Original Article

Nonclinical safety assessment for the sensitizing potential of K-115

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ABSTRACT — K-115, a strong and selective inhibitor of Rho-associated coiled coil-forming protein kinase (ROCK), was developed as a therapeutic drug for glaucoma. In long-term phase 3 clinical studies, blepharitis and allergic conjunctivitis were observed. Nonclinical studies were conducted to determine if these adverse effects were caused by the sensitizing potential of K-115. In mouse local lymph node assays (LLNA), sensitizing reactions were not observed at 8.17% w/v of K-115, but weak positive skin sensitivity with a 2.0% K-115 ophthalmic solution was observed. K-115 ophthalmic solution was classified as having slight sensitizing potential. No photosensitization potential was observed with UV-LLNA studies and skin photosensitization studies. In a guinea pig conjunctivitis model study, K-115 had no immediate allergic effect on conjunctivitis, and did not function as an antigen. Furthermore, in ocular toxicity studies in rabbits (26 weeks) and monkeys (52 weeks), no abnormalities were observed in ophthalmic and pathological examinations. Considering the results of nonclinical sensitization and ocular toxicity studies, the clinical adverse effects were possibly not caused by K-115 sensitization potential.

Key words: ROCK inhibitor, Sensitization, Nonclinical, Blepharitis, Conjunctivitis

INTRODUCTION

K-115 is a strong and selective inhibitor of Rho-associated coiled coil-forming protein kinase (ROCK). K-115 ophthalmic solution was developed as a therapeutic for glaucoma, to lower intraocular pressure (IOP) by promoting aqueous humor excretion through Schlemm's canal, via the trabecular meshwork (Isobe *et al.*, 2014; Honjo *et al.*, 2001; Nakajima *et al.*, 2005). In three long-term phase 3 clinical studies of K-115 ophthalmic solution (K-115-OP; 0.4%), ocular blepharitis and allergic conjunctivitis, and localized adverse effects related to allergy and inflammation, were observed, and were possibly related to K-115-OP treatment.

The blepharitis was thought to be a contact dermatitis classified into the following reaction types; irritant con-

tact dermatitis, allergic contact dermatitis (ACD), phototoxic and photoallergic contact dermatitis, and systemic contact dermatitis (Takayama *et al.*, 2009; Bourke *et al.*, 2009). The blepharitis observed in clinical studies could be caused by drug induced allergic and photoallergic reactions. Contact allergy is considered to be a form of delayed type (type 4) hypersensitivity. In common with other forms of allergy, ACD develops in two phases, which are defined operationally as induction and elicitation (Kimber *et al.*, 2002). The induction of sensitization and the elicitation of allergic contact reactions are dependent upon, and are orchestrated by, T-cell type lymphocytes. Chemical allergens are thought to be haptens and immunogenicity must be acquired by stable association with protein and the formation of hapten-protein conjugates. The hapten-protein conjugate is recognized

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and processed for subsequent presentation to the immune system. This is primarily the responsibility of epidermal Langerhans cells (LCs) and cutaneous dendritic cells (DCs) (Lappin *et al.*, 1996). Their functions are to recognize and internalize antigens encountered and to transport the antigen via afferent lymphatic vessels to regional lymph nodes. During migration from the skin, LCs are induced to differentiate from antigen processing cells to mature immunostimulatory DCs. DCs displaying the allergenic epitope activate responsive T lymphocytes. As a result, clonal expansion of allergen-reactive T cells including the chemical allergen, when encountered again, provide an accelerated and more aggressive secondary immune response. To confirm the allergic potential of K-115 in nonclinical studies localized guinea pig lymph node assays (LLNA) were used and provided an appropriate method to determine the mechanism of sensitization. Furthermore, the possibility of photoallergic contact dermatitis was examined using the UV-irradiated LLNA assay and the guinea pig photosensitizing model.

Allergic conjunctivitis, another adverse reaction, is a form of immediate type (type 1) hypersensitivity. In allergic conjunctivitis, antigen is processed by antigen presenting cells before being recognized by T lymphocytes. This process causes secretion of cytokines, controlling basophil, eosinophil, and mast cell activity involved in the inflammatory or allergic response, and also influences B cells to produce IgE. Antigens also crosslink with membrane bound IgE on sensitized mast cells in the conjunctival epithelium to cause cell activation and histamine (mediator) release. As a result, the released histamine bound to H1 receptors in the surrounding tissues causes itching sensations induced at nerve endings. Vascular permeability was increased in vascular endothelial cells, and hyperemia was induced by relaxation of vascular smooth muscle (McGill *et al.*, 1998). The increased vascular permeability induced by antigen may be similar to clinically observed adverse events and thus these permeability changes were determined quantitatively in the guinea pig model for evaluation of the potential of K-115 to induce immediate type hypersensitivity.

MATERIALS AND METHODS

Chemicals

K-115 was manufactured by Fuji Chemical Industry Co., Ltd. (Toyama, Japan) and K-115-OP solutions at 0.4, 1.0, and 2.0% were manufactured by Kowa Company, Ltd. (Aichi, Japan). K-115-OP placebo was manufactured by Kowa Company, Ltd. and by Nitto Medic Company, Ltd. (Tokyo, Japan). Hexyl cinnamic aldehyde (HCA) was purchased from Sigma-Aldrich Co. Ltd. (Gillingham, UK), tetra- chlorosalicylanilide (TCSA) was from Acros (Geel, Belgium), and 1-chloro-2,4-dinitrobenzene (DNCB), and 6-methylcoumarin (6-MC) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Freund's complete adjuvant (FCA) and sodium lauryl sulfate were from DIFCO Laboratories (Franklin Lakes, NJ, USA) and Wako Pure Chemical Industries, Ltd. ^{3}H-methyl thymidine was purchased from Amersham Scientific Co. (Buckinghamshire, UK). Patanol sterile ophthalmic solution (0.1%) was manufactured by Alcon Japan Ltd. (Tokyo, Japan), and chicken egg white albumin (OVA) was from Sigma-Aldrich Japan G.K. (Tokyo, Japan).

Animals and housing conditions

Female mice (CBA/Ca) were obtained from Harlan UK Ltd. (Bicester, UK). After quarantine and acclimatization, only healthy animals showing favorable body weight gain and good clinical signs were used for the studies. Mice were housed individually in polycarbonate cages, with wood flake bedding, under controlled conditions of temperature (19-23°C), humidity (40-70%), and ventilation (about 19 times/hr) with 11 hr illumination. Mouse studies were performed under the authority of the UK Home Office project license.

Male guinea pigs (Slc: Hartley) were obtained from Japan SLC, Inc. (Shizuoka, Japan). After quarantine and acclimatization, only healthy animals, showing favorable body weight gain and good clinical signs, were used for the studies. Animals were housed individually in stainless steel wire mesh cages, in animal rooms, under controlled conditions of temperature (20-26°C), humidity (40-70%), and ventilation (14-19 times/hr), with 12 hr illumination.

Male and female rabbits (Kbl: Dutch) were obtained from Kitayama Labs Co., Ltd. (Nagano, Japan), and weighed 1.3-1.8 kg at initiation of dosing. After quarantine and acclimatization, only healthy animals showing favorable body weight gain and good clinical signs, were used for the studies. The animals were housed, individually in aluminum cages, in an animal room under controlled conditions of temperature (19-25°C), humidity (40-70%), and ventilation (12 times/hr), and with 12 hr illumination. Feed and sterilized tap water was available *ad libitum*.

Male and female cynomolgus monkeys were obtained from Japan Laboratory Animals, Inc. (Tokyo, Japan), and weighed 2.3-4.2 kg at initiation of dosing. After quarantine and acclimatization, only healthy animals, showing favorable body weight gain and good clinical signs, were used for the studies. The animals were housed individually, in stainless steel cages, in an animal room under controlled conditions of temperature (22-28°C), humidity

(40-80%), and ventilation (15-17 times/hr, with 12 hr illumination. The feed was given at 100 g/animal /day, and sterilized tap water was available *ad libitum*.

The animal studies, using guinea pigs, rabbits and monkeys, were conducted in compliance with the "Partial Amendments to the Law for the Humane Treatment and Management of Animals (Law No. 68, Jun. 22, 2005, Japan)."

Skin sensitization study using the local lymph node assays in the mouse (LLNA study)

The study was conducted in accordance with OECD guideline No.429, Skin Sensitization: Local Lymph Node Assay (Kimber *et al.*, 1994; Basketter *et al.*, 1999). Groups of five mice were treated with one of three concentrations [0.82, 4.08, and 8.17% w/v of K-115, in dimethyl formamide (DMF)], groups of five mice were treated with HCA (25% v/v) as positive controls and another five mice were treated with DMF as controls. Five days following the first application, all mice were injected via the tail vein with 250 µL of phosphate-buffered saline containing ^{3}H-methyl thymidine (^{3}HTdR: 80 µCi/mL). Five hours following the administration, all mice were humanely killed and the draining auricular lymph nodes excised. One mL of phosphate-buffered saline was added to the lymph nodes for each animal. A single-cell suspension of lymph node cells (LNC) was prepared by gentle mechanical disaggregation. Then LNC were washed twice with phosphate-buffered saline and resuspended in 5% trichloroacetic acid (TCA). After overnight incubation with TCA at 4°C, the precipitate was recovered by centrifugation and resuspended in 5% TCA and transferred to Ultima gold scintillation fluid. ^{3}HTdR incorporation was measured by β-scintillation counting. The proliferative response of LNC was expressed as radioactive disintegrations per minute per lymph node (dpm/node) and as the ratio of ^{3}HTdR incorporation into LNC of test nodes relative to that recorded for control nodes (test/control ratio).

Skin sensitization study of K-115-OP in guinea pigs (adjuvant and patch study)

The study was conducted in accordance with established and previously reported guidelines (Sato *et al.*, 1981). K-115-OP (2.0%) was used as a test concentration, K-115-OP placebo was used as a control and 1-chloro-2, 4-dinitrobenzene (DNCB) was used as a positive control. The skin was observed at 24 and 48 hr after challenge according to the Draize scale for scoring (Draize, 1959).

Skin photosensitization study using a modified local lymph node assay in mice (UV-LLNA study)

Following UV irradiation was conducted in addition to the LLNA study. At approximately 30 min after dosing, the animals in the UV irradiation groups were exposed to a minimum of 10 J/cm^2 UVa light (5 mW/cm^2 × 40 min), followed by a separate exposure to 0.1 J/cm^2 UVb light (2 mW/cm^2 × 1 min). The light source was a UV 400W flood lamp mounted above the animals. Subsequent treatments were the same as in the LLNA studies.

Skin photosensitization study of K-115-OP in guinea pigs (adjuvant and strip study)

The study was conducted in accordance with previously reported guidelines (Ichikawa, 1984; Sato *et al.*, 1980). K-115-OP (2.0%) was used as the test compound, K-115-OP placebo was used as the control and 6-methylcoumarin (6-MC) provided the positive control. The irradiated (10-11 J/cm^2 UVa) and non-irradiated skin was observed according to the Draize scale for scoring, at 24 and 48 hr after termination of the challenge (after termination of UVa irradiation).

Effects of K-115-OP on increased vascular permeability induced by antigen in the guinea pig model (conjunctivitis model)

The study was conducted with reference to previously reported method (Hashimoto *et al.*, 2003). In the OVA sensitized group, 50 µL of aluminum hydroxide gel, containing 2 µg of OVA, was injected as the sensitizer into conjunctiva of guinea pigs under anesthesia. Two weeks after sensitization, K-115-OP at 0.4 and 2.0%, and Patanol sterile ophthalmic solution at 0.1%, were topically instilled into both eyes, and the K-115-OP placebo was instilled into the control animals (n = 8/group). One hour after instillation, evans blue solution was intravenously administered (50 mg/mL/kg) then 10 µL of 2.5% OVA solution was instilled as the initiator to increase vascular permeability. Thirty min after OVA instillation, the conjunctiva was extracted and dissolved by 1 hr incubation in 1 mL of 1 M KOH solution. Then 1.5 mL of 0.6 M H_3PO_4 solution and 4.5 mL of acetone was added and mixed completely. After centrifugation (1800 × *g*, 20°C, 15 min), the supernatant was collected and measured by spectrophotometry at 620 nm to calculate the leakage of evans blue in the conjunctiva of each eye. Averages of the right and left eye values were used as individual leakage measurements.

In the K-115-OP sensitized group (n = 8), 50 µL of aluminum hydroxide gel containing 10 µg of K-115 was injected into the conjunctiva as a sensitizer, and 10 µL of K-115-OP (2.0%) was instilled as the initiator.

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A 26-week ocular toxicity study of K-115-OP in rabbits

K-115-OP at 1.0 and 2.0% were topically instilled four times per day (at 2-3 hr intervals) for 26 weeks (n = 10) and K-115-OP placebo was instilled, using the same protocol as in the control group. The ophthalmic solutions were instilled into the left eye in a volume of 50 μL/unit time using micropipettes. The right eye of each animal was left untreated. Ophthalmological examinations were conducted at predose and at weeks 6, 15, and 26 for each animal to determine irritations in the anterior parts of the eyes (cornea, conjunctivae, and iris) by slit lamp microscopy (cornea, conjunctiva, anterior chamber, and iris) and by fluorescein staining to assess damage to the corneal epithelium. Histopathologic examinations were conducted of optic nerves, lacrimal glands, and harderian glands. The removed eyes were promptly immersed in ice-chilled 0.1 mol/L phosphate-buffered 2.5% glutaraldehyde fixative for 1 hr. Approximately 0.2-0.3 mL of the same fixative was injected to replace the anterior chamber humor. Then, the eyes were reimmersed into ice-chilled 0.1 mol/L phosphate-buffered 2.5% glutaraldehyde fixative for 1 hr. The other tissues were fixed in 10% neutral buffered formalin. All the above tissues were routinely processed and stained with hematoxylin and eosin (HE). Specimens were examined by light microscopy.

A 52-week ocular toxicity study of K-115-OP in cynomolgus monkeys

K-115-OP at 1.0 and 2.0% were topically instilled in the twice daily dose groups at 7-8 hr intervals. K-115-OP (2.0%) and placebo were instilled in the four times daily groups at 2-3 hr intervals for 52 weeks (n = 5). The ophthalmic solutions were instilled into the left eye in a volume of 20 μL/unit time using micropipettes. The right eye of each animal was left untreated. Ophthalmological examinations were conducted at predose, and at weeks 13, 25, and 52, on each animal, similar to what was described for the rabbit study. Histopathologic examinations were conducted on optic nerves, eyelids, and lacrimal glands and methods of fixation and staining were the same as described for the rabbit study. Specimens were examined by light microscopy.

Justification for selection of the concentrations, frequency, and dose volume

In a preliminary study of LLNA and UV-LLNA dosage, two female mice were treated with either 4.08 or 8.17% w/v of the test substance in DMF. The mice were treated on the dorsal surface of each ear for 3 consecutive days. As a result, wet fur around the cranial region,

white dose residue on the ears and partially closed eyelids were all noted post dose from day 1. Therefore, the 8.17% w/v dose was selected as a high dose. Frequency and dose volumes complied with these guidelines.

In skin sensitization and skin photosensitization studies, K-115-OP at 2.0% was selected because this dose was the maximum concentration available as an ophthalmic solution. Frequency and dose volume complied with the guidelines.

Likewise, for the study of effects of the compounds on increased vascular permeability, K-115-OP at 2.0% was selected because it was the maximal concentration available for use as an ophthalmic solution.

Finally, in rabbit and monkey ocular toxicity studies, performed to test severe conditions, K-115-OP at 2.0% was the maximum concentration available as an ophthalmic solution and therefore was selected. Instilled volumes were the same for rabbit and monkey studies, and the frequency was set as used clinically (four times per day).

Statistical analysis

The data from the LLNA and UV-LLNA studies were analyzed using SAS 8.2 software (SAS Institute, Cary, NC, USA). Analysis of variance was used for treatment groups as the factor of interest. If Bartlett's test for homogeneity of variance was significant at the 1% level, the data were logarithmically transformed prior to analysis to stabilize the variances (Bartlett, 1937). Comparisons were made between the control and K-115 treated groups using Dunnett's test (Dunnett, 1955, 1964). To investigate the nature of the dose-response curve, the linear contrast was isolated. Analysis of variance was also used to compare the positive control group with the vehicle control group.

In the study of effects on increased vascular permeability, the data were analyzed using Stat Preclinical 1.1 (Takumi Information Technology Inc., Tokyo, Japan), and SAS 9.1 software (SAS Institute). Analysis of variance was used to analyze the data. If Bartlett's test for homogeneity of variance was significant at the 5% level, comparisons were made between the control and treated groups using Steel's test (Steel, 1959; Kobayashi *et al.*, 2000).

RESULTS

LLNA study

Group dpm/node and test/control ratios in LLNA studies are shown in Table 1. The test/control ratios obtained for 0.82, 4.08 and 8.17% w/v K-115 were 1.7, 2.2 and 1.8 respectively. As a test/control ratio of 3 or more was not recorded for any of the concentrations tested, K-115 was considered not to have the potential to cause skin sensiti-

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Table 1. Group dpm/node and test/control ratios in LLNA study

Concentration	dpm/node	Stimulation index	Result + = positive - = negative
Vehicle (DMF)	113.78	n/a	n/a
K-115 0.82% w/v	195.98	1.7	-
K-115 4.08% w/v	249.88	2.2	-
K-115 8.17% w/v	202.23	1.8	-
HCA 25% v/v	2550.98	22.4**	+

n/a = not applicable; (n = 5); dpm = disintegrations per minute
DMF = dimethyl formamide (vehicle control)
HCA = hexyl cinnamic aldehyde (positive control)
** $P > 0.001$

Table 2. Skin sensitization study of K-115-OP in guinea pigs (adjuvant and patch test)

Group	Substance for induction	Substance for challenge	Number of animals	Time after the end of challenge (hr)	Mean of score	Positive ratio[a]
Non-sensitization	-	Base [b]	5	24	0.0	-
				48	0.0	-
		K-115-OP -2.0%	5	24	0.0	-
				48	0.0	-
K-115-OP 2.0%	K-115-OP 2.0%	Base [b]	10	24	0.0	0/10
				48	0.0	0/10
		K-115-OP- 2.0%	10	24	0.0	0/10
				48	0.1	1/10
Positive control	1% DNCB	Acetone	5	24	0.0	-
				48	0.0	-
		0.1% DNCB	5	24	5.2	5/5
				48	6.0	5/5

[a] Number of animals with positive / Number of animals tested; hr = hours
[b] K-115-OP placebo
DNCB: 1-chloro-2,4-dinitrobenzene (positive control)

zation. The stimulation index for the positive control substance HCA was 22.4, which demonstrated the reliability and sensitivity of this assay to detect skin sensitization potential.

Skin sensitization study of K-115-OP in guinea pigs (adjuvant and patch study)

Skin reactions to K-115-OP, in skin sensitization studies in guinea pigs, are shown in Table 2. In the test drug group no skin reactions were observed at any application site of K-115-OP or in any of the 10 animals with buffer only at 24 hr after the challenge. Very slight erythema (score 1) was observed at the application site of K-115-OP in one animal at 48 hr after the challenge (mean group score 0.1, positive ratio 1/10). No skin reactions were

observed at any application site from the buffer. In the non-sensitization group, no skin reactions were observed at any application site of K-115-OP or from the buffer in any animal. In the positive control group, erythema and edema were observed at the application sites of DNCB in all five animals at 24 and 48 hr after the challenge. At the application sites of acetone, no skin reactions were observed in any animal at either observation time point.

UV-LLNA study

Group dpm/node and test/control ratios in the UV-LLNA study are shown in Table 3. In this assay, the stimulation indexes obtained for 0.82, 4.08 and 8.17% w/v K-115 with UV light were 1.1, 1.2 and 1.4 respectively with no statistically significant differences from control or

the group receiving 8.17% w/v K-115 without UV light. This indicated that K-115 did not induce skin photosensitization. The study included two positive control groups receiving 1% w/v TCSA with and without UV light. The group receiving UV light had a higher mean dpm count compared with the non-UV group, and was statistically significant. Furthermore, the UV exposed group also had a higher stimulation index.

Skin photosensitization study of K-115-OP in guinea pigs (adjuvant and strip study)

The data from reactions in skin photosensitization studies of K-115-OP in guinea pigs are shown in Table 4. In the test and non-sensitization groups, no skin reactions were observed in any UV-irradiated or non-irradiated area of any animal at 24 or 48 hr after termination of challenge

with the K-115 ocular formulation. In the positive control group, well defined erythema and very slight or some slight edema were observed in all five animals in the UV-irradiated areas at 24 and 48 hr. The mean group scores were 3.6 and 4.0 at 24 and 48 hr after termination of the challenge, respectively. The positive ratios were all 5/5. No skin reactions were observed in any UV-non-irradiated areas.

Effect of K-115-OP on increased vascular permeability induced by antigen in guinea pigs

Leakage data for evans blue in the conjunctiva after sensitization by OVA, are shown in Fig. 1. The inhibition percentage of leakage for K-115-OP at 0.4 and 2.0% and Patanol sterile ophthalmic solution (0.1%) versus the control group were 18.1, -30.3 and 54.9% respec-

Table 3. Group dpm/node and test/control ratios in the UV-LLNA study

Treatment	Without exposure to UV light		With exposure to UV light	
	dpm/node	Stimulation index	dpm/node	Stimulation index
Vehicle (DMF)	133.73	n/a	234.63	n/a
K-115 0.82%w/v	-	-	246.88	1.1
K-115 4.08%w/v	-	-	276.03	1.2
K-115 8.17%w/v	279.98	2.1	323.13	1.4
TCSA 1.0% w/v	5859.48	43.8	13962.43*#	59.5

n/a, Not applicable; (n = 5)
DMF: Dimethyl formamide (vehicle control)
TCSA: Tetrachloro salicylanilide (positive control)
* Statistically significant ($P = 0.001$) compared with the group receiving DMF and UV light
# Statistically significant ($P = 0.021$) compared with the group receiving 1% w/v TCSA without UV light

Table 4. Skin photosensitization study of K-115-OP in guinea pigs (adjuvant and strip method)

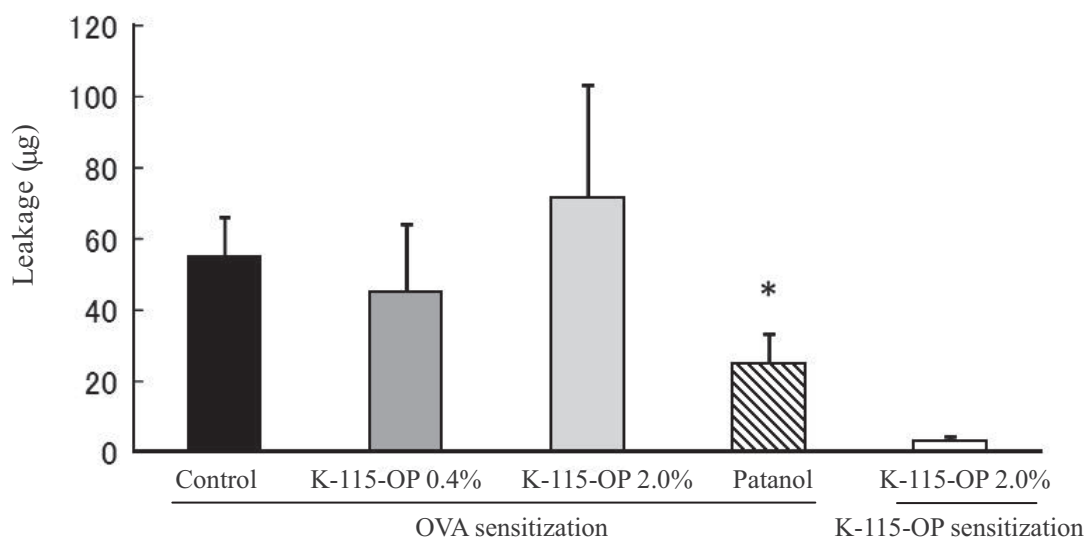| Group	Substance for induction	Substance for challenge	Number of animals	Time after the end of challenge (hr)	UVA+		UVA-
					Mean of score	Positive ratio[a]	Mean of score
Non-sensitization	-	Base [b]	5	24	0.0	-	0.0
				48	0.0	-	0.0
		K-115-OP 2.0%	5	24	0.0	-	0.0
				48	0.0	-	0.0
K-115-OP 2.0%	K-115-OP 2.0%	Base [b]	9	24	0.0	0/9	0.0
				48	0.0	0/9	0.0
		K-115-OP 2.0%	9	24	0.0	0/9	0.0
				48	0.0	0/9	0.0
Positive control	5% 6-MC	Acetone	5	24	0.0	-	0.0
				48	0.0	-	0.0
		1% 6-MC	5	24	3.6	5/5	0.0
				48	4.0	5/5	0.0

a) Number of animals with positive/number of animals tested
b) K-115-OP placebo
6-MC: 6-methylcoumarin in acetone (positive control)
hr - hours

Fig. 1. Leakage amount of evans blue into conjunctiva of guinea pigs sensitized by OVA (Control, K-115-OP 0.4, -2.0% and Patanol). The leakage of Patanol showed significant decreases versus the control group. The K-115-OP 0.4 and 2.0% groups did not show significant changes. In the K-115-OP sensitized group, increase leakage was not observed. (mean ± S.E., n = 6-8) *$P < 0.05$, Steel's test

tively. Though the leakage with Patanol sterile ophthalmic solution (0.1% group) showed significant decreases versus the control group, leakage of K-115-OP (0.4 and 2.0% groups) did not show significant changes. Leakage data after sensitization with K-115-OP are shown in Fig. 1 but increases of leakage were not observed.

A 26-week ocular toxicity study of K-115 in rabbits

In ophthalmological examinations, no abnormalities were observed in any treated animal for irritations in the anterior part of the eyes by slit lamp tests and by observations for damage in the corneal epithelium. In pathological examinations, no abnormalities were observed in any treated animal eyes including the optic nerves, lacrimal glands and harderian glands.

A 52-week ocular toxicity study of K-115 in cynomolgus monkeys

In ophthalmological examinations, redness of the bulbar or palpebral conjunctiva (score 1, vessels definitely injected above normal) of the treated eyes were observed in three males and one female in the 2.0% K-115 (four times daily) group during week 25 or 51 (mean total score, 0.4 or 1.2 respectively) in observations to determine irritations in the anterior parts of the eyes. No irritations in the anterior part of the eyes, as assessed by slit lamp tests and observations for damage in the corneal epithelium, were observed. In pathological examinations, no abnormalities were found in any treated animal eyes including the optic nerves, eyelids, and lacrimal glands.

DISCUSSION

K-115 is a strong and selective inhibitor of ROCK, and its ophthalmic solution has been developed as a therapeutic to lower IOP during glaucoma by promotion of aqueous humor excretion through Schlemm's canal via the trabecular meshwork. In long-term phase 3 clinical studies, blepharitis, and allergic conjunctivitis, adverse and localized effects related to allergy and inflammation, were observed and possibly were related to K-115 ophthalmic solution treatment.

In mice LLNA studies, sensitizing reactions were not observed at concentrations of 8.17% w/v of K-115, the maximum concentration examined. However in skin sensitization studies (adjuvant and patch study) of K-115-OP, one of ten animals showed weak positive reactions (very slight erythema) in a 2% K-115-OP treated group. Using a currently accepted strength of sensitizing potential protocol for chemical substances (Kimber *et al.*, 2003), K-115-OP was classified as slight to less sensitizing. It is common to classify chemical substances as positive sensitizers that show more than 30% positive reac-

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tion in skin sensitization studies compared with adjuvant effects in guinea pigs (Ministry of Economy, Trade and Industry, Japan, 2010; OECD test guideline No.406). On this basis, K-115 was found to have slight or less sensitizing potential. No photosensitization potential was shown from UV-LLNA studies and skin photosensitization studies. The slight erythema seen in 1 of 10 animals was possibly due to weak irritant contact dermatitis.

Data from the conjunctivitis model study showed statistically significant inhibition of vascular permeability at a concentration of 0.1% olopatadine ophthalmic solution. However, K-115 did not show any significant effects when used at 2.0% ophthalmic solution. The immunogenicity of K-115 was examined by injection of K-115-OP with aluminum hydroxide gel into conjunctiva and by instillation of K-115-OP at 2.0% and it did not increase vascular permeability in either case. From these findings, it was concluded that K-115 had no effect on conjunctivitis through an immediate type allergic reaction. Furthermore, allergic airway inflammation in a murine model was shown to cause an immediate type (type one) allergy and ROCK 1 was found mainly in the lungs (Nakagawa *et al.*, 1996), whereas, Y-27632 inactivated eosinophils, and caused hyperreactivity (Zhu *et al.*, 2011; Henry *et al.*, 2005). These results suggested that K-115 did not function as an antigen of type 1 allergies and had no influence on increased vascular permeability.

In the rabbits given 2.0% K-115-OP four times daily for 26 weeks, hyperemia, conjunctival and eye lid margin swelling were not observed in ophthalmic exams. In pathological examinations, infiltrations of mast cells or basophils in conjunctiva and lymphocytes in palpebra were not observed. In monkey studies, 2.0% K-115-OP given four times daily, caused slight hyperemia of the conjunctiva, but no other abnormality was observed. Likewise, no pathological abnormalities were observed. Though hyperemia of conjunctiva could be due to the pharmacological action of ROCK inhibitors known to produce vasodilation by relaxation of vascular smooth muscles (Shimokawa and Takeshita, 2005), no findings related to inflammation were observed in monkeys in our study.

In summary, our nonclinical sensitization studies and ocular toxicity studies demonstrated that any clinical adverse effects of K-115 were not due to its sensitization potential.

ACKNOWLEDGMENTS

Conflict of interest---- The authors declare that there is no conflict of interest.

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