



Bepotastine besilate, a highly selective histamine H₁ receptor antagonist, suppresses vascular hyperpermeability and eosinophil recruitment in in vitro and in vivo experimental allergic conjunctivitis models

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ABSTRACT

To elucidate the ocular pharmacological properties of bepotastine besilate, a selective histamine H₁ receptor antagonist, when compared with other histamine H₁ receptor antagonists, using guinea pig allergic conjunctivitis models and in vitro models of eosinophil recruitment and mast cell membrane stabilization. Conjunctival vascular hyperpermeability was studied in guinea pigs passively sensitized with anti-ovalbumin antiserum or following subconjunctival injection of histamine. Modulation of eosinophil recruitment was evaluated for both platelet-activating factor (PAF)-induced eosinophil infiltration in guinea pigs and leukotriene B₄-induced in vitro chemotaxis of guinea pig peritoneal eosinophils. Membrane-stabilizing effects of bepotastine also were studied with rat peritoneal mast cells stimulated with the ionophore A23187. Histamine H₁ receptor antagonists including bepotastine besilate were topically administered before ovalbumin, histamine or PAF challenges for in vivo experiments or were added directly to mast cell and eosinophil medium in vitro. Bepotastine besilate significantly inhibited conjunctival vascular hyperpermeability in a dose-dependent manner with maximal effect for bepotastine besilate 1.5%. In separate in vivo experiments, bepotastine besilate 1.0% was significantly more effective than levocabastine 0.025% in the passive sensitization model or olopatadine 0.1% in the histamine-induced hyperpermeability model. Bepotastine besilate 1.0% further suppressed PAF-induced eosinophil infiltration into conjunctival tissue more effectively than ketotifen 0.05%. Chemotaxis of guinea pig peritoneal eosinophils and histamine release from rat peritoneal mast cells in vitro were also inhibited by addition of bepotastine. Olopatadine had a weak effect as compared to that of bepotastine on eosinophil chemotaxis and no effect on mast cell histamine release in our study conditions. Bepotastine besilate was more potent than olopatadine, ketotifen, or levocabastine in reducing vascular hyperpermeability in various animal models of allergic conjunctivitis. Mast cell function and eosinophil chemotaxis were also inhibited in vitro with bepotastine, suggesting bepotastine acts as an inhibitor of allergic response through multiple mechanisms: histamine H₁ receptor antagonism, mast cell stabilization, and inhibition of eosinophil migration to ocular inflammatory sites.

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1. Introduction

Allergic conjunctivitis affects approximately one fifth of the population, and more than 90% of these cases are seasonal or perennial (Abelson and Chapin, 2000). Seasonal allergies are brought on by environmental triggers such as trees, grasses, or ragweed pollens, while perennial allergies are caused by dusts, molds, or animal dander. Signs and symptoms of these common types of allergic conjunctivitis include itching, redness, tearing, chemosis, and eyelid swelling.

Seasonal and perennial allergic conjunctivitis (SAC and PAC) are closely related to a type I hypersensitivity reaction in patient's

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conjunctiva, and is associated with immunoglobulin E (IgE)-mediated mast cell activation in the conjunctiva, followed by the release of mast cell-derived chemical mediators (e.g., histamine and cytokines) that recruit eosinophils and lymphocytes to the ocular surface (Calder and Lackie, 2004; Abelson et al., 2003). Histamine binding to its receptors plays a central role in the induction of major allergic symptoms such as itching, swelling, and redness.

Bepotastine besilate (TAU-284; formerly designated betotastine besilate, (+)-(S)-4-[4-[(4-chlorophenyl)(2-pyridyl)methoxy]piperidino]butyric acid monobenzenesulfonate) is a second-generation histamine H₁ receptor antagonist possessing a high binding selectivity to its receptor and low liability of sedative adverse effects (Kato et al., 1997; Narita et al., 1997). In addition, bepotastine possesses antiallergic activity due to effects on multiple allergen effector pathways, including the inhibition of eosinophil infiltration induced by platelet-activating factor (PAF) and antigens in the airway (Ueno et al., 1998; Sakai et al., 1997), the inhibition of interleukin-5 production by human peripheral blood mononuclear cells (Kaminuma et al., 1998) and the suppression of leukotriene B₄ (LTB₄)-dependent allergic response (Andoh and Kuraishi, 2006). Oral bepotastine also was reported to inhibit the frequency and duration of scratching behavior induced by compound 48/80 in mouse models including itching and atopic dermatitis in NC/Nga mice (Tanizaki et al., 2008). Oral bepotastine additionally inhibited the acceleration of histamine-induced vascular permeability and homologous passive cutaneous anaphylaxis (PCA) (Yato et al., 1997; Honda et al., 1997; Sakamoto et al., 1997; Murata et al., 1997). The antiallergic activity of bepotastine was demonstrated in these *in vitro* and *in vivo* studies using a number of animal models of allergic diseases, although none were models of ocular allergy.

To date, various ocular allergic animal models which mimic symptoms of allergic conjunctivitis have been utilized to examine the possible effectiveness of histamine H₁ receptor antagonists for the treatment of ocular allergic symptoms such as conjunctival edema, hyperemia, eyelid swelling, itching behavior, and/or inflammatory cell infiltration (Groneberg et al., 2003). In particular, antigen-induced conjunctivitis models involving passive sensitization in rats or guinea pigs are based on IgE-dependent degranulation of mast cells by allergens and release of endogenous histamine, giving rise to a typical early phase response of type I allergy. Furthermore, these models replicate the spectrum of SAC and PAC symptoms seen in allergy patients. For these reasons, this model has been widely used to estimate the efficacy of H₁ receptor antagonists. Further, conjunctival vascular hyperpermeability alternatively induced by exogenous histamine has the same allergic response properties and has been used for estimating the effectiveness of various H₁ receptor antagonists including ketotifen (Schoch, 2005) and olopatadine (Yanni et al., 1996).

The late phase conjunctival allergic response occurs within several hours after initial mast cell activation, and is characterized by the infiltration of inflammatory cells such as neutrophils, lymphocytes, basophils, and eosinophils onto the ocular surface (Leonardi et al., 2007). This cytokine-driven cellular response is seen in allergic conjunctivitis and also is one of the important pathophysiological elements of vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis. Among inflammatory cells, eosinophils have a pivotal role in allergic inflammation of the eye because they release cytotoxic proteins and cytokines which lead to prolonged inflammation. Eosinophils are readily attracted to conjunctiva by several chemical mediators including PAF and LTB₄. We therefore investigated whether bepotastine suppressed eosinophil infiltration *in vivo* and chemotaxis activity *in vitro* induced by PAF and LTB₄, respectively.

Currently, the most commonly prescribed drugs for allergic conjunctivitis have dual mechanisms, competitive inhibition of

histamine binding to cognate H₁ receptors and inhibition of histamine release from mast cells. Moreover, some of these drugs are known to modulate the activity of eosinophils (Bielory et al., 2005). Although bepotastine, ketotifen and olopatadine are known drugs in this class (Abelson et al., 2003; Yato et al., 1997), it remains unclear which of these drugs may be the most potent for treating ocular allergy.

The aim of this study is to supplement the known pharmacological profile of bepotastine in ophthalmic use by comparing bepotastine besilate ophthalmic solutions with the commonly used ophthalmic antihistamines ketotifen 0.05%, olopatadine 0.1% and levocabastine 0.025% in *in vivo* experimental allergic conjunctivitis models, focusing on vascular hyperpermeability induced by antigens or histamine that induce type I early allergic response. Eosinophil infiltration into the conjunctiva induced by PAF was also studied as a manifestation of late phase allergic response. In addition, *in vitro* experiments using mast cells and eosinophils were conducted to directly compare the potency of several H₁ receptor antagonists including bepotastine.

2. Materials and methods

2.1. Animals

All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the institutional committee for animal care and use. Male Hartley guinea pigs (Slc:Hartley) aged 4–5 weeks and male Wistar rats (Slc:Wistar) weighing approximately 200 g were obtained from Japan SLC, Inc. (Shizuoka, Japan), and these animals were housed in controlled temperature (23 ± 2 °C) and humidity ($55 \pm 10\%$) environments under a 12-h light/12-h dark cycle and specific pathogen-free conditions. Animals were fed standard chow diet and given sterilized water to drink *ad libitum*.

2.2. Test articles

Bepotastine besilate was provided from Mitsubishi Tanabe Pharma Corporation (Osaka, Japan) and Ube Industries, Ltd. (Ube, Japan). Olopatadine hydrochloride was prepared from ALLELOCK® tablets (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) and its chemical identity was confirmed by nuclear magnetic resonance spectroscopy, melting point measurement (248.5 – 249.0 °C) in our laboratory, and its purity determined by high performance liquid chromatography was 99.9%. Ketotifen fumarate was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). All other reagents used were commercially available and were reagent-grade.

For *in vivo* experiments, bepotastine besilate ophthalmic solutions containing benzalkonium chloride (BAK) 0.005% were prepared at concentrations of 0% (vehicle), 0.01%, 0.1%, 0.3%, 1.0%, and 1.5% (w/v) in our laboratory. Ketotifen ophthalmic solution 0.05% (Zaditen®, Novartis Pharma K.K., Tokyo, Japan), olopatadine ophthalmic solution 0.1% (Patanol®, Alcon Laboratories, Inc., Fort Worth, TX, USA), and levocabastine ophthalmic suspension 0.025% (Livostin®, Janssen Pharmaceutical K.K., Tokyo, Japan) were used as positive control drugs. These commercially available ophthalmic formulations were containing BAK as a preservative.

For *in vitro* experiments, bepotastine besilate, ketotifen fumarate and olopatadine hydrochloride were dissolved in Hanks' balanced salt solution (Sigma–Aldrich Co.) containing 0.25% bovine serum albumin (BSA, Sigma–Aldrich Co.), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (HBSS/BSA, pH 7.4) and 0.2% ethanol for an *in vitro* eosinophil chemotaxis experiment, or Tyrode's solution (137 mM NaCl, 2.7 mM KCl,

0.41 mM NaH₂PO₄, 1.6 mM CaCl₂, 1 mM MgCl₂, 0.1% D-Glucose, 10 mM HEPES, and 0.05% gelatin, pH 7.4) containing 0.2% ethanol for an in vitro histamine release experiment, before use. These buffers were inactive in cell responses in the experiments.

2.3. Antigen-induced conjunctival vascular hyperpermeability in passively sensitized guinea pigs

Anti-ovalbumin (OVA) antiserum containing IgE antibody was prepared by the method of Mota and Perini (1970) using male guinea pigs weighing 400–500 g. The PCA titer of the antiserum was estimated as 1:4096 by 48-h PCA in guinea pigs.

Anesthesia of guinea pigs (6-week-old) was carried out by intramuscular injection with an equal volume of 5% ketamine (Ketalar® for Intramuscular Injection 500 mg, Daiichi Sankyo Co., Ltd., Tokyo, Japan) and 2% xylazine (Rompun®, Bayer HealthCare AG, Monheim, Germany) at 0.5 mL/kg. Guinea pigs were passively sensitized with 50 µL of anti-OVA antiserum diluted with saline (1:600) by the upper subconjunctival injection in one eye under anesthesia. Forty-eight hours after the sensitization, the animals were subjected to challenge by an intravenous injection of OVA (3 mg/kg, Grade V, Sigma–Aldrich Co.) and Evans blue dye solution (20 mg/kg, Merck KGaA, Darmstadt, Germany). After 30 min, they were euthanized, and the conjunctiva colored in blue was excised. Extravasated dye was extracted from the conjunctiva and its absorbance at 620 nm was determined with a spectrophotometer (U-3000, Hitachi, Ltd., Tokyo, Japan) (Katayama et al., 1978). Thirty minutes before the intravenous antigen challenge, animals were topically instilled with 10 µL of test article or saline (Otsuka Normal Saline, Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) to the eye.

2.4. Histamine-induced conjunctival vascular hyperpermeability

Guinea pigs (5-week-old) were anesthetized with ketamine and xylazine as described above. After 5 min, 50 µL of 0.2% histamine dihydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in saline was injected into the upper conjunctival sac of guinea pigs immediately after the intravenous injection of Evans Blue dye solution (20 mg/kg). Thirty minutes before the histamine injection, the animals were topically instilled with 10 µL of each test article or saline in the affected eye. Thirty minutes after the histamine injection, the animals were euthanized and the conjunctiva colored in blue was excised. Extravasated dye was determined by the method described above.

2.5. PAF-induced eosinophil infiltration into guinea pig conjunctiva

Guinea pigs (6-week-old) topically received 10 µL of each test article in one eye 3 times at intervals of 20 min. At 20 min after the last instillation of each test article, 10 µL of PAF (Cayman Chemical Company, Ann Arbor, MI, USA) dissolved in saline at a final concentration of 0.1% (w/v) was topically applied into their conjunctiva to induce eosinophil infiltration. Six hours after PAF instillation, the animals were euthanized and each conjunctiva was removed for determination of eosinophil peroxidase (EPO) activity as an index of eosinophil infiltration as previously described by Pettipher et al. (1994) with slight modification. Briefly, each conjunctiva was homogenized in 5 mL of 0.5% n-hexadecyltrimethylammonium bromide dissolved in phosphate-buffered saline (pH 7.4) and subjected to two freeze–thaw cycles. The homogenates were centrifuged at 3000 rpm for 10 min and a portion (50 µL) of supernatants was incubated with 100 µL of 5 mM o-phenylenediamine dihydrochloride in 50 mM Tris–HCl (pH 8.0) containing 0.005% hydrogen peroxide for 5 min at room

temperature, and the reaction was terminated by adding 100 µL of 4 M sulfuric acid. The absorbance was measured with a microplate reader at 492 nm (Powerscan HT, DS Pharma Biomedical Co., Ltd., Osaka, Japan).

2.6. LTB₄-induced chemotaxis in vitro of guinea pig peritoneal eosinophils

Guinea pigs (350–500 g) received an intraperitoneal injection of 2 mL of normal horse serum (DS Pharma Biomedical Co., Ltd.) every 4 or 5 days for 6 weeks. At 16 h after the final injection, the animals were sacrificed by decapitation and exsanguination, and 50 mL of phosphate-buffered saline (PBS) was injected into the peritoneal cavity. After massaging the abdomen for 15 s, the intraperitoneal lavage fluid was carefully aspirated and filtered through sterile gauze to remove cellular aggregates. After hypotonic treatment to hemolyze erythrocytes, the cell pellet was washed with PBS containing 0.01% BSA (PBS/BSA) by centrifugation at 150 × g for 5 min at 4 °C and resuspended in 15 mL of PBS/BSA. The suspension was layered onto 20 mL of Ficoll–Paque™ PLUS (GE Healthcare UK Ltd., Buckinghamshire, England) and centrifuged at 400 × g for 50 min at 18 °C. The lower layer containing granulocytes was collected and resuspended in 3.75 mL of PBS/BSA, then the suspension was layered onto 5 mL of Ficoll–Paque™ PLUS and centrifuged at 380 × g for 70 min at 18 °C. Eosinophils were obtained from the lower layer in the centrifuge tube and suspended in HBSS/BSA, pH 7.4. To remove contaminated macrophages, the suspension was incubated in a plastic dish for 30 min at 37 °C in a humidified atmosphere with 5% CO₂, and the eosinophils in the supernatant were collected and placed on ice until use. The purity of eosinophil population exceeded 90% as determined by staining with Hinkelman's reagent (Kanto Chemical Co., Inc., Tokyo, Japan).

Eosinophil chemotaxis to LTB₄ was measured by a modified Boyden chamber technique using a 48-well microchemotaxis chamber with a polycarbonate filter (5 µm pore) which divides the chamber into upper and lower wells (Neuro Probe, Inc., Gaithersburg, MD, USA) as described by Kikuchi et al. (1998) with a slight modification. Eosinophils (2 × 10⁵ cells/mL) were pre-incubated with or without each histamine H₁ receptor antagonist at final concentrations of 10, 100, and 1000 µM in HBSS/BSA containing 0.1% ethanol for 20 min at 37 °C in a humidified atmosphere with 5% CO₂. A portion (50 µL) of cell suspension then was added to the microchemotaxis chamber upper wells and incubated with 25 µL of 0.1 µM LTB₄ (Cayman Chemical Co.) dissolved in HBSS/BSA in lower wells for 90 min at 37 °C in a humidified atmosphere with 5% CO₂. The filter was removed from the chamber and non-migrated cells were scraped from the upper side of the filter. The migrated eosinophils were fixed and stained with Diff-Quick (Sysmex Corporation, Kobe, Japan) on the lower side of the filter and counted with a light microscope. The rates of chemotaxis were defined as the number of migrated eosinophils with treatment of each histamine H₁ antagonist divided by that without treatment.

2.7. Histamine release from cultured rat peritoneal mast cells

Rats (220–260 g) were sacrificed by decapitation and exsanguination. Rat peritoneal mast cells (RPMCs) were isolated by intraperitoneal injection of 20 mL Tyrode's solution containing 5 IU/mL heparin, and the abdomen was massaged about 2 min. Thereafter, the peritoneal cavity was opened, and the fluid containing RPMCs was collected. The collected mast cells were washed twice by centrifugation at 100 × g for 5 min at 4 °C and then resuspended in 1 mL of Tyrode's solution. RPMCs were separated by layering on 12 mL of 60% Percoll (density 1.09 g/mL) (GE Healthcare UK Ltd.) dissolved in Tyrode's solution, and centrifuging at 60 × g

for 15 min at 4 °C. After the upper layer was discarded, the remaining cell pellet was washed twice with and resuspended in Tyrode's solution, and purified RPMCs were diluted in Tyrode's solution (2×10^4 cells/mL) and kept on ice until use. RPMC preparations were over 90% viable as assessed by toluidine blue staining.

For the assay of histamine release, 1 mL of RPMC suspension (2×10^4 cells/mL) was mixed with 1 mL of Tyrode's solution containing 0.2% ethanol with or without an H_1 receptor antagonist at final concentrations of 10, 100, or 1000 μ M, and the mixture was pre-incubated for 120 min at 37 °C in a humidified atmosphere (5% CO_2). After preincubation, 10 μ L of the calcium ionophore A23187 (EMD Chemicals, Inc., Gibbstown, NJ, USA) in dimethylsulfoxide was added to each well to make a final concentration of 0.01 μ M A23187 and the plates were incubated for 10 min. The plates were then placed on ice to terminate the reaction and each cell suspension was centrifuged at $200 \times g$ for 8 min at 4 °C. The supernatant was collected to determine the amount of histamine release from RPMCs. Histamine in the precipitant was determined by adding Tyrode's solution and homogenizing by sonication. The sonicated samples were centrifuged at $800 \times g$ for 10 min at 4 °C and the supernatant was collected to determine the amount of residual histamine.

Both released and residual histamine amounts were determined by an enzyme-linked immunosorbent assay (IBL International GmbH, Hamburg, Germany) according to the manufacture's standard protocol. Total histamine in RPMCs was calculated as the sum of the released and residual histamine contents in RPMCs.

2.8. Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (S.E.M.), and processed using SAS Release 8.2 (SAS Institute Inc., Cary, NC, USA) software for statistical analysis. For in vivo experiments, the dose-dependent suppression of bepotastine besilate ophthalmic solution was analyzed by the Shirley–Williams test (Shirley, 1977) (1-sided) followed by linear-regression analysis. The differences of the mean values of extravasated dye or absorbance of EPO activity between the drug-treated groups and the saline-treated group were analyzed by the Dunnett's test (1-sided). For comparison of efficacy of bepotastine besilate ophthalmic solutions with other ophthalmic drugs, the Dunnett's test (2-sided) and/or the Student's *t*-test (2-sided) were applied. For in vitro experiments, the differences of the mean numbers of migrated eosinophils or the mean amounts of released histamine between drug-treated groups and the corresponding control treatment group were analyzed by Dunnett's test (1-sided). A *P*-value less than *P* = 0.05 was considered statistically significant.

3. Results

3.1. Antigen-induced conjunctival vascular hyperpermeability in passively sensitized guinea pigs

A marked increase in the amount of extravasated dye provoked by local anaphylactic reaction was observed in the conjunctiva of anti-OVA antiserum sensitized guinea pigs which intravenously received the OVA antigen and Evans blue dye. This increase is apparent when comparing results for sensitized animals treated with vehicle of bepotastine besilate ophthalmic solution or saline ("Vehicle" in Fig. 1, "Saline" in Fig. 2) to non-sensitized ("Normal") animals (*P* < 0.01). When bepotastine besilate concentrations from 0.1% to 1.5% were instilled into guinea pig eyes 30 min before antigen challenge, extravasated dye was dose-dependently suppressed at all concentrations, with the greatest suppression seen

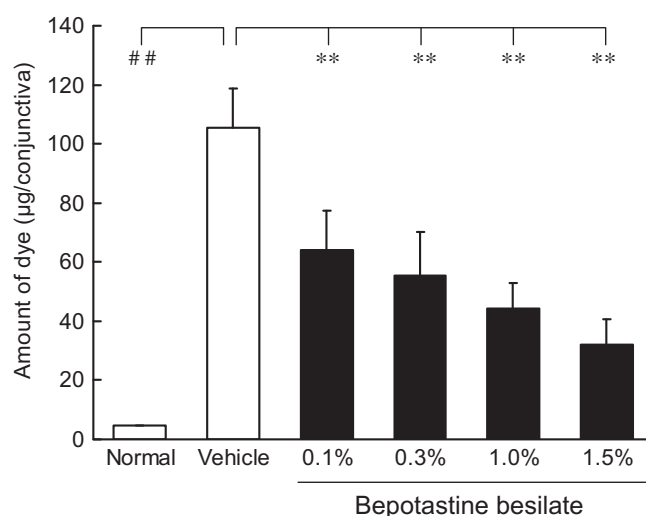


Fig. 1. Dose-responsive effects of topically applied bepotastine besilate ophthalmic solution on the ovalbumin-induced conjunctival vascular hyperpermeability in passively sensitized guinea pigs. Each concentration of bepotastine besilate ophthalmic solution was topically administered 30 min before the intravenous injection of ovalbumin (OVA) with Evans blue dye. The animals in the Normal group were treated with saline instead of anti-OVA serum during passive sensitization procedures. The animals in the Normal and Vehicle groups were instilled with the vehicle of bepotastine besilate at the time of drug treatment. Data are represented as the mean $\pm$ S.E.M. of 9 or 10 animals. ##*P* < 0.01, compared with Normal (Student's *t*-test, 1-sided), ***P* < 0.01, compared with Vehicle (Shirley–Williams test following linear-regression analysis, 1-sided).

with bepotastine besilate 1.0% and 1.5% (*P* < 0.01) (Fig. 1 and Table 1). Fig. 2 and Table 1 represent the effect of bepotastine besilate 1.0% when directly compared with other ophthalmic H_1 receptor antagonists in this guinea pig model. Bepotastine besilate 1.0% as

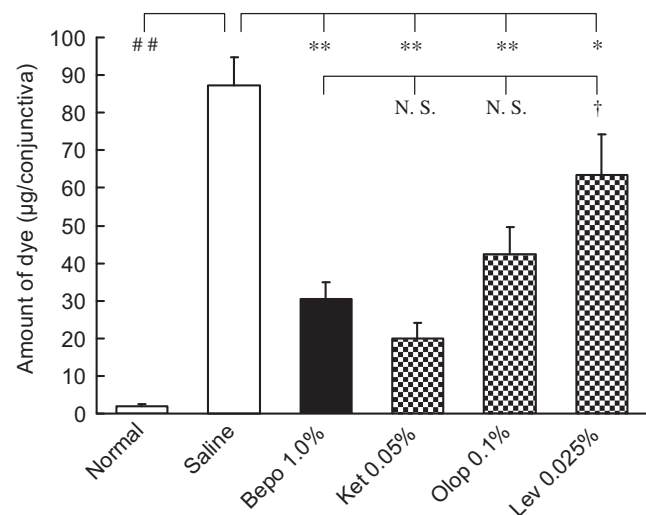


Fig. 2. Effect of topically applied bepotastine besilate ophthalmic solution 1.0% compared with ophthalmic formulations of H_1 receptor antagonists on ovalbumin-induced conjunctival vascular hyperpermeability in passively sensitized guinea pigs. Bepotastine besilate ophthalmic solution (Bepo) 1.0%, ketotifen ophthalmic solution (Ket) 0.05%, olopatadine ophthalmic solution (Olop) 0.1% and levocabastine ophthalmic suspension (Lev) 0.025% were topically administered 30 min before the intravenous injection of ovalbumin with Evans blue dye, respectively. The animals in the normal group received the same treatment at the sensitization described in Fig. 1. The animals in the Normal and Saline groups were instilled with saline instead of active drug. Data are represented as the mean $\pm$ S.E.M. of 10 or 11 animals. ##*P* < 0.01, compared with Normal (Student's *t*-test, 1-sided), ***P* < 0.01, **P* < 0.05, compared with Saline (Dunnett's test, 1-sided), †*P* < 0.05, compared with Bepo 1.0% (Dunnett's test, 2-sided), N.S., not significant.

Table 1

Tabular summary of the inhibition rate of bepotastine besilate ophthalmic solution and other ophthalmic H₁ receptor antagonists in in vivo allergic conjunctivitis models.

	Inhibition rate (%)						
	Bepo		Ket		Olop	Lev	
	0.1%	0.3%	1.0%	1.5%	0.05%	0.1%	0.025%
Ovalbumin-induced vascular hyperpermeability in Fig. 1	40.9 ^b	49.4 ^b	60.7 ^b	72.8 ^b			
Ovalbumin-induced vascular hyperpermeability in Fig. 2			66.6 ^c	78.8 ^c	52.6 ^c	27.9 ^{d,f}	
Histamine-induced vascular hyperpermeability in Fig. 3			77.6 ^c		60.1 ^{c,g}		
PAF-induced eosinophil infiltration in Fig. 4			71.0 ^{a,c}	51.0 ^{a,c,e}	75.6 ^{a,c}		

Bepo, Ket, Olop and Lev abbreviations are defined as in Fig. 2. Each inhibition rate was calculated unless otherwise noted by the equation Inhibition Rate (%) = $\{1 - (X - N)/(C - N)\} \times 100\%$, where X = mean value of the group treated by each drug, N = mean value of the Normal group, and C = mean value of the group treated by saline or the vehicle of bepotastine besilate.

^a Calculated by the equation; Inhibition Rate (%) = $\{1 - X/C\} \times 100\%$.

^b $P < 0.01$ vs. Vehicle (Shirley–Williams test following linear-regression analysis, 1-sided).

^c $P < 0.01$.

^d $P < 0.05$ vs. Saline (Dunnett's test, 1-sided).

^e $P < 0.01$.

^f $P < 0.05$ vs. Bepo 1.0% (Dunnett's test, 2-sided).

^g $P < 0.01$ vs. Bepo 1.0% (Student's *t*-test, 2-sided).

well as ketotifen 0.05%, olopatadine 0.1%, and levocabastine 0.025% significantly suppressed the extravasated dye in the conjunctiva ($P < 0.05$ for levocabastine 0.025%, $P < 0.01$ for the others). Notably, the effect of bepotastine besilate 1.0% was significantly superior to that of levocabastine 0.025% ($P < 0.05$).

3.2. Histamine-induced conjunctival vascular hyperpermeability

Both bepotastine besilate 1.0% and olopatadine 0.1%, applied to the eye 30 min before histamine challenge significantly inhibited histamine-induced vascular hyperpermeability in guinea pig conjunctiva ($P < 0.01$, respectively) (Fig. 3 and Table 1). Moreover, the inhibitory effect of bepotastine besilate 1.0% was significantly more potent than that of olopatadine 0.1% ($P < 0.01$).

3.3. PAF-induced eosinophil infiltration into guinea pig conjunctiva

The topical instillation of PAF caused a significant increase in extractable EPO activity ($P < 0.01$) as an index of eosinophil infiltration in guinea pig conjunctiva for the saline-treated control group (Fig. 4 and Table 1). This increase was significantly attenuated by pretreatment with all tested drugs including bepotastine besilate 1.0%, ketotifen 0.05% and olopatadine 0.1% ($P < 0.01$). The inhibitory effect of bepotastine besilate 1.0% was significantly different from that of ketotifen 0.05% ($P < 0.01$) and almost the same as that of olopatadine 0.1%.

3.4. LTB₄-induced chemotaxis in vitro of guinea pig peritoneal eosinophils

Bepotastine dose-dependently inhibited chemotaxis of cultured eosinophils induced by LTB₄ at 100 μ M (81.4% of control, $P < 0.05$) and 1000 μ M (30.7%, $P < 0.01$), respectively (Fig. 5). Ketotifen also inhibited chemotaxis at a concentration of 100 μ M (79.3%, $P < 0.05$) and 1000 μ M (1.8%, $P < 0.01$). Olopatadine inhibited chemotaxis only at 1000 μ M (68.5%, $P < 0.05$), a weak effect compared to that of bepotastine or ketotifen at 1000 μ M.

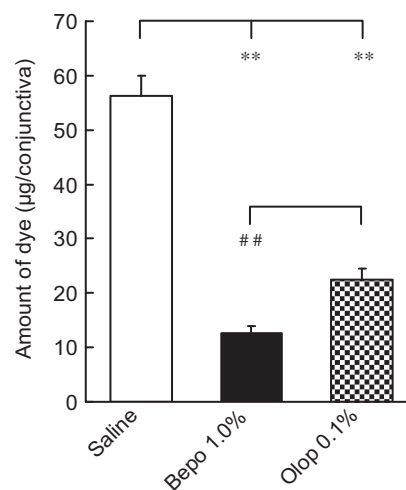


Fig. 3. Effect of topically applied bepotastine besilate ophthalmic solution 1.0% compared with olopatadine ophthalmic solution 0.1% on histamine-induced conjunctival hyperpermeability in guinea pigs. Bepotastine besilate ophthalmic solution (Bepo) 1.0%, olopatadine ophthalmic solution (Olop) 0.1% or saline were topically administered 30 min before subconjunctival injection of histamine in guinea pigs and intravenous administration of Evans blue dye, respectively. Data are represented as the mean $\pm$ S.E.M. of 8 or 10 animals. ** $P < 0.01$, compared with Saline (Dunnett's test, 1-sided), ## $P < 0.01$, compared with Bepo 1.0% (Student's *t*-test, 2-sided).

3.5. Histamine release from cultured RPMCs

The amount of released histamine induced by A23187 treatment of cultured RPMCs gradually decreased with exposure to bepotastine and reached a statistically significant reduced level at 1000 μ M (43.7% of control, $P < 0.01$) (Fig. 6). Ketotifen inhibited histamine release only at 100 μ M (40.9%, $P < 0.01$), but it did not exert any inhibitory effect at 1000 μ M. Olopatadine did not inhibit histamine release at any concentration tested in this in vitro model up to 1000 μ M.

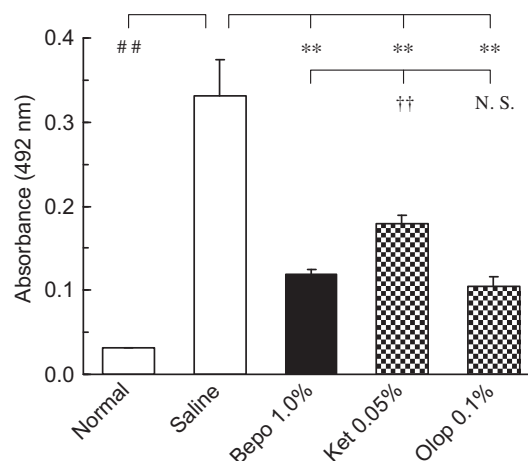


Fig. 4. Effect of topically applied bepotastine besilate ophthalmic solution (Bepo) 1.0% compared with ophthalmic formulations of H₁ receptor antagonists on platelet-activating factor (PAF)-induced eosinophil infiltration in conjunctiva of guinea pigs. Bepo 1.0%, ketotifen ophthalmic solution (Ket) 0.05% and olopatadine ophthalmic solution (Olop) 0.1% were topically applied at 20, 40 and 60 min before a topical PAF challenge. Eosinophil peroxidase activity, an index of eosinophil infiltration, was determined in isolated conjunctiva by spectrophotometry. Data are represented as the mean $\pm$ S.E.M. of 5 or 6 animals. ## $P < 0.01$, compared with Saline (Student's *t*-test, 1-sided), ** $P < 0.01$, compared with Saline (Dunnett's test, 1-sided), †† $P < 0.01$, compared with Bepo 1.0% (Dunnett's test, 2-sided), N.S., not significant.

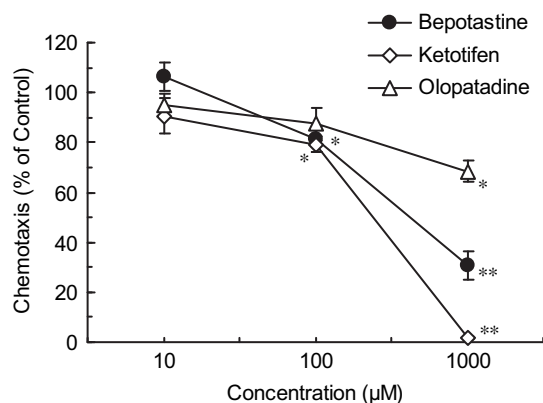


Fig. 5. Effects of bepotastine, ketotifen, and olopatadine on leukotriene B₄ (LTB₄)-induced chemotaxis of guinea pig peritoneal eosinophils. The results were normalized to the cell content values in the presence of LTB₄ in the lower part of Boyden's chamber representing 100% for negative (untreated) controls. The cell numbers of migrated eosinophils in the control group were 778.3 ± 41.4 cells/well ($n = 12$). Data are represented as the mean $\pm$ S.E.M. from 4 to 6 independent experiments. ** $P < 0.01$, * $P < 0.05$, compared with control group (Dunnett's test, 1-sided). Bepotastine, ketotifen, and olopatadine at a concentration of 1000 μ M are equivalent to 0.039%, 0.031%, and 0.034% in corresponding approved ophthalmic solutions, respectively, for these antihistamines.

4. Discussion

Allergic conjunctivitis is a typical IgE-mediated disease associated with ocular symptoms such as itching, lacrimation, hyperemia, and chemosis. Histamine H₁ receptor antagonists are effective for treating early phase symptoms, but often ineffective for severe and/or chronic states of these symptoms due to inflammation mediated by inflammatory cells such as eosinophils, neutrophils and Th2 cells. In this study, we investigated efficacy in in vivo ocular allergic models characterized by early phase conjunctival responses or late phase eosinophil infiltration to conjunctiva in order to estimate therapeutic potential of bepotastine besilate compared with other ophthalmic histamine H₁ receptor antagonists. We also examined in vitro activity of several ophthalmic preparations of antihistamines including bepotastine besilate for reducing properties of

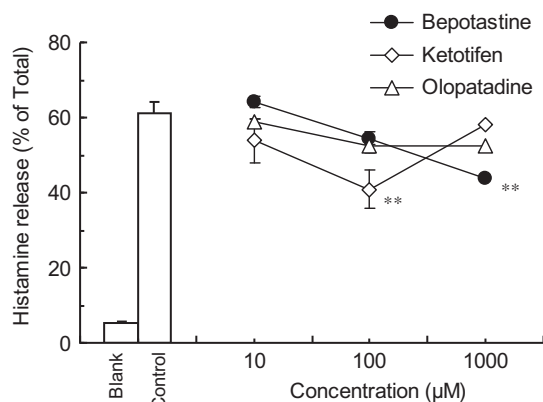


Fig. 6. Effects of bepotastine, ketotifen, and olopatadine on A23187-induced histamine release from rat peritoneal mast cells. The total histamine contents (Total) in mast cells were calculated as the sum of released histamine and remaining histamine amounts in mast cells for each data point. The results were normalized to the values in the total histamine contents (Total) representing 100%. Data are represented as the mean $\pm$ S.E. M. from 3 independent experiments. ** $P < 0.01$, compared with control group (Dunnett's test, 1-sided). Bepotastine, ketotifen, and olopatadine at a concentration of 1000 μ M are equivalent to 0.039%, 0.031%, and 0.034% in corresponding approved ophthalmic solutions, respectively, for these antihistamines.

inflammatory cells related to the pathophysiology of allergic inflammation.

Bepotastine besilate ophthalmic solution gradually increased its inhibitory effect up to a concentration of 1.5% in an OVA-based passive sensitization guinea pig model. In the OVA-induced model, although the effect of bepotastine besilate 1.0% was approximately the same as that of olopatadine 0.1%, the effect of bepotastine besilate 1.5% would likely have been better than that of olopatadine 0.1% because of observed in vivo dose-response of bepotastine besilate. In general, the effect of bepotastine besilate 1.5% was equal to that of ketotifen 0.05% and likely stronger than the effects of olopatadine 0.1% and levocabastine 0.025% based on results seen in the passive sensitization and histamine-induced guinea pig models. Abelson et al. (2009) have recently reported that both bepotastine besilate ophthalmic solutions 1.0% and 1.5% were associated with clinically and statistically significant reductions in ocular itching when instilled 15 min or 8 h prior to an ocular allergen challenge in the conjunctival allergen challenge (CAC) clinical model of allergic conjunctivitis. Furthermore, it was suggested by these authors that the 1.5% formulation may be slightly more effective than 1.0% formulation for the relief of ocular itching. Our data showing superior efficacy of the higher strength 1.5% formulation in an animal study (Fig. 1) support the findings of this phase III clinical trial.

Eosinophils are a major inflammatory cell type in the pathophysiology of ocular allergy to contribute to the severe and prolonged symptoms seen in SAC and PAC patients. In fact, infiltrated eosinophils are commonly identified in biopsy specimens of conjunctiva and tear fluid of allergy patients, especially those with more chronic SAC, PAC, or VKC. These eosinophils produce ocular surface impairment due to eosinophil-derived cytotoxic proteins such as eosinophil cationic protein, major basic protein, and EPO (Bielory, 2000; Bonini et al., 1997). In the present study, bepotastine besilate 1.0% demonstrated significant inhibition of PAF-induced conjunctival eosinophil infiltration and was more potent than ketotifen 0.05%. Furthermore, LTB₄-induced eosinophil chemotaxis in vitro was suppressed by treatment with bepotastine, ketotifen, and olopatadine. The mechanism(s) of action underlying the suppressive effect on eosinophil infiltration in vivo remain unclear. However, the impact of bepotastine on eosinophil activation and migration within the conjunctiva may at least partially be explained by the observations that bepotastine inhibited the production of interleukin-5, which is an important factor for the activation and the survival of eosinophil (Kaminuma et al., 1998), and bepotastine also suppressed the expression of CD54, intercellular adhesion molecule-1, and proinflammatory cytokines/chemokines in human epidermal keratinocyte (Kobayashi et al., 2009).

In an in vitro model of late stage allergic response, ketotifen was superior to bepotastine in reducing LTB₄-induced chemotaxis of cultured guinea pig peritoneal eosinophils, while bepotastine in turn was superior to olopatadine at the highest concentration tested (1000 μ M). It has been reported that ketotifen has cytotoxic properties at high concentrations, while no cytotoxicity was observed with high concentrations of olopatadine (Yanni et al., 1996; Lee et al., 2008). These prior findings raise the meaningful possibility that the apparent inhibitory effect of 1000 μ M ketotifen on in vitro chemotaxis of eosinophils may be ascribed to injured eosinophils that cannot perform normal cell functions.

Several histamine H₁ receptor antagonists such as bepotastine (Yato et al., 1997), ketotifen (Schoch, 2003), and olopatadine (Yanni et al., 1996) are known to possess membrane-stabilizing activity in mast cells. In experiments with RPMCs, the activity of bepotastine on mast cell release of histamine was directly compared with that of ketotifen and olopatadine. Bepotastine was found to be effective at a concentration of 1000 μ M, but not at 100 μ M, while ketotifen

was active at 100 μM , and olopatadine had no activity at any concentration tested. Although Yanni et al. (1996) reported that the IC_{50} value of olopatadine for histamine release was 559 μM in a different mast cell tissue culture system, the present investigation did not show any olopatadine effect. Any inconsistency in the outcomes of the two studies is likely attributable to the difference in mast cell types studied and the differences in experimental conditions, including the method of inducing histamine release. On the other hand, the bell-shaped response in which ketotifen exerted its activity only at 100 μM but not at higher concentration was observed in this study as was also reported for ketotifen by Yanni et al. (1996). As previously mentioned, a likely cause of this bell-shaped response for ketotifen was the induction of cellular damage by ketotifen at high concentration.

The present study confirmed that bepotastine besilate 1.0% and 1.5% are effective in *in vivo* allergic conjunctivitis models of allergic early phase and late phase reactions. Further, the *in vitro* studies showed that bepotastine possesses membrane-stabilizing activity in instances when it was not observed with ketotifen or olopatadine, including the observation that the inhibitory effect of bepotastine on eosinophil infiltration was stronger than that of olopatadine. Taken altogether, bepotastine was generally more consistently effective than olopatadine, ketotifen or levocabastine in the nonclinical models of vascular hyperpermeability and eosinophil migration reported here. The bepotastine nonclinical data also suggest bepotastine operates on multiple allergic effector pathways, with effectiveness in antagonizing histamine H_1 receptor interactions, stabilizing mast cell membranes, and suppressing eosinophil function.

Various types of inflammatory cells, cytokines and chemical mediators are produced by inflammatory cells and are involved spatially and temporally in the pathophysiology of allergic conjunctivitis (Abelson et al., 2003). Therefore, for the treatment of ocular allergic symptoms, drugs which affect multiple cellular and biochemical mechanisms including histamine H_1 receptor antagonism as well as other pathways leading to allergic responses are optimal. On the basis of the experiments described here, bepotastine is such an antihistamine and it can be anticipated that bepotastine besilate 1.0% or 1.5% may be effective in SAC and PAC patients suffering mainly from early phase symptoms such as chemosis and itching. Our nonclinical results demonstrating histamine H_1 receptor antagonism, mast cell membrane stabilization, and suppression of eosinophil function, in addition to the CAC clinical results (Abelson et al., 2009) strongly support that bepotastine besilate ophthalmic solutions 1.0% and 1.5% may successfully treat the signs and symptoms of allergic conjunctivitis.

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