E6201, a Novel Kinase Inhibitor of Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Kinase-1 and Mitogen-Activated Protein Kinase Kinase/Extracellular Signal-Regulated Kinase Kinase Kinase-1: In Vivo Effects on Cutaneous Inflammatory Responses by Topical Administration

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ABSTRACT

E6201 [(3S,4R,5Z,8S,9S,11E)-14-(ethylamino)-8,9,16-trihydroxy-3,4-dimethyl-3,4,9,10-tetrahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione] is a novel anti-inflammatory agent that has potent inhibitory effects on the production of proinflammatory cytokines from leukocytes and antiproliferative activity on keratinocytes. To characterize the in vivo pharmacological activity of E6201, topically administered E6201 was evaluated in several different animal models of dermatitis. E6201 formulated as an ointment or cream showed dose-dependent inhibition of croton oil-induced acute edema formation and neutrophil infiltration into mouse skin. In addition, E6201 cream inhibited the 1-fluoro-2,4-dinitrobenzene-induced contact hypersensitivity reaction mediated by T cells in mice. In this model, E6201 cream also suppressed the migration of neutrophils and lymphocytes into the inflammatory site. Pretreatment with E6201 cream attenuated phorbol-12 myristate 13-acetate-induced ornithine decarboxylase activity, a marker of proliferation in epidermis. Furthermore, E6201 ointment showed inhibitory effects on both mezerein-induced and interleukin (IL)-23-induced epidermal hyperplasia. E6201 also suppressed T cell receptor-stimulated IL-17 production from human T cells. These results indicate that topically administered E6201 may be a useful agent for the prevention and treatment of cutaneous inflammatory and hyperproliferative diseases such as psoriasis.

Introduction

A potent bioactive natural product, f152A1 [(3S,5Z,8S,9S,11E)-8,9,16-trihydroxy-14-methoxy-3-methyl-3,4,9,10-tetrahydro-1H-benzo[c][1]oxacyclotetradecine-1,7(8H)-dione], was isolated from the fermentation broth of the fungus Curvularia verruculosa and found to be an inhibitor of tumor necrosis factor α transcription (Goto et al., 2009). f152A1 inhibits the production of proinflammatory cytokines in several cell based-assays at nanomolar concentrations, but, because of instability in biological fluids (plasma and liver microsomes), its anti-inflammatory activity in various in vivo models is not remarkable. To address the metabolic instability of f152A1, a medicinal chemistry program was undertaken, resulting in the generation of more than 400 analogs of f152A1. E6201 [(3S,4R,5Z,8S,9S,11E)-14-(ethylamino)-8,9,16-trihydroxy-3,4-dimethyl-3,4,9,10-tetrahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione] was

ABBREVIATIONS: E6201, (3S,4R,5Z,8S,9S,11E)-14-(ethylamino)-8,9,16-trihydroxy-3,4-dimethyl-3,4,9,10-tetrahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione; f152A1, (3S,5Z,8S,9S,11E)-8,9,16-trihydroxy-14-methoxy-3-methyl-3,4,9,10-tetrahydro-1H-benzo[c][1]oxacyclotetradecine-1,7(8H)-dione; FK-506, hexadecahydro-5,19-dihydroxy-3-[2-{(4-hydroxy-3-methoxy)cyclohexyl}-1-methylthethyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8(2-propenyl)-15,19,19-epoxy-3H-pyrido[2,1-c][1,4]oxazacyclotetraene-1,7,20,21(4H,23H)-tetrone; PMA, phorbol 12-myristate 13-acetate; ODC, ornithine decarboxylase; DNFB, 1-fluoro-2,4-dinitrobenzene; IL, interleukin; MPO, myeloperoxidase; CHS, contact hypersensitive; H&E, hematoxylin and eosin; PBMC, peripheral blood mononuclear cell; ELISA, enzyme-linked immunosorbent assay; AP-1, activator protein-1.
identified as a promising analog with superior pharmacological characteristics.

We have reported the in vitro pharmacological properties of E6201 (Goto et al., 2009), including potent inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1 and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1, and suppression of the activation of NF-κB and AP-1 in various cells. In human monocytes and lymphocytes, E6201 suppresses the production of various proinflammatory cytokines. In human keratinocytes, E6201 inhibits cell proliferation and IL-8 production. These studies suggest that E6201 may have therapeutic potential in inflammatory, immunologically active, and hyperproliferative diseases such as psoriasis.

Psoriasis is a major chronic inflammatory skin disease characterized by severe hyperplasia in the epidermis and marked infiltration of leukocytes into both dermis and epidermis (Schön and Boehncke, 2005). Although there is no known naturally occurring disorder in laboratory animals that mimics the complex phenotype of psoriasis, it is possible to evaluate antipsoriasis drug candidates on several animal models. Each model may shed light on specific aspects implicated in the pathophysiology of psoriasis. The acute croton oil dermatitis model (Berg et al., 1995, Wilmer et al., 1994, Schottelius et al., 2002) and the contact hypersensitivity (CHS) model (Friedmann, 1989) provide insight regarding drug candidate activities on inflammatory and immunological aspects of the disease, respectively. In regard to drug candidates’ activity on hyperproliferation, experimental models of phorbol 12-myristate 13-acetate (PMA)-induced ornithine decarboxylase (ODC) activation in epidermis (Russell, 1973) and mezerein-induced epidermal hyperplasia (Mufson et al., 1979) were used.

The present studies were conducted to evaluate the in vivo activity of topically administered E6201 in these dermal inflammatory models of psoriasis.

Materials and Methods

Animals. All animal studies were performed with the approval of the Animal Ethics Committee at Eisai according to Laboratory Animal Welfare guidelines. BALB/c mice (male, 6–8 weeks old) were purchased from Charles River Laboratories, Inc. (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME). Hox:HR-1 mice (female, 7 weeks old) were purchased from Charles River Laboratories, Inc. (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME). Hox:HR-1 mice (female, 7 weeks old) were purchased from Charles River Laboratories, Inc. (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME).

Test Compounds. E6201 was synthesized at Eisai Inc. (Andover, MA). The chemical structure of E6201 is shown in Fig. 1. As a prototype formulation, a petrolatum-based E6201 ointment (20% propylene glycol monostearate containing petrolatum) was prepared at Eisai Co., Ltd., Tsukuba Research Laboratories (Ibaraki, Japan). E6201 cream was manufactured at Eisai, Inc. (Research Triangle Park, NC). Each formulation was made on the percentage basis. Betamethasone (Sigma-Aldrich, St. Louis, MO) was formulated in a petrolatum-based ointment, or a commercially available betamethasone ointment (0.064%) was purchased from Schering-Plough Corporation (Kenilworth, NJ). FK-506 [hexadecahydro-5,19-dihydroxy-3-[2-(4-hydroxy-3-methoxy cyclohexyl)-1-methylhexyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(2-propenyl)-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxazacyclotricosine-1,7,20,21(4H,23H)-tetrone] ointment (0.1%) (Protopic) was purchased from Astellas Pharma Inc (Tokyo, Japan). Tazarotene (0.1%) (Tazorac) was purchased from Al-lergan, Inc., (Irvine, CA). Devonex (0.005%) was purchased from Teikoku Seiyaku (Kagawa, Japan).

Reagents. The petrolatum (Vaseline) for a prototype ointment formulation was purchased from Fluka (Buchs, Switzerland). Croton oil, PMA, mezerein, o-dianisidine, pyridoxal 5’-phosphate, L-ornithine hydrochloride, hematoxylin, eosin hexadecyltrimethylammonium bromide, and human myeloperoxidase (MPO) were obtained from Sigma-Aldrich. 1-Fluoro-2,4-dinitrobenzene (DNFB) was obtained from Wako Pure Chemicals (Osaka, Japan). Calcein was obtained from Invitrogen (Carlsbad, CA). dL-[1-14C]ornithine and NCS II tissue solubilizer were obtained from Amersham Biosciences (Ontario, Canada). Ficol-Paque was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). T cell expander was obtained from Invitrogen. Recombinant mouse IL-23 and human IL-17 ELISA kits were obtained from R&D Systems (Minneapolis, MN).

Croton Oil-Induced Inflammation. In a prophylactic experiment, E6201 or betamethasone (0.01–1%) formulated in petrolatum-based ointments (approximately 15 mg/ear) or the vehicle ointment were topically applied onto the right ear of each BALB/c mouse. Four hours after drug or vehicle administration, dermal inflammation was induced by applying croton oil (150 μg/ear: 15 μl of 10 mg/ml acetone solution per ear) to the right ear. In a therapeutic experiment, E6201 (0.003–0.3%) and betamethasone formulated in the ointments or placebo ointment were topically administered to the right ear 30 min after croton oil application. Six hours after croton oil application, mice were euthanized, and ear thickness was measured with a dial thickness gauge (Ozaki Mfg. Co., Kanagawa, Japan) as an indicator of swelling. In addition, MPO activity in homogenates from inflamed ears was measured as an indicator of neutrophil infiltration.

Measurement of MPO Activity. Measurement of MPO enzyme activity was carried out according to a previously reported method (Bradley et al., 1982). In brief, mouse ear tissues were homogenized in 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0, using a Polytron homogenizer. Homogenates were sonicated, freeze-thawed three times, and then centrifuged at 40,000g for 15 min. MPO activity in the resulting supernatant was measured spectrophotometrically with 180 μl of 50 mM potassium phosphate buffer, pH 6.0, using a Polytron homogenizer. Homogenates were sonicated, freeze-thawed three times, and then centrifuged at 40,000g for 15 min. MPO activity in the resulting supernatant was measured spectrophotometrically with 180 μl of 50 mM potassium phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured with a SpectraMax tunable spectrophotometer (Molecular Devices, Sunnyvale, CA). Equipped with Softmax software kinetics mode and slope calculation. The MPO enzyme activity was reported as units per milligram of protein (BCA protein assay) calculated from the slope of the MPO enzymatic reaction of each homogenate by using a standard curve generated with standard human MPO (unit of MPO activity is defined by Sigma-Aldrich).

Contact Hypersensitivity Model. BALB/c mice were sensitized on 2 successive days (days 0 and 1) by the application of 25 μl of the 0.4% DNFB solution (acetone/olive oil, 4:1) to the abdomen of the mice. On the fifth day, placebo cream, E6201 cream (0.03–1%), betamethasone ointment, Devonex ointment, or FK-506 ointment were applied to the ear at 5 min and 4 h after rechallenge with the same
antigen (20 μl of 0.2% DNFB in acetone) to the right ear of each mouse. To avoid vehicle effect, these ointments were wiped off by cotton with 70% ethanol. Twenty-four hours after antigen challenge, the thickness of each ear was measured with a thickness gauge. The intensity of the elicited CHS reaction (edema formation) was calculated on the basis of the difference in the thickness between the DNFB-treated (right) and the untreated (left) ear of the same mouse.

**T Cell Infiltration in the CHS Model.** T cells were separated from the spleens of DNFB-sensitized mice by nylon fiber column and then labeled with calcein according to the manufacturer’s instructions (Wako Pure Chemicals). BALB/c mice were sensitized on 2 successive days (days 0 and 1) by the application of 25 μl of the 0.4% DNFB solution (acetone/olive oil, 4:1) to the abdominal skin. On the fifth day, placebo cream, E6201 cream (0.1 and 1%), or betamethasone ointment was topically applied onto the same region. After approximately 4.5 h, the animals were euthanized and the treated area of the dorsal skin was removed. The weight of the skin was measured as an indicator of edema. After weighing the skin, each sample was placed in saline at approximately 55°C for 30 s and then immersed in ice-cold saline. The epidermis was separated from the skin with a surgical knife and homogenized in 500 μl of homogenization buffer (50 mM phosphate buffer pH 7.2, 0.4 mM pyridoxal 5'-phosphate, 5 mM dithiothreitol). The homogenate was then centrifuged at 10,000g for 10 min, and the supernatant was recovered and stored at 4°C. The next day, 100 μl of the supernatant was mixed with 50 μl of ODC reaction buffer [0.5 mM L-ornithine hydrochloride, 370 MBq/liter (0.179 mM) DL-[1-14C]ornithine, 40 mM phosphate buffer, pH 7.2, 0.32 mM pyridoxal 5’-phosphate, 4 mM dithiothreitol] in a microtiter tube. The tube was treated with 2.5 μl of PMA (170 μM) or vehicle solution for 10 min, and the resultant ornithine decarboxylase (ODC) activity was measured.

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**PMA-Induced Ornithine Decarboxylase Activity in Epidermis.** PMA-induced ODC activity in epidermis was measured by using a previously reported method with slight modification (O’Brien et al., 1975). In brief, 100 μl of PMA (170 μM) or vehicle solution (1.7% dimethyl sulfoxide in acetone) was applied to the dorsal skin surface of Hos:HR-1 mice or Hr-/Kud mice over an area of approximately 5 (2 x 2.5) cm². After drying the solution, E6201 cream (0.01–0.3%), betamethasone ointment, or placebo cream was topically applied onto the same region. After approximately 4.5 h, the animals were euthanized and the treated area of the dorsal skin was removed. The weight of the skin was measured as an indicator of edema. After weighing the skin, each sample was placed in saline at approximately 55°C for 30 s and then immersed in ice-cold saline. The epidermis was separated from the skin with a surgical knife and homogenized in 500 μl of homogenization buffer (50 mM phosphate buffer pH 7.2, 0.4 mM pyridoxal 5’-phosphate, 5 mM dithiothreitol). The homogenate was then centrifuged at 10,000g for 10 min, and the supernatant was recovered and stored at 4°C. The next day, 100 μl of the supernatant was mixed with 50 μl of ODC reaction buffer [0.5 mM L-ornithine hydrochloride, 370 MBq/liter (0.179 mM) DL-[1-14C]ornithine, 40 mM phosphate buffer, pH 7.2, 0.32 mM pyridoxal 5’-phosphate, 4 mM dithiothreitol] in a microtiter tube. The tube was treated with 2.5 μl of PMA (170 μM) or vehicle solution for 10 min, and the resultant ornithine decarboxylase (ODC) activity was measured.

**Fig. 2.** Inhibitory effects of topically applied E6201 and betamethasone on croton oil-induced dermatitis in mice. A, prophylactic treatment. E6201 (0.01–1%) and betamethasone or the vehicle ointment were topically applied onto the ear 4 h before croton oil application. Six hours after croton oil application, the thickness of the inflamed ear (left) and migration of neutrophils (right) were measured. B, therapeutic treatment. Thirty minutes after croton oil application, E6201 (0.003–0.3%) or the vehicle ointment was topically applied onto the ear. Six hours after croton oil application, the thickness of the inflamed ear (left) and the migration of neutrophils (right) were measured. Data show mean ± S.E.M. (n = 6–8). ∗, P < 0.05 by Dunnett’s multiple comparison test.
placed in a sealed scintillation vial containing a circular GF/C filter soaked with 100 μl of NCS II tissue solubilizer. The mixture was incubated at 37°C for 1 h. The reaction was stopped by injecting 300 μl of 2 M citric acid, and incubation was continued for an additional hour to ensure the complete absorption of 14CO2 by the GF/C filter. The radioactivity of the sample was measured with a liquid-scintillation counter (TRI-CARB 2700TR; Packard Japan, Tokyo, Japan). The result was expressed as nanomols of 14CO2 production per milligram of protein.

Mezerein-Induced Epidermal Thickening. Mezerein is known to induce epidermal hyperplasia (Mufson et al., 1979) and is used in animal models of epidermal hyperproliferation for drug evaluation (Schottelius et al., 2002). The dorsal skin of female CD-1 mice (8 weeks old) was shaved 7 days before the beginning of the experiment.

Fig. 3. E6201 and reference drugs inhibit contact hypersensitivity reaction in mice. A, effect of E6201 on DNFB-induced contact hypersensitivity reaction. Five days after DNFB sensitization to the abdomen, E6201 (0.03–1%) cream or vehicle cream were topically applied to the ear of each mouse before a second challenge with DNFB solution. Twenty-four hours after the second challenge with antigen, the thicknesses of both ears were measured as described. Data are mean ± S.E.M. of eight mice in each group. *, P < 0.05 by Dunnett’s multiple comparison test. B, comparison of the activity of E6201 and reference drugs in DNFB-induced contact hypersensitivity reaction. E6201 cream (0.1–1%) and ointments of reference drugs (Dovonex ointment: VD3; Protopic ointment: FK-506) were painted on the ear at 5 min and 4 h after the application of DNFB solution. To evaluate systemic effects of E6201, 1% E6201 cream was applied to the nonelicited ear (NE). Twenty-four hours after the second challenge with antigen, the thicknesses of the ears were measured. Data are mean ± S.E.M. of eight mice in each group. *, P < 0.05 by Dunnett’s multiple comparison test.

Fig. 4. Effect of E6201 and betamethasone on infiltration of lymphocytes and neutrophils in a contact hypersensitivity model. A, lymphocyte infiltration. B, neutrophil infiltration. Five days after DNFB sensitization to the abdomen, E6201 (0.1 and 1%) cream, betamethasone ointment (Beta) or vehicle cream (vehicle) were topically applied to the ear of each mouse before the second challenge with DNFB solution. Calcein-labeled lymphocytes were intravenously injected in mice just before the second challenge with DNFB. Six hours after the second challenge with antigen, the number of infiltrated lymphocytes and neutrophils in the ear was measured. Data are mean ± S.E.M. of eight mice in each group. *, P < 0.05 by Dunnett’s multiple comparison test.
Mezerein was dissolved in acetone at a concentration of 55 μg/ml with or without E6201 (0.01–1%), and 100 μl of the mixture was applied onto the dorsal skin to a 9 (3 × 3) cm² area that was premarked. The mice were euthanized 30 h after treatment with mezerein, and the inflamed dorsal skin [4 (2 × 2) cm² within the mezerein-applied area] was excised immediately after euthanasia. Skin tissues were fixed in 10% buffered formalin. The tissues were processed for histological analysis of hematoxylin and eosin (H&E)-stained sections at Charles River Laboratories. The interfollicular epidermal thickness was measured at 200× magnification by using an ocular micrometer, and the number of nucleated cell layers was counted at the same location according to previously described methods (Mufson et al., 1979).

IL-23-Induced Acanthosis (Hyperplasia of Epidermis). An IL-23-induced acanthosis model in mice was performed as described previously (Zheng et al., 2007). Recombinant mouse IL-23 (500 ng/20 μl/site) was injected into the ears of C57BL/6 mice (8 weeks old) intradermally every other day. Twenty milligrams of E6201 cream (0.01 and 0.03%) or vehicle cream was applied onto the IL-23-injected ear once a day. On day 10, ears were harvested for H&E-stained sections at Charles River Laboratories. The interfollicular epidermal thickness was measured at 200× magnification by using an ocular micrometer, and the number of nucleated cell layers was counted at the same location according to previously described methods (Zheng et al., 2007). Recombinant mouse IL-23 (500 ng/20 μl/site) was injected into the ears of C57BL/6 mice (8 weeks old) intradermally every other day. Twenty milligrams of E6201 cream (0.01 and 0.03%) or vehicle cream was applied onto the IL-23-injected ear once a day. On day 10, ears were harvested for H&E staining. Stained ear skin images were acquired and analyzed by Coolscope (Nikon, Tokyo, Japan), and epidermal thickness was measured according to a previously reported method (Zheng et al., 2007).

IL-17 Production from Human T Cells. Human peripheral blood mononuclear cells (PBMCs) were separated from freshly harvested human peripheral blood by Ficoll-Paque. These cells (1 × 10⁵ cells/well) were cultured in RPMI medium 1640 containing 10% fetal bovine serum and 50 μM 2-mercaptoethanol in a 96-well microtiter plate. Serially diluted E6201 (3–300 nM) or vehicle solutions were added to each well, then T cell expander (anti-CD3 and anti-CD28; Dynabeads CD3/CD28) was added. After incubation for 48 h, the IL-17 concentration in the supernatant was measured by ELISA.

Data Analysis. Results were expressed as the mean ± S.E.M. Statistical analysis was performed by Dunnett-type multiple comparison test or paired t test. A value of p < 0.05 (two-sided) was considered to be statistically significant.

Results

Croton Oil-Induced Inflammation. The effects of topically administered E6201 on croton oil-induced ear inflammation in BALB/c mice were examined by using prophylactic or therapeutic regimens. Croton oil-induced swelling and neutrophil infiltration detected by MPO activity in the inflamed ear were used as the parameters. In a prophylactic study, topically administered E6201 ointment showed significant inhibition of both ear swelling and neutrophil infiltration into the ear from doses of 0.01 to 1.0% in a dose-dependent manner comparable with betamethasone (Fig. 2A).

When E6201 ointment was administered 30 min after croton oil application in a therapeutic regimen, E6201 showed...
significant dose-dependent inhibition of both ear swelling and neutrophil infiltration into the ear from doses of 0.003 to 0.3% (Fig. 2B). The clinically used topical formulations of Tazarotene (retinoid derivative) and Dovonex (vitamin D3 derivative) slightly inhibited ear swelling but did not affect neutrophil infiltration in this acute therapeutic model (Supplemental Fig. 1). A cream formulation of E6201 showed similar potency to petrolatum-based ointment in both prophylactic and therapeutic studies (data not shown). These results indicated that topically applied E6201 has potent anti-inflammatory activity in croton oil-induced acute skin inflammation. The effects of vehicle were not significant in both prophylactic and therapeutic schedules when tested in separate experiments in advance.

DNFB-Induced Contact Hypersensitivity. To evaluate the effect of E6201 on lymphocyte response in vivo, the effect of topically applied E6201 on DNFB-induced CHS in mice was examined. To optimize the treatment schedule in the CHS model, a 1× (application 5 min after rechallenge) and a 2× treatment of E6201 cream (applications 5 min and 4 h after rechallenge) were compared (Supplemental Fig. 2). The ED50 value was ~0.3% with the 1× treatment and 0.1% with the 2× treatment. Thus the 2× treatment schedule was used for the CHS model. It should be noted that the ED50 value for the 1× treatment of E6201 cream in CHS (24-h model) was clearly higher than the ED50 value in the croton oil-induced inflammation (6-h model) (Fig. 2A), suggesting that tissue residence time of E6201 cream in mice skin is not long enough to explain differences in long-term activities (prophylactic versus therapeutic schedules).

Topically applied E6201 cream dose-dependently inhibited the increase in ear thickness, and statistically significant inhibition was observed at concentrations of 0.03% and above (Fig. 3A). In this model, Dovonex ointment and FK-506 ointment also showed significant inhibitory effects (Fig. 3B). Betamethasone ointment, but not Tazarotene cream, suppressed the CHS reaction (Supplemental Fig. 3). Application of E6201 cream onto the nonelicited opposite ear did not inhibit the contact hypersensitivity at all in the DNFB-challenged ear, indicating that the inhibitory effect of E6201 in this model is not caused by systemic absorption (Fig. 3B).

We analyzed the effect of E6201 cream on the migration of T cells and neutrophils into skin in a CHS experiment. The infiltration of calcine-labeled T cells into the inflamed ear was inhibited by treatment with E6201 cream and was comparable with that observed with betamethasone ointment (Fig. 4A). Furthermore, E6201 cream showed suppression of neutrophil infiltration as assessed by MPO activity was comparable with that observed with betamethasone ointment (Fig. 4B). These results indicate that topically applied E6201 inhibits the lymphocyte response in DNFB-induced CHS in mice, and E6201 exhibits a suppressive effect on T cell and neutrophil migration. In this model, vehicle cream did not affect the CHS reactions (Supplemental Fig. 3).

PMA-Induced Elevation of Ornithine Decarboxylase Activity in the Epidermis. To study the in vivo effect of E6201 on the proliferation of the epidermis, topically administered E6201 was tested in a model of PMA-induced ODC activity in hairless mice. Skin weight and ODC activity were significantly elevated approximately 4.5 h after the application of PMA solution onto the skin. Topical application of E6201 cream dose-dependently inhibited the increase in both skin edema and ODC induction (Fig. 5A). Statistically significant inhibition by E6201 cream was observed at doses of 0.01% and above in skin edema and doses of 0.03% and above in ODC. Betamethasone strongly inhibited skin edema formation, but did not affect ODC

Fig. 6. E6201 inhibits mezerein-induced epidermal hyperproliferation. Mezerein with or without E6201 was applied onto the dorsal skin. After 30 h, the skin tissues were analyzed by H&E staining. Typical photo images of the skin tissues are shown. A, vehicle control. B, mezerein control. C, mezerein + 0.1% E6201-treated mice. D, mezerein + 1% E6201-treated mice. Arrows indicate interfollicular epidermis.
induction (Fig. 5B). These results indicated that E6201 attenuated both PMA-induced epidermal proliferation and skin inflammation. In this model, Dovonex ointment (Supplemental Fig. 4) and FK-506 ointment (Supplemental Fig. 5) did not affect either edema formation or ODC induction. On the other hand, Tazarotene cream strongly inhibited the elevation of ODC activity (Supplemental Fig. 5).

**Mezerein-Induced Epidermal Thickening.** To evaluate the antiproliferative activity of E6201 on epidermis, histopathological evaluation was used in a mezerein-induced epidermal hyperplasia model in mice. We examined the effect of topically coadministered E6201 on mezerein-induced epidermal hyperproliferation in mice. Compared with the typical histological image of skin from a vehicle-treated control mouse (Fig. 6A), mezerein induced marked epidermal hyperplasia (red arrow in Fig. 6B) detectable by either epidermal thickness or the number of nucleated cell layers of interfollicular epidermis. Coadministration of E6201 with mezerein suppressed the hyperplasia (Fig. 6, C and D) in a dose-dependent manner (Fig. 7). Statistically significant suppression was observed at 0.1% E6201, and almost complete suppression was observed at 1% E6201 (Fig. 7).

**IL-23-Induced Acanthosis (Hyperplasia of Epidermis).** An IL-23-induced acanthosis model was used to evaluate the effect of E6201 on epidermal hyperplasia. Repeated injection of IL-23 into the ear every other day induced approximately 4-fold epidermal thickening compared with untreated controls. Daily topical administration of E6201 cream (0.01 or 0.03%) or vehicle cream were applied onto the IL-23-injected ear once a day. On day 10, the ear thickness was measured as described under Materials and Methods. Data show mean ± S.E.M. (n = 6), *P < 0.05 by Dunnett’s multiple comparison test.

**Discussion**

We have previously reported the in vitro pharmacological properties of E6201, including the following: 1) E6201 suppressed the activation of AP-1 via inhibitory effects on mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1 and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-; 2) E6201 decreased activation of NF-κB; 3) E6201 suppressed the production of various proinflammatory cytokines in human monocytes and leukocytes; and 4) E6201 attenuated hyperproliferation and IL-8 production in human keratinocytes in vitro (Goto et al., 2009). These pharmacological properties of E6201 indicate that this compound represents a promising strategy for the treatment of psoriasis, because psoriasis is a chronic inflammatory skin disease characterized by severe hyperplasia in the epidermis and marked infiltration of leukocytes into dermis and epidermis. In this article, we investigated the therapeutic effects of topically administered E6201 in several dermatitis models that mimic some aspects of human psoriasis.
First, in an acute inflammation model, we examined the effect of E6201 on croton oil-induced ear inflammation. Croton oil is a phorbol ester-containing oil extracted from the seed of a plant, *crotoniglium*, that induces an acute inflammatory reaction through protein kinase C activation (Berg et al., 1995, Schottelius et al., 2002). The ear edema formation induced by croton oil application to the ear skin is an acute skin inflammatory response and seems to be T cell-independent because this reaction does not require prior sensitization (Wilmer et al., 1994). Biosyntheses of eicosanoids and inducible nitric-oxide synthase are involved in the responses to croton oil, and anti-inflammatory steroids are effective in suppressing this type of inflammation (Lloret and Moreno, 1995, Sala et al., 2003). E6201 showed inhibitory effects even when it was dosed at 30 min after croton oil application. Thus, the inhibitory effects of E6201 in this model are likely to be mediated by inhibition of inflammatory processes induced by croton oil, rather than by blocking penetration of croton oil. As observed with betamethasone, topical application of E6201 strongly inhibited the acute inflammatory response in this model, as assessed by inhibition of neutrophil infiltration and edema.

E6201 suppressed lymphocyte activation and proliferation in vitro (Goto et al., 2009). To evaluate the effects of E6201 on lymphocyte-mediated responses in vivo, we examined the effect of E6201 on DNPB-induced CHS responses in a widely used model of type IV allergic reaction (Friedmann, 1989). CHS responses are mediated by T cells after the epicutaneous application of chemically reactive compounds (haptens) to the skin. E6201 cream showed a dose-dependent inhibition of edema in this model. Similar inhibitory effects were observed with the ointments containing either FK-506 or Dovonex.

CD4+ and CD8+ T cells play an important role in this allergic reaction and neutrophils also infiltrate inflammatory sites (Gocinski and Tigelaar, 1990). Several groups have demonstrated that CHS is mediated by migrated T lymphocytes (Gautam et al., 1991, Miller and Jenkins, 1985). E6201 cream suppressed T cell infiltration, and the inhibitory effect was comparable with that observed with betamethasone ointment. In addition, E6201 inhibited neutrophil migration in this CHS model. These results suggest that inhibitory effects on the migration of both T cells and neutrophils into inflammatory sites contribute to the mechanism of suppression of CHS by E6201 cream. Because E6201 inhibited IL-8 production from activated human keratinocytes (Goto et al., 2009), the inhibition of migration observed here may reflect inhibition of chemokine production from keratinocytes in the CHS model.

E6201 inhibits the proliferation of epidermal growth factor-stimulated human keratinocytes in vitro (Goto et al., 2009). Increased ODC activity is known to be one of the earliest responses that precede hyperproliferation in mouse epidermis (Russell, 1973), and it has been reported that ODC is activated in PMA-stimulated epidermis (O’Brien et al., 1975). Topical application of E6201 cream dose-dependently inhibited both skin edema and ODC activation. Betamethasone inhibited edema (an indicator of inflammation) completely, but did not suppress ODC induction in the epidermis. Tazarotene showed significant inhibitory effects on ODC induction in this model, but Dovonex did not. It has been reported that the activation of p38, c-Jun NH2-terminal kinase, and NF-κB are involved in this model (Chung et al., 2007). The activation of AP-1 could result in the activation of ODC transcription (Tseng and Verma, 1995). E6201 has inhibitory effects on the phosphorylation of c-Jun NH2-terminal kinase, p38 mitogen-activated protein kinase, and IκBα in human lymphocytes (Goto et al., 2009), and the suppressive effect of E6201 on PMA-induced ODC activity may be explained by inhibition of one or more of these intracellular signals.

Mezerein induces epidermal hyperplasia (Mufson et al., 1979) and is used in animal models of epidermal hyperproliferation for drug evaluation (Schottelius et al., 2002). E6201 suppressed mezerein-induced epidermal hyperproliferation in a dose-dependent manner. Thus, in addition to the inhibitory effects on PMA-induced ODC induction, the antiproliferative activity of E6201 on epidermis was further demonstrated histopathologically.

Genotyping of single-nucleotide polymorphisms in patients with psoriasis has been reported, and IL-23A, IL-23R, and IL-12B were confirmed as loci associated with psoriasis (Nair et al., 2009). Intradermal injection of IL-23 induced epider-
mal hyperplasia (acanthosis) in mice (Zheng et al., 2007). Topical treatment with E6201 significantly inhibited epidermal hyperplasia in IL-23-treated mice in a dose-dependent manner. Th17 cells are reported to play an important role in the IL-23-induced acanthosis model (Zheng et al., 2007). E6201 suppressed T cell receptor-stimulated IL-17 production from human T cells, suggesting that E6201 can suppress the production of effector molecules resulting from T cell receptor stimulation of Th17 cells. These results suggest that E6201 has inhibitory effects not only on epidermal hyperplasia induced by PMA and PMA-related agents, but also on Th17-mediated hyperplasia.

The comparative effects of E6201 and the clinical dose of reference antipsoriasis drugs in three different types of dermatitis models are summarized in Table 1. Tazarotene, a retinoic acid derivative, strongly inhibited epidermal hyperproliferation, but was only partially effective on the acute inflammatory response monitored in the croton oil-induced inflammation model and was not effective on the T cell-mediated immune response monitored in the DNPB-induced CHS model. Dovonex, a vitamin D3 derivative, inhibited T cell-mediated immune responses, but was only partially effective on the acute inflammatory response monitored in the croton oil-induced inflammation model and was not effective on the T cell-mediated immune response monitored in the DNPB-induced CHS model. Dovonex, a vitamin D3 derivative, inhibited T cell-mediated immune responses, but it was not effective in inhibiting croton oil-induced neutrophil migration and PMA-induced epidermal hyperproliferation. Be	amethasone was effective on acute edema formation and T cell-mediated immune responses, but it was not effective in inhibiting croton oil-induced neutrophil migration and PMA-induced epidermal hyperproliferation. In contrast, E6201 showed a wider inhibitory profile in these three different types of dermal inflammation models. Our results suggest that E6201 may be a useful agent for the prevention and treatment of cutaneous inflammatory and hyperproliferative diseases such as psoriasis.

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References


decine-1,7(8f)-dione, a novel kinase inhibitor of mitogen-activated protein ki-
nase/extracellular signal-regulated kinase kinase (MEK)-1 and MEK kinase-1, in vitro characterization of its anti-inflammatory and anti-hyperproliferative activi-

Lloret S and Moreno JJ (1995) Effects of an anti-inflammatory peptide (antiflam-

min 2) on cell influx, eicosanoid biosynthesis and sedema formation by arachidonic acid and tetradecanoyl phorbol dermal application. Biochem Pharmacol 50:347–353.


Mufson RA, Fischer SM, Verma AK, Gleason GL, Slaga TJ, and Boutwell RK (1979) Effects of 12-


O’Brien TG, Simmsman RC, and Boutilier RK (1975) Induction of the polyamine-


Tseng CP and Verma AK (1985) Lack of 12-O-tetradecanoylphorbol-13-acetate re-
sponsiveness of ornithine decarboxylase introns which have Ap-1 consensus se-
quences. Mol Cell Biochem 146:7–12.


Comparison of the activity of E6201 and reference drugs on croton oil-induced dermatitis in mice

Normal
Vehicle
E6201
0.003 %
0.01 %
0.03 %
0.1 %
0.3 %
Reference
Beta 0.064 %
FK506 0.1 %
Taza 0.1 %
VD3 0.005 %

Increasing of ear thickness (x10^{-2} mm)
MPO activity (mUnits /mg protein)
Comparison of efficacy of single and twice treatment of E6201 on DNFB-induced contact hypersensitivity reaction.
Fig. S3
Comparison of the activity of E6201 and reference drugs in DNFB-induced contact hypersensitivity reaction.
**Fig. S4**

Comparison of the activity of E6201 and Dovonex on PMA-induced ear edema and the elevation of ODC activity in epidermis.
Fig. S5

Comparison of the activity of E6201 and reference drugs on PMA-induced ear edema and the elevation of ODC activity in epidermis.