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The *In Vitro* and *In Vivo* Ocular Pharmacology of Olopatadine (AL-4943A), an Effective Anti-Allergic/Antihistaminic Agent

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ABSTRACT

Olopatadine (AL-4943A; KW-4679) [(z)-11-[3-(dimethylamino)propylidene]-6,11-dihydrodibenz[b,e]oxepine-2 acetic acid hydrochloride] is an anti-allergic agent which inhibits mast cell mediator release and possesses histamine H_1 receptor antagonist activity. Studies were conducted to characterize the *in vitro* and *in vivo* pharmacological profile of this drug relevant to its topical ocular use. AL-4943A inhibits histamine release in a concentration-dependent fashion (IC_{50} = 559 μM) from human conjunctival mast cell preparations *in vitro*. Histamine release was not stimulated by AL-4943A at concentrations as high as 10 mM. In contrast, ketotifen stimulated histamine release at concentrations slightly higher than effective inhibitory concentrations. AL-4943A did not display any *in vitro* cyclooxygenase or 5-lipoxygenase inhibition. Topical ocular application of AL-4943A effectively inhibits antigen- and histamine-stimulated conjunctivitis in guinea pigs. Passive anaphylaxis in guinea pig conjunctiva was attenuated by AL-4943A applied 30 min prior to intravenous or topical ocular antigen challenge (ED_{50} values 0.0067% and 0.0170%, w/v, respectively). Antihistaminic activity *in vivo* was demonstrated using a model of histamine-induced vascular permeability in guinea pig conjunctiva. AL-4943A applied topically from 5 min to 24 hrs prior to histamine challenge effectively and concentration-dependently inhibited the vascular permeability response, indicating the compound has an acceptable onset and a long duration of action. Drug concentrations 5-fold greater than those effective against histamine-stimulated conjunctival responses failed to inhibit vascular permeability responses induced with either serotonin or Platelet-Activating-Factor. These data indicate that the anti-histaminic effect observed with AL-4943A is specific. These anti-allergic/antihistaminic activities of AL-4943A observed in preclinical model systems have been confirmed in clinical trials in allergic patients.

INTRODUCTION

Olopatadine (AL-4943A; KW-4679) [(z)-11-[3-(dimethylamino) propylidene]-6,11-dihydrodibenz-[b,e]oxepine-2 acetic acid hydrochloride] is an anti-allergic/antihistaminic agent, synthesized

allergic rhinitis and chronic urticaria. AL-4943A's anti-allergic efficacy following systemic administration has been demonstrated in rat and guinea pig models of immediate hypersensitivity (1,2,3). Inhibition of passive anaphylaxis persisted for 9 hrs following oral administration of AL-4943A (3) indicating a prolonged duration of action. *In vitro* receptor binding studies have demonstrated the affinity of AL-4943A for the H_1-histamine receptor (1,4,5). Additionally, these studies have shown selectivity of AL-4943A for the H_1 receptor and demonstrated a lack of significant interaction with alpha adrenergic, muscarinic, dopaminergic and numerous other receptors (5).

Because of the reported long duration of anti-allergic activity, coupled with selective anti-histaminic activity, studies were undertaken to evaluate the utility of this compound for use in allergic conjunctivitis. AL-4943A's effects on human conjunctival mast cell mediator release, on cyclooxygenase and 5-lipoxygenase enzymes, and in models of allergen- and histamine-stimulated conjunctivitis following topical ocular administration were characterized. The results of those investigations are reported below.

METHODS AND MATERIALS

Inhibition of Histamine Release from RBL Rat Basophils

RBL-2H3 is a rat basophilic leukemia cell line which secretes both histamine and serotonin upon degranulation. RBLs were passively sensitized overnight with IgE specific for dinitrophenol (DNP) tagged to bovine serum albumin according to Benacerraf and Levine (6). Cells were washed with PIPES buffer, and incubated for 15 min at 37°C with AL-4943A prior to challenge with antigen (DNP, 10 ng/ml). Supernatants were analyzed for histamine content using a commercially available radioimmunoassay (RIA) system (AMAC, Inc., Westbrook, ME).

Histamine Release from Human Conjunctival Mast Cells

Human conjunctival tissue was obtained from organ/tissue donors. Upper and lower palpebral conjunctivas were aseptically collected within 8 hrs of death (average time 4.5 hrs) and transported in Dexsol® corneal preservation medium (Chiron Ophthalmics, Irvine, CA). Conjunctival tissue was weighed and placed into a glass 20 ml screw-cap bottle containing culture medium (RPMI 1640) supplemented with heat inactivated fetal bovine serum (20%), L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 mg/ml), amphotericin B (2.5 mg/ml), and HEPES (10 mM). Tissues and medium were transferred to sterile petri dishes for overnight equilibration at 37°C prior to enzymatic digestion.

Modification of a previously reported method for obtaining monodispersed cell suspensions containing mast cells (7) was employed using human conjunctival tissue. Briefly, tissues were transferred to Tyrode's buffer (in mM: 137 NaCl, 2.7 KCl, 0.35 NaH_2PO_4, 1.8 $CaCl_2$, 0.98 $MgCl_2$, 11.9 $NaHCO_3$, 5.5 glucose) containing 0.1% gelatin (TGCM) for enzymatic treatment. Tissues were incubated with 200 U each of collagenase (Type IV) and hyaluronidase (Type I-S) per gram of tissue for 30 min at 37°C. Following enzyme digestion, tissues were washed with an equal volume of TGCM over Nitex® filter cloth (Tetko, Briarcliff Manor, NY). Two digestions, completed as described above, were followed by additional digestion steps using 2000 U each of collagenase and hyaluronidase per gram of tissue for 30 min at 37°C.

The filtrate obtained from each digestion was centrifuged (825 g, 7 min), and pelleted cells were resuspended in calcium/magnesium free Tyrode's buffer (TG). Pooled cells from all digestions were centrifuged (825 g, 30 min) over a 1.058 g/L Percoll® cushion. Mast cell enriched cell pellets were resuspended and washed in TG buffer. Viability and number of mast cells were determined by vital dye (trypan blue) exclusion and toluidine blue O staining of the harvested cell suspensions. Cell

treated with test compound 15 min prior to challenge with anti-human IgE (10 μg/ml). The final volume per reaction tube was 1.0 ml. The release reaction was terminated by the addition of ice-cold TGCM and centrifugation (500 g, 7 min). Supernatants were collected and stored at -20°C until histamine analysis (described above).

Inhibition of Cyclooxygenase *In vitro*

Inhibition of cyclooxygenase activity from sheep vesicular glands by AL-4943A was assayed as follows: Lipid-depleted sheep vesicular gland microsomal powder (10 mg) was homogenized in ice-cold 50 mM phosphate buffer (pH 7.4) supplemented with 1% Tween-20, 2 μM hematin, and 5 mM diethyldithiocarbamic acid. The solubilized microsomal preparation (10 μg) was added to the incubation chamber containing 3.0 ml 50 mM phosphate buffer (pH 7.4), 0.5 mM phenol, and 0.5 μM hematin and pre-equilibrated to 30°C. AL-4943A, dissolved in dimethyl sulfoxide (DMSO), was added to the incubation chamber. The mixture was stirred for 2 min before initiating the reaction by the addition of 30 μl of a freshly prepared aqueous solution of 10 mM ammonium arachidonate. Indomethacin, also in DMSO (≤ 30 μl), served as reference compound. Cyclooxygenase activity was determined polarographically by monitoring the rate of oxygen consumption, due to the conversion of arachidonic acid to prostaglandin H_2 (8).

Inhibition of 5-HETE and LTB_4 Formation *In vitro*

The potency of AL-4943A to suppress 5-hydroxy eicosatetraenoic acid (5-HETE) and leukotriene B_4 (LTB_4) formation was investigated in calcium ionophore (A_{23187})-stimulated neutrophils isolated from rabbit peripheral blood. Neutrophils from peripheral blood were isolated by standard procedures. Briefly, heparinized/calcium chelated blood was obtained from five New Zealand Albino rabbits by heart puncture. Red cells were removed at 4°C by dextran sedimentation (9). White cells, contained in the supernatant fraction, were sedimented by centrifugation and contaminating red cells removed by hypotonic lysis. The white cell pellet obtained following hypotonic red cell lysis and centrifugation was resuspended in Dulbecco's phosphate buffered saline (Ca^{2+}/Mg^{2+}-free). The cell suspension was layered onto a 60% Histopaque-1083/40% Histopaque-1119 cushion. The neutrophil pellet at the bottom of the tube following centrifugation was washed and resuspended in 1/25 the original blood volume. Aliquots of the cell suspension were pretreated for 5 min at 37°C with either carrier (DMSO) or test article dissolved in DMSO. Immediately thereafter, $CaCl_2$ was added to the cell suspension and cells stimulated by the addition of 5 μl of a mixture containing [1-^{14}C]-arachidonic acid and calcium ionophore (A_{23187}) in DMSO. The final concentrations of $CaCl_2$, [1-^{14}C]-arachidonic acid and calcium ionophore were 5.0 mM, 52 μM and 5.0 μM, respectively. After 3 min of incubation at 37°C, reactions were terminated by the addition of 2 volumes of acetone. Extraction and reversed phase (C_{18}-5 μ) HPLC analysis of [1-^{14}C]-labelled arachidonic acid metabolites were conducted as previously described by Graff and Anderson (10).

Passive Anaphylaxis in Conjunctiva

Guinea pigs or rats (5-8/group) were passively sensitized with anti-ovalbumin serum injected subconjunctivally in one eye. Twenty-four (24) hrs after passive sensitization, ovalbumin (OA) was administered either intravenously (i.v.) or topically onto the eye.

The anti-allergic effect of AL-4943A following i.v. antigen administration was determined as follows: Thirty (30) min prior to i.v. antigen challenge, the animals received 20 μl of AL-4943A or saline applied topically to the eye. The animals were then challenged i.v. via the marginal ear vein or lateral tail vein with 1.0 ml of an OA:Evans Blue solution (100 μg:1 mg; guinea pigs; 1 mg:2.5

For assessment of the allergic response following topical ocular antigen challenge, 20 µl of ovalbumin (1.0%, w/v) was administered to the sensitized eye 5 min after topical ocular application of AL-4943A or saline (20 µl). During dose response studies, the order of compound administration was randomized. Thirty (30) min later, the reaction was quantitated, using the following scoring scheme (maximum score per animal = 10):

Congestion (refers to palpebral and bulbar conjunctiva)
- 0- Normal
- 1 - Pink conjunctiva
- 2 - Red conjunctiva
- 3 - Dark red conjunctiva; petechiae present

Swelling
- 0- None
- 1 - Any swelling on lower lid only
- 2 - Swelling upper and lower lid, lids partially closed
- 3 - Lids everted, very swollen, lids at least half closed
- 4- Swelling of both lids and side of face

Discharge
- 0- None
- 1 - Glazed, glassy appearance
- 2 - Moist lids and surrounding hair
- 3 - Moist lids and surrounding hair, thicker mucous-like

Histamine-induced Vascular Permeability in Guinea Pig Conjunctiva

Male Dunkin Hartley Viral Antibody Free outbred guinea pigs (Charles River Labs, Portage, MI), 250-350 grams, (6/group) were injected i.v. via the marginal ear vein with 1.0 ml of Evans Blue dye (1.0 mg/ml). Forty-five (45) min post dye injection, 20 µl of test compound or saline vehicle was applied topically onto one eye of each experimental animal. Thirty min following topical drug application, the guinea pigs were anesthetized and challenged subconjunctivally with histamine (300 ng/10 µl). Responses were quantitated as previously described (11).

All modifications of the pretreatment interval between compound administration and histamine challenge are noted in the Results section.

Platelet Activating Factor (PAF)- or Serotonin (5-HT)-induced Vascular Permeability in Rat Conjunctiva

Male Sprague Dawley rats (Harlan Sprague Dawley, Houston, TX), 125-200 grams, (6/group) were injected i.v. via the lateral tail vein with 1.0 ml Evans Blue dye (2.5 mg/ml). Approximately 20 min post dye injection, 20 µl of test compound or vehicle was applied topically onto one eye of the experimental animal. Thirty (30) min later, each animal was anesthetized and challenged subconjunctivally with PAF (30 ng/10 µl) or 5-HT (100 ng/10 µl) in the treated eye. Thirty (30) min after challenge, the animals were killed, and responses were quantitated as noted for histamine.

Compounds

AL-4943A (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan); levocabastine (Janssen

hydrochloride, Percoll®, goat IgG, ketotifen fumarate, bovine serum albumin, dinitrofluorobenzene, cyproheptadine hydrochloride (Sigma Chemical Co., St. Louis, MO); Evans Blue dye (Aldrich Chemical Co., Milwaukee, WI); PAF (Biomol, Plymouth Meeting, PA); anti-human IgE (goat-derived IgG) (Cortex Biochem, San Leandro, CA).

Immediately prior to *in vivo* use, all compounds were prepared as solutions or suspensions in saline (0.9% NaCl in water) at the concentrations noted in the Results section. All solutions and suspensions were prepared on a weight/volume basis to reflect the percentage of free acid or base for all *in vivo* experiments.

Statistical Analyses

Dunnett's t-test (12) was used to compare the mean of each treatment group with the mean of the vehicle control group. Linear regression was used to analyze dose response and calculate effective doses (ED_{50}).

RESULTS

The anti-allergic activity of AL-4943A, defined as inhibitory activity on basophil and mast cell degranulation, was assessed *in vitro*. The addition of AL-4943A 15 min before antigen challenge to cultured RBL cells inhibited histamine release in a concentration dependent manner. The IC_{50} value was calculated to be 803 μM. When human conjunctival mast cells were treated with AL-4943A for 15 min prior to anti-IgE challenge, histamine release was also significantly reduced in a concentration dependent fashion. The IC_{50} value obtained in human conjunctival mast cells was 559 ± 277 μM. Dose response curves obtained using both cell types are presented in Figs. 1A and 1B. The reference drug, ketotifen, examined in the human conjunctival mast cell also significantly inhibited histamine release. However, at concentrations 3 times greater than the maximally effective concentration, ketotifen caused a significant release of histamine from these cells (Fig. 1B). Additional *in vitro* evaluations of AL-4943A for cyclooxygenase or 5-lipoxygenase activity failed to detect significant activity (data not presented).

Anti-allergic activity noted *in vitro* was confirmed *in vivo* using two models of passive conjunctival anaphylaxis. Significant inhibition of i.v. antigen-stimulated allergic conjunctivitis was observed in both rats and guinea pigs when AL-4943A was applied topically onto the eye prior to antigen challenge. Significant concentration dependent inhibition was noted in both species (Table 1). AL-4943A was more efficacious when evaluated in guinea pigs compared to rats (80% inhibition vs. 40% inhibition, respectively, at 0.1%).

The anti-allergic activity observed following i.v. antigen challenge was further evaluated using topical ocular antigen challenge of passively sensitized guinea pigs. The application of AL-4943A onto the eye of sensitized animals 30 min, 4 hrs or 8 hrs before allergen instillation significantly attenuated the allergic response (Table 2). The ED_{50} values determined in these experiments were 0.017%, 0.053% and 0.100%, respectively.

When evaluated for topical ocular antihistaminic activity *in vivo,* AL-4943A potently inhibited histamine-induced vascular permeability in the conjunctiva. The compound was applied topically 5, 15 or 30 min, 2, 4, 8 or 24 hrs prior to histamine injection. Concentration dependent inhibition was observed (Table 3). The ED_{50} values derived from these data are 0.019%, 0.004%, 0.002%, 0.0014%, 0.0056%, 0.035% and 0.114%, respectively.

The ability of AL-4943A to affect the response of the contralateral eye to histamine challenge following topical ocular administration was also assessed. AL-4943A or levocabastine (0.05%) was applied to one eye and histamine was subsequently injected subconjunctivally in the contralateral eye. The drugs' effects were quantified as described above. AL-4943A (0.1%) applied 30 min before histamine challenge failed to decrease the response in the contralateral eye (197 ± 40, vehicle vs. 199

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