left eye was considered as a control. The cornea, iris and conjunctiva were observed at intervals of 1 h, 24 h, 48 h and 72 h post-dose by macroscopic examination using a slit lamp. The score of ocular irritation was obtained according to the Draize test: no irritation (score 0–3); slight irritation (score 4–8); moderate irritation (score 9–12); and severe irritation (score 13–16) [34].

2.6. *Statistical analysis*

Statistical analysis was carried out using SPSS software version 24. All experiments were performed in triplicate and the results were presented as mean ± standard deviation (SD). The Student *t*-test, Mann-Whitney *U* test or One-way analysis of variance (ANOVA) were used to analyze the data in case of two groups or multiple groups, respectively. A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. *Incompatibility between timolol maleate and netarsudil dimesylate*

The safety and effectiveness of the fixed-concomitant multiple drugs were influence by the incompatibility among different drugs [22]. In current study, we firstly found that maleic acid, the salt of the commercial timolol maleate, was not compatible with the commercialized netarsudil dimesylate in pH 4.8–5.2 solution, since white precipitate appeared in the solution and the content of netarsudil dimesylate was below 50% when stored in refrigeration condition (5 ± 3 °C) for 7 d as shown in Fig. S1. Therefore, homologous salt engineering strategy was used to make netarsudil dimesylate soluble in pH 4.8–5.2 solution by changing timolol maleate into timolol mesylate.

3.2. *Timolol mesylate characterization*

3.2.1. *The chemical structure identification of timolol mesylate*

^{1}H NMR spectra of timolol, timolol maleate and timolol mesylate was measured in DMSO–d_6 solution (400 MHz). The ^{1}H NMR chemical shift [d, parts per million (ppm)] data of timolol were δ 5.00 (s, 1H), 4.42–4.28 (m, 2H), 3.82 (m, 1H), 3.80 (t, 4H), 3.46 (t, 4H), 2.59–2.54 (m, 2H), 1.01 (s, 9H) (Fig. S2). For timolol maleate, the chemical shift [d, parts per million (ppm)] data were 6.04 (s, 2H), 4.44–4.37 (m, 2H), 4.22–4.19 (m, 1H), 3.70 (t, 4H), 3.46 (t, 4H), 3.16–3.12 (m, 1H), 2.93–2.87 (m, 1H), 1.30 (s, 9H) (Fig. S3). As for timolol mesylate, the chemical shift [d, parts per million (ppm)] data were 5.93 (s, 1H), 4.43–4.36 (m, 2H), 4.21 (s, 1H), 3.70 (t, 4H), 3.46 (t, 4H), 3.16–3.13 (m, 1H), 2.94–2.86 (m, 1H), 2.34 (s, 3H), 1.29 (s, 9H) (Fig. S4). The ^{1}H NMR chemical shift data of timolol and timolol maleate were in agreement with those previously reported [35]. The signals of the protons assigned to the olefin carbon of maleic acid at 6.04 ppm disappeared, whereas signals at 5.93 ppm and 2.34 ppm associated with methanesulfonic acid protons were observed in the spectra of timolol mesylate. These results demonstrated that the desired salt was formed.

Timolol mesylate in the ESI mode of MS was represented by a molecular $[M+H]^+$ ion peak at 317.1 *m/z* (Fig. S7), corresponding to the molecular weight of timolol (Fig. S5), which was also the same as that of timolol maleate (Fig. S6), demonstrating the integrity of the parent molecule after salt formation.

As shown in Fig. S8, the FTIR spectrum of timolol (red line), timolol maleate (green line) and timolol mesylate (blue line) showed a broad band between 3500 and 3300 cm^{-1} due to the N–H and O–H stretching vibrations, the bands around at 2963 and 2855 cm^{-1} due to the aliphatic C–H stretching vibrations, the band around at 1498 cm^{-1} due to the C–N stretching, and the bands around at 1262, 1119 and 954 cm^{-1} due to the =C–O-C and morpholino C–O-C stretching vibrations, respectively [23,36,37], also proving that the formation of salt did not destroy the integrity of the parent molecule.

The FTIR spectra of timolol mesylate showed the characteristic peaks of methanesulfonic acid at 2920 cm^{-1} corresponded to the -CH_3 stretching vibrations, at 1226 and 1045 cm^{-1} corresponded to the SO_2 symmetric vibration [38], accompanied by the disappearance of the characteristic peaks associated with maleic acid at 3046, 1705, 1589, 1452 and 1229 cm^{-1} [23,36,37]. These results demonstrated that methanesulfonic acid was incorporated in the parent molecule.

3.2.2. *The morphological analysis of timolol mesylate*

Morphological analysis of timolol maleate and timolol mesylate shape and surface was firstly carried out by SEM assessment. Fig. S9 showed obviously distinct surface

morphologies between timolol maleate and timolol mesylate, which demonstrated that their crystalline phases varied among each other.

Meanwhile, the thermal and hygroscopicity behaviors of timolol salt were also variable with their different crystal structure and packing modes [39]. The DSC curve showed that timolol mesylate had a sharp endotherm peak at 142–146 °C (Fig. S10B), which was distinct difference to timolol maleate (199–203 °C) (Fig. S10A). In addition, the TGA curves showed that both timolol mesylate and timolol maleate did not show any weight loss before the melting point.

Futhermore, the PXRD patterns of timolol mesylate revealed numerous sharp and high intensity peaks at 2θ (°) = 14.5, 15.1, 18.4, 19.2, 24.0, 25.0, 27.4, 28.4, and 31.9, whereas the characteristic peaks of timolol maleate were at 2θ (°) = 7.1, 9.4, 14.1, 17.6, 19.1, 19.5, 20.0, 20.3, and 22.0, confirming the different crystallinity between timolol mesylate and timolol maleate (Fig. S11).

3.3. *Preparation of FC-TNL*

Due to the similar prescription and preparation method among the three monotherapy agents, FC–NL and FC-TNL, the preparation process of FC-TNL would be detailedly illustrated as an example. The decisive factors for the preparation of FC-TNL included the following three aspects:

Firstly, due to the poor water solubility of latanoprost [26], BAK is an key ingredient to promote latanoprost dissolution [27]. Meanwhile, stirring speed and heating time also influenced the degree of latanoprost dissolved in water. As shown in Fig. S12, the optimal conditions for the complete dissolution of latanoprost in water were that BAK concentration was 0.02%, stirring speed was 160±5 rpm and heating time was 2.0–2.5 h in 80 ± 2 °C water bath.

Secondly, the osmolality of the ophthalmic solution is preferably similarity to tear to prevent eye irritation, pain and serious side effects [25,40]. Mannitol is used as tonicity agent according to the prescribing information of Rhopress® and Rocklatan®. Consequently, 5.0% mannitol was used to prepare FC-TNL with the osmolality of 295±5 mOsmol/kg, which was isotonic with tear [41].

Futhermore, pH is not only associated with local irritation, but also influence the stability of FC-TNL. Rhopress® and Rocklatan® were provided as a sterile borate buffered aqueous solution (containing 0.05% borate acid) with a pH of 4.8–5.2. Meanwhile, in both netarsudil dimesylate solution and FC-TNL with pH 5.4–5.6, the content of netarsudil dimesylate was lower than 50% and white precipitate appeared after being stored in refrigeration condition (5 ± 3 °C) for 7 d shown in Fig. S13, indicating that netarsudil dimesylate was not stable when the pH of the borate buffer solution was over 5.4, which was consistent with the commercial products (Rhopress®, Rocklatan®). Therefore, FC-TNL was prepared in borate buffer solution containing 0.05% borate acid and the pH of the ophthalmic solution was adjusted to 4.8–5.2 with sodium hydroxide solution (10%) according to the prescribing information and above study results.

3.4. *In vitro stability study of FC-TNL*

Storage stability is an important index to evaluate the quality of ophthalmic solution, including refrigeration temperature condition (5 ± 3 °C), room temperature condition (25 ± 2 °C), and transportation temperature condition (40 ± 2 °C). According to the prescribing information of those three commercial monotherapy agents, Timoptic® can be stored at 2–25 °C for 24 months. However, Rhopress® and Xalatan® must be stored at 2–8 °C until opening and can be stored at 2–25 °C for up to 6 weeks after opening. During shipment at temperature up to 40 °C, Rhopress® should be maintained for not exceeding 14 d, but Xalatan® can only be maintained for 8 d

The stability profiles of the ophthalmic solutions exposed to the above temperature conditions for a period of time were presented in Fig. S14. The content of all active pharmaceutical ingredients in FC-TNL was not significantly different from the monotherapy agents ($P > 0.05$), and all ophthalmic solutions still contained more than 98% of the initial timolol mesylate, netarsudil dimesylate, and latanoprost content in refrigeration storage for 6 months (Fig. S14A-S14C). To evaluate if the stability of FC-TNL is consistent with the commercial medicines (Timoptic®, Rhopress®, Xalatan®), the experiment of FC-TNL stored at 5 ± 3 °C for 24 months is currently underway. And no significant change in the content of the three active pharmaceutical ingredients in FC-TNL was observed at room temperature for 6 weeks (Fig. S14D- S14F), which met the storage requirement of ophthalmic solution after opening. In addition, FC-TNL was still stable when stored up 14 d at 40 °C (Fig. S14G-S14I), meeting the shipment requirement for the ophthalmic solutions [27]. Futhermore, the parameters of pH and osmolality were not significantly different from Day 0, and visible particles were not observed during study storage in all FC-TNL and three monotherapy agents ophthalmic solutions shown in Table S2.

3.5. *In vivo pharmacokinetic study of FC-TNL*

To evaluate if FC-TNL could influence intraocular penetration of each active compounds, a pharmacokinetic study in vivo was performed among the three monotherapy agents and FC-TNL in healthy male Japanese white rabbits. The cornea is a tight barrier that forms tight junctions in multiple layers to restrict the movement of water and solutes [42]. And the penetration of the drugs into the AH from the cornea is crucial to lower IOP [43]. Thus, it is of utmost importance to evaluate the concentration of the active compounds in the cornea and AH. Latanoprost and netarsudil are carboxyl ester prodrugs, which can be rapidly hydrolyzed to LA and netarsudil-M1 by corneal esterases after ocular administration [17,31]. Therefore, the concentration of timolol, netarsudil, netarsudil-M1, latanoprost and LA was determined in the cornea and AH. However, the concentration of netarsudil and latanoprost in the cornea and AH were below the lower limit of quantitation (1 ng/ml) at all the monitored time points in monotherapy groups and FC-TNL group, indicating that latanoprost and netarsudil were hydrolyzed

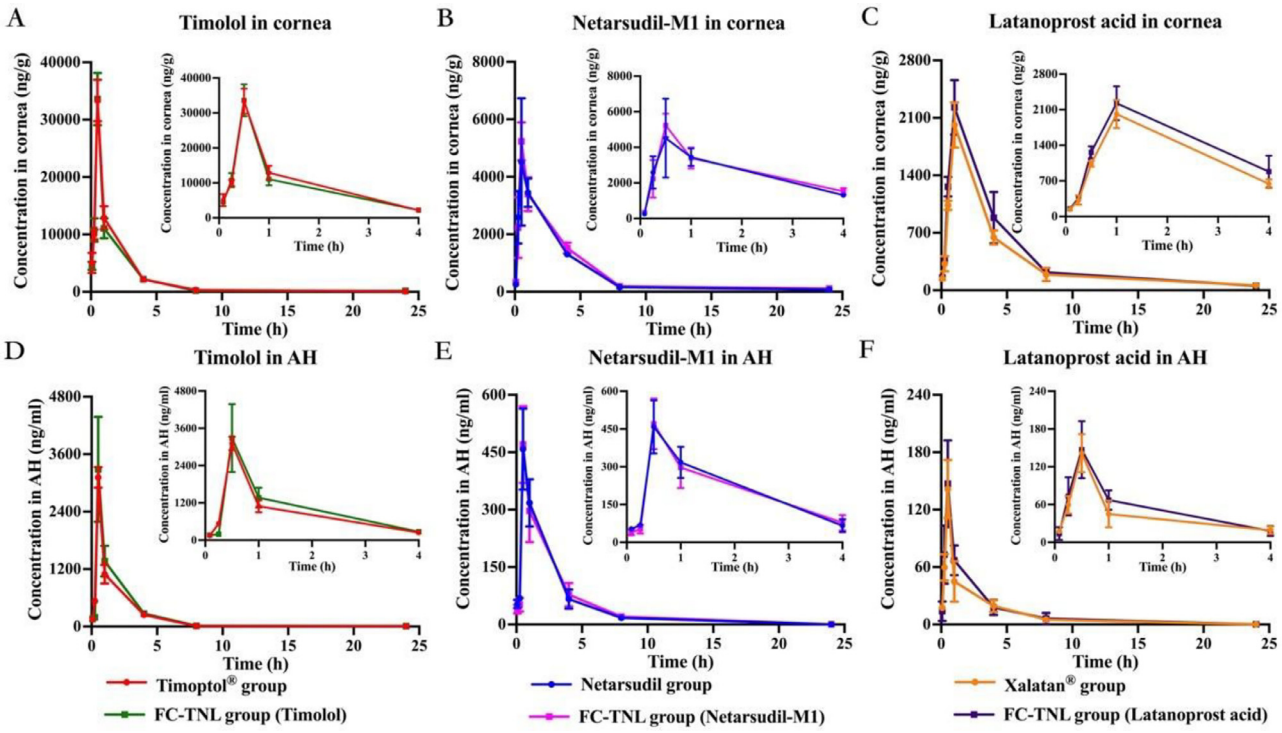


Fig. 2 – AH and cornea concentration-time curves of (A, D) timolol, (B, E) netarsudil-M1, and (C, F) latanoprost acid following a single 40 μl Timoptol®, netarsudil dimesylate ophthalmic solution, Xalatan®, and FC-TNL in healthy male Japanese white rabbits. Data are presented as mean ± SD ($n = 3$). There was no significant difference in AH and cornea concentration of timolol, netarsudil-M1 and latanoprost acid in any time points among monotherapy groups and FC-TNL group ($P > 0.05$).

by corneal esterases, which was consistent with previous studies [17,44].

Table S3 summarized the pharmacokinetic parameters of timolol, netarsudil-M1, and LA in AH and cornea. In the AH, the C_{max} of timolol in Timoptic® group was 3115 ± 215 ng/ml at 0.5 h, and the $AUC_{0-24\,h}$ was 3990 ± 513 ng·h/ml (Fig. 2A). In netarsudil group, the C_{max} of netarsudil-M1 was 458.3 ± 77.9 ng/ml at 0.5 h, and the $AUC_{0-24\,h}$ was 972.6 ± 246.7 ng·h/ml (Fig. 2B). For LA, the C_{max} was 141.6 ± 30.4 ng/ml at 1.0 h and $AUC_{0-24\,h}$ was 299.0 ± 75.8 ng·h/ml in Xalatan® group (Fig. 2C). Importantly, no significant difference was observed in the pharmacokinetic parameters of each active compounds in AH among the monotherapy groups and the FC-TNL group ($P > 0.05$). Additionally, the pharmacokinetic behavior of FC-TNL in cornea was also consistent with the monotherapy agents ($P > 0.05$) (Fig. 2D-2F). These pharmacokinetic results in the cornea and AH indicated that FC-TNL did not influence the intraocular penetration of each active compounds.

3.6. *IOP-lowering efficacy of FC-TNL*

To verify whether the change of timolol from maleate to mesylate affects its pharmacological activity, the IOP-lowering efficacy of timolol mesylate ophthalmic solution was compared with Timoptic® using a rabbit model with the compound carbomer solution-induced ocular hypertension. Although, IOP is not the sole factor causing the damage of the glaucomatous optic nerve, it represents a modifiable risk factor and a quantitative mean of measuring the treatment effect [5,45]. When the IOP of the right eye was higher than 21 mmHg, ophthalmic solutions were administered in each treatment group for 10 d consecutively. Rabbits did not receive any treatment in model group and control group.

The mean actual IOP and mean IOP change from the baseline in each groups were shown in Table 1, Table S4 and Table S5. During the study period, the level of IOP in model group gradually increased and over 21 mmHg, suggesting that rabbit ocular hypertension model was established successfully. On the 10th d, all treatment groups presented a significant reduction in IOP vs baseline IOP ($P < 0.001$). As shown in Fig. S15, the actual IOP at 9:00 PM in the rabbit with the compound carbomer solution-induced ocular hypertension treated with timolol mesylate ophthalmic solution was 22.52 ± 0.87 mmHg, which was no significant difference to that in Timoptic® group (22.45 ± 1.08 mmHg) ($P > 0.05$). Meanwhile, mean IOP reduction in timolol mesylate group (−2.98±0.87 mmHg) was similar to that in Timoptic® group (−2.93±1.08 mmHg) ($P > 0.05$). Therefore, the change of timolol salt form may not affect its IOP lowering effect.

Based on the similar pharmacokinetic behavior among the three monotherapy agents and FC-TNL, the efficacy of the FC-TNL was compared with the three monotherapy agents using a high IOP rabbit model. As shown in Fig. S16, the actual IOP at 9:00 PM in FC-TNL group was approximately 1.91-fold, 1.89-fold and 1.88-fold less than the values in Timoptic® group, netarsudil dimesylate group and Xalatan® group. And

Table 1 – Pharmacodynamic parameters after topical application of Xalacom®, FC–NL, and FC-TNL for 10 d in Japanese white rabbits with the compound carbomer solution-induced ocular hypertension.

Group	9:00 AM (12 h post-dose)			9:00 PM (24 h post-dose)		
	Baseline IOP	Actual IOP on Day 10	IOP change on Day 10	Baseline IOP	Actual IOP on Day 10	IOP change on Day 10
Control	6.00±1.00	5.50±0.50***, ###	−0.50±0.50***, ###	6.00±1.00	8.25±1.25***	2.25±1.25***, ###
Model	26.75±0.41	35.33±2.87	8.33±2.87	25.00±0.50	36.00±2.16	11.00±2.16
Xalacom®	24.33±0.47	20.00±0.71***, ##	−4.33±0.71***, ###	23.67±0.47	19.00±0.82***, ##	−4.67±0.85***, ###
FC-NL	24.83±0.85	20.33±2.05**, ##	−4.50±2.05***, ##	26.00±1.41	21.17±1.25***, ###	−4.83±1.25***, ###
FC-TNL	25.67±0.94	12.67±1.47***	−13.00±1.47***	23.33±0.47	10.00±1.82***	−13.33±0.82***

Baseline IOP: prior to treatment for 3 d consecutively. IOP change: the difference in IOP between the value on Day 10 and that at the baseline in the same eye. IOP values in mmHg. Data are presented as mean ± SD ($n = 3$).

** $P < 0.01$, *** $P < 0.001$ compared with model group.

$P < 0.01$, ### $P < 0.001$ compared with FC-TNL group.

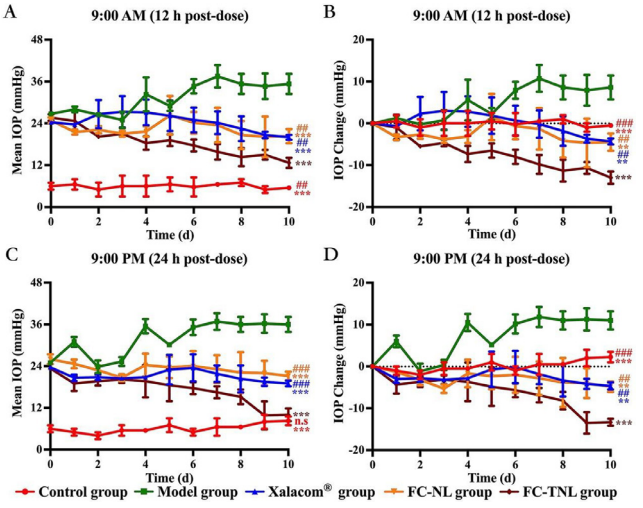


Fig. 3 – The mean actual IOP and mean IOP change from baseline at (A, B) 9:00 AM (12 h post-dose) and (C, D) 9:00 PM (24 h post-dose) in control group, model group, Xalacom® group, FC–NL group, and FC-TNL group in rabbits with the compound carbomer solution-induced ocular hypertension for 10 d consecutively. Data are presented as mean ± SD ($n = 3$). **$P < 0.01$, ***$P < 0.001$ compared with model group. (n.s) $P > 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with FC-TNL group.

the mean IOP-reduction in high IOP rabbit treated with FC-TNL (14.03±1.53 mmHg) was significantly more than that in Timoptic® group (3.00±1.21 mmHg, $P < 0.001$), netarsudil dimesylate group (2.90±1.51 mmHg, $P < 0.001$), and Xalatan® group (3.60±1.20 mmHg, $P < 0.001$). Hence, FC-TNL had better IOP-lowering effect than monotherapy agents.

Based on above pharmacodynamic results, the efficacy of the FC-TNL was then compared with Xalacom® and FC–NL in a high IOP rabbit model. The actual IOP at 9:00 AM (Fig. 3A) in high IOP rabbit treated with FC-TNL, Xalacom® and FC–NL was reduced to approximately 49.4%, 82.2%, and 81.9%, respectively. Meanwhile, the mean IOP reduction in FC-TNL group was approximately 3.0-fold and 2.9-fold more than the values in Xalacom® group and FC–NL group (Fig. 3B). Furthermore, the IOP-lowering effect in each groups at 24 h post-dose (9:00 PM) (Fig. 3C-3D) was consistent with the tendency of that at 12 h post-dose (9:00 AM). These results demonstrated that FC-TNL had a superior IOP-lowering effect and persistent IOP-controlling effect for 24 h in rabbit ocular hypertension model. Therefore, the FC-TNL might represent a great potential in the long-term treatment of glaucoma.

3.7. *Protective effect on RGCs of FC-TNL*

Glaucoma is a neurodegenerative disease and eventually leads to blindness due to the permanent death of RGCs and the loss of the optic nerve fibers [46]. Thus, the protection of RGCs from death is of vital importance for glaucoma patients. Extensive studies reported that rho kinase inhibitors can increase the ocular blood flow, thus slowing the progression of the glaucomatous optic neuropathy by directly increasing the perfusion of the retina and the optic disk [18,33]. Therefore, the number of surviving RGCs in control group, model group, Xalacom® group, FC–NL group, and FC-TNL group was evaluated by H&E staining of the retina after pharmacodynamic study to evaluate the potential effect of FC-TNL on rabbit's optic nerve.

Figs. S17 and S18 showed that the number of surviving RGCs was significantly less in the model group (7.67±1.7 cells) than that in the control group (32.00 ± 1.63 cells) ($P < 0.001$), indicating that persistent high IOP resulted in the death of RGCs in ocular hypertension rabbits. Among the treatment groups, the number of surviving RGCs in FC-TNL group was approximately 2.8-fold and 1.2-fold more than that in Xalacom® group and FC–NL group, demonstrating that FC-TNL could provide better protection for the optic nerve than fixed-concomitant two agents. Moreover, no significant difference in the number of surviving RGCs was observed between control group and FC-TNL group ($P > 0.05$), suggesting that the FC-TNL might be highly effective in protecting the optic nerve and delaying vision loss.

3.8. *Ocular irritation of FC-TNL*

Ocular irritation is the main issue associated with the topical delivery of ophthalmic drugs, which is closely related to the tolerance and compliance of patients [47]. Therefore, the right eye of healthy male Japanese white rabbits was treated

with normal saline, blank ophthalmic solution, netarsudil dimesylate ophthalmic solution, Xalacom®, or FC-TNL, and all the left eyes without any treatment were used as a control. According to the Draize Eye Test, six components are used to evaluate the ocular irritation, including the density and area of corneal opacification, severity of iritis, conjunctival redness, edema, and discharge. The score of ocular irritation was obtained by weighting and summing the above six components. As shown in Fig. S19, the symptoms of ocular irritation appeared at 1 h after instillation in blank group, netarsudil group, Xalacom® group and FC-TNL group, but the evaluation scores of each groups were less than 3, demonstrating that FC-TNL did not cause any moderate or severe irritation. Moreover, all symptoms of ocular irritation disappeared completely after 24 h administration. Thus, the FC-TNL was safe and could be potentially considered for clinical application.

4. Conclusion

To date, many commercial IOP-lowering ophthalmic solutions suffer from poor patient compliance because of the frequent daily instillations, expensive costs, poor therapeutic effect and adverse effects. Once-daily FC-TNL has the ability to treat glaucoma by lowering the IOP with great efficacy and improving patient compliance. However, the commercialized netarsudil dimesylate precipitated when the pH of the solution was above 5.4, or when maleic acid, the salt of commercial timolol maleate, was mixed with netarsudil dimesylate. Consequently, the homologous salt engineering strategy was used to make netarsudil dimesylate soluble in pH 4.8–5.2 solution by synthesizing timolol mesylate. Next, the chemical structure of timolol mesylate was identified by ^{1}H NMR, MS, and FTIR. The morphology of timolol mesylate was analysed by SEM, DSC, TGA, and PXRD. The prepared FC-TNL showed good stability during refrigeration storage. Additionally, FC-TNL exerted no influence on the intraocular penetration of each active compounds in the pharmacokinetic study. And FC-TNL did not cause any sign of moderate or severe ocular irritation. More importantly, once-daily FC-TNL exerted great IOP-lowering effect and persistent IOP-controlling effect for 24 h, and it could slow down RGCs death to potentially protect eyesight. In conclusion, the FC-TNL with good stability and safety has a great potential in controlling IOP and protecting the optic nerve from damage, thus representing an alternative to the current treatments of glaucoma and being more meaningful for glaucoma management.

Conflicts of interest

The authors report no conflicts of interest.

Acknowledgements

This work was financially supported by the LiaoNing Revitalization Talents Program (XLYC1902061).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ajps.2022.11.001.

REFERENCES

[1] Russo R, Nucci C, Adornetto A. The promise of neuroprotection by dietary restriction in glaucoma. Neural Regen Res 2022;17(1):45–7.
[2] Mansouri K, Weinreb RN. Meeting an unmet need in glaucoma: continuous 24-h monitoring of intraocular pressure. Expert Rev Med Devices 2012;9(3):225–31.
[3] Babic N. Fixed combinations of glaucoma medications. Srp Arh Celok Lek 2015;143(9–10):626–31.
[4] Bourlais CL, Acar L, Zia H, Sado PA, Needham T, Leverge R. Ophthalmic drug delivery systems–recent advances. Prog Retin Eye Res 1998;17(1):33–58.
[5] Machen L, Razeghinejad R, Myers JS. Fixed-combination topical anti-hypertensive ophthalmic agents. Expert Opin Pharmacother 2020;21(10):1269–82.
[6] Garcia-Lopez A, Paczka JA, Jimenez-Roman J, Hartleben C. Efficacy and tolerability of fixed-combination bimatoprost/timolol versus fixed-combination dorzolamide/brimonidine/timolol in patients with primary open-angle glaucoma or ocular hypertension: a multicenter, prospective, crossover study. BMC Ophthalmol 2014;14:161.
[7] Goldberg I, Crowston JG, Jasek MC, Stewart JA, Stewart WCGroup ASI. Intraocular pressure-lowering efficacy of brinzolamide when added to travoprost/timolol fixed combination as adjunctive therapy. J Glaucoma 2012;21(1):55–9.
[8] Hartleben C, Parra JC, Batoosingh A, Bernstein P, Goodkin M. A masked, randomized, phase 3 comparison of triple fixed-combination bimatoprost/brimonidine/timolol versus fixed-combination brimonidine/timolol for lowering intraocular pressure. J Ophthalmol 2017;2017:4586763.
[9] Hatanaka M, Reis A, Sano ME, Susanna R Jr. Additive intraocular pressure reduction effect of fixed combination of maleate timolol 0.5%/dorzolamide 2% (Cosopt) on monotherapy with latanoprost (Xalatan) in patients with elevated intraocular pressure: a prospective, 4-week, open-label, randomized, controlled clinical trial. J Glaucoma 2010;19(5):331–5.
[10] Konstas AG, Boboridis KG, Kapis P, Marinopoulos K, Voudouragkaki IC, Panayiotou D, et al. 24-Hour efficacy and ocular surface health with preservative-free tafluprost alone and in conjunction with preservative-free dorzolamide/timolol fixed combination in open-angle glaucoma patients insufficiently controlled with preserved latanoprost monotherapy. Adv Ther 2017;34(1):221–35.
[11] Conlon R, Saheb H, Ahmed II. Glaucoma treatment trends: a review. Can J Ophthalmol 2017;52(1):114–24.
[12] Shukla AG, Razeghinejad R, Myers JS. Balancing treatments for patients with systemic hypertension and glaucoma. Expert Opin Pharmacother 2020;21(18):2225–30.
[13] Sah AK, Suresh PK. Medical management of glaucoma: focus on ophthalmologic drug delivery systems of timolol maleate. Artif Cells Nanomed Biotechnol 2017;45(3):448–59.
[14] Feldman RM. An evaluation of the fixed-combination of latanoprost and timolol for use in open-angle glaucoma and ocular hypertension. Expert Opin Pharmacother 2004;5(4):909–21.
[15] Jayanetti V, Sandhu S, Lusthaus JA. The latest drugs in development that reduce intraocular pressure in ocular hypertension and glaucoma. J Exp Pharmacol 2020;12:539–48.

[16] Diaconita V, Quinn M, Jamal D, Dishan B, Malvankar-Mehta MS, Hutnik C. Washout duration of prostaglandin analogues: a systematic review and meta-analysis. J Ophthalmol 2018;2018:3190684.
[17] Lin CW, Sherman B, Moore LA, Laethem CL, Lu DW, Pattabiraman PP, et al. Discovery and preclinical development of netarsudil, a novel ocular hypotensive agent for the treatment of glaucoma. J Ocul Pharmacol Ther 2018;34(1–2):40–51.
[18] Tanna AP, Johnson M. Rho kinase inhibitors as a novel treatment for glaucoma and ocular hypertension. Ophthalmology 2018;125(11):1741–56.
[19] Konstas AG, Mocan MC, Katsanos A, Voudouragkaki IC, Irkec M. Latanoprost/timolol fixed combination for the treatment of glaucoma. Expert Opin Pharmacother 2013;14(13):1815–27.
[20] Sinha S, Lee D, Kolomeyer NN, Myers JS, Razeghinejad R. Fixed combination netarsudil-latanoprost for the treatment of glaucoma and ocular hypertension. Expert Opin Pharmacother 2020;21(1):39–45.
[21] Wang T, Cao L, Jiang Q, Zhang T. Topical medication therapy for glaucoma and ocular hypertension. Front Pharmacol 2021;12:749858.
[22] Abdelkader A, Fathi HA, Hamad MA, Elsabahy M. Nanomedicine: a new paradigm to overcome drug incompatibilities. J Pharm Pharmacol 2020;72(10):1289–305.
[23] Gaikwad SS, Thombre SK, Kale YK, Gondkar SB, Darekar AB. Design and *in vitro* characterization of buccoadhesive tablets of timolol maleate. Drug Dev Ind Pharm 2014;40(5):680–90.
[24] Abd-Elal RMA, Elosaily GH, Gad S, Khafagy ES, Mostafa Y. Full factorial design, optimization, *in vitro* and *ex vivo* studies of ocular timolol-loaded microsponges. J Pharm Innov 2020;15(4):651–63.
[25] Arbelaez-Camargo D, Roig-Carreras M, Garcia-Montoya E, Perez-Lozano P, Minarro-Carmona M, Tico-Grau JR, et al. Osmolality predictive models of different polymers as tools in parenteral and ophthalmic formulation development. Int J Pharm 2018;543(1–2):190–200.
[26] Rodriguez-Aller M, Guinchard S, Guillarme D, Pupier M, Jeannerat D, Rivara-Minten E, et al. New prostaglandin analog formulation for glaucoma treatment containing cyclodextrins for improved stability, solubility and ocular tolerance. Eur J Pharm Biopharm 2015;95(Pt B):203–14.
[27] Halder A, Khopade AJ. Physiochemical properties and cytotoxicity of a benzalkonium chloride-free, micellar emulsion ophthalmic formulation of latanoprost. Clin Ophthalmol 2020;14:3057–64.
[28] Walsh K, Jones L. The use of preservatives in dry eye drops. Clin Ophthalmol 2019;13:1409–25.
[29] Asada H, Kimura A. Clear ophthalmic solution comprising latano prostas active ingredient. USA, US 8 183 291 B2[P].
[30] Matthews BR, Wall GM. Stability storage and testing of ophthalmic products for global registration. Drug Dev Ind Pharm 2000;26(12):1227–37.
[31] Sjoquist B, Stjernschantz J. Ocular and systemic pharmacokinetics of latanoprost in humans. Surv Ophthalmol 2002;47(Suppl 1):S6–12.
[32] Ou-Yang Y, Liu ZL, Xu CL, Wu JL, Peng J, Peng QH. miR-223 induces retinal ganglion cells apoptosis and inflammation via decreasing HSP-70 *in vitro* and *in vivo*. J Chem Neuroanat 2020;104:101747.
[33] Moura-Coelho N, Tavares Ferreira J, Bruxelas CP, Dutra-Medeiros M, Cunha JP, Pinto Proenca R. Rho kinase inhibitors-a review on the physiology and clinical use in Ophthalmology. Graefes Arch Clin Exp Ophthalmol 2019;257(6):1101–17.
[34] Wilhelmus KR. The Draize eye test. Surv Ophthalmol 2001;45(6):493–515.
[35] Zielinska-Pisklak MA, Pisklak DM, Wawer I. ^{1}H and ^{13}C NMR characteristics of beta-blockers. Magn Reson Chem 2011;49(5):284–90.
[36] Agnihotri SA, Aminabhavi TM. Chitosan nanoparticles for prolonged delivery of timolol maleate. Drug Dev Ind Pharm 2007;33(11):1254–62.
[37] Joshi GV, Kevadiya BD, Patel HA, Bajaj HC, Jasra RV. Montmorillonite as a drug delivery system: intercalation and in vitro release of timolol maleate. Int J Pharm 2009;374(1–2):53–7.
[38] Renuga Devi TS, Sharmi kumar J, Ramkumaar GR. DFT analysis on the molecular structure, vibrational and electronic spectra of 2-(cyclohexylamino)ethanesulfonic acid. Spectrochim Acta A Mol Biomol Spectrosc 2015;137:761–77.
[39] Peng B, He H, Li M, Wang JR, Mei X. Comparison of the crystal structures and physicochemical properties of novel resveratrol cocrystals. Acta Crystallogr B Struct Sci Cryst Eng Mater 2019;75(Pt 6):1186–96.
[40] Rahman Z, Xu X, Katragadda U, Krishnaiah YS, Yu L, Khan MA. Quality by design approach for understanding the critical quality attributes of cyclosporine ophthalmic emulsion. Mol Pharm 2014;11(3):787–99.
[41] Craig JP, Simmons PA, Patel S, Tomlinson A. Refractive index and osmolality of human tears. Optom Vis Sci 1995;72(10):718–24.
[42] Gipson IK, Argueso P. Role of mucins in the function of the corneal and conjunctival epithelia. Int Rev Cytol 2003;231:1–49.
[43] Isobe T, Kasai T, Kawai H. Ocular penetration and pharmacokinetics of ripasudil following topical administration to rabbits. J Ocul Pharmacol Ther 2016;32(7):405–14.
[44] Ichhpujani P, Katz LJ, Hollo G, Shields CL, Shields JA, Marr B, et al. Comparison of human ocular distribution of bimatoprost and latanoprost. J Ocul Pharmacol Ther 2012;28(2):134–45.
[45] Leske MC, Heijl A, Hussein M, Bengtsson B, Hyman L, Komaroff E, et al. Factors for glaucoma progression and the effect of treatment: the early manifest glaucoma trial. Arch Ophthalmol 2003;121(1):48–56.
[46] Almasieh M, Wilson AM, Morquette B, Cueva Vargas JL, Di Polo A. The molecular basis of retinal ganglion cell death in glaucoma. Prog Retin Eye Res 2012;31(2):152–81.
[47] Zhang X, Vadoothker S, Munir WM, Saeedi O. Ocular surface disease and glaucoma medications: a clinical approach. Eye Contact Lens 2019;45(1):11–8.